グリホサートカリウム塩

要旨及び評価結果

（ヒトに対する毒性）

検索期間：2006 年 7 月 1 日～2009 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

Short summary of relevant and reliable articles from category a) with / without restrictions)

3031 Toxicokinetics of glyphosate and its metabolite aminomethyl phosphonic acid in rats .

Author	Anadon A; Martinez-Larranaga M R; Martinez M A; Castellano V J; Martinez M; Martin M T; Nozal M J; Bernal J L
Source	Toxicology letters, (2009 Oct 08) Vol. 190, No. 1, pp. 91-5. Electronic Publication Date: 14 Jul 2009
Title	Toxicokinetics of glyphosate and its metabolite aminomethyl phosphonic acid in rats .
Unique ID	3031

Short Summary with assessment and conclusion:
The publication is providing additional information on blood plasma concentrations of glyphosate and AMPA, as well as elimination after oral and i.v. application of glyphosate.
Rats were divided into two groups of 80 animals each, one group (Group 1) received a single oral gavage administration of 400 mg/kg bw glyphosate and the other group (Group 2) received a single i.v. injection of 100 mg/kg bw glyphosate.
Intravenous injection is more rapidly excreted than oral simply because of lag time for gastro intestinal tract absorption and entry into the blood stream. That reported, half-lives for i.v (0.345 and 9.99 hours for α and β phases respectively) versus oral (4.17 and 14.38 hours for α and β phases respectively) are all very rapid elimination kinetics, emphasizing the low duration of any systemic exposure to glyphosate, irrespective of the route.
Study report meets basic scientific principles, and is comparable to actual kinetics guidelines (large number of test animals used, standard deviations and mass balance not reported).

Short summary of relevant and reliable articles from category a) with / without restrictions)

140 Agricultural pesticide use and pancreatic cancer risk in the Agricultural Health Study Cohort.

Author	Andreotti, Gabriella (Correspondence); Freeman, Laura E. Beane; Coble, Joseph; Silverman, Debra T.; Alavanja, Michael C. R.
Source	International Journal of Cancer, (15 May 2009) Vol. 124, No. 10, pp. 2495-2500. Refs: 25 ISSN: 0020-7136; E-ISSN: 1097-0215 CODEN: IJCNAW
Title	Agricultural pesticide use and pancreatic cancer risk in the Agricultural Health Study Cohort.
Unique ID	140

Short Summary with assessment and conclusion:

This was a case-control study conducted in the Agricultural Health Study cohort in Iowa and North Carolina, USA. The study population comprised licensed private and commercial pesticide applicators and their spouses. The health outcome of interest was incident pancreatic cancer, and was captured from population-based state cancer registries; it was defined as codes C25.0 – C25.9 in the International Classification of Diseases 10th Revision (ICD-10). The exposure of interest was glyphosate use, and was captured by self-administered questionnaire; exposure status was classified in two different ways: ever vs. never exposure, and number of exposure days. Data for 93 pancreatic cancer cases and 82,503 controls were analyzed using logistic regression. The reported an odds ratio (OR) and 95% confidence interval (CI) of 1.1 (0.6, 1.7) for participants who self-reported to have ever used glyphosate compared to those who self-reported to have never used it. Age group, cigarette smoking, diabetes, and applicator type were adjusted for in this analysis. The reported OR and 95% CI for participants with ≤184 days of exposure compared to those who self-reported to have never used it was 1.9 (0.9, 3.8). The OR and 95% CI for participants with ≥185 days compared to those who self-reported to have never used it was 1.2 (0.6, 2.6). The reported value for p-trend was 0.85. Age group, cigarette smoking, and diabetes were adjusted for in this analysis. These results suggest that there is not a statistically significant difference in pancreatic cancer incidence among exposure groups at the 0.05 significance level. This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because exposure classification was based on self-reported glyphosate use, an indirect method by which to estimate exposure status of study participants. No biological evidence is provided to support exposure classification of study participants. Misclassification of exposure is possible in this study.

Short summary of relevant and reliable articles from category a) with / without restrictions)

1697 In utero pesticide exposure and childhood morbidity.

Author	Weselak, Mandy; Arbuckle, Tye E.; Wigle, Donald T.; Krewski, Daniel
Source	Environmental Research, (JAN 2007) Vol. 103, No. 1, pp. 79-86.
Title	In utero pesticide exposure and childhood morbidity.
Unique ID	1697

Short Summary with assessment and conclusion:

This was a retrospective cohort study conducted in Canada as part of the Ontario Farm Family Health Study (OFFHS). The study population comprised children of married couples, or couples living as married, who lived year-round on a farm with reported agricultural product sales of at least $50,000 in 1986. Wives were aged 44 years or younger. The health outcomes of interest were chronic bronchitis or cough, asthma, hayfever or allergies in children, and was captured via questionnaire; it was defined as self-reported physician diagnosis. The exposure of interest was reported farm use of glyphosate during pregnancy, and was self-reported via questionnaire. Data from 3,405 children were analyzed using logistic regression and generalized estimating equations. The reported adjusted ORs and 95% CIs for children with reported glyphosate use during pregnancy compared to those without was 0.71 (0.21, 2.35) for persistent cough or bronchitis, 0.82 (0.35, 1.90) for asthma, and 0.98 (0.46, 2.10) for hayfever or allergies. Analyses for persistent cough or bronchitis were adjusted for child's age at time of questionnaire, father's age at conception, income, breast feeding, and mother's age at conception. Analyses for asthma were adjusted for child's age at time of questionnaire, and father's education level. Analyses for hayfever or allergies were adjusted for child's age at time of questionnaire, father's age at conception, mother's age at conception, and father's education level. These results suggest that there is not a statistically significant difference in odds of persistent cough or bronchitis, asthma, or hayfever or allergies among exposure groups at the 0.05 significance level. This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because exposure classification was based on self-reported glyphosate use, an indirect method by which to estimate exposure status of study participants. No biological evidence is provided to support exposure classification of study participants. Additionally, outcome classification was based on self-report of physician diagnosis. Misclassification of either exposure, outcome, or both is possible in this study.

Short summary of relevant and reliable articles from category a) with / without restrictions)

2196 Pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis.

Author	Eriksson Mikael; Hardell Lennart; Carlberg Michael; Akerman Mans
Source	International journal of cancer, (2008 Oct 01) Vol. 123, No. 7, pp. 1657-63.
Title	Pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis.
Unique ID	2196

Short Summary with assessment and conclusion:

This was a case control study conducted in Sweden. The study population comprised consecutive patients aged 18-74 years with newly diagnosed Non-Hodgkin lymphoma (NHL), and population controls selected from the same health service region. The health outcome of interest was NHL and its subtypes; diagnosis was confirmed by a pathologist. The exposure of interest was self-reported exposure or non-exposure to glyphosate, and number of days exposed; it was self-reported via questionnaire and, in some cases, interview. Data from 910 NHL cases and 1,016 controls were analyzed using logistic regression. The reported OR and 95% CI for participants exposed to glyphosate compared to those not exposed was 2.02 (1.10, 3.71). The reported OR and 95% CI for participants with 10 or fewer exposure days compared to those not exposed was 1.69 (0.70, 4.07), and the reported OR and 95% CI for participants with more than 10 exposure days compared to those not exposed was 2.36 (1.04, 5.37). When comparing participants with self-reported exposure and those with self-reported non-exposure reported ORs and 95% CIs were 1.87 (0.998, 3.51) for B-cell lymphomas, 3.35 (1.42, 7.89) for lymphocytic lymphoma/B-CLL (SLL/CLL), 1.89 (0.62, 5.79) for follicular, grade I-III (FL), 1.22 (0.44, 3.35) for diffuse large B-cell lymphoma (DLBCL), 1.63 (0.53, 4.96) for other specified B-cell lymphoma, 1.47 (0.33, 6.61) for unspecified B-cell lymphoma, 2.29 (0.51, 10.4) for T-cell lymphomas, and 5.63 (1.44, 22.0) for unspecified non-Hodgkin lymphoma. Controls were frequency matched on 10 year age and sex; analyses were adjusted for year of diagnosis for cases and of enrollment in controls. These results suggest that there is a statistically significant difference in odds of glyphosate exposure between NHL and non-NHL cases at the 0.05 significance level. Results were statistically significant for participants with more than 10 exposure days, but not for those with 10 or fewer compared to those who were non-exposed. This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because exposure classification was based on self-reported glyphosate use, an indirect method by which to estimate exposure status of study participants. No biological evidence is provided to support exposure classification of study participants. Misclassification of exposure is possible in this study.

Short summary of relevant and reliable articles from category a) with / without restrictions)

2206 Pesticide use and colorectal cancer risk in the Agricultural Health Study.

Author	Lee, Won Jin; Sandler, Dale P.; Blair, Aaron; Samanic, Claudine; Cross, Amanda J.; Alavanja, Michael C. R.
Source	Int. J. Cancer, Volume 121, Issue 2, Page 339-346, Publication Year 2007
Title	Pesticide use and colorectal cancer risk in the Agricultural Health Study.
Unique ID	2206

Short Summary with assessment and conclusion:
This was a case-control study conducted in the Agricultural Health Study cohort in Iowa and North Carolina, USA. The study population comprised certified pesticide applicators and their spouses. The health outcome of interest was incident colorectal cancer, and was captured from a cancer registry. The exposure of interest was self-reported ever or never exposure to glyphosate, and was captured by questionnaire. Data for 305 colorectal cancer cases, and 56,508 non-cases were analyzed using unconditional multivariate logistic regression. The reported ORs and 95% CIs were 1.2 (0.9, 1.6) for colorectal cancer, 1.0 (0.7, 1.5) for colon cancer, and 1.6 (0.9, 2.9) for rectum cancer. Analyses were adjusted for age, state of residence, smoking history, and total pesticide application days for any pesticide. These results suggest that there is no statistically significant difference between odds of glyphosate exposure between cases and non-cases of colorectal cancer, colon cancer, or rectum cancer at the 0.05 significance level. This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because exposure classification was based on self-reported glyphosate use, an indirect method by which to estimate exposure status of study participants. No biological evidence is provided to support exposure classification of study participants. Misclassification of exposure is possible in this study.

Short summary of relevant and reliable articles from category a) with / without restrictions)

2209 Pesticides and adult respiratory outcomes in the agricultural health study.

Author	Hoppin, Jane A.; Umbach, David M.; London, Stephanie J.; Lynch, Charles F.; Alavanja, Michael C. R.; Sandler, Dale P.
Source	Ann. N. Y. Acad. Sci., Volume 1076, Issue Living in a Chemical World, Page 343-354, Publication Year 2006
Title	Pesticides and adult respiratory outcomes in the agricultural health study.
Unique ID	2209

Short Summary with assessment and conclusion:
This was a cross-sectional study conducted in the Agricultural Health Study cohort in Iowa and North Carolina, USA. The study population comprised private and commercial pesticide applicators and their spouses. The health outcome of interest was self-reported wheeze, and was captured by self-administered questionnaire; it was defined as having had at least one reported episode of wheezing or whistling in the chest in the past 12 months. The exposure of interest was self-reported glyphosate use in the year before enrollment, and was captured by self-administered questionnaire. Data from 17,920 farmers and 2,255 commercial applicators were analyzed using logistic regression. Reported ORs and 95% CIs comparing participants with self-reported glyphosate use and those without were 1.05 (0.94, 1.17) among farmers, and 1.14 (0.83, 1.57) among commercial applicators. Analyses were adjusted for age, BMI, smoking, asthma/atopy, and previous use of pesticide. Analyses among commercial applicators also adjusted for chlorimuron-ethyl, and those among farmers also adjusted for state. These results suggest that there is no statistically significant difference in odds of self-reported glyphosate use and wheeze among either farmers or commercial applicators at the 0.05 significance level. This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because exposure classification was based on self-reported glyphosate use, an indirect method by which to estimate exposure status of study participants. No biological evidence is provided to support exposure classification of study participants. Additionally, outcome classification was based on self-report. Misclassification of either exposure, outcome, or both is possible in this study.

グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2006 年 7 月 1 日～2009 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

Short summary of relevant and reliable articles from category a) with / without restrictions)

100 Acute and chronic toxicity of glyphosate compounds to glochidia and juveniles of Lampsilis siliquoidea (Unionidae).

Author	Bringolf Robert B; Cope W Gregory; Mosher Shad; Barnhart M Chris; Shea Damian
Source	Environmental toxicology and chemistry, (2007 Oct) Vol. 26, No. 10, pp. 2094-100.
Title	Acute and chronic toxicity of glyphosate compounds to glochidia and juveniles of Lampsilis siliquoidea (Unionidae).
Unique ID	100

Short Summary with assessment and conclusion:
The article describes acute and chronic toxicity tests with different forms of glyphosate and different glyphosate formulations on juveniles and glochidia of the freshwater mussel Lampsilis siliquoidea. Acute 48 EC50 values for the different glyphosate forms (4.6 - 200 mg/L) and glyphosate formulations (2.9 - 148 mg/L) are reported, which are partially considered relevant for risk assessment. As no raw data are provided, the test doses used for the acute test are not reported and the analytical methods are not clearly described, the article is regarded reliable with restrictions.

Short summary of relevant and reliable articles from category a) with / without restrictions)

413 Combination effects of herbicides on plants and algae: do species and test systems matter?

Author	Cedergreen, Nina; Kudsk, Per; Mathiassen, Solvejg K.; Streibig, Jens C.
Source	Pest Management Science (2007), 63(3), 282-295
Title	Combination effects of herbicides on plants and algae: do species and test systems matter?
Unique ID	413

Short Summary with assessment and conclusion:

In this study, the toxicity of technical grade glyphosate (95%) on the terrestrail plants Tripleurospermum inodorum (ED50 = 18 ± 1 g a.s./ha) and Stellaria media (ED50 = 16 ± 3 g a.s./ha), the aquatic plant Lemna minor (EC50 = 17.2 ± 1.1 mg a.s./ha) and the alga Pseudokirchneriella subcapitata (EC50 = 55.1 ± 2.5 mg a.s./ha) was calculated. As no analytical verifications were conducted for any of the tests with either terrestrial or aquatic species, some important methodological details are insufficiently reported and no raw data are available to verify ED/EC50 values, the article is considered reliable with restrictions.

Short summary of relevant and reliable articles from category a) with / without restrictions)

834 Effect of glyphosate on growth of four freshwater species of phytoplankton: a microplate bioassay.

Author	Vendrell E; Ferraz D Gomez De Barreda; Sabater C; Carrasco J M
Source	Bulletin of environmental contamination and toxicology, (2009 May) Vol. 82, No. 5, pp. 538-42. Electronic Publication Date: 6 Mar 2009
Title	Effect of glyphosate on growth of four freshwater species of phytoplankton: a microplate bioassay.
Unique ID	834

Short Summary with assessment and conclusion:
In this article the effect of technical glyphosate (97.5%) on the growth of four freshwater algae species was tested in a 72 hour exposure test. EC50 values based on area under the growth curve are reported, which ranged between 24.5 and 41.7 mg/L. As the test concentrations were not analytically verified and no raw data are presented, the article is considered reliable with restrictions.

Short summary of relevant and reliable articles from category a) with / without restrictions)

1124 Evaluating the Effects of Forestry Herbicides on Fish Development Using Rapid Phenotypic Screens

Author	Stehr, Carla M; Linbo, Tiffany L; Baldwin, David H; Scholz, Nathaniel L; Incardona, John P
Source	North American Journal of Fisheries Management [N. Am. J. Fish. Manage.]. Vol. 29, no. 4, pp. 975-984. Aug 2009. ISSN: 0275-5947 E-ISSN: 1548-8675 DOI: 10.1577/M08-173.1 Published by: American Fisheries Society, 5410 Grosvenor Ln. Bethesda MD 20814-2199 U
Title	Evaluating the Effects of Forestry Herbicides on Fish Development Using Rapid Phenotypic Screens
Unique ID	1124

Short Summary with assessment and conclusion:
In this article the developmental effects (anatomy, morphology, body length, touch response) of glyphosate on zebra fish embryos after 5 days of exposure were assessed. No significant effects were observed at the highest test concentration of 100 mg/L. As the test concentrations were not analytically verified and the raw data are not presented, the article is considered reliable with restrictions.

Short summary of relevant and reliable articles from category a) with / without restrictions)

3012 Toxicity and effects of a glyphosate -based herbicide on the Neotropical fish Prochilodus lineatus.

Author	Langiano Vivian Do Carmo; Martinez Claudia B R
Source	Comparative biochemistry and physiology. Toxicology and pharmacology : CBP, (2008 Mar) Vol. 147, No. 2, pp. 222-31. Electronic Publication Date: 26 Sep 2007
Title	Toxicity and effects of a glyphosate -based herbicide on the Neotropical fish Prochilodus lineatus.
Unique ID	3012

Short Summary with assessment and conclusion:
In this article acute and sub-lethal endpoints for the neotropical fish Prochilodus lineatus after exposure to the glyphosate formulation Roundup® are reported. The determined 96 h LC50 of 13.69 mg/L is considered relevant for risk assessment. As the test concentrations were not analytically verified, no raw data are presented and no confidence intervals are given for the 96 h LC50, the article is considered as reliable with restrictions (NOTE: probably not relevant because of the formulation used).

Short summary of relevant and reliable articles from category a) with / without restrictions)

3016 Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient.

Author	Pereira Joana L; Antunes Sara C; Castro Bruno B; Marques Catarina R; Goncalves Ana M M; Goncalves Fernando; Pereira Ruth
Source	Ecotoxicology (London, England), (2009 May) Vol. 18, No. 4, pp. 455-63. Electronic Publication Date: 10 Feb 2009
Title	Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient.
Unique ID	3016

Short Summary with assessment and conclusion:
In this article, acute endpoints for Daphnia magna and the green algae Pseudokirchneriella subcapitata after exposure to the glyphosate formulation Spasor® or the active substance glyphosate are reported, i.e. 307 mg/L (> 2000 mg/L) for Daphnia magna and 71 mg/L (129 mg/L) for P. subcapitata. As the test concentrations were not analytically verified and no raw data are presented, the article is considered reliable with restrictions.

Short summary of relevant and reliable articles from category a) with / without restrictions)

3084 Understanding the net effects of pesticides on amphibian trematode infections.

Author	Rohr Jason R; Raffel Thomas R; Sessions Stanley K; Hudson Peter J
Source	Ecological applications : a publication of the Ecological Society of America, (2008 Oct) Vol. 18, No. 7, pp. 1743-53.
Title	Understanding the net effects of pesticides on amphibian trematode infections.
Unique ID	3084

Short Summary with assessment and conclusion:

This study showed no significant effects of technical grade glyphosate (98%) on all the species involved in an amphibian trematode infection (i.e. the trematode Echinostoma trivolvis and its first and second intermediate hosts, the snail Planorbella trivolvis and tadpoles of the green frog Rana clamitans) when applied at 3.7 mg/L separatedly. A general NOEC of 3.7 mg/L can therefore be established. As some general methodological information is lacking and the tested individuals for all species were taken from natural sources with unknown previous history of chemical exposure/applications, which could make up the glyphosate effects, the article is considered reliable with restrictions.

グリホサートカリウム塩

要旨及び評価結果

（ヒトに対する毒性）

検索期間：2010 年 1 月 1 日～2019 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 5.4
Report author	Adler-Flindt S. *et al.*
Report year	2019
Report title	Comparative cytotoxicity of plant protection products and their active ingredients
Document No	Toxicology in Vitro (2019) Vol. 54, 354-366
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study PPPs for the correlation of GHS classifications was studies resulting mainly from in vivo LD50-values with classifications obtained from calculated LD50-values using the CM. Accordingly, the CM predicted 80% of the PPPs correctly. However 31% of classified products were not identified revealing a considerable inaccuracy of this method. Based on these results ten PPPs and corresponding ASs were further tested in a cytotoxicity assay employing 3T3 and hFF cells (one PPP and corresponding AS were tested in HepaRG cells).

Materials and methods

Chemicals - Glyphosate isopropylamine salt (MON 0138) and RoundUP LB Plus (360 g/L A.I., MON 52276) was purchased from Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany. Glyphosate was received dissolved in water at a concentration of 620 g/L.

Culture of Balb/3T3 cells, hFF cells and HepaRG cells – Mouse fibroblast cells (Balb/3T3) and human foreskin fibroblast cells (hFF) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 50 U/mL penicillin/streptomycin, and passaged every 3 to 4 days at a split-ratio of approximately 1:20 for 3T3 cells and 1:8 for hFF cells after enzymatic dissociation with trypsin-EDTA. Differentiated HepaRG cells were received in 96-well plates and were grown for 2 weeks in Williams's medium containing 10% foetal calf serum (FCS), 100 U/mL penicillin/streptomycin, 0.05% human insulin and 50 µM hydrocortisone hemisuccinate. For differentiation, cells were incubated for a another 2 weeks in differentiation medium containing in addition to the above components 1.7% DMSO.

Proliferation test - To determine the optimal seeding density for the 48-hour toxicity assay, proliferation tests were performed in 2 independent runs. 3T3 and hFF cells were seeded in test medium (DMEM) supplemented with 10% (v/v) Panexin NTA serum substitute and 50 U/mL penicillin/streptomycin, into 96-well plates, in a 2-fold dilution series, with the highest cell density being 1.6×10^4 cells/well. After 48 hours the viability of the cells was assessed by measuring the reduction of resazurin to the fluorescent resorufin. The fluorescence signal was measured at 530 nm (excitation) and at 590 nm (emission) using a multimode plate reader.

Cytotoxicity test - 3T3 and hFF cells were dissociated into single cells and seeded into 96-well plates in 100 µL per well of DMEM supplemented with 10% (v/v) Panexin NTA serum substitute and 50 U/mL penicillin/streptomycin, at a density of 2,000 cells per well for 3T3 cells and 4,000 cells per well for hFF cells. Differentiated HepaRG cells were received in 96-well plates already seeded in a density of 9,000 cells per well. The test medium for HepaRG cells was based on phenol-red-free Williams's medium containing 2% foetal calf serum (FCS), 100 U/mL penicillin/streptomycin, 0.05% human

insulin and 50 µM hydrocortisone hemisuccinate. After 24 hours, 100 µL of test medium (containing the double required final concentration of the test substance) were added to each well (day 0). After 48 hours cell viability was assessed by measuring the reduction of resazurin to the fluorescent resorufin. The fluorescence signal was measured at 530 nm (excitation) and at 590 nm (emission) using a multimode plate reader.

Testing of glyphosate and MON 52276 - At least two independent runs of each experiment were performed. The highest tested concentration for glyphosate isopropylamine salt was 1000 µg/mL.

Results

Proliferation assay - Proliferation tests were performed to determine the optimal seeding density for the 48-hour toxicity assay. The cells were seeded in a 2-fold dilution series into 96-well plates, and reduction of resazurin into the fluorescent resorufin was measured after 48 hours. The proliferation assay revealed an optimal seeding concentration for the cytotoxicity test of 2,000 cells per well for 3T3 cells and 4,000 cells per well for hFF cells.

Testing of glyphosate and MON 52276 - Two controls, saccharin as the negative control and 5-FU as the positive control, were tested for 48 hours on 3T3 and hFF cells. The treatment of both cell types with saccharin did not significantly reduce cell viability up to a concentration of 1,000 µg/mL. In contrast, the treatment with 5-FU resulted in a noticeable reduction of viability, with different IC_{50} values for the two cell types, i.e. 0.06 ± 0.01 µg/mL for 3T3 cells and 0.14 ± 0.05 µg/mL for hFF cells. The IC_{50} for glyphosate isopropylamine salt was 954.8 ± 117.1 µg/mL for 3T3 cells and 1211 ± 885.7 µg/mL for hFF cells. The IC_{50} for MON 52276 was 313.2 ± 29.3 µg/mL for 3T3 cells and 361.6 ± 612 µg/mL for hFF cells. The ratio of the AUC under the % viability vs concentration curve of glyphosate isopropylamine salt over MON 52276 is 1.7 for 3T3 cells and 1.3 for hFF cells. This indicates that the treatment of hFF cells with glyphosate and its formulation Roundup did not result in significant differences between cytotoxicity curves. The ratio of the AUCs of glyphosate over MON 52276 for both cell types was below a factor 2 and could thus be regarded as minor.

Conclusion

In this study, glyphosate isopropylamine salt, amongst other pesticides, and its corresponding formulation MON 52276 were tested for cytotoxicity in 3T3 cells and hFF cells. The IC_{50} for glyphosate isopropylamine salt was 954.8 ± 117.1 µg/mL for 3T3 cells and 1211 ± 885.7 µg/mL for hFF cells and the IC_{50} for MON 52276 was 313.2 ± 29.3 µg/mL for 3T3 cells and 361.6 ± 612 µg/mL for hFF cells. The ratio of the AUCs of glyphosate over MON 52276 for both cell types was below a factor 2 and could thus be regarded as minor.

3. Assessment and conclusion

Assessment and conclusion by applicant:

It was the intention of this study to evaluate the GHS classification of pesticide formulations for acute toxicity based on calculated LD_{50} values using the CLP calculation method (CM). Because of the considerable inaccuracy of this method the *in vitro* cytotoxicity of 10 pesticide formulations was compared against that of the active ingredient using mouse (3T3) and human (hFF) fibroblasts. In this exercise the IC_{50} for glyphosate isopropylamine salt was found to be 954.8 ± 117.1 µg/mL for 3T3 cells and 1211 ± 885.7 µg/mL for hFF cells and the IC_{50} for MON 52276 was 313.2 ± 29.3 µg/mL for 3T3 cells and 361.6 ± 612 µg/mL for hFF cells. The difference in cytotoxicity (expressed as the AUC of the % viability vs concentration curve) between glyphosate and MON 52276 could be regarded as minor.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate isopropylamine salt used was not sufficiently characterized and

the standard deviation of the IC_{50} of glyphosate (1211 ± 885.7 ug/mL) and MON 52276 (361.6 ± 612 µg/mL) for human fibroblasts is too large.

Reliability criteria for *in vitro* toxicology studies

Publication: Adler-Flindt *et al.*, 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Glyphosate isopropylamine salt, purity not reported. Source: Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany.
Only glyphosate acid or one of its salts is the tested substance	N	MON 52276 (RoundUP LB Plus, 360 g/L A.I.). Source : Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany. Other pesticides and their formulations were tested as well.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Mouse (3T3) and human (hFF) fibroblasts.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y (for local contact)	Test concentrations up to 1000 µg/mL which is beyond the systemic physiological range but not when applied dermally.
Cytotoxicity tests reported	Y	
Positive and negative controls	N	Saccharin was used as the negative control and 5-FU as the positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	IC_{50} were calculated.

Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate isopropylamine salt used was not sufficiently characterized and the standard deviation of the IC_{50} of glyphosate (1211 ± 885.7 ug/mL) and MON 52276 (361.6 ± 612 µg/mL) for human fibroblasts is too large.		

1. Information on the study

Data point:	CA 5.5
Report author	Andreotti, G. et al.
Report year	2018
Report title	Glyphosate Use and Cancer Incidence in the Agricultural Health Study
Document No	Journal of the National Cancer Institute (2018), Vol. 110, No. 5, pp. 509-516
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable/Reliable with restrictions

2. Full summary of the study according to OECD format

In 2005, an evaluation of glyphosate and cancer risk was conducted in the Agricultural Health Study (AHS) (DeRoos et al. 2005). This evaluation considered glyphosate use reported at enrollment (1993–1997) and included 2,088 cancers diagnosed between enrollment and 2001. No statistically significant associations were found for any cancer site. For NHL, the relative risk (RR) adjusted for age, personal factors and other pesticides was 1.1 (95% CI 0.7-1.9) and there was no trend of increasing RRs with increasing frequency of glyphosate. For multiple myeloma, the overall adjusted RR was 2.6 (95% CI 0.7 – 9.4) and the assessment of RR by frequency of glyphosate use did not evidence a significant trend. Andreotti et al. 2018 updated the 2005 AHS publication by DeRoos et al. (2005), extending cancer incidence follow-up through 2012 in North Carolina and 2013 in Iowa and incorporating additional exposure information from a follow-up questionnaire. The authors also dealt with missing information through imputation and conducted sensitivity analyses to address the potential for various types of bias in their primary analyses. This 2018 publication includes a total of 7,290 cancers, 3.6 times as many as in the earlier publication. The median lifetime days of glyphosate use for cohort members who reported glyphosate use (83% of the cohort) was 48 days (interquartile range (IQR) 20 to 166 days). The authors found no evidence of an association between glyphosate use and risk of any solid tumour, NHL (RR 0.87 (95% CI 0.64, 1.20 in the highest intensity weighted exposure quartile, p_{trend} 0.95), or multiple myeloma (RR 0.87, 95% CI 0.45, 1.69 highest quartile, , p_{trend} 0.84). They found a moderately elevated RR for acute myelogenous leukaemia that was not statistically significant (RR 2.44, 95% CI 0.94, 6.32, p_{trend} = 011). The findings for cancer types were consistent across different exposure metrics, in various sensitivity analyses, and for lagged exposure analyses meant to address cancer induction-latency.

Materials and methods

Study design

Briefly, 57 310 individuals seeking licenses to apply restricted-use pesticides were enrolled between 1993 and 1997. Of the enrolled participants, 63% completed a follow-up phone interview approximately five years after enrollment (1999–2005). Incident cancer diagnoses were ascertained via linkage to cancer registries in Iowa (through 2013) and North Carolina (through 2012). Cancer diagnoses were classified according the International Classification of Disease– Oncology, 3rd Revision. Subtypes of lymphoid malignancies were defined according to the Surveillance, Epidemiology, and End Results Program Lymphoma Subtype Recodes. According to this updated classification of lymphoid

malignancies, multiple myeloma was included in the analyses as a subtype of non-Hodgkin's lymphoma. Vital status was ascertained via state mortality registries and the National Death Index, and state of residence was regularly updated using various government databases.

Exposure assessment

Lifetime use of glyphosate and 49 other pesticides was ascertained at enrollment and in the follow-up questionnaire. At enrollment, applicators reported the number of years and days per year each pesticide was used, while at follow-up applicators reported the number of days each pesticide was used in the most recent year farmed. Using this information, three metrics of cumulative lifetime exposure were created for each pesticide: ever/never use, lifetime days of use (days per year multiplied by the number of years), and intensity-weighted lifetime days (lifetime days multiplied by an intensity score). The intensity score was derived from an algorithm based on literature-based measurements and information provided by the applicator, specifically whether the participant mixed or applied pesticides, repaired pesticide-related equipment, used personal protective equipment, and application method used. For participants who did not complete the follow-up questionnaire (37%), multiple imputation was used to impute pesticide use since enrollment. Factors used to impute pesticide use included demographic data and medical history, as well as factors related to farm characteristics and reported pesticide use at enrollment.

Statistical analysis

For this analysis, individuals who had a history of cancer at enrollment (n=1096), did not live in North Carolina or Iowa (n=343), or did not report whether they had used glyphosate or not at enrollment (n=1620) were excluded, resulting in an analytic sample of 54,251 licensed farmers and applicators. Individuals accumulated person-time from enrollment until the earliest of the following events: movement out of state, diagnosis of cancer, death, or end of the follow-up period (December 31, 2012 in NC, December 31, 2013 in IA). Poisson regression was used to calculate incidence rate ratios (RRs) and 95% confidence intervals (CIs), and PROC MIANALYZE was used to obtain the appropriate variance for the imputed data. All statistical significance tests were two-sided and considered to be statistically significant when $p \leq 0.05$. RRs for total cancer and for cancer sites with at least 20 exposed cases were evaluated. For analyses by exposure level, based on the distribution among all cancer cases, cumulative lifetime days and intensity-weighted lifetime days of glyphosate exposure into quartiles, tertiles, or the median were categorized, such that there were at least five exposed cases in each category. Linear trend was evaluated according to the Wald test using the median of each exposure category as a continuous variable. Risk estimates were adjusted for attained age (continuous), cigarette smoking status (never, former, current), alcohol drinks per month (none, ≤ 6 per month, ≥ 7 per month), family history of any cancer (yes, no), state of recruitment (North Carolina, Iowa), and the five pesticides most highly correlated with glyphosate based on lifetime days and intensity-weighted lifetime days ($r > 0.4$: atrazine, alachlor, metolachlor, trifluralin, 2,4-D). Lagged exposure was also evaluated allowing for 5, 10, 15, or 20 years to address the induction-latency period for specific cancers. Other potential confounding factors were calculated, including body mass index (BMI; <25, 25–<30, _30 kg/m^2) and pack-years of cigarettes smoked (tertiles of use among former and current smokers). The numbers of women and nonwhites were small, precluding adjustment for sex and race for most cancer sites; in sensitivity analyses, the risks in men and whites alone were assessed. For lymphohematopoietic cancers, RRs were additionally adjusted for occupational exposure to solvents, gasoline, x-ray radiation, and engine exhaust, and pesticides linked to lymphohematopoietic malignancies in previous AHS analyses (lindane, DDT, diazinon, terbufos, and permethrin). The risk of NHL excluding multiple myeloma was calculated for comparison

with previously published studies. Lastly, sensitivity analyses were conducted to evaluate the impact of including additional exposure information.

Results

Among 54 251 participants, 44,932 (82.8%) reported ever using glyphosate at enrollment or during follow-up. Among the participants who used glyphosate, the median lifetime days of use was 48 (interquartile range [IQR] = 20–166 days), and the median lifetime years of use was 8.5 years (IQR = 5–14 years). A total of 7,290 incident cancers were diagnosed during the follow-up period. Among the participants who used glyphosate and were diagnosed with cancer during follow-up (n=5,779), the median lifetime days of use was 38.75 (IQR = 13.75–108.5 days), and the median lifetime years of use was 8.0 (IQR = 3.5–13.0). Selected characteristics of the study participants by glyphosate use are presented in Table 1. Risk ratios for intensity-weighted lifetime days of glyphosate use and cancer risk are shown in Table 2. Glyphosate use was not associated with total cancer or with lymphohematopoietic malignancies. There also was no evidence for positive associations with NHL (RR in the highest intensity weighted days of glyphosate use quartile = 0.9 (95% CI 0.6 – 1.2)), multiple myeloma ($RR_{quartile\ 4}$ = 0.9, 95% CI 0.5 – 1.7) or for any NHL subtype. Although not statistically significant, the authors observed an elevated RR for acute myeloid leukemia (AML; n = 57 exposed cases) among applicators in the highest quartile of intensity weighted glyphosate use compared with never users (n = 18 cases, RR.2.4, 95% CI = 0.9 to 6.3, P_{trend} = .11). The results based on intensity weighted days of use were very similar to results based on unweighted days of use.

The impact of lagging exposure on risk estimates for lymphohematopoietic cancers was evaluated for intervals of 5, 10, 15 and 20 years. The patterns of risk for lagged exposures were similar to those for unlagged exposures.

Conclusion

In conclusion, the authors found no evidence of an association between glyphosate use and risk of any solid tumor, NHL, or multiple myeloma. They found an elevated RR for AML that was not statistically significant, but that merits evaluation in AHS updates or other studies. This findings across cancer types were consistent across different exposure metrics, in various sensitivity analyses, and for lagged exposure analyses meant to address cancer induction-latency.

Table 1. Selected characteristics of the Agricultural Health Study population by glyphosate use

Characteristics*	Never-used glyphosate No. (%)	Lifetime days of glyphosate use†	
		< Median No. (%)	≥ Median No. (%)
Total	9319 (100.0)	19 714 (100.0)	24 727 (100.0)
Age at enrollment, y			
<30	814 (8.7)	1726 (8.8)	2372 (9.6)
30–39	1730 (18.6)	4293 (21.8)	6612 (26.7)
40–49	2217 (23.8)	5304 (26.9)	7437 (30.1)
50–59	2051 (22.0)	4261 (21.6)	4759 (19.2)
60–69	1797 (19.3)	3043 (15.4)	2738 (11.1)
70+	710 (7.6)	1087 (5.5)	809 (3.3)
Sex			
Male	8887 (95.4)	19 220 (97.5)	24 203 (97.9)
Female	432 (4.6)	494 (2.5)	524 (2.1)
Race			
White	8838 (94.8)	19 128 (97.0)	24 267 (98.1)
Black and other	441 (4.7)	538 (2.7)	404 (1.6)
Missing	40 (0.4)	48 (0.2)	56 (0.2)
State of recruitment			
Iowa	6692 (71.8)	12 668 (64.3)	15 756 (63.7)
North Carolina	2627 (28.2)	7046 (35.7)	8971 (36.3)
Applicator type			
Private (farmer)	8476 (91.0)	18 717 (94.9)	21 932 (88.7)
Commercial	843 (9.0)	997 (5.1)	2795 (11.3)
Highest level of education			
High school or less	6528 (70.1)	11 409 (57.9)	12 005 (48.6)
Beyond high school	2569 (27.6)	7884 (40.0)	12 213 (49.2)
Missing	222 (2.4)	421 (2.1)	509 (2.1)
Body mass index, kg/m^2			
<25	1656 (17.8)	3779 (19.2)	4168 (16.9)
25–<30	3044 (32.7)	7123 (36.1)	8492 (34.3)
30+	1435 (15.4)	3175 (16.1)	3985 (16.1)
Missing	3184 (34.2)	5637 (28.6)	8082 (32.7)
Cigarette smoking status			
Never	4987 (53.5)	10 371 (52.6)	12 876 (52.1)
Former	2621 (28.1)	6004 (30.5)	7295 (29.5)
Current	1526 (16.4)	3147 (16.0)	4355 (17.6)
Missing	185 (2.0)	192 (1.0)	201 (0.8)
Cigarette smoking pack-years			
Never	4987 (53.5)	10 371 (52.6)	12 876 (52.1)
Former, tertile 1	896 (9.6)	2004 (10.2)	2471 (10.0)
Former, tertile 2	791 (8.5)	1865 (9.5)	2198 (8.9)
Former, tertile 3	741 (8.0)	1748 (8.9)	2109 (8.5)
Current, tertile 1	548 (5.9)	1037 (5.3)	1513 (6.1)
Current, tertile 2	453 (4.9)	975 (4.9)	1399 (5.7)
Current, tertile 3	461 (4.9)	1076 (5.5)	1376 (5.6)
Missing	442 (4.7)	638 (3.2)	785 (3.2)
Usual number of alcohol drinks per month in year prior enrollment			
Never	3150 (33.8)	6406 (32.5)	6946 (28.1)
≤6/mo	3036 (32.6)	6646 (33.7)	8240 (33.3)
≥7/mo	2492 (26.7)	5631 (28.6)	8646 (35.0)
Missing	641 (6.9)	1030 (5.2)	895 (3.6)
Family history of cancer			
No	5452 (58.5)	10 846 (55.0)	13 866 (56.1)
Yes	3226 (34.6)	7700 (39.1)	9876 (39.9)
Missing	641 (6.9)	1168 (5.9)	985 (4.0)

*Data from the enrollment questionnaire.
†Based on median cumulative lifetime days of glyphosate use among all cancer cases (38.75 days)

Table 2. Cancer incidence in relation to intensity-weighted lifetime days of glyphosate use in the Agricultural Health Study

Cancer site*	Glyphosate use†	No.	RR (95% CI)‡	P_{trend}‡
All cancers				
	None	1511	1.00 (reference)	
	Q1	1445	0.99 (0.91 to 1.07)	
	Q2	1443	0.99 (0.91 to 1.07)	
	Q3	1440	1.04 (0.96 to 1.13)	
	Q4	1451	0.99 (0.91 to 1.08)	.91
Oral cavity				
	None	33	1.00 (reference)	
	Q1	36	0.95 (0.56 to 1.60)	
	Q2	35	0.92 (0.54 to 1.57)	
	Q3	35	0.96 (0.56 to 1.65)	
	Q4	35	0.84 (0.48 to 1.46)	.54
Colon				
	None	116	1.00 (reference)	
	Q1	104	1.00 (0.74 to 1.35)	
	Q2	102	1.03 (0.76 to 1.39)	
	Q3	102	1.06 (0.78 to 1.44)	
	Q4	96	1.01 (0.74 to 1.38)	1.00
Rectum				
	None	50	1.00 (reference)	
	Q1	43	0.81 (0.51 to 1.28)	
	Q2	55	1.16 (0.76 to 1.76)	
	Q3	39	0.80 (0.50 to 1.29)	
	Q4	46	0.84 (0.52 to 1.34)	.43
Pancreas				
	None	25	1.00 (reference)	
	Q1	42	1.80 (1.05 to 3.08)	
	Q2	42	1.69 (0.98 to 2.94)	
	Q3	24	1.09 (0.59 to 2.02)	
	Q4	23	1.06 (0.57 to 1.97)	.14
Lung				
	None	144	1.00 (reference)	
	Q1	117	0.92 (0.70 to 1.22)	
	Q2	138	1.19 (0.91 to 1.56)	
	Q3	159	1.39 (1.07 to 1.82)	
	Q4	131	1.00 (0.76 to 1.33)	.78
Melanoma				
	None	56	1.00 (reference)	
	Q1	59	1.00 (0.67 to 1.50)	
	Q2	67	1.18 (0.80 to 1.74)	
	Q3	69	1.12 (0.75 to 1.67)	
	Q4	78	1.17 (0.78 to 1.74)	.53
Prostate				
	None	579	1.00 (reference)	
	Q1	571	0.99 (0.87 to 1.12)	
	Q2	564	0.95 (0.83 to 1.08)	
	Q3	559	1.03 (0.91 to 1.18)	
	Q4	571	0.99 (0.86 to 1.13)	.89
Testicular				
	None	7	1.00 (reference)	
	T1	17	1.28 (0.49 to 3.34)	
	T2	12	0.74 (0.26 to 2.09)	
	T3	11	0.57 (0.20 to 1.67)	.07
Bladder				
	None	66	1.00 (reference)	
	Q1	86	1.29 (0.91 to 1.82)	
	Q2	68	1.04 (0.72 to 1.51)	
	Q3	66	1.09 (0.75 to 1.59)	
	Q4	79	1.26 (0.87 to 1.82)	.42

(continued)

Table 2. (continued)

Cancer site*	Glyphosate use†	No.	RR (95% CI)‡	P_{trend}‡
Kidney				
	None	54	1.00 (reference)	
	Q1	54	1.13 (0.74 to 1.71)	
	Q2	50	0.91 (0.59 to 1.41)	
	Q3	45	0.87 (0.55 to 1.38)	
	Q4	53	1.03 (0.66 to 1.61)	.95
Lymphohematopoietic				
	None	161	1.00 (reference)	
	Q1	136	0.87 (0.64 to 1.19)	
	Q2	126	0.88 (0.66 to 1.17)	
	Q3	137	0.93 (0.71 to 1.23)	
	Q4	144	1.00 (0.74 to 1.34)	.43
Hodgkin lymphoma				
	None	7	1.00 (reference)	
	M1	7	0.59 (0.17 to 2.11)	
	M2	11	0.90 (0.25 to 3.24)	.94
Non-Hodgkin lymphoma				
	None	135	1.00 (reference)	
	Q1	113	0.83 (0.59 to 1.18)	
	Q2	104	0.83 (0.61 to 1.12)	
	Q3	112	0.88 (0.65 to 1.19)	
	Q4	111	0.87 (0.64 to 1.20)	.95
Non-Hodgkin lymphoma B cell				
	None	128	1.00 (reference)	
	Q1	102	0.79 (0.55 to 1.13)	
	Q2	93	0.76 (0.56 to 1.05)	
	Q3	106	0.88 (0.64 to 1.21)	
	Q4	103	0.86 (0.62 to 1.19)	.86
Chronic lymphocytic lymphoma, small lymphocytic leukemia				
	None	36	1.00 (reference)	
	Q1	28	0.75 (0.40 to 1.41)	
	Q2	26	0.76 (0.41 to 1.41)	
	Q3	26	0.90 (0.50 to 1.62)	
	Q4	27	0.87 (0.48 to 1.58)	.71
Diffuse large B cell lymphoma				
	None	27	1.00 (reference)	
	Q1	28	1.11 (0.60 to 2.07)	
	Q2	23	0.94 (0.49 to 1.80)	
	Q3	30	1.13 (0.59 to 2.17)	
	Q4	22	0.97 (0.51 to 1.85)	.83
Marginal-zone lymphoma				
	None	4	1.00 (reference)	
	M1	6	0.39 (0.06 to 2.45)	
	M2	5	0.44 (0.09 to 2.17)	.67
Follicular lymphoma				
	None	16	1.00 (reference)	
	T1	21	0.89 (0.37 to 2.15)	
	T2	11	0.61 (0.23 to 1.60)	
	T3	20	0.85 (0.36 to 2.03)	.95
Multiple myeloma				
	None	30	1.00 (reference)	
	Q1	19	0.70 (0.36 to 1.36)	
	Q2	26	0.94 (0.50 to 1.76)	
	Q3	19	0.78 (0.39 to 1.56)	
	Q4	24	0.87 (0.45 to 1.69)	.84
Non-Hodgkin lymphoma T cell				
	None	2	1.00 (reference)	
	M1	14	4.25 (0.73 to 24.64)	
	M2	6	1.53 (0.23 to 10.38)	.31

(continued)

Table 2. (continued)

Cancer site*	Glyphosate use†	No.	RR (95% CI)‡	P_{trend}‡
Acute myeloid leukemia				
	None	9	1.00 (reference)	
	Q1	13	1.62 (0.60 to 4.38)	
	Q2	14	1.70 (0.61 to 4.73)	
	Q3	12	1.46 (0.49 to 4.37)	
	Q4	18	2.44 (0.94 to 6.32)	.11
Chronic myeloid leukemia				
	None	7	1.00 (reference)	
	M1	5	0.36 (0.09 to 1.43)	
	M2	11	0.82 (0.23 to 2.98)	.36

*Cancer sites are based and presented in order of Surveillance, Epidemiology, and End Results Site Recode ICD-O-3. CI = confidence interval; RR = rate ratio.
†Quartiles: Q1: 1–598.9; Q2: 599–1649.9; Q3: 1650–4339.9; Q4: ≥4340.0. Tertiles: T1: 1–866.24; T2: 866.25–2963.9; T3: ≥2964.0. Median: M1: 1–1649.9; M2: ≥1650.0.
‡Poisson regression was used to model rate ratios and confidence intervals, and P values were calculated using a two-sided Wald test. All models adjusted for age, state of recruitment, education, cigarette smoking status, alcohol per month, family history of cancer, atrazine, alachlor, metolachlor, trifluralin, 2,4-D.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The AHS is an ongoing prospective cohort study of glyphosate and other pesticides. It was initiated in 1993 and has been ongoing for more than 25 years. Researchers from the US National Cancer Institute and other government agencies initiated the AHS as a prospective cohort study to eliminate the possibility of case-recall bias – an intractable potential bias in case control studies that rely on self-reported exposure information. Crump (Risk Analysis DOI:10.1111/risa.13440) has recently illustrated that the results from the glyphosate case-control studies align closely with what would be expected from case recall bias.

In addition to obviating concerns about case-recall bias, the Andreotti et al. publication is noteworthy on several counts. First, the frequency of glyphosate use by participants (median = 48 days, IQR 20 to 166 days) vastly exceeds that in the glyphosate case-control studies. In those studies the most frequent days of use category is > 10 days (Eriksson M, et al. Int J Cancer. 2008; 123:1657-1663), while most of the case control studies' primary analyses were based on 1 day or more of use in a lifetime. Second, the participants in the AHS were licensed pesticide applicators who were considered by the authors to be very capable to report pesticide use accurately compared with other study populations. Third, the analyses by Andreotti et al. controlled for a multitude of personal factors and for other pesticides in addition to incorporating a wide range of sensitivity and lagged analyses (allowing for up to 20+ years induction-latency). No other study has evaluated the relationship between glyphosate use and cancers as extensively. The AHS is, by far, the most informative and relevant study epidemiologic study for glyphosate to date.

Accordingly, given the AHS results for NHL among those with extensive glyphosate use (n = 111 exposed cases, RR = 0.9, 95% CI 0.6 – 1.2), it is unlikely that the positive associations for glyphosate and NHL in some case control studies are valid. As follow-up of the AHS cohort continues, it remains to be seen whether subsequent results will identify relationships between individual cancers and glyphosate use that are relevant for risk evaluations.

Reliability Criteria: Epidemiology studies

Publication: Andreotti G. *et al.,* 2018	**Criteria met? Y/N/?**	**Comments**
Study Design		
Adequate study design given study objectives	Yes	
Appropriate study population to address potential glyphosate-related health outcomes	Yes	Most appropriate population studied to date. Highest frequency of glyphosate use by far.
Exposure studied		
Exposure to formulations with glyphosate as a.i.	Yes	
Exposure to formulations with other a.i.	Yes	
Exposure to other farm exposures	Yes	
Study Conduct/analysis		
Adequate description of study population	Yes	

Publication: Andreotti G. *et al.,* 2018	**Criteria met? Y/N/?**	**Comments**
Adequate description of exposure circumstances	Yes	
Comparable participation by groups being compared	Yes	
Information provided by proxy respondents	No	
Adequate statistical analysis	Yes	Very comprehensive
Adequate consideration of personal confounding factors	Yes	Very comprehensive
Adequate consideration of potentially confounding exposures	Yes	Very comprehensive
Overall assessment		
Reliable without restrictions	Yes	Most reliable epidemiology study for glyphosate users versus non-users.
Reliable with restrictions	Yes	Certain analyses are limited: dose is not known, only frequency of use. So, “dose response” analyses must be interpreted cautiously.
Not reliable	No	

1. Information on the study

Data point:	CA 5.5
Report author	Biserni M. *et al.*
Report year	2019
Report title	Quizalofop-p-ethyl induces adipogenesis in 3T3-L1 Adipocytes
Document No	Toxicological Sciences (2019), Vol. 170(2), 452–461
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

In this study glyphosate, among other pesticide active ingredients, was investigated for its effect on lipid accumulation in differentiated adipocytes *in vitro* at concentrations ranging from 0.1 to 1000 µM. The results indicated that at the concentrations tested glyphosate scored negative for lipid accumulation.

Materials and methods

Chemicals – Glyphosate (purity ≥ 96%) purchased from Sigma-Aldrich, Gillingham, UK. Stock solutions of glyphosate were prepared in serum-free medium and adjusted to pH 7.2.

Cell culture - The murine fibroblast 3T3-L1 cell line was purchased from ZenBio (Cambridge Bioscience, Cambridge, UK) and was not used past passage 10. Undifferentiated 3T3-L1 cells were grown at 37°C under 5% CO_2 in a maintenance medium composed of phenol red free Dulbecco's Modified Eagle Medium (DMEM), 10% newborn calf serum, 2 mM glutamine and 10 µg/mL penicillin/streptomycin. Cells were released from the flask substratum using 0.05% trypsin-EDTA and counted using a hemocytometer prior to seeding.

Adipocyte differentiation - For the differentiation of murine 3T3-L1 cell cultures to adipocytes, cells were seeded into 96-well plates at a density of 20,000 cells per well in 100 µL maintenance medium. Following a 2-day stabilization period, cells were switched to differentiation medium consisting of DMEM, 2 mM glutamine, 10 µg/mL penicillin/streptomycin, 10% fetal bovine calf serum, 500 µM 3-isobutyl-1-methyl-xanthine and 100 nM insulin from bovine pancreas. After a further 2 days of culture, the medium was refreshed to start the incubation with concentrations of glyphosate ranging from 0.1 to 1000 µM. Dexamethasone was used as a positive control. Media were replenished every 2 days for a further 6 days. Lipid accumulation was visualized on day 8 using fluorescent Nile Red staining in accordance with the manufacturer's instructions and quantified using a microplate reader. The fluorescence was measured with a filter giving an excitation at 490 nm and emission at 510-570 nm. The adipogenic effect was expressed as a fold change in emission signal intensity between untreated differentiated and treated differentiated 3T3-L1 cells.

Cell viability assay - Cell viability was assessed using a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which indirectly measures cell number by testing for activity of mitochondrial succinate dehydrogenase. The 3T3-L1 cells were seeded into 96-well plates and differentiated as described above for 8 days. Cells were then incubated with 100 mL of MTT solution (1 mg/mL) for 2 hours and the test terminated by adding 100 µL DMSO. As a measure of cell number, the optical density of the cell lysate was determined at 570 nm using a GloMax Multi Microplate Multimode Reader. The number of cells was directly proportional to the intensity of the signal. Cell viability was expressed as a percentage of the control samples.

Intracellular lipid staining - The 3T3-L1 cells were seeded into 96-well clear bottom black tissue culture treated plates and differentiated as described above for 8 days. Medium was then removed and cells fixed by addition of 100 µL 4% paraformaldehyde. Cells were then stained for intracellular lipid accumulation by adding 50 µL of 1 mg/mL Nile Red and 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in 0.2% Triton X-100-PBS for 15 minutes in the dark. Nile Red staining for lipid droplets and DAPI staining for cell nuclei were imaged at 530 and 405 nm, respectively, using fluorescence imaging on a Nikon Eclipse Ts2 microscope at 40 x.

Statistical analysis - The statistical analysis of the dose-response results from the adipogenesis assay was performed by ANOVA. Pair-wise comparisons were made using a Mann-Whitney test. Nonlinear regression analysis was performed using 5-parameter logistic dose-response curve models. These statistical analyses were performed using GraphPad Prism version 7.00 for MAC OS X.

Results

With dexamethasone as the positive control the adipogenic assay using murine 3T3-L1 cells to undergo differentiation to adipocytes was shown to be a sensitive assay system causing a maximum of 21-fold increase in lipid accumulation when compared to untreated differentiated cells. The dose response relationship of dexamethasone was used to determine the concentration that caused a 50% response (EC_{50}). The EC_{50} for dexamethasone was 9.4 pM and some of the tested compounds showed an effect on lipid accumulation with different dose response patterns. Treatment with glyphosate scored negative.

Discussion

Commonly used herbicide active substances were tested in an adipogenesis assay to evaluate their obesogenic potential. Using the 3T3-L1 cell assay system, which measures lipid accumulation following differentiation to adipocytes, glyphosate scored negative at all concentrations tested. This study uses the well-established murine 3T3-L1 cell line model system for obesogenic screening. However, this cell line can only address a limited number of possible modes of action. The 3T3-L1 cells consist of unipotent pre-adipocytes, which can only differentiate into mature adipocytes. Another important limitation of 3T3-L1 cells is that they are of murine origin, and may not fully be representative of human metabolism. In addition, stocks of 3T3-L1 cells from different sources can have different metabolic capabilities.

Conclusion

Amongst the pesticide active ingredients tested for effects on lipid accumulation in differentiated adipocytes glyphosate scored negative.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study glyphosate, among other pesticide active ingredients, was investigated for its effect on lipid accumulation in differentiated adipocytes *in vitro* at concentrations ranging from 0.1 to 1000 µM. The results indicated that at the concentrations tested glyphosate scored negative for lipid accumulation. This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions.

Reliability criteria for *in vitro* toxicology studies

Publication: Biserni *et al*., 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of ≥ 96%. Source: Sigma-Aldrich, Gillingham, UK
Only glyphosate acid or one of its salts is the tested substance	N	Other pesticide active ingredients were tested as well.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Concentration range *in vitro* from 0.1 to 1000 µM.
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	Glyphosate was not tested in all tests described.
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	No effect of glyphosate over the entire concentration range tested.
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions.		

1. Information on the study

Data point:	CA 5.7
Report author	Chorfa A. *et al.*
Report year	2013
Report title	Specific pesticide-dependent increases in α-synuclein levels in human neuroblastoma (SH-SY5Y) and melanoma (SK-MEL-2) cell lines.
Document No	Toxicological sciences: (2013) Vol. 133, No. 2, pp. 289-97.
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The objective was to precisely assess changes in α-syn levels in human neuroblastoma (SH-SY5Y) and melanoma (SK-MEL-2) cell lines following acute exposure to glyphosate using Western blot and flow cytometry. The study was conducted using an in vitro test system. Glyphosate did not have any impact on the endpoints measured in this study.

Materials and methods

Cell culture: SH-SY5Y (a human dopaminergic neuroblastoma cell line) and SK-MEL-2 (a human cutaneous melanoma cell line) obtained from American Type Culture Collection (ATCC, Rockville, MD) were maintained in Dulbecco's modified Eagle's (DMEM-F12-GlutamaxI) medium containing 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin in an incubator at 37°C and 5% CO2.

Recombinant AdV-mediated overexpression of α-syn. Cell transduction was performed as previously described (Mougenot et al., 2010). Briefly, a recombinant adenoviral genome containing the full-length complementary DNA encoding human WT and mutant A53T α-syn in frame with a C-terminal myc-His epitope tag was generated by homologous recombination. Cells were infected with α-syn AdV or GFP AdV. On day 2 of infection, the medium was replaced with AdV-free DMEM.

Pesticides exposure: Cells at 70% confluence were exposed to glyphosate (N-(phosphonomethyl)-glycine) (Sigma-Aldrich). Purity was 99.5%. Glyphosate was dissolved in ultrapure water. Glyphosate concentrations for cell exposures (at 75 and 50% viability) were chosen based on the evaluation of toxicity following exposure of the SH-SY5Y cell line (0.005-800 µM) using the 3-(3,4-dimethylthiazol-2-yl)-2,5-diphe- nyltetrazolium bromide (MTT) assay. The protocol used for cell exposure and/ or transduction with recombinant AdVs is shown in Figure 1.

Cell death and viability assays: The MTT assay was performed with the Celltiter96 nonradioactive kit (Promega, France). MTT is metabolically converted into formazan by mitochondrial dehydrogenases of healthy, living cells (Denizot and Lang, 1986). Briefly, 5×10^4 cells per well were seeded into 96-well plates in duplicate and then treated with different concentrations of glyphosate. Cell death was assessed after 72 h of pesticide exposure. Then, 15 µl of "dye solution" was added to each well, and the plates were incubated at 37°C, 5% CO_2, for 4 h. Finally, 100 µl of "solubilization/stop solution" was added. After incubation for 1 h at 37°C, the optical density of the dissolved formazan grains within the cells was measured spectrophotometrically at 560 nm (BioTek ELx808, France). Results were expressed as percentage of the control. The IC_{50}, half-maximal (50%) inhibitory concentration, was determined for each pesticide from the graph of cell viability.

Protein extraction and Western blotting: After pesticide exposure, the cells were harvested and lysed in Laemmli buffer and then heated for 10 min at 100°C. The cell extract was then centrifuged (15,000 × g, 30 min at 4°C) before being loaded on to 12% gel for SDS-PAGE. Western blots were performed as previously described (Lee and Kamitani, 2011). Blots were hybrid- ized with monoclonal antibodies against β-actin (Abcam, dilution 1:1000) and against α-syn (clone 42, BD Biosciences, dilution 1:2000) overnight at 4°C. The membranes were then washed and further hybridized with goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase–conjugated antibody (Pierce, dilution 1:1000) for 30 min at room temperature. Protein bands were detected with chemiluminescent reagents (SuperSignal West Dura Extended Duration Substrate Kit, Pierce), then exposed to autoradiographic films or to a CCD camera (Versadoc system 5000, Bio-Rad), and quantified by Quantity One soft- ware (Bio-rad).

Flow cytometry: The cells were harvested by centrifugation at 1000 × g for 5 min at 4°C. The cell pellets were resuspended in a blocking solution (2% BSA-PBS) at 4°C. The cells were simultaneously permeabilized and fixed for 20 min at 4°C with BD Cytofix/Cytoperm Kit (BD Biosciences). All steps were carried out with permwash solution (BD Permwash Kit). After another centrifugation step, the cell pellets were incubated with α-syn clone 42 antibody in permwash solution for 30 min at 4°C. They were then rinsed with permwash solution and incubated with goat anti-mouse IgG R-phycoerythrin conjugate (Invitrogen) at 4°C for 30 min. The specific fluorescence intensities were measured with a BD FACS LSRII analyzer (BD Biosciences). Data were acquired using Diva software (BD Biosciences) and analyzed with FlowJo software (v7.6.5-TreeStar, Ashland, Oregon).

Statistical analysis: The results represent means ± SEM from at least 3 and up to 15 independent experiments. The effects of glyphosate on cell survival, following exposures of the four cell types (SH-SY5Y ± WT α-syn AdV and ± A53T α-syn AdV and ± GFP AdV) to different concentrations, were determined from an analysis of covariance. The results from Western blot studies were subjected to a Wilcoxon's test. For the flow cytometry studies of AdV-transduced cells, the mean fluorescence intensities of cells exposed or not exposed to glyphosate were compared using Student's test. Then, we realized a Levene's analysis in order to verify the hypothesis of homogeneity of variances. If the hypothesis was not verified, the Welch's analysis was used.

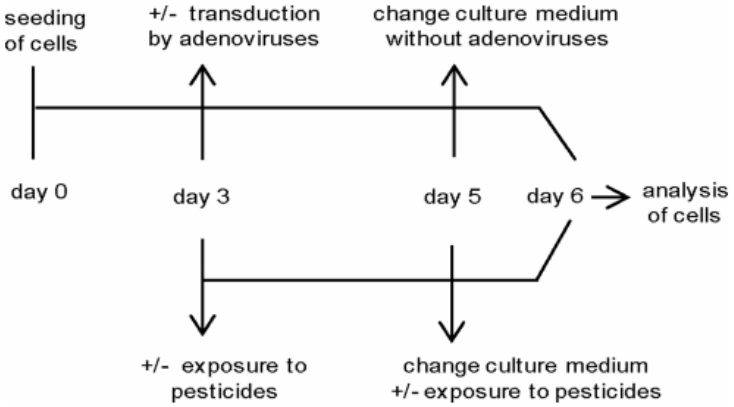


Figure 1. Experimental protocol of exposure to pesticides and recombinant AdV transduction of SH-SY5Y neuroblastoma and SK-MEL-2 melanoma cell lines. At approximately 70% cell confluence (day 3), cells were transduced with recombinant AdVs for protein overexpression (WT α-syn, A53T α-syn, GFP) and/or exposed to pesticides (rotenone, paraquat, maneb, and glyphosate) at concentrations corresponding to the IC50 determined on the SH-SY5Y cell line. For certain experiments, these two steps (pesticides exposure/adenoviral transduction) were combined. After 48 h (day 5), two protocols were followed. When the cells were only transduced with AdV, the culture medium was replaced by fresh AdV-free culture medium. When the cells were exposed to pesticides, the culture medium was replaced by fresh AdV-free culture medium supplemented with pesticides. Experiments were ended 24 h later (day 6), and adherent cells were collected for analyses by flow cytometry or Western blot.

Results

The impact of in vitro exposure to glyphosate on α-syn levels was assessed in two human cell lines of neuronal (SH-SY5Y) or melanocytic (SK-MEL-2) origin. The levels of endogenous α-syn or of recombinant α-syn levels after transduction with recombinant AdVs (WT and A53T α-syn AdV) (Mougenot et al., 2010) were analyzed by Western blot and flow cytometry.

Cytotoxicity Associated With Pesticide Exposure and/or Adenoviral Transduction

The SH-SY5Y neuronal cell line was first exposed to various concentrations of glyphosate (0.005-800 μM) for 72 h. The respective cytotoxicity of glyphosate (relative amounts of living and metabolically active cells) was estimated by MTT assay (Fig. 2). This showed that glyphosate had a distinct effect on the survival of SH-SY5Y cells after 72 h of exposure. The half-maximal (50%) inhibitory concentrations (IC50) for glyphosate was 9 μM. It was 11 μM for glyphosate for the SK-MEL-2 cell line. The IC50 value, determined after exposure of the SH-SY5Y cell line for 72 h, was chosen for further investigations of the effects on α-syn levels. The same concentration was used in experiments with the SK-MEL-2 melanoma cell line.

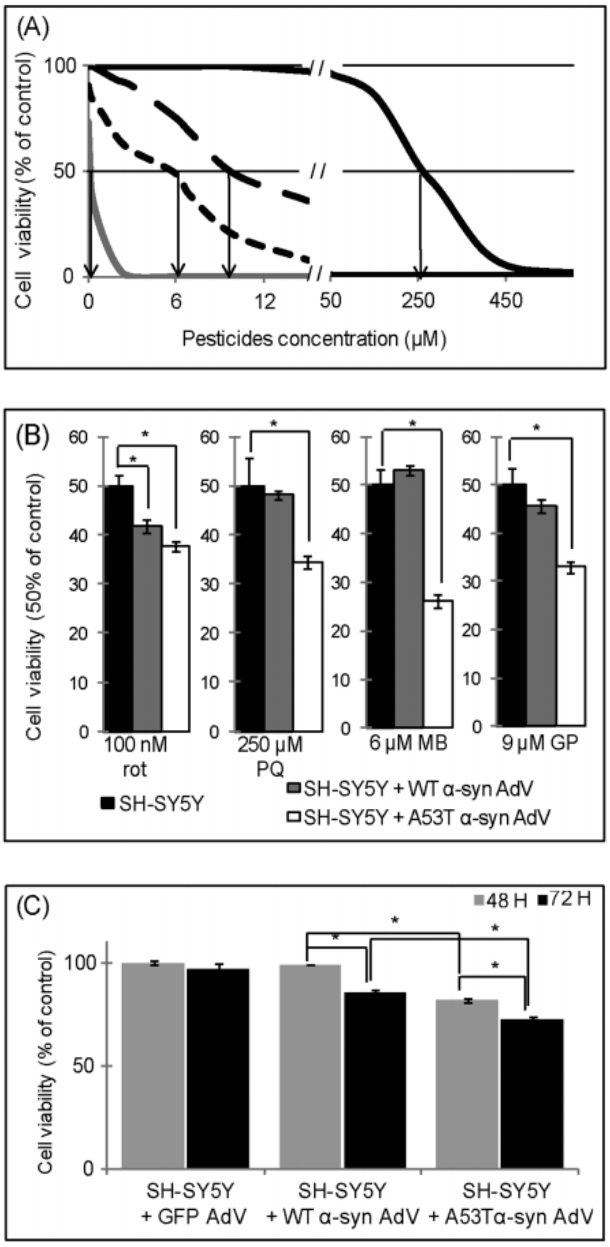


Figure 2. Effects of pesticides and adenoviral transduction on viability of SH-SY5Y neuroblastoma cells. Cell viability was assessed using the MTT assay. Data represent mean ± SEM numbers of viable treated cells/numbers of viable untreated cells from three separate experiments (*$p < 0.05$). The p values were determined by Wald's test. We measured the cell viability percen tage after 72-h treatment with increasing concentrations (0.005-800µM) of pesticide: rotenone (grey curve), maneb (dashed black curve), glyphosate (black curve em dash), or paraquat (black solid curve)(A). Part of the concentration range has been removed to facilitate comparison of the results. We then used the IC_{50} previously determined on SH-SY5Y cells (100nM and 6, 9, and 250µM) to quantify the viability of cells transduced with WT or A53T α-syn AdV after pesticide exposure for 72 h, as shown in Figure 1, in comparison to untransduced SH-SY5Y cells (B). The cytotoxicities associated with the recombinant Advs transduction alone, with either the GFP protein, WT or A53T α-syn AdV, were measured at 48 h (grey bars) and 72 h (black bars)(C).

Glyphosate cytotoxicity, following exposure for 72 h of SH-SY5Y cells transduced with recombinant AdVs, was then analyzed using the protocol summarized in Figure 1. The MTT assay did not reveal a significant decrease in the viability of WT α- syn AdV-transduced cells after exposure to glyphosate [-2% [$p = 0.6$]) at the previously determined IC50 (Fig. 2B). Viability was significantly decreased (-17%) with glyphosate ($p < 0.01$) when the cells were transduced with A53T α-syn AdV. Thus, glyphosate

induced a greater reduction of viability in cells transduced with A53T α-syn AdV than in nontransduced SH-SY5Y cells.

It should be noted, however, that viability was also consistently decreased in the absence of pesticide exposure, after transduction with WT α-syn AdV (-14%)($p < 0.05$) and even more so with A53T α-syn AdV (-38%)($p < 0.05$) at 72 h, as shown in Figure 2C representing three different experiments. Viability was already decreased 48 h after transduction with A53T α-syn AdV (-18%) ($p < 0.05$). Cytotoxicity seemed to be related to α-syn overexpression, as viability was unchanged 48 or 72 h after transduction with the GFP AdV used as a control in these experiments.

Specific Increase of Endogenous α-syn in Human Neuroblastoma and Melanoma Cells Exposed to Pesticides

Both SH-SY5Y neuroblastoma and SK-MEL-2 melanoma cell lines express α-syn (Pan et al., 2011). We therefore assessed endogenous α-syn expression using Western blot and flow cytometry (Fig. 3) and examined the ability of glyphosate exposure to modulate changes in α-syn levels. The Western blot experiments indicated that endogenous α-syn levels (Fig. 3A) were significantly increased by 100nM rotenone (~1.81×) ($p < 0.001$), whereas no change was observed in the closely related, but nonamyloidogenic, β-synuclein protein (Fig. 3C). The impacts of glyphosate on the level of endogenous α-syn in SH-SY5Y neuroblastoma cells was measured in comparison (Fig. 3A). A significant increase in α-syn levels was not observed with glyphosate up to 9μM ($p = 0.6553$) (Fig. 3A).

Quantification of Recombinant α-Syn Levels by Flow Cytometry Following Pesticide Exposure

We then attempted to quantify the changes in α-syn levels following transduction with AdV designed to overexpress α- syn. As shown in Figure 4A, transduction of SH-SY5Y cells with WT or A53T α-syn AdV resulted in similar levels of α-syn expression as measured by flow cytometry. In comparison to the isotype control, a 5.1% increase in fluorescence intensity was observed compared with 1.7% for the endogenous protein (Fig. 4A). Similar levels of α-syn were produced in α-syn AdV-transduced SK-MEL-2 cells (data not shown). The Western blot revealed that transduction with recombinant α-syn AdV was associated with a predominant band at 22.5 kDa, resulting from the presence of the myc-His tag epitope (Mougenot et al., 2010), in addition to the 19 kDa band representing the endogenous α-syn (Fig. 4B).

We then confirmed the effects of pesticides on AdV-associated α-syn levels by Western blot (Fig. 4C). In AdV-transduced SH-SY5Y cells, 1.38- and 1.70-fold increases were observed with WT (left panel) and A53T (right panel) α-syn, respectively, following exposure to 100nM rotenone ($p < 0.001$).

Pesticide impact was then assessed by flow cytometry after 72-h exposure of SH-SY5Y cells transduced with WT or A53T α-syn AdV to the IC_{50} (determined on the SH-SY5Y cell line) in comparison to a GFP AdV control (Fig. 5A). The observed increases in α-syn levels were specific, as no change in GFP fluorescence was apparent after exposure to glyphosate (data not shown) of cells transduced with a GFP AdV control.

No increase in α-syn levels was detected after exposure of SH-SY5Y cells transduced with WT or A53T α-syn AdV to glyphosate.

No significant increase in α-syn levels was found after the exposure to glyphosate of SK-MEL-2 cells transduced with WT or A53T α-syn AdV (compared with those transduced with GFP AdV).

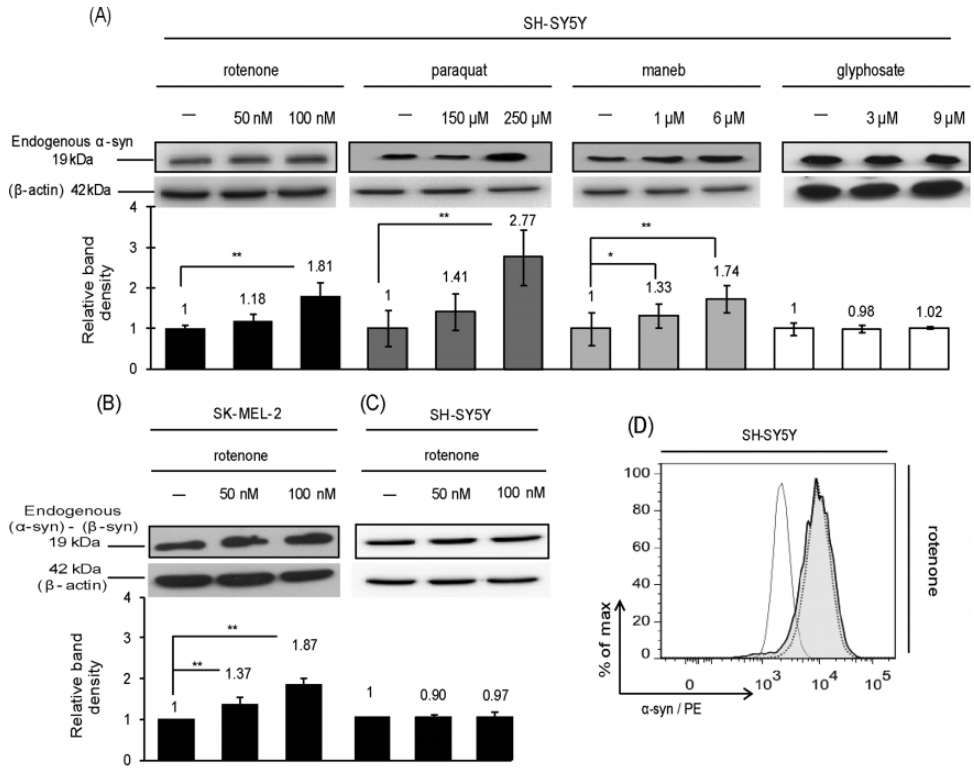


Figure 3. Characterization of the impact of pesticides on endogenous α-syn levels in SH-SY5Y and SK-MEL-2 cells by Western blot and flow cytometry. he levels of endogenous α-syn were estimated by Western blot after exposure of SH-SY5Y cells to different pesticides at concentrations corresponding to 25 and 50% of the IC_{50} (50 and 100nM rotenone; 150 and 250µM paraquat; 1 and 6µM maneb; 3 and 9µM glyphosate)(A). Similarly, the levels of endogenous α-syn in SK-MEL-2 cells exposed to 50 and 100nM rotenone were also assessed (B). In parallel, we estimated the amounts of β-synuclein in SH-SY5Y cells after rotenone exposure (C). β-actin was used as a loading control. Data represent the means ± SEM from four to six independent experiments obtained by Wilcoxon's test (**$p < 0.001$; *$p < 0.05$). The fold increase is indicated above the histogram for each experimental condition. Finally, the fluorescence curves corresponding to the levels of endogenous α-syn (dotted curve) in SH-SY5Y cells observed by flow cytometry after exposure to 100nM rotenone (black curve, shaded area) are compared with SH-SY5Y cells immunostained with an isotype control (grey curve)(D). Data represent the means ± SEM from three independent experiments.

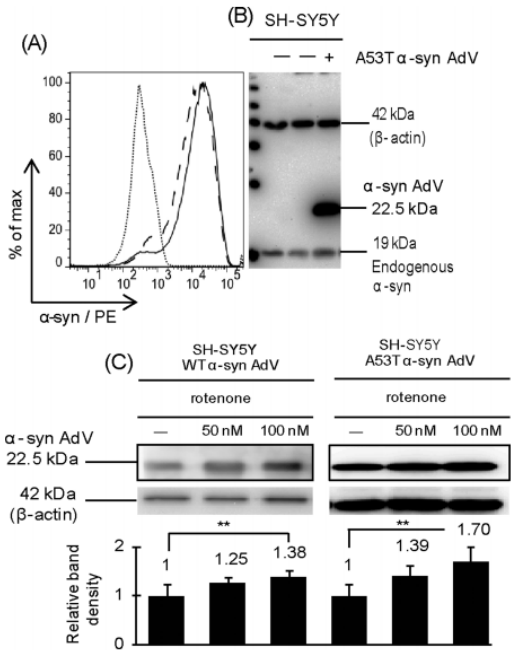


Figure 4. Characterization of the impact of pesticides on recombinant α-syn levels in SH-SY5Y cells by Western blot and flow cytometry. Three days after adenoviral transductions, the cells were permeabilized and immunostained with clone 42 antibody raised against α-syn. The flow cytometry histogram shows the levels of recombinant α-syn in SH-SY5Y cells overexpressing either WT (dashed line) or A53T (solid line) α-syn AdV, in comparison to endogenous α-syn (grey line) (A). Western blot detection of recombinant α-syn in comparison to endogenous α-syn is shown (B). Finally, the levels of recombinant α-syn were quantified in SH-SY5Y cells transduced with either WT or A53T α-syn AdV following rotenone exposure at 50 and 100nM (C). Data represent the mean ± SEM α-syn/β-actin ratios from four independent experiments (**$p < 0.001$ obtained by Wilcoxon's test).

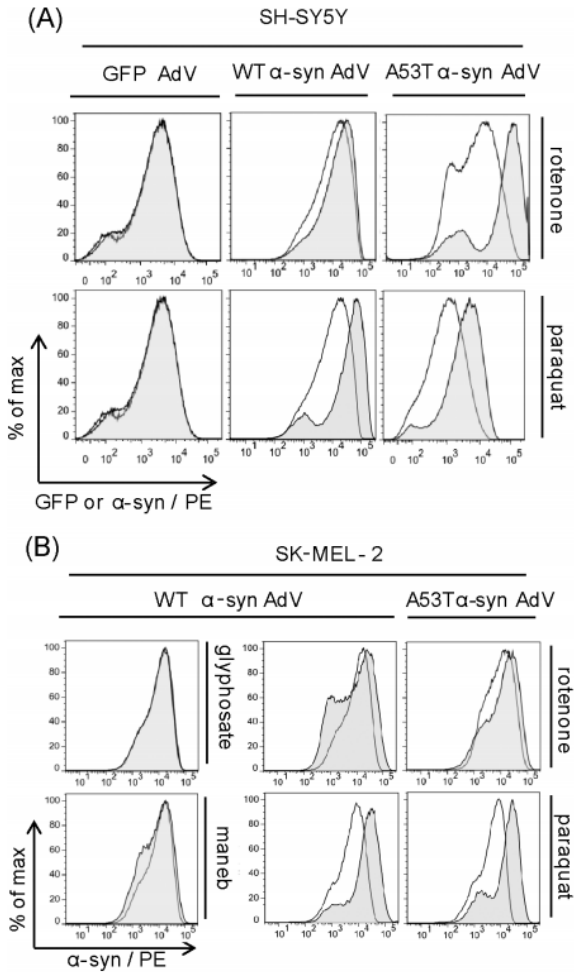


Figure 5. Characterization of the impact of pesticides on α-syn levels in AdV-transduced SH-SY5Y and SK-MEL-2 cells by flow cytometry. Fluorescence curves correspond to the α-syn levels following AdV transduction of SH-SY5Y cells by GFP AdV (left column, solid curve not shaded), WT α-syn AdV (center column, solid curve not shaded), and A53T α-syn AdV (right column, solid curve not shaded) alone or following exposure to either 100nM rotenone (upper panel, solid curve, grey shaded) or 250µM paraquat (lower panel, solid curve, grey shaded)(A). Fluorescence curves correspond to the α-syn levels in SK-MEL-2 after transduction with WT α-syn AdV (left and center column, solid curve not shaded) or A53T α-syn AdV (right column, solid curve not shaded) alone or following exposure to 9µM glyphosate (solid curve, grey shaded), 6µM maneb (solid curve, grey shaded), 100nM rotenone (upper panel, solid curve, grey shaded), or 250µM paraquat (lower panel, solid curve, grey shaded)(B). Data represent the means ± SEM of 6 to 12 separate experiments for each of the different pesticide exposures.

Discussion & Conclusion

Overall, the specific effects of the pesticides were quite consistent for both endogenous and AdV-produced α-syn. The mechanisms involved in the pesticide-induced increases were not examined but could reflect a decreased efficiency of the cellular mechanisms involved in the degradation of misfolded proteins such as the proteasome pathway, and/or, for the endogenous protein, increased synthesis of the protein (Tanner et al., 2011; Ulusoy and Di Monte, 2012). The changes in protein levels were specific to α-syn, as no changes were observed for a GFP protein encoded by recombinant AdVs or for the endogenous β-synuclein expressed in the SH-SY5Y cell line. β-Synuclein shares a high homology sequence with α-syn but is less prone to aggregation in relation with the absence of 11 amino acids in the central region of the protein (Sung and Eliezer, 2007).

No effect of the pesticide glyphosate on α-syn levels was detected. Although a case of parkinsonian syndrome was reported following acute poisoning with glyphosate (Barbosa et al., 2001), it was

concluded in a new study that there is little evidence to suggest a causal relationship between glyphosate exposure and noncancer diseases, including PD (Mink et al., 2011).

We found that paraquat, and to a lesser extent rotenone, but not maneb or glyphosate, also increased the α-syn levels in SK-MEL-2 cells, whether produced endogenously or after adenoviral transduction. In summary, we provide an approach based on commonly used methods, which allow the quantification of cellular α-syn levels, and we show here that these levels are greatly increased following exposure to certain pesticides, which have been specifically associated with PD. This experimental strategy could provide useful and readily available information specific to each pesticide so that the neurotoxic potential of the chemical can be assessed prior to large scale use in the field. On this basis, further studies are now focusing on the development of analytical methods in microplate format, which would be more convenient for large scale screening. To what extent such in vitro observations reflect specific events involved in the molecular pathogenesis of human α-syn associated diseases remains to be determined.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective was to precisely assess changes in α-syn levels in human neuroblastoma (SH-SY5Y) and melanoma (SK-MEL-2) cell lines following acute exposure to glyphosate using Western blot and flow cytometry. The study was conducted using an *in vitro* test system. Glyphosate did not have any impact on the endpoints measured in this study. This is not a guideline study, nor did this study evaluate an endpoint used in risk assessment. Therefore, this study in not usable for quantitative human health risk assessment or hazard assessment.

This publication is considered reliable with restrictions (no positive control was included and only 2 test concentrations were used) but is not relevant for the risk assessment of glyphosate.

Reliability criteria for *in vitro* toxicology studies

Publication: Chorfa *et al*., 2013	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 99.5%. Source: Sigma-Aldrich.
Only glyphosate acid or one of its salts is the tested substance	N	Also other pesticides tested (rotenone, paraquat, maneb).
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Human neuroblastoma (SH-SY5Y) and melanoma (SK-MEL-2) cell lines.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0, 3, and 9 µM.

Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive controls used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate isopropylamine salt used was not sufficiently characterized and the standard deviation of the IC_{50} of glyphosate (1211 ± 885.7 ug/mL) and MON 52276 (361.6 ± 612 µg/mL) for human fibroblasts is too large.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Connolly A. *et al.*
Report year	2018
Report title	Characterising glyphosate exposures among amenity horticulturists using multiple spot urine samples
Document No	International journal of Hygiene and Environmental Health (2018) Vol. 221, 1012-1022
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The study aimed to characterise occupational exposures to glyphosate among amenity horticulturists through the collection and analysis of urine samples following pesticide application. The impact of work practices on personal exposure, as well as suitability of collecting multiple spot urine samples as a sampling strategy for the assessment of occupational exposure for glyphosate were also examined.

Materials and methods

Site description and study population - The monitoring campaign took place from September 2016 to September 2017. In this study 3 similar exposure groups (SEGs) were considered for glyphosate applications: (1) manual knapsack (Roundup Biactive XL, 360 g/L; Clinic Ace, 360 g/L; Roundup Biactive, 360 g/L; Pistol, 250 g/L; Roundup XL, 360 g/L), (2) pressurized handheld lance (Roundup Biactive XL, 360 g/L; Pistol, 250 g/L; Glyfos, 360 g/L; Rambo, 360 g/L; Roundup Gold, 450 g/L), (3) controlled droplet applicator (Nomix Dual, 120 g/L; Roundup XL, 360 g/L). Recruitment was completed in coordination with the OPW Health and Safety Unit. The lead researcher explained the project background and objectives and circulated project information leaflets to potential study participants. Participation in the study was voluntary. After having given informed consent the participants completed a self-administered questionnaire providing relevant personal and work related details, as well as information on their use of pesticides outside of work and dietary habits. Biomonitoring protocols were developed based on established protocols. Project ethical approval was received from the National University of Ireland, Galway Research Ethics Committee.

Urine collection - Biomonitoring exposure assessments took only place on the days workers were using glyphosate based pesticide products. The researcher observed all work tasks on site, and collected information such as SEG, personal protective equipment (PPE) worn, climatic conditions, and any activities or work duties performed between samples and the duration of these activities. Individual full urinary void spot samples were collected over the exposure assessment period. The void spot samples were kept and analyzed separately. Time and date were mentioned on each sample container which was stored in a cooler box until collection by the researcher the morning following each task. A minimum of 3 spot urine samples were provided: a pre-task sample, a post-task sample taken within one hour of task completion and a sample taken on the following morning after the first morning void. Participants were also given the option to provide urine samples for all additional voids over the exposure assessment period (pre-task to the following first morning void sample). Participants provided more than the minimum required 3 spot samples for 59% (17) of the tasks sampled ranging between 3 and 7 samples per task. Of the spot urine samples provided, a peak urinary sample was identified for each task and defined as the highest urinary glyphosate concentration measured during the sampling period. Following sample collection, the researcher measured and recorded the volume of each urinary spot sample. A 20 mL aliquot of the well mixed spot urine sample was then transferred into a 20 mL Sterilin™ pot and

labelled with a unique identifier number, date and time. Care was taken to avoid any contamination. All samples were stored at −18°C within 24 hours pending analysis.

Analysis of glyphosate in urine samples - Glyphosate was extracted from diluted urine samples (200 µL urine added to 800 µL deionised water) using anion exchange solid phase extraction (SPE) with 10% formic acid in methanol as the eluent. The eluent was evaporated under a stream of nitrogen and reconstituted in 100 µL of 0.1% formic acid for quantitative analysis by LC-MS/MS. Chromatographic separation was achieved on a Zorbax XDB-C8, 150×4.6 mm, 5 µm (Agilent, Stockport, UK) column with 0.1% formic acid and acetonitrile with a gradient elution as the mobile phase. The method was linear over the range of 0–20 µg/L and the limit of quantification (LOQ) was 0.5 µg/L. Creatinine was determined in all urine samples with a Pentra C400 clinical analyzer using the alkaline picrate method.

Statistical analysis - Prior to analysis, all glyphosate concentration levels below the LOQ were imputed, using SAS v 9.4. Differences in urinary glyphosate concentrations between the pre-task samples versus the post-task and the peak urinary samples were both analyzed using paired Student t-tests. Determinants of exposure on glyphosate urine concentrations were evaluated using Pearson's correlation coefficients and linear regression. A multivariate mixed effect model was elaborated to compare the glyphosate concentrations between post-task and following first morning void samples. In these models, worker identity was entered as a random effect to account for the presence of correlations between repeated measurements from the same individuals.

Results

Descriptive and summary statistics of demographic and task characteristics - 18 male and 2 female amenity horticultural workers applying pesticides as part of their work duties consented to participate in the study. Urine samples were collected for 29 work tasks involving glyphosate based pesticides. The concentration of glyphosate in 14 (48%) of the pre-task samples could have been influenced by work tasks performed in the days prior to this study and by starting the work task before giving the pre-task sample. For 6 (21%) of the pre-task samples, workers reported performing work tasks involving handling of glyphosate based products on the previous day to the measurement period. Similarly, for 10 (35%) of the pre-task samples (including 2 who were also involved with spraying the previous day), workers reported collecting, checking or handling potentially contaminated spraying equipment or other work equipment prior to providing the first sample (pre-task sample). A large proportion of workers reported using pesticides outside of their job (90%), corresponding to 27 (93%) of the 29 tasks included in this study. However, none reported using glyphosate based pesticides at home on the days before the sampling. The majority of the workers (60%) reported using pesticides at work for 6–7 months per year. 100% of the workers wore gloves, 90% a Tyvec suit and 97% RPE.

Urinary glyphosate concentrations - 125 spot urinary samples were collected and analyzed for 29 work tasks. Participants provided between 3 and 7 individual spot samples per exposure assessment period. Participants giving more than 3 spot urine samples over the exposure assessment period (n=17) allowed for a more accurate estimation of the urinary concentrations over time. There was no statistically significant difference in the average log transformed peak urinary concentrations where only the minimum of 3 samples were provided versus those tasks where multiple samples were provided (Student t-test; $p = 0.14$). For 13 (45%) of the 29 tasks, the peak concentrations were identified within the samples that were collected in addition to the minimum required (pre-task, post-task and following first morning void) samples. Another 31% of the peak samples were identified in post-task samples, whereas 24% comprised of following first morning void samples. The geometric mean of the glyphosate concentrations measured in urine samples of the combined glyphosate SEGs were 0.68 µg/L for pre-task samples, 1.17 µg/L for post-task samples and 0.83 µg/L for following morning void samples. The geometric mean of the peak samples was 1.9 µg/L. Glyphosate concentrations were less than the LOQ in 34 (27%) urinary samples, of which 11 (38%) were pre-task samples and a further 11 (38%) were following first morning void samples. Two (7%) of the 29 work tasks had peak samples with urinary glyphosate concentrations below the LOQ, both belonging to the manual knapsack SEG.

Conclusion

This study provides information on occupational exposures to glyphosate among amenity horticulturalists and suggests that the collection and analysis of urine samples given up to 3 hours after task completion can be a suitable sampling strategy for estimating potential occupational exposures to glyphosate. The findings suggest that amenity horticulturists, largely complying with workplace exposure controls, have low levels of glyphosate exposures.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study the exposure of amenity horticulturalists to glyphosate was assessed. Three similar exposure groups (SEGs) were considered for the application of various glyphosate based herbicides: one using a manual knapsack, one using a pressurized handheld lance and one using a controlled droplet applicator. Urine samples were taken pre-task, post-task and the morning after the task and analyzed for glyphosate using and LC-MS/MS method with an LOQ of 0.5 µg/L. Glyphosate concentrations were found to be less than the LOQ in 27% of the urinary samples, of which 38% were pre-task samples and 38% were following morning void samples. Two of the 29 work tasks had peak samples with urinary glyphosate concentrations below the LOQ, both belonging to the manual knapsack SEG. The geometric means of the glyphosate concentrations measured in urine samples of the combined glyphosate SEGs were 0.68 µg/L for pre-task samples, 1.17 µg/L for post-task samples and 0.83 µg/L for following morning void samples. 100% of the workers wore gloves, 90% a Tyvec suit and 97% RPE.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because more detail could have been provided on the validation of the analytical method. Also the number of participants per exposure scenario was rather limited.

Reliability criteria of exposure studies

Publication: Connolly *et al.,* 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.	Y	
Exposure to formulations with glyphosate combined with other a.i.	N	
Exposure to various formulations of pesticides	N	
Study		
Study design clearly described	Y	
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y?	Not complete.
Monitoring results reported	Y	

Publication: Connolly *et al.*, 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because more detail could have been provided on the validation of the analytical method. Also the number of participants per exposure scenario was rather limited.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Connolly A. *et al.*
Report year	2019
Report title	Exploring the half-life of glyphosate in human urine samples
Document No	International Journal of Hygiene and Environmental Health (2019) Vol. 222, 205-210
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study aimed to estimate the human half-life of glyphosate from human urine samples collected from amenity horticulture workers using glyphosate based pesticide products.

Materials and methods

Site description and study population - An occupational urinary biomonitoring study for glyphosate was carried out from September 2016 to September 2017. Sample collection took place at the Irish Commissioner for Public Works (OPW) field sites. The tasks completed by the workers sampled were subdivided into three similar exposure groups (SEGs) based on the application method used by the workers to apply glyphosate based pesticide products: manual knapsacks, pressurized applicators and controlled droplet applicators, all of which involved the use of a handheld lance.

Urine collection - Full void urinary spot samples were collected from the participants for 29 work tasks and collected and analyzed separately. A minimum of 3 urine samples were collected from each participant: one pre-task sample, one post-task sample and the first morning void sample obtained the day after completing the work task (following first morning void). Participants had an option to provide individual spot urine samples for all urinary voids from the start of the pesticide task to the following first morning void. A pre-labelled sample container was given to every participant for each void and they were asked to write the time and date on the container label. The volume of each urine sample was recorded and the sample was well mixed before taking a 20 mL aliquot to be transferred into a Sterilin™ pot, labelled with a unique identifier number, date and time. Care was taken to avoid any contamination. All samples were stored frozen at -18°C within 24 hours of collection pending analysis.

Urine sample analysis - Glyphosate was extracted from diluted urine samples (200 µl added to 800 µL deionized water) using anion exchange solid phase extraction (SPE) using 10% formic acid in methanol as an eluent. Quantitative analysis was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Chromatographic separation was achieved on a Zorbax XDB-C8, 150×4.6 mm, 5 µm column with 0.1% formic acid and acetonitrile with a gradient elution as the mobile phase. The analytical method was linear over the range 0–20 µg L^{-1} and intra- and inter-assay coefficient of variations (CVs) of 3.54% (n = 10) and 9.96% (n = 40, over 4 runs) were achieved. The analytical limit of quantification (LOQ) was 0.5 µg L^{-1}. Creatinine analysis was performed on all urine samples with a Pentra 400 clinical analyzer using the alkaline picrate method.

Estimation of the half-life time of glyphosate - To explore the elimination rate and to estimate the potential human biological half-life of glyphosate, only work tasks with at least two spot urine samples collected after the peak exposure were included for excretion profile analysis. The peak urinary exposure value was defined as the highest urinary glyphosate concentration measured in a spot sample after application per task. The elimination time and the estimation of the half-life of glyphosate was assessed

using three different measurement metrics: unadjusted glyphosate concentrations (µg/L), creatinine corrected concentrations (µmol/mol creatinine) and Urinary Excretion Rates (UER). The UER (µg/hr) was calculated by taking the glyphosate concentration of the spot urine sample and multiplying it by the volume of the void and dividing it by the time that elapsed between this urine void and the last urine void.

Statistical analysis - All statistical analyses were performed on Microsoft Excel and Stata Software. Glyphosate concentrations were log transformed as the data showed a log normal distribution. The period of peak sample collection was taken as the start time (t = 0) and the time period from the sample collection time (t = 0) to each proceeding sample was calculated. The slope of the glyphosate urine concentration by the time duration (time passed from the start time) was calculated for each task. Linear interpolation using regression analysis was also performed for each of the included tasks. The mean values, as well as the 95% confidence interval of the half-lives were calculated to estimate the half-life range for each measurement metric.

Results

Urine samples from 7 participants performing 8 work tasks involving glyphosate based products were analyzed. Data from 6 males and one female worker is included in this study. One male participated twice on two consecutive days. The age range was from 32 to 60 years, with an arithmetic mean (AM) of 48 years. Workers carried out work tasks that involved the application of glyphosate based pesticide products within one of the SEGs, which lasted between approximately 1 and 6 hours daily. The total sampling time duration of the selected 8 work tasks included in the data analysis, ranged from approximately 19 to 26 hours. In total, 28 individual spot urine samples were analyzed for the 8 work tasks included in this study (3 to 4 spot urine samples per sample set). Each sample set was analyzed to evaluate the relationships between the measured urinary glyphosate concentrations (µg/L, µmol/mol creatinine or UER) and the duration. The duration started from the peak concentration sample (start time) to each of the subsequent samples. Correlations and linear regression analysis were performed for each sample set. Four sample sets were excluded from the analysis: two creatinine corrected samples sets (µmol/mol creatinine) and two UER calculated sample sets. One creatinine corrected sample set was excluded due to low creatinine levels (< 3 mmol/L) in individual spot urine samples and another because there was no association between concentrations and duration of sampling. This lack of association could relate to a number of factors like gender, diet and hydration.
Each of the sample sets showed a moderate to strong relationship between concentration and duration for all samples (R^2 = 0.42–1.00), with an estimated half-life ranging approximately from 1.5 to 10 hours for unadjusted values or from 4.75 to 20 hours for creatinine corrected values. When the results were restricted to sample sets which showed very strong relationships (R^2 > 0.90), the estimated half-life average (range) was 4.5 (1.5 - 7) hours and 7.5 (4.75 – 9.25) hours for unadjusted and creatinine corrected values, respectively. UER calculated samples showed moderate to strong relationship (R^2 = 0.60–0.95), with an estimated half-life average (range) of 7.25 (3 and 9.50) hours. The average glyphosate half-life including all measuring metrics was approximately 5.5 to 10 hours. The average and range of the half-life on sample sets (numbers 2, 12, 19 and 30) that had all three measuring metrics included was calculated. Sensitivity analysis on the four sample sets, common across all measuring metrics, had an estimated half-life average (range) of approximately 6.5 (4–10), 11.75 (7.25 - 20), and 6.5 (3–7.75) hours for the unadjusted glyphosate concentrations, creatinine corrected concentrations and urinary excretion rate. The limitations of this study are the lack of standardization (pesticide products used, quantity of pesticides applied per task, different application methods and different sampling times). The small sample size prevented the use of more elaborate statistical tests to identify differences due to sex or age. The pharmacokinetic analysis revealed first order kinetics but due to the collection of urine samples over a limited period of time (19-26 hours) multi-phasic kinetics may not have been identified.

Conclusion

The results from this study provide new information on the elimination rate and estimated human biological half-life of glyphosate based on the analysis of urine samples collected during an exposure assessment study. This information can be helpful in the design of sampling strategies, as well as

assisting in the interpretation of results for human biomonitoring studies involving glyphosate. The data could also contribute to the development or refinement of Physiologically Based PharmacoKinetic (PBPK) models for glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Analytical data for glyphosate obtained from spot urine samples collected during a glyphosate exposure study (Connolly et al., International journal of Hygiene and Environmental Health (2018), Vol. 221, 1012-1022) were used to estimate the human biological half-life of glyphosate. To that end only work tasks with at least two spot urine samples collected after the peak exposure were included for excretion profile analysis. Glyphosate concentrations were log transformed and the slope of the glyphosate urine concentration by the time duration (time passed from the start time) was calculated for each task. When the results were restricted to sample sets which showed very strong relationships ($R^2 > 0.90$), the estimated half-life average (range) was 4.5 (1.5 - 7) hours and 7.5 (4.75 - 9.25) hours for unadjusted and creatinine corrected values, respectively. UER calculated samples showed moderate to strong relationship ($R^2 = 0.60–0.95$), with an estimated half-life average (range) of 7.25 (3 and 9.50) hours. This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions in view of the limitations of the study such as the lack of standardization (pesticide products used, quantity of pesticides applied per task, different application methods and different sampling times). The small sample size prevented the use of more elaborate statistical tests to identify differences due to sex or age. The pharmacokinetic analysis revealed first order kinetics but due to the collection of urine samples over a limited period of time (19-26 hours) multi-phasic kinetics may not have been identified.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions in view of the limitations of the study such as the lack of standardization (pesticide products used, quantity of pesticides applied per task, different application methods and different sampling times). The small sample size prevented the use of more elaborate statistical tests to identify differences due to sex or age. The pharmacokinetic analysis revealed first order kinetics but due to the collection of urine samples over a limited period of time (19-26 hours) multi-phasic kinetics may not have been identified.

Reliability criteria of exposure studies

Publication: Connolly *et al.*, 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	Urine monitoring data from a glyphosate exposure study were used to study the urinary excretion kinetics of glyphosate. There are limitations in the study approach.
Test substance		
Exposure to formulations with only glyphosate as a.i.	Y	
Exposure to formulations with glyphosate combined with other a.i.	N	

Publication: Connolly *et al.*, 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Exposure to various formulations of pesticides	N	
Study		
Study design clearly described	N	Based on the glyphosate exposure study.
Population investigated sufficiently described	N	Based on the glyphosate exposure study.
Exposure circumstances sufficiently described	N	Based on the glyphosate exposure study.
Sampling scheme sufficiently documented	Y	Based on the glyphosate exposure study.
Analytical method described in detail	Y	
Validation of analytical method reported	Y?	Could be more elaborate.
Monitoring results reported	Y	
Statistical analysis	Y	
Pharmacokinetic analysis	Y	To some extent, supposing first order one-compartment pharmacokinetics.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions in view of the limitations of the study such as the lack of standardization (pesticide products used, quantity of pesticides applied per task, different application methods and different sampling times). The small sample size prevented the use of more elaborate statistical tests to identify differences due to sex or age. The pharmacokinetic analysis revealed first order kinetics but due to the collection of urine samples over a limited period of time (19-26 hours) multi-phasic kinetics may not have been identified.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Connolly A. et al.
Report year	2017
Report title	Exposure assessment using human biomonitoring for glyphosate and fluroxypyr users in amenity horticulture
Document No	International Journal of Hygiene and Environmental Health (2017) Vol. 220, 1064–1073
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

This study aims to measure occupational exposures to amenity horticuturalists using pesticides containing the active ingredients, e.g. glyphosate by urinary biomonitoring. A total of 40 work tasks involving glyphosate were surveyed over the period of June – October 2015. Pesticide concentrations were measured in urine samples collected pre and post work tasks using liquid chromatography tandem mass spectrometry. Pesticide urinary concentrations were higher than those reported for environmental exposures and comparable to those reported in some agricultural studies. Log-transformed pesticide concentrations were statistically significantly higher in post-work samples compared to those in pre-work samples. Urinary pesticide concentrations in post-work samples had a geometric mean / geometric standard deviation of 0.66 / 1.11 μg L^{-1}. Linear regression revealed a statistically significant positive association to exist between the time-interval between samples and the log-transformed adjusted (i.e. post-minus pre-task) pesticide urinary concentrations ($\beta = 0.0039$; $p < 0.0001$).

Materials and Methods

Site description and study population - This study was conducted over the period of June – October 2015 in the Republic of Ireland, at field sites managed by the Office of Public Works (OPW). A walk through survey was performed by the researcher at the selected OPW sites including national parks, ornamental gardens and historic monuments, to collect information on the frequency of use of pesticides containing glyphosate and spraying methods used. In this study 3 similar exposure groups were considered for glyphosate applications: glyphosate with a manual knapsack (8 participants using Roundup Biactive XL, 360 g/L; Pistol, 250 g/L; Destrol Amenity, 250 g/L; Glyfos, 360 g/L; Rambo 360, 360 g/L), glyphosate with a controlled droplet applicator (4 participants using Nomix Dual, 120 g/L), and glyphosate with a pressurized handheld lance (5 participants using Roundup Biactive XL, 360 g/L; Pistol, 250 g/L; Glyfo, 360 g/L; Rambo 360, 360 g/L). Amenity horticulturists (age ranging from 33–66 years) who used glyphosate and worked with the OPW at the designated sites, were invited to participate in the study. Participation in the study was voluntary and recruitment was done in coordination with the OPW Health and Safety Unit. Prior to the commencement of the study, the workers were informed of the sampling protocols and methods and completed a self-administered questionnaire to collect information on the participants including their work activities, out-of-work use of pesticides, dietary habits and smoking status.

Biological Monitoring

Urine collection - On the day workers participated in the study, they were asked to provide a pre-work and a post-work sample of up to 50 mL each. The post-work sample was taken within one hour after completion of the task. The samples were stored at -18°C until laboratory analysis. The sampling time

in this study was defined as the time interval between the pre- and post-sample collection. Following information was collected for each task: sampling time, application method, pesticide used, personal protective equipment (PPE) used and climatic conditions. Any changes in PPE, change in weather conditions, breaks taken and problems were also recorded.

Urine sample analysis - Glyphosate standard and glyphosate internal standard (2-$^{13}C^{15}N$-glyphosate) were purchased commercially. 500 µL of urine sample was diluted with 500 µL of deionised water for the analysis of glyphosate. The prepared samples were spiked with 10 µL of internal standard (500 µg/L). Glyphosate was extracted from urine by solid phase extraction (SPE) with subsequent analysis by liquid chromatography tandem mass spectrometry (LC–MS/MS) using a Zorbax SB-C3 150 × 4.6 mm, 5 µm column equipped with a C18 guard column. Mass spectrometry analysis was performed in negative MRM mode. The method was linear over the calibration range 0–20 µg/L. The intra and inter assay coefficients of variation were 3.54% (n = 10) and 9.96% (n = 40, over 4 runs) for glyphosate. The limit of detection (LOD) for glyphosate was set at 0.5 µg/L. Creatinine analysis was performed on all urine samples. Statistical analysis for this study was performed using STATA software. Urinary glyphosate concentrations are expressed as geometric means (GM) and geometric standard deviations (GSD) along with arithmetic mean (AM), minimum and maximum levels.

Results

Demographic and working characteristics - The study population consisted of 18 horticulture amenity gardeners (17 men and one woman), who applied pesticides as part of their work. One participant stated that he was a smoker. In 58% of the tasks sampled, a pesticide task had also been completed on the previous day. Of the 40 tasks analyzed in this study, 93% of the workers applied glyphosate by spraying with 53% being involved with mixing and loading. The vast majority of the workers wore PPE for each task: 94% wore gloves, 83% respiratory protective equipment (RPE), and 67% Tyvek suits. 89% of the workers reused their PPE and 78% of the workers did not take breaks during the task. The average duration of exposure to glyphosate (from pre-work to post-work sampling time) was 110 minutes for application with a manual knapsack, 235 minutes for application with a large droplet applicator, and 219 minutes for application with a pressurized lance applicator.

Urinary glyphosate concentrations - A total of 80 samples were collected in this study, 40 pre-work and 40 post-work samples. 58% (23 samples) of the pre-work samples and 43% (17 samples) of the post-work samples had glyphosate concentrations below the LOD. For the combined exposure groups the geometric mean was 0.42 µg/L for the pre-work samples and 0.66 µg/L for the post-work samples. The geometric means of the glyphosate urinary concentrations of the knapsack and pressurized lance applicators were comparable (0.62 µg/L and 0.57 µg/L, respectively) whereas the geometric mean of the post-work samples of the controlled droplet applicators was higher (1.00 µg/L) although not statistically significantly different from the other exposure groups. The urinary glyphosate concentrations (both simple and corrected for creatinine values) of the pre- and post-work samples were statistically significantly different. When the effects of the time between the collection of the pre- and post-work samples on the urinary concentrations were considered, a positive statistically significant association was observed. Similar results were found with the creatinine corrected values Similar but somewhat less pronounced associations were observed for the controlled droplet applicator and pressurized lance groups, but not for measurements performed during spraying with the manual knapsack. Pesticide urinary concentrations from workers who took breaks during the task were significantly higher by a factor 1.7 on average, compared to those from workers who did not take breaks. Trends were similar when analysis was repeated using the creatinine corrected values. Similarly, median exposure durations were statistically significantly longer for the measurements where workers took breaks compared to those who did not (273 versus 105 minutes), as well as those samples that had pesticide concentrations above the LOD compared to those with concentrations below the LOD (170 versus 98 mins).

Discussion

Studies quantifying occupational exposure to pesticides in amenity horticulture are very sparse. The sensitivity of the analytical method used for glyphosate (LOD of 0.5 µg/L) was comparable to those previously reported. The study results suggest that amenity workers have elevated urinary pesticide levels for glyphosate, above what would be expected from dietary exposures. In 43% of the post-work samples, pesticide concentrations were lower than the LOD. Although very low, the pesticide concentrations in almost all of the post-work samples were higher than those in the pre-work samples. Although no human biomonitoring data are available for the Irish population, the arithmetic mean of the urinary concentrations in the post-work samples (1.35 µg/L) is higher than the mean of 0.21 µg/L reported in an European environmental exposure study. Similarly, both mean and maximum values reported in the current study (1.35 µg/L and 10.66 µg/L, respectively) are also higher than the maximum urinary glyphosate concentrations (0.41 µg/L) reported for German adults with non-occupational exposures to glyphosate. Compared to previous studies among working populations, glyphosate urinary concentrations are comparable to those reported on agricultural exposures and slightly lower but within the range of urinary concentrations reported in studies among farm families in Iowa and farmers in Minnesota and South Carolina. The mean urinary glyphosate concentrations were comparable across all exposure groups although the exposure data from the controlled droplet applicator group appeared to be somewhat more variable than those from the other groups. The observed variation in exposure within this group may reflect inconsistent use of PPE and complacent work practices. Protective coveralls were only worn for 29% of the tasks in this group compared to at least 50% in the other groups.

Previous studies have associated higher pesticide exposures with the mixing and loading of pesticide concentrate. In this study, the majority of the participants performed mixing and loading of the pesticide concentrate as part of the overall task assessed with the exception of the controlled droplet applicators who used a pre-mixed solution. It was therefore not possible to evaluate pesticide exposure during the mixing and loading of the pesticide concentrate. A strong association was found between urinary glyphosate concentrations and exposure duration. The duration of exposure for work involving spraying with the manual knapsack and controlled droplet applicator groups ranged from 5 to 115 min and 33–195 min, respectively. No association between urinary pesticide levels and exposure duration was found for manual knapsack applicators. These study results show that there is a potential for exposure during tasks in horticulture and amenity gardening that typically involve small volumes of pesticides, ranging from 100 mL to 2 L, which warrants further investigation. 89% of the workers reused PPE such as coveralls, gloves, disposable face masks and this may have contributed to the level of exposure. Higher levels of urinary pesticide levels were found among workers who took breaks during the task. Workers who encountered problems during pesticide application such as adjusting the nozzle, leaks or spills, change in climatic conditions or issues with PPE, had higher average urinary concentrations of glyphosate. The sampling strategy adopted in this study (spot sampling pre- and post-work), most likely underestimates the exposure potential. A larger study incorporating 24–72 h urine samples would provide more reliable estimates of exposure and allow comparison with ESFA's designated ADIs and AEOLs.

Conclusions

The results from this study provide evidence of occupational exposure to glyphosate among amenity horticultural workers. The measured levels of urinary concentrations are comparable to those reported for agricultural workers. Urinary concentrations appeared to be dependent on the duration of exposure and the levels measured were higher among workers who took breaks or performed longer tasks, such as the use of controlled droplet applicators. Further research is currently underway to investigate 24 h exposures, evaluate dermal and inadvertent ingestion exposure and their contribution to total body burden of the pesticides.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Biological monitoring has previously been used in studies evaluating occupational exposures to pesticides in both the agricultural and horticultural sectors. The aim of this study was to characterise the occupational exposures in amenity horticultural workers using a biomonitoring method for glyphosate in urine. The geometric mean of the urinary glyphosate concentrations in the post-work samples of all exposure groups combined was found to be 0.66 µg/L. When the relationship between urinary concentrations of glyphosate and systemic dose as established by Acquavella et al. (Acquavella et al. (2004) Environmental Health Perspectives, 112(3), 321-326) is taken into consideration, the daily systemic dose for the workers in this study is estimated to be 0.000021 mg/kg bw/day. The corresponding daily oral external dose is about 0.0001 mg/kg bw/day when an oral bioavailability of 20% is taken into account. This is 5,000 times lower than the ADI of 0.5 mg/kg bw/day.

This publication is considered relevant for glyphosate risk assessment and reliable without restrictions because it complies with all the reliability criteria of an exposure study.

Reliability criteria for exposure studies

Publication: Connolly *et al.*, 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.		
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	
Study		
Study design clearly described	Y	
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with all the reliability criteria of an exposure study.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Connoly A. *et al.*
Report year	2018
Report title	Glyphosate in Irish adults – A pilot study in 2017
Document No	Environmental Research 165 (2018) 235-236
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

The objective of this study was to conduct an exploratory glyphosate exposure assessment study among Irish adults, who were non-occupational users of glyphosate. A biomonitoring survey involving the collection and analysis of 20 ml spot urine samples from 50 Irish adults was conducted in June 2017. Participants completed a short questionnaire to collect information on demographics, dietary habits and lifestyle. Glyphosate was extracted using solid phase extraction (SPE) and analysed by liquid chromatography tandem mass spectrometry (LC-MC/MS). Of the 50 samples analysed, 10 (20%) contained detectable levels of glyphosate (0.80 – 1.35 µg/L). Exposure concentrations were higher than those reported in comparable studies of European and American adults.

Materials and methods

Using a convenient sampling method, 50 Irish adults (> 18 years) were recruited from 16 counties across the Republic of Ireland to participate in the study over June – August 2017. Participants with specific dietary habits (vegetarian/vegan) and those whose occupation involved the use of pesticides were excluded from the study, to prevent dietary exposure bias, due to a proportionally increased intake of wheat products, fruits and vegetables or from occupational use of glyphosate-based pesticide products. Participants completed a project questionnaire and provided one, 20 mL first morning void urine sample. A questionnaire was designed to collect information on participant demographics, dietary habits and lifestyle.

Glyphosate was extracted using solid phase extraction (SPE) and analysed by liquid chromatography tandem mass spectrometry (LC-MC/MS). The method was linear over the calibration range 0 – 20 µg/L, the limit of detection (LOD) was set at 0.5 µg/L (signal to noise ratio of ≥ 3:1). Creatinine analysis was completed on all samples using an automated alkaline picrate method.

Results

Of the 50 study participants, 60% (n = 30) were female, the mean age was 42 (range 18 − 82), 60% (n = 30) were pet owners and only 10% (n = 5) were smokers. Participants reported that 70% (n = 35) of them consumed few or no portions of organic food. A large proportion of participants, 80% (n = 40), reported consuming 2 portions or less of bread per day.

Home use of pesticides was reported by 40% (n = 20) of participants and 16 of the 20 reported using glyphosate-based products such as Roundup, Gallup, and Weedol, they also reported wearing personal protective equipment when using pesticides (gloves, facemasks or both).

Of the 50 samples analysed, 10 samples (20%) contained detectable levels of glyphosate (0.79 – 1.32 µg/L). Based on results from urinary creatinine analysis, 47 samples were valid (creatinine values < 3.0 or > 30 mmol/L). The three invalid samples had no detectable level of glyphosate. Six of the detectable samples were from females, similar to the gender spread in the full data set. Of the detectable results, 3 out of the 10 participants used glyphosate-based products at home but none of them had used pesticides for at least a month previous (Table 1).

Table 1. Summary of the urinary glyphosate concentrations expressed as µg/L and as µmol/mol creatinine among 50 Irish adults sampled in 2017

Variable		
Samples	**Glyphosate**	
No. of samples analysed *n (%)*	50 (100)	
No. of valid samples *n (%)*	47 (94)	
No. of samples > LOD *n (%)*	10 (20)	
*Sample analysis of 10 samples > 0.5 µg L^{-1} (LOD)	µg L^{-1}	µmol/mol creatinine
Median	0.87	0.41
Maximum	1.35	0.89
Minimum	0.80	0.21

* 20% (10) of the samples analysed had detectable levels.

Conclusion

The proportion of detectable urinary glyphosate concentrations for samples collected from 50 Irish adults is low (20%), which could be due to less localised use of pesticides. A European study that had an LOD of 0.1 µg/L, reported low detection rates in Germany (32%), whereas in the USA, 93% of samples were above 0.5 µg/L (despite an LOD of 0.1 µg/L). The detection rate could possibly have underestimated the true population exposure proportion due to the small sample size and the higher limit of detection of the analytical method used in this study.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study is newly submitted for purpose of review. A biomonitoring survey involving the collection and analysis of 20 ml spot urine samples from 50 Irish adults on non-occupational setting was conducted. The LC-MC/MS analyses of urinary samples revealed that 10 out of 50 samples analysed (i.e. 20%) contained detectable levels of glyphosate (0.80 – 1.35 µg/L). The low proportion of detectable glyphosate levels could be due to lower localised use of pesticides, having a small sample size or the higher analytical detection limit used in this study (0.5 µg/L).

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good exposure study.

Reliability criteria of exposure studies

Publication: Connolly *et al*., 2018.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	?	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.	Y	
Exposure to formulations with glyphosate combined with other a.i.	Y	
Exposure to various formulations of pesticides	Y	
Study		
Study design clearly described	Y	
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	First morning urine void sample.
Analytical method described in detail	Y	To some extent, ref. to other paper.
Validation of analytical method reported	Y	To some extent, ref. to other paper.
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good exposure study.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Connolly A. *et al.*
Report year	2019
Report title	Evaluating glyphosate exposure routes and their contribution to total body burden: a study among amenity horticulturalists
Document No	Annals of Work Exposures and Health, 2019, 63 (2), 133–147
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

The purpose of this study was to evaluate determinants of dermal and inadvertent ingestion exposure and assess their contribution to total body burden among amenity horticultural users using glyphosate-based pesticide products. A dermal and inadvertent ingestion exposure assessment was completed alongside a biomonitoring study among amenity horticultural workers. Linear mixed effect regression models were elaborated to evaluate determinants of exposure and their contribution to total body burden.

A total of 343 wipe and glove samples were collected from 20 workers across 29 work tasks. Geometric mean (GM) glyphosate concentrations of 0.01, 0.04 and 0.05 µg/cm^2 were obtained on wipes from the workers' perioral region and left and right hands, respectively. For disposable and reusable gloves, respectively, GM glyphosate concentrations of 0.43 and 7.99 µg/cm^2 were detected. The combined hand and perioral region glyphosate concentrations explained 40% of the variance in the urinary (µg/L) biomonitoring data. Data show the dermal exposure is the prominent route of exposure in comparison to inadvertent ingestion but inadvertent ingestion may contribute to overall body burden.

Materials and methods

Site description and study population

Exposure assessments were conducted at sites managed by the Irish Commissioner for Public Works (OPW) from September 2016 to September 2017. Workers were grouped into three similar exposure groups (SEGs): manual knapsack, pressurised lance and controlled droplet applicator, based on the applicator used to apply glyphosate-based pesticide products. The manual knapsack applicator SEG (typical capacity of 10 – 15 L), was carried as a knapsack with the pesticide product being applied with a handheld lance. The pressurised lance SEG applied the pesticide product using a handheld lance connected to a motorised knapsack. The controlled droplet applicator SEG, similar to the manual knapsack, but with a capacity 5 L, was purchased with a pre-mixed solution (eliminating the mixing and loading task) and had an adaptable applicator that could increase the droplet size, thus reducing the spray drift.

Study participants were recruited via oral presentation and circulated project information leaflets. Participation was voluntary and all participants gave informed consent. Ethics approval for this project was received from the National University of Ireland, Galway Research Ethics Committee (Ref: 16 July 2019) on the 5th September 2016.

Biomonitoring samples

A biomonitoring study involving the collection of individual full urinary void spot samples was completed and previously been reported (Connolly *et al*., 2018). A minimum of three urine samples were collected from each participant: a sample before the task began, within 1 h of task completion and the following first morning void. For 59% (n = 17) of tasks, participants gave samples for each void over the exposure assessment period (pre-task to the following first morning void). Urine samples were analysed separately for glyphosate, so the sample with the highest glyphosate concentration could be identified for each task. The urine sample with the highest glyphosate concentration measured during the sampling period was selected and referred to as the peak urinary sample for that participant.

Dermal and inadvertent ingestion sampling strategy

Wipe samples of the hands, perioral region and of potentially contaminated work surfaces (pesticide product container, worker mobile phones and steering wheels of work vehicles) were collected. Wipe sampling was conducted using Ghost Wipes™, pre-packaged polyvinyl alcohol wipes wetted with deionised water by the manufacturer. Dermal and perioral wipe samples were collected from the workers before and after the sampling task. Pre-work task wipe samples were required to evaluate whether detectable data collected post-task was as a result of the observed pesticide application task. Workers' glove and surface wipe samples were collected after the pesticide application tasks. The researcher wore disposable nitrile gloves for collecting samples and changed these gloves with each sample obtained. An appropriate number of Ghost Wipes™ and glove field blanks were also collected.

Following sampling, wipes were placed in 100 mL plastic pots and appropriately labelled. Samples were extracted and aliquots were frozen to −18°C within 24 h of collection, shipped and chemical analysis completed at the Health and Safety Executive's Laboratory, Buxton, UK.

Dermal sampling

Hand wipes were collected, using two wipes per hand. The front of the hand was wiped with five strokes from the base of the hand to the top of the palm and then five strokes across the palm, starting from the base of the palm of the small finger. The wipe was folded in half and the same sequence repeated on the back of the hand. The wipes were then folded once more and each individual finger was wiped, starting at the small finger to the thumb, going between the fingers and including the finger web areas. Followed by the tips of each finger wiped in a circular motion. The same procedure was completed again with a second wipe and repeated for both hands. Dermal wipe samples were collected from the hands when workers removed their gloves, either during the task (i.e. lunch break) or after the pesticide application task.

Glove contamination samples

PPE use varied from disposable to reusable chemical resistant nitrile gloves as per company policy. After the work task, disposable glove samples were collected for glyphosate analysis, while only some participants provided their reusable gloves for analysis. Worker gloves were collected after the pesticide application tasks or within the task if gloves were changed during the pesticide task. At the end of the pesticide application task, dermal wipe samples were collected of each hand after the gloves were removed.

Inadvertent ingestion sampling

Perioral wipes were collected starting from the upper lip area and wiped in a clockwise motion around the upper lip and philtrum area and down around to the mentolabial fold to the edge of the mouth of the lower lip area. The wipe was folded in half and similarly wiped in an anti-clockwise direction, starting at the lower lip area and finishing at the upper lip area. One wipe was used for the perioral region.

An inadvertent ingestion observational study was also conducted. The frequency of worker contacts per task, (which in the current study included all surfaces contacted by the worker), frequency of worker hand to mouth contacts, contacts with the body and surrounding area (i.e. potentially contaminated surfaces) were recorded. The frequency of contacts was recorded during only the pesticide task. Worker contacts pre- and post-work task or during work breaks were not recorded.

Potentially contaminated work surface sampling

Potentially contaminated work surfaces were wiped using one Ghost Wipe™, according to an object-specific sampling protocol developed within the study. Specifically, mobile phones were first wiped on the front of the phone, from the top to the bottom of the screen in one stroke. The wipe was folded in half and wiped on the back from top to bottom in one stroke. Finally, the wipe was folded once more and the edge of the phone was wiped, starting at the top right hand corner and completing the full edge in a clockwise motion.

Similarly, work vehicle steering wheel wipe samples were wiped from the top of the steering wheel in a clockwise direction, then folded it in half and repeated in an anti-clockwise direction. The wipe was folded once more and the centre of the wheel was wiped in a clockwise direction and the spokes were wiped from the edge to the base of the steering wheel.

Pesticide product containers were wiped from top to bottom for the full width of the container. The bottom of the container was wiped from right to left in one stroke. The wipe was folded and the top of the container was wiped in a clockwise motion. The container handle and surrounding area were wiped from top to bottom afterwards. The wipe was folded once more and the area around the lid was wiped, and then the lid itself, in a clockwise motion.

Chemical analysis

Wipe samples from the hands and perioral region, as well as the disposable glove samples, were placed into a 100 mL plastic pot and extracted by adding 50 mL of deionised water, shaken vigorously for 30 seconds, then placed on the Denley Spriamax 5 roller mixer for an hour. A 20 mL aliquot was transferred to a labelled Sterilin™ pot for storage and transport before analysis. Glyphosate was extracted from large reusable gloves at the laboratory. One glove was placed into a grip seal bag with 100 mL deionised water. Bags were placed on a gyratory rocker for 1 h with bags being turned over at 30 min. The liquid contents were then transferred to Sterilin™ pots for storage before analysis. The solubility of glyphosate in water (11.6 g/L at 25°C) made it an appropriate extraction solvent.

All samples were prepared and analysed for glyphosate. Glyphosate was extracted from dermal wipe, surface wipe and glove extracted water samples (100 µL diluted with 900 µL deionised water) using strong anion exchange solid phase extraction eluting into 10% formic acid in methanol. The eluent was evaporated under a stream of nitrogen and reconstituted in 200 µL of 0.1% formic acid. Quantitative analysis was performed by liquid chromatography tandem mass spectrometry. Chromatographic separation was achieved on a Zorbax XDB-C8, 150 x 4.6 mm, 5 µm (Agilent, Stockport, UK) column with mobile phases of 0.1% formic acid and acetonitrile with a gradient elution. The method was linear over the range 1–1000 µg/L and the LOQ was 1 µg/L and the LOD was 0.5 µg/L. Where results exceeded the top of the linear range (1000 µg/L) the samples were repeated with dilutions. The method was reproducible with an intra assay CV of 2.9% (n = 12) and an inter assay CV of 4.2% (n = 42, over four runs).

Data processing and analysis

All 23 wipe field blanks had non-detectable glyphosate concentrations. Seventeen glove field blanks were collected from the sites (as some workers used reusable gloves), six having non-detectable glyphosate concentrations, whereas detectable levels were found in the remainder. For each task with detectable glyphosate levels found on the blank glove, each glove sample within that task was field blank corrected. All the samples were corrected for the sample volume and for the surface area wiped. Although samples were not corrected for recovery efficiencies, the mean recovery percentage for plastic containers, disposable chemical resistant nitrile gloves and mobile phones, spike at 20 µg, was 122, 104 and 125%, respectively. Ghost wipes™ have a mean recovery for three samples spiked at 200 µg of 106%.

The average hand surface area measurements were assigned according to published US EPA guidance (US EPA, 2011). The glove adjustments were assigned in the same manner as the surface area for hands. This assumes a 1070 cm^{-2} for both male hands, or 535 cm^{-2} surface per hand and 890 cm^{-2} for both female hands, or 445 cm^{-2} per hand. Average surface area measurements for the perioral region were assigned as 40 cm^{-2}.

Surface area calculations for the steering wheel were assigned as 1100 cm^{-2} surface area. An average mobile phone surface area of 202 cm^{-2} was calculated using the physical phone dimension measurements, based on the phone type sampled. Similarly, for the product containers, an average surface area value of 2300 cm^{-2} was calculated.

Statistical analysis

Before the conducting statistical analysis, all concentration levels below the LOQ were imputed, in SAS v 9.4 (SAS Institute, North Carolina, USA). A single random imputation method based on maximum likelihood estimation was used. The remainder of the statistical analysis was performed using Stata Statistical Software 15 (StataCorp, 2015).

The data were log normally distributed and thus all statistical analysis was performed with log transformed exposure concentrations. Summary and descriptive analysis was performed on the work demographics and glyphosate concentrations levels for the combined dataset and by SEG. The results for the potentially contaminated surfaces are only shown for the combined dataset.

Pearson's correlation coefficients were estimated to evaluate relationships between glyphosate concentrations on the right and left hand, the dominant hand and both hands combined. Similar tests were performed on the glove data.

A linear mixed effect regression model was elaborated based on exposure determinants for inadvertent ingestion previously identified and evaluated in regression analysis against measurements of metals (Gorman Ng *et al.*, 2017). In this model, the hand contamination and the frequency of contacts per task were entered as fixed effects whereas the worker's id was entered as a random effect to account for correlations between repeated measurements from the same worker. This model had some differences to Gorman Ng *et al.*, 2017 model, including that respiratory protective equipment (RPE) was not considered as it was used by all workers participating in the study and that we used frequency of contacts per task, not just hand to perioral/oral contacts per task. Further models using a forward model-built approach were elaborated to examine the robustness of the derived model as well as to identify determinants for inadvertent ingestion and dermal exposure and their relative contribution to overall body burden. In these models, parameters were entered sequentially based on their level of significance and kept within the model if they had a statistical significance of ($P < 0.1$).

Results

Demographic and working characteristics

Twenty amenity horticulturists who applied glyphosate-based pesticide products as part of their daily duties participated in the study (18 males and 2 females) were grouped into 3 SEGs. The pesticide task duration ranged from approximately a half hour to 6 hours. Work tasks involving the manual knapsack, controlled droplet applicator and the pressurised applicator were, on average, ~3, 3½ and 6 h, respectively.

Good worker compliance with PPE use was observed, with workers using PPE for most of the work tasks sampled; gloves, Tyvek suits and RPE were used for 29 (100%), 26 (90%) and 28 (97%) of the observed tasks, respectively.

Levels of glyphosate concentrations on wipes, gloves and contaminated surfaces

A total of 343 wipe and glove samples across 29 work tasks were collected and analysed for glyphosate. A minimum of seven sets of wipe samples were collected for each task sampled. A sample set consists of a blank wipe, perioral sample and each hand sample (two wipes per hand), before and after each work task.

Glyphosate concentration data for perioral and hand wipes (μg/cm), collected pre and post the work tasks are presented in Table 1 for overall samples and per SEG. Table 2 details the glyphosate concentration data for the disposable and reusable gloves samples. Seventeen pairs of disposable gloves and seven pairs of reusable gloves were analysed in this study. For three of the work tasks analysed, workers wore disposable gloves over a pair of reusable gloves and gave both sets of gloves for analysis. For eight of the tasks, the workers refused to give their gloves. Glyphosate was detected in all the pre-

and post-work task hand wipe samples, as well as on the post-work task glove samples. Only 11 (38%) of the pre-work task and 6 (21%) post-work task perioral wipes had non-detectable glyphosate concentrations. For a third of work tasks sampled (n = 10), workers had started the pesticide task prior to the collection of pre-work task samples. Detectable glyphosate concentrations were also found on 11 (65%) of the blank gloves samples, collected from PPE field stores. Field observations suggest that cross contamination may occur when storing new gloves in close proximity of the pesticide chemical storage area or when handling unused gloves with contaminated hands.

In the current study, arithmetic mean (range) glyphosate concentrations of 2708 (3.0–21 845) μg wipe^{-1} and 2797 (5.0–27 354) μg wipe^{-1}, (right and left hand, respectively) were found on worker hand wipe samples collected after the pesticide application task. Glyphosate concentration levels of 41 (< LOQ–321) μg per wipe were detected on wipes collected from the perioral region. Values reported for hand wipes in this study were higher than those found for agricultural pesticide users 647 (83–2081) μg (Christopher, 2008). However, the perioral glyphosate concentrations were within a range of that reported in Christopher (2008), with an arithmetic mean and range of 39.5 (2.6 – 91) μg. Christopher (2008) also detected glyphosate in worker saliva samples with an arithmetic mean and range of 140 (56–440) ng, which could suggest comparable inadvertent ingestion levels for the current study workers.

Strong positive associations were found between left and right hands, and between the dominant hand, the individual hands and the combination of both hands. Between SEGs, similar glyphosate concentrations were detected on perioral wipes, with the highest geometric mean (GM) and maximum value found in the pressurised lance SEG. Within SEGs, similar glyphosate wipe concentrations were found on both the right and left hands in the manual knapsack group and the pressurised lance applicator. Glyphosate concentrations on reusable gloves were two orders of magnitudes higher than those on disposable gloves.

On some occasions, additional dermal and gloves samples were collected for workers who took a break during the pesticide application task. For three of the work tasks, dermal wipe samples were collected from three participants before taking a break and the glyphosate concentrations for the perioral and both hands (combined) ranged from 0.01 to 0.15 and 0.002 to 0.41 μg/cm, respectively. Similarly, three participants provided their disposable gloves during the break for five of the work tasks, which included two of these participants giving samples on two consecutive days and found glyphosate concentrations that ranged from 1.27 to 20.89 μg/cm. As concentrations were similar to the post-work task samples and due to the limited sample numbers, this data were not included in further analysis. The additional samples collected could only have had a negligible effect on the post-work task samples due to similar detection levels. The assumption would be that workers would wash hands and dispose of contaminated work gloves before their break, removing the contaminant, thus no accumulation of glyphosate concentration occurs on the post-work task samples.

Wipe samples data from pesticide product containers (n = 21), work vehicle steering wheels (n = 10) and participant's personal mobile phones (n = 18) are presented in Table 3. Of all the potentially contaminated work surfaces sampled in this study (n = 49), the highest glyphosate concentrations were detected on the pesticide product containers. A pesticide product container is considered to be handled up to 50 times by the worker before disposal.

Glyphosate was also detectable on all wipes from work vehicle steering wheels (n = 21), with a mean and range of 1928 (478–5984) μg wipe^{-1} (unadjusted values). These included small tractors and vehicles (e.g. vans, cars) used to transport equipment to and around field sites. Participating workers drove the work vehicle, on some occasions to travel to multiple sites within a day, and performed the required pesticide application tasks. Only two (11%) of the mobile phone samples had non-detectable glyphosate concentrations. Most mobile phones were personal use phones.

Table 1. Glyphosate wipe concentration data (µg/cm) for pre- and post-work task perioral and hand (left and right) measurements. Results are presented as for the overall sample and per similar exposure group concerned

Variable	*k*	*N*	<LOQ *N* (%)	GM	GSD	Min	Max	<LOQ *N* (%)	GM	GSD	Min	Max
Combined SEGs				Pre-work task samples					Post-work task samples			
Perioral	20	29	11 (38%)	2.1×10^{-03}	12.93	8.2×10^{-06}	0.15	6 (21%)	0.01	9.05	1.7×10^{-04}	0.40
Hand left	20	29	0	6.1×10^{-03}	8.77	9.4×10^{-05}	0.31	0	0.04	9.21	4.7×10^{-04}	2.56
Hand right	20	29	0	6.5×10^{-03}	9.09	9.4×10^{-05}	0.22	0	0.05	8.73	2.8×10^{-04}	2.04
Manual knapsack				Pre-work task samples					Post-work task samples			
Perioral	10	12	6 (50 %)	1.1×10^{-03}	21.30	8.2×10^{-06}	0.15	4 (33%)	0.01	11.30	1.7×10^{-04}	0.23
Hand left	10	12	0	2.2×10^{-03}	8.47	9.4×10^{-05}	0.07	0	0.01	4.64	4.7×10^{-04}	0.08
Hand right	10	12	0	2.7×10^{-03}	11.00	9.4×10^{-05}	0.14	0	0.01	5.26	2.8×10^{-04}	0.09
Pressurised lance				Pre-work task samples					Post-work task samples			
Perioral	6	10	2 (20 %)	3.9×10^{-03}	8.56	5.8×10^{-05}	0.05	0	0.04	4.68	2.5×10^{-03}	0.40
Hand left	6	10	0	2.2×10^{-02}	10.01	3.7×10^{-04}	0.31	0	0.19	9.17	2.6×10^{-03}	2.56
Hand right	6	10	0	1.8×10^{-02}	10.62	4.7×10^{-04}	0.22	0	0.21	7.41	3.7×10^{-03}	2.04
Controlled droplet				Pre-work task samples					Post-work task samples			
Perioral	5	7	3 (43 %)	2.6×10^{-03}	9.44	1.1×10^{-04}	0.03	2 (29%)	0.01	8.34	6.0×10^{-04}	0.10
Hand left	5	7	0	5.9×10^{-03}	2.39	2.4×10^{-03}	0.03	0	0.04	5.89	3.5×10^{-03}	0.50
Hand right	5	7	0	6.8×10^{-03}	2.09	3.0×10^{-03}	0.02	0	0.06	5.73	7.9×10^{-03}	0.85

k is the number of participants in the group. *N* is the number of samples (one sample per task) in the group. Number of samples below the limit of quantification (<LOQ) (1 µg l^{-1}) by number (*N*) and percentage (%). GM is the geometric mean, GSD the geometric standard deviation and the range (min–max).

Table 2. Glyphosate wipe concentration data (µg/cm) for the post-work task glove samples. Results are presented for the overall sample and per similar exposure group concerned

Variable	*k*	*N*	GM	GSD	Min	Max	*k*	*N*	GM	GSD	Min	Max
Combined SEGs			Post-work task disposable gloves						Post-work task reusable gloves			
Glove left	12	17	0.18	6.14	3.9×10^{-03}	2.78	6	6	4.49	4.68	0.81	58.87
Glove right	12	17	0.20	6.96	2.4×10^{-03}	10.62	6	6	4.52	4.43	0.73	66.27
Glove both	12	17	0.43	5.67	0.02	13.41	6	7	7.99	4.14	1.53	125.1
Manual knapsack			Post-work task disposable gloves						Post-work task reusable gloves			
Glove left	7	8	0.06	5.39	3.9×10^{-03}	0.63	1	1	—	—	6.11	6.11
Glove right	7	8	0.07	6.56	2.4×10^{-03}	0.49	1	1	—	—	6.43	6.43
Glove both	7	8	0.15	4.46	0.02	1.12	1	1	—	—	12.54	12.54
Pressurised lance			Post-work task disposable gloves						Post-work task reusable gloves			
Glove left	3	4	0.39	2.42	0.19	1.23	2	2	—	—	6.64	58.87
Glove right	3	4	0.36	1.44	0.26	0.58	2	2	—	—	6.11	66.27
Glove both	3	4	0.80	1.94	0.46	1.76	2	2	—	—	12.74	125.1
Controlled droplet			Post-work task disposable gloves						Post-work task reusable gloves			
Glove left	3	5	0.55	5.83	0.04	2.78	2	3	1.51	1.75	0.81	2.39
Glove right	3	5	0.75	7.29	0.05	10.62	2	3	1.49	1.86	0.73	2.17
Glove both	3	5	1.35	6.76	0.09	13.41	3	4	3.19	1.64	1.53	4.56

k is the number of participants in the group. *N* is the number of samples (one sample per task) in the group. There was no samples in this table that were below the limit of quantification (<LOQ) (1 µg l^{-1}). GM is the geometric mean, GSD the geometric standard deviation and the range (min–max).

Table 3. Glyphosate concentration data (µg/cm) for wipe samples collected from work surfaces. Sampling from those was performed post-work task completion and results are presented as geometric mean (GM), geometric standard deviation (GSD) and range (min–max) for the overall sample

Variables	*k*	*N*	<LOQ*N* (%)	GM	GSD	Min	Max
Combined SEGs							
Product container	15	21	0	2.06	7.48	0.01	27.7
Steering wheel	7	10	0	0.06	2.44	0.02	0.27
Mobile	15	18	2 (11%)	0.004	6.50	1.0×10^{-4}	0.12

k is the number of participants in the group. *N* is the number of samples (one sample per task) in the group. Samples that are below the limit of quantification (<LOQ) (1 µg l^{-1}) by number (*N*) and percentage (%). GM is the geometric mean, GSD the geometric standard deviation and the range (min–max).

Differences in exposure levels across SEGs, sampling and working parameters

Disposable worker gloves had the highest glyphosate concentrations, followed by worker hands (post-work task). Glyphosate concentrations were lowest on perioral wipes (Fig. 1a). The highest glyphosate concentrations were detected on wipe samples collected from the pesticide product containers, followed by much lower levels on the work vehicle steering wheel. The lowest concentrations were detected on the worker mobile phones (Fig. 1b). Three of the participants in this study had their mobile phone wiped on two separate occasions, and on both occasions, glyphosate concentrations were detected.

A strong positive relationship was found between urinary glyphosate concentrations (µg/L) and glyphosate wipe concentrations (µg/cm) on the worker perioral wipe samples (Fig. 2a). Similarly, perioral region and worker hands glyphosate concentrations (µg/cm) correlated positively and strongly (Fig. 2b). Some of the participants started the work task before monitoring began. Although this has no influence on the dermal exposure assessments measurements as samples were taken before and after the work task but it could potentially impact on the urinary concentrations which may not accurately reflect the full day's exposure. The precision of peak urinary concentration estimates used within our analysis may be dependent on the number of samples available for participants. However, no statistically significant differences were found in peak urinary concentrations between participants.

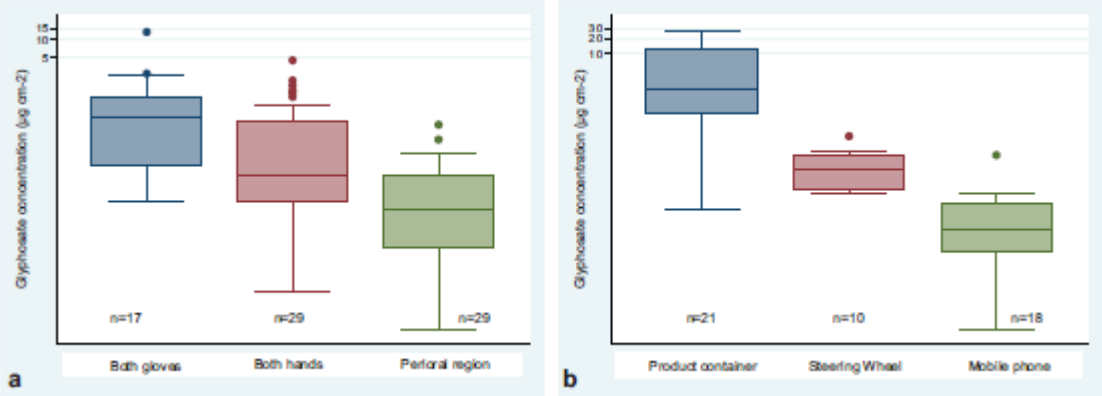


Figure 1. Boxplot showing the post-work task glyphosate concentrations for (a) disposable gloves, the workers hands (under the glove) and the perioral region and (b) for potentially contaminated work surfaces, pesticide product containers, the steering wheel of work vehicles and participants mobile phone (µg/cm). The box is the 25th to the 75th percentile, the line within the box is the median and the whiskers the lower and the upper adjacent values. Single points indicate outliers. *n is the number of samples. Reusable gloves were not included in the boxplot (a) due to the low number of samples. Mean measured concentrations were statistically different between all types of samples (*t*-tests; P < 0.001).

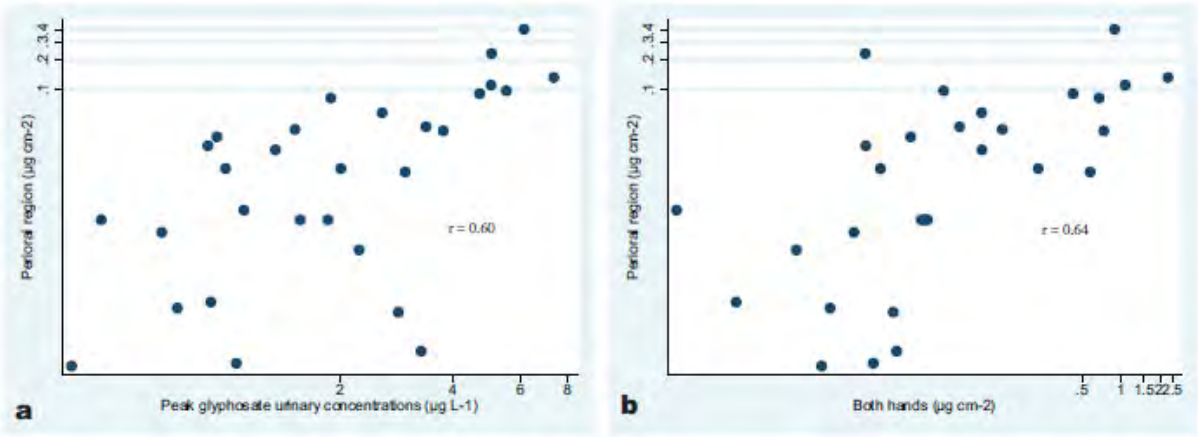


Figure 2. Scatter graph showing moderate relationships between glyphosate perioral glyphosate wipe contamination levels (µg/cm) and (a) peak urinary glyphosate concentrations (µg/L) and (b) both hands glyphosate surface loading contamination levels after a pesticide application task (µg/cm).

Elaborated linear mixed effect regression models are presented in Table 4. The model evaluating previously documented determinants of inadvertent ingestion exposure Gorman Ng *et al.* (2017), explained 45% of the variability for the glyphosate concentrations found on the perioral region. In this model (Model 1), an increase in the frequency of contacts per task and post-task hand contamination was significantly associated with an increased in perioral glyphosate concentrations.

Forward building of the same model on the basis of improvement of the fit parameters resulted with the same parameters being included alongside sampling time. The effects for hand contamination and

frequency of contacts per task were comparable to those of the Model 1, whereas perioral concentrations also increased with increasing sampling time ($\beta = 0.01$; $P < 0.08$). This model explained ~50% of the total variability in perioral glyphosate concentrations.

The forward built model examining determinants of participants' hand contamination (Model 2) comprised of the task sampling time, the age of the participant and the SEG and explained 62% of the total variability of glyphosate concentrations measured on the hands.

All the workers who participated in this study wore gloves when applying pesticides. Gloves were used throughout the task however it was observed that on a number of occasions workers would remove their gloves for various reasons (e.g. checking mobile phones, to drive work vehicles, when going on a break, etc.). All had detectable glyphosate concentrations on their hands. Field observations suggest that poor glove doffing procedures or removing gloves during the work task (e.g. answering the phone, adjusting facemasks) may be responsible for hand contamination.

Table 4. Mixed effect models with participants' identification number included in the models as a random effect. Model 1 results are describing the effects of hand contamination and the frequency of contacts per task on glyphosate concentrations measured on the perioral region. Model 2 results describe the effects of sampling time, the age of the workers and work task characteristic on glyphosate surface loading concentrations measured on the hands. Measurements are given as on the log-transformed glyphosate concentrations per surface area (µg cm−2) and were taken from 20 workers over 29 pesticide application tasks.

Covariate	Model 1			Model 2		
	β	SE	*P*	β	SE	*P*
Intercept	−3.91	0.70	0.00	−10.05	1.67	0.00
Glyphosate conc. of both hands, post-work task (µg cm^{-2})	0.56	0.16	0.00			
Sampling time of the work task (mins)				0.01	0.003	0.01
Total frequency of contacts per task	0.02	0.01	0.02			
Age of the participant (years)				0.12	0.04	0.00
Similar exposure groups						
Pressurised lance				1.41	0.67	0.04
Controlled droplet applicator				0.69	0.67	0.30
Manual knapsack				Ref	—	—
Between variance (naive model)		1.18 (2.32)			0.00 (3.94)	
Within variance (naive model)		1.55 (2.62)			1.85 (0.97)	
Total variance explained		45%			62%	

β, regression coefficient for log-transformed exposure data; SE, standard error. The naive model is the results from the model without the inclusion of any fixed effects.

To identify the relative contribution of the routes of exposure to the total uptake of glyphosate, a separate model was forward built using the log-transformed results of the biomonitoring exposure measurements as the dependent covariate. The final elaborated model comprised of only the combined hand and perioral region glyphosate concentration (Table 5). Overall, the model explained 40% of the total variance in urinary concentrations. The hands and perioral data were not entered as separate covariates in the model, as they were highly correlated ($r = 0.64$; $P < 0.001$). Hand and perioral glyphosate concentrations are important determinants of total glyphosate body burden (glyphosate urinary concentrations), explaining 40% of variance in the urine data. Discriminating the individual contribution for each route to the total body burden was not possible. However, hand glyphosate concentrations alone explained approximately one third of the variance in the glyphosate urine concentrations, which would indicate that dermal exposure was the predominant route but that inadvertent ingestion may contribute to overall body burden since the presence of glyphosate contamination in the perioral region may result in ingestion and/or dermal absorption.

Table 5. Mixed effect model describing the effects of glyphosate concentration of the combined hands and the perioral region on the log-transformed glyphosate concentrations (µg/L) measured of 20 workers over 29 pesticide application tasks. Mixed models build with participants' identification number as a random effect.

Covariate	β	SE	*P*
Intercept	1.20	0.19	0.00
Ln concentrations in hand and perioral region surfaces	0.26	0.06	0.00
Between variance (naive model)		0.15 (0.36)	
Within variance (naive model)		0.22 (0.27)	
Total variance explained		40%	

β, regression coefficient for log-transformed exposure data; SE, standard error. The naive model is the results from the model without the inclusion of any fixed effects.

Conclusion

The analysis of a total of 343 wipe and glove samples were collected from 20 workers across 29 work tasks revealed the GM glyphosate concentrations of 0.01, 0.04 and 0.05 µg/cm^2 on wipes from the workers' perioral region and left and right hands, respectively. For disposable and reusable gloves, respectively, GM glyphosate concentrations of 0.43 and 7.99 µg/cm^2 were detected. The combined hand and perioral region glyphosate concentrations explained 40% of the variance in the urinary (µg/L) biomonitoring data. The results of the study show dermal to be a prominent route of exposure but support inadvertent ingestion potential contribution to the total body burden among this worker group. The study also identified a potential for the spread of contamination among non-pesticide users in the workforce and para-occupational exposures. Study results also showed that PPE practice is an important determinant of both inadvertent ingestion and dermal exposure. An implementation of PPE management and work practices policies for pesticide use could potentially reduce both occupational exposures and para-occupational exposures.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study is newly submitted for purpose of review. The total uptake of glyphosate was assessed in parallel with dermal and inadvertent exposure routes, using urine, wipes and glove samples collected from 20 workers across 29 work tasks. The average hand surface area measurements were assigned according to published US EPA guidance. Geometric mean (GM) glyphosate concentrations of 0.01, 0.04 and 0.05 µg/cm^2 were obtained on wipes from the workers' perioral region and left and right hands, respectively. For disposable and reusable gloves, respectively, GM glyphosate concentrations of 0.43 and 7.99 µg/cm^2 were detected. The combined hand and perioral region glyphosate concentrations explained 40% of the variance in the urinary (µg/L) biomonitoring data. Data show the dermal exposure is the prominent route of exposure in comparison to inadvertent ingestion, but inadvertent ingestion may contribute to overall body burden. The study also identified potential exposure to non-pesticide users in the workplace and para-occupational exposures.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good exposure study.

Reliability criteria of exposure studies

Publication: Connolly *et al.*, 2019.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.	Y	
Exposure to formulations with glyphosate combined with other a.i.	Y	
Exposure to various formulations of pesticides	Y	
Study		
Study design clearly described	Y	
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good exposure study.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Conrad U. *et al.*
Report year	2017
Report title	Glyphosate in German adults – Time trend (2001 to 2015) of human exposure to a widely used herbicide
Document No	International Journal of Hygiene and Environmental Health 220 (2017) 8-16
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

The purpose of this study was to elucidate the internal exposure of the general German population to glyphosate and aminomethylphosphonic acid (AMPA) and its change over time.

The broadband herbicide glyphosate (N-[phosphonomethyl]-glycine) and its main metabolite AMPA were analysed by GC-MS-MS in 24 h-urine samples cryo-archived by the German Environmental Specimen Bank (ESB). Samples collected in 2001, 2003, 2005, 2007, 2009, 2011, 2012, 2013, 2014, and 2015 were chosen for this retrospective analysis. All urine samples had been provided by 20 to 29 years old individuals living in Greifswald, a city in north-eastern Germany.

Out of the 399 analysed urine samples, 127 (= 31.8%) contained glyphosate concentrations at or above the limit of quantification (LOQ) of 0.1 g/L. For AMPA this was the case for 160 (= 40.1%) samples. The fraction of glyphosate levels at or above LOQ peaked in 2012 (57.5%) and 2013 (56.4%) after having discontinuously increased from 10.0% in 2001. Quantification rates were lower again in 2014 and 2015 with32.5% and 40.0%, respectively. The overall trend for quantifiable AMPA levels was similar. Glyphosate and AMPA concentrations in urine were statistically significantly correlated (spearman rank correlation coefficient = 0.506, $p \leq 0.001$). Urinary glyphosate and AMPA levels tended to be higher in males. The possible reduction in exposure since 2013 may be due to changes in glyphosate application in agricultural practice.

Materials and Methods

Sampling and study group; This retrospective monitoring study was based on 24 h-urine specimen collected in the annual sampling of the German ESB. To reduce the risk of contamination, all containers needed for sampling and aliquoting were carefully cleaned before use according to standard operating procedures. All samples have been provided by young adults (predominantly students) aged 20 to 29 years. To follow the time trend of human exposure to glyphosate and AMPA, cryo-preserved urine samples collected in 2001, 2003, 2005, 2007, 2009, 2011, 2012, 2013, 2014, and 2015 were analysed. All urine samples were collected from individuals living in Greifswald, a city in north-eastern Germany. Annual ESB sampling in Greifswald was regularly carried out in the period of March and April. From each of the ten study years, 24 h-urine samples donated by 20 male and 20 female participants were randomly selected for analyses. The only inclusion criterion for this main study sample was that no specifically restricted diet – mainly vegetarian or vegan – had been reported by the sample provider in the self-administered ESB questionnaire. In 2001 the questionnaire item on dietary restrictions had not yet been implemented. Therefore, some samples from 2001 may have been provided by vegetarians or vegans. The fraction of vegetarian or vegan ESB participants, however, remained roughly between 2 and 14% from 2002 to 2014 followed by fractions up to 18% in 2015. Therefore, it can be assumed that,

if any, only very few participants with restricted diets might have erroneously been included in the 2001 sample. One 2013 measurement had to be excluded from the main study sample, as the participant was later identified not to fulfil the inclusion criterion.

Hence, the main sample of this study consisted of 399 participants living in the ESB sampling location Greifswald with virtually equal sample sizes and sex ratios in each study year (cf. Table 1).

Table 1. Description of sample composition (ESB participants from Greifswald analysed for glyphosate and AMPA concentrations in 24 h-urine (no self-reported specific dietary restrictions)

Year	Sample size (male/female)	Age [years] AM (range)	24 h-urine volume [mL] AM (range)	Creatinine in urine [g/L] AM (range)	BMI [kg/m^2] AM (range)
2001	40 (20/20)	23.2 (20–28)	1757 (490–3000)	0.98 (0.38–3.73)	22.3 (17.8–27.4)
2003	40 (20/20)	24.6 (20–28)	1793 (770–2850)	1.06 (0.42–2.11)	23.4 (17.3–31.2)
2005	40 (20/20)	23.1 (20–29)	1910 (895–2841)	0.90 (0.29–2.38)	22.6 (18.1–33.2)
2007	40 (20/20)	23.8 (20–28)	1937 (771–3047)	0.96 (0.36–2.92)	23.3 (18.0–34.6)
2009	40 (20/20)	24.3 (20–29)	1959 (701–3438)	0.85 (0.37–2.39)	22.7 (17.8–34.6)
2011	40 (20/20)	24.3 (20–29)	1893 (783–3045)	0.87 (0.26–2.17)	23.6 (18.0–39.1)
2012	40 (20/20)	24.4 (20–29)	1802 (768–3076)	0.97 (0.32–2.27)	22.8 (17.5–29.8)
2013	39 (20/19)	24.7 (20–29)	1973 (924–3081)	0.75 (0.20–1.60)	23.9 (19.3–40.9)
2014	40 (20/20)	24.1 (20–28)	1958 (760–3069)	0.82 (0.28–2.07)	23.2 (17.9–44.8)
2015	40 (20/20)	24.3 (20–28)	1759 (588–2956)	0.93 (0.18–2.02)	23.0 (17.9–36.8)
Total	399	24.1 (20–29)	1874 (490–3438)	0.91 (0.18–3.73)	23.1 (17.3–44.8)
Male	200	24.4 (20–29)	1881 (490–3076)	1.09 (0.20–3.73)	24.1 (17.4–36.8)
Female	199	23.8 (20–29)	1866 (600–3438)	0.73 (0.18–2.02)	22.0 (17.3–44.8)

Notes: AM = arithmetic mean, BMI = body mass index.

Male ESB participants tended to have higher BMI values and urinary creatinine levels than females. To investigate possible regional/seasonal differences of glyphosate and AMPA levels 40 urine samples collected in January 2005 and 2015 at the ESB sampling location Muenster (a city in north-western Germany) have additionally been analysed. Moreover, 20 urine samples from vegetarian or vegan participants have been analysed as well, in order to investigate differences due to diet. These samples were collected in Greifswald in the years 2007 (10 females) and 2015 (5 males and 5 females) and represent all available samples from vegetarian or vegan participants. A description of the two additionally analysed comparative ESB sub-populations is provided in Table 2. Participants in Muenster tended to have slightly lower BMI values. The other sub-population of self-reported vegetarians/vegans also exhibited lower average BMI values as well as higher 24 h-urine sample volumes and lower urinary creatinine concentrations.

Table 2. Description of two sub-populations from Muenster (no self-reported specific dietary restrictions) and Greifswald (self-reported vegetarians/vegans) analysed for comparison with the main study sample

Year	Sample size (male/female)	Age [years] AM (range)	24 h-urine volume [mL] AM (range)	Creatinine in urine [g/L] AM (range)	BMI [kg/m^2] AM (range)
ESB sampling location Muenster (no self-reported specific dietary restrictions)					
2005	40 (20/20)	23.6 (20–28)	1790 (691–2962)	1.03 (0.19–2.41)	22.2 (17.4–29.3)
2015	40 (20/20)	23.6 (20–28)	1991 (271–4601)	0.75 (0.35–1.73)	22.1 (18.3–28.6)
Total	80 (40/40)	23.6 (20–28)	1891 (271–4601)	0.89 (0.19–2.41)	22.1 (17.4–29.3)
Male	40	24.0 (20–28)	1934 (797–2952)	0.98 (0.19–2.41)	23.1 (19.6–28.6)
Female	40	23.3 (20–28)	1847 (271–4601)	0.80 (0.35–1.65)	21.1 (17.4–29.3)
Self-reported vegetarians/vegans (ESB sampling location Greifswald)					
2007	10 (0/10)	24.5 (23–28)	2293 (457–3011)	0.67 (0.21–2.51)	23.0 (19.9–28.1)
2015	10 (5/5)	24.3 (20–28)	1831 (773–2993)	0.72 (0.24–1.40)	22.1 (17.7–25.1)
Total	20 (5/15)	24.4 (20–28)	2062 (457–3011)	0.69 (0.21–2.51)	22.5 (17.7–28.1)
Male	5	25.4 (24–27)	1915 (1135–2813)	0.80 (0.31–1.36)	22.6 (21.3–23.9)
Female	15	24.1 (20–28)	2111 (457–3011)	0.66 (0.21–2.51)	22.5 (17.7–28.1)

Notes: *AM = arithmetic mean, BMI = body mass index.*

Analytical procedure; The chemical analysis was based on the method by Alferness and Iwata (1994) initially developed for trace analysis of Glyphosate and AMPA in food which uses gas chromatography (GC) coupled with a single quadrupole mass selective detector (MSD). The newly developed method applied in the present study used GC with tandem mass spectrometry (GC-MS-MS) to reach a low limit of quantification (LOQ) in human urine along with high selectivity. Isotope labelled internal standards have been used for further increasing the method's performance.

Standards and reagents; All chemicals were of analytical grade unless stated otherwise. Reference compounds (glyphosate and AMPA) and internal standards (1,2-13C2-15N-glyphosate and 13C-15N-AMPA) were obtained from Dr. Ehrenstorfer (Augsburg, Germany) as solutions in water (10 g/mL each). 2,2,2-trifluoroethanol (99%), trifluoroacetic anhydride (99%) and acetonitrile were purchased from Sigma-Aldrich (Seelze, Germany). Methanol was obtained from Merck (Darmstadt, Germany). Water was purified by an ultra-water purification system from ELGA (Ransbach-Baumbach, Germany).

Sample preparation; 50 L of urine sample and 25 L of the internal standard (IS) solution (containing 4 ng/mL of each IS) were transferred to 10 mL screw-capped glass tubes containing 1 mL of acetonitrile. After evaporation to dryness in a vacuum centrifuge, 0.5 mL of 2,2,2-trifluoroethanol and 1 mL of freezing cold (-40 °C) trifluoroacetic anhydride were added cautiously to the residue. The derivatization of the analytes was started by heating the closed tubes to 85 ◦C for 1 h in a heating block. After cooling down to room temperature the mixture was cautiously evaporated to dryness. The oily residue was then dissolved in 100 L of methanol and transferred into a microvial. This final solution was used for GC-MS-MS analysis. Mixed glyphosate and AMPA calibration solutions were prepared by serial dilution of a stock solution (each 5 ng/mL) in solutions of 50 L water in 1 mL acetonitrile containing 25 L of the IS-solution. These solutions were processed in the same way as described for human urine samples and represent sample concentrations from 0.1 to 10 g/L.

GC-MS-MS analysis; The derivatised analytes were separated by gas chromatography using a GC system7890 equipped with a split/split less injector (Agilent) and a MPS2 autosampler (Gerstel). The GC column was a HP INNOWAX, 30 m length, 0.25 mm internal diameter and 0.25 m film thickness (Agilent). The mass spectrometric parameter and ion transitions used are summarised in Table 3. While the primary transitions are well suitable for quantification of glyphosate and AMPA at low environmental internal exposure levels, the secondary transitions of glyphosate and AMPA only worked well at urine concentrations beyond approx. 20 g/L to confirm the identity of analytes. As the method was clearly focused on reaching the lowest quantification limits in human urine, the secondary transitions were not considered. The high specificity of the primary ion transition was evaluated during the validation of the analytical method.

Table 3. Mass spectrometric parameter and ion transitions used in glyphosate and AMPA analyses

Mass spectrometric parameters	
Instrument	Agilent 7000
Ion source temperature	150 °C
Ionization type	Negative chemical ionization (NCI)
Chemical ionization gas	Methane 4.5
Collision gas	Argon 5.0
Electron multiplier	Relative +200 V

Mass transfers of analytes and internal standards

Transition	Precursor ion [m/z]	Product ion [m/z]	Collision energy [V]	Use
Glyphosate 1st transition	370	245	10	Analyte quantifier
Glyphosate 2nd transition	351	268	5	Of limited suitability
AMPA 1st transition	351	268	5	Analyte quantifier
AMPA 2nd transition	271	188	5	Of limited suitability
1,2-$^{13}C_2$-^{15}N-glyphosate	371	246	10	Internal standard
^{13}C-^{15}N-AMPA	353	270	5	Internal standard

Notes: The secondary transitions of glyphosate and AMPA are listed for sake of completeness only. As they provide reliable confirming information only at concentrations beyond 20 µg/L, they have not been used in this study.

Validation and quality assurance measures of analytical method; For evaluation of the method performance the requirement of SANCO guideline 825 (European Commission, 2010) were considered which are mandatory for analytical methods in the context of pesticide registration and monitoring. Specificity, linearity, working range, accuracy, precision and LOQ were investigated for method evaluation. It can be concluded that the primary transition was very selective for a reliable quantification of glyphosate and AMPA.

The specificity of the analytical method was checked by the chromatography of unfortified human urine samples which showed no other interfering peaks besides the analytes. Further, the sample solutions of 44 unfortified human urine samples containing residues of glyphosate were analysed in parallel using

separation columns with phases of different selectivity. Analysed concentrations of glyphosate (n = 44 > LOQ) and AMPA (n = 25 > LOQ) ranged from 0.2 to 5 µg/L on both columns and correlated well: The respective slopes of the linear regression lines were close to unity (1.03 for glyphosate and 1.12 for AMPA) and the coefficients of determination (R2) reached satisfactory values (0.9968 for glyphosate and 0.9893 for AMPA). Therefore, it could be concluded that the primary transition was very selective for a reliable quantification of glyphosate and AMPA.

Basic calibration was performed by the measurement of 8 calibration solutions with concentrations ranging from 0.1 to 10 µg/L. A linear relationship between concentration and the ratio of the peak area of glyphosate and AMPA and its internal standards was observed. All calibration curve points were within 15% of their respective theoretical value. The linear correlation coefficients were typically > 0.99. Calibration curves for glyphosate and AMPA based on water and pooled human urine were both linear (each R2 > 0.99) and ran parallel. The slopes differed only by approximately 2%. This indicates that possible matrix effects are well compensated by the internal standards and matrix matched calibration solutions are not required for accurate determination of glyphosate and AMPA. The LOQ for glyphosate and AMPA was determined by fortification of human urine samples. The lower level at 0.1 µg/L demonstrated sufficient recovery (86 to 115%) and precision (8.9to 9.1%) for both analytes. This concentration was set as the LOQ of the GC-MS-MS method. The urine samples were analysed in a randomised order. Blank values (urine substituted by water) were measured during the analysis of urine samples regularly every 15th sample. All blank values were below the LOQ of 0.1 µg/L. Evaluation of the accuracy and precision of the method was performed through recovery experiments. Pooled human urine samples with no detectable amount of glyphosate and AMPA (each <0.1 µg/L) were fortified at 0.5, 1.0, 2.5, and 5 µg/L on 8 replicates each level. The recovery values ranged from 81 to 106% with are lative standard deviation (RSD) below 8.3%. Further, we performed recovery experiments using individual human urine samples to check for possible matrix effects caused by variations in the composition of the samples. Ten individual urine samples free of glyphosate and AMPA (each < 0.1 µg/L) were spiked at 0.5, 2.5 and 5 µg/L and were analysed in triplicate. The recoveries ranged from 87 to 110% proving that possible matrix effects were compensated by the internal standards $^{13}C_2$-^{15}N-glyphosate and ^{13}C-^{15}N-AMPA. In addition, the performance of the method was checked by measuring of control samples spiked at 0.5 and 2.5 µg/L during the analysis of the samples from this study (about every 33rd sample). A summary of the results of the control samples is provided in Table 4.

Table 4. Results of control samples concurrently analysed with the study samples

Analyte	Spiking level [µg/L]	Mean recovery [%]	Range [%]	RSD [%]	Number of samples
Glyphosate	0.5	103.0	84.4–113.3	8.6	15
	2.5	102.0	94.2–111.3	5.1	15
AMPA	0.5	102.1	82.4–112.4	9.4	15
	2.5	101.3	91.2–111.0	5.7	15

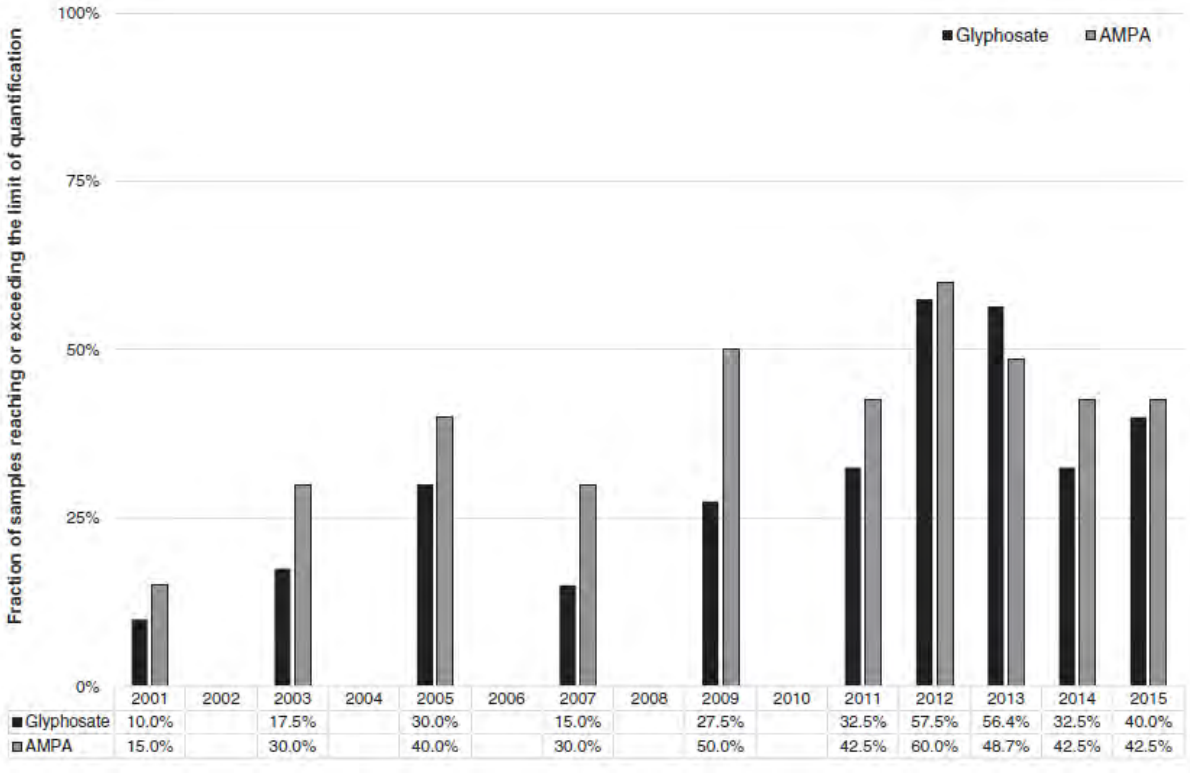
Statistical analysis; Glyphosate and AMPA concentrations below the LOQ were set to LOQ/2 prior to statistical evaluation. All data analyses were carried out in SPSS Statistics Version 20 (IBM Corporation, 2011). Differences between frequencies were tested with Pearson's Chi^2 test of independence after cross tabulation. Correlations between variables were quantified by Spearman's rank correlation coefficients, as concentration and other data mostly contained few extreme values. Box-plots were created in R Version 3.2.3 (R Core Team, 2015) displaying the 25th, median and 75th percentile as a box. The whiskers were set to extend to the minimum and maximum value, due to considerable skewness and obvious non-normality of the data. All p-values of 0.05 or lower were considered to indicate statistical significance.

Results

Urinary concentrations of glyphosate and AMPA in the main study sample

Frequency of quantifiable concentrations; Out of the 399 analysed urine samples, 127 (= 31.8%) contained glyphosate concentrations that reached or exceeded the LOQ of 0.1 g/L. For AMPA this was

the case for 160 (= 40.1%) of all samples. The fraction of samples at or above LOQ varied significantly over the years investigated, both for glyphosate ($p \leq 0.001$) and AMPA ($p = 0.005$). As displayed in Table 5 and Fig. 1, years with the highest quantification rates were 2012 (57.5%) and 2013 (56.4%) after rates having discontinuously increased from 10.0% in 2001. Fractions of at least 0.1 g/L were lower again in 2014 and 2015 with 32.5% and 40.0%, respectively. The overall trend for quantifiable AMPA levels was quite similar. The highest fraction of samples reaching or exceeding the LOQ was observed for samples taken in 2012 (60.0%). The fractions of quantifiable levels of glyphosate and AMPA per year were generally higher in males. Especially for glyphosate, the principally increasing trend in urine concentrations was mainly due to samples provided by males (cf. Table 5).



	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Glyphosate	10.0%		17.5%		30.0%		15.0%		27.5%		32.5%	57.5%	56.4%	32.5%	40.0%
AMPA	15.0%		30.0%		40.0%		30.0%		50.0%		42.5%	60.0%	48.7%	42.5%	42.5%

Fig. 1. Temporal trend of glyphosate and AMPA in human 24 h-urine (fraction of samples at or above limit of quantification of 0.1 µg/L, ESB sampling location Greifswald , no self-reported specific dietary restriction)

Fractions of quantifiable glyphosate levels in samples from females were particularly high only in 2012 (55.0%) and 2013 (47.4%). The same difference between males and females was also apparent for AMPA, although the difference was less pronounced. Glyphosate sales in Germany have increased substantially from approximately 3300 t in 2000 to approximately 5400 t in 2014. The interim peak of approximately 7600 t in 2008 might be interrelated with the abolishment of EU set-aside requirements announced in 2007. Against the background of these data, the increase in quantifiable glyphosate and AMPA concentrations in analysed ESB urine samples were in agreement with expectations. Although the internal exposure to glyphosate and AMPA seems to have decreased again since 2013, there was a clear increase in comparison to 2001. The possible reduction in exposure since 2013 indicated by ESB data may be due to changes in application of glyphosate in agriculture: Austria, for example, banned the pre-harvest use of glyphosate in 2013. Also in Germany, intended glyphosate uses as pre-harvest treatment have been restricted (e.g. to partial applications instead of whole field treatments) from 2014 onwards. Currently, no German sales data are available for the year 2015.

Distribution of concentrations; The 50th, 75th, and 95th percentiles and maximal values for glyphosate and AMPA levels by sex and study year are provided in Table 5. Only in 2012 and 2013 the median concentration of glyphosate was slightly above the LOQ of 0.1 g/L. The 75th percentile exceeded the LOQ in all study years after 2007, reaching highest values in 2012 and 2013. The 95th percentiles of glyphosate concentrations in 24 h-urine were substantially higher in 2013 (1.25 g/L) and 2014 (0.80

g/L) compared to all other years. Also the maximum concentrations of glyphosate peaked in these two years (2013: 2.80 g/L, 2014: 1.78 g/L). The median urinary AMPA concentration only slightly exceeded the LOQ in 2012. With the exception of the first year of the study, 2001, all 75th percentiles exceeded the LOQ with the highest level observed in 2013. The 95th percentiles of AMPA levels peaked in 2013. The two highest AMPA concentrations were observed in samples from 2013 (1.88 g/L and 1.54 g/L). The observed urinary glyphosate and AMPA concentrations were in good agreement with findings from other studies. In view of these results, ESB data was considered to provide a reliable indication of the background exposure to glyphosate and AMPA in Germany and its change from 2001 to 2015. Comparability with other studies was limited, partly due to differences in the study population and in the type of urine samples.

Table 5. Summary statistics for glyphosate and AMPA concentrations in 24 h-urine samples (µg/L) by sex and year of sampling at ESB sampling location Greifswald (no self-reported specific dietary restrictions)

			Glyphosate					*AMPA*				
		N	% ≥ LOQ	P 50	P 75	P 95	Max.	% ≥ LOQ	P 50	P 75	P 95	Max.
2001	Male	20	15.0	<LOQ	<LOQ	0.26	0.40	15.0	<LOQ	<LOQ	0.25	0.29
	Female	20	5.0	<LOQ	<LOQ	<LOQ	0.11	15.0	<LOQ	<LOQ	0.21	0.22
	Total	40	10.0	<LOQ	<LOQ	0.12	0.40	15.0	<LOQ	<LOQ	0.22	0.29
2003	Male	20	20.0	<LOQ	<LOQ	0.25	0.37	40.0	<LOQ	0.14	0.18	0.18
	Female	20	15.0	<LOQ	<LOQ	0.16	0.20	20.0	<LOQ	<LOQ	0.19	0.20
	Total	40	17.5	<LOQ	<LOQ	0.16	0.37	30.0	<LOQ	0.13	0.18	0.20
2005	Male	20	40.0	<LOQ	0.14	0.26	0.26	45.0	<LOQ	0.19	0.24	0.24
	Female	20	20.0	<LOQ	<LOQ	0.19	0.24	35.0	<LOQ	0.13	0.26	0.29
	Total	40	30.0	<LOQ	0.11	0.25	0.26	40.0	<LOQ	0.17	0.24	0.29
2007	Male	20	10.0	<LOQ	<LOQ	0.20	0.26	35.0	<LOQ	0.14	0.23	0.23
	Female	20	20.0	<LOQ	<LOQ	0.14	0.14	25.0	<LOQ	<LOQ	0.19	0.20
	Total	40	15.0	<LOQ	<LOQ	0.14	0.26	30.0	<LOQ	0.13	0.21	0.23
2009	Male	20	40.0	<LOQ	0.11	0.22	0.30	55.0	0.11	0.18	0.55	0.81
	Female	20	15.0	<LOQ	<LOQ	0.12	0.12	45.0	<LOQ	0.16	0.20	0.20
	Total	40	27.5	<LOQ	0.10	0.13	0.30	50.0	<LOQ	0.17	0.26	0.81
2011	Male	20	50.0	<LOQ	0.14	0.38	0.51	60.0	0.13	0.22	0.48	0.65
	Female	20	15.0	<LOQ	<LOQ	0.11	0.11	25.0	<LOQ	0.11	0.32	0.37
	Total	40	32.5	<LOQ	0.11	0.25	0.51	42.5	<LOQ	0.18	0.34	0.65
2012	Male	20	60.0	0.12	0.22	0.48	0.57	65.0	0.15	0.21	0.61	0.66
	Female	20	55.0	0.11	0.16	0.44	0.63	55.0	0.11	0.19	0.46	0.50
	Total	40	57.5	0.11	0.20	0.48	0.63	60.0	0.12	0.21	0.56	0.66
2013	Male	20	65.0	0.18	0.29	0.55	0.63	60.0	0.18	0.35	1.03	1.54
	Female	19	47.4	<LOQ	0.16	2.80	2.80	36.8	<LOQ	0.16	1.88	1.88
	Total	39	56.4	0.11	0.27	1.25	2.80	48.7	<LOQ	0.29	1.54	1.88
2014	Male	20	55.0	0.11	0.20	1.12	1.78	60.0	0.13	0.19	0.25	0.26
	Female	20	10.0	<LOQ	<LOQ	0.63	1.15	25.0	<LOQ	<LOQ	0.60	0.97
	Total	40	32.5	<LOQ	0.11	0.80	1.78	42.5	<LOQ	0.16	0.25	0.97
2015	Male	20	70.0	0.16	0.22	0.45	0.49	50.0	<LOQ	0.18	0.38	0.41
	Female	20	10.0	<LOQ	<LOQ	0.37	0.57	35.0	<LOQ	0.13	0.38	0.39
	Total	40	40.0	<LOQ	0.16	0.45	0.57	42.5	<LOQ	0.16	0.38	0.41

Notes: N = sample size, LOQ = limit of quantification, P = percentiles, Max. = maximum value.

As displayed in Figs. 2 and 3 glyphosate and AMPA concentrations were generally higher in samples from male ESB participants compared to samples from female participants. From 2011 onwards, median levels and 75th percentiles for glyphosate were higher in males. Box-plots for AMPA concentrations showed the same pattern. The maximum values for glyphosate and AMPA concentrations in urine, however, were observed in samples from female ESB participants. The differences in urinary glyphosate might be due to differences in exposure patterns between males and females or to sex-related differences in physiological determinants of glyphosate and AMPA in urine.

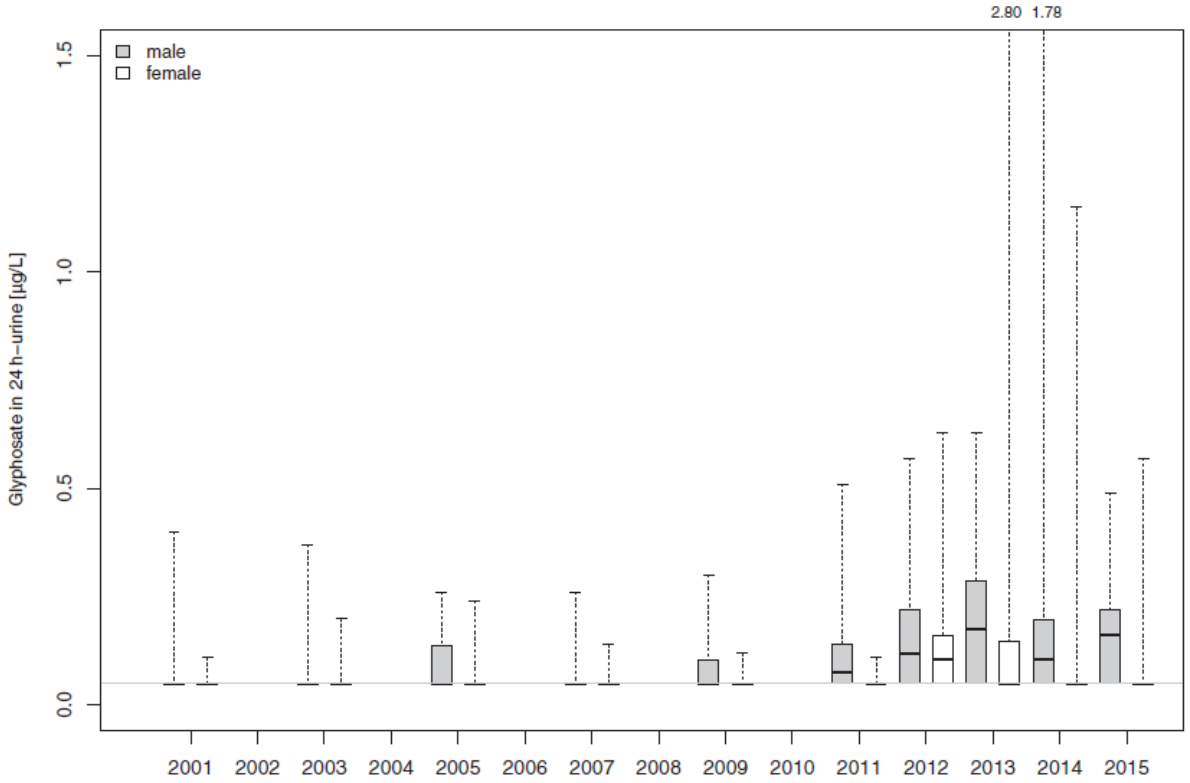


Fig. 2. Box-plots of glyphosate concentrations in 24 h-urine samples by study year and sex (ESB sampling location Greifswald, no self-reported specific dietary restriction, concentrations below LOQ of 0.1 µg/L set to LOQ/2 = horizontal solid line, box displays 25th, median and 75th percentile, whiskers extend to minimum and maximum value)

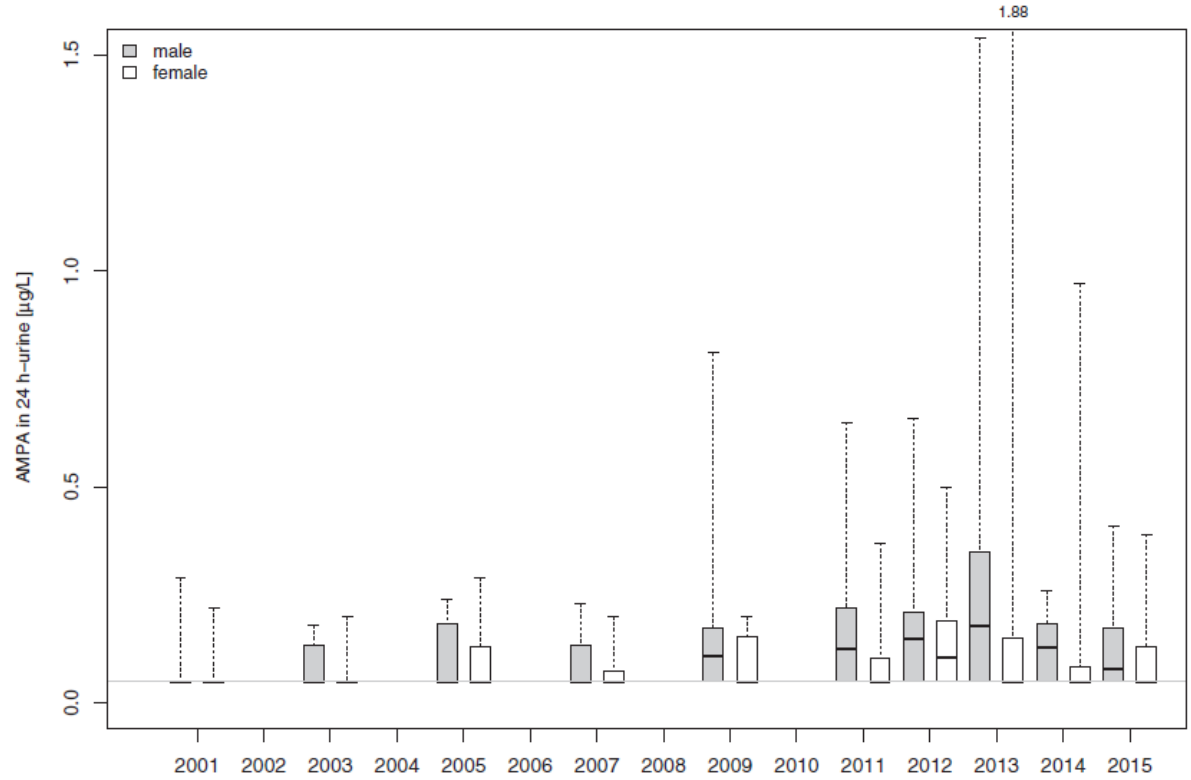


Fig. 3. Box-plots of AMPA concentrations in 24 h-urine samples by study year and sex (ESB sampling location Greifswald, no self-reported specific dietary restriction, concentrations below LOQ of 0.1 µg/L set to LOQ/2 = horizontal solid line, box displays 25th, median and 75th percentile, whiskers extend to minimum and maximum value

Correlations between glyphosate, AMPA and physiological parameters; Spearman rank correlations between glyphosate and AMPA levels in urine and physiological parameters observed in the main study sample are summarised in Table 6.

Table 6. **Spearman rank correlation coefficients between glyphosate and AMPA concentrations in 24 h-urine and physiological parameters**

		AMPA in 24 h-urine [µg/L]	Body mass index [kg/m²]	Volume of 24 h-urine sample [mL]	Creatinine in 24 h-urine [g/L]
Glyphosate in 24 h-urine [µg/L]	*Corr. coeff.*	**0.506**	**0.161**	**−0.278**	**0.347**
	p-value	≤0.001	0.001	≤0.001	≤0.001
	N	399	399	398	398
AMPA in 24 h-urine [µg/L]	*Corr. coeff.*		0.079	**−0.327**	**0.373**
	p-value		0.114	≤0.001	≤0.001
	N		399	398	398
Body mass index [kg/m²]	*Corr. coeff.*			0.020	**0.252**
	p-value			0.692	≤0.001
	N			398	398
Volume of 24 h-urine sample [mL]	*Corr. coeff.*				**−0.760**
	p-value				≤0.001
	N				397

Notes: N = sample size, statistically significant correlation coefficients ($p \leq 0.05$) highlighted in bold

Glyphosate and AMPA concentrations in urine were statistically correlated (spearman rank correlations coefficient $r_S = 0.506$, $p \leq 0.001$). When calculating coefficients of rank correlation separately for each study year, glyphosate and AMPA levels correlated statistically significantly in all years except for the first two, 2001 and 2003. For the following eight years of the study, r_S ranged between 0.360 and 0.616 (all p-values $\leq$ 0.05). A statistically significant association between glyphosate and AMPA concentrations in urine was also observed when cross tabulating all quantifiable and non-quantifiable levels for both analytes as well as when calculating the Pearson product-moment correlation coefficient (data not shown). There were, however, urine samples with comparatively high glyphosate and quite low AMPA concentrations, and vice versa. The coefficients of correlation of glyphosate and AMPA with BMI were comparatively low and statistically significant only for glyphosate. Correlations between BMI and glyphosate concentrations in urine were only statistically significant at the 5% level in 2011 ($r_S = 0.344$) and 2015 ($r_S = 0.365$). For AMPA, only the correlation of the concentrations in urine with the participants' BMI in 2015 reached statistical significance ($r_S = 0.346$). Glyphosate and AMPA concentrations in urine were consistently negatively correlated with the urine sample volume (r_S = -0.278 and -0.327) and positively correlated with urinary creatinine levels ($r_S = 0.347$ and 0.373). All these coefficients of correlation were statistically significant ($p \leq 0.001$). The BMI was positively correlated with the creatinine concentration in 24 h-urine samples ($r_S = 0.252$, $p \leq 0.001$). Glyphosate and AMPA concentrations in urine were positively associated with urinary creatinine in all study years. The coefficients of correlation were statistically significant at the 5% level in almost all study years. Examined for the individual years of the study, the r_S of urine sample volume and glyphosate as well as AMPA levels were consistently negative. The correlation, however, was often not statistically significant. These results warrant a further discussion on options for a combined consideration of glyphosate and AMPA in exposure assessment. The quite low, but statistically significant correlation between BMI and glyphosate deserves attention when further investigating glyphosate exposure via food consumption. The negative association of glyphosate and AMPA concentrations with 24 h-urine sample volumes and positive association with urinary creatinine concentrations were in line with expectations, as both parameters reflect the individual urinary diluteness. 24 h creatinine excretion was usually higher in males.

Comparison with other ESB sub-populations

To get a first insight into differences in exposures due to the place of residence and season of sampling, 40 urine samples collected in 2005 and 2015 at the ESB sampling location Muenster were also analysed for glyphosate and AMPA. In contrast to samples being taken in April/May in Greifswald, the annual

Muenster sampling is carried out in January. The summary statistics for glyphosate and AMPA in this sub-population are given in Table 7.

Table 7. Summary statistics for glyphosate and AMPA concentrations in 24 h-urine samples (µg/L) by sex and year of sampling in two sub-populations from Muenster (no self-reported specific dietary restrictions) and Greifswald (self-reported vegetarians/vegans) analysed for comparison with the main study sample

			Glyphosate					*AMPA*				
		N	% ≥ LOQ	P 50	P 75	P 95	Max.	% ≥ LOQ	P 50	P 75	P 95	Max.
ESB sampling location Muenster (no self-reported specific dietary restrictions)												
2005	Male	20	0.0	<LOQ	<LOQ	<LOQ	<LOQ	30.0	<LOQ	0.12	0.20	0.22
	Female	20	10.0	<LOQ	<LOQ	0.34	0.54	25.0	<LOQ	<LOQ	0.28	0.30
	Total	40	5.0	<LOQ	<LOQ	<LOQ	0.54	27.5	<LOQ	0.11	0.24	0.30
2015	Male	20	15.0	<LOQ	<LOQ	0.23	0.31	30.0	<LOQ	0.11	0.34	0.45
	Female	20	15.0	<LOQ	<LOQ	0.17	0.17	40.0	<LOQ	0.17	0.28	0.31
	Total	40	15.0	<LOQ	<LOQ	0.17	0.31	35.0	<LOQ	0.15	0.28	0.45
Self-reported vegetarians/vegans (ESB sampling location Greifswald)												
2007	Male	0										
	Female	10	10.0	<LOQ			0.14	0.0	<LOQ			<LOQ
	Total	10	10.0	<LOQ			0.14	0.0	<LOQ			<LOQ
2015	Male	5	60.0	0.26			0.61	40.0	<LOQ			0.33
	Female	5	20.0	<LOQ			0.53	20.0	<LOQ			0.43
	Total	10	40.0	<LOQ			0.61	30.0	<LOQ			0.43

Notes: N = sample size, LOQ = limit of quantification, P = percentiles, Max. = maximum value.

In 2005 and 2015 the percentage of quantifiable glyphosate levels was significantly higher in the main study sample (Greifswald) than in Muenster (2005: 30.0% vs. 5.0%, p = 0.003 and 2015: 40.0% vs. 15.0%, p = 0.012). For AMPA no statistically significant differences between Greifswald and Muenster samples were observed in 2005 (40.0% vs. 27.5%, p = 0.24) and 2015 (42.5% vs. 35.0%, p = 0.49). Also the 75th and 95th percentile of urinary glyphosate concentrations in the main study sample were higher than in samples collected in Muenster. For AMPA these percentiles were quite similar for both populations. A second comparative subsample analysed for glyphosate and AMPA consists of 10 samples provided in 2007 and 2015 by self-reported vegetarians/vegans taking part in Greifswald (cf. Table 7). There was virtually no difference between self-reported vegetarians/vegans and the main study sample concerning quantifiable percentages of glyphosate in 2007 and 2015. For AMPA the fractions of samples with levels of at least 0.1 g/L tended to be lower for vegetarians/vegans (2007: 0.0% vs. 30.0%, p = 0.047 and 2015: 30.0% vs. 42.5%, p = 0.47), being statistically significant only in 2007. In that year, all self-reported vegetarians/vegans who participated in Greifswald were female. When limiting the comparison to samples collected from women, the difference observed in 2007 was less pronounced and no longer statistically significant (0.0% vs. 25.0%, p = 0.083). Glyphosate concentrations in urine seem slightly higher in the main study sample in comparison to the Muenster sub-population. Although there were virtually no differences in urinary AMPA, this result indicated possible regional or seasonal differences in exposure. Against expectations, the results of this study did not considered to advocate urinary glyphosate and AMPA levels being higher in vegetarian/vegan participants. No equal sex distribution could be achieved for the sub-population of self-reported vegetarians/vegans, due to a low participation rate of male vegetarians/vegans. This might have reduced comparability of this sub-population, as males showed a tendency to exhibit higher glyphosate and AMPA concentrations in urine. Another limitation of this comparison was that vegetarian/vegan participants exhibit on average higher 24 h-urine sample volumes than in the main study sample without self-reported specifically restricted diet. In general, the sample sizes of the two sub-populations analysed for comparison were possibly too small to draw general conclusions on seasonal or regional effects and on effects of dietary preferences.

Health-relevance of observed internal exposure; The acceptable daily intake (ADI) for glyphosate derived by the European Food Safety Authority (EFSA) is 0.5 mg/kg/d (EFSA, 2015). Assuming a bodyweight of 60 kg, an oral absorption of 20% with fast elimination via urine, and a daily urine excretion of 1500 to 2000 mL, the concentration in 24 h-urine associated with this ADI resulted in 3000 to 4000 g/L. This concentration was higher than the maximum concentration observed in this study (2.8 g/L) by a factor of 1000. Considering EFSA's risk assessment, none of glyphosate concentration measured in ESB samples was considered problematic for human health. The International Agency for

Research on Cancer (IARC), however, classified glyphosate in Group 2A ("probably carcinogenic to humans"; IARC,). Taking this assessment into account, especially the increasing trend in internal glyphosate exposure documented by ESB samples needs an attention with regard to human health.

Conclusion
Retrospective GC-MS-MS analyses of the general German population urinary samples collected during a period covering 2001 – 2015 revealed that 31.8% of analysed samples contained detectable level of glyphosate. For AMPA this was the case for 40.1% samples analysed. A peak of detectable glyphosate level was observed in 2012 (57.5%) and 2013 (56.4%), followed by a decrease in 2014 (32.5%) and 2015 (40.0%), which may be due to changes in glyphosate application in agricultural practice. Urinary glyphosate levels tended to be higher in males. Overall, the urinary level of AMPA showed a similar trend as glyphosate, with a statistically significantly correlation.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The internal exposure levels of glyphosate and its main metabolite AMPA were analysed using the general German population urinary samples collected during a period covering 2001 – 2015 with similar sample sizes and sex distributions. Retrospective GC-MS-MS analyses revealed that 31.8% of analysed samples contained detectable level of glyphosate. For AMPA this was the case for 40.1% samples analysed. A peak of detectable glyphosate level was observed in 2012 (57.5%) and 2013 (56.4%), followed by a decrease in 2014 (32.5%) and 2015 (40.0%), which may be due to changes in glyphosate application in agricultural practice. Urinary glyphosate levels tended to be higher in males. Overall, the urinary level of AMPA showed a similar trend as glyphosate, with a statistically significantly correlation.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good monitoring study.

Reliability criteria of exposure studies

Publication: Conrad *et al.* 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	Retrospective population monitoring study of glyphosate and AMPA in urine.
Test substance		
Exposure to formulations with only glyphosate as a.i.	NA	Exposure to glyphosate and AMPA mainly through the diet.
Exposure to formulations with glyphosate combined with other a.i.	NA	
Exposure to various formulations of pesticides	NA	
Study		
Study design clearly described	Y	
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	General population.
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y	

Reliability criteria of exposure studies

Publication: Conrad *et al.* 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good monitoring study.		

1. Information on the study

Data point:	CA 5.5
Report author	Crump K.
Report year	2019
Report title	The potential effects of recall bias and selection bias on the epidemiological evidence for the carcinogenicity
Document No	Risk Anal. (2020); 40(4):696-704
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

The principal human data for glyphosate and non-Hodgkin's lymphoma (NHL) come from five case–control studies and two (related) cohort studies. The case–control studies are at risk of recall bias resulting from information on exposure to pesticides being collected from cases and controls based on their memories; cases being deemed likely by textbook authors to have a greater motivation than controls for remembering or reporting past exposures. In addition, two of the case–control studies are additionally at risk of a form of selection bias that can exacerbate the effect of recall bias. Both biases are in the direction of making glyphosate appear carcinogenic (viz. causing odds ratios (ORs) to be >1 in the absence of a true causal relationship). If ORs are not biased and a pesticide plays no role in causing NHL, the probability that an OR for that pesticide is greater than 1.0 is approximately 0.5. The fractions of ORs for pesticides other than glyphosate that are greater than 1.0 in the five case–control studies are 0.90 (n = 92), 0.90 (n = 152), 0.93 (n = 59), 0.76 (n = 140), and 0.53 (n = 54), the first two from studies that are at risk for both types of bias. In the two cohort studies, which are not subject to these biases, the comparable fractions for relative risks for all cancers are 0.51 (n = 70) and 0.48 (n = 158). Thus, this analysis provides evidence that at least four of the five case–control studies of glyphosate exposure and NHL are contaminated by statistical bias, likely stemming in the main from recall bias, exacerbated by selection bias in two of the studies. This suggests that these case control studies of glyphosate are not reliable evidence for a relationship between glyphosate and NHL.

Materials and methods

Two of the case–control studies exclude from some analyses of glyphosate (as well as analyses of other pesticides) the unexposed cases and controls who report exposures to pesticides not the subject of the analysis, which raises the possibility of selection bias if recall bias is also operating. The potential effect of selection bias in the presence of recall bias on ORs is illustrated by simulating sets of case–control data in which increasing amounts of recall bias are introduced, and the effects of adding selection bias to these analyses are noted. Each simulation involves 500 cases and 1,500 controls. Controls are randomly assigned exposure to pesticides according to the percentages of controls reporting exposure to nine pesticides reported by McDuffie et al. (2001) (Table II). Cases are randomly assigned exposures in the same way except the exposed percentages are multiplied by increasing factors in different simulations, which introduces increasing amounts of recall bias. In each of six simulations, 10,000 sets of data are simulated and the 10,000 simulated ORs for glyphosate are averaged, (1): using all data so that recall bias but not selection bias is present and (2): after removing from the unexposed group both cases and controls that are exposed to any pesticide other than glyphosate (so that both recall bias and selection bias are present). In interpreting this simulation study, it needs to be understood that the McDuffie data and glyphosate are used only to make the simulations more realistic, and that the simulations say nothing about the McDuffie study or about the risk from glyphosate exposure, as any such data or a herbicide other than glyphosate could have been used to illustrate the same points. The likelihood that statistical bias (from any source) may be responsible for the elevated ORs for glyphosate is evaluated by tabulating ORs and RRs from the studies and cross-classifying them by pesticide groups

and the fraction that exceed 1.0. If all types of pesticides have an elevated percentage of ORs greater than 1.0, this suggests that bias may be the cause of the elevations rather than any carcinogenic effect of the pesticides. The ORs and RRs included in the tabulations were selected from the original papers according to the following rules:

(1) In some instances, the exact same OR calculation is reported in two or more separate tables (e.g., the category "herbicide" in tables I, II, and V of Hardell et al., 2002). Only one of the identical calculations is tabulated.

(2) Similarly, some tables contain two sets of OR or RR calculations for testing the same hypotheses, but using different statistical methods (e.g., controlling for different sets of potential confounders). In these instances, only one set of calculations is tabulated, namely the set of ORs or RRs whose method of calculation agrees most closely with methods used in in the remainder of the article or in other articles. For example, De Roos et al. (2003) reported ORs calculated using both logistic regression and hierarchical regression. The ORs computed using logistic regression were selected for tabulation because this was the only study that employed hierarchical regression and logistic regression was the most common method used in the remaining studies.

(3) In De Roos et al. (2003), the category "potentially carcinogenic pesticides" apparently was formed post hoc and included those pesticides that gave greatest evidence of a carcinogenic effect in initial analyses. Such an approach would almost guarantee an OR greater than 1. In fact, the three ORs from this category were all greater than 1. ORs from this analysis were not included in order to avoid biasing the tabulation. (This does not imply that De Roos et al. erred in computing these ORs, only that they were not suitable for inclusion in our analysis.)

(4) Otherwise, all OR or RR calculations reported in the publications were tabulated. A complete listing of the ORs from each study contained in the tabulation is provided in the Supporting Information. The results of these tabulations are summarized in graphs and in tabular form.

Results

Specific results part not given in this article. Results and discussion are merged.

Discussion

Results of the simulation exercise to demonstrate the effect of selection bias are shown in Table I. The first row in the table verifies that, as expected, selection bias does not affect the expected OR in the absence of recall bias. The remaining rows assume increasing amounts of recall bias as indicated in the first column. The effect of that recall bias on the expected ORs for glyphosate are shown in the second column. The third column shows the expected ORs when selection bias is added to the recall bias present by removing from the unexposed (to glyphosate) groups cases and controls exposed to any herbicide other than glyphosate, just as was done in Hardell et al. (2002) and Eriksson et al. (2008).

Table I. Results of Simulations[a] to Demonstrate the Effect of Recall Bias[b] Alone and with Selection Bias[c] on Average OR

Recall Bias[b]	Average OR for Glyphosate with Recall Bias Only	Average OR for Glyphosate with Both Recall Bias and Selection Bias
0	1.01	1.01
0.05	1.07	1.10
0.1	1.12	1.18
0.3	1.35	1.59
0.5	1.59	2.10
1	2.24	3.95

[a]10,000 simulations, 500 cases, 1,500 controls, each row. Herbicides assumed present and the percentage of controls assumed to report exposure: (glyphosate, 8%), (2,4-D, 19%), (mecoprop, 5.4%), (MCPA, 3.1%), (diclofopmethyl, 1.7%), (thiocarbamates, 3.3%), (bromoxynil, 3.2%), (dicamba, 8.7%), (dinitroaniline, 2.1%). Thus, for example, the probability that a particular control claimed exposure to MCPA was 0.031. Exposures to different herbicides are assumed to occur independently.

[b]Recall bias is introduced into each simulation by computing the prevalence of each herbicide exposure among cases as [prevalence among controls] × [1+ Recall bias]

[c]Selection bias is introduced by eliminating from OR calculations both unexposed (to glyphosate) cases and controls exposed to herbicides other than glyphosate.

Crump notes that the effect of selection bias increases with the increase in recall bias. This simulation demonstrates that selection bias can cause ORs to be inflated by important amounts above that due solely to recall bias when recall bias is also present. Fig. 1 shows plots of the tabulated ORs or RRs by study, with the pesticide groupings upon which the ORs are based (including ORs derived for both individual pesticides and groups of pesticides), classified as to fungicides, herbicides not containing glyphosate, impregnating agents, insecticides, and pesticide groupings that include glyphosate. The individual pesticides and groupings for each study are listed in the footnote to Table II. The tabulated RRs from the two cohort studies are similarly classified into cancer groupings not containing NHL and groupings containing NHL. Since the logarithms of ORs are plotted, the focus is on the proportion of log-transformed ORs that are greater than 0.0 (equal to the proportion of untransformed ORs greater than 1.0). These figures show that ORs in McDuffie et al. (2001), Hardell et al. (2002), and Eriksson et al. (2008) are nearly all greater than 1.0.

Table II. Counts of ORs and RRs Categorized by Study, Pesticide, and Whether Greater than 1.0

Case–Control Studies	Fungicides		Herbicides (No Glyphosate)		Impregnating Agents		Insecticides		Glyphosate-Containing		Glyphosate Only		Totals (No Glyphosate)		Totals (Incl. Glyphosate)	
	N	% > 1	*N*	% > 1	*N*	% > 1	*N*	% > 1	*N*	% > 1	*N*	% > 1	*N*	% > 1	*N*	% > 1
Hardell et al. (2002)[a]			31	93.5%	42	84.5%	19	94.7%	13	96.2%	1	100.0%	92	89.7%	106	90.6%
Eriksson et al. (2008)[b]	10	100.0%	44	92.0%	43	86.0%	55	89.1%	22	100.0%	11	100.0%	152	89.8%	185	91.6%
McDuffie et al. (2001)[c]	18	83.3%	19	94.7%			22	100.0%	6	100.0%	3	83.3%	59	93.2%	68	93.4%
Orsi et al. (2009)[d]	27	83.3%	36	72.2%			77	75.3%	27	66.7%	9	66.7%	140	76.1%	176	74.1%
De Roos et al. (2003)[e]			20	47.5%			34	55.9%	6	33.3%	1	100.0%	54	52.8%	61	51.6%
Totals	55	86.4%	150	82.0%	85	85.3%	207	80.2%	74	81.8%	25	86.0%	497	82.3%	596	82.4%

Cohort Studies	No NHL		NHL + Subtypes		Totals	
	N	% > 0	*N*	% > 0	*N*	% > 0
De Roos et al. (2005)[f]	55	53.6%	15	43.3%	70	51.4%
Andreotti et al. (2018)[g]	86	51.2%	72	43.1%	158	47.5%
Totals	141	52.1%	87	43.1%	228	48.7%

[a]ORs for NHL and hairy cell leukemia computed for herbicides; phenoxyacetic acids; MCPA; 2,4,5-T + 2,4-D; glyphosate; other herbicides; insecticides; DDT; mercurial seed dressing; pyrethrins; fungicides; impregnating agents; chlorophenols; pentachlorophenol; arsenic; creosote; other impregnating agents; organic solvents.

[b]ORs for NHL, including subtypes, as well as several subcategories of NHL were computed for herbicides, total; phenoxyacetic acids; MCPA; 2,4,5-T and/or 2,4-D; other phenoxyacetic acids; herbicides except phenoxyacetic acids; glyphosate; other herbicides; insecticides, total; DDT; mercurial seed dressing; pyretrine; permetrine; other insecticides; fungicides; impregnating agents; chlorophenols; arsenic; creosote; tar; other impregnating agents; rodenticides.

[c]ORs for NHL computed for phenoxyherbicides; 2,4-D; mecoprop; MCPA; diclofopmethyl; phosphonic acid; glyphosate (Roundup); thiocarbamates; diallate; phenols: bromoxynil; dicamba; dicamba (Banvel or Target); dinitroaniline; trifluralin; carbamates; carbaryl; carbofuran; methomyl; organochlorine; chlordane; lindane; aldrin; organochlorine diphenylchlorides; DDT; organophosphorus; malathion; dimethoate; diazinon; amide; captan; vitavax; aldehyde; formaldehyde; mercury containing; mercury dust; mercury liquid; sulfur compounds.

[d]ORs for NHL, including subtypes, and other categories of lymphoid neoplasms computed for occupational pesticide use, insecticides, organochlorine, organophosphate, pyrethrin, fungicides, carbamates, imide, triazole, herbicides, phenoline, phenoxy, picoline, triazine, amide, urea, quaternary ammonium, glyphosate, garden pesticide use, insecticides, fungicides, herbicides, domestic insecticide use.

[e]ORs for NHL computed for aldrin; bufencarb; carbaryl; carbofuran; chlordane; copper; acetoarsenite; coumaphos; DDT; diazinon; dichlorvos; dieldrin; dimethoate; ethoprop; famphur; fly, lice, or tick spray; fonofos; heptachlor; lead; arsenate; lindane; malathion; methoxychlor; nicotine; phorate; pyrethrins; rotenone; tetrachlorvinphos; toxaphene; terbufos; alachlor; atrazine; bentazon; butylate; chloramben; cyanazine; 2,4-D; dicamba; EPTC + protectant; glyphosate; linuron; MCPA; metolachlor; metribuzen; paraquat; propachlor; sodium; chlorate; 2,4,5-T; trifluralin; any pesticide; any insecticide; any herbicide; chlordane and DDT; carbofuran and atrazine; diazinon and atrazine; alachlor and atrazine; atrazine and dicamba.

[f]RRs computed for glyphosate exposure in relation to the following cancers: all cancer, lung, oral cavity, rectum, pancreas, kidney, bladder, prostate, melanoma, lymphohematopoietic cancer, NHL, lukemia, and multiple myeloma.

[g]RRs computed for glyphosate exposure in relation to the following cancers: all cancer; oral cavity; colon; rectum; pancreas; lung; melanoma; prostate; testicular; bladder; kidney; lymphohematopoietic; Hodgkin lymphoma; non-Hodgkin lymphoma; non-Hodgkin lymphoma B cell; chronic lymphocytic lymphoma or small lymphocytic leukemia; diffuse large B cell lymphoma; marginal-zone lymphoma; follicular lymphoma; multiple myeloma and non-Hodgkin lymphoma T cell.

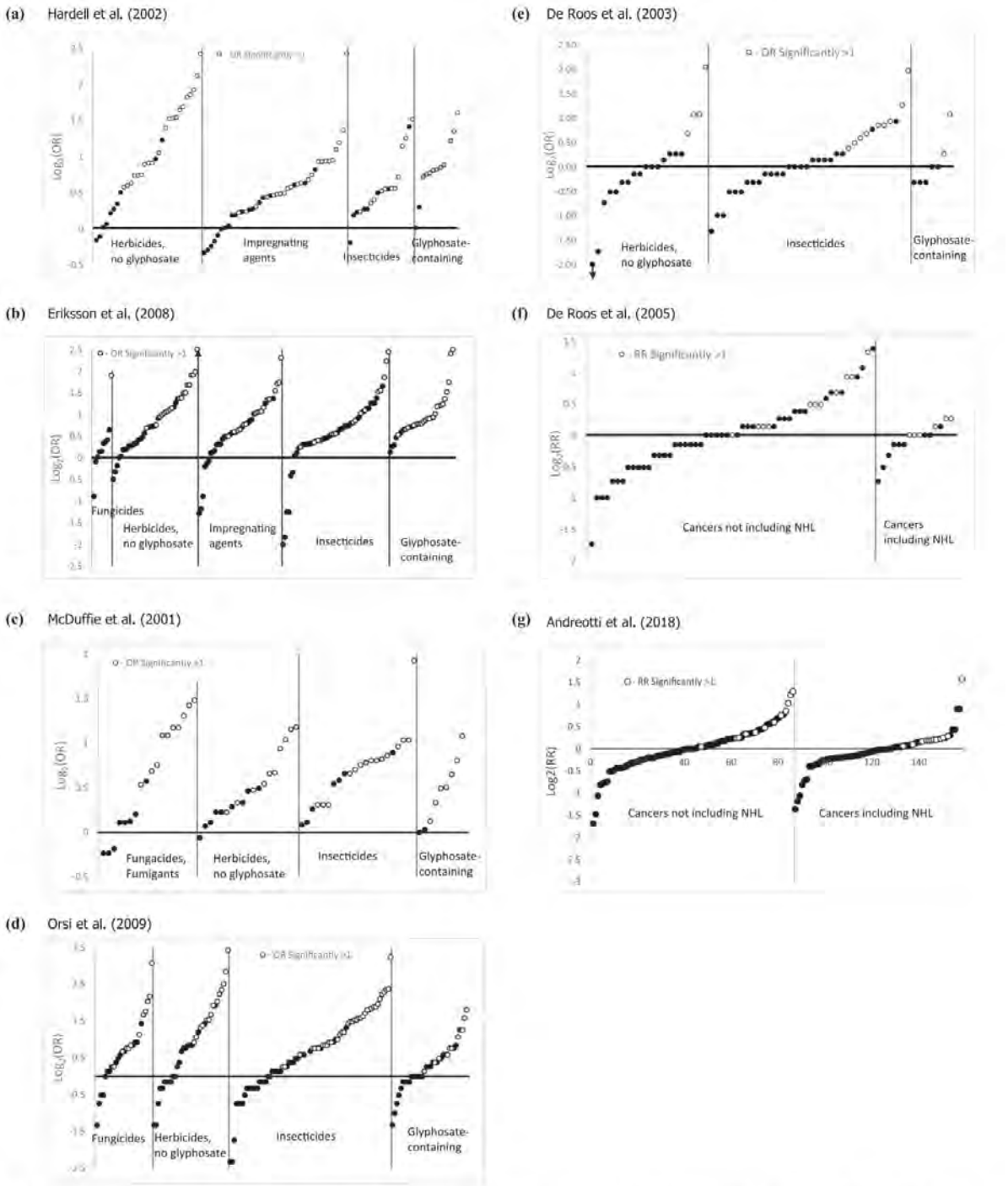


Fig. 1. Graphs of tabulated ORs and RRs. (a) Hardell et al. (2002), (b) Eriksson et al. (2008), (c) McDuffie et al. (2001), (d) Orsi et al. (2009), (e) De Roos et al. (2003), (f) De Roos et al. (2005), (g) Andreotti et al. (2018).

Also, there is an excess of ORs greater than 1.0 in Orsi et al. (2009). These excesses of ORs greater than 1.0 occur in all categories of pesticides considered in these studies. On the other hand, there appears to be roughly a balance between the numbers of ORs greater than and less than 1.0 in all categories of pesticides in the case–control study of De Roos et al. (2003). Similarly, in both cohort studies (Andreotti et al., 2018; De Roos et al., 2005), there seems to be roughly an equal number of RRs greater than and less than 1.0, both for cancer groupings that include NHL and those that do not. These graphs also display those ORs and RRs that are statistically significantly greater than 1.0, with statistical significance defined by the lower bound on the 95% confidence interval being greater than or equal to 0.8 (apparently the decision rule used in De Roos et al., 2003 to define "potentially carcinogenic pesticides.") This shows that many statistically significantly elevated ORs occur in every pesticide category with little notable difference between categories that contain glyphosate and those that do not. Table II, which

summarizes the tabulated ORs and RRs, provides confirmation of the impressions obtained from the graphs. In this table OR and RR reported in the original papers as equal to 1.0 (which are mostly due to roundoff in reported values) each contribute 0.5 to the counts of ORs and RRs greater than 1.0. In the case–control studies of Hardell et al. (2002), Eriksson et al. (2008), and McDuffie et al. (2001), 90% or more of all ORs from pesticide groups not containing glyphosate are greater than 1.0. In these three studies, the percentage of ORs greater than 1.0 exceeds 80% in all pesticide groupings (fungicides, herbicides not including glyphosate, impregnating agents, and insecticides, as well as groupings that contain glyphosate). The percentages of ORs from Orsi et al. (2009) that exceed 1.0 are also elevated, although not to the same extent as in the other three studies. By contrast, the percentages of ORs from the case–control study of De Roos et al. (2003) that are greater than 1.0 are all fairly close to 50% in all pesticide categories (52.8% in categories combined than do not include glyphosate and 51.6% if glyphosate-containing categories are included). Thus, of the five case control studies, the study of De Roos et al. (2003) presents considerably less evidence of recall bias resulting from an excess of ORs greater than 1.0. In the two cohort studies, the percentages of RRs greater than 1.0 in cancer groupings not containing NHL in both studies are 54%, and overall, with NHL included, are 49.5% (Table II). Thus, these results from the two cohort studies, which are not subject to recall bias or selection bias, are reasonably consistent with what would be expected if these studies are free of statistical bias and glyphosate has no effect upon cancer rates. If the ORs are not biased, the results in Table II pertaining to the case–control studies suggest that all types of pesticides investigated in these studies are having a role in causing NHL, including fungicides, herbicides other than glyphosate, impregnating agents and insecticides. It should also be kept in mind that the category NHL contains many types of lymphoma, not all of which are likely to share common risk factors. Thus, it seems unlikely (at least to this investigator) that pesticides within each of these types of pesticides would be causing NHL, and particularly to an extent to be responsible for the evidence seen in Table II. It seems much more likely that the preponderance of ORs greater than 1.0 seen in all pesticide categories in most of the case–control studies is simply the result of recall bias, which is a well-known problem with these types of case–control studies, possibly augmented in two studies by selection bias. Such a conclusion is further supported by the fact that in the two cohort studies, which are not subject to these biases, the overall percentage of RRs greater than 1.0 is 49.6% that is in excellent agreement with the theoretical value of 0.5, assuming no bias and no effect of glyphosate on any cancer. Given this evidence, one could reasonably conclude that at least four of the case–control studies of glyphosate and NHL are contaminated by statistical bias, and consequently are not suitable for reaching conclusions about the potential ability of glyphosate to cause NHL.The potential for case–control studies to be affected by recall bias is well known and has been discussed in many publications. The potential for the case–control studies of glyphosate, in particular, to be subject to recall bias, along with concerns about glyphosate studies not controlling for exposure to farm animals, and for the use of proxy respondents were discussed previously. These same issues were raised by some panelists in an EPA FIFRA scientific advisory panel.

Conclusion

In summary, the potential for these types of case–control studies to be contaminated by bias from the use of exposure information based on the memories of both cases and controls (recall bias) is well known. This article provides evidence that at least four of the five case–control studies of glyphosate exposure and NHL are contaminated by statistical bias, likely stemming in the main from recall bias, exacerbated by selection bias in two of the studies. This suggests that the case control studies of glyphosate are not reliable for determining whether glyphosate is carcinogenic. However, the two cohort studies (Andreotti et al., 2018; De Roos et al., 2005) do not present evidence of bias. If further study of the potential relationship between glyphosate exposure and NHL is needed, it would best come from cohort or other studies that are not at risk of recall bias resulting from quantifying exposures by questioning subjects. Of course, cohort studies have other potential problems that must be evaluated, including incomplete follow-up, the healthy worker effect and poor information on exposures. The potential for recall bias identified herein could affect, not just case–control studies of the potential carcinogenicity of glyphosate, but any such study that involves quantifying exposures occurring in the distant past based on participant's memories.

3. Assessment and conclusion

Assessment and conclusion by applicant:

It is well known that recall bias is a potentially important bias in cancer case control studies where study participants are asked to recall their past exposures. In an ideal study, information about exposures for cases and controls would be collected under exactly the same circumstances. However, circumstances are quite different for cases and controls. Cancer cases have suffered a grievous illness and it is only natural for them to be deeply introspective about what might have caused their cancers. Controls have no such motivation that would augment their recall (or reporting). So, the concern expressed in many textbooks is that recall bias tends to produce false positive results. The purpose of this analysis by Crump was to evaluate the evidence for recall bias in the overall pattern of results in five case control studies and two cohort studies that comprise the main part of the glyphosate-NHL literature.

In evaluating the case control studies, Crump reasoned that the percentage of odds ratios > 1 for non-glyphosate exposures should be approximately 50% if recall bias was not operative and those exposures did not cause NHL. Yet, it turned out that the percentages of ORs >1 for non-glyphosate exposures were 90% for Hardell et al. (2002), 90% for Erikson et al. (2008), 93% for McDuffie et al. (2001), 76% for Orsi et al. (2009), and 53% for DeRoos et al. (2003). These extreme departures from 50% for 4 of the 5 case control studies is consistent with recall bias, perhaps augmented by a type of selection bias in the analyses by Hardell et al. (2002) and Eriksson et al. (2008). In contrast, in the most recent publication from the Agricultural Health Study (Andreotti et al. 2018), only 48% of the relative risks (RR) calculated were >1 – a percentage in the range expected with a true probability of 50%. While the evaluation of Andreotti et al. (2018) concerned glyphosate and other cancer sites and not other exposures and NHL, the principle is the same: under the null hypothesis the proportion of ORs or RRs > 1 should be roughly 50% absent bias.

We agree with Crump's conclusion that the 4 case-control studies with a high proportion of ORs > 1 are "contaminated" by statistical bias and are not reliable as evidence of a relationship between glyphosate and NHL. Of course, there are also other types of bias that may contribute to the high proportion of positive ORs (e.g., lack of control for confounding, lower participation for controls than cases (traditional selection bias), proxy respondents, etc.) (see Acquavella et al. 2016). Nonetheless, Crump's point is well taken that ORs for glyphosate in 4 of the 5 case control studies should be interpreted as unreliable because the vast majority of ORs for other exposures are >1.

Reliability Criteria: Epidemiology studies

Publication: Crump K., 2019	**Criteria met? Y/N/?**	**Comments**
Study Design		
Adequate study design given study objectives	Yes	For a methodologic evaluation of recall bias in existing studies
Appropriate study population to address potential glyphosate-related health outcomes	Not applicable	
Exposure studied		
Exposure to formulations with glyphosate as a.i.	Yes	
Exposure to formulations with other a.i.	Yes	

Publication: Crump K., 2019	**Criteria met? Y/N/?**	**Comments**
Exposure to other farm exposures	Yes	
Study Conduct/analysis		
Adequate description of study population	Not applicable	
Adequate description of exposure circumstances	Not applicable	
Comparable participation by groups being compared	Not applicable	
Information provided by proxy respondents	Not applicable	
Adequate statistical analysis	Yes	To illustrate bias
Adequate consideration of personal confounding factors	Not applicable	
Adequate consideration of potentially confounding exposures	Not applicable	
Overall assessment		
Reliable without restrictions	Yes	As methodologic work. Clearly illustrates recall bias in the glyphosate case control studies.
Reliable with restrictions	No	
Not reliable	No	

1. Information on the study

Data point:	NA Zebra fish cell line
Report author	da Silva N. D. G. *et al.*
Report year	2020
Report title	Interference of goethite in the effects of glyphosate and Roundup® on ZFL cell line
Document No	Toxicology in Vitro 65 (2020) 104755
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Goethite (α-FeOOH) can adsorb a wide variety of compounds, including glyphosate. This study aimed to evaluate the effects of goethite nanoparticles (NPs), glyphosate (Gly), Roundup® (Rd), and co-exposures (Gly + NPs and Rd + NPs) on zebrafish liver cell line (ZFL). ZFL cells were exposed to NPs (1, 10, and 100 mg L^{-1}), Gly (3.6 mg L^{-1}), Rd (10 mg L^{-1}), and co-exposures (Gly + NPs and Rd + NPs), or only to saline for 1, 6, and 12 h. Cell viability was assessed by Trypan blue, MTT, and neutral red assays. The generation of reactive oxygen species and total antioxidant capacity were also determined, while genotoxicity was quantified by the comet assay. Both NPs and Rd in isolation produced cytotoxic effects at 6 h and genotoxic effects at 1 and 6 h. Rd + NPs resulted in synergistic effects, intensifying the toxicity. Cells exposed to Gly did not present toxic effects and Gly + NPs resulted in the suppression of toxic effects observed for NPs.

Materials and methods

Preparation of goethite nanoparticles (NPs): A solution containing goethite NPs was dispersed in ultra-pure water and sonicated. The concentration of NPs in the stock solution was estimated by dry weight and the corresponding iron concentration. This solution was autoclaved and used for the preparation of exposure solutions at final concentrations of 1, 10, and 100 mg L^{-1} of NPs diluted in Dulbecco's phosphate buffered saline with calcium, magnesium and glucose (Dulbecco's PBS: 136.9 mM NaCl; 2.68 mM KCl; 0.90 mM $CaCl_2$; 0.49 mM $MgCl_2 \cdot 6H_2O$; 7.58 mM Na_2HPO_4; 1.47 mM KH_2PO_4; 5.55 mM glucose; pH 7.4). Considering the lack of data reporting the effects of goethite NPs at cellular level, the concentrations used in the present work were based on cytotoxicity results found in the literature for different iron oxide nanoparticles. Furthermore, previous tests (MTT and NR) were performed in a range of 1–200 mg L^{-1} to select appropriate concentrations. Based on these results, the concentrations of 1, 10, and 100 mg L^{-1} were chosen to further investigate the toxic effects of goethite NPs in ZFL cells.

Quantification of iron (Fe): Total and dissolved Fe concentrations were analyzed in the exposure solutions. For the analysis of total Fe, the solutions were fixed in nitric acid (HNO_3 0.5% - Fmaia, Brazil) and for dissolved Fe solutions, they were filtered (0.45 μm) and then fixed. The samples were analysed using an atomic absorption spectrophotometer (EAA - Analyst 700, Perkin Elmer®, USA) through flame atomization.

ZFL cell line: The ZFL cell line was grown in 25 cm^2 flasks using medium containing 50% Leibovitz L-15 (Gibco®), 40% RPMI 1640 (Gibco®), and 10% fetal bovine serum (FBS) (Gibco®). The flasks were kept in a dry oven without addition of CO_2 at 28 °C.

In vitro exposures: Concentrations of the selected herbicides, Roundup® (360 g glyphosate L^{-1}, Monsanto do Brasil LTDA) and glyphosate (CAS no. 1071-83-6, Milenia Agrociências S/A), were

defined taking into account previous studies that our research group have already developed with the neotropical fish *P. lineatus* exposed to Roundup Transorb®, Roundup®, and glyphosate. Stock solutions of Roundup® and glyphosate were prepared at a concentration 100 times higher (100 and 36 mg L^{-1}, respectively) than the final exposure and diluted in Dulbecco's PBS before distribution to the plate wells to reached 10 and 3.6 mg L^{-1}, respectively. Exposure concentrations of Roundup® and glyphosate were based on the concentration of the active ingredient present in the formulation (360 g glyphosate L^{-1}). All the experimental solutions were prepared in Dulbecco's PBS to avoid any interactions between the treatments and the compounds present in the culture medium, which could influence the results. In addition, preliminary tests demonstrated that Dulbecco's PBS was able to maintain ZFL cell viability (mitochondrial and lysosomal activity) for at least twelve hours, which corresponds to the maximum exposure time used in this study. ZFL cells were seeded at a density of 10^6 cells mL^{-1} on a transparent 96-well plate (TPP®) for the cytotoxic assays, on a black 96-well plate with a clear bottom (Perkin Elmer®) for the biochemical assays, and on a transparent 24-well plate (TPP®) for the genotoxic assay. After 24 h of cell attachment at 28 °C, the cells were exposed for 1, 6, and 12 h to the following treatments: goethite NPs at 1 mg L^{-1} (N1), 10 mg L^{-1} (N2), and 100 mg L^{-1} (N3); glyphosate at 3.6 mg L^{-1} (Gly), and co-exposures to Gly plus goethite NPs (GlyN1, GlyN2, GlyN3); Roundup® at 10 mg L^{-1} (Rd); and Rd plus goethite NPs (RdN1, RdN2, RdN3). The cells of the control groups (CTR) were only exposed to Dulbecco's PBS. Three independent experiments were performed, and for each assay four plates were assembled per experimental time and, depending on the assay, the number of replicates per treatment was variable (cytotoxic and biochemical assays: eight replicates per treatment; genotoxic assay: two replicates per treatment).

Cytotoxic assays: Cytotoxicity was evaluated using different assays. To verify the integrity of the plasma membrane, the Trypan blue exclusion test (TB) was used prior to the comet assay. Cell viability was expressed as the percentage of viable cells and the treatments with cell viability equal to or > 80% were selected for ROS, ACAP, and comet assay. Mitochondrial activity was assessed through the reduction in 3–4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) salt. After exposure, the MTT salt was added to the wells for 4 h at a final concentration of 0.80 mM. Subsequently, the plate was centrifuged for 5 min at 200 g, dimethyl sulfoxide (DMSO, 99.5%) was added for solubilization of the formazan crystals, and the absorbance was determined on a microplate reader (Victor 3, Perkin Elmer®) at 540 nm. Lysosomal integrity was also assessed by the neutral red (NR) retention assay. After exposure, the cells were incubated with NR dye (40 μg mL^{-1}) for 3 h. The absorbance was determined on a microplate reader (Victor 3, Perkin Elmer®) at 540 nm and, for MTT and NR assays, the results of the different treatments were given in relation to the CTR, which was considered as 100% cell viability.

Biochemical assays: The generation of reactive oxygen species (ROS) and the total antioxidant capacity against peroxyl radicals (ACAP) were determined. After the exposures, the solutions were withdrawn and reaction medium (30 mM HEPES, 200 mM KCl, 1 mM $MgCl_2$, pH 7.2) was added to all wells. For each experimental treatment, eight replicates were performed per plate; four wells were treated with potassium phosphate buffer (0.1 M, pH 7.4) and the other four received peroxyl, 2,2′-azobis radicals (2-methylpropinamide) dihydrochloride (10 mM ABAP, pH 7.2). The autofluorescence reading was performed on a microplate reader (Victor 3, Perkin Elmer®). To quantify the ROS and ACAP, the fluorescence data were adjusted to a second-order polynomial function and the integral value was calculated. For ROS quantification, the values of the integrals of the samples treated with potassium phosphate buffer in isolation were analyzed and the results expressed as unit area of fluorescence over time (FU x min). To evaluate the ACAP, the difference in the area values of the samples treated and non-treated with ABAP were calculated. A greater difference between the areas indicated lower antioxidant capacity of the sample. To facilitate visualization of the results, ACAP data were inverted (1/relative area).

Genotoxic assay: To evaluate DNA damage, the comet alkaline test was performed. For this assay, a positive control (PC) was also performed using 0.5 mM methyl methanesulfonate (MMS). After exposure, aliquots of 20 μL of each sample were homogenized with low melting point agarose (5%) for the preparation of slides previously prepared with normal melting point agarose (1.5%), covered with coverslips, and placed in a refrigerator for 40 min. After this period, the coverslips were removed and

the slides were immersed in lysis solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10) for 2 h. After the lysis, the slides were transferred to an electrophoresis cube, containing fresh and ice-cold alkaline buffer (1 mM EDTA and 300 mM NaOH, pH > 13) for 35 min. The electrophoresis was conducted at 25 V and 300 mA for 20 min. The slides were then neutralized (0.4 M Tris, pH 7.5) for 15 min and fixed with 100% ethanol for 10 min. The slides were stained with GelRed (Biotium®) and 100 nucleoids per slide were analyzed in a blinded test under a fluorescence microscope (Leica®, DM 2500) in a 40× objective. DNA damage was visually classified, according to the migration of the DNA fragments into four classes: class 0 (nucleoid without tail with few fragments around), class 1 (tail smaller than the nucleoid diameter), class 2 (tail with a length one to two times the diameter of the nucleoid), and class 3 (tail with a length greater than twice the diameter of the nucleoid). The damage score was obtained by multiplying the number of nucleoids observed in each class of damage analyzed by the value of the class.

Statistical analyses: For each parameter evaluated, the results were compared between the different treatments CTR x N1 x N2 x N3, CTR x Gly x GlyN1 x GlyN2 x GlyN3, CTR x Rd x RdN1 x RdN2 x RdN3, and CTR x PC (where applicable) for each experimental time (1, 6, and 12 h) through parametric (ANOVA) and nonparametric analysis of variance (KruskalWallis), according to distribution of data normality and homogeneity of variance). When necessary, the differences were identified by the Student-Newman-Keuls (SNK) multiple comparisons test. Values of $P < .05$ were considered significant and the results are expressed as mean ± standard error (SE).

Results

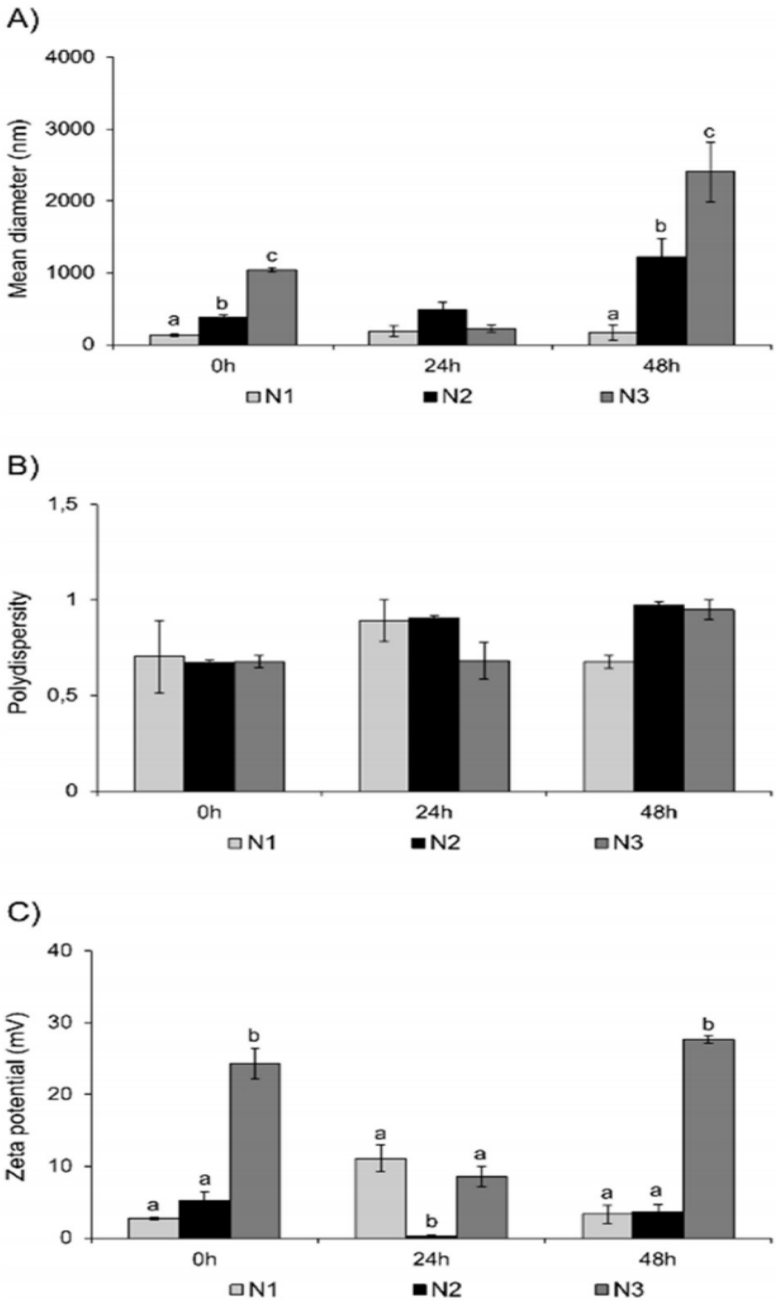


Fig. 1. Mean diameter (A), polydispersity (B), and zeta potential (C) of the goethite NPs present in the exposure solutions of 1, 10, and 100 mg L^{-1} (N1, N2, and N3) for 0, 24, and 48 h. Results are mean ± SE ($n = 3$). Different letters indicate significant differences between treatments for the same experimental time ($P < .05$).

Quantification of iron: The results showed an increase in total Fe concentrations as the NPs concentrations increased both in isolation and in co-exposure with glyphosate and Roundup® when compared to the respective CTR. Although these values were lower than the nominal values of the concentrations of 1, 10, and 100 mg L^{-1} of goethite, the intended gradient was produced. Similarly, the concentrations of dissolved Fe found in the different solutions containing NPs were lower when compared with the total Fe concentrations analyzed, and the highest concentrations of dissolved Fe were for solutions containing the highest concentration of NPs (N3, GlyN3, and RdN3) when compared to

the respective CTR. In addition, the concentration of dissolved Fe found for N3 (8.36 mg L^{-1}) was approximately 12 to 16 times higher, and for GlyN3 (7.10 mg L^{-1}) and RdN3 (4.97 mg L^{-1}), approximately 7 to 10 times higher when compared to the dissolved Fe values of the different treatments.

Cytotoxicity: The integrity of the plasma membrane (i.e. viability), assessed by TB, was > 90% for all times and experimental treatments tested. No statistical differences were found for NPs, Gly and co-exposures, and Rd and co-exposures in this parameter. Despite a significant decrease at 6 h for treatment GlyN3, the viability was still > 90% (Fig. 2A, B, and C). When the MTT assay was employed, the results indicated that the N1 and N2 treatments were cytotoxic at 6 h, resulting in a significant decrease in mitochondrial activity of the cells in relation to the CTR, with no change in this parameter at 1 and 12 h (Fig. 2D). For glyphosate alone (Gly) or in combination (GlyN1, GlyN2, and GlyN3), no significant alterations in viability were found. The concentrations of NPs which demonstrated cytotoxic effects when isolated (N1 and N2) showed that in association with glyphosate the effect was suppressed (Fig. 2E). For Roundup®, all treatments (Rd, RdN1, RdN2, and RdN3) at 6 h produced cytotoxic effects for ZFL cells, with a significant reduction in the viability of these organelles. In addition, the association of NPs with the herbicide did not reverse the cytotoxicity caused by the Roundup®. In contrast, the concentrations of NPs used seemed to have negatively influenced this cytotoxicity, since N1 and N2 treatments also resulted in a decrease in viability, with a pronounced decrease in the co-exposure RdN2. No alteration in mitochondrial activity was observed for times of 1 and 12 h (Fig. 2F).

The results of lysosomal integrity through the NR retention test indicated that N1, N2, and N3 promoted a significant increase in viability of the cells at 1 h and this pattern of increase in viability was maintained in the N3 treatment at 6 h. In contrast, N1 and N2 were cytotoxic at 6 h, and this cytotoxicity was more pronounced for N2. No alteration in this parameter was observed at 12 h (Fig. 2G). When glyphosate-containing treatments were evaluated, the same pattern for the MTT assay was repeated: glyphosate in isolation or in combination did not induce alterations in the viability of the ZFL line for any period tested, and although the isolated NPs were cytotoxic, when associated with glyphosate this cytotoxic effect disappeared (Fig. 2H). In the treatments containing Roundup®, it was possible to verify that only the co-exposure RdN2 resulted in a significant reduction in the viability of these organelles at 6 h. In this case, it is possible to suggest that N2, which was cytotoxic to ZFL cells at the same experimental time, appears to negatively influence the co-exposure cytotoxicity. For 1 and 12 h, no alterations in this parameter were found (Fig. 2I).

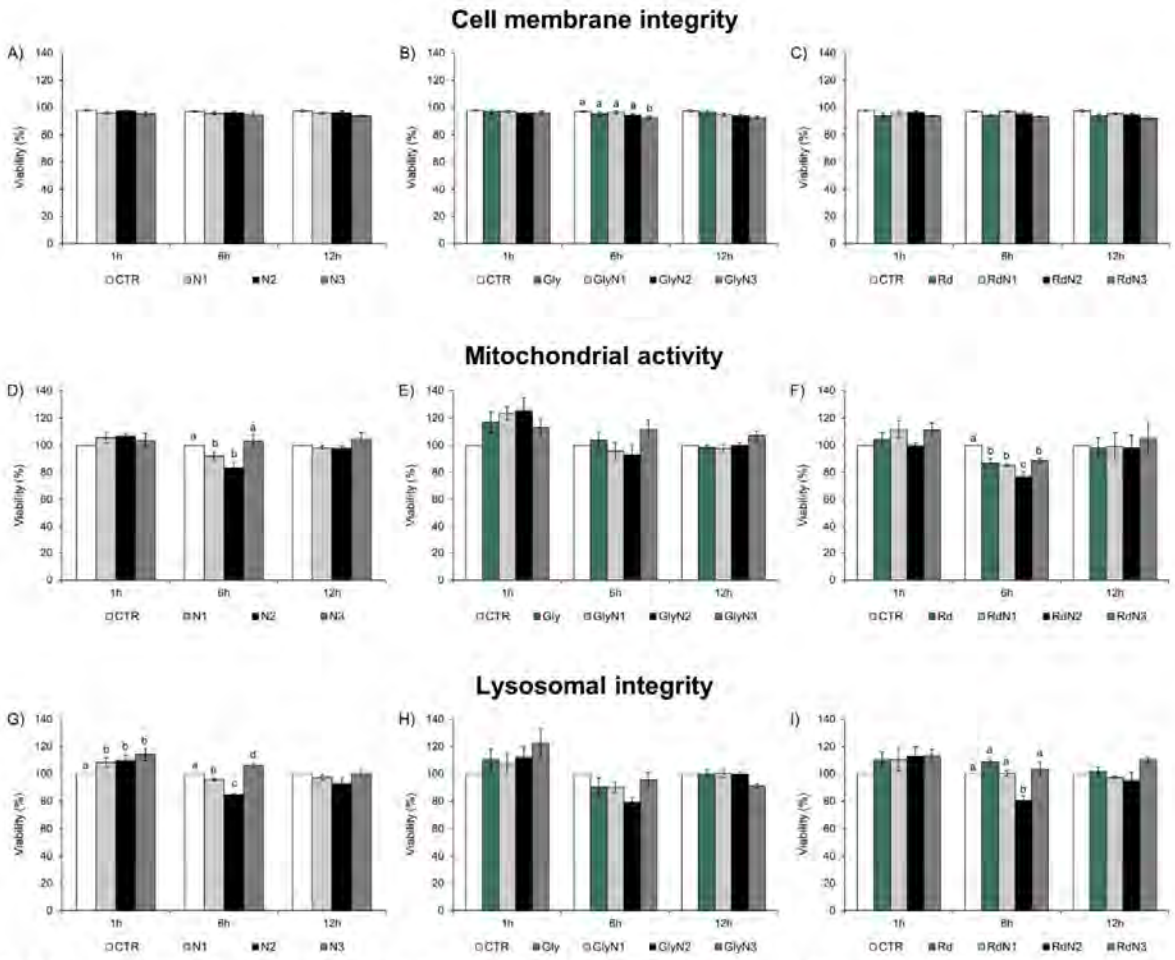


Fig. 2. Cell viability (%) based on plasma membrane integrity (A-C), mitochondrial activity (D-F), and lysosomal integrity (G-I) for ZFL cells exposed to: 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3) and 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco's PBS (CTR) for 1, 6, and 12 h. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time (P < .05).

ROS and ACAP: Regarding the generation of reactive oxygen species (ROS), no significant difference was found for NPs or Gly and co-exposures in all experimental times evaluated (Figs. 3A and B). The results indicated that only the co-exposure RdN3 resulted in a significant increase in ROS at 6 h and no significant alterations were observed in this parameter at 1 h and 12 h (Fig. 3C). Concerning total antioxidant capacity against peroxyl radicals (ACAP), no significant difference between the treatments was found for NPs in the three different times of exposure (Fig. 4A). When glyphosate-containing treatments were evaluated, only treatment GlyN1 showed a significant increase in ACAP at 1 h and no significant alteration was observed for 6 and 12 h. (Fig. 4B). Similarly, in the treatments containing Roundup®, only RdN1 and RdN3 produced a significant increase in this parameter at 1 h and no alterations were found for 6 and 12 h (Fig. 4C).

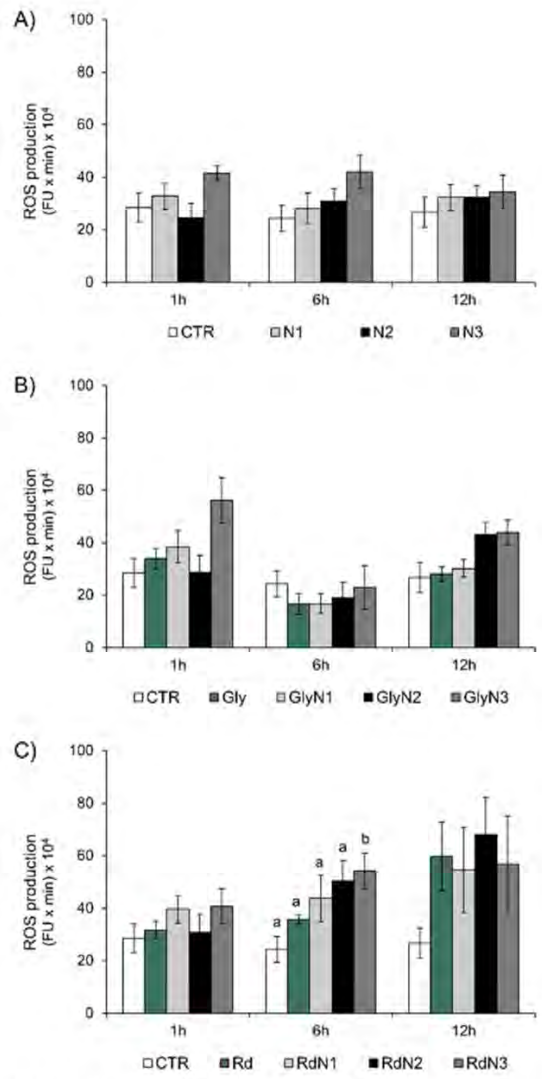


Fig. 3. Production of reactive oxygen species (ROS) in ZFL cells exposed to: A) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); B) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and C) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco's PBS (CTR) for 1, 6, and 12 h. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time ($P < .05$).

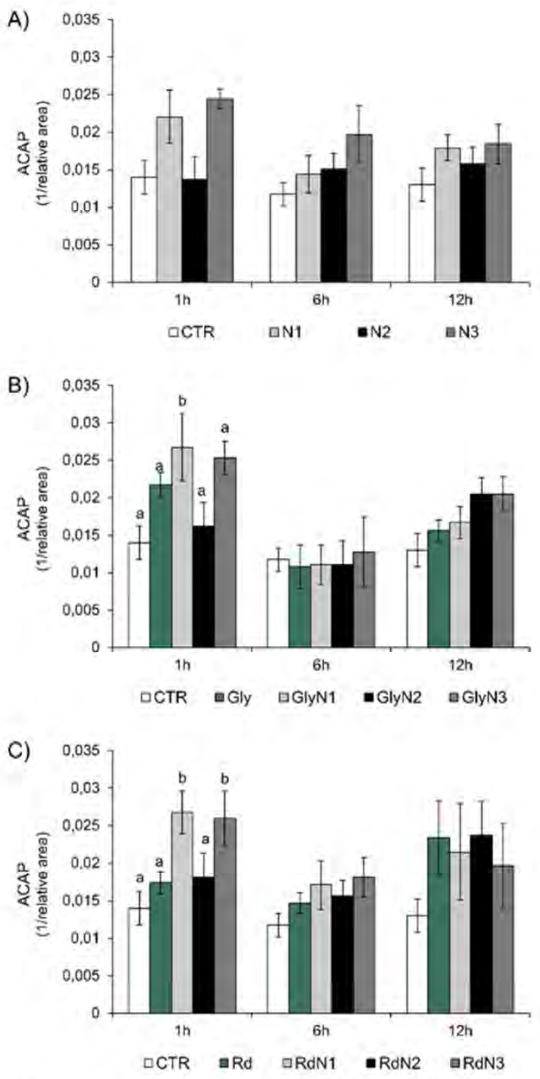


Fig. 4. Antioxidant capacity against peroxyl radicals (ACAP) of ZFL cells exposed to: A) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); B) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and C) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco's PBS (CTR) for 1, 6, and 12 h. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time ($P < .05$).

Genotoxicity: The DNA damage in ZFL cells exposed to the concentration of 0.5 mM MMS (PC) were significantly higher than their respective CTR at the three times tested (Fig. 5A), ensuring the efficiency of the procedure and validation of the methodology used. The results for goethite NPs showed a significant increase in the DNA damage score, compared to the respective CTR, for the ZFL cells exposed to N1 and N2 after 1 h, and the N3 cells after 6 h. After 12 h, no alteration in DNA score was observed. (Fig. 5B). When the ZFL line was exposed to glyphosate in isolation (Gly) or in combination (GlyN1, GlyN2, and GlyN3), no significant alteration in the DNA damage score was identified (Fig. 5C). All the treatments containing Roundup® (Rd, RdN1, RdN2, and RdN3) resulted in a significant increase in the DNA damage score in relation to the CTR after 1 h, with a more pronounced genotoxic effect for the co-exposures. After 6 h, only Rd and RdN3 promoted a significant increase in DNA damage of the ZFL. On the other hand, after 12 h, all co-exposures of Rd with NPs resulted in a significant increase in damage score, with RdN1 and RdN3 causing the greatest damage (Fig. 5D). Different comet classes observed in ZFL cells were shown in Fig. 5E.

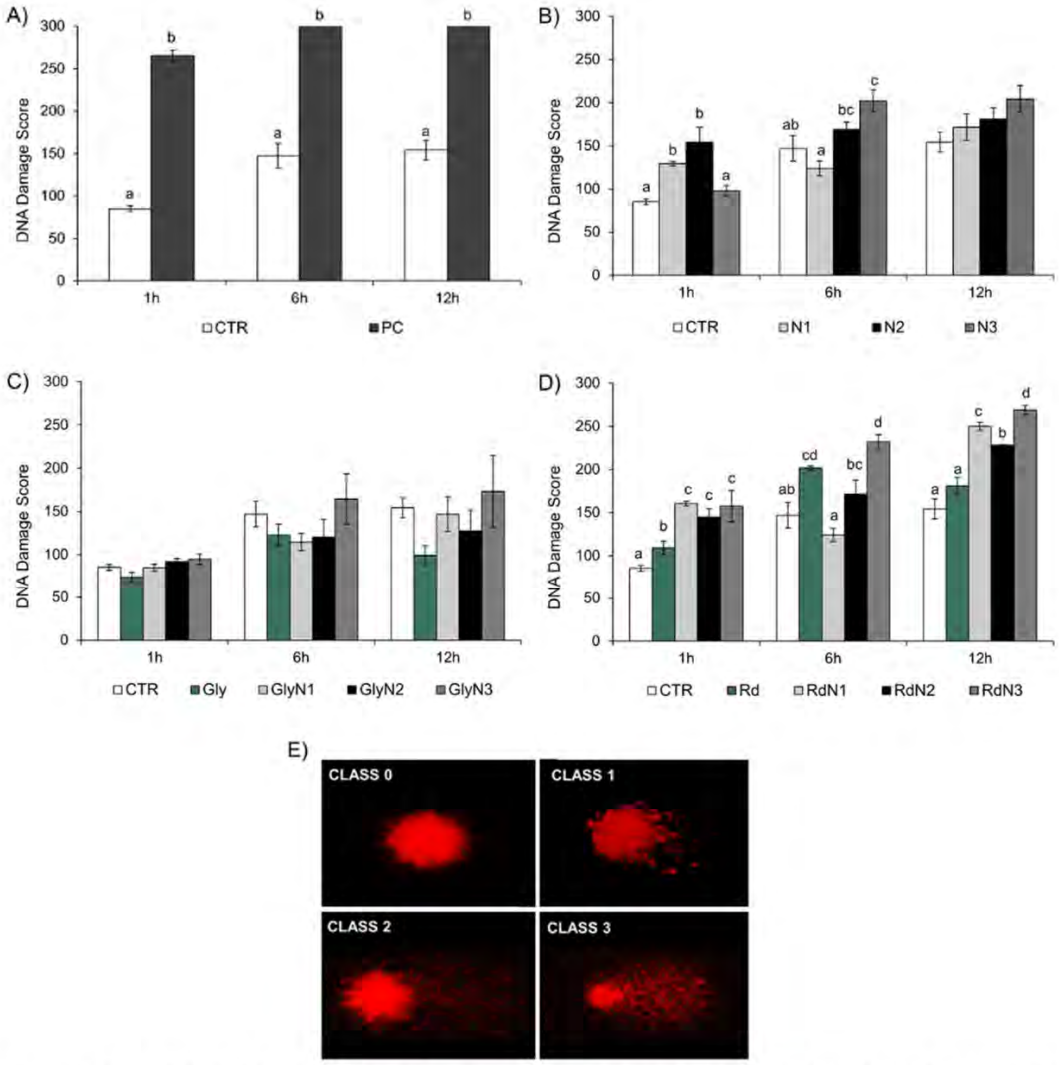


Fig. 5. A) Score of DNA damage in ZFL cells exposed to 0.5 mM of MMS (Positive Control or PC); B) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); C) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and D) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco's PBS (CTR) for 1, 6, and 12 h, quantified by the comet assay. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time ($P < .05$). E) Photomicrographs of ZFL cells processed for comet assay showing increasing degrees of DNA damage (original magnification: 1000×).

Discussion

Considering the potential application of goethite NPs in environmental remediation, the present study evaluated the effects of this iron oxide and its interference in the effects of glyphosate and Roundup® on the hepatocyte cell line of D. rerio (ZFL), through different cellular assays for 1, 6, and 12 h. The results showed that the effects of isolated goethite NPs on ZFL cells were different from the effects produced in association with the selected herbicides: co-exposure with glyphosate suppressed the effects of goethite NPs; and co-exposure with the formulated product, intensified the effects of iron oxide NPs. Regarding cell viability, the concentrations of 1 and 10 mg L^{-1} of goethite NPs were cytotoxic to the ZFL line after 6 h of exposure, interfering in mitochondrial metabolism and lysosomal integrity, without changes in the plasma membrane. Several in vitro studies have already demonstrated the ability of different iron oxide NPs to cause cytotoxic damage in lymphocyte cells, lung cell lines, neural cells, and keratinocytes and dermal microvascular endothelial human cells in different cellular targets (plasma membrane, mitochondria, and lysosomes). Iron oxide NPs are more likely to cross biological membranes due to their large surface area and high reactivity. Once internalized, these NPs can undergo the acid dissolution process within lysosomes, with subsequent liberation of free Fe ions (Fe2+). As a result, the excess of these ions in cells can lead to iron imbalance and cause direct damage to the mitochondria, such as morphological alterations or decreases in mitochondrial membrane potential. In the latter case, the free iron can react with hydrogen peroxide and oxygen produced by the mitochondria to produce highly reactive hydroxyl radicals and ferric ions (Fe3+) via the Fenton reaction. Another

important factor to be considered is that depending on the surface charge of nanomaterials, it may interact with the inner surface of the lysosomal membrane, which could result in damage to this organelle. From assessment of the concentrations of dissolved iron in the exposure solutions, it can be inferred that the positive cytotoxicity results are not related to the excess of free Fe ions. Although treatment N3 presented the highest values of dissolved iron, no cytotoxic damage was observed in ZFL cells exposed to 100 mg L^{-1} of goethite NPs. In contrast, the lowest dissolved iron values found in goethite NPs exposure solutions were at concentrations that resulted in damage to mitochondrial and lysosomal viability (N1 and N2). To evaluate the possible DNA damage to ZFL cells, the comet assay alkaline version was used. This technique is widely used due to its high sensitivity to detect DNA molecule damage, such as single- and doublestrand breaks, alkali-labile sites, incomplete excision repair sites, and cross-links. The results of this assay demonstrated the genotoxic potential of goethite NPs at concentrations of 1 and 10 mg L^{-1} after 1 h and the concentration of 100 mg L^{-1} after 6 h. DNA damage caused by iron oxide NPs has been reported in different cell lines and the concentrations of these NPs are quite varied. Moreover, the majority of studies report that DNA damage caused by exposure to nanoparticles is indirect, resulting from ROS formation by Fenton reaction. However, in the present study, the establishment of oxidative stress was not observed in any period of exposure and, in this particular case, it is suggested that dissolved iron values found for treatment N3 are related to the reported genotoxicity. In addition, it is possible that the DNA damage of the ZFL cells is due to the direct action of goethite NPs. Super magnetic iron oxide nanoparticles can cause direct damage in the DNA by mechanisms still unknown. Although the toxic potential glyphosate has been demonstrated for mussels Perna perna, and fishes D. rerio and Jenynsia multidentata in vitro, in the present work the herbicide was not cytotoxic and it did not promote oxidative stress or cause DNA damage to ZFL cells. In agreement, another study reported the absence of cytotoxic effects on the same cell line exposed to glyphosate at concentrations of 0.65 and 3.25 mg L^{-1} for 24 and 48 h. In turn, when associated with glyphosate, the observed toxicity effects of goethite NPs in ZFL cells disappeared. Glyphosate forms innersphere surface complexes on goethite by a ligand exchange mechanism, resulting in the formation of inner-sphere surface complexes, where the phosphonate group of Gly binds Fe+3 ions at the surface. Considering the great adsorption capacity of goethite to glyphosate, it is possible that the formation of this complex resulted in a decrease in available Fe+3 ions and, consequently, the suppression of toxic effects on ZFL cells exposed only to goethite NPs. The type of contaminant and its interaction with iron-based NPs has been previously reported by a study, which showed that different effects could be obtained depending on the chemical properties of the pollutant. On the other hand, several authors have reported that glyphosate based formulations may cause alterations in mitochondrial function, such as: inhibition of succinate dehydrogenase enzyme activity, transmembrane reduction capacity, and inhibition of ATP synthesis. In the present study, the results demonstrated that Roundup® was cytotoxic, with a decrease in mitochondrial viability at 6 h and no effect on lysosomal and plasma membrane viability parameters. In agreement with the results found, a study, using the ZFL lineage, observed a significant reduction in mitochondrial metabolic activity after exposure to Roundup® (3.25 mg L^{-1}) at 24 and 48 h, whereas lysosomal integrity was reduced only after 24 h exposure, with no alteration in membrane viability at the two experimental times tested. Among the effects described for fish, the genotoxicity of Roundup® has been pointed out as one of the most harmful. In agreement with previous studies, the present work confirmed the genotoxic potential of Roundup® with a significant increase in DNA damage after 1 and 6 h of exposure. After 12 h, the DNA damage of the ZFL cells returned to CTR levels, which could indicate possible activation of the DNA repair system, in order to restore the breaks caused by exposure to the formulated product. According to the results found for isolated goethite NPs or isolated Roundup®, we found that both caused cytotoxic and genotoxic damage to the ZFL cells. When associated, the effects produced were more pronounced, even for the highest concentration of goethite NPs (100 mg L^{-1}), demonstrating cytotoxic characteristics for mitochondrial and lysosomal metabolism, ROS promotion, and ACAP activation, as well as genotoxic potential, even for the longest exposure time. In relation to this intensified toxicity demonstrated by the co-exposure, it is possible to infer that both substances could act together, producing a synergistic effect and intensifying the toxicity for the ZFL cells. The entry of toxic molecules, such as Roundup®, into cells is facilitated due to the adsorbent capacity of nanomaterial. Thus, NPs can serve as carriers, even for contaminants, increasing the intracellular concentration of these compounds and, consequently, their potential toxicity. Moreover, the presence of the other substances in the formulated product, such as surfactants, could favor the entrance of NPs to the ZFL cells, causing the damage observed in this study. Finally,

considering the absence of toxic effects produced by glyphosate and the cytotoxic and genotoxic damage produced by Roundup® to ZFL cells in the present work, it is possible to suggest that the presence of other compounds in its composition may play an important role in the toxicity of this herbicide. The different compounds present in the Roundup® formulation have already been compared in several aquatic organisms and the order of toxicity of the chemical agents found was: POEA>Roundup® > glyphosate >glyphosate isopropylamine salt (IPA), indicating that toxicity of the formulated product can be attributed to the surfactant POEA. In another study, it was pointed out that the genotoxic potential of Roundup® for the fish Anguilla anguilla is directly related to the genotoxicity of surfactant POEA. Although the toxicity of the surfactant POEA has been demonstrated in several studies, our data cannot support that the increase in Roundup® toxicity for ZFL cells is exclusively associated with POEA.

Conclusion

In summary, these data lead us to conclude that the concentrations of goethite NPs used were not safe for the ZFL lineage. In addition, it was shown that goethite NPs and Roundup®, both isolated, presented cytotoxic and genotoxic effects and, when co-exposed, produced a synergistic effect, intensifying the previously reported damage. On the other hand, glyphosate did not promote cytotoxic, biochemical, or genotoxic damage to ZFL cells and, in association, the toxic effects produced by isolated goethite NPs were suppressed. In comparison, given the lack of toxic effects of glyphosate, it is possible to suggest that the presence of other compounds in the formulated product favors the toxicity of this herbicide when compared to the active ingredient glyphosate. Considering the lack of studies investigating the possible toxic effects of goethite NPs, especially for aquatic organisms, our study is extremely significant, as we evaluated the mechanism of action of nanomaterial at the cellular level, contributing to knowledge of the toxic effects generated by iron oxide NPs. In addition, the results may favour discussion and investigation of strategies to determine environmentally safe NPs concentrations that result in an effective tool for removing contaminants from polluted aquatic ecosystems.

3. Assessment and conclusion

Assessment and conclusion by applicant:

It was shown in this *in vitro* study that goethite NPs and Roundup® presented cytotoxic and genotoxic effects in ZFL cells and, when co-exposed, produced a synergistic effect. Glyphosate did not promote cytotoxic, biochemical, or genotoxic damage to ZFL cells and, in association, the toxic effects produced by isolated goethite NPs were suppressed by glyphosate. It is concluded therefore that these findings indicate that the presence of other compounds in the formulated product may be responsible for the aquatic organism toxicity of this herbicide when compared to the active ingredient glyphosate.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test system and glyphosate were not sufficiently characterized and only one concentration of glyphosate was used for testing, preventing any characterisation of dose-response.

Reliability criteria for *in vitro* toxicology studies

Publication: Da Silva *et al*., 2020	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	

Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity of glyphosate is not reported, only source (Milenia Agrociencias S/ A)
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	N	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Only one concentration of glyphosate tested: 3.6 µg/mL.
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	N	Only one concentration of glyphosate tested
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test system and glyphosate were not sufficiently characterized and only one concentration of glyphosate was used for testing.		

1. Information on the study

Data point:	CA 5.6
Report author	Dai P. *et al.*
Report year	2016
Report title	Effect of glyphosate on reproductive organs in male rat
Document No	Acta Histochemica (2016) Vol. 118, 519–526
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Many studies have been published already on reproductive toxicity of glyphosate-based formulations, but few investigated toxicity of glyphosate alone on the male reproductive system. In this study SD rats were Lavaged with glyphosate at doses of 5, 50, 500 mg/kg to detect the toxicity of glyphosate on rat testis. Glyphosate significantly decreased the average daily feed intake at dose of 50 mg/kg, the weight of seminal vesicle gland, coagulating gland as well as the total sperm count at dose of 500 mg/kg. Immunohistochemistry of androgen receptor (AR) has no difference among all groups. As to testosterone, estradiol, progesterone and oxidative stress parameters, the level of them has no differences amidst all doses. Taken together, it had been concluded that glyphosate alone has low toxicity on male rats reproductive system.

Materials and Methods

Chemicals - Isopropylamine salt of glyphosate (90% w/w purity) was purchased from Shanghai Ryon Biological Technology Co. Ltd., China.

Animals - 32 sexually mature 56-day old Sprague-Dawley (SD) male rats were raised in an animal house and maintained in an air-conditioned room at approx. 21^{o}C with a 12 h/12 h light/dark cycle. A balanced mixture of pelleted food and water were available to the rats.

Experimental design and treatment – 32 rats were randomly divided into 4 groups, 3 groups were orally given glyphosate as an aqueous solution by gavage once a day. The control group was treated in the same way with deionized water. The doses administered were 5, 50, and 500 mg/kg bw. All rats were treated for 5 weeks continuously. After the last treatment the rats were sacrificed and testis, epididymis, prostate gland and seminal vesicle removed and weighed.

Epididymal sperm parameters – Epididymal sperm was used for the measurement of total sperm count. The epididymis was minced in PBS and filtered using a nylon mesh screen. The filtrate was treated with 10 mL PBS and the number of sperm was counted using a standard hemo-cytometric method.

Hormone measurement - Serum hormones were measured by radioimmunoassay using a ^{125}I-labeled ligand double-antibody RIA Kit for total testosterone, estradiol and progesterone. The minimum sensitivity of the method was 0.02 ng/mL for testosterone, less than 0.2 ng/mL for progesterone and less than 5 pg/mL for estradiol. The intra- and inter-assay coefficients of variation (CV) were less than 10%.

Testicular, epididymal and seminal vesicle gland histology - Following fixation of the tissues, the samples were passed through a graded series of ethanol and xylene solutions and embedded in paraffin wax. Paraffin-embedded tissues were serially sectioned at 5 μm thickness. For each rat, two non-serial sections were stained with hematoxylin/eosin (HE).

Antioxidant status analysis - The levels of catalase (CAT, U/mg protein), superoxide dismutase (SOD, U/mg protein) and malondialdehyde (MDA, µmol/g protein) were determined by the absorbance of samples in multiskan spectrum. SOD activity was determined by an SOD assay kit with absorbance measured at 560 nm. CAT activity was determined by the H_2O_2 consumption (µmol/g protein) with absorbance measured at 405 nm. Lipid peroxidation was determined by measurement of MDA by the TBA test with absorbance at 532 nm.

Immunohistochemistry - Sections of the testes were deparaffinized with xylene and rehydrated in graded ethanol before being washed with twice-distilled water. To increase epitope exposure, the sections were heated for 15 minutes in sodium citrate buffer (0.01 M, pH 6.0) in a microwave oven. The sections were then cooled and washed with 0.01 M PBS at pH 7.2 and then blocked with 10% bovine serum albumin (BSA) in TBST (20 mM Tris-buffered saline, 0.05% Tween 20, pH 7.5) for 1 hour at room temperature. The sections were incubated overnight at 4ºC with diluted (1:400) polyclonal antibodies against androgen receptor (N-20; rabbit anti-human AR). The secondary antibody was goat anti-rabbit IgG. The binding of the antibodies were visualized using a SABC Kit Elite and 0.05% 3,3-diaminobenzidine tetrachloride in 0.01 M PBS at pH 7.2, containing 0.01% H_2O_2 for 2 minutes. The sections were counter stained with hematoxylin and mounted with cover slips. The specificity of the antibody was examined using 1% BSA rather than the primary antibody.

Data analysis - All results are means ± SEM. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by Tukey's multiple comparison test. Significant differences with controls were considered when $p < 0.05$.

Results

Average daily weight gain and average daily feed intake - Daily exposure to glyphosate caused a statistically significant decrease in average daily feed intake at 50 mg/kg bw/day only. Although not statistically significant there was a dose-dependent decrease in daily weight gain.

Reproductive organ weights and sperm parameters - Seminal vesicle and coagulating gland absolute weight showed a statistically significant changes amongst treatment groups whereas no such change was observed in other reproductive organs. No significant differences were observed in relative reproductive organ weights. Total sperm count was statistically significantly decreased at 500 mg/kg bw. .

Concentrations of testosterone, estradiol and progesterone in serum - Although there was a trend towards decreased serum concentrations with dose for testosterone and progesterone no statistically significant changes were noted in the serum concentrations of testosterone, estradiol and progesterone.

SOD and CAT activity, H_2O_2 and MDA levels in testes - There were no statistically significant changes in SOD and CAT activity and H_2O_2 and MDA levels in testes.

Testicular, epididymal and seminal vesicle gland histology - No statistically significant changes were observed in the histopathology of the testis, epididymis and seminal vesicles.

Immunohistochemical localization of androgen receptor in the testis - No statistically significant changes were observed in androgen receptor (AR) immunoreactivity localized in the nuclei of cells, including Sertoli cells, peritubular myoid cells and Leydig cells.

Discussion and conclusions

The present study provides information on the potential effects of glyphosate on the reproductive system of the male rat. Average daily weight gain showed no substantial decrease whereas average daily feed intake was significantly decreased at 50 mg/kg bw but not at 500 mg/kg bw. It is therefore suggested that the decrease of average daily feed intake is independent of glyphosate treatment. Although there are no statistically significant differences in average weight gain, the trend is a decrease. At 500 mg/kg

bw the absolute weight of seminal vesicle gland and coagulating gland and total sperm count decreased substantially. There was no significant change in oxidative stress parameters after oral administration of glyphosate at doses up to 500 mg/kg bw. Testosterone, estradiol and progesterone serum levels, as well as AR in testis presented no significant change at all dose levels tested when compared to controls.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The potential toxicity of glyphosate to the male reproductive system of the rat has been investigated after oral treatment with glyphosate for 5 weeks at dose levels up to 500 mg/kg bw. The endpoints studied were body weight, food intake, daily weight gain, absolute and relative reproductive organ weight, serum hormone levels, oxidative stress parameters, testicular histopathology and expression of AR in testis. The effects found were a significant decrease in absolute (but not relative) weight of the seminal vesicle gland and coagulating gland and a decrease in sperm count at the highest dose tested.

This publication is considered relevant but reliable with restrictions because there are deviations from regulatory guidelines for reproductive toxicology studies and the reproductive effects seen are not corroborated by the results from regulatory studies at similar dose levels.

Reliability criteria for *in vivo* toxicology studies

Publication: Dai *et al.*, 2016	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	Incomplete study
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 90% as isopropylamine salt. Source Shanghai Ryon Biological Technology Co. Ltd., China.
Only glyphosate acid or one of its salts is the tested substance	Y	Isopropylamine salt
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y?	
Route and mode of administration described	Y	
Dose levels reported	Y	
Number of animals used per dose level reported	Y	8 males per group
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y?	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	Results are not concordant with outcome of regulatory

		reproduction toxicology studies
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant but reliable with restrictions because there are deviations from regulatory guidelines for reproductive toxicology studies and the reproductive effects seen are not corroborated by the results from regulatory studies at similar dose levels.		

1. Information on the study

Data point:	CA 5.4
Report author	De Almeida L. K. S. *et al.*
Report year	2018
Report title	Moderate levels of glyphosate and its formulations vary in their cytotoxicity and genotoxicity in a whole blood model and in human cell lines with different estrogen receptor status
Document No	3 Biotech (2018) Vol. 8(10), 438 (1-15)
Guidelines followed in study	None
Deviations from current test guideline	NA
Previous evaluation	No
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In vitro studies were conducted to determine the short-term cytotoxic and genotoxic effects of pure glyphosate and two glyphosate formulations (Roundup® and Wipeout®) at concentrations relevant to human exposure using whole blood (cytotoxicity) and various cancer cell lines (cytotoxicity and genotoxicity). Pure glyphosate (pure glyph) and Roundup® (Ro) showed similar non-monotonic toxicological profiles at low dose exposure (from 10 µg/ml), whereas Wipeout® (Wo) demonstrated a monotonic reduction in cell viability from a threshold concentration of 50 µg/ml, when tested in whole blood. We evaluated whether using various cancer cells (the estrogen-E2-responsive HEC1A, MCF7 and the estrogen-insensitive MDA-MB-231) exposed to moderate doses (75–500 µg/ml) would indicate varied toxicity and results indicated significant effects in the HEC1A cancer cells. A non-monotonic reduction in cell viability was observed in HEC1A exposed to pure glyph (75–500 µg/ml) and proliferative effects were observed after exposure to Wo (75, 125 and 250 µg/ml). Genotoxicity assessment (test concentration 500 µg/ml) demonstrated DNA damage in the HEC1A and MDA-MB-231 cells.

Materials and methods

Chemicals - Glyphosate (99.5% purity) was purchased from Supelco Analytical (USA).

Whole blood cell culture and exposure - Blood from 5 healthy volunteers was collected by venipuncture in heparin-containing tubes. Blood samples were diluted 1:10 in RPMI 1640 media supplemented with 50 µg/ml streptomycin and 50 U/ml penicillin. The 1 mL whole blood samples were exposed to glyphosate at various concentrations up to 500 µg/mL for 18 hours at 37 °C. Lipopolysaccharide (LPS, 5 µg/mL) was used as the positive control and pyrogen-free water as the negative control.

Breast cancer (MCF7 and MDA-MB-231) and endometrial cancer (HEC1A) cell line culture - MCF7 (hormone responsive) and MDA-MB-231 (hormone independent) cell lines were obtained from the American Type Culture Collection (ATCC), USA and HEC1A cells were obtained from Nelson Mandela Metropolitan University, Eastern Cape, South Africa. All cell lines were grown in DMEM supplemented with 5% heat inactivated fetal calf serum, 50 U/mL penicillin and 50 µg/mL streptomycin. To maintain a stable estrogen-sensitive phenotype, cells were cultured in phenol-free medium after the removal of phenol red. All data were normalized against untreated controls. Cell lines were routinely maintained under standard cell culture conditions at 37 °C, 5% CO2 and 90% humidity. The different cell lines were exposed to glyphosate at varying concentrations up to 500 µg/mL for 24 hours at 37 °C. Camptothecin (100 µM), a DNA topoisomerase inhibitor, was used as the positive control in this study.

Cytotoxicity assay (MTT assay) - Following exposure to glyphosate, samples were incubated in 0.5 mg/mL MTT reagent for 3 hours for the cancer cell lines and for 30 minutes for whole blood at 37 °C. After incubation, the MTT reagent was aspirated and 1 mL of DMSO was added to solubilize the formazan product formed. Purple color formation was determined spectrophotometrically at 560 nm using a Biotek Powerwave XS microplate reader.

Single-cell gel electrophoresis (comet assay) - The exposure concentrations used for this study were chosen based on results obtained in the cell viability assay and the positive control reference concentration was chosen based on cytotoxicity results reported in the HepG2 human liver cell line. The MCF7, MDA-MB-231 and HEC1A cancer cell lines (100,000 cells/well) were incubated in 24-well plates for 4 hours at 37 °C, in the presence 500 and 1000 µg/mL glyphosate. Camptothecin was used as a positive control in this study (100 µM). The preparation of the samples and the method used for the comet assay were conducted according to the instructions described in the OxiSelect™ Comet Assay Kit (Cell Biolabs, Inc).

Preparation of cell samples for the comet assay - Trypsinized cells were pooled (three sample wells) and centrifuged at 700×g for 2 minutes. The supernatant was discarded and the pellet washed with ice cold PBS and centrifuged at 700×g. The cells (1×10^5 cells/mL) were then resuspended in ice cold PBS before the assay was conducted.

Comet assay sample slide preparation and cell lysis - Resuspended cells (10 µL) were combined with 100 µL molten comet agarose and the mixture (75 µL/well) was immediately placed onto an OxiSelect™ comet slide. Slides with the cell agarose mixture were incubated at 4 °C in the dark for 15 minutes to allow the agarose to set. Slides were treated in pre-chilled lysis buffer (pH 10) for 60 minutes at 4 °C in the dark followed by treatment in alkaline solution (pH 13) for 30 minutes at 4 °C in the dark.

Alkaline electrophoresis - Slides were subjected to alkaline electrophoresis for 18 minutes at 300 mA, neutralized in pre-chilled deionised water and washed in 70% cold ethanol for 5 minutes. The slides were then air-dried and incubated with 100 µL of 1 × Vista Green dye prepared in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) for 15 minutes at room temperature in the dark.

Comet assay sample visualization and data analysis - Slides were visualized with fluorescence microscopy (5×) using a FITC filter (7%). Images were obtained using an AxioCam MR3 Camera and stored in the Axio vision Rel.4.8 program. Image J Macro was used to calculate the tail length, % tail moment and % tail DNA. 50 cells were analyzed per slide (replicates of 3) from pooled cell cultures, per experimental treatment (n = 3).

Statistical analysis - All data were presented as mean ± standard error of the mean (SEM). ANOVA single-factor analysis (Microsoft Excel) was used to determine significant differences ($P \leq 0.05$, $P \leq 0.01$). Non-linear least square regression models were applied to cytotoxicity data in human whole blood using the Graphpad Prism 6 software package. Goodness of fit was assessed using R2, R2 adjusted values and by the assessment of upper and lower 95% confidence limits associated with the model fit. The Wald–Wolfowitz (runs) test was used to determine the deviation of the nonlinear regression model from the experimental data. The models were used to estimate the concentration of glyphosate required to illicit a half maximal response (half maximal effective concentration, EC50). Based on the observed biphasic nature of the curves obtained for glyphosate and Roundup, a model describing a seven-parameter bell-shaped dose (combines two sigmoidal responses)-response curve was selected.

Results

Cell viability in human whole blood – Glyphosate cytotoxicity in human whole blood at concentrations from 0.1 to 500 µg/mL was determined using the MTT reduction assay. A statistically significant reduction in cell viability of whole blood was observed for glyphosate at 10, 50 and 250 µg/mL but not at 500 µg/mL. LPS was a suitable positive control for this study.

Cell viability (MTT assay) in human cancer cell lines - A statistically significant reduction in cell viability was noted for glyphosate in the HEC1A cell line at 75, 125, 250 and 500 µg/mL. No change in viability was seen with the MCF7 and MDA-MB-231 cell lines. The positive control, camptothecin, reduced significantly cell viability in all three cell lines.

Single cell gel electrophoresis (comet assay) - The test concentrations selected for genotoxicity studies were based on the results of the cell viability study and reference concentrations were chosen based on glyphosate concentrations reported in the literature to incur genotoxic damage in human cell lines. Glyphosate was tested in the comet assay at 500 and 1000 µg/mL where a statistically significant increase in tail length and tail moment was observed at both concentrations in the HEC1A and MDA-MB-231 cell lines. The positive control, camptothecin, increased tail length significantly in the HEC1A and MDA-MB-231 cell lines but not in the MCF-7 cell line. The positive control increased tail moment significantly in all 3 cell lines.

Discussion and conclusions

When tested at concentrations ranging from 0.1 to 500 µg/mL, a statistically significant reduction in cell viability was observed in whole blood at glyphosate concentrations of 10, 50 and 250 µg/mL but not at 500 µg/mL. When tested for cytotoxicity glyphosate showed a statistically significant reduction in cell viability in the endometrial cancer cell line HEC1A at 75, 125, 250 and 500 µg/mL. No effect on cell viability was seen on hormone responsive (MCF7) and hormone independent (MDA-MB-231) breast cancer cell lines at concentrations up to 500 µg/mL. When glyphosate was tested for DNA damage in the single cell gel electrophoresis assay (comet assay) a statistically significant increase in tail length and tail moment was observed at 500 and 1000 µg/mL in the endometrial cancer cell line HEC1A and the hormone independent breast cancer cell line MDA-MB-231. No DNA damage was observed in the hormone responsive breast cancer cell line MCF7 up to concentrations of 1000 µg/mL. Cytotoxicity results at concentrations relevant to occupational and residential exposure to glyphosate observed in the three cancer cell lines suggest that toxicity varies depending on cell type, with the most significant results observed in the HEC1A cancer cell line exposed to glyphosate. Moderate concentrations of glyphosate (500 µg/mL) induced genotoxic effects in the HEC1A and MDA-MB-231 cancer cell lines, which suggests that glyphosate may display various mechanisms of toxicity.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The cytotoxicity of glyphosate has been investigated in whole blood, in hormone independent (MDA-MB-231) and in hormone responsive (MCF7) cell lines and in a an endometrial cancer cell line (HEC1A). The capacity of glyphosate to produce DNA damage was investigated in MCF7, MDA-MB-231 and HEC1A cells in the Comet assay. Glyphosate was found to reduce cell viability in whole blood at the intermediate concentrations (10-250 µg/mL) but not at the highest concentration tested (500 µg/L). A concentration related reduction in cell viability was seen with glyphosate in HEC1A cells but not in the two other cell lines. When glyphosate was tested at 500 and 1000 µg/mL an increase in tail length and tail moment was observed in HEC1A and MDA-MB-231cells but not in the hormone responsive breast cancer cell line MCF7. The *in vitro* concentrations of glyphosate at which DNA damage was observed were 500 and 1,000 µg/mL which are systemic concentrations that cannot be reached in *in vivo* toxicology studies.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the Comet assay was only conducted at concentrations that are physiologically not feasible in *in vivo* toxicology studies (> 1mM).

Reliability criteria for *in vitro* toxicology studies

Publication: De Almeida et al., 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 99.5%. Source: Supelco Analytical USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also glyphosate-based formulations were tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Whole blood from volunteers, breast cancer cells (MCF7 and MDA-MB-231) and endometrial cancer cells (HEC1A).
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	N	For cytotoxicity testing glyphosate concentrations from 0.1 to 500 µg/mL were used. For comet testing only glyphosate concentrations of 500 and 1000 µg/mL were used (> 1 mM).
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Was studied but not established.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the Comet assay was only conducted at concentrations that are physiologically not feasible in *in vivo* toxicology studies (> 1mM).		

1. Information on the study

Data point:	CA 5.5
Report author	Duforestel M. *et al.*
Report year	2019
Report title	Glyphosate primes mammary cells for tumorigenesis by reprogramming the epigenome in a TET3-dependent manner
Document No	Frontiers in genetics, (2019) Vol. 10, pp. 885.
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The herbicide glyphosate has been scrutinized for an impact on cancer incidence, but reports demonstrate the difficulty of linking estimates of exposure and response analysis. An approach to better apprehend a potential risk impact for cancer is to follow a synergistic approach, as cancer rarely occurs in response to one risk factor. The known influence of glyphosate on estrogen-regulated pathway makes it a logical target of investigation in breast cancer research. In this study, non-neoplastic MCF10A cells in a repeated glyphosate exposure pattern over 21 days were used. Glyphosate triggered a significant reduction in DNA methylation, as shown by the level of 5-methylcytosine DNA; however, in contrast to strong demethylating agent and cancer promoter UP peptide, glyphosate-treated cells did not lead to tumor development. Whereas UP acts through a DNMT1/PCNA/UHRF1 pathway, glyphosate triggered increased activity of ten-eleven translocation (TET)3. Combining glyphosate with enhanced expression of microRNA (miR) 182-5p associated with breast cancer induced tumor development in 50% of mice. Culture of primary cells from resected tumors revealed a luminal B (ER+/PR-/HER2-) phenotype in response to glyphosate-miR182-5p exposure with sensitivity to tamoxifen and invasive and migratory potentials. Tumor development could be prevented either by specifically inhibiting miR 182-5p or by treating glyphosate-miR 182-5p-cells with dimethyloxallyl glycine, an inhibitor of TET pathway. Looking for potential epigenetic marks of TET-mediated gene regulation under glyphosate exposure, we identified MTRNR2L2 and DUX4 genes, the hypomethylation of which was sustained even after stopping glyphosate exposure for 6 weeks. The findings reveal that low pressure but sustained DNA hypomethylation occurring via the TET pathway primes cells for oncogenic response in the presence of another potential risk factor. These results warrant further investigation of glyphosate-mediated breast cancer risk.

Materials and methods

Cell Culture and Transfection; MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum (Invitrogen, Cergy Pontoise, France), 500 ng/ml hydrocortisone (Sigma-Aldrich, France), 100 ng/ml cholera toxin (Sigma-Aldrich, France), 10 µg/ml insulin (TermoFisher, France) and 20 ng/ml epidermal growth factor (EGF, SigmaAldrich, France), penicillin (100 U/ml), and 2 mmol/L L-glutamine. MCF7 and MDA-MB-231 cells were cultured in DMEM medium (Invitrogen) all supplemented with 5% FCS and 2 mM l-glutamine. Glyphosate (CAS 1071-83-6, sc-211568) was purchased from Santa-Cruz (France), and a 10^{-8}-M stock solution was prepared in DMSO every week. Glyphosate was diluted directly in fresh cell culture medium and was fed to the cells at the time points indicated in the results section. For the transfection of RNAs, we used miRCury LNA miR mimics for the has-miR-146a, has-miR-182-5p, has-miR-27a, has-miR-500a-5p, has-miR-30a, and has-miR-495 (Qiagen, France), siRNA for siRNA-T ET3 (sc94636) and control siRNA-A (sc94636) and HIPerfect Transfection Reagent (Qiagen, France). All miRs showed similar transfection efficiency (10- to 15-fold change, as measured by RTqPCR).

DNA Extraction, 5mC ELISA, and qMSRE; A QIAcube automate and QIAmp DNA Mini QiaCube kit (Qiagen, France) were used to isolate DNA. The quantification of 5mC was performed using the 5mC DNA ELISA Kit (Zymo Research-Euromodex, France) according to the manufacturer's instructions. The 5mC DNA ELISA Kit estimates the number of 5mC on DNA without distinction of localization; therefore, the term of global DNA methylation level when referring to results obtained via this mode of quantification was used. Next, DNA methylation was quantified by qMSRE. Digestions were performed with adequate restriction enzymes, HpaII and AciI (NEB, France). Typically, 1 ng of genomic DNA was digested with 40 U of enzymes at 37°C for 2 h in 50 µl of reaction. Control samples were treated in the same way but without addition of the enzyme. Five microliters of digestion mixture were used for qPCR. The QuantiFast SYBR Green PCR Kit and Rotor-Gene Q (Qiagen, France) were used to perform the qPCR. Primers were MSH3: TTTCTCCAG GGCTGGGACTTTG and CCCGACTGGATTCCCCTTTTCT; DHFR: AAACCTCAGCGCTTCACCCAAT and TGATAGG GCTGGAGGAGGAAG; DUX4: CGACACCCTCGGACAGCA and TCAAAGCAGGCTCGCAG; COL23A1: TCTCCAGG CCAGAAACAGTCTT and ATTTAGAGAGGCAGGGTC GAGA; and MTRNR2L2: ACCCCACCTGTTTACCAA and GCTACCTTTGCACGGTTAGGG.

Tumor Xenografts in Nude Mice; Cells were harvested by trypsinization, washed and resuspended in saline buffer. Cell suspensions were injected subcutaneously into the flank of 7 to 8-week-old mice (Janvier, France) in 100 µl of sterile PBS. Tumor volume based on caliper measurements was calculated using the modifed ellipsoidal formula [Tumor volume = 1/2 (length × width^2)] according to previously published work. At the end of the observation period, the mice with xenograf tumors were euthanized, and the tumor tissues were removed for analysis. The experimental procedures with animals were in accordance with the guidelines of Institutional Animal Care and the French National Committee of Ethics. In addition, all experiments were conducted according to the Regulations for Animal Experimentation at the Plateforme Animalerie in the Institut de Recherche en Santé de l'Université de Nantes (IRS-UN) and approved by the French National Committee of Ethics. The number of mice was restricted to four per condition to limit the number of animals to the necessary minimum as in previous studies based on the fact that we anticipated to detect a highly frequent tumorigenic event (frequency superior to one to four for tumorigenesis).

Establishment of Tumor Cells; From Xenografts (PCTCdX); PCTCdX (here named Glypho-iBPCTC) were obtained after mechanical dissociation. Briefly, resected tumor tissue from mice was cut into pieces of 1–5 mm^3 and plated in a 60-mm^2 tissue culture dish with DMEM containing 10% FBS and antibiotics. Minced pieces of tumor were incubated with 200 U/ml collagenase I (Sigma) and 500 U/ml DNaseI (Sigma) in PBS for 1 h at 37°C with vigorous constant agitation. The single cell suspension was filtered through a 70-mm cell strainer (BD Falcon), washed with PBS, and then placed in DMEM-10% FBS. Cell cultures were split 1:5 when confluent.

Migration Assay; Cells (3×10^5) were seeded in six-well plates, cultured until they reached 80–90% confluence, and treated with 10 µg/ml of mitomycin C (Sigma, France) for 2 h (to prevent cell proliferation). The monolayer of cells was scratched using a two-well silicone insert (Ibidi, Germany). Cell migration was monitored by microscopy (Incellis Cell Imager, Bertin, France). The images acquired at different time points (0, 4, 8, 24, 28, 32, and 48 h) for each sample were analyzed quantitatively. For each image, distances between one side of the wound and the other side were measured with ImageJ software; the mean value of 10 measurements all along the wound was recorded. The average migration speed was obtained by calculating the ratio distance/time along the time course.

Invasion Assay; All of the procedures were performed according to the manufacturer's instructions (QCM 24-Well Collagen-Based Cell Invasion Assay, Millipore, France). In brief, 200 µl of serum-free medium containing 2×10^5 cells were added into the invasion chamber, with the bottom well of the 24-well plate containing 500 µl of complete medium. After 72 h of incubation at 37°C, the medium was removed, and the cells were stained by placing the chamber in staining solution for 20 min at room temperature. Cells that did not invade were carefully removed from the top side of the chamber using a cotton swab. The stained chamber was inserted into a clean well containing 200 µl of extraction buffer for 15 min at room temperature. A total of 100 µl of extracted (stained) solution from the chamber was transferred into a 96-well plate, and the optical density was measured 570 nm using a spectrophotometer.

Viability Assay: MTT and XTT Tests; A cell suspension containing 105 cells was prepared, and 100 µl was distributed in sixplicates in a 96-well plate. After 24 h of incubation at 37°C and 5% CO_2, cells

were exposed to tamoxifen for 48 h. Tamoxifen was first diluted 10 times in dimethyl sulfoxide (DMSO) and then further diluted in DMEM containing 4.5 g/L glucose, 1% SVF, 1% glutamine, 1% penicillin-streptomycin at the desired concentrations. Following treatment, 10 µl of MTT (10 µg/ml) (VWR Chemicals, France) was added in each well, and the cells were incubated for 3 h. Finally, the medium containing MTT was removed, and 200 µl/well of DMSO was added to measure the optical density at 570 nm using a spectrophotometer. For the XTT test, the XTT Assay Kit was used (ab232856, Abcam, France) according to the manufacturer's instructions. Briefly, 10^5 cells were seeded in 100 µl of culture medium in each well of a 96-well plate. After 24 h of incubation at 37°C and 5% CO_2, cells were treated with adequate drugs. Then, 10 µl/well of XTT mixture was added for an incubation of 2 h at 37°C and 5% CO_2. Finally, absorbance was measured at 450 nm.

Breast Tissue and Urine Samples; Human samples were collected from the Réseau des tumorothèques du Cancéropole Grand-Ouest and Institut de Cancérologie de l'Ouest. In accordance with regulations, all subjects signed a specifc informed consent form for this biocollection approved by an Ethics Committee (CPP OUEST IV, n°18/16), the French State Department for National Education, Higher Education and Research (Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche, N° DC-2015-2457) and the Commission Nationale de l'Informatique et des Libertés (CNIL) (compliance commitment to MR 001). The glyphosate concentration in urine samples was obtained using Glyphosate kit (Novakits, France).

mMTase and TET Activities; TET activity was determined using the Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit (Colorimetric; Epigentek/Euromedex, France) according to the manufacturer's instructions. Dnmts-magnetic beads (DMB) assays were performed to estimate mMTase, such as initially described. Briefly, a typical methylation reaction required 50 µg of nuclear extract (Nuclear extract kit, Active Motif, France), 125 nM DNA oligonucleotides (probes), and 900 nM tritium-labeled AdoMet (1 mCi/ml; #NET155V001MC; PerkinElmer, France) in reaction buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). After incubation at 37°C for 1 h, reactions were quenched with an equal volume of magnetic beads suspension and incubated for 15 min at room temperature. Next, the beads were magnetically isolated from the reaction mix, and tritium incorporation was measured by scintillation counting.

In-Cell ELISA; In-cell ELISA was performed using the In-Cell ELISA Kit (Abcam, France) according to the manufacturer's instructions and after a fixation step performed with 4% of paraformaldehyde solution (10 min at room temperature). Primary antibodies were incubated overnight at 4°C. Adequate HRP-conjugated secondary antibodies were incubated for 1 h at room temperature. Detection was performed at 450 nm. After the washes, cells in each well were incubated with 1X Janus Green Stain for 5 min at room temperature, according to the manufacturer's instructions. Data were expressed in normalized unit, according to the following calculation: (HRPsignal 'minus' HRPsignal in absence of primary antibody)/(Janus Green signal 'minus' Janus Green signal in absence of cells). Antibodies used were anti-TET1 (sc163446, Santa Cruz, France), anti-TET2 (sc398535, Santa Cruz), anti-TET3 (sc139186, Santa Cruz), anti-ERα (sc8002, Santa Cruz), anti-PR (sc130071, Santa Cruz), and anti-HER2 (sc-393712, Santa Cruz).

ChIP Analyses; ChIP was performed using the ChIP-IT Express kit (Active Motif, France) according to the manufacturer's instructions. The cross-linking step was performed by treating the cells with 37% formaldehyde solution for 15 min at room temperature. Sonication was performed with the Bioruptor Plus (eight cycles 30 s on/90 s off) (Diagenode, France). The QuantiFast SYBR Green PCR Kit and Rotor-Gene Q (Qiagen, France) were used to perform the qPCR. Antibodies used were Anti-IgG (Abcam, AB2410) and anti-TET3 (sc139186, Santa Cruz).

Statistical Analysis; All experiments were done at least in biological triplicates. Differences in means were assessed using Student t test, and the degree of correlation between two parameters was calculated using Pearson's test. $P < 0.05$ was considered significant.

Results

Exposure to Glyphosate Promotes TET3-Mediated Global DNA Hypomethylation in MCF10A Cells; DNA hypomethylation has been shown to play a determining role in cancer development. To verify the

impact of glyphosate exposure on the global level of DNA methylation, non-neoplastic breast epithelial MCF10A cells were treated with a low dose (10-11 M) of this herbicide every three to four days over 21 days, covering three passage numbers; whereas control cultures were treated with vehicle DMSO (Figure 1A). Several articles analyzing the effect of glyphosate on human cells have reported using 10-11 M. Indeed, 90% of MCF10A cells were viable as measured by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay at this concentration. Importantly, glyphosate 10-11 M is below the concentration detected in biological fluids (milk, serum, urine). As a control performed in parallel, MCF10A cells were exposed to carcinogenic UP peptide (0.5 μM) previously described to promote global DNA hypomethylation via the disruption of the DNMT1/PCNA/UHRF1 complex. As expected, there was a decrease in the level of 5mC-DNA in MCF10A cells treated with the UP peptide (Figure 1B). There was also a reduction in 5mC content in cells treated with glyphosate (Figure 1B), hence suggesting that glyphosate promotes a global DNA hypomethylation as per the definition given in the introduction. The origin of glyphosate-mediated decrease in DNA methylation was assessed by measuring the levels of activity of maintenance methyltransferase (mMTase) and Ten-eleven translocation (TET), since a decrease of mMTase activity and an increase of TET activity are both causes of DNA hypomethylation. The mMTase activity remained unchanged in MCF10A cells treated with glyphosate (Figure 1C) while TET activity significantly increased in these cells (Figure 1D). Specifically, an ELISA-based assessment of the amount of the three TET family members, TET1, TET2 and TET3, revealed an overexpression of TET3 in MCF10A cells following exposure to glyphosate (Figure 1E). To confrm that glyphosate promotes TET3-mediated global DNA hypomethylation in MCF10A cells, we analysed the level of DNA methylation in MCF10A cells with siRNAmediated TET3 down-regulation. ELISA results show that the presence of siRNA-TET3 abrogates TET3 overexpression and prevents DNA hypomethylation in cells exposed to glyphosate (Figure 1F).

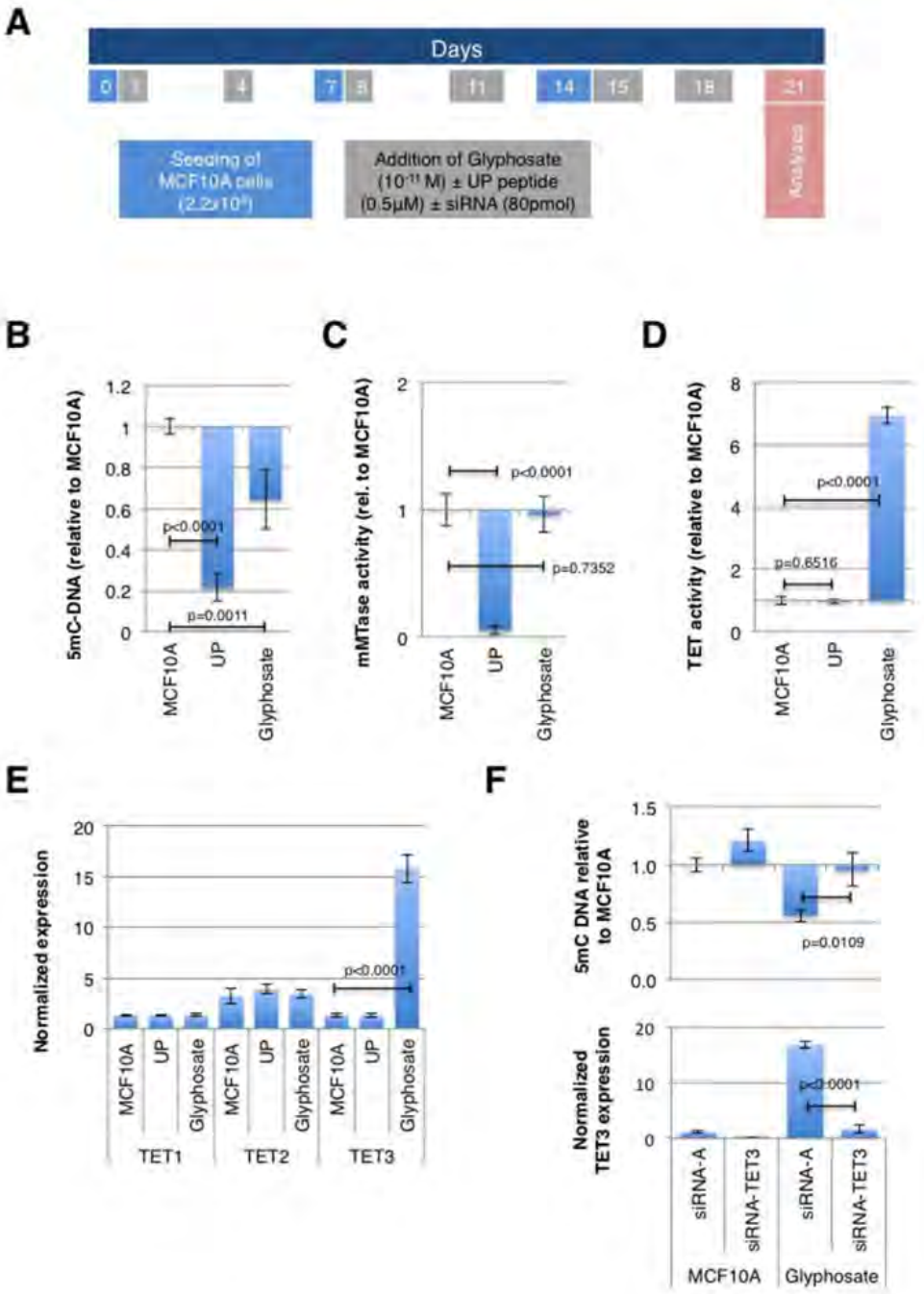


FIGURE 1 | Glyphosate exposure promotes a TET3-mediated global DNA hypomethylation. MCF10A cells were treated according to a timetable shown in **(A)** and analyzed on day 21 of culture. (Explanations for color-coded days are located in corresponding color rectangles underneath the timeline. UP peptide promotes DNMT1/PCNA/UHRF1 disruption). **(B)** ELISA was used to measure the level of 5-methylcytosine (5-mC). **(C)** DMB assay was used to measure maintenance methyltransferase (mMTase). **(D)** TET assay. **(E)** In-Cell ELISA was used to quantify TET proteins. **(F)** MCF10A cells were transfected either with siRNA for TET3 or with control siRNA (siRNA-A) and treated with glyphosate (Glyphosate) or vehicle DMSO (MCF10A) according to a timetable shown in **(A)**. ELISA was used to measure the level of 5mC, and TET3 levels were determined by In-Cell ELISA and normalized to Janus Green staining intensity to account for differences in cell seeding density. For all assays, the bar graph displays the average ± standard deviation values of three independent experiments.

Glyphosate Exposure Is Tumorigenic for MCF10A Cells in a Two-Factor Hit Model; Global DNA hypomethylation is potentially tumorigenic. Therefore, MCF10A cells exposed to glyphosate were injected subcutaneously in Swiss nude mice. No tumors developed, whereas the control experiment with MCF10A cells exposed to the UP peptide led to visible tumor growth within 21 days in 100% of the mice (Figure 2A). The Knudson's hypothesis for cancer initiation suggests that several oncogenic hits cooperate to promote cancer. This hypothesis initially based on mutations can be transposed to risk factors beyond genetic alterations. Indeed, several microRNAs (miR) have been associated with cancer either as oncomiR (one hit) or suspected to promote cancer phenotype in light of their overexpression

in cancers. To investigate the possibility of a two factor hit oncogenic impact with glyphosate, six miRs associated with poor prognosis of breast cancer [miR-182-5p, miR-27a, miR-500a-5p, miR-30a, miR-495, and miR-146a] were transfected individually in MCF10A cells. For this purpose, miRs mimics were used, and their increased expression was confirmed by RTqPCR. Tumor nodules were observed in two out of the four mice with subcutaneous injection of glyphosate-exposed MCF10A overexpressing miR-182-5p, whereas none of the other five miRs were associated with tumor formation (Figure 2B). Moreover, no tumor nodules were observed with subcutaneous injection of glyphosate/miR-182-5p/siRNA-TET3-exposed MCF10A, confirming that TET3 is implicated in glyphosate-mediated tumorigenic pathway (Figure 2C). The use of the Pan-cancer RNA-seq data available from the KM plotter database revealed that although TET3 overexpression is associated with a favorable overall survival in head and neck squamous cell carcinoma, thymoma, and thyroid carcinoma, it is associated with an unfavorable overall survival in breast cancer, as well as cervical squamous cell carcinoma, kidney renal papillary cell carcinoma, liver hepatocellular carcinoma, pheochromocytoma, paraganglioma, and uterine corpus endometrial carcinoma. We next compared several molecular signatures and phenotypic traits of primary cultures of tumor cells (PCTC) from glyphosate-induced breast tumors (Glypho-iBPCTC) with the ones of luminal A (MCF-7) and triple negative (MDA-MB-231) breast cancer cells. Only one of the two tumors led to viable Glypho-iBPCTC. In-cell ELISA confirmed that MCF7 and MDA-MB-231 cells were ERα+/PR+/HER2- (luminal A) and ERα-/PR-/HER2- (triple negative), respectively, and revealed that Glypho-iBPCTC were ERα+/PR-/HER2-, hence corresponding to a luminal B type of breast cancer with poorer outcome compared to ER+/PR+/HER2-subtype (Figure 3A). Tamoxifen/IC50 in MCF-7 and Glypho-iBPCTC were similar (Figure 3B). The QCM™ 24-Well Collagen-based cell invasion assay revealed that all cell strains had similar invasion capacity (Figure 3C), although scratch test indicated that Glypho-iBPCTC had the lowest migration ability compared to MCF-7 ($p = 0.0137$) and MDA-MB-231 cells ($p = 0.0002$) (Figure 3D). These results confirm that Glypho-iBPCTC display phenotypic traits associated with breast cancer cells in vitro.

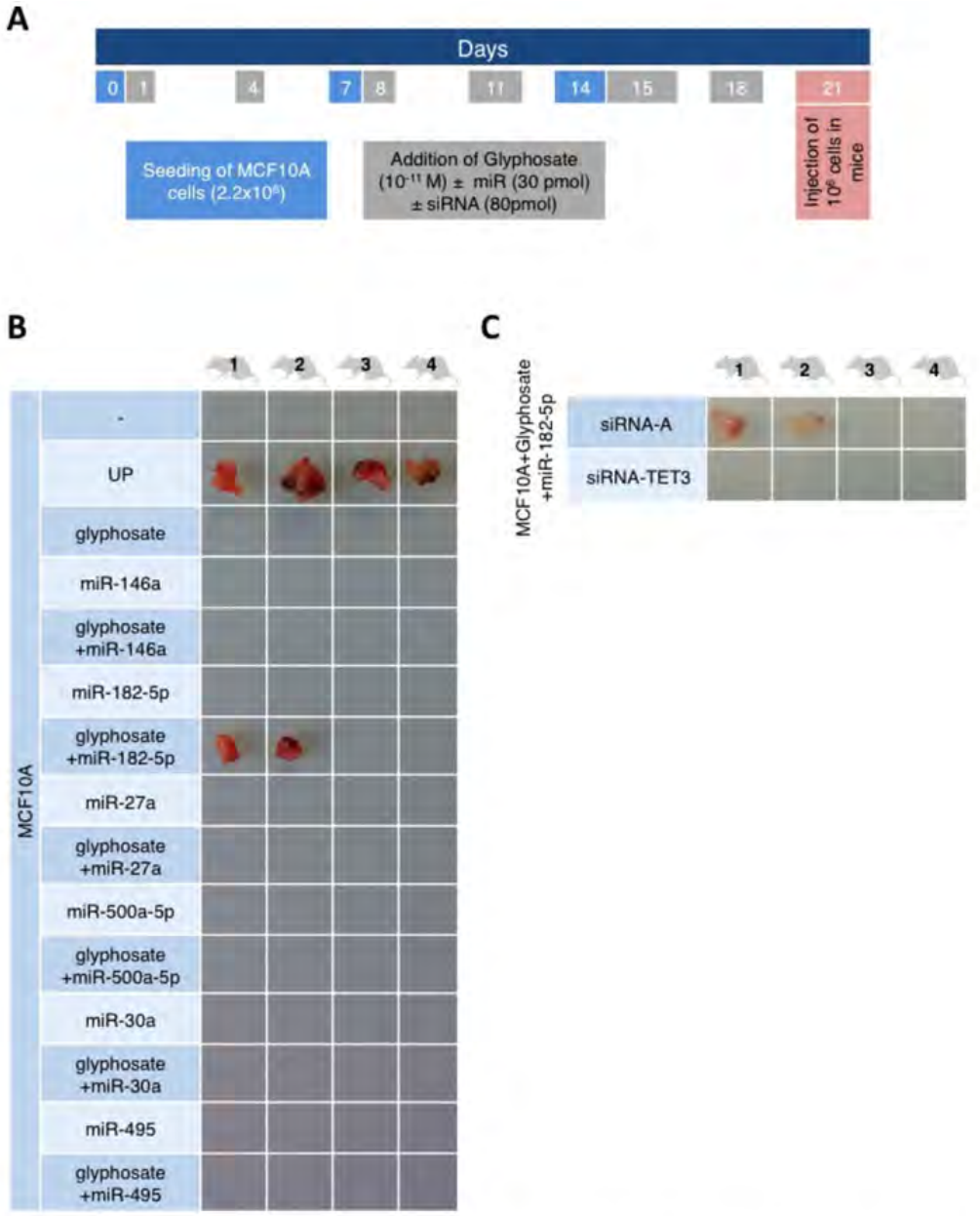


FIGURE 2 | The combination of glyphosate exposure and miR-182 overexpression is tumorigenic for MCF10A cells in a two-factor hit model. **(A)** The timetable illustrates the experiment design. Explanations for color-coded days are located in corresponding color rectangles underneath the timeline. **(B** and **C)** Four mice were injected per condition. miRCury LNA miR mimics and siRNA for TET3 were used to overexpress miRs or siRNA in MCF10A cells. Mice were euthanized 21 days after the injection of cells, and the tumors were resected. The pictures show the resected tumors.

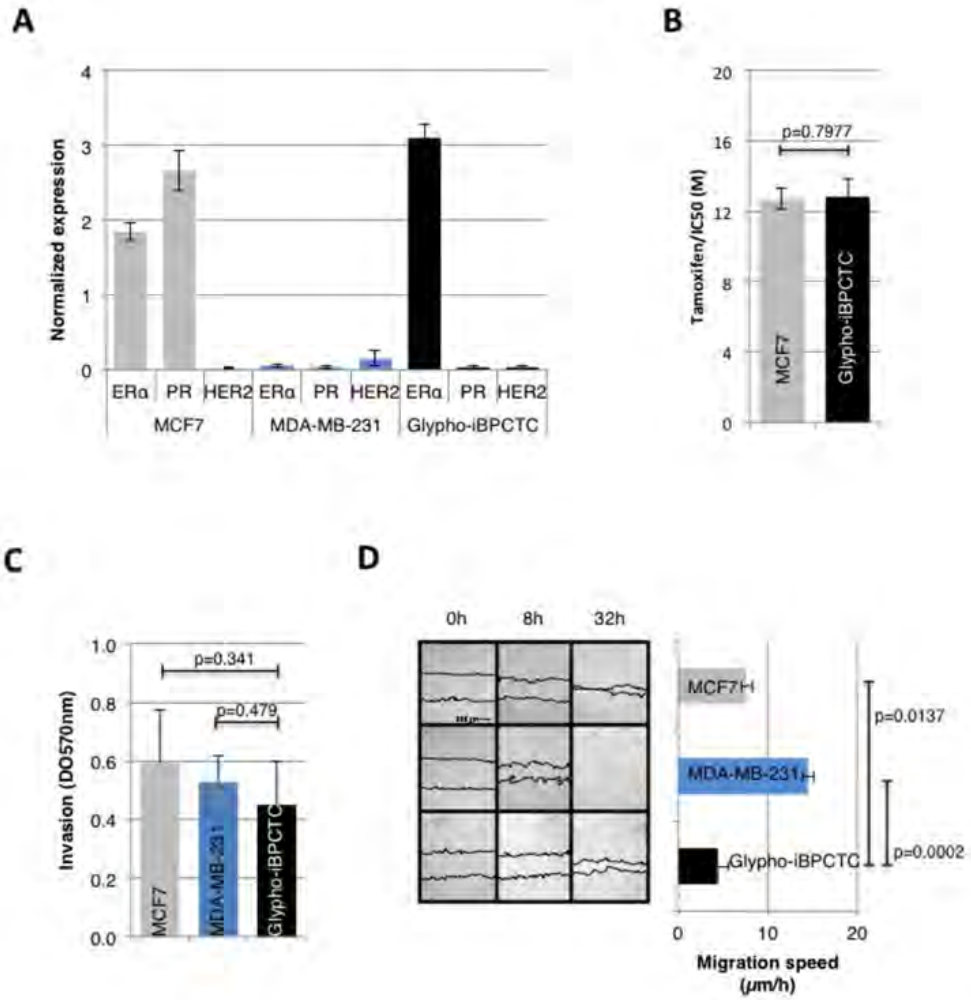


FIGURE 3 | Primary cells from glyphosate-induced breast tumor display characteristics of malignant cells. **(A)** The expression levels of ERα, PR, and HER2 were estimated in MCF7 cells, MDA-MB-231 cells, and Glypho-iBPCTC primary cells using In-Cell ELISA. Normalization to Janus Green staining intensity was performed to account for differences in cell seeding density. The bar graph displays the average ± standard deviation values of three independent experiments. **(B)** Bar graph of the viability of MCF-7 and Glypho-iBPCTC cells treated with increasing doses of tamoxifen (0, 2, 4, 6, 8, 10, 16, 19, 22 µM). Viability was measured by an MTT test, and the results represent the average ± standard deviation values of six independent experiments. The IC50 for each cell type was calculated using the IC50 Calculator (ATT Bioquest). **(C)** Bar graph showing the invasion capacity of MCF-7, MDA-MB-231, and Glypho-iBPCTC cells measured by optical density (absorbance at 570 nm). n = 3. **(D)** Confluent cultures of MCF-7, MDA-MB-231, and Glypho-iBPCTC cells were subjected to the wound healing test. The average migration speed was obtained by calculating the ratio distance/time between each acquisition time. Left: Pictures were acquired immediately after seeding (0 h) and after 8 and 32 h of culture. The bar graph represents the average ± standard deviation values of three independent experiments.

DMOG, a TET Inhibitor, Prevents Tumor Formation in Glyphosate-Challenged Cells; Some of the nutraceuticals/alicaments currently available target epigenetic pathways involved in normal homeostasis, notably those controlling DNA methylation. Like established epigenetic drugs, these sources of epigenetic modifiers offer great potentials to help determine the epigenetic path targeted by environmental factors and possibly revert the risk of tumorigenesis. MCF10A cells were transfected with miR-182-5p and exposed to 10-11 M of glyphosate (MCF10Aglyphosate/miR-182-5p) every 3 to 4 days over a 21-day period. They were also simultaneously treated with 40 µg/ml folate, a promoter of DNA methylation, or with 250 µM ascorbic acid, an activator of TET, 24 h after every glyphosate +/- miR treatment (Figure 4A). MCF10Aglyphosate/miR-182-5p cells were also treated in a similar manner with two therapeutic agents, an anti-miR-182-5p (50 nM) and dimethyloxallyl glycine (DMOG, 1 mM), a compound that blocks TET enzymatic activity (Figure 4A). For all of these conditions, the global level of DNA methylation and tumor incidence compared to untreated MCF10Aglyphosate/miR-182-5p cells (control) at the end of the 21-day treatment sequence was measured. As expected, folate and DMOG prevented glyphosate-induced DNA demethylation, whereas ascorbic acid further reduced DNA methylation in MCF10Aglyphosate/miR-182-5p cells, as shown by the level of 5mC (Figure 4B). Treatment with anti-miR-182-5p did not modify significantly the level of 5mC compared to control. Both folate and DMOG treatments were confirmed to indeed induce hypermethylation in several cell

lines. Of the two hypermethylating agents, DMOG and folate, only DMOG prevented tumor formation; there was no difference between folate and control treatments (50% of the mice displayed tumors). Ascorbic acid and glyphosate acting synergistically on DNA hypomethylation led to a 50% increase in tumor incidence. In contrast, although without an obvious impact on glyphosate-induced DNA hypomethylation, anti-miR-182-5p was able to prevent tumor formation (Figure 4C). These results confirm that both DNA demethylation and miR-182-5p are necessary for tumor onset. Importantly, the extent of DNA demethylation appears to set a threshold for tumor onset (i.e., the more hypomethylated, the higher the risk for tumor development).

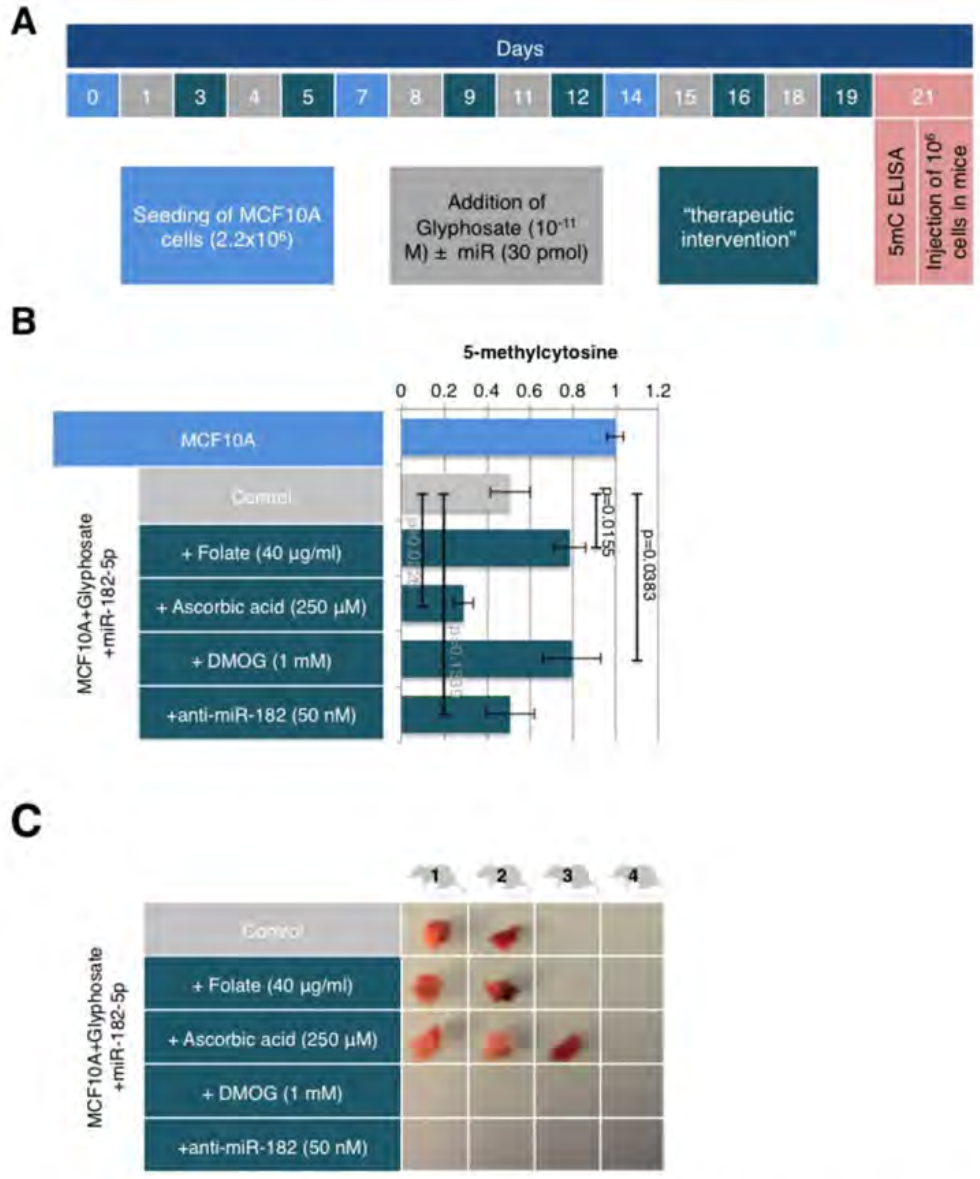


FIGURE 4 | DMOG and anti-miR-182 prevent tumor onset but differentially impact 5-meC level. **(A)** The timetable illustrating the experiment design. Explanations for color-coded days are located in corresponding color rectangles underneath the timeline. Therapeutic interventions on MCF10A cells treated with glyphosate and miR as indicated were performed on days 3, 5, 9, 12, 16, and 19 with folate (40 µg/ml), ascorbic acid (250 µM), DMOG (1 mM), or anti-miR-182 (50 nM). **(B)** MCF10A cells were treated as shown in schedule A. DNA was extracted at day 21 and used in 5mC ELISA. The bar graph illustrates the levels of 5mC for the different conditions. **(C)** Mice were injected with the cells following the treatment schedule A and euthanized 21 days later. Shown are pictures of the resected tumors.

Glyphosate Exposure Induces Sustained TET3-Mediated Gene Demethylation; The hypomethylation induced by glyphosate treatment is sufficient for tumor onset when using a two-factor hit model with induced overexpression of miR-182-5p. Therefore, the possibility that an epimark of hypomethylation might be imprinted in the DNA was investigated. It was postulated that the putative epimark induced by glyphosate might be the hypomethylation of TET3-targeted genes because TET3 mediates glyphosate-induced DNA hypomethylation. The chromatin immunoprecipitation (ChIP) atlas database identifies

MTRNR2L2, COL23A1, MSH3, DHFR, and DUX4 as the most frequently present in TET3-ChIP hits. According to this predictive finding, ChIP experiments using anti-TET3 antibody were performed for chromatin obtained from MCF10A cells treated or not with glyphosate for 21 days, such as described in Figure 1A. Interestingly, only MTRNRL2 and DUX4 genes were immunoprecipitated by TET3 in MCF10A cells treated with glyphosate. COL23A1, MSH3, and DHFR genes were not immunoprecipitated in both untreated and treated MCF10A cells. Thus, the prediction made by the ChIP atlas database was validated for MTRNRL2 and DUX4 genes and not for the COL23A1, MSH3, and DHFR genes, suggesting a context-dependent accessibility for this set of TET3-controled genes. Accordingly, quantitative methylation-sensitive restriction enzyme (qMSRE) revealed that MTRNRL2 and DUX4 genes were strongly methylated in control cells and became hypomethylated in MCF10A cells exposed to glyphosate (Figure 5A). The involvement of TET3 in the glyphosate-induced hypomethylation of DUX4 and MTRNR2L2 was confirmed by the abrogation with siRNA-TET3 of the glyphosate-induced hypomethylation of these genes (Figure 5B). Preliminary investigation of available breast tissue from breast cancer-free women confirmed the demethylation of DUX4 and MTRNR2L2 in a woman showing glyphosate exposure based on urinary test. However, the methylation status of the five genes immunoprecipitated by TET3, MTRNR2L2, DUX4COL23A1, MSH3, and DHFR, should be kept in consideration in the future because a woman with low glyphosate exposure displayed methylation on the five genes, hence suggesting that an epimark should consider the methylation status of all these genes in future investigations (Supplementary Figure S5). The stability of epigenetic changes is an important factor for long-term risk determination. MCF10A cells were exposed to glyphosate for 21 days (as previously described; Figure 1A) and then cultured without glyphosate for 1 and 6 weeks. The DUX4 and MTRNRL2 hypomethylations remained stable, as shown by qMSRE, even after exposure to glyphosate has seized (Figure 5C). bc-GenExMiner and KM plotter indicated that a high expression of DUX4 is associated with a poor prognosis, suggesting that genes controlled by TET3 might deserve additional scrutiny in breast cancer pathogenesis.

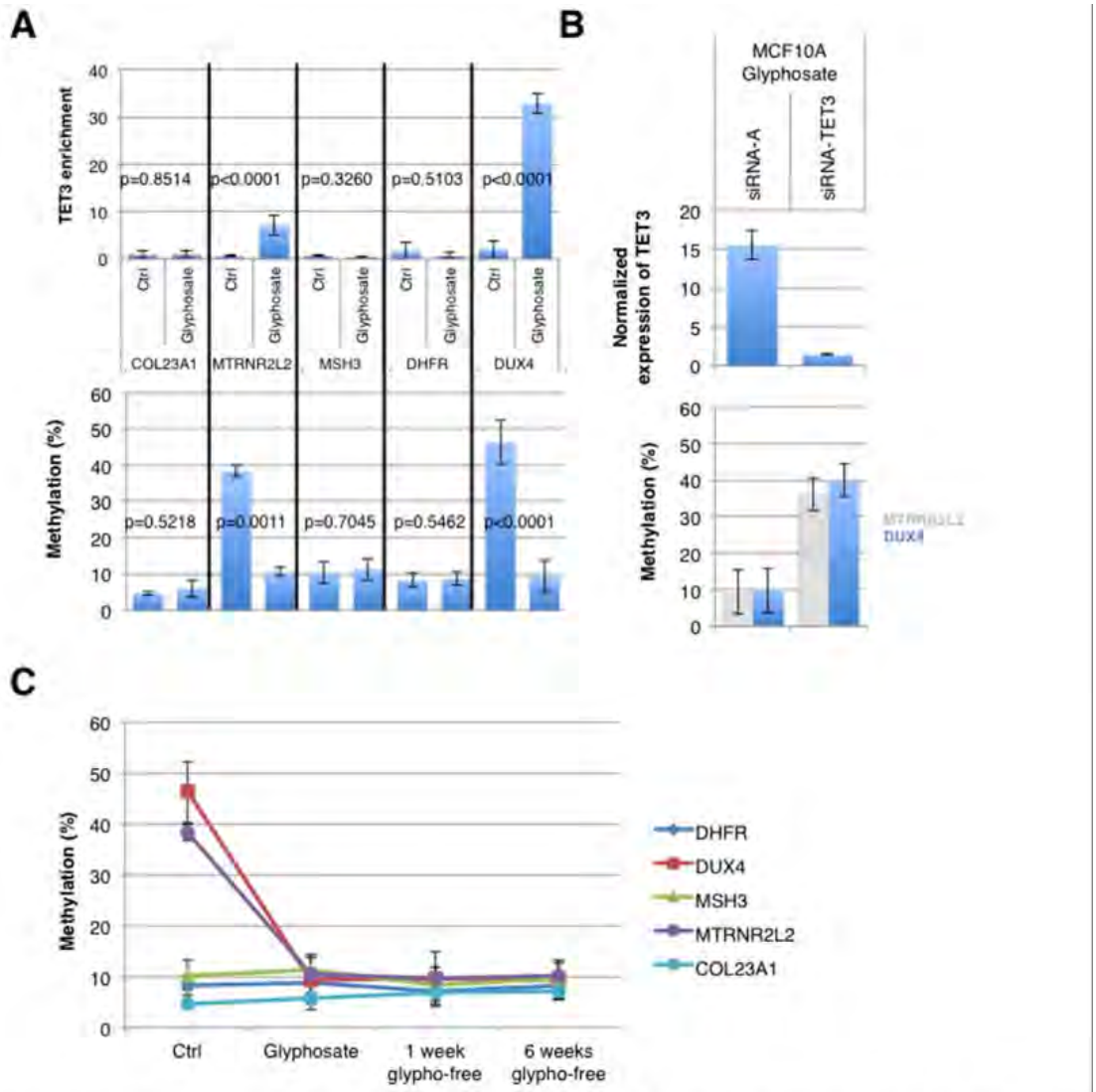


FIGURE 5 | Glyphosate-induced TET3-mediated demethylation affects *MTRNR2L2* and *DUX4* genes. **(A)** MCF10A cells were treated with glyphosate for 21 days as in the schedule shown in **Figure 2**. The graphs illustrate TET3 enrichment (top) following chromatin immunoprecipitation (ChIP) and the methylation level measured by qMSRE (bottom) of five genes defined by the ChIP atlas as being TET3-targeted genes. **(B)** MCF10A cells were treated with glyphosate for 21 days (according to the timetable of **Figure 2**), with siRNA added concomitantly to glyphosate. Bar graph (top) of TET3 expression measured with In-Cell ELISA after treatment with siRNA-TET3 (sc94636) or control siRNA-A (sc94636). Normalization to Janus Green staining intensity was performed to account for differences in cell seeding density. Bar graph (bottom) of methylation levels of *DUX4* and *MTRNR2L2* genes as measured by qMSRE. **(C)** MCF10A cells were treated with glyphosate for 21 days (glyphosate) according to the schedule shown in **Figure 1** and then cultured in glyphosate-free medium for another 1 (1 week glypho-free) or 6 (6 weeks glypho-free) weeks. Shown is the graph of the methylation level of five TET3-dependent genes. "Ctrl" represents MCF10A cells without glyphosate exposure.

Discussion

The impact of glyphosate on human health has been analysed and discussed for several years now. Recently, glyphosate exposure was correlated with shortened gestational lengths, and the level of glyphosate excretion was associated with steatohepatitis and advanced liver fibrosis in patients with fatty liver disease. However, the multiple research studies that investigated the tumorigenic effect of glyphosate as the sole risk factor had not led to convincing evidence of its implication. It is assumed that only 5–10% of cancers are directly caused by inherited genetic abnormalities. The remaining 90% of cancers are linked to environmental factors that directly or indirectly affect DNA, possibly triggering genetic defects or aberrations in the reading and/or expression of DNA. Environmental and lifestyle factors are pleiotropic and include diet, tobacco, infections, obesity, alcohol, radiation, stress, physical activity, exposure to heavy metals and other pollutants, such as glyphosate. This study is reporting that glyphosate exposure is not oncogenic by itself, but it acts as an oncogenic hit factor that, combined with another oncogenic hit, promotes the development of mammary tumors. At the molecular level, these findings demonstrate that glyphosate exposure can predispose breast cells to tumorigenesis via epigenetic reprogramming occurring via TET3-mediated global and local DNA hypomethylation. this

study and others have identified that global DNA hypomethylation promoting tumorigenesis may be caused by a deficiency of the DNMT1/PCNA/UHRF1 complex or of DNMT1 expression as shown in astrocytes, pulmonary fibroblasts, mesothelial cells, and breast cells. This study shows that glyphosate-mediated DNA hypomethylation is associated with TET3 overexpression instead of the DNMT1 pathway. The lower degree of DNA hypomethylation reached via the glyphosate TET3 path compared to that reached via UP peptide-DNMT1 path that is capable of inducing tumor onset suggests that a great intensity of global DNA hypomethylation could act as an oncogenic event, while a moderate intensity of global DNA hypomethylation might be considered a predisposing factor to cancer. The fact that active DNA demethylation orchestrated by TET can occur in resting (non-dividing) cells representing the majority of breast cells (in contrast to DNMT activity that requires cell proliferation) confers to TET-mediated mechanism a potentially higher degree of danger for cancer development. The implication of TET proteins in breast cancer growth and metastasis has been strongly documented, and the level of hypomethylation of triple-negative breast cancer has been associated with TET1 DNA demethylase activity. In the latter article, it is proposed but not shown that TET1 might act as an oncogene by leading to aberrant hypomethylation. These findings demonstrate that the hypothesis of an involvement of TET-mediated DNA hypomethylation in cancer onset was correct. Notably, siRNA-TET3 abolished the presence of glyphosate-induced global and local DUX4 and MTRNR2L2 hypomethylation, as well as tumorigenesis. The data from this study feed the ongoing debate regarding whether TET3 exerts an oncogenic role or a tumor suppressor role. For the latter role, TET3 might act by inhibiting epithelial-to-mesenchymal transition in ovarian and melanoma cancers. But the current analysis with KM plotter database revealed a potentially unfavorable outcome for breast cancers when TET3 is overexpressed. This work shows that two epigenetic events (global DNA hypomethylation and overexpression of a miR) cooperate to promote breast cancer. Other epigenetic events described to be involved in breast cancer development include the reduction of H3K9 acetylation via TIP60 downregulation that promotes ER-negative tumors. Histone acetyltransferase p300 activity and BIM1- mediated histone H2A ubiquitination that remodel chromatin are also two epigenetic events described as promoters for the development of aggressive breast tumors. A body of literature reports that miRs also play a crucial role in mammary tumorigenesis. In addition to oncogenic miRs, there are also miRs acting as tumor suppressors. For example, loss of miR-10b delays oncogene-induced mammary tumorigenesis overexpression of miR-489 inhibits HER2/neu-induced mammary tumorigenesis. Since the expression of miR depends on epigenetic control, it seems that either an extensive global hypomethylation of DNA (like with UP peptide) or a less extensive global hypomethylation associated with local epigenetic alterations affecting a miR might lead to tumor onset. The mechanisms associated with specific targeting of miR expression remain to be understood. Breast cancer susceptibility has been statistically linked to epigenetic age acceleration and CpG island methylation. An important question is whether exposure to pollutants that are detrimental to epigenetic homeostasis might replace or synergize with age-related epigenetic changes and thus lead to the increase in earlier onset of breast cancer that is now documented. Tis possibility is further supported by our preliminary observation that the luminal B subtype of tumor (ER+/PR-/HER2-) triggered by glyphosate exposure combined with miR-182-5p overexpression is associated with poorer outcomes than the frequent ER+/PR+/HER2-luminal A type of tumor. Indeed, luminal B type of tumors have been found to be most common in young patients. Tis phenotype obtained from one tumor produced in mice will have to be confirmed with additional means; in any case, epigenetic markers of risk would be a prime resource to help curve the incidence. There exist already DNA methylation markers that add to the prediction of tertiary and secondary outcomes over and beyond standard clinical measures. In the MCF10A model, glyphosate-induced DNA hypomethylation can be detected via the methylation level of only two of the five genes predicted to be controlled by TET3, MTRNR2L2 and DUX4 genes. Even if several other factors than glyphosate-induced TET3-mediated DNA hypomethylation (such as chromatin structure, other epimark, etc.) can govern the methylation status of the five genes, MTRNR2L2, DUX4, COL23A1, MSH3, and DHFR, this preliminary data with human samples support the idea that the study of the methylation status of these five genes might be important to obtain a marker of risk based on a MethylGlypho score. The current study is pursuing this direction of research by detecting and analyzing this 5-gene TET3-dependent epimark in blood samples. Possibly, glyphosate induced methylome reprogramming might be used for the detection of an increased risk for breast cancer in women living in an environment conducive to this type of pollution. Due to their concomitant expression during tumorigenesis associated with glyphosate-induced DNA hypomethylation, DUX4 and MTRNR2L2 may appear as players in this process instead of only be

considered potential biomarkers. Results with KM plotter and bc-GenExMiner indicate that DUX4 level is negatively associated with breast cancer prognosis. No data seems available on MTRN2L2 in these databases. Based on the literature, DUX4 could act as an oncogene in various sarcomas and hematological malignancies, while information could not be found in the literature revealing a putative oncogenic role for MTRNR2L2. These TET3-controlled genes are worth further investigation to establish their causal effect in mammary tumorigenesis in future work. Knowing the epigenetic pathway involved in glyphosate-mediated risk increase might lead to prevention strategies to follow detection of the epigenetic risk. The current findings suggest that TET-specific inhibitor DMOG might be a plausible therapeutic intervention since it gave a satisfactory response on both DNA methylation and tumor incidence. It would act by limiting TET3-mediated global DNA hypomethylation. In contrast, global remethylation of DNA by folate that has been considered for possible preventive effect is insufficient to prevent tumor incidence in the case of glyphosate exposure. Another interesting direction would be to limit the intake of ascorbic acid since it not only further reduced DNA methylation but also increased tumor incidence in mice. The epigenetic pathway leading to DNA hypomethylation is an important aspect to consider for further translational work on breast cancer risk.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to investigate DNA hypomethylation in MCF10A cells, tumorigenic response for MCF10A Cells in a two-factor hit model, prevention of tumor formation in glyphosate-challenged cells, and TET3-Mediated Gene Demethylation following glyphosate exposure. This study was conducted in vitro using only one level of glyphosate. Glyphosate was not correlated to environmental exposures. In the in vivo portion of the study, a sufficient number of animals were not used to determine a carcinogenic response for statistical analysis. While this study is acceptable as supplemental information on the in vitro effects of glyphosate, it is not appropriate for endpoint derivation in human health risk assessment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was insufficiently characterized and only one and extremely low concentration of glyphosate was used.

Reliability criteria for *in vitro* toxicology studies

Publication: Duforestel *et al.*, 2019.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity was not reported, source: Santa-Cruz, France.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Non-neoplastic breast epithelial MCF10A cells.
Test conditions clearly and completely described	Y	

Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	One test concentration at 10^{-11}M, 10^{-5} µM (extremely low concentration) applied every 3 to 4 days over 21 days.
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	N	Not possible with one concentration
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was insufficiently characterized and only one and extremely low concentration of glyphosate was used.		

1. Information on the study

Data point:	CA 5.6
Report author	Forgacs A. L. *et al.*
Report year	2012
Report title	BLTK1 Murine Leydig Cells: A Novel Steroidogenic Model for Evaluating the Effects of Reproductive and Developmental Toxicants
Document No	Toxicological Sciences (2012) Vol.127(2), 391–402
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of this study was to evaluate the effect of several structurally diverse endocrine disrupting compounds (EDCs) on steroidogenesis in a novel BLTK1 murine Leydig cell model. It was demonstrated that BLTK1 cells possess a fully functional steroidogenic pathway that produces low basal levels of testosterone (T) and express all the necessary steroidogenic enzymes including Star, Cyp11a1, Cyp17a1, Hsd3b1, Hsd17b3, and Srd5a1. Recombinant human chorionic gonadotropin (rhCG) and forskolin (FSK) elicited concentration- and time-dependent induction of 3',5'-cyclic adenosine monophosphate, progesterone (P), and T, as well as the differential expression of Star, Hsd3b6, Hsd17b3, and Srd5a1 messenger RNA levels. The results demonstrated that BLTK1 cells can be used to screen substances that alter intracellular cAMP, steroidogenic gene expression, and sex steroid levels. When tested in this system glyphosate was not found to induce testosterone production or alter rhCG induction of testosterone.

Materials and Methods

Chemicals - Purity and origin of the glyphosate sample tested was not reported.

Cell culture and treatment - Mouse Leydig BLTK1 (BLT-1 cells, clone K1) cells were isolated from a testicular tumor that developed in a transgenic mouse expressing the mouse inhibin α promoter/simian virus 40 T-antigen fusion gene. Cells were maintained in phenol red–free DMEM/F-12 media with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin and incubated in 5% CO_2 at 37°C). For the evaluation of steroidogenic enzyme and receptor expression, cells were grown to 80% confluency and harvested without any treatment. For the determination of 3',5'-cyclic adenosine monophosphate (cAMP), progesterone (P), testosterone (T), and estradiol (E2), cells were grown to 80% confluency, transferred into 24-well tissue culture plates and incubated overnight. Cells were treated with DMSO, or with 0.1, 0.3, 1, 3, 10, 30, or 100 ng/mL recombinant human chorionic gonadotropin (rhCG) or with 0.1, 0.3, 1, 3, 10, 30, or 100 µM Forskolin (FSK) and media were collected at indicated times. Time course studies were conducted with DMSO, 3 ng/mL rhCG or 10 µM FSK, and media were collected after 1, 2, 4, 8, 12, 24, or 48 hours of incubation. Gene expression studies used the same study design, concentrations and time points with cells seeded into T-25 flasks.

MTT assay - BLTK1 cells placed in 96-well plates were treated with 1, 3, 10, 30, 100, 300, or 600 ng/mL rhCG, 1, 3, 10, 30, 100, 300, or 600 µM FSK or 1, 3, 10, 30, 100, 300, or 600 µM of test compound in triplicate. Media were aspirated after 24 hours and replaced with 50 µL of fresh MTT reagent (5 mg/mL thiazolyl blue tetrazolium bromide in PBS). Following 3 hours of incubation, MTT reagent was removed and replaced with 150 µL DMSO. Cells were incubated for 2 hours followed by absorbance measurements at 595 and 650 nm using an Emax precision microplate reader. Results are reported as

percentage of control calculated from the relative absorbance of treated versus DMSO controls where 100% indicates no cytotoxicity.

RNA isolation and gene expression - Total RNA was extracted from cell pellets using RNeasy Mini Kits with an additional RNase-free DNase digestion. RNA was quantified at 260 nm and purity assessed using the A260/A280 ratio, as well as by denaturing gel electrophoresis. First-strand complementary DNA (cDNA) was synthesized from RNA (1 µg) using SuperScript II reverse transcriptase and anchored oligo-dT primer. For real-time PCR (RT-PCR) evaluation of steroidogenic enzyme and receptor expression, cDNA was used as a template for PCR amplification with gene-specific primers. Quantitative RT-PCR (QRT-PCR) was used to quantify concentration- and time-dependent expression of specific genes. Reactions in 96-well plates consisted of 30 µL, including 1 µL of cDNA template, 0.1 µM forward and reverse gene-specific primers using an Applied Biosystems PRISM 7500 Sequence Detection System. Dissociation curve analysis assured single product amplification. To control for differences in RNA loading, quality and cDNA synthesis, samples were standardized to the geometric mean of three housekeeping genes: *ActB*, *Gapdh*, and *Hprt*. Results were quantified using a standard curve generated on the same 96-well plate and amplified by using purified cDNA product as template specific for each gene (serial 10-fold dilutions from 10^8 to 10^1 copies). The slope of the standard curve was used to assess amplification efficiency with all amplification efficiencies > 90%. Fold changes were calculated relative to time-matched vehicle. Relative expression was scaled such that time-matched vehicle control expression equaled one for graphing purposes.

Dose-response modeling and statistical analyses - The ToxResponse modeler uses particle swarm optimization to identify the best fit across five model classes: sigmoidal, exponential, linear, quadratic, and Gaussian. The best fitting model was then used to calculate half maximal effective concentration (EC_{50}) values. All statistical analyses were carried out using SAS v9.1 by ANOVA, with Dunnett's or Tukey's *post hoc* tests for concentration-response and time course data, respectively. Differences between treatment groups were considered significant when $p < 0.05$ relative to time-matched DMSO control.

Results

Steroidogenic Enzyme Expression in BLTK1 Cells - Steroidogenic enzyme messenger RNA (mRNA) and protein were detected in BLTK1 cells by RT-PCR and/or Western blotting, confirming the expression of all required steroidogenic enzymes. In addition, mRNA for several potential regulatory factors including LHCGR, estrogen receptor (ER), androgen receptor (AR), and steroidogenic factor 1 (SF-1), peroxisome proliferator-activated receptors (PPARa and PPARc), the pregnane X receptor (PXR), and the aryl hydrocarbon receptor (AhR) were also detected. However, mRNA for progesterone receptor, glucocorticoid receptor, or the liver receptor homolog 1 was not detected in BLTK1 cells despite verification of RT-PCR primer specificity and functionality in mouse Hepa1c1c7 cells.

Induction of Steroidogenesis by FSK and rhCG - Temporal profiles of intracellular cAMP as well as P and T levels in media were evaluated in response to 3 ng/mL rhCG or 10 µM FSK by enzyme immunoassay (EIA). Intracellular cAMP was induced by FSK after 30 minutes (~120 pmol/mL, ~10-fold) and after 1 hour in response to rhCG (635 pmol/mL, 60-fold). However, levels quickly diminished such that no intracellular cAMP was detected by 8 hours. Maximum P levels (200 ng/mL, 8-fold) were observed after 2 hours in response to rhCG and FSK, followed by a steady decline due to metabolism to androgens and estrogens. In contrast, T levels gradually increased reaching a maximum of ~200 pg/mL (7-fold) after 48 hours, with significant increases as early as 1 hour post treatment. Concentration-dependent induction of intracellular cAMP and secreted P and T was evaluated after 4 hours when cAMP could still be detected. 17β-Estradiol (E2) was evaluated after 48 hours as it was not consistently detected after 4 hours. cAMP, P, and T were induced 25-, 10-, and 4-fold, respectively, after 4 hours, whereas E2 was induced ~4-fold by 48 hours. The EC_{50} for cAMP induction was greater than 24 ng/mL for rhCG and greater than 29 µM for FSK. Meanwhile, EC_{50} values of 1 ng/mL rhCG and 9 µM FSK were conserved for both P and T induction, whereas E2 EC_{50} values were 10 ng/mL for rhCG and 9 µM for FSK. Intracellular cAMP levels are not only regulated by synthesis but also by degradation, which is regulated by cyclic nucleotide phosphodiesterase enzymes. The phosphodiesterase inhibitor IBMX maximizes cAMP levels in order to further induce steroidogenesis. However, IBMX co-treatment

with rhCG or FSK did not increase T levels further, albeit rhCG and FSK potencies were greater with and without IBMX by FSK: 0.1 µM vs. 9.4 µM vs. 0.9 ng/mL, respectively. When tested in this system glyphosate at 300 µM did not induce T production nor alter rhCG induction of T.

Conclusion

Current test protocols and models are inadequate to screen the universe of chemicals, metabolites, and mixtures that may alter steroidogenesis. BLTK1 cells are a novel complementary rhCG-inducible Leydig-based model that can be used to assess effects on steroidogenic gene expression, intracellular cAMP, and P, T, and E2 levels in media. Their consistent response characteristics and inducibility over 30 passages also make this cell line attractive for high-throughput screening. Comprehensive characterization of effects on intermediate steroid biosynthesis, including pregnenolone, 17-hydroxyprogesterone, DHEA, androstenedione, estrone, and DHT, as well as the differential expression of steroidogenic enzymes will also facilitate the elucidation of modes of action relevant to adverse outcome pathways in humans and other relevant species. When tested in this system glyphosate at 300 µM did not induce testosterone production or alter rhCG induction of testosterone.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study, recombinant human chorionic gonadotropin (rhCG) and forskolin (FSK) were used as positive controls for the induction of steroidogenesis, as measured by increases in progesterone, testosterone and 17β-estradiol levels in culture media. Murine BLTK1 Leydig cells were investigated as a novel model for evaluating the effects of chemicals on steroidogenesis. The results demonstrated that BLTK1 cells can be used to screen substances that alter intracellular cAMP, steroidogenic gene expression, and sex steroid levels. When tested in this system glyphosate was not found to induce testosterone production or alter rhCG induction of testosterone.

This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the test substance was not characterized and the results of only one concentration level were reported.

Reliability criteria for *in vitro* toxicology studies

Publication: Forgacs *et al*., 2012	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity and source not reported.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)		

Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	N	Only one concentration was tested (300 μM) Glyphosate, did not induce or alter rhCG induction of T. Glyphosate also had no effect on T levels in BLTK1 cells
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the test substance was not characterised and only one concentration level was tested.		

1. Information on the study

Data point:	CA 5.8.2
Report author	Forsythe S. D. *et al.*
Report year	2018
Report title	Environmental Toxin Screening Using Human-Derived 3D Bioengineered Liver and Cardiac Organoids
Document No	Frontiers in Public Health (2018) Vol. 6, 103
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study the toxicity of glyphosate for liver and cardiac organoids was investigated in the concentration range from 25 μM to 25 mM. The endpoints considered were cell viability, ATP activity and beating rate of the cardiomyocytes. Glyphosate was shown to reduce organoid integrity and viability at doses from 250 μM to 2.5 mM. The IC50 values based on ATP activity of liver and cardiac organoids were found to be 10.53 and 10.85 mM, respectively. When cardiac organoids were exposed to glyphosate at 0.25 mM a non-statistically significant effect was found on beating rate. Exposure to 2.5 mM for 2 days resulted in all organoids stopping beating.

Materials and methods

Chemicals – The purity and source of glyphosate were not reported.

Liver and cardiac cell sources, culture and organoid formation - For liver organoids, all cells used were commercially sourced, human primary cells. Hepatic stellate cells (HSCs) were expanded in culture for two passages before cryopreservation for use in organoid formation. During expansion, HSCs were cultured in 90% high glucose DMEM and 10% fetal bovine serum on a rat tail collagen I coating (10 g/cm^2) at 37°C with 5% CO_2. Primary human hepatocytes were thawed according to the manufacturer's instructions using hepatocyte thawing medium. Kupffer cells were also thawed following the manufacturer's instructions. After thawing, primary human hepatocytes were plated on collagen coated (10 g/cm^2) 6-well culture plates, using hepatocyte plating medium at a density of ~ 150,000 cells/cm^2. Cells were incubated at 37°C with 5% CO_2 for 4 hours before adding matrigel as an overlay. Following further incubation for 24 hours, fresh HCM medium was added. For cardiac organoids, induced pluripotent stem cell-derived cardiomyocytes (iPSC CMs) were commercially sourced from Axiogenesis (cat. #COR.4U Cardiomyocytes). Human primary cardiac fibroblasts were commercially sourced from ScienCell (cat. #6330). Prior to organoid formation, iPSC CMs were cultured on tissue culture plastic for 48 hours in COR.4U medium until cells began beating spontaneously. At this point, iPSC CMs were harvested using trypsin-EDTA. The liver cells were combined in a cell seeding mixture comprised of 90% HCM medium, 10% heat-inactivated fetal bovine serum, and rat tail collagen I (10 ng/μL). Liver organoids were produced with a mixture of 80% hepatocytes, 10% hepatic stellate cells, and 10% Kupffer cells. Approximately 1,500 cells per 40 μL medium were used to form aggregates in each well of non-adherent, round-bottom, 96-well plates to produce spherical organoids. Cardiac organoids were produced similarly. IPSC CMs were suspended in cardiomyocyte maintenance medium. Fibroblasts were added as 10% of the total cell number, and the volume was adjusted to reach a cell density of 10,000 cells/mL. 100 μL of cell suspension was pipetted into each well of non-adherent, round-bottom, 96-well plates to produce spherical organoids resulting in approximately 1,000 cells/organoid. Well plates were incubated and observed daily until organoid formation, and then immediately used in experiments.

Preparation of test item stock solutions – Glyphosate was dissolved in DI H_2O for environmental toxins to reach 50 mM. Serial dilutions were performed in media for each cell type until all concentrations were created at 2X final concentration. Glyphosate was assessed in the concentration range from 25 μM to 25 mM.

Live/dead staining - Organoids were isolated from 96-well low adhesion round bottom plates, suspended in Hystem hydrogel in a 20 μL construct, and placed into 12-well plates to immobilize organoids in a 3D extracellular matrix environment. Each concentration of glyphosate premixed at 1X concentration in media according to organoid type was added to individual wells and organoids were allowed to incubate under their respective conditions for 2 days at 37°C with 5% CO_2. Studies were performed using $n = 5$ or higher for all conditions. Medium was then removed and organoids were assessed by LIVE/DEAD® Viability/Cytotoxicity Kit assays. Specifically, 2.0 μM calcein AM and 4.0 μM ethidium homodimer in PBS was added to each well and was allowed to incubate for 1 hour. Imaging was then performed by macro-confocal microscopy and composite images were created with ethidium bromide red fluorescence representing dead nuclei and calcein AM green fluorescence representing live cells.

ATP activity assays – Glyphosate was added in a premix 2× concentration of 100 μL solution to each well of a 96-well plate containing an organoid with 100 μL of medium and allowed to incubate for 2 days at 37°C with 5% CO_2 with n = 6 or higher. Medium was then removed from each well leaving 100 μL of media remaining along with the organoid. Next, 100 μL of Cell-Titer Glo Luminescent Cell Viability Assay solution was added to each well along with 100 μL added to 100 μL DMEM in a Costar Black Polystrene 96-well assay plate and allowed to incubate for 10 minutes at room temperature shielded from light. The entire contents of the wells containing organoids were then added to the Costar Black Polystrene 96-well assay plate wells and the contents were read on a Vertias Microplate Luminometer using default settings. Values were then averaged among the different groups and graphed for analysis using Graph Pad Prism© software.

Heart beat assay - Fully formed cardiac organoids in the wells of a 96-well non-adhesive round bottom plate were placed under a Leica DMIL LED microscope to allow for the recording of natural beat rates in 20 s videos ($n = 3$). The plate was then returned to the 37°C, 5% CO_2 incubator for 5 minutes to ensure that organoids did not experience significant temperature decreases, which can detrimentally impact beating rates. The process was repeated until all experimental subject organoids under the test compound concentrations described above, but at varying time points, had been recorded. Glyphosate was added as 2 × 100 μL concentration to each well of a 96-well low adhesion round bottom plate containing 100 μL of normal medium with a cardiac spheroid, and allowed to incubate for 30 minutes at 37°C. The plate was then recorded, 3 organoids at a time, in the process listed above until all organoids were recorded and the plate was returned to the incubator. The process was then repeated 24 and 48 hours later. The 20 s videos were then analyzed by counting the beats for each video and multiplied by 3 to scale values to beats per minute. A beat was defined as the beginning of the contractile movement of the organoid. A beat did not need reach conclusion to be counted. If multiple beating regions were observed then the beating of the largest multi-cell structure was used to calculate the beating rate.

Statistical analysis - The data are generally presented as the means of number of replicates ± SD. All data were graphed and analyzed for significance using a Student's T-test. For ATP activity assays p-values were considered significant when < 0.01. For beating kinetic assays p-values were considered significant when < 0.05. Data samples were eliminated from the experimental groups if they fell outside of two SDs from the experimental group averages. Sample sizes (generally n = 5 or n = 6, depending on the experiment as described) were determined based on preliminary experiments. These sample sizes, with the typically observed SDs, allowed statistical significance at $\alpha = 0.05$ with statistical power greater than 80%. The IC_{50} was calculated using the Graph Pad Prism© software.

Results

Organoid production and viability - Liver organoids were successfully formed after 4 days whereas it took 7 days for the cardiac organoids to allow for self-propagating beating to occur. Viability for both was confirmed to be greater than 95% using live/dead imaging.

Organoid viability - Live/dead staining of liver organoids was used to visualize indications of cytotoxicity due to exposure to glyphosate. Integrity and viability of liver organoids began to show a steady reduction when exposed to glyphosate concentrations from 250 µM to 2.5 mM. In contrast, cardiac organoids maintained integrity during all testing, but cell death occurred when exposed to glyphosate concentrations from 2.5 to 25 mM.

Organoid ATP activity - After testing original 3 doses ($n \geq 6$) centered on doses described in the literature, 2 further trials narrowed the range containing the IC_{50}. The IC_{50} values were found to be similar for cardiac and liver organoids for glyphosate, i.e. 10.85 and 10.53 mM, respectively. For liver organoids, 10 mM of glyphosate resulted in a statistically significant decrease in ATP activity ($p < 0.001$) and for cardiac organoids, 5 mM of glyphosate resulted in statistically significant decrease in ATP activity ($p < 0.001$).

Cardiac organoid beating rates – The time points to test physiological reactions to glyphosate exposure were chosen based on previous studies testing calcium channel impairment in 2D cardiomyocyte populations and defined as immediate (30 minutes), short term (1 day), and long term (2 days). Although not statistically significant, glyphosate demonstrated toxicity at 250 µM and higher, with beat rates at 250 µM slowing on day one among all organoids and 2 of the 3 organoids ceasing beating on day 2. At 2.5 mM, exposure for one day caused two spheroids to cease beating entirely, with one-third showing a 50% reduction in beat rate. By day 2 all organoids ceased beating.

Conclusion

Glyphosate live/dead imaging varied greatly, with significant cell death in liver organoids and little cell death in cardiac organoids at 2.5 mM. ATP values recorded at 10 mM showed a large difference between liver and cardiac organoids (0.568 and 0.804, respectively), displaying a more gradual decrease in cell viability for the liver organoids as compared to the cardiac organoids. The IC_{50} values of liver and cardiac organoids were found to be similar i.e. 10.53 and 10.85 mM, respectively. When cardiac organoids were exposed to glyphosate at concentrations from 0.25 mM on an effect on beating rate was observed with a complete stop of beating at 2.5 mM after 2 days of exposure.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study the toxicity of glyphosate for liver and cardiac organoids was investigated in the concentration range from 25 µM to 25 mM. The endpoints considered were cell viability, ATP activity and beating rate of the cardiomyocytes. Glyphosate was shown to reduce organoid integrity and viability at doses from 250 µM to 2.5 mM. The IC_{50} values based on ATP activity of liver and cardiac organoids were found to be 10.53 and 10.85 mM, respectively. When cardiac organoids were exposed to glyphosate at 0.25 mM a non-statistically significant effect was found on beating rate. Exposure to 2.5 mM for 2 days resulted in all organoids stopping beating.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not characterized, no positive controls were used to validate the organoid test systems and the concentrations at which most of the effects have been observed are physiologically not feasible in *in vivo* experimental models.

Reliability criteria for *in vitro* toxicology studies

Publication: Forsythe *et al*., 2018	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity and source were not reported.
Only glyphosate acid or one of its salts is the tested substance	N	Also lead, mercury and thallium were assessed.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y?	Liver and cardiac organoids. Origin of hepatic cells not sufficiently documented.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Partly	Test concentrations at which effects were seen were > mM.
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive controls used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not characterized, no positive controls were used to validate the organoid test systems and the concentrations at which most of the effects have been observed are physiologically not feasible in *in vivo* experimental models.		

1. Information on the study

Data point:	CA 5.3
Report author	Gao H. *et al.*
Report year	2019
Report title	Activation of the N-methyl-D-aspartate receptor is involved in glyphosate-induced renal proximal tubule cell apoptosis
Document No	Journal of Applied Toxicology (2019) Vol. 39(8), 1096-1107
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate-based herbicides have been used worldwide for decades and have been suggested to induce nephrotoxicity, but the underlying mechanism is not yet clear. In this study, a human renal proximal tubule cell line (HK-2) was treated with glyphosate for 24 hours at concentrations of 0, 20, 40 and 60 μM. A cell culture model and an animal exposure model were used to investigate the influences of glyphosate on renal proximal tubule cells and the potential role of NMDAR in response to glyphosate exposure.

Materials and methods

Chemicals – Glyphosate monoisopropylamine salt (purity 96%), as a 40% w/w solution in water, purchased from Millipore Sigma, St. Louis, USA.

Cell culture and treatment - The human renal proximal tubular epithelial cell line HK-2 was obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C. Glyphosate monoisopropylamine salt solution (40% w/w in water) was used to prepare a glyphosate stock solution (200 mM), which was then was diluted in complete medium to the final concentrations. HK-2 cells were exposed to glyphosate at 0, 20, 40, 50, 60, 70, 80, 90 or 100 μM for 24 hours. For intervention experiments, HK-2 cells were pretreated with MK-801 at 100 μM for 12 hours, BAPTA-AM at 2 μM for 12 hours or NAC at 2 mM for 12 hours followed by treatment with glyphosate at 40 μM.

Cytotoxicity assays - Cell viability and death were evaluated by Cell Counting Kit-8 (CCK-8) and lactate dehydrogenase (LDH) cytotoxicity assay kit following the manufacturer's instructions. To determine cell viability, HK-2 cells were seeded into a 96-well plate (1×10^4 cells/mL) and then exposed to various concentrations of glyphosate for 24 hours. Cell-free medium and cells treated with water served as the blank and solvent controls, respectively. A mixture of 10 μL of CCK-8 solution and 90 μL of culture medium was added to each well and then incubated at 37°C for 2 hours. Optical density at 450 nm was measured with a Synergy™ HT Microplate Reader. During the detection of glyphosate cytotoxicity, cell supernatants from each well were collected and then incubated with the LDH assay solutions for 30 minutes at 25°C. Cell-free medium and cells treated with water served as the blank and solvent controls, respectively. Cells treated with lysis buffer and lysis buffer alone served as the positive and positive blank controls, respectively. Optical density at 490 nm was measured using a Synergy™ HT Microplate Reader.

Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis assay - Cell apoptosis was measured using an Annexin V-fluorescein isothiocyanate apoptosis detection kit. Glyphosate- or vehicle-treated HK-2 cells were harvested and suspended in binding buffer. Annexin V-fluorescein isothiocyanate (5

μL) was added to the cells, followed by the addition of propidium iodide (PI, 5 μL). Water served as the solvent control, and apoptosis inducer A (Apopida) in the Apoptosis Inducer Kit served as the positive control. Cells without Annexin V and PI were used as negative controls. Subsequently, the cells were labeled for 15 minutes at 37°C. The fluorescence intensity of Annexin V and PI was recorded using a flow cytometer. Data from 10,000 events per sample were analyzed using FlowJo™ software.

Cellular reactive oxygen species measurement - Intracellular ROS production was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) using flow cytometry. HK-2 cells were incubated with 100 nM DCFH-DA for 30 minutes at 37°C. Cells were then harvested and resuspended in basal medium. Water and hydrogen peroxide were used as the solvent and positive controls, respectively. Cells without the DCFH-DA probe were used as a negative control. The fluorescence intensity of 3 × 104 cells per sample was acquired using a flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data were analyzed using FlowJo™ software.

Detection of intracellular Ca^{2+} levels -
Intracellular Ca^{2+} concentration was analyzed using a Fluo-4/AM fluorescent probe. The cells were incubated with 2 μM Fluo-4/AM in Hanks' balanced salt solution at 37°C for 30 minutes and then suspended in Hanks' balanced salt solution and incubated at 37°C for an additional 20 minutes. H_2O and ionomycin (5 μM) were used as the solvent and positive controls, respectively. Cells without Fluo-4/AM probe were used as a negative control. Cell analysis was performed on a flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The $[Ca^{2+}]_i$ value is represented by the mean fluorescent intensity.

Western blotting - Untreated and treated cells were washed three times with ice-cold PBS and then lysed by RIPA lysis buffer containing 1% protease inhibitors. The cell lysate was centrifuged and the supernatant was collected. The total cellular protein concentration was determined using a BCA assay kit. Equal amounts of total proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis through a 12% gel and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in PBS + Tween 20 (pH 7.5) at room temperature for 2 hours. Subsequently, the membranes were washed with PBS + Tween 20 and incubated with primary antibodies against human NMDAR1, Bcl-2, Bax, Bcl-xl, Bad, cleaved caspase-3 or β-tubulin overnight at 4°C. The membranes were then incubated with antirabbit horseradish peroxidase-conjugated IgG antibodies for 2 hours at room temperature. The protein bands were visualized using an ECL detection kit. β- Tubulin served as an internal loading control.

Animals and treatment - Adult male ICR mice (aged 8 weeks) were obtained from the Shanghai Jiesijie Laboratory Animal Company (Shanghai, China) with license number SCXK (Hu)2013-0006. The mice were provided food and water ad libitum and maintained in a controlled environment at a temperature of 24 ± 1°C, humidity of 45% ± 5% and a 12-hour light/dark cycle. The animals were randomly assigned to the control group or the glyphosate exposure group (6 mice per group). The mice in the glyphosate exposure group received 400 mg/kg bw/day glyphosate via oral gavage once per day for a period of 28 days. The mice in the control group received distilled water. Body weight and food intake were measured daily. Urine was collected once a week using metabolic cages. At the end of the exposure period, blood was collected from the orbital venous plexus under anesthesia to prepare serum. Then, the animals were sacrificed and kidneys excised and washed with saline. The kidney samples were fixed by immersion in a 4% paraformaldehyde solution for 24 hours at 4°C for histology, immunohistochemistry and TUNEL examinations. The remaining samples were snap-frozen in liquid nitrogen and maintained at −80°C for subsequent laboratory analysis.

Urine biomarker measurement - Urine and serum were frozen and stored at −80°C immediately after collection and centrifugation. Urine creatinine, uric acid, urea and serum creatinine levels were determined using the respective assay kits. Urine β2-microglobulin and albumin levels were measured using enzyme-linked immunosorbent assay kits.

Superoxide dismutase, catalase, glutathione peroxidase and malondialdehyde measurements - At the end of glyphosate treatment, the culture supernatants of treated and untreated HK-2 cells for the *in vitro*

experiment or renal tissue for the *in vivo* experiment were freshly collected on ice for measurements of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) activities using the corresponding assay kits. SOD activity was determined according to the xanthine oxidase method, CAT activity was determined using the colorimetric method, GSH-Px activity was determined according to the ultraviolet spectrophotometric method, and MDA levels were determined using the thiobarbituric acid method. All experiments were performed following the manufacturer's instructions.

Kidney histopathology – Kidney tissue fixed for histopathological examination was dehydrated and embedded in paraffin. Tissue sections of 5 μm were mounted on poly-L-lysine-coated slides, deparaffinized with xylene, and stained with hematoxylin and eosin. A trained pathologist blinded to the treatments evaluated the tissue slides using an optical microscope.

Immunohistochemistry - Because the chemical structure of glyphosate and that of its metabolite AMPA are similar to glycine and glutamate, which are agonists of the N-methyl-D-aspartate receptor (NMDAR), the potential role of the NMDAR pathway in mediating the proapoptotic effect of glyphosate on proximal tubule cells was investigated. The kidney samples fixed for immunohistochemical examination were dehydrated, embedded in paraffin and cut in 5-μm thick slices. The slides were then deparaffinized and incubated with rabbit monoclonal anti-NMDAR1 primary antibodies at 4°C overnight, washed in PBS and incubated with goat antirabbit IgG-horseradish peroxidase secondary antibodies for 50 minutes at room temperature. Next, 50 mL of 3,3'- diaminobenzidine was added to each kidney section, which was stained for 5 minutes. After the slides were washed, they were counterstained with hematoxylin for 3 minutes. The slides were then mounted and examined under a microscope. NR1 expression was quantified using Image-Pro Plus 6.0 software. Two negative controls were used: PBS treatment in place of primary antibody and an isotype-matched nonspecific antibody (normal rabbit IgG). Brain tissue from mice was used as a positive control.

TUNEL assay - Apoptotic cells were detected with a TUNEL assay kit according to the manufacturer's instructions. 4'-6-diamidino-2-phenylindole was used for counterstaining. Kidney tissue samples fixed for TUNEL examination were dehydrated, embedded in paraffin and cut in tissue sections of 5 μm. The sections were then deparaffinized, rehydrated and treated with Protease K, after which they were incubated with the TUNEL reaction mixture in a humidified chamber at 37°C for 2 hours. The sections were washed with PBS (pH 7.4) to terminate the reaction and then treated with 4'-6-diamidino-2-phenylindole. Sections treated with DNase before TUNEL examination served as a positive control, and sections without terminal deoxynucleotidyl transferase were used as a negative control. From each section, 5 randomly selected fields (200 × magnification) were photographed with a fluorescence microscope. The number of TUNEL-positive cells in each field was counted using Image-Pro Plus 6.0 software and divided by the field area. The average apoptotic cell density of the 5 fields was then obtained for each group.

Results

Cytotoxicity and apoptosis in HK-2 cells – Glyphosate reduced statistically significantly the number of viable cells at ≥ 40 μM and increased LDH release from 40 μM on. In addition, the cytotoxic effect of glyphosate on HK-2 cells was found to be dose- and time-dependent upon prolongation of the exposure times. Cell viability of both the 40 and 60 μM exposure groups was reduced but still greater than 50% after 24 hours of exposure. Therefore, subsequent experiments were conducted with 0, 20, 40 and 60 μM with 24-hour exposure periods. In comparison with the control group, the glyphosate exposure groups showed increased percentages of apoptotic cells (Annexin V+, PI+/−), with significant differences occurring at concentrations greater than 40 μM. Furthermore, the expression of apoptosis-related proteins was investigated using Western blotting where in comparison to the control group glyphosate exhibited upregulated proapoptotic proteins (Bax and Bad) at 20 and 40 μM while downregulated antiapoptotic proteins (Bcl-2 and Bcl-xl) were evident at 40 and 60 μM. Cleaved caspase-3 levels were significantly upregulated at 40 and 60 μM.

Oxidative stress, NMDAR1 expression and calcium influx in HK-2 cells - In comparison with the control group, glyphosate increased statistically significantly cellular ROS levels at 40 and 60 μM. SOD, CAT and GSH-Px levels were statistically significantly reduced whereas there was a statistically significant increase in the MDA level. Glyphosate exposure increased NMDAR1 expression in a dose-dependent manner. Because NMDAR is involved in calcium influx and calcium homeostasis, $[Ca^{2+}]_i$ levels were determined after glyphosate exposure using flow cytometry-based measurement with the Ca^{2+}- sensitive probe Fluo-4/AM. It was found that glyphosate exposure increased $[Ca^{2+}]_i$ levels.

Effects after pretreatment with MK-801 - To examine the role of NMDAR in glyphosate-induced calcium influx and apoptosis, HK-2 cells were pretreated with the NMDAR inhibitor MK-801 at 100 μM for 12 hours followed by treatment with 40 μM glyphosate for 24 hours. MK-801 attenuated the upregulation of $[Ca^{2+}]_i$ in the HK-2 cells after 24 hours of glyphosate treatment. Inhibition of NMDAR was found to attenuate ROS increase in these cells and significantly decrease glyphosate-induced apoptosis.

Effects after pretreatment with BAPTA-AM and N-acetylcysteine - To examine the relationship between glyphosate-induced calcium influx and apoptosis, HK-2 cells were pretreated with 2 μM of an intracellular calcium chelator (BAPTA-AM) for 12 hours to decrease $[Ca^{2+}]_i$. Cells pretreated with BAPTA-AM and exposed to 40 μM glyphosate had lower $[Ca^{2+}]_i$ levels than cells exposed to glyphosate alone. BAPTA-AM pretreatment also significantly decreased glyphosate-induced apoptosis and ROS. To determine the role of ROS in glyphosate-induced apoptosis of HK-2 cells, cells were pretreated with 2 mM of a ROS scavenger (N-acetyl cysteine, NAC) for 12 hours before glyphosate treatment. NAC pretreatment was found to reduce glyphosate-induced ROS levels and apoptosis.

NMDAR1 expression and kidney damage - Glyphosate was administered orally to ICR mice for 28 days at a daily dose of 400 mg/kg bw to investigate its effects on the kidney *in vivo*. No statistically significant difference was found in body weight gain and relative liver and kidney weight between the control and the glyphosate exposure groups. Histopathological examination of the kidney identified exfoliation of renal tubular cells. The TUNEL assay confirmed the increase in renal tubular cell apoptosis in mice exposed to glyphosate. No statistically significant changes were observed in urine creatinine, uric acid, urea nitrogen, serum creatinine and blood urea nitrogen levels. A transient increase in urine albumin was observed after 7 and 14 days of treatment and urinary β2- microglobulin levels were statistically significantly increased after 7, 21 and 28 days of treatment. In addition, statistically significant reductions in the levels of SOD, CAT and GSH-Px, and a statistically significant increase in MDA was observed in the kidneys of the glyphosate treated group. Besides, the average optical density for NMDAR1 was found to be increased in kidneys from glyphosate treated mice.

Discussion and conclusion

Glyphosate exposure was found to increase the production of ROS. In *in vitro* and *in vivo* it was shown that MDA levels increased and that the activities of the major endogenous antioxidant enzymes SOD, CAT and GSH-Px decreased as a result of glyphosate exposure which is indicative of the disturbance of the pro-oxidant/antioxidant balance. To understand how this balance is disturbed the role of the N-methyl-D-aspartate receptor (NMDAR) was investigated because the structure of glyphosate is similar to that of glycine and glutamate which are both agonists of this receptor. NMDAR has been reported to mediate some renal diseases, such as hyperhomocysteinemia-induced glomerulosclerosis, gentamicin nephrotoxicity and lipopolysaccharide-induced renal insufficiency. In this study, using the human renal tubular epithelial cell line HK-2, the increased expression of NMDAR1 protein was demonstrated upon glyphosate exposure. In the animal tests, a clear upregulation of NMDAR1 in renal tissue was observed in tubular epithelial cells using immunohistochemical staining. Accompanied by the increase in NMDAR1 upon glyphosate exposure, [Ca2+]i, oxidative stress markers and apoptosis were all increased. Blocking NMDAR not only ameliorated glyphosate-induced increases in [Ca2+]i and ROS levels but also attenuated apoptosis. The increase in β2-microglobulin in the urine as observed in the *in vivo* study is indicative of an impairment of tubular reabsorption. From the results of this study it can be concluded that glyphosate could affect renal tubule epithelial cells via the NMDAR1/$[Ca^{2+}]_i$/ROS pathway both *in vitro* and *in vivo*. These findings provide a theoretical basis and reference data to assess the risk of

glyphosate and to explore the ethiology of chronic kidney disease.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study the effect of glyphosate on human proximal tubular epithelial cells was studied *in vitro* and on kidney *in vivo*. Tubular epithelial cells (HK-2) *in vitro* were exposed to glyphosate at concentrations ranging from 20 to 100 µM whereas mice were orally treated with glyphosate at 400 mg/kg bw/day for 28 days. The endpoints investigated for the *in vitro* study were cell viability, apoptosis, oxidative stress, intracellular Ca^{2+}, expression of the N-methyl-D-aspartate (NMDA) receptor and expression of proteins involved in apoptosis. The endpoints explored in the *in vivo* study in mice were kidney pathology biomarkers, oxidative stress in kidney tissue, kidney histopathology, NMDA receptor immunochemistry and apoptosis in kidney tissue. The results were that glyphosate was found to reduce cell viability, increase the incidence of apoptotic cells with an increase in the expression of apoptosis-related proteins, increase of oxidative stress in a concentration-related manner, increase of the expression of the NMDA receptor and increase Ca^{2+} influx. Kidney histopathology in mice treated with glyphosate at 400 mg/kg bw/day for 28 days revealed the exfoliation of renal tubular cells. It is postulated by the authors that glyphosate could affect renal tubule epithelial cells via the NMDAR1/$[Ca^{2+}]_i$/ROS pathway.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because some of the biochemical methods are not completely described, only one dose was used in the *in vivo* study and the pathology results from the *in vivo* study are not corroborated by regulatory 90-day repeated dose toxicity studies where no renal effects were seen in rats dosed up to more than 4,000 mg/kg bw/day and mice dosed up to more than 7,000 mg/kg bw/day.

Reliability criteria for *in vitro* toxicology studies

Publication: Gao *et al.*, 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 96% as glyphosate monoisopropylamine salt. Source: Millipore Sigma, St. Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Concentration range *in vitro* from 20 to 100 µM. Only one dose (400 mg/kg bw/day) was used in the oral

		toxicity study in mice.
Cytotoxicity tests reported	Y	
Biochemical methods described	Y?	Some could be better documented.
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	*In vitro* but not *in vivo* (only one dose used).
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because some of the biochemical methods are not completely described, only one dose was used in the *in vivo* study and the pathology results from the *in vivo* study are not corroborated by regulatory 90-day repeated dose toxicity studies where no renal effects were seen in rats dosed up to more than 4,000 mg/kg bw/day and mice dosed up to more than 7,000 mg/kg bw/day.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Gigante P. *et al.*
Report year	2018
Report title	Glyphosate affects swine ovarian and adipose stromal cell functions
Document No	Animal Reproduction Science (2018) Vol. 195, 185–196
Guidelines followed in study	None
Deviations from current test guideline	NA
Previous evaluation	No
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The effect of glyphosate (GLY) at different doses (0.2, 4 and 16 μg/mL) was evaluated on granulosa cells growth (BrDU incorporation and ATP production), steroidogenesis (17-β estradiol and progesterone secretion) and redox status (superoxide and nitric oxide production and non-enzymatic scavenging activity). GLY has been shown to inhibit cell growth, 17-β estradiol and non-enzymatic scavenging activity and to increase progesterone and nitric oxide secretion ($P < 0.05$). In addition, GLY significantly decreased the viability of ASCs ($P < 0.001$), and inhibited their adipogenic differentiation. These data indicate that GLY alters the main features of granulosa cells and ASCS thus suggesting that GLY could affect both reproductive function and adipose tissues homeostasis.

Materials and methods

Chemicals – Glyphosate was purchased from Sigma Chemical Co, St Louis, USA. The purity was not reported.

Isolation and culture of granulosa cells - Swine ovaries were collected at a local slaughterhouse, placed in PBS at 4°C supplemented with penicillin (100 Ul/mL), streptomycin (100 Ul/mL) and amphotericin B (2.5 μg/mL) and transported to the laboratory within 1 hour. The samples were immersed for 1 minute in ethanol 70% before processing. Cystic or hemorrhagic follicles were discarded and the granulosa cells were harvested by aspiration of the follicles in the later state of maturation. The granulosa cells were then centrifuged at 450xg for 10 minutes and the cell pellet obtained treated with ammonium chloride to remove red blood cells. The cell number was estimated after vital staining with trypan blue whereafter the cells were plated and cultured in a validated serum free system composed of DMEM/Ham's F12 medium supplemented with penicillin (100 μg/mL), amphotericin B (2,5 μg/mL), streptomycin (100 μg/mL), sodium selenite (5 ng/mL) and transferrin (5 μg/mL) (CM). CM medium maintains the characteristics of granulosa cells and avoids luteinization.

Cell proliferation - Cell proliferation is measured using the BrdU cell proliferation ELISA assay. The granulosa cells were plated into 96-well plates (10^4 cells/100 μL CM) and incubated overnight with glyphosate at 0, 0.2, 4 or16 μg/mL. At the end, plates were centrifuged for 10 minutes at 400×g, the supernatants discarded and the cells dried and fixed. The DNA was denatured before the addition of the anti-BrdU antibody, conjugated with horseradish peroxidase (POD). The POD substrate used was tetramethyl-benzidine (TMB). Absorbance at 450 nm of the blue color formed is proportional to the amount of newly synthesized DNA. To quantify the viable cell number, the absorbance of each sample was related to a standard curve prepared by culturing, in quintuplicate, granulosa cells at different plating densities for 48 hours. The curve was repeated in four different experiments. The cell number/well was estimated from the resulting linear regression equation and was used to correct experimental data. The assay detection limit was 10^3 cell/well and the variation coefficient was less than 5%.

Cell viability - Granulosa cell viability was assessed using the ATP-lite bioluminescent assay based on the reaction of ATP luciferase and luciferin. The light emitted is proportional to the ATP concentration in the cells. The test was validated by plating different viable cell numbers (from 2.5×10^3 to 4×10^6/100 µL) three times. The relationship between cell number and luminescence was linear (r = 0.95). 2×10^5 cells/100 µL CM were seeded in 96-well plates and treated with glyphosate at 0, 0.2, 4 or 16 µg/mL for 48 hours. Kit reagents were added according to the instructions and luminescence was measured.

Granulosa cell steroid production - Granulosa cells at 10^4/200 µL in CM supplemented with androstenedione at 28 ng/mL were seeded in 96-well plates and treated with glyphosate at 0.2, 4 or16 µg/mL. After 48 hours incubation, culture media were collected, frozen and stored at −20 °C until determination of progesterone (P4) and 17-β estradiol (E2) by a validated RIA. P4 assay sensitivity and ED_{50} were 0.24 and 1 nmol/L, respectively. E2 assay sensitivity and ED_{50} were 0.05 and 0.2 nmol/L. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

Granulosa cell superoxide (O_2^-) *production* - O_2^--production was measured using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate) test which is based on the cleavage of the water soluble tetrazolium salt, WST-1, to the water soluble formazan. A total of 10^4 cells/200 µL CM were seeded in 96-well plates and incubated with glyphosate at 0, 0.2, 4 or 16 µg/mL for 48 hours. During the last 4 hours of incubation, 20 µL of WST-1 was added to the cells, and the absorbance determined at 450 nm against 620 nm. The coefficients of variation were less than 3%.

Granulosa cell nitric oxide (NO) production - 10^5 viable cells/200 µL CM were seeded in 96-well plates and treated with glyphosate at 0.2, 4 or 16 µg/mL for 48 hours. At the end, plates were centrifuged for 10 minutes at 400×g, the supernatants were collected and NO levels assessed by measuring nitrite after incubation with Greiss reagent (1% sulfanilamide, 5% phosphoric acid and 0.1% N-[naphtyl] ethylenediaminedihydrochloride). The absorbance was measured at 540 nm against 620 nm. A calibration curve ranging from 25 to 0.39 µM was prepared by diluting sodium nitrite in CM.

Non-enzymatic scavenging activity - The Ferric Reducing Activity of Plasma (FRAP) assay is a colorimetric method based on the ability of the antioxidant molecules present in a biological matrix to reduce ferric-tripiridyltriazine (Fe^{3+} TPTZ) to a ferrous form (Fe^{2+} TPTZ). 2×10^5 cells/200 µL CM were seeded in 96-well plates and treated with glyphosate at 0, 0.2, 4 or 16 µg/mL. At the end, the plates were centrifuged for 10 minutes at 400×g, the supernatants discarded and the cells lysed by adding, Triton 0.5% + PMSF in PBS at 200 µL/well in an ice bath for 30 minutes. The test was performed on 40 µL of cell lysates added to Fe^{3+} TPTZ reagent. After 30 minutes incubation at 37°C, absorbance of Fe^{2+} TPTZ was determined at 595 nm. The ferric reducing ability of cell lysates was calculated by plotting a standard curve of absorbance against $FeSO_4 .7H_2O$ standard solution.

Isolation and culture of adipose stromal cells (ASCs) from swine adipose tissue - Samples of about 5 g of subcutaneous abdominal adipose tissue were collected from pigs at a local slaughterhouse and placed in 20 mL of PBS supplemented with penicillin (100 Ul/mL), streptomycin (100 Ul / mL) and amphotericin B (2.5 µg/mL), transferred to Petri dishes in sterile conditions and shredded in fragments of about 3 mm^3. Thereafter, the adipose tissue fragments were distributed in 6-well plates, at a density of 10 fragments/well and then carefully covered with a minimal quantity (1 mL) of culture medium (CM_{ASC}) to avoid floating. CM_{ASC} was composed of low glucose DMEM + GlutaMax, supplemented with 10% FBS, penicillin (100 UI/mL), streptomycin (100 UI/mL) and amphotericin B (2.5 µg/mL). The plates were maintained at 37° in a humidified atmosphere at 5% CO_2 and 19% O_2. Every 48 hours CM_{ASC} was replaced. After 5 to 7 days, the adipose tissue fragments were removed from the culture plates and the remaining adherent cells, growing in monolayer, were cultured in fresh CM_{ASC} for 5 to 7 days, until 80% confluence was reached. The ASCs were the trypsinized and cultured in 25 cm^2 flasks with 5 mL CM_{ASC}. The ASCs obtained were subsequently either used to evaluate the expression of adipogenenesis-related marker genes or plated to study the effects of glyphosate at 4 µg/mL on cell viability and adipogenic differentiation.

Adipose stromal cells (ASCs) viability - Cell viability was evaluated using the MTT assay. ASCs were seeded in 96-well plates at 10^4 cells/well in 200 µL CM_{ASC} and incubated at 37°C in humidified atmosphere at 5% CO_2 and 19% O_2 for 48 and 72 hours with glyphosate at 0 or 4 µg/mL. At the end of each incubation period, 20 µL of 5 mg/mL of MTT were added and incubated for 4 hours. Subsequently, media were discarded and 100 µL of lysis solution (SDS10% in HCl 0.01 N) were added to the wells

and left overnight at 37°C. Absorbance was measured at 540 nm.

Adipose stromal cells (ASCs) adipogenic differentiation - ASCs were seeded in CM_{ASC} and incubated for 48 hours at 37°C in a humidified atmosphere at 5% CO_2 and 19% O_2 to permit cell adhesion. Adipogenic differentiation was performed by exposing cells to 3 cycles of 4 days each, of the following treatments:

- 3 days with induction medium: CM_{ASC} supplemented with dexamethasone (DEX) at 1 μM, insulin (INS) at 1.7 μM, 3-iso-butyl-1-methylxanthine (IBMX) at 0.5 mM and indomethacin at 250 μM.
- 1 day with differentiation maintaining medium: CM_{ASC} supplemented with insulin (INS) at 1.7 μM.

Negative controls were performed using ASCs cultured in CM_{ASC}. Cell differentiation was evaluated by Oil Red O Staining to detect cytoplasmic lipid vacuoles as markers of adipogenesis.

Expression of adipogenic marker genes - In order to evaluate the expression of adipogenic marker genes crucial for the function of adipose tissue, PPARγ and Leptin total RNA was extracted from ASCs pellets (7×10^5 cells), subjected or not to adipogenic differentiation, using NucleoSpin RNA II. cDNA was then obtained by reverse transcription of 2 μg of the extracted RNA using the High-capacity cDNA reverse transcription kit. The expression of the genes was evaluated by PCR (Polymerase Chain Reaction) using the primers for PPARγ, leptin and GADPH. The occurrence of the amplification was verified by 2.5% agarose gel electrophoresis in TAE buffer (Tris base, glacial acetic acid, EDTA 0,5 M, pH 8), where the correct length of the amplicons was verified by comparison with Gene Ruler™ 100 bp DNA LaddeR markers. DEPC treated water was used instead of cDNA as a negative control for all reactions. The DNA patterns were displayed under UV light and the images were captured with a PowerShot A610 Canon camera.

Solubilisation and quantification of Oil Red O production - To evaluate intracellular lipid accumulation, 20×10^4 ASCs were plated in 24 wells plates and subjected to a differentiation process with glyphosate at 0 or 4 μg/mL. After 12 days the cells were washed 3 times with PBS, fixed with 4% formaldehyde for 1 hour and washed with 60% isopropanol. The cells were then dried and stained with Oil Red O staining solution for 30 minutes. After multiple washings, Oil Red O was solubilized in each well with 60% isopropanol for 2 hours and the absorbance measured at 540 nm. Absorbance was considered to be proportional to the amount of differentiated cells.

Adipose cell counting - 5×10^4 MSCs were seeded on a cover slip in 6-well plates and subjected to the adipose differentiation process with glyphosate at 0 or 4 μg/mL. After 12 days, cover slips were fixed with formalin (4%) and stained with Oil Red O. Then, cells were stained in Mayer haematoxylin for 3 minutes and images made by light microscopy (total magnification 10x) and quantified by counting the stained cells in 10 different fields.

Statistical analysis - The experiments were performed five times on granulosa cells and three times on ASCs and six replicate wells were used for the assessment of the effect of glyphosate. Every time the adipose tissue was collected from 3 pigs. Data are presented as the mean ± SEM. Statistical differences were calculated by ANOVA using Statgraphics software. In the presence of a significant difference ($p < 0.05$), the means were subjected to Scheffè' F test for multiple comparisons.

Results

The effect of glyphosate on swine granulosa cells - Glyphosate statistically significantly decreased cell proliferation ($p < 0.001$) as evaluated by BrdU incorporation and cell viability ($p < 0.05$) as measured by ATP production without a concentration-effect relationship. Granulosa cell steroidogenesis was affected by glyphosate. E2 secretion was statistically significantly inhibited ($p < 0.05$) whereas P4 secretion was statistically significantly increased ($p < 0.05$) both at all tested concentrations but without a concentration-effect relationship. While O_2^--production was not statistically significantly modified by glyphosate at any of the concentrations tested, NO production was significantly stimulated ($p < 0.001$) at all the tested concentrations although with no statistically significant differences amongst them. Scavenging activity, represented by non-enzymatic antioxidant power, was significantly inhibited ($p < 0.05$) by glyphosate at all the concentrations tested but also without significant differences among them.

The effect of glyphosate on swine adipose stromal cells (ASCs) - The histological analysis of the isolated fragments obtained from pig fat showed typical characteristics of adipose tissue. In particular, adipocytes containing a single large lipid droplet with thin cytoplasm and flattened peripheral nucleus are surrounded by a microvasculature dispersed in the stromal tissue. After 3 to 4 days of *in vitro* culture of the fragmented explants, there was a migration of cells characterized by typical mesenchymal fibroblast-like morphology, which proliferated in adhesion to the bottom of the well. After 5 to 7 days from fragment removal, ASCs reached 80% confluence in monolayer, maintaining their morphological characteristics. The proliferation of ASCs under basic culture conditions was statistically significantly increased ($p < 0.001$) (454 ± 12 vs 476 ± 7), while glyphosate treatment at 4 μg/mL significantly decreased ($p < 0.001$) the viability of proliferating ASCs both after 48 (432 ± 10) and 72 hours (451 ± 7). The expression of PPARγ and LEP adipogenic markers was not observed in undifferentiated ASCs, but was detected following adipogenic differentiation. Adipogenic differentiation of ASCs was achieved, as shown by the appearance of red lipid droplets in the differentiated cell cytoplasm. Differentiated cell counts showed a significant inhibition ($p < 0.05$) of the adipogenic process by glyphosate at 4 μg/mL. These data were confirmed by spectrophotometric evaluation of solubilized Oil RedO.

Discussion

This study shows that glyphosate is able to significantly stimulate P4 production. This is a critical effect for granulosa cells because P4 is a hallmark of luteinization. Ovarian steroidogenesis is a key function for the survival of GCs, and is essential for follicular development and growth. To verify the potential effects of glyphosate on follicular physiology, the present study also analyzed the possible effect of glyphosate on granulosa cell proliferation and metabolic activity. The data show an inhibition of DNA replication activity and ATP production at all concentrations analyzed suggesting a disruption of follicular development in vivo. Since endothelial cell characteristics have been recently attributed to granulosa cells, it is interesting to evaluate the increased NO production in granulosa cells following glyphosate exposure. It can be hypothesized that glyphosate exerts direct pro-angiogenic effects on pigs GCs at all tested concentrations as a result of a significant increase in NO, which is a key molecule of this process. The stimulation of NO production can also be associated with an increase in oxidative stress which seems to be supported by the glyphosate-induced reduction of the non-enzymatic antioxidant power in GCs at all concentrations tested. Adipose stromal cells (ASCs) were isolated from abdominal subcutaneous fat tissue of pig and characterized. Their ability to differentiate into adipocytes under appropriate stimuli has been evaluated on the basis of the expression of PPARγ and leptin genes that was not detected in undifferentiated ASCs. These results were essential to define an adequate experimental model to investigate potential effects of glyphosate on the expression of these adipogenic markers. The inhibitory effect of glyphosate during induction of ASCs adipogenic differentiation suggests that this substance, once accumulated as a consequence of environmental exposure, can interfere with adipose tissue biology *in vivo*. Moreover, glyphosate was found to significantly decrease the viability of ASCs after 48 and 72 hours of exposure. Such evidence appears to be critical since it suggests a direct interference of glyphosate with adipose tissue function.

Conclusion

In this *in vitro* study it is shown that glyphosate inhibits cell growth, 17-β estradiol production and non-enzymatic scavenging activity and increased progesterone production and nitric oxide secretion in granulosa cells. In addition, glyphosate significantly decreased the viability of adipose stromal cells (ASCs) and inhibited their adipogenic differentiation. These data are indicative of the interference of glyphosate with the main functional parameters of granulosa cells and ASCs and could affect both reproductive function and adipogenic processes *in vivo*.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effects of glyphosate on functional parameters of granulosa cells and adipose stromal cells from swine were investigated *in vitro*. In granulosa cells the effect of glyphosate was studied on cell proliferation, cell viability, steroid production, superoxide anion production, NO production and ferric reducing activity. In adipose stromal cells the effect of glyphosate was studied on cell viability, adipogenic differentiation, adipogenic marker genes (PPARγ and leptin), intracellular lipid accumulation and adipose cell count. Glyphosate was found to significantly decrease cell proliferation, cell viability, estrogen production and ferric reducing capacity and increase progesterone and NO production in granulosa cells when tested at concentrations ranging from 0.2 to 16 µg/mL. However, in none of the assays with granulosa cells a concentration-effect relationship was established. Glyphosate treatment at 4 µg/mL significantly decreased ($p < 0.001$) the viability of proliferating adipose stromal cells after 48 and 72 hours. Differentiated cell counts showed a significant inhibition ($p < 0.05$) of the adipogenic process by glyphosate at 4 µg/mL. Since only one concentration of glyphosate was tested it was not possible to establish a concentration-effect relationship. In this publication it is suggested that glyphosate interferes with the main functional parameters of the granulosa cell which could affect reproductive function. No effects on female reproductive function were reported in the rat in regulatory reproductive toxicology tests at doses beyond 2,000 mg/kg bw/day producing systemic glyphosate concentrations that are higher than those tested in this study.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized, no positive controls were included in the assays and only one dose level was used for the testing of adipose stromal cells.

Reliability criteria for *in vitro* toxicology studies

Publication: Gigante *et al.,* 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity is not reported. Source: Sigma Chemical Co, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	

Study		
Test system clearly and completely described	Y	Granulosa cells from swine ovaries, adipose stromal cells from swine adipose tissue.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.2, 4 or 16 µg/mL.
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized, no positive controls were included in the assays and only one dose level was used for the testing of adipose stromal cells.		

1. Information on the study

Data point:	CA 5.6
Report author	Gorga A. *et al.*
Report year	2020
Report title	In vitro effects of glyphosate and Roundup on Sertoli cell physiology
Document No	Toxicology in Vitro 62 (2020) 104682
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of the present study was to analyze whether glyphosate (G) and Roundup (R) are able to affect Sertoli cell functions, such as energy metabolism and blood-testis barrier (BTB) integrity. Sertoli cell cultures from 20-day-old rats were exposed to 10 and 100 ppm of G or R, doses which do not decrease cell viability. Neither G nor R caused impairment in lactate production or fatty acid oxidation. G and R decreased Transepithelial Electrical Resistance, which indicates the establishment of a Sertoli cell junction barrier. However, neither G nor R modified the expression of claudin11, ZO1 and occludin, proteins that constitute the BTB. Analysis of cellular distribution of claudin11 by immunofluorescence showed that G and R induced a delocalization of the signal from membrane to the cytoplasm.

Materials and methods

Materials: [2,6-3H]-2-deoxy-D-glucose (2-DOG) and [9,10(n)-3H] palmitic acid were purchased from NEN (Boston, MA, USA). Culture media, glyphosate and all other drugs and reagents were purchased from SigmaAldrich (St Louis, MO, USA). The glyphosate formulation used in this work was the formulation available on the market called Roundup Full II (Monsanto Argentina S.A.I.C.), which contains 54% w/v acid glyphosate.

Sertoli cell (SC) isolation and culture: Twenty-day-old Sprague-Dawley rats (Rattus norvegicus) were obtained from the Animal Care Laboratory, Facultad de Ciencias Veterinarias, Buenos Aires, Argentina. Animals were killed by CO2 asphyxiation according to protocols for animal laboratory use following the principles and procedures outlined in the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Comité Institucional de Cuidado y Uso de Animales de Laboratorio (CICUAL) from the Hospital de Niños “Dr. Ricardo Gutiérrez”. SC were isolated as previously described. Decapsulated testes were digested with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in Hanks' balanced salt solution (HBSS) for 5 min at room temperature by manual agitation. The enzymatic action was stopped by dilution with four volumes of HBSS. Seminiferous tubules were collected by sedimentation and washed twice with HBSS. Then seminiferous tubules were cut with a razor and submitted to 1M glycine-2mM EDTA (pH 7.4) treatment for 10 min to remove peritubular cells. At the end of the incubation period, nine volumes of HBSS were added and a 30 min sedimentation was performed. The washed tubular pellet was then digested again with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in HBSS for 10 min at room temperature by continuous pipetting. The enzymatic action was stopped by dilution with four volumes of HBSS. The cell suspension was collected by centrifugation at 200 x g for 3 min. The cell suspension was diluted with HBSS and submitted to a 10 min sedimentation to remove germ cells. The pellet containing SC was filtered through a nylon mesh and SC were recovered by centrifugation at 200 x g for 3 min. SC were resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 10mM HEPES, 100 IU/ml penicillin, 2.5 μg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 μg/ml transferrin, 5 μg/ml insulin, 5 μg/ml

vitamin E and 4 ng/ml hydrocortisone. SC were cultured on 6-, 24- or 96-multiwell plates (5 μg DNA/cm2), on Matrigel-coated cell culture inserts (15 μg DNA/cm2) placed on 24- multiwell plates or on glass coverslips coated with laminin at 34 °C in a mixture of 5% CO2:95% v/v air. No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to SC cultures using a specific antiserum to smooth muscle α actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5% after 48 h in culture as examined by phase contrast microscopy.

Culture conditions: SC were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. Treatment with glyphosate (G) and Roundup (R) was performed with variable doses and for variable periods of time. Cells incubated for 48 h with 10, 100 and 1000 ppm of G and R harvested on day five were used to evaluate cell viability and LDH leakage. The cells treated for 48 h with 100 ppm of G and R were harvested on day five and used to evaluate GLUT1, FAT/CD36 and CPT1 mRNA levels, glucose uptake and fatty acid (FA) oxidation and the 48 h-conditioned media were utilized to evaluate lactate production. For Western blot studies, cells cultured for 4 days under basal conditions and pretreated for 30 min with 10 and 100 ppm of G or R were used. To quantify Transepithelial Electrical Resistance (TER), SC were cultured at high cell density (15 μg DNA/cm2, corresponding to 1.2 × 106 cells/cm2) on Matrigel-coated (1,6 dilution with F12/DMEM v/v) cell culture inserts (Millicell HA inserts) (Millipore, Billerica, MA, USA) placed on 24-multiwell plates. On day 3 in culture, testosterone was added and TER across the SC monolayer was recorded every 24 h in culture. On day 5, when the tight junction barrier had been formed, different doses of G or R were added and TER was recorded until day 8. To study the distribution and localization of claudin11, the cells were cultured on glass coverslips coated with laminin and treated with 100 ppm G or R in the presence or absence of testosterone for 48 h and harvested on day 5.

Evaluation of Sertoli cell energetic metabolism: Energetic metabolism in SC has been considered to have features of its own. Lactate, produced by SC, provides the energetic substrate to germ cells in the adluminal compartment. Consequently, it has been postulated that SC utilizes FA as their energy source. In this context, lactate production, glucose uptake, FA oxidation and the expression of genes involved in these processes were evaluated.

a) Lactate determination: Conditioned media obtained from cells cultured in 24-multiwell plates were used to determine lactate production. Lactate was measured by a standard method involving conversion of NAD+ to NADH.

b) Measurement of 2-deoxyglucose (2-DOG) uptake: Glucose transport was studied using the uptake of the labelled non-metabolizable glucose analogue 2-DOG on cells cultured in 24-multiwell plates as previously described.

c) Fatty acid oxidation assay: FA oxidation was performed measuring the release of 3H2O to the incubation medium from [3H]-palmitate on SC cultured in 24-multiwell as previously described

Evaluation of BTB function: The main component of the BTB is the presence of tight junctions between neighboring SC. In order to evaluate BTB function, Transepithelial Electrical Resistance (TER), claudin11 cellular distribution and the expression of proteins that participate in tight junction assembly were evaluated.

a) Transepithelial Electrical Resistance (TER) measurement: The establishment of the SC junction barrier was assessed daily from day 3 to day 8 by measurement of TER across the SC monolayer by a Millicell electrical resistance system (Millipore), as described previously. Briefly, a short (~2 s) 20-μA pulse of current was passed through the epithelial monolayer between 2 silver-silver chloride electrodes and electrical resistance was measured. Electrical resistance was then multiplied by the surface area of the insert to yield the area of resistance in ohms.cm2. The net value of electrical resistance was then computed by subtracting the background, which was determined by Matrigel-coated cell-free inserts.

Each time point had quadruplicate bicameral units. This experiment was run four times on different batches of cells.

b) Immunofluorescent (IF) detection of claudin11 protein: Monolayers were fixed with methanol for 10 min at -20 °C. After washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature. After 3 washes with PBS for 1 min each, the cells were blocked with 5% bovine serum albumin (BSA). Then, the coverslips were incubated with a 1:50 dilution of polyclonal antibody against claudin11 in PBS overnight at 4 °C. After 3 washes with PBS for 1 min each, coverslips were incubated with an anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated (1,25; Vector Laboratories, Burlingame, CA, USA). For negative controls, primary antibodies were replaced by PBS. Finally, the coverslips were washed 3 times with PBS for 1 min each, mounted in buffered glycerine and observed using an Axiophot fluorescent microscope with epi-illumination (Carl Zeiss Inc., Oberkochen, Germany).

RT-Real-time PCR (RT-qPCR): The expression of genes that participate in energetic metabolism (GLUT1, FAT/CD36 and CPT1) and in BTB organization (occludin, claudin11 and ZO-1) was evaluated by RT-qPCR. Total RNA was isolated from SC cultured in 6-multiwell plates with TRI Reagent (Sigma-Aldrich). The amount of RNA was estimated by spectrophotometry at 260 nm. Amplification was carried out as recommended by the manufacturer: 25 µl reaction mixture containing 12.5 µl of SYBR Green PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µl of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalized to HPRT1. The amplification program included the initial denaturation step at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative ΔΔCt method was used to calculate relative gene expression.

Western blot analysis: Cells cultured in 6-multiwell plates were washed once with PBS at room temperature. Then, 200 µl of PBS containing 2 µl of protease inhibitor cocktail (P-8340; Sigma-Aldrich), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50 nM okadaic acid and 2 mM PMSF was added to each well. Cells were then placed on ice and disrupted by ultrasonic irradiation. Western blot analysis was performed as previously described. Membranes were probed with antibodies that allow specific recognition of total Akt and mTOR, phosphorylated p38-MAPK and ERK1/2 (Cell Signaling Technology, Inc., Danvers, MA, USA), claudin11 (Zymed Lab. Inc.), androgen receptor and GAPDH (Santa Cruz Biotechnology, Inc., USA). A 1:1000 dilution of primary antibodies, as indicated by the manufacturer, was used. For chemiluminescent detection of the blots, a commercial kit from Cell Signaling Technology was used. The intensities of the autoradiographic bands were estimated by densitometry scanning using NIH Image Software (Scion Corporation). Levels of the corresponding total Akt, mTOR and GAPDH served as loading controls.

Cytotoxicity: A cell viability test (MTT assay) was performed in cells cultured in 96-multiwell using a commercial kit (CellTiter 96® AQueous NonRadioactive Cell Proliferation Assay; Promega Corporation). Cell cytotoxicity was determined by measuring the activity of LDH enzyme leaked from the cytosol of damaged cells into the medium as previously described. Results were expressed as the percentage of activity detected in the media over the sum of the activities in the media and in cells.

Statistical analysis: All experiments were run in triplicates and repeated 3–4 times. One way ANOVA and post hoc analysis using Tukey-Krämer's multiple comparisons test were performed using InfoStat versión 2016 (Grupo InfoStat, FCA, UNC, Argentina). P values < 0.05 were considered statistically significant.

Results

Effects of glyphosate and Roundup on SC cell viability: SC cultures were exposed for 48 h to glyphosate (G) and Roundup (R) at concentrations ranging from 10 to 1000 ppm, corresponding to 0.01 to 1 g/L respectively. Cell viability was analyzed by MTT assay and by measuring LDH leaked from the cytosol of damaged cells into the medium. The highest dose tested for R (1000 ppm) caused a cell death (Fig.

1). Therefore, in the present investigation destined to analyze G and R effects on SC functions only doses of 10 and 100 ppm were utilized.

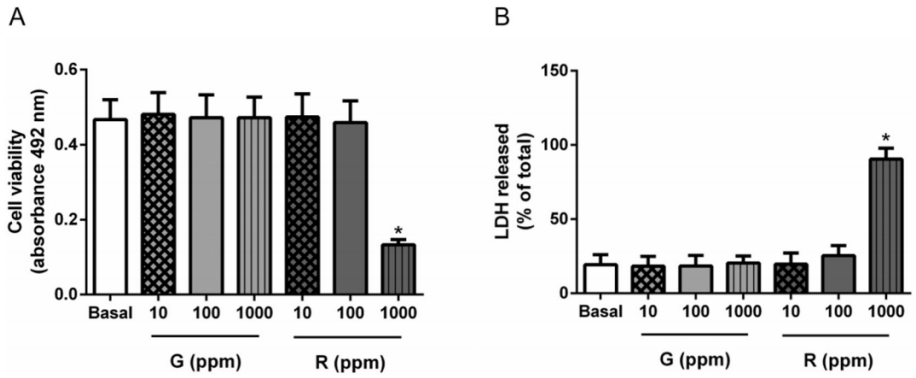


Fig. 1. Effect of G and R on SC cytotoxicity.
SC were maintained under Basal conditions or incubated with 10, 100 or 1000 ppm of G or R for 48 h. (A) Cell viability was determined by MTT assay. (B) LDH activity was determined in SC monolayer and in the culture medium. Values represent mean ± S.D. of one representative experiment out of three. *$p < 0.05$ versus Basal.

Effects of glyphosate and Roundup on SC energetic metabolism: SC cultures were exposed for 48 h to 100 ppm of G and R. Fig. 2 shows the results obtained for lactate production, glucose uptake and GLUT1 mRNA levels. The exposure to G or R did not modify lactate production neither glucose uptake nor GLUT1 expression.

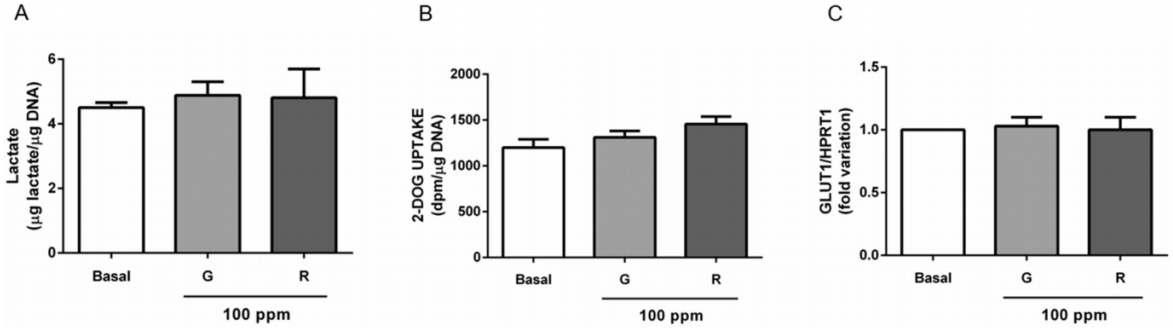


Fig. 2. Effect of G and R on lactate production, glucose uptake and on *Glut1* mRNA levels in SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. (A) Lactate levels were determined in the conditioned media. (B) Glucose uptake assay (2-DOG uptake) was performed after the 48 h incubation period. (C) Total RNA was extracted and RT-qPCR was performed to detect *Glut1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

Effects of glyphosate and Roundup on blood-testis barrier integrity: SC supply germ cells with a microenvironment preserved by the BTB. The main component of the BTB is the presence of tight junctions between neighboring SC. The establishment of these junctions between SC in culture was assessed daily from day 3 to day 8 by measuring TER across the SC monolayer. When SC were plated, junctions begin to assemble and an increase in TER was observed. On day 5, when SC completed barrier assembly, G or R were added to the culture medium. Compared with Control (92.3±3.1), a significant decline in TER was produced by the addition of 100 ppm of G (58.0±1.5) or 10 and 100 ppm of R (67.8±2.8 or 62.2±4.9, respectively)(Fig. 4 and Table 2).

Table 2
Effect of 48-h treatment with G and R on TER.

	TER ($\Omega \cdot cm^2$)
Control	92,3 ± 3,1
G 10 ppm	85,5 ± 4,5
G 100 ppm	58,0 ± 1,5*
R 10 ppm	67,8 ± 2,8*
R 100 ppm	62,2 ± 4,9*

SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G or R on day 5 for 48 h. Results are presented as means ± SD of four independent experiments (*$p < 0.05$).

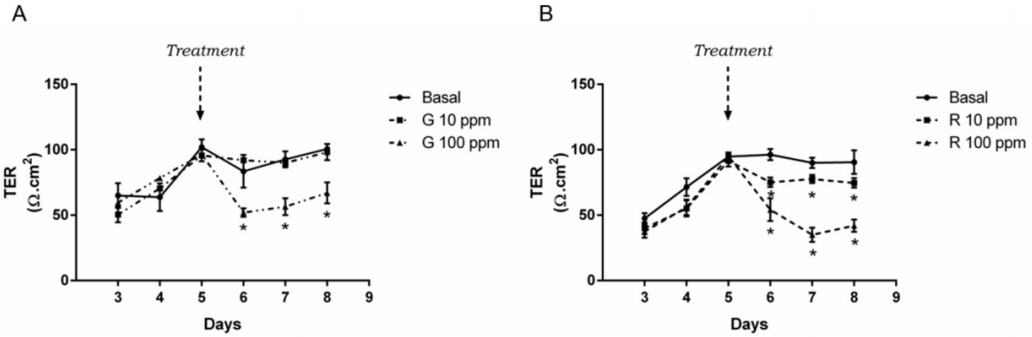


Fig. 4. Effect of G and R on TER across SC.
SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G (A) or R (B) on day 5. TER across SC monolayer was measured from day 3 to 8. Values represent mean ± S.D. of triplicate wells from the same culture of one representative experiment out of four. Asterisks indicate significant differences from basal cultures for each particular day, $p < 0.05$.

Considering that it had been demonstrated that p38-MAPK and ERK1/2 signaling pathways were involved in the disruption of BTB integrity by xenobiotics, the possible alteration of these pathways by G or R treatment was evaluated. R increased P-p38-MAPK and P-ERK1/2 levels. G did not modify P-p38-MAPK and P-ERK1/2 levels at any dose tested. 100 ppm G or R treatment did not modify *claudin11*, neither *occludin* nor *ZO-1* mRNA levels. Claudin11 protein levels were not modify by G or R treatment. In control conditions, claudin11 was detected at the zone of contact between adjacent cells, in a linear and continuous pattern that delineated cell borders in basal conditions. Addition of 100 ppm G or R induced redistribution of claudin11 since immunofluorescence became discontinuous and was redistributed from the cell surface into the cytoplasm.

Effects of glyphosate and Roundup on testosterone regulation of bloodtestis barrier integrity; It is well known that testosterone is the main regulator of BTB function, and that G or R can act as endocrine disruptors. In order to elucidate a possible mechanism responsible for adverse effects of G or R, we decided to evaluate whether herbicides can interfere with androgen action in BTB. Fig. 8A and B shows that 100 ppm G or R treatment did not modify androgen receptor mRNA or protein levels. Fig. 9 shows that, similar to what was observed under basal conditions, 100 ppm G or R treatment induced a redistribution of claudin11 from cell membrane to cytoplasm under the presence of testosterone.

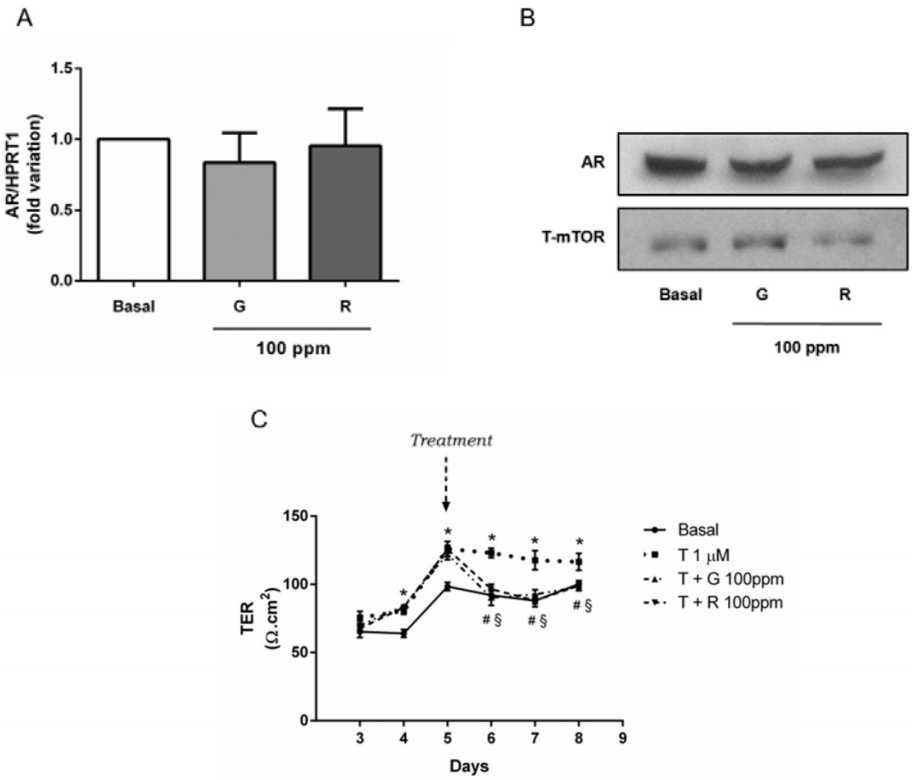


Fig. 8. Effect of G and R on androgen receptor (AR) expression and testosterone regulation of TER across SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. A) Total RNA was extracted and RT-qPCR was performed to detect AR mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal. B) Western blot analysis was performed utilizing antibodies for AR or total Akt (T-Akt). Results are representative of three independent experiments performed/treatment group. C) SC monolayers were maintained under Basal conditions or stimulated with testosterone (T) since day 3. On day 5, SC monolayers were treated with 100 ppm of G or R. TER across SC monolayer was measured from day 3 to 8. Values represent mean ± S.D. of triplicate wells from the same culture of one representative experiment out of three. Symbols indicate significant differences for each particular day: *p < 0.05 T vs Basal; #p < 0.05 T vs T + G; §p < 0.05 T vs T + R.

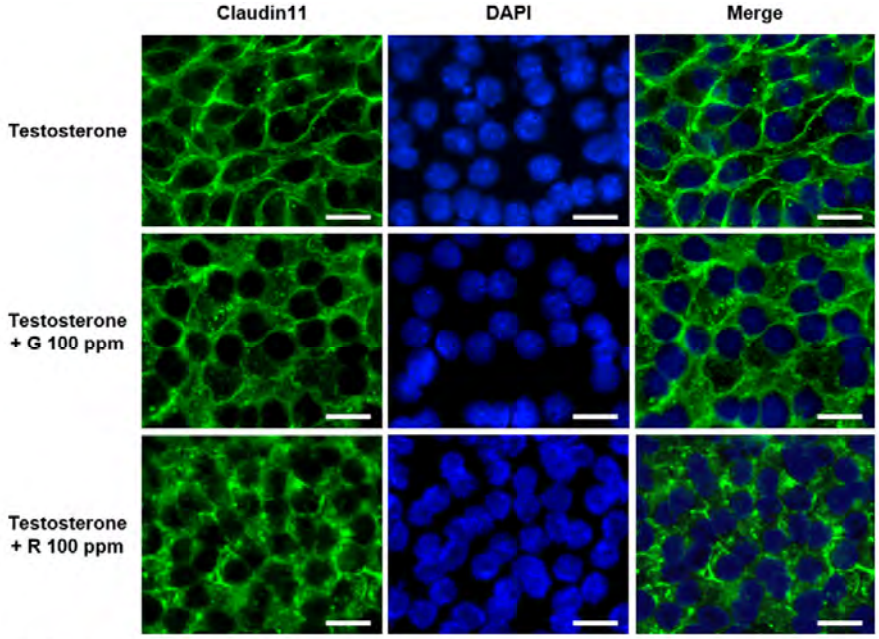


Fig. 9. Effect of G and R on claudin11 localization in the presence of testosterone in SC.
SC were incubated with 100 ppm of G or R in the presence of testosterone for 48 h. Claudin11 was revealed by IF. Bars: 50 μm.

Discussion

Over the last 60 years a progressive decrease in male reproductive function has been observed. Epidemiological and experimental studies suggest that one of the main causes is exposure to environmental toxicants. As previously stated, several studies have shown that G or R can potentially

cause adverse effects in male reproduction. Despite the compelling documented evidence proving the existence of adverse effects on testis function, little is known about direct effects on Sertoli cell function and the possible mechanisms involved. Primary cultures of Sertoli cells are a good and reliable model to assess the direct effects of xenobiotic exposure on this cell type. Regarding G and its commercial formulation R, initial studies on Sertoli cell cultures utilizing doses from 1 to 10,000 ppm of G and R were performed. In these experiments, treatment with 1000 ppm R produced Sertoli cell apoptosis with a maximal effect in 24 h-incubation period. However, in the same study G did not induce apoptosis in isolated Sertoli cells. Other studies, which measured apoptosis and/or cell viability, have also demonstrated that several human cell lines are more sensitive to R than to G. Coincidentally, the results presented herein show that 1000 ppm R decreases cell viability while G does not. These differences on the actions of both agents can be interpreted by the presence of various adjuvants in R. These adjuvants change human cell permeability and amplify toxicity by G, which may explain the differences observed between R and G effects on Sertoli cell viability. As we were focused on analyzing possible effects on Sertoli cell function, in this study we utilized 10 and 100 ppm doses which do not decrease cell viability. As mentioned in the introduction, two essential functions of mature Sertoli cells are the provision of nutrients to germ cells and the maintenance of a favorable microenvironment for spermatogenesis. As for Sertoli cell nutritional function, it has been well documented that Sertoli cell glycolysis provides lactate to satisfy germ cell energy demands while FA oxidation supplies energy to fulfill Sertoli cell energetic demands. Consequently, it can be predicted that an alteration in Sertoli cell metabolism can lead to a perturbation in normal germ cell development. The results obtained in the present investigation show that neither G nor R modify lactate production, glucose uptake and GLUT1 expression in Sertoli cells. Additionally, the results show that G and R do not modify FA oxidation and FAT/CD36 and CPT1 expression, proteins that are essential for FA utilization. Altogether, the results presented herein lead us to conclude that G and R effects on testicular function are not mediated by impairment of Sertoli cell metabolism. Noticeably, other reproductive toxicants, including phthalate esters, nitro-benzene, gossypol and certain heavy metal ions, alter Sertoli cell lactate production and show that toxicant effects strongly depend on the chemical nature of the toxicant. Regarding lipid metabolism, it has been demonstrated that 24 h exposure to 5000 ppm G or R induce lipid droplet accumulation in the Sertoli cell line TM4 cytoplasm. This increase in lipid droplet accumulation was interpreted as storage of potentially deleterious lipophilic formulants in the cytoplasm of TM4 cells and was considered a sign of the cytotoxic effect. An alternative explanation for the above results would be a modification in lipid metabolism, however, the authors did not explore this possibility. We have explored the latter possibility and the results presented herein show that 100 ppm G and R, doses that do not affect Sertoli cell viability, do not alter FA oxidation. It is worth mentioning that Sertoli cells, in addition to the nourishment provided to germinal epithelia, supply germ cells with a suitable microenvironment for successful meiosis and completion of spermatogenesis. This microenvironment is sustained by the BTB, whose main components are the tight junctions between neighboring Sertoli cells. The BTB is highly dynamic and is regulated by an array of intriguingly coordinated signaling pathways and molecules. Several studies have shown that many environmental toxicants, such as cadmium, bisphenol A, fluoride and sulfur dioxide exert their effects by targeting Sertoli and germ cell junctional proteins, as well as the permeability of the BTB. TER is a widely accepted quantitative technique that measures the integrity and permeability of BTB in vitro. The results presented in this investigation show that 100 ppm G and 10 and 100 ppm R decrease TER after 24 and 48 h-treatment. Notable, the disrupting effect of G on the permeability of other barriers had already been observed. It was shown that exposure to 10 mg/ml (10,000 ppm) G reduces TER and increases permeability to mannitol in Caco-2 and IEC-18 intestinal cell lines. In addition, 1 and 10μM G treatment (0.16 and 1.6 ppm) decreases TER and increases permeability to fluorescein in iPSC-derived brain microvascular endothelial cells cell line (BMECs). Results from other authors and our own let us postulate that one of the mechanisms by which G exerts toxic effects is related to disruption of barrier properties in different important organs such as intestine, brain and testis. It has been shown that p38-MAPK and ERK1/2 pathways play central roles in the dynamics of BTB. For example, TGFb3-induced physiological effect on Sertoli cell BTB dynamics is mediated via the p38-MAPK pathway. As for toxicant effects, it has been demonstrated that the polychlorinated biphenyls (PCBs), such as congener Aroclor1254, and the perfluorooctane sulfonate (PFOS) can disrupt the BTB integrity by activating the p38-MAPK pathway. The present investigation shows that R increases P-p38-MAPK and PERK1/2 levels. Therefore, it might be suggested that R decreases TER through a p38-MAPK and ERK1/2 dependent pathways. However, the same reasoning

cannot be applied to the effects of G considering that it lowers TER while it does not modify P-p38-MAPK and P-ERK1/2 levels. Hence, it seems that there is no direct relationship between P-p38- MAPK and P-ERK1/2 levels and TER levels, at least as a consequence of G and R exposure. We then decided to look for further mechanisms that may be involved in the disruption of BTB integrity by exposure to G and R. The next set of experiments was devoted to analyze possible alterations in the expression of some tight junction proteins, such as occludin, claudin11 and ZO-1, in response to G or R. An increased expression of these proteins, at the time when the junctions are assembled as manifested by a stable TER across the Sertoli cell epithelia, was demonstrated. It is worth mentioning that certain agents that perturb BTB permeability, such as cytokines or toxicants, alter the expression of cell junction proteins. The results obtained in the present investigation show that neither occludin nor claudin11 or ZO-1 expression was modified by G or R treatment. Finally, we decided to investigate whether the cellular localization of a tight junction protein such as claudin11 could shed some light on the mechanism underlying the decrease in TER. Claudin11 is a key molecule that provides functional integrity to the BTB. It is located in functional Sertoli cell tight junctions and its intracellular distribution pattern changes when gonadotropins are suppressed, coincident with a dysfunctional barrier. Claudin11 null mice are sterile highlighting the importance of this protein for BTB integrity. It is worth mentioning that in men with testicular disorders such as intraepithelial neoplasia, hypospermatogenesis, spermatogenic arrest, and Sertoli cell only testes, claudin11 is located in the cytoplasm, away from the tight junctions reinforcing the idea that localization of claudin11 in cytoplasm can be considered a sign of BTB dysfunction. In in vitro studies, a direct relationship between TER and localization of claudin11 in membrane junctions has been observed. The results presented herein show that although G and R treatment does not modify claudin11 protein levels, a redistribution of claudin11 from membrane to cytoplasm is observed. This alteration in the distribution of claudin11 may be interpreted as the result of an increase in membrane protein recycling from cell surface to cytoplasm. This last result may explain, at least in part, the effects of G and R in the integrity of BTB and the deleterious effect of these toxicants at testicular level. Disruption of the Sertoli cell permeability barrier by loss of usual distribution patterns of other junctional proteins such as occludin, ZO-1, and Cx43 induced by other toxicants has also been demonstrated and these results indicate that redistribution of these proteins can also contribute to alter barrier permeability. Further studies will be necessary to definitively assign a role to this phenomenon in the alteration of BTB dynamics by G or R. Several lines of evidence obtained from in vivo and in vitro approaches highlight the importance of testosterone in the regulation of BTB assembly and function. In vivo studies showed that reestablishment of testicular androgen levels by hCG or testosterone treatment leads to a restoration of claudin11 localization and BTB function. In vitro studies showed that Sertoli cell permeability barrier formation and claudin11 localization are promoted by androgen treatment. Furthermore, numerous studies describe G or R as endocrine disruptors. On the one hand, it was demonstrated that in vivo treatment of drakes with R decreases the expression of the androgen receptor (AR) in Sertoli cells while in rats, G treatment does not modify AR expression in the same cell type. On the other hand, it was shown that in vivo treatment of rats decreases serum testosterone levels, and furthermore, it was demonstrated that the effect on testosterone levels may be attributed to a decrease in the expression of STAR in Leydig cells. Therefore, it is tempting to speculate that G or R effects on BTB integrity can be partially attributed to the interference with androgen action. The results presented herein show that neither G nor R treatment modified androgen receptor mRNA and protein levels. They also show that the effects of the herbicides on TER and on claudin11 localization in the presence of testosterone were similar to those observed under basal conditions. Thus, G and R are able to disrupt BTB function in the presence of testosterone. Further in vivo experiments will be necessary to determine a possible role of G or R as endocrine disruptors.

Conclusion

In summary, this investigation shows that G and R alter the Sertoli cell junction barrier permeability. This study also shows that, at least in part, the loss of location of claudin11 at the interface between neighboring Sertoli cells might be responsible for the disassemble of the barrier. We postulate that BTB integrity is a sensitive target for the adverse effects of G or R on male reproductive function.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This *in vitro* investigation showed that exposure to G and R at 100 ppm alters Sertoli cell junction barrier permeability, measured by decreased TER, and also decreased testosterone-stimulated TER. This study also showed that, at least in part, the loss of location of claudin11 at the interface between neighboring Sertoli cells might be responsible for the disassembly of the barrier. G or R did not modify androgen receptor mRNA or protein levels, nor did G modify P-p38-MAPK and P-ERK1/2 signalling pathways involved with BTB integrity at any doses tested, or affect the expression of intercellular junction proteins (claudin11, occludin and ZO-1). However, G and R induced redistribution of claudin11 at the zone of contact between cells. Neither G nor R modified lactate production, glucose uptak, GLUT1, FA oxidation, or *FAT/CD36* and *CPT1* expression in SC, thus indicating no effect of G or R on SC nutritional function or metabolism.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not sufficiently characterized, there was no positive control and most of the endpoints were tested at only 2 concentrations preventing any dose response evaluations, with the highest concentration exceeding that which is physiologically relevant.

Reliability criteria for *in vitro* toxicology studies

Publication: Gorga *et al*., 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup Full II (Monsanto, Argentina)
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described		
Test concentrations in physiologically acceptable range (< 1 mM)	Y	3 test concentrations used of which the highest was > 1 mM (1000 µg/mL).
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control reported.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	

Dose-effect relationship reported		Explored but not found (only 2 concentration levels tested for most endpoints)
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not sufficiently characterized and most of the endpoints were tested at only 2 concentrations.		

1. Information on the study

Data point:	CA 5.5
Report author	Hao Y., Xu W. et al
Report year	2019
Report title	Roundup-Induced AMPK/mTOR-Mediated Autophagy in Human A549 Cells
Document No	Journal of agricultural and food chemistry (2019) Vol. 67(41), 11364-11372
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate-based herbicide (GBH) is one of the most widely used pesticides based on a 5-enolpyruvylshikimate-3-phosphate synthase target, which does not exist in vertebrates. Here, the autophagic effects of the most famous commercial GBH Roundup (RDP) on human A549 cells in vitro has been studied. Intracellular biochemical assay indicated opening of mitochondrial permeability transition pore, LC3-II conversion, up-regulation of beclin-1, down-regulation of p62, and the changes in the phosphorylation of AMPK and mTOR induced by RDP in A549 cells. Further experimental results indicated that all the effects induced by RDP were related to its adjuvant polyethoxylated tallow amine, not its herbicidal active ingredient glyphosate isopropylamine salt. All these results showed that RDP has the ability to induce AMPK/mTOR-mediated cell autophagy in human A549 cells.

Materials and methods

Chemicals - Glyphosate isopropylamine salt (≥ 95%) was obtained from Weihai Hanfu Biochemical Pharmaceutical Co., Ltd., Weihai, China.

Cell culture - DMEM supplemented with 1% antibiotics (streptomycin and penicillin) and 10% fetal bovine serum was used to culture human alveolar carcinoma A549 cells (ATCC, CCL-185). The cells were placed in an incubator at 37 °C with a humidified atmosphere of 5% CO_2.

Cell viability - The MTT assay was performed to evaluate the cytotoxicity of glyphosate in A549 cells. 1.0×10^5 cells in 100 μL of fresh DMEM medium were plated in 96-well plates. After incubation for 24 hours, medium was replaced with fresh medium containing glyphosate at 100 μg/mL. Fresh medium served as the control. Three replicates were used per test group. After incubation for 2 hours, the MTT reagent (20 μL/well, 5 mg/mL) was added to form formazan crystals during incubation for 4 hours at 37 °C. The medium was removed and the formazan crystals were dissolved in DMSO (150 μL/well). Optical density (OD) was measured with a microplate reader at 570 nm. Percent cell viability inhibition was calculated as follows: $cell\ inhibition(\%) = (OD_{control} - OD_{treatment})/OD_{control} \times 100$. SPSS version 17.0 was used to calculate IC_{50} values.

Monodansylcadaverine (MDC) staining – MDC staining was used to mark autophagic vacuoles. A549 cells (1×10^5 cells/well) were grown in 24-well plates. The cells were exposed to glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After rinsing twice with PBS at pH 7.4, the cells were incubated with 10 μM MDC in the dark for 20 minutes at 37 °C. After washing with PBS at pH 7.4, the treated cells were photographed and analyzed by fluorescence microscopy at 488 nm at a magnification of 200×. Three photos were taken per group and analyzed to determine the number of autophagosomes.

Visualization of double-membrane autophagosomes - Visualization of the ultrastructure of A549 cells was performed by transmission electron microscopy (TEM). A549 cells were harvested after being treated for 2 hours with glyphosate at 100 μg/mL. Fresh medium served as the control. The cells were then rinsed twice with PBS at pH 7.4 and placed in a 2.5% glutaraldehyde solution and kept overnight at 4 °C. Subsequently, the cells were post-fixed in 1% osmium tetroxide (OsO_4) after rinsing with PBS at pH 7.4. The cells were then dehydrated with an ascending series of ethanol solutions and embedded with Epon 812. Ultrathin sections were stained with 2% uranyl acetate and lead citrate solutions. The images were recorded using a JEOL JEM-2100 transmission electron microscope.

Autophagic flux - Ad-mCherry-GFP-LC3B is an adenovirus that expresses the mCherry-GFP-LC3B fusion protein, which is used to analyze autophagic flux after infection of cells. A549 cells were grown in 3 cm glass bottom cell culture dishes and infected with adenovirus (40 MOI) for 24 hours. After incubation for another 24 hours, the cells were exposed to glyphosate at 100 μg/mL and incubated for 2 hours and then rinsed twice with PBS at pH 7.4. Fresh medium served as the control. The expression of mCherry and green fluorescent protein (GFP) was visualized and analyzed by a Nikon confocal microscope at 488 and 561 nm at a magnification of 100× by taking three photos per group. Diffuse yellow fluorescence indicates no occurrence of autophagy whereas yellow spots indicate autophagy. Autophagic flux was evaluated by the accumulation of mCherry-GFP-LC3B on the autophagosome membrane.

Colocalization of mitochondria and lysosomes - MitoTracker Green was used for mitochondria-specific fluorescent staining of live cells. LysoTracker Red was used for lysosomal-specific fluorescent staining of live cells. The two probes were used for mitochondrial and lysosomal colocalization imaging. A549 cells were treated with glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After treatment and rinsing, the cells were incubated with MitoTracker Green (0.5 μM) and LysoTracker Red (0.5 μM) for 30 minutes. Afterwards, the cells were photographed and analyzed by fluorescence microscopy at 488 and 561 nm at a magnification of 200×. Three photos were taken per group and analyzed. The counterstaining cells were photographed by a Nikon confocal microscope at a magnification of 100× and emission recorded at 488 nm for MitoTracker Green and 561 nm for LysoTracker Red. The related plugins (JACoP, colocalization threshold) of ImageJ v1.8.0 software were used to obtain a relative coefficient of colocalization between Mito and Lyso.

Opening of mitochondrial permeability transition pore (mPTP) - Opening of mPTP was measured by using calcein and $CoCl_2$. A549 cells were grown in 12-well plates and treated with glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After rinsing twice with PBS at pH 7.4, the cells were incubated with 1 mM calcein in the dark for 20 minutes at 37 °C and then exposed to 1 mM $CoCl_2$ for 30 minutes. The control group was not incubated with $CoCl_2$. After rinsing with PBS at pH 7.4, the treated cells were analyzed by fluorescence microscopy at 488 nm at a magnification of 200× by taking three photos per group.

Immunoblotting of proteins linked with autophagy - The relative protein expression levels of LC3, beclin-1, p62, p-AMPK, p-mTOR, and p-p70s6k in A549 cells were determined by immunoblot analysis to explore the underlying mechanisms of induced autophagy. A549 cells were treated with glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After treatment and rinsing the cells were lysed by a mixture of 50 μL immunoprecipitation assay lysis buffer with 0.5 μL protease inhibitor (100 mM). The total protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit. Equal amounts of lysate proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes with the proteins were blocked in Tris-buffered saline containing 5% nonfat dried milk and 0.05% Tween-20 for 2 hours. Blocked membranes were incubated with rabbit polyclonal antibodies for LC3, beclin-1, p62, p-AMPK, p-mTOR, p-p70s6k, and β-actin overnight at 4 °C. After being washed three times in Tris-buffered saline containing 0.05% Tween-20, membranes were incubated with anti-rabbit IgG secondary antibodies for 2 hours. The antibody-bound proteins were detected by Electro-Chemi-Luminescence kit and scanned by the chemiluminescent gel imaging system. The bands' grayscale values were quantified by ImageJ v1.8.0 software.

Adenosine triphosphate (ATP) content - The levels of intracellular ATP were determined by the ATP Assay Kit from Beyotime following the manufacturer's instructions. After treatment with glyphosate at 100 μg/mL or fresh medium as the control for 2 hours, A549 cells were lysed in the lysis buffer for 30 minutes at 4 °C. After centrifugation at 12,000 rpm for 15 minutes, a part of the supernatant was transferred into a light-protected 96-well plate for determination of the activity of ATP. The other part was used to determine the protein concentrations by the Pierce BCA Protein Assay Kit. The ATP concentrations (μmol/L) were converted to the protein concentrations (mg/L).

Statistical Analysis - Three separate replicates were performed for each assay. The statistical analysis process runs under SPSS version 17.0, statistical program (SPSS Inc). Data are presented as the means ± standard deviation (SD). Three independent experiments of MTT assay were analyzed by two-way ANOVA followed by Tukey post hoc testing. Different small alphabets indicate significant differences ($P \leq 0.05$). Others were subjected to one-way analysis of variance (ANOVA) followed by Dunnet's test for determining the differences with control ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$).

Results

Viability - Inhibition of cell viability (as % of control) of A549 cells was 5.36 ± 0.82 for glyphosate at 100 μg/mL after 2 hours of treatment.

Monodansylcadaverine (MDC) staining - MDC staining in A549 cells treated with glyphosate at 100 μg/mL was not significantly different from the control.

Visualization of double-membrane autophagosomes - The visualization of autophagosomes by TEM showed an homogeneous cytoplasm with normal mitochondria in A549 cells treated with glyphosate at 100 μg/mL.

Autophagic flux - In the control and in the cells treated with glyphosate at 100 μg/mL, mCherry-GFP-LC3B is present in the form of diffuse yellow fluorescence which is indicative of the absence of autophagy.

Colocalization of mitochondria and lysosomes - The relative coefficient of colocalization between Mito and Lyso was 0.22 ± 0.01 for the control and 0.21 ± 0.02 for the cells treated with glyphosate at 100 μg/mL.

Opening of the mitochondrial permeability transition pore (mPTP) - When compared with the $CoCl_2$-treated control group, no significant increase in fluorescence was noted in the cells treated with glyphosate at 100 μg/mL which is indicative of the absence of mPTP opening.

Immunoblotting of proteins linked with autophagy - The effect of glyphosate on the autophagy related proteins beclin-1, LC3-II/I, and p62 was studied by immunoblotting. When compared with the control no difference was found in the expression ratio of LC3-II/I as well as protein expression of beclin-1 in cells treated with glyphosate at 100 μg/mL. Immunoblotting was also used to examine the phosphorylation of mTOR, AMPK, and p70s6k. No difference in phosphorylation levels of mTOR, p70s6k, and AMPK could be demonstrated in cells treated with glyphosate at 100 μg/mL when compared to controls. These results indicate that glyphosate does not contribute to the activation of the AMPK/mTOR pathway.

Adenosine triphosphate (ATP) content - When compared with the control, no difference was found in the levels of cellular ATP of cells treated with glyphosate at 100 μg/mL.

Conclusion

All commercial glyphosate formulations are more toxic than glyphosate so that the increased toxicity may be attributed to the adjuvants. The herbicidal active ingredient of Roundup is glyphosate isopropylamine salt and its main adjuvant is polyethoxylated tallow amine (POEA). To explore whether

the herbicidal active ingredient or the adjuvant contributes Roundup's ability of inducing autophagy in human A549 cells, TEM, MDC, immunoblotting, and Ad-mCherry-GFP-LC3B analyses were performed on 4 concentrations of Roundup from 50 to 125 µg glyphosate acid eq./mL and one concentration of glyphosate (100 µg/mL) and POEA (35 µg/mL). This study revealed that the commercial formulation Roundup has the ability to cause autophagy in A549 cells via the AMPK/mTOR signaling pathway. Previous studies showed that compared with glyphosate, commercial glyphosate based herbicides are more toxic, and the toxic effect is highly correlated with the adjuvant POEA. This study indicates that the adjuvant POEA in Roundup contributes to its ability of inducing autophagy in A549 cells, and that the herbicidal active ingredient glyphosate has no contribution.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The effect of glyphosate, POEA and a herbicidal formulation containing POEA as a co-formulant on the AMPK/mTOR signaling pathway was studied in human alveolar carcinoma A549 cells *in vitro*. Only the results of exposure to glyphosate at 100 µg/mL are reported and discussed in this summary. The endpoints selected to study the effect of glyphosate on autophagy are inhibition of viability, monodansylcadaverine (MDC) staining to mark autophagic vacuoles, visualization of double-membrane autophagosomes by TEM, autophagic flux, colocalization of mitochondria and lysosomes, opening of the mitochondrial permeability transition pore (mPTP), expression of proteins involved in the AMPK/mTOR signaling pathway, and ATP content. No effect could be demonstrated of glyphosate on any of these endpoints indicating that glyphosate, in contrast to POEA and Roundup, does not contribute to the activation of the AMPK/mTOR signaling pathway and has thus no role in autophagy.

This publication is relevant for the risk assessment of glyphosate but reliable with restrictions because only one glyphosate concentration was tested and no positive controls were used.

Reliability criteria for *in vitro* toxicology studies

Publication: Hao, Xu *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of ≥95%. Source: Weihai Hanfu Biochemical Pharmaceutical Co., Ltd. ,China.
Only glyphosate acid or one of its salts is the tested substance	N	Also GBH (Monsanto, St. Louis, USA) and POEA tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Human alveolar carcinoma A549 cell line.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Glyphosate only at one concentration tested: 100 µg/mL.

Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	N	Only one concentration for glyphosate.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is relevant for the risk assessment of glyphosate but reliable with restrictions because only one glyphosate concentration was tested and no positive controls were used.		

1. Information on the study

Data point:	CA 5.4
Report author	Ilyushina N. A. *et al.*
Report year	2018
Report title	Comparative investigation of genotoxic activities of glyphosate technical products in the micronucleus test in vivo
Document No	Toksikologicheskii Vestnik, (2018) No. 4, pp. 24-28.
Guidelines followed in study	OECD Test Guideline 474
Deviations from current test guideline	Positive control animals were included but the data are not reported. No ratio of PCE to NCE was reported. No evidence of bone marrow exposure Data have been presented per group rather than per animal
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, the induction of micronucleus formation in vivo in polychromatophilic erythrocytes of bone marrow of CD-I mice was assessed by the action of three different technical glyphosate products on the market in the Russian Federation. It was found that the tested samples of technical products showed different cytogenetic activity, while only one of them caused a statistically significant, dose-dependent increase in the frequency of induction of micronuclei compared to the negative control.

The analysis of the composition of the studied product samples showed that the cytogenetic activity may depend on the content of potentially mutagenic impurities, formaldehyde in particular.

The acquired data provides some additional grounds for lowering the upper limit of formaldehyde content in technical glyphosate products and also indicates the need to assess the genotoxic activity of analogue pesticides entering the market of plant protection products.

Materials and methods

Three glyphosate technical products were used that were manufactured at different factories and that contained the active substance (glyphosate acid) in amounts of 96.6%, 95.8% and 95.7% in technical products I, II and III, respectively.

Chromosomal disturbances were revealed through the use of in vivo micronucleus analysis [OECD Test No. 474: Mammalian Erythrocyte Micronucleus Test. 2016] of the bone marrow erythrocytes in CD-I laboratory mice obtained from the husbandry center at the Andreyevka Branch of the Federal State Budget Research Institution “Biomedical Technology Science Center” of the Russian Federal Medicobiological Agency.

Five mice per group were used. Technical glyphosate was administered intragastrically in 1% starch in doses of 500, 1,000 and 2,000 mg/kg of body weight (the maximum dose according to OECD Test No. 474) twice, twenty-four hours apart, and the animals were euthanized 22 hours following the second administration. At the same time, an excipient (1% starch) was given as a negative control following the same pattern as was used for the technical glyphosate. Cyclophosphamide was used as a positive control, given intragastrically in the amount of 40 mg/kg once, at the same time as the second dose of glyphosate.

Bone marrow preparations from each specimen were studied microscopically (on a Japan-made Nikon

Eclipse Ci-L), counting 4,000 polychromatophilic erythrocytes (PCEs) for measuring micronucleus frequency), and the portion among the erythrocytes as a whole made up by PCEs, counting no fewer than 500 erythrocytes for each animal.

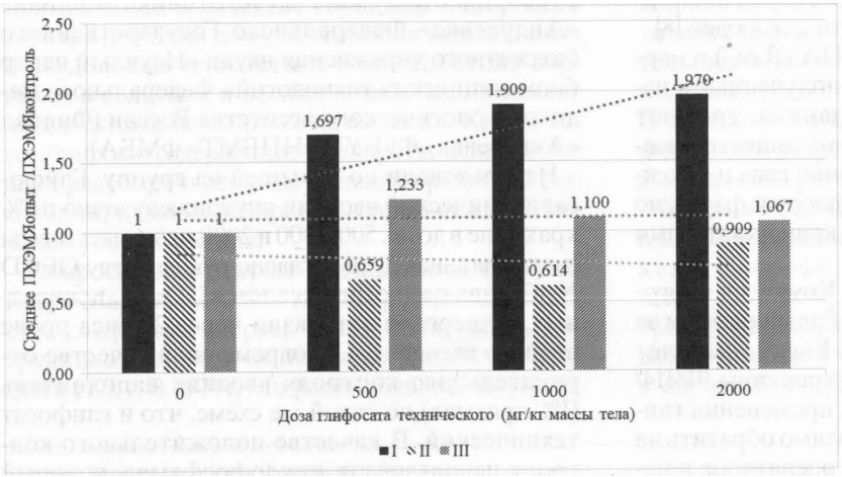
A statistical analysis of the study results was carried out using SPSS Statistics v. 22.0 (IBM, New York, USA). Frequencies of micronuclei in the PCEs were compared by building a generalized log-linear model of the Poisson Regression ($\alpha = 0.05$). The data were also checked for dependencies between micronucleus frequency and the dose of the technical product being tested using the Mantel-Haenszel method.

Results

All the research was carried out in accordance with OECD Manual No. 474 and Methodological Instructions MU-1.2.3365-16. Each experiment used positive and negative controls simultaneously. The frequency of PCEs containing micronuclei in the case of the positive control was in a range from 1.58 to 2.95%, while in the case of the negative control it was from 0.06 to 0.12%. All the values were within the range of the distribution boundaries of historical laboratory controls.

The quantification of the portion of total erythrocytes made up by polychromatophilic erythrocytes show that in none of the analyses was erythropoiesis suppressed, i.e. there were no toxic effects on mouse bone marrow.

Varying effects were revealed during the study of the mutagenic activity of the glyphosate technical products through micronucleus analysis. Technical products II and III did not significantly increase the PCE frequency in mouse bone marrow compared to the simultaneous negative controls (Table 1, Figure 1).



Technical Glyphosate Dose (mg/kg of body weight)

Figure 1. Frequencies of increased occurrence of PCEs compared to the negative control depending on the dose of glyphosate technical products. I, II, III – Reference numbers for different technical products. $PCEMN_{exp}$ denotes the frequency of PCEs with micronuclei in groups of mice that received different glyphosate doses. $PCEMN_{control}$ denotes the frequency of PCEs with micronuclei in groups of mice that received an excipient with 1% starch (negative control).

When administering technical Product I, a low, but statistically significant genotoxic effect was observed at all doses (Table 2). Moreover, a statistically significant linear dependency was observed of the frequency of PCEs with micronuclei upon the dose of the compounds under study ($p= 0.002$) (Table 1).

A comparison was made between the content of ingredients in the study samples based on the available batch analysis certificates for the technical products. Two ingredients were of special interest from the point of view of genotoxicity: nitroso-glyphosate and formaldehyde. According to the provided certificates, Ni-trosoglysophate, was not present in any of the batches of technical products (its content, in accordance with FAO specifications, must not exceed 1 mg/kg). Formaldehyde content in products I, II and III amounted to 0.13%, 0.024% and 0.06%, respectively. A formaldehyde level in the glyphosate product of 0.13% is the maximum permissible concentration in glyphosate technical products in accordance with the FAO specifications[14]. However, it should be noted that, according to Annex I of Directive 91/414/EEC, as amended in 2017, the partial content of formaldehyde in glyphosate must not exceed 1 mg/kg [10].

A comparison of the results we have obtained on the mutagenic activity and formaldehyde concentrations in glyphosate technical samples showed that statistically significant genotoxic effects and a linear dependence of the frequency of PCEs with micronuclei upon the dose of the compound under study were only observed in the case of the product with 0.13% formaldehyde. Lower concentrations of this ingredient did not induce the statistically significant formation of PCEs with micronuclei in bone marrow cells.

Thus, the observed mutagenic effects were most likely not related to the active ingredient, but rather to the formaldehyde contained as an ingredient in the products.

Table 1: Probable profile (95%) of confidence intervals for the frequency of polychromatophilic erythrocytes with micronuclei (MN) under the effects of different doses of glyphosate technical products relative to the negative control

Glysophate dose (mg/kg of body weight)	Sample I		Sample II		Sample III	
	Number of PCEs with MN per 40,000 PCEs	95% confidence intervals	Number of PCEs with MN per 40,000 PCEs	95% confidence intervals	Number of PCEs with MN per 40,000 PCEs	95% confidence intervals
0	33	1	44	1	30	1
500	56	1.111 1.697 2.634	29	0.408 0.659 1.048	37	0.763 1.233 2.009
1000	63	1.263 1.909 2.941	27	0.376 0.614 0.984	33	0.671 1.100 1.812
2000	65	1.306 1.970 3.028	40	0.591 0.909 1.395	32	0.647 1.067 1.762
Assessment of linear trend using the Mantel-Haenszel method	1 (146)		II (189)		III (341)	
	Value	Asymptomatic significance p (one-dimensional)	Value	Asymptomatic significance p (one-dimensional)	Value	Asymptomatic significance p (one-dimensional)
	9.791	0.002	0.28	0.597	0.06	0.938

The results obtained are in agreement with data from the literature. In the majority of studies conducted using the Ames test, glyphosate did not induce gene mutations in bacteria [Li A.P., Long T.J., 1988]. In both *in vivo* and *in vitro* micronucleus analyses carried out on different subjects, contradictory data were obtained. An increased frequency of the appearance of micronuclei was noted in several studies on cultivated human buccal epithelial cells [Koller V.J. et al, 2012], in mouse bone marrow [Manas F., 2009], and in human lymphocytes having undergone been affected by glyphosate-containing preparations during crop spraying [Bolognesi C. eta la, 2009]. However, the majority of the studies gave negative results in terms of micronucleus analyses [Kier L.D., 2013]. However, none of the studies analyzed the content of possible mutagenic ingredients in technical products and glyphosate-containing preparations and their contribution to the observed effects.

Conclusion

The comparative investigation of the genotoxic activity of various glyphosate technical products through the analysis of micronuclei in mouse bone marrow erythrocytes showed that cytogenetic activity may depend on the content in the product of potentially mutagenic ingredients, and in particular of formaldehyde. The contradictory results obtained in different studies of the mutagenic activity of glyphosate and preparations containing it may be connected to the different concentrations of ingredients that may exhibit effects of intracellular genetic structures.

The obtained data comprise additional substantiation for reducing the upper limit for formaldehyde content in glyphosate technical products, and also provide evidence of the need to assess the genotoxic activity of analogous pesticides entering the Russian Federation's market.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes the results of three different technical batches of glyphosate tested in a mouse micronucleus test. The study followings the recommendations of OECD 474, with some deficiencies, mostly regarding the reporting of data rather than test methodology. However, the reliability of the reported conclusions are unknown due to a lack of clarity and accuracy of the reported data. Individual animal data and group mean±SD frequency of micronucleated (MN) PCE have not been reported. Instead the results per group appear to be presented as the total number of MN PCE found per 40,000 PCE, together with the frequency of MN PCE values, expressed as relative to control, for the group with calculated 95% upper and lower limits (no explanation for how these data awere derived is provided). The text describes the vehicle control MN PCE frequencies as ranging between 0.06% and 0.12% (within HCD) but similar detail is not provided for the treated groups. Consequently, there is no indication of animal variability within the groups and it is unknown if any of the treated animals fall outside of HCD. Furthermore, the total number of MN PCE is described as being per 40,000 PCE, however, only 4000 PCE were scored per animal and with 5 animals per group this would result in total of 20,000 PCE per group.

The authors postulate that the positive results observed for technical batch I are likely to be due to the presence of 0.13% formaldehyde in the material, although they provide no data to support their hypothesis.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the 3 glyphosate batches was not revealed although the concentrations of the most important impurities were given for each batch. Although reference was made to OECD test guideline 474 too little detail was given on the conduct of the MN assay to conclude to reliability without restrictions and the data are inadequately reported.

Reliability criteria for *in vivo* toxicology studies

Publication: Ilyushina *et al*., 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	OECD test guideline 474 according to the authors.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	

Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Technical glyphosate from 3 sources with purity of 96.6%, 95.8%, and 95.7%. Sources were not reported but the concentration of impurities (nitroso-glyphosate and formaldehyde) was given.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	CD-1 mouse.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	0, 500, 1,000, and 2,000 mg/kg bw twice, twenty-four hours apart.
Positive control	Y	Cyclophosphamide.
Number of animals used per dose level reported	Y	5/dose group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	Y	Referred to but not presented.
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the 3 glyphosate batches was not revealed although the concentrations of the most important impurities were given for each batch. Although reference was made to OECD test guideline 474 too little detail was given on the conduct of the MN assay to conclude to reliability without restrictions, and the data are inadequately reported.		

1. Information on the study

Data point:	CA 5.4
Report author	Ilyushina N. *et al.*
Report year	2019
Report title	Maximum tolerated doses and erythropoiesis effects in the mouse bone marrow by 79 pesticides' technical materials assessed with the micronucleus assay
Document No	Toxicology Reports (2019) Vol. 6, 105-110
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Effects of technical materials of pesticide active ingredients, belonging to various chemical classes, on erythropoiesis in mouse bone marrow were studied as part of the research on the pesticide mutagenic activity in micronucleus test. The purpose of the present study was to estimate the toxic action of the test substances on the target organ and the validity of the results of the micronucleus assay under conditions of erythropoiesis suppression.

Materials and methods

Chemicals - Four glyphosate batches were tested with a purity of respectively 95.7, 98.3, 95.1, and 95.8%.

Animals – CD-1 mice were purchased from "Andreevka" Branch of the Federal Government Budgetary Establishment of Science "Scientific Center of Biomedical Technologies" of the Federal Bio-Medical Agency of the Russian Federation. The acclimation period was 7 days. Mice had access to drinking water and feed ad libitum, and were maintained under a 12:12-hour light/dark photoperiod at 22 – 22.5 °C and 36 - 40% humidity.

Mammalian Erythrocyte Micronucleus Test (OECD TG 474) - At least 5 groups of minimum 5 mice per sex were used. Each assay included a positive control group (40 mg/kg bw cyclophosphamide), a negative control group (1% potato starch in water), and 3 treatment groups. Glyphosate was administered orally by gavage once a day for 2 subsequent days (24 hours apart) at a volume of 10 mL/kg bw. The maximum dose in the main experiment was either 2,000 mg/kg bw or the MTD as determined in a preliminary dose-finding experiment. Mice were sacrificed 22 hours after the second administration by cervical dislocation, then femurs were removed and bone marrow was harvested. To assess the effect of glyphosate on erythropoiesis, the ratio of polychromatic erythrocytes (PCEs) to the sum of PCEs and normochromatic erythrocytes (NCEs) was determined by counting at least 500 cells (PCEs + NCEs) per animal (at least 250 cells for each slide) under a Nikon Eclipse Ci-L microscope. At least 4,000 PCEs were counted per animal by two different researchers.

Statistical analysis - Statistical analysis was performed using SPSS Statistics v. 22.0 software. The statistical significance of the difference in the proportion of PCEs/(PCEs + NCEs) between the highest dose group and the concurrent negative control group was evaluated using the independent samples t-test for each study.

Results

It should be noted that the negative control values slightly varied from experiment to experiment, and that the historical negative controls of the laboratory were 0,50 ± 0,06 and 0,52 ± 0,06 PCEs/(PCEs+NCEs) females and males, respectively. Cyclophosphamide did not cause a significant decrease in the proportion PCEs/(PCEs+NCEs) in comparison with the negative control. The 4 glyphosate batches tested at the limit dose of 2,000 mg/kg bw in CD-1 mice according to the protocol of the *in vivo* micronucleus assay compliant with OECD test guideline 474 did not reveal any effect on erythropoiesis in the bone marrow.

Conclusion

Glyphosate tested at the limit dose of 2,000 mg/kg bw in mice in the *in vivo* micronucleus assay did not show any effect on erythropoiesis in the bone marrow.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Along with 51 other pesticides 4 batches of glyphosate with purities ranging from 95.1 to 98.3% were investigated for their effect on erythropoiesis in mice. To assess the toxicity of glyphosate on the bone marrow the *in vivo* micronucleus test in the mouse according to OECD test guideline 474 was conducted at the limit dose of 2,000 mg/kg bw. No effect of glyphosate on erythropoiesis was found.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the glyphosate batches used was not reported and no suitable positive control was used in the micronucleus test to assess bone marrow toxicity. The test conducted was in compliance with OECD test guideline 474.

Reliability criteria for *in vivo* toxicology studies

Publication: Ilyushina *et al*., 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	OECD TG 474, in vivo MN assay only used with the purpose to assess toxicity to erythropoiesis in the bone marrow. The positive control used (cyclophosphamide) is not suitable as a positive control for bone marrow toxicity.
Study performed according to GLP	N	Not stated.
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y?	4 batches were tested with purity of 95.7, 98.3, 95.1, and 95.8%. Source was not mentioned.
Only glyphosate acid or one of its salts is the tested substance	N	51 other pesticides

		were tested as well.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	
Dose levels reported	Y	Only limit dose of 2,000 mg/kg bw was considered.
Number of animals used per dose level reported	Y	At least 5 groups of minimum 5 mice per sex.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y?	Only effect on erythropoiesis was reported.
Statistical methods described	Y	
Historical control data of the laboratory reported	Y	
Dose-effect relationship reported	N	Not possible since only one dose was used.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the glyphosate batches used was not reported and no suitable positive control was used in the micronucleus test to assess bone marrow toxicity. The test conducted was in compliance with OECD test guideline 474.		

1. Information on the study

Data point:	CA 5.6
Report author	Johansson H. K. L. *et al.*
Report year	2018
Report title	Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis
Document No	Reproductive Toxicology (2018) Vol. 82, 25-31
Guidelines followed in study	None
Deviations from current test guideline	NA
Previous evaluation	No
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate has been suggested to be an endocrine disrupting chemical capable of disrupting male reproduction. There are conflicting data, however, with studies reporting effects from exposure to either glyphosate alone or to herbicide formulations, making comparisons difficult. Rat testis histopathology and androgen function following two weeks exposure to either glyphosate at 2.5 and 25 mg/kg bw/day (5x and 50x Acceptable Daily Intake, ADI, respectively), or equivalent high dose of glyphosate in a herbicide formulation; Glyfonova, were assessed.

Materials and methods

Chemicals - Glyphosate (purity ≥ 96%) was purchased from Sigma-Aldrich, St Louis, USA.

Animals - Four-week old male Sprague-Dawley rats were randomly caged in pairs and acclimatized for 7 days. Cages were subsequently distributed into four exposure groups based on animal weight, but following exposure only one animal per cage was used for analyses. Ten animals per dose group were treated with glyphosate orally by gavage at 2.5 and 25 mg/kg bw. Water was used as the control. After 2 weeks of exposure, the animals were decapitated under CO_2/O_2 sedation and the testes collected. From each of 10 males per dose group, one testis, selected at random, was snap frozen in liquid nitrogen and the other was fixed in 10% formalin.

Testosterone assay - One half of each testis was used for hormone extraction. The testes were cut into two equally sized pieces. After removal of the testis cap the inner tissue (seminiferous tubules and interstitial cells) was transferred immediately to a glass vial containing 0.5 mL sterile water and reweighed to determine total tissue weight per sample. Subsequently, 2.5 mL heptane was added and the sample homogenized by manual disruption and then frozen solid. The heptane fraction was transferred and the process repeated to yield a second 2.5 mL heptane fraction that was pooled together with the first fraction. The extracts were dried under nitrogen. Before hormone analyses, the dry extracts were dissolved in EIA buffer at 4 °C overnight, then vortexed and placed in a 42 °C water bath for 10 minutes. Intra-testicular testosterone assays were performed using the Testosterone ELISA kit according to the manufacturer's instructions. Reads were obtained using 96-well plates and absorbance read at 405 nm using a microplate reader. Each sample was assayed in duplicates and result means presented as pg testosterone/g testis.

Histology - Testes were fixed in 10% formalin, processed for paraffin embedding and sectioned at 5 μm for histological assessments. Hematoxylin & Eosin (H&E) staining was performed following standard protocols.

Immunohistochemistry - Immunohistochemistry with peroxidase was carried out on sections of formalin-fixed testes. Sections were dewaxed in petroleum and rehydrated by immersion in ethanol

solutions and finally water. Antigen retrieval was carried out in 0.01M citrate buffer (pH 6) in a microwave and then allowed to cool at room temperature. Samples were washed in PBS and blocked with 1% bovine serum albumin (BSA) and then incubated overnight at 4 °C with primary antibody. The following day, samples were washed in PBS, blocked with 3% H_2O_2 for 10 minutes and again washed in PBS. The samples were incubated with EnVision+System (Dako) for 30 minutes, washed, then with Liquid DAB+System (Dako) for 15 minutes and washed again. To visualize all cell nuclei, sections were counterstained with Meyers' hematoxylin. Following washing and rehydration, samples were mounted with Eukitt. The primary antibody used was against the Androgen receptor.

Immunofluorescence – Five µm sections (4/group) were dewaxed in petroleum and washed in ethanol and dehydrated. Antigen retrieval was done by heat treatment in Tris-EDTA buffer (pH 9) and then cooled at room temp before washing in PBS and blocking in 5% bovine serum albumin (BSA). The samples were incubated with primary antibodies overnight at 4 °C. The next day, slides were brought to room temperature and incubated with secondary antibodies for 1 hour in the dark. Samples were then counterstained with 4,6-diamidino-2-phenylindole (DAPI). The primary antibodies were goat anti-HSD3β, rabbit anti-DDX4, goat anti-CYP11A1 and rabbit anti-STAR. Secondary antibodies were donkey anti-goat AlexaFluor-488 and donkey anti-rabbit AlexaFluor-568.

RNA extraction, cDNA synthesis and quantitative RT-PCR - Total RNA was extracted from a ~100 mg cross-sectional piece from each testis (n=10/group), of which 500 ng RNA was used for each cDNA synthesis. TaqMan Gene Expression Assays were Ar (Rn00560747), Ddx4 (Rn01489814), Cyp11a1, Cyp17a1, Insl3 (Rn00586632), Pcna (Rn01514538), Star and Hsd3b1 (Rn017747410). RT-qPCR assays were run on a QuantStudio 7 Flex Real-Time PCR System in a 384-well format using 3 µL diluted (1:20) cDNA as template in each 20 µL reaction. Relative transcript abundance was calculated by the comparative Ct-method with the geometric mean of the reference genes Sdha (Rn00590475) and Rpl13a (Rn00821946).

Apoptosis - One section per testis (n=5/group) was stained with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, Apoptag®Peroxidase in Situ Apoptosis Detection Kit) according to the manufacturer's instructions with one exception i.e. pre-dilution (1:80) of the TdT enzyme in MilliQ water before dilution with reaction buffer. Color was developed for 15 minutes using DAB+substrate. Lastly, the nuclei were stained using Mayer Hematoxylin prior to mounting using Eukitt. All positively stained cells within the seminiferous tubules on a cross-section were counted. Cells that were clearly stained as a result of cell division rather than apoptosis were excluded, as were stained cells at the outer border of the tissue sections. The relative percentage of seminiferous epithelium to whole testis was calculated using a 15 point-grid count on three separate fields of view from each sample. Second, whole testis section areas were calculated in Adobe Photoshop CC 2017 using the measurement tool. Third, apoptotic cells were defined relative to percentage seminiferous epithelium to whole section. Data and statistical analyses are presented as number of apoptotic cells per seminiferous tubuli area.

Statistical analysis - Data were tested for normal distribution and homogeneity of variance and logarithmic transformation was applied if required. ANOVA with Dunnett's post-test was applied and $p < 0.05$ was considered statistically significant. Statistical software GraphPad Prism 5 was used for analysis.

Results

Testosterone levels - No statistically significant increase in testicular testosterone levels was observed in rats treated orally at 2.5 and 25 mg/kg bw/day for 2 weeks.

Quantitative gene and protein expression analysis - In the testis from animals orally exposed to glyphosate at 2.5 or 25 mg/kg bw/day for 2 weeks no significant differences in gene expression were observed for the Leydig cell specific genes Cyp11a1, Cyp17a1, Insl3, Hsd3β1 and Star and the somatic marker gene Ar or germ cell marker gene Ddx4.

Testis histopathology - No adverse histopathology in any of the glyphosate exposed groups was observed when compared with controls. The seminiferous tubules were intact and displayed active spermatogenesis with comparable spermatogenic cycling between examined specimens (qualitative assessment). Neither were there any differences between exposed and control animals with regard to

missing germ cell layers or multinucleated germ cells. The interstitial space was comparable between glyphosate treated groups and controls.

Qualitative protein expression analysis - The Leydig cell-specific steroidogenesis factors CYP11A1 and STAR were both expressed at comparative levels between the glyphosate treated animals and the controls. Also no difference in expression and distribution was noted between glyphosate treated groups and controls for the steroidogenic enzyme HSD3B1 and the germ cell-specific factor DDX4.

Apoptosis - The number of apoptotic cells was comparable between control testes and all glyphosate treated groups (5 per group).

Discussion and conclusions

Glyphosate at 2.5 mg/kg bw/day (5-fold ADI) and 25 mg/kg bw/day (50-fold ADI) did not show significant effects on intra-testicular testosterone levels, testicular gene and protein expression, testicular histopathology and apoptosis in the testes of rats treated orally for 2 weeks. This suggests that the active compound glyphosate may not contribute to previously reported endocrine disrupting effects, but rather other constituents in the commercial formulated products. This is in agreement with conclusions from the most recent expert opinion review on glyphosate commissioned by the European Food Safety Authority which conclude that there is no evidence of endocrine mode of action of glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The effects of glyphosate on intra-testicular testosterone levels, expression of Leydig cell specific genes Cyp11a1, Cyp17a1, Insl3, Hsd3β1 and Star and expression of somatic marker gene Ar or germ cell marker gene Ddx4, expression of Leydig cell-specific steroidogenesis factors CYP11A1 and STAR, testicular histopathology and apoptosis were investigated in male rats treated orally at 0, 2.5 and 25 mg/kg bw/day for 2 weeks. No effects were found on either of the testicular parameters tested suggesting that glyphosate does not contribute to endocrine disrupting effects of the male reproductive system.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only two dose levels were used to explore the dose-effect relationship for the endpoints assessed.

Reliability criteria for *in vivo* toxicology studies

Publication: Johansson *et al*., 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity ≥ 96%. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance		Also formulations were tested: Glyfonova 450 Plus, FMC corporation.
AMPA is the tested substance	N	

Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	Only 2 dose levels for glyphosate (2.5 and 25 mg/kg bw)
Number of animals used per dose level reported	Y	10 animals/group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	Only 2 dose levels tested.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only two dose levels were used to explore the dose-effect relationship for the endpoints assessed.		

1. Information on the study

Data point:	CA 5.4
Report author	Kasuba V. *et al.*
Report year	2017
Report title	Effects of low doses of glyphosate on DNA damage, cell proliferation and oxidative stress in the HepG2 cell line
Document No	Environ Sci Pollut Res (2017) Vol. 24, 19267–19281
Guidelines followed in study	Not mentioned
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The toxic effects of glyphosate in vitro on HepG2 cells exposed for 4 and 24 h to low glyphosate concentrations likely to be encountered in occupational and residential exposures [the acceptable daily intake (ADI; 0.5 μg/mL), residential exposure level (REL; 2.91 μg/mL) and occupational exposure level (OEL; 3.5 μg/mL)] were studied. The assessments were performed using biomarkers of oxidative stress, CCK-8 colorimetric assay for cell proliferation, alkaline comet assay and cytokinesis-block micronucleus (CBMN) cytome assay. The results obtained indicated effects on cell proliferation, both at 4 and 24 h. The levels of primary DNA damage after 4-h exposure were lower in treated vs. control samples, but were not significantly changed after 24 h. Using the CBMN assay, a significantly higher number of MN and nuclear buds at ADI and REL after 4 h and a lower number of MN after 24 h were found. The obtained results revealed significant oxidative damage. Four-hour exposure resulted in significant decrease at ADI [lipid peroxidation and glutathione peroxidase (GSH-Px)] and OEL [lipid peroxidation and level of total antioxidant capacity (TAC)], and 24-h exposure in significant decrease at OEL (TAC and GSH-Px). No significant effects were observed for the level of reactive oxygen species (ROS) and glutathione (GSH) for both treatment, and for 24 h for lipid peroxidation. Taken together, the elevated levels of cytogenetic damage found by the CBMN assay and the mechanisms of primary DNA damage should be further clarified, considering that the comet assay results indicate possible cross-linking or DNA adduct formation.

Materials and Methods

Chemicals - Glyphosate was purchased as analytical standard purity grade (≤100%) as Pestanal®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany.

Cell line - HepG2 cell line (ATCC® HB8065™) was purchased from the American Type Culture Collection (Rockville, MD, USA), cultivated in EMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U/mL) and maintained in a humidified atmosphere (95% relative humidity) with 5% CO_2 at 37 °C. Testing was performed when the duplication time has been established to be constant, and the passage step was more than 3 but less than 10 cell passages.

Treatment conditions - The cells in culture were exposed to glyphosate concentrations of 0.5 μg/mL, 2.91 μg/mL and 3.5 μg/mL in PBS corresponding with the systemic concentrations at the acceptable daily intake (ADI), the residential exposure level (REL) and the occupational exposure level (OEL). The calculation of the concentrations to be tested was based on the average male human body weight of 60 kg and the total volume of 36 L of extracellular liquid. HepG2 cells were grown until 80% confluence, trypsinized and transferred in cell culture flasks for the micronucleus and comet assays and in 96 well plates for the cell proliferation assay and the determination of markers of oxidative stress. Prior to treatment, the culture medium was removed, cells were washed with PBS and fresh complete medium

with glyphosate was added. Cells were treated for 4 or 24 hours at 37 °C in a humidified atmosphere (95% relative humidity) with 5% CO_2. Negative and positive controls were tested in parallel.

Cell proliferation assay - Proliferation of HepG2 cells after 4 and 24 hours of exposure to glyphosate was studied by means of the CCK-8 colorimetric assay, based on the use of Cell Counting Kit-8. This kit uses 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, sodium salt (WST-8) that is bioreduced by cellular dehydrogenases to an orange formazan product, soluble in tissue culture medium in the presence of the electron carrier, 1-methoxy PMS. The amount of formazan produced is directly proportional to the number of living cells. At the end of treatment cells were washed with PBS and 10 µL of WST-8 solution added. After 4 and 24 hours of incubation at 37 °C in a CO_2 incubator, the optical density at 450 nm was determined for each well using a Victor3™ Multilabel Plate Reader. Data were expressed as a percentage absorbance compared to relevant negative controls. The positive control was 10% DMSO in the complete nutrient medium. The experiment was done twice independently with 4 repeated measurements in each.

Alkaline comet assay - After 4 or 24 hours of treatment, cells were washed twice with PBS, detached by trypsinization, centrifuged and resuspended in complete EMEM. 7 µL of single cell suspension at 10^4 cells/mL was mixed with low melting point agarose and layered on slides. Duplicate slides were prepared for each time period and concentration together with negative and positive controls. Hydrogen peroxide was used as the positive control. Following preparation of all microgels, they were immersed in fresh cold lysis solution for 24 hours. Afterwards, slides were washed with bidistilled water and denatured in freshly prepared cold denaturation/electrophoresis buffer. After 20 min at 4 °C, cells were placed in the electrophoretic chamber and subjected to electrophoresis. Afterwards the slides were neutralized, dehydrated and stained with ethidium bromide (20 µg/mL) for image analysis with the fluorescent microscope Olympus BX51 (×200 magnification) using the Comet Assay IV software. A total of 100 nucleoids (50 per slide) were measured per each experimental point. The level of DNA damage was estimated based on tail intensity (TI), indicating %DNA in the comet tail. The experiment was performed once with two replicates that were compared, and if no statistically significant difference was found they were combined.

Cytochalasin B-blocked micronucleus cytome assay (CBMN cytome assay) - Cells were seeded at 10^4 cells/mL in complete EMEM medium. After 4 or 24 hours of treatment with glyphosate, cells were washed twice with PBS and fresh complete medium was added. At the 44^{th} hour, cytochalasin B (3 µg/mL) was added and the cell cultures harvested 24 hours thereafter. The positive control, cyclophosphamide, was incubated simultaneously. After harvesting, the medium was discarded, and cells were washed twice with PBS, detached by trypsinization, rinsed and resuspended in complete EMEM medium. Cell suspensions in complete EMEM were centrifuged and the pellet resuspended in PBS, centrifuged and the pellet fixed and put on slides for staining with 2% Giemsa stain and air-dried. The experiment was performed once with two replicates. Micronuclei (MNi), nucleoplasmic bridges (NPBs), nuclear buds (NBUDs) and apoptotic and necrotic cells were scored in binucleated (BN) cells. A total of 2000 BN cells per each experimental point were scored to determine the parameters of the CBMN Cytome assay.

Lipid peroxidation - Malondialdehyde (MDA), and endproduct of lipid peroxidation, was measured using the thiobarbituric acid reactive substance (TBARS) assay with some modification. 0.5 mL of sample was added to 0.5 mL of thiobarbituric-trichloroacetic acid (TBA-TCA) reagent and heated at 90 °C for 30 min and then cooled in an ice bath and centrifuged. Absorbances were measured with a microplate spectrophotometric system (Victor3™ Multilabel Plate Reader) at 530 nm. Two independent experiments were performed and each sample was measured in duplicate. The TBARS concentration in unknown samples was calculated using a standard curve constructed with 1,1,3,3- tetrametoxypropane (0.3–6.07 µM) and expressed as µM.

Total antioxidant capacity - Total antioxidant capacity (TAC) was investigated using the ferric-reducing ability of plasma (FRAP) assay, based on the reduction of Fe3+-TPTZ complex under acidic conditions. The FRAP assay used in this study was slightly modified. 100 µL of cell suspension (10^4 cells in total) was added to 1.0 mL of FRAP reagent. Absorbance was measured after 4 minutes of incubation at 593

nm using the FRAP working solution as a blank. Two independent experiments were performed and each sample was measured in duplicate. The results were calculated on a basis of a standard curve using $Fe_2SO_4 \cdot 7H_2O$ (0.05-4.0 mM).

ROS detection - The amount of intercellular reactive oxygen species (ROS) was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is deacetylated by cellular esterases to form a non-fluorescent compound which is then oxidized in the presence of hydroxyl, peroxyl and other ROS to the fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). All measurements were performed in quadruplicate in dark-sided 96-well microplates in which each well was added 100 µL of cell suspension containing 10^4 cells. Cells were grown in the same medium and conditions as for the cell proliferation assay and treated with the same concentrations of glyphosate for 4 and 24 hours. After treatment the cells were washed with PBS and 100 µL of 50 µM DCFH-DA dye diluted in PBS was added and kept in contact with the cells for 30 minutes at 37 °C in a CO_2 incubator. The control for dye autofluorescence was prepared without addition of dye. Negative (non-treated) cell controls in EMEM were included in each experiment. The positive control was a solution of 100 mM H_2O_2. Measurements were made using a Victor3™ Multilabel Plate Reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Data were expressed as fluorescence arbitrary units (AUs) and later transformed into percentages compared to control values.

Quantification of glutathione - The fluorogenic bimane probe, monochlorobimane (MBCl), reacts specifically with glutathione (GSH) and forms a fluorescent product equal to the amount of GSH that can be measured fluorometrically. Cell cultures in 96-well plates were prepared with 100 µL of cell suspension containing 10^4 cells and each well was treated with glyphosate for 4 and 24 hours. After washing with PBS, cells were incubated with 100 µL of 50 µM MBCl in PBS for 20 minutes at 37 °C in a CO_2 incubator. Measurements were made using a Victor3™ Multilabel Plate Reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Negative cell controls were included in each experiment. Data were expressed as fluorescence AUs and later transformed into percentages compared to control values. All of the measurements were performed in quadruplicate.

Glutathione peroxidase activity - Glutathione peroxidase (GSH-Px) activity in HepG2 cells was measured according to the European standardized method. To increase assay sensitivity for the measurement of GSH-Px, samples were prediluted 60 times instead of a recommended 110-fold dilution as used for blood samples. The amount of glutathione oxidized by *t*-butyl hydroperoxide was determined by following the decrease in β-NADPH concentration at 340 nm. Two independent experiments were performed and each sample was measured in duplicate. One unit of GSH-Px was expressed as the amount of enzyme that oxidizes 1 µmol β-NADPH/min at 37 °C. Activity of GSH-Px was expressed in U/g total protein. The protein concentration of cell lysates was measured using a Total Protein Kit, Micro. Samples, standards and blanks were tested in triplicate, and absorbance was measured in 96-well plates at 570 nm**.**

Statistical analysis - Statistical analysis was performed using StatSoft Dell Statistica 13 package program. Descriptive statistics were used to determine the basic statistical parameters (mean, standard error and deviation, median and minimum and maximum values). Data gathered with the Comet Assay IV software were logarithmically transformed prior to statistical evaluation, with the aim of normalizing distribution and equalizing variances. The intra- and intergroup comparisons between samples were performed using one-way analysis of variance (ANOVA) with post hoc Scheffé's test. Comparisons between values obtained with the CBMN Cytome assay and HepG2 proliferation kinetics measured with CBMN were made by Pearson's $\chi 2$ test for two-by-two contingency tables. For the biochemical and cell proliferation assays descriptive statistics and *t* test for comparisons between independent samples were used. The level of statistical significance was set at $p < 0.05$.

Results

Cell proliferation measured by the CCK-8 assay - Although without statistical significance a slight increase of cell proliferation was observed in HepG2 cells after a 4-hour treatment of about 9% at the ADI and almost 8% at the REL. The values recorded for the OEL-treated cells were not different from

control. After 24 hours of treatment, HepG2 cells showed a not statistically significant increase in cell proliferation of 3% at the REL and of 1% at the OEL when compared to the control. Proliferation of ADI-treated cells did not differ from control.

Primary DNA damage measured by the alkaline comet assay - After 4 hours of exposure, the TI was statistically significantly decreased at all glyphosate concentrations. After 24 hours of exposure, the TI at ADI, OEL and REL were not statistically significantly different from control. Throughout comet measurements, apoptotic and necrotic nucleoids were also counted but there were no significant deviations from control.

Cytogenetic damage measured using CBMN cytome assay - After 4 hours of exposure, a not statistically significant increase in MN was found in HepG2 cells treated with glyphosate at all 3 concentrations. In HepG2 cells treated with glyphosate at ADI and REL, a statistically significant increase in nuclear buds (NBUD) frequency was observed. The incidence of nucleoplasmic bridges (NPB) was highest in cells treated with glyphosate at ADI (6 per 2000 BN cells). Only 2 apoptotic cells per 2000 cells were found in the sample treated with glyphosate at OEL. The nuclear division index (NDI) in glyphosate-treated cells was different from that of the control. In the OEL-treated sample, there were statistically significant changes in the distribution of M1–M4 cells and OEL treatment resulted with more cells in the M2 phase. The same effect was observed when ADI-treated and OEL-treated samples were compared, as well as when REL-treated and OEL-treated samples were compared. After 24 hours of exposure, a lower number of micronucleated BN cells and a lower frequency of BN MN was found at all 3 glyphosate concentrations when compared to the control. Also the frequency of BN NBUDS was statistically significantly lower at all 3 concentrations. Control and glyphosate-treated cells did not significantly differ in the number of NPBs. At ADI 7 and at REL 5 apoptotic cells per 2000 BN cells were found. There was no statistically significant difference in NDI values between glyphosate-treated samples and control. A slightly changed incidence of M2 and M3 cells was found in treated samples as compared to control.

Lipid peroxidation - After 4 hours of exposure, TBARS concentrations in samples treated with glyphosate at ADI and OEL were statistically significantly lower than control values. Treatment of HepG2 cells for 24 hours with glyphosate resulted in lower TBARS concentrations at REL, but the difference was not statistically significant.

GSH level - The level of GSH was not statistically significantly different from the control value for all concentrations and exposure times. After 4 hours of treatment, the ADI-treated sample showed a 4% higher level of GSH than the control whereas REL and OEL-treated cells showed GSH levels similar to control values. The 24-hour ADI-treated sample had the lowest GSH concentration, which was 5% lower than the levels measured in the control samples, and REL- and OEL-treated cells showed GSH levels similar to control values.

GSH-Px activity - GSH-Px activity was statistically significantly decreased in HepG2 cells exposed to glyphosate at the ADI concentration after 4 hours. After 24 hours of treatment, a statistically significant decrease in the GSH-Px activity was observed at the OEL concentration.

Discussion

The present study evaluated how low-dose exposure for 4 and 24 hours affected cytotoxic, genotoxic and biochemical parameters in the human hepatoma cell line HepG2. Glyphosate increased not statistically significantly cell proliferation in HepG2 cells after 4 and 24 hours of exposure. The effect was more pronounced after 4 hours (8–9% as compared to control), than after 24 hours (3% as compared to control). The results of this study have demonstrated that exposure to glyphosate for 4 hours led to a slightly increased cytogenetic damage in terms of MN. Despite the non-significant increase in MN frequencies at ADI and REL, significant increases in nuclear bud frequency were found after 4 hours of exposure. Since nuclear budding represents a mechanism of MN formation, these results indicate that even a low dose of glyphosate as the ADI influences the level of DNA damage and cell stability. After 24 hours of exposure, all of the treated cells showed a lower frequency of micronuclei, nuclear buds

and nucleoplasmic bridges than the control. The results obtained suggest that glyphosate exerts the highest DNA-damaging potential after 48 or even after 72 hours of treatment. Considering that the shorter exposure lasted only 4 hours, while the total cell culture lasted for 72 hours before harvesting, there was an opportunity to better examine the effects of low-dose exposure on DNA damage. On the contrary, in the case of an exposure of 24 hours it seems that this effect only started to be slightly visible. Serum in the cell culture can mask the development of DNA damage observed in micronucleus assay results. This can be the explanation, why the standard time period used in this experiment did not demonstrate an effect, while in the comet assay the primary DNA damage demonstrated higher damage levels.

Conclusion

All cytogenetic, molecular and biochemical methods used in this study indicate that glyphosate applied at low concentrations, possesses a toxic potential towards HepG2 cells, which has to be further explained. It seems that the theory of different effects of low-dose vs. high-dose exposure, and more deleterious effects at low doses, is true. Although at ADI, REL and OEL, no drastically different levels of oxidative damage were seen, the elevated level of permanent DNA damage found with the micronucleus assay calls for concern, especially if leading to adduct formation, as shown by comet assay results. Based on the obtained results, it cannot be said without doubt whether glyphosate acts as an aneugen or a clastogen, but there are indications from previous studies that the aneugenic effect plays an important role in the formation of micronuclei. This study did not evaluate the effects of glyphosate on cytoskeleton and proteins, but this would be a direction for future evaluations of glyphosate toxicity, together with the clarification of its effects on cell membrane level, especially in different phases of the cell cycle.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to examine the effects of acute exposure (4 and 24 hours) of human hepatoma HepG2 cells to glyphosate at concentrations equivalent to the systemic concentrations at the ADI, REL and OEL. The endpoints investigated in this study are cell proliferation, DNA damage, MN formation and oxidative stress. A non-statistically significant increase in cell proliferation was seen in the CCK-8 test with no dose-effect relationship. The comet assay showed a statistically significant decrease in tail intensity after 4 hours with no difference from control after 24 hours. In the CBMN cytome assay a non-statistically significant increase in BN MN frequency was seen after 4 hours without a dose-effect relationship. After 24 hours, a decrease instead of an increase in BN MN frequency was reported. The nuclear bud frequency was statistically significantly elevated after 4 hours of exposure but was statistically significantly lower than control after 24 hours of exposure. The indicator tests for oxidative stress did not show a substance related effect.

This publication is considered relevant but reliable with restrictions because the cytogenetic damage found *in vitro* at a systemic concentration corresponding with the ADI (0.5 µg/mL which should have been 0.17 µg/mL) was not confirmed in *in vivo* regulatory MN studies with doses up to 2000 mg/kg bw corresponding with a systemic concentration of about 50 µg/mL.

Reliability criteria for *in vitro* toxicology studies

Publication: Kasuba *et al.,* 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines		
Study performed according to GLP		
Study completely described and conducted following scientifically acceptable standards		
Test substance		

Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of ≤100% as Pestanal®. Source: Sigma-Aldrich Laborchemikalien GmbH, Germany.
Only glyphosate acid or one of its salts is the tested substance		As "Pestanal"
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	Culture concentration not in correspondence with ADI of 0.5 mg/kg bw.
Metabolic activation system clearly and completely described	N	HepG2 cells used
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	N	Very low concentrations used
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	?	No significant effects were observed for the level of ROS and GSH for 4h and 24h incubation, and for 24 h for lipid peroxidation. Elevated levels of cytogenetic damage were found in the CBMN assay and the comet assay results indicate possible cross-linking or DNA adduct formation. These data were obtained at in vitro test concentrations that correspond with an external dose of 0.5 mg/kg bw/day (ADI) whereas there are regulatory studies with no effects at doses up to 2,000 mg/kg bw/day
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant but reliable with restrictions because the cytogenetic damage found in vitro at a systemic concentration corresponding with the ADI (0.5 µg/mL which should have been 0.17 µg/mL) was not confirmed in *in vivo* regulatory MN studies with doses up to 2000 mg/kg bw corresponding with a systemic concentration of about 50 µg/mL.		

1. Information on the study

Data point:	CA 5.4
Report author	Koller V. J. *et al.*
Report year	2012
Report title	Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells
Document No	Arch Toxicol (2012) Vol. 86, 805–813
Guidelines followed in study	SCGE assays were performed according to the guidelines described by Tice et al. (2000) and the CBMN Cytome assay according to Fenech (2007). The corresponding Guidance Documents are OECD 489 and OECD 487
Deviations from current test guideline	Cell type (buccal cells)
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

Aim of this study was to investigate the cytotoxic and genotoxic properties of glyphosate (G) and Roundup (R) (UltraMax) in a buccal epithelial cell line (TR146), as workers are exposed via inhalation to the herbicide. R induced acute cytotoxic effects at concentrations [40 mg/l after 20 min, which were due to membrane damage and impairment of mitochondrial functions. With G, increased release of extracellular lactate dehydrogenase indicative for membrane damage was observed at doses [80 mg/l. Both G and R induced DNA migration in single-cell gel electrophoresis assays at doses [20 mg/l. Furthermore, an increase of nuclear aberrations that reflect DNA damage was observed. The frequencies of micronuclei and nuclear buds were elevated after 20-min exposure to 10 -20 mg/l, while nucleoplasmatic bridges were only enhanced by R at the highest dose (20 mg/l). R was under all conditions more active than its active principle (G).

Materials and Methods

Chemicals - Glyphosate (purity 95% w/w) and Roundup (Roundup Ultra Max, 450 g/L glyphosate acid) were obtained from Monsanto Europe S.A.

Storage and cultivation of the indicator cells - The human cell line TR146 was cultured under standard conditions in DMEM supplemented with 10% heat-inactivated fetal calf serum. The medium was changed every 2–3 days. When the cultures reached confluency, the cells were washed with Dulbecco's PBS, detached with TrypLE Express, centrifuged, and sub-cultured. TR146 cells express ultrastructural characteristics of normal human buccal epithelial cells, e.g. intermediate filaments, microvilli-like processes, and lack of complete keratinization.

Cytotoxicity assays - For cytotoxicity experiments, $5x10^4$ indicator cells were seeded in 96-well plates and exposed to concentrations of glyphosate and Roundup ranging from 0 to 200 mg glyphosate equivalents/L for 20 minutes. Toxicity due to damage of the cell membrane was determined with the extracellular LDHe assay that is based on the measurement of the oxidation of NADH to NAD. Alterations of mitochondrial functions were studied in XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) assays that measure succinate dehydrogenase activity of viable cells. The SRB test was used to monitor total protein synthesis as a marker of cell proliferation. SRB binds to cellular proteins and can be quantified after solubilization. The neutral red assay was conducted to evaluate membrane integrity and lysosomal activity of the cells. All experiments are based on spectrophotometric measurements and were evaluated with an automated microplate reader. In all experiments, three measurements were performed per dose and repeated at least once.

Single-cell gel electrophoresis (SCGE) assay - SCGE assays were performed to determine the effect of glyphosate and Roundup on DNA stability. The concentrations of glyphosate and Roundup used in this assay ranged from 0 to 2000 mg glyphosate equivalents/L. TR146 cells were seeded in 24-well plates and allowed to attach. Thereafter, the culture medium was replaced by 400 µL of different concentrations of glyphosate dissolved in serum-free medium. After incubation in the dark at 37ºC and 5% CO_2 for 20 minutes, the medium was discarded and the cells washed twice with DPBS and detached. After two washing steps with DMEM and centrifugation, the pellets were resuspended in low melting point agarose, spread onto slides precoated with normal melting point agarose and lysed in the dark at 4ºC for at least 1 hour. After 20 minutes unwinding in an alkaline electrophoresis solution electrophoresis was carried out. Air-dried slides were stained with ethidium bromide and the percentage of tail DNA was measured using a computer-aided image analysis system (Comet IV, Perceptive Instruments Ltd., Haverhill, UK). For each experimental point, 3 cultures were made in parallel and from each culture, one slide was prepared and 50 randomly distributed cells evaluated.

Cytokinesis-block micronucleus (CBMN) cytome assay - The CBMN assay using the cytochalasin B technique was performed to determine the effect of glyphosate and Roundup on chromosome integrity. The concentrations of glyphosate and Roundup used in this assay ranged from 0 to 20 mg glyphosate eq./L. Into 6-well plates, 4.5 x 10^5 cells were seeded and allowed to attach overnight. After treatment for 20 minutes with the test compounds or 100 µg/mL methyl methanesulfonate as the positive control, the cells were washed twice with PBS and cultured in DMEM containing 10% FCS and cytochalasin B for 48 hours. Subsequently, the cells were washed twice with DPBS and harvested. The slides were made by cytocentrifugation and subsequently air-dried, fixed, and stained with Diff Quick stain. The total number of micronuclei (MNi) in binucleated cells (BN) as well as the number of binucleated cells with micronuclei (BN-MNi), nuclear buds (NB), nucleoplasmic bridges (NPB), and apoptotic and necrotic cells was determined. Cells that divided after addition of cytochalasin B were recognized as binucleated. The nuclear division index (NDI) was determined in 500 cells. For each experimental point, TR146 cultures were prepared in triplicate. From each culture, >1,000 binucleated cells were evaluated under 400-fold magnification (Nikon Photophot-FXA, Tokyo, Japan).

Statistical analysis - Data analyses of the cytotoxicity and SCGE assays were performed with the GraphPad Prism 5 Project software system. Results are reported as means ± standard deviations (SD). The results were analyzed using one-way ANOVA and Dunnett's test and p values ≤0.05 were considered as statistically significant. The chi-square test with Yate's correction was used for the evaluation of the CBMN experiments and p values ≤0.05 were considered statistically significant.

Results

Cytotoxicity - Clear differences have been found between the effects of glyphosate and Roundup. Roundup was in all cases more cytotoxic than the active ingredient. In the LDHe test Roundup NADH consumption was statistically significantly increased from 10 mg glyphosate eq./L on whereas this was the case with glyphosate from 80 mg/L. In the XTT test, a statistically significant decrease in cellular integrity was seen with Roundup from concentrations of 40 mg glyphosate eq./L on while there was no effect with glyphosate up to 200 mg/L. In the SRB test there was a statistically significant decrease in cellular integrity from 100 mg glyphosate eq./L on whereas there was no effect with glyphosate up to 200 mg/L. The same difference in cytotoxicity was evident in the NR assay where Roundup produced a statistically significant decrease in cell integrity from 100 mg glyphosate eq./L on whereas there was no effect with glyphosate. The LC_{50} values for the cytotoxicity of Roundup were about 100 mg glyphosate eq./L for the XXT test, 140 mg/L for the NR assay and 150 mg/L for the SRB assay.

SCGE assay - In the SCGE assay, glyphosate produced at statistically significant increase in tail intensity from 20 mg/L on without a dose-effect relationship from 40 to 2,000 mg/L. A statistically significant and dose dependent increase in tail intensity was observed with Roundup from 20 mg glyphosate eq./L up to 200 mg glyphosate eq./L, a dose level with 0% cell integrity.

CBMN assay - The endpoints recorded in this assay were the frequency of binucleated (BN) cells with micronuclei (MNi), the total number of MNi, nuclear buds (NB) and nucleoplasmic bridges (NPB). After addition of cytochalasin B for 48 h, more than 75% of the cells were binucleated (BN) and the nuclear division index (NDI) in untreated cultures was 1.89 ± 0.09. Treatment of the cells with 100 µg/mL of the positive control MMS for 20 minutes produced a significant induction of BN cells with MNi, total number of MNi, NB and NPB. Exposure of the cells to glyphosate and Roundup at 10, 15 and 20 mg glyphosate eq./L for 20 minutes led to a statistically significant and dose-dependent increase of the frequency of MN, BN-MN, NB and NPB. The number of necrotic cells was statistically significantly increased for glyphosate and Roundup at 20 mg/L. The number of apoptotic cells was statistically significantly increased with glyphosate at 20 mg/L but not with Roundup.

Discussion

The results show that Roundup but not glyphosate causes pronounced cytotoxic effects in human-derived buccal epithelial cells. The genotoxicity tests show that glyphosate as well as its formulation induce DNA strand breaks as well as nuclear anomalies that reflect DNA instability including chromosomal damage. Glyphosate did not induce effects in the NR, SRB, and in the XTT assay up to concentrations of 200 mg/L, while a clear effect was seen in the LHDe assay from 80 mg/L on. On the contrary, statistically significant cytotoxic effects were observed with Roundup in all four assays, and significant changes were seen in the LDHe assay from 10 mg glyphosate eq./L and in the XTT test from 40 mg glyphosate eq./L on. Comparisons of the sensitivity of the different toxicity tests indicate that Roundup causes membrane damage at lower concentrations than the inhibition of mitochondrial functions in embryonic and placental-derived cells (JEG3), while umbilical vein cord endothelial cells (HUVEC) were equally sensitive to both endpoints. High concentrations of glyphosate affected in these experiments primarily mitochondrial functions while no effects were seen in an assay detecting changes of cell membrane functions.

The results of the SCGE assays show that glyphosate as well as Roundup induce comet formation under alkaline conditions. The effects increased as a function of the exposure and DNA migration was also observed under conditions that did not affect the cellular integrity. Also in earlier investigations with human-derived cells, SCGE assays were used to monitor the DNA-damaging effects of glyphosate. Significant induction of DNA migration was seen in human hepatoma cells (HepG2) with Roundup 400 with glyphosate levels of more than 5 mg/L after 24 hours of treatment. Other cell lines such as normal human fibroblasts (GM38) and the human fibrosarcoma line HT1080 were less sensitive in long-term (72 h) exposure studies. Taken together, these effects show that distinct differences exist in the sensitivity of cells from different organs and indicate that drug-metabolizing enzymes, which are represented in the enzyme activation mix and in HepG2 cells, increase the DNA-damaging properties of glyphosate. The most relevant finding of the present study is the observation of a statistically significant, dose-dependent induction of MNi, BN-MNi, and NB reflecting the genomic damage by glyphosate and its formulation. The most sensitive endpoint was MNi induction. Treatment of the cells with highest level (20 mg glyphosate eq./L) of Roundup caused a 3-fold increase over the background. A weaker effect was seen with the corresponding concentration of glyphosate. NPB was a less responsive endpoint with a statistically significant increase with glyphosate and Roundup at all dose levels. These results indicate that the damage seen in the SCGE assays is not completely repaired but leads to persisting alterations of the genetic material. MNi reflect numerical as well as structural chromosomal aberrations, while NB are formed as a consequence of gene amplification or expulsion of intact chromosomes or fragments; NPB are caused by formation of dicentric chromosomes.

The findings of the present study suggest that buccal epithelial cells are more sensitive toward the cytotoxic and DNA-damaging effects of glyphosate and Roundup than cells from the hematopoietic system. The Roundup formulation that was tested contains 450 g/L of glyphosate and should be diluted according to the instructions of the manufacturer to 1–3% before use (final concentration 4,500–13,500 mg/L). The fact that we found significant acute and genotoxic effects at levels between 10 and 20 mg/L after 20 min indicates that short contact with a 225–1,350-fold dilution of the formulation may cause adverse effects in cells from the oral cavity (and possibly also in other respiratory epithelia).

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of the present study was to find out whether exposure of human-derived buccal epithelial TR146 cells to glyphosate and Roundup causes adverse effects. In cytotoxicity experiments, four different endpoints were used, which reflect different modes of action. To assess the effect of glyphosate and Roundup on DNA stability, single cell gel electrophoresis assays (SCGE) were conducted under standard alkaline conditions reflecting the formation of single- and double-strand breaks. Additionally cytokinesis-block MN cytome assays have been conducted in which different nuclear anomalies were measured. This study demonstrated that there is a big difference in cytotoxicity between glyphosate and Roundup. This is not surprising since the surfactants present in glyphosate formulations decrease the integrity of cell and mitochondrial membranes causing toxicity and ensuing DNA instability. Glyphosate was found to significantly increase tail intensity as of 20 mg/L but without any further increase with dose from 40 to 2000 mg/L. Roundup increased in a dose dependent manner the tail intensity from 20 mg glyphosate eq./L up to 200 mg glyphosate eq./L with increasing cytotoxicity and 0% cell integrity at 200 glyphosate eq. mg/L. This indicates that there is a relationship between the cytoxicity of Roundup and DNA instability. This study has demonstrated a greater sensitivity of buccal epithelial cells for glyphosate and its formulations than hematopoietic cells where no effects have been noted in *in vivo* MN tests with doses up to 2,000 mg/kg bw. Since there is no direct exposure of the buccal epithelium with the Roundup formulation (unless it is swallowed) during application and the inhalation of aerosol of the spray dilution during application is negligible (Jauhiainen A *et al*. (1991) Am. Ind. Hyg. Assoc. J. 52, 61–64) the likelihood of DNA damage in epithelial cells of the GI and the respiratory tract remains very low.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria of an *in vitro* toxicology study.

Reliability criteria for *in vitro* toxicology studies

Publication: Koller *et al.*, 2012.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95%. Source: Monsanto Europe S.A.
Only glyphosate acid or one of its salts is the tested substance	Y	Also formulation (Roundup Ultra Max) was tested
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	10-1000 µg/mL
Cytotoxicity tests reported	Y	

Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Roundup, but not glyphosate causes pronounced cytotoxic effects in human-derived buccal epithelial cells. The alkaline comet test results show that glyphosate as well as Roundup induce comet formation that reflect strand breaks and apurinic sites. 20 µg/mL glyphosate in Roundup caused a 3-fold increase over the background, with glyphosate alone a weaker effect was seen.
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria of an *in vitro* toxicology study.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Kongtip P. *et al.*
Report year	2017
Report title	Glyphosate and Paraquat in Maternal and Fetal Serums in Thai Women
Document No	Journal of Agromedicine 2017, vol. 22 no. 3, 282-289
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This longitudinal study measured the glyphosate concentrations found in maternal and umbilical cord serum in 82 pregnant women who gave birth in three provinces of Thailand. Through questionnaires and biological samples collected at childbirth, factors such as personal characteristics, family members occupation, agricultural activities, and herbicide use in agricultural work were evaluated as predictors of glyphosate levels in the pregnant women. Statistical analysis used univariate and binary multiple logistic regression, where the outcome was the probability of exposure to glyphosate above the limit of detection associated with occupation and household factors. The glyphosate concentrations in the pregnant women's serum at childbirth (median: 17.5, range: 0.2–189.1 ng/mL) were significantly higher ($P < .007$) than those in the umbilical cord serum (median: 0.2, range: 0.2–94.9 ng/mL). Women with glyphosate levels >limit of Detection (LOD) in serum at childbirth were 11.9 times more likely to report work as an agriculturist ($P < .001$), 3.7 times more likely to live near agricultural areas ($P = .006$), and 5.9 times more likely to have a family member who worked in agriculture ($P < .001$).

Materials and Methods

Subjects for a pilot birth cohort were recruited from pregnant women who came for prenatal care at three hospitals in Thailand: Amnatchareon Hospital in Amnatchareon Province in the northeast, Sawanpracharak Hospital in Nakhorn Sawan Province in the lower north, and Paholpolpayuhasena Hospital in Karnjanaburi Province in the west of Thailand, from May to December 2011. To be recruited, the women had to be in their 7th month of pregnancy, 19–35 years of age, not have diabetes or hypertension, and plan to give birth and have follow-up infant care at the recruiting hospital. The 82 subjects for this study included 81 full-term normal birth neonate and 1 full-term cesarean birth neonate.

During their 7th month of pregnancy, the women were interviewed about their general health, diet, and work exposures, including agricultural work, as well as about use of pesticides at home and work. Several questionnaires, each with several sections, constituted the data collection. The questionnaire was based on the type of information collected in previous studies of agricultural workers and modified for the conditions of agriculture and types of pesticide exposures experienced in Thailand. The questionnaires were reviewed by staff at each hospital, then piloted and revised based on comments. One questionnaire collected data on demographics and the mother's general health, whereas another collected birth data. The pesticide exposure questionnaire consisted of six sections; the first section contained 12 items related to pesticide use in the home or outside the home, as well as sources of drinking water. The second section contained 9 items about the woman's work history outside the home. The third section, with 10 items, covered agricultural activities if conducted by the woman. The fourth section, with 3 items, covered agricultural activities conducted by the woman during pregnancy. The fifth section, with 6 items, asked about the agricultural work of family members. The sixth section, with

30 items, collected detailed information only from those who were agricultural workers or had family members who were agricultural workers and included detailed information on the mixing and spraying of pesticides. To summarize, the pesticide exposure questionnaire had 40 items for all subjects and 30 items only for those who were self-identified as agricultural workers or who had family members who were agricultural workers. For comparisons with agriculturists, women with other occupations were used as the control group. The nurses at the prenatal clinics in the three study hospitals were trained to recruit and interview subjects. The maternal and umbilical cord serum was collected during delivery by the delivery nurses and was frozen at -45°C until analysis. This study was reviewed and approved by the Ethics Committee on Human Rights Related to Human Experimentation, Mahidol University, and the University of Massachusetts Lowell Institutional Review Board.

Glyphosate was obtained from Sigma-Aldrich Inc., Singapore. Acetonitrile, trimethylamine, methylene chloride, dichloromethane (all high-performance liquid chromatography [HPLC] grade), and sodium dodecyl sulfate (SDS) (gas chromatography [GC] grade) were purchased from Apex Chemical, Bangkok, Thailand. Other chemicals were of analytical grade. Analysis was performed with an HPLC system (Agilent 1200 Series) with a fluorescence detector for glyphosate. The serum sample was analyzed on Luna 5 μm C18 (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA) with guard column at 45°C. A calibration curve was developed using hospital serum samples of non-subjects tested for the non-presence of glyphosate and then spiked with glyphosate to yield final concentrations of 12.5, 25, 50, 100, 150, and 200 ng/mL (n = 3 replicates). Samples of glyphosate were prepared using 250-μL of serum and 30 μL of derivatized glyphosate and injected into the HPLC. Evaluation of the detection limit was performed following the National Institute for Occupational Safety and Health method. For determination of accuracy and precision, concentrations of hospital serum samples of non-subjects tested for the non-presence of pesticide were used to prepare concentrations of 50, 100, and 150 ng of glyphosate/mL. Three replicates of each concentration were analyzed on three separate days. The calibration curve for glyphosate was linear over the concentration range of 12.5–200 ng glyphosate/mL serum with the correlation coefficient of 0.998. The detection limit for the analysis of glyphosate in serum was0.4 ng/mL. Values below the detection limit for serum glyphosate were used as the detection limit divided by 2 since the data were highly skewed. The recovery of the method ranged from 94.33% to 99.03% with a relative standard deviation (RSD) of <3% for glyphosate concentrations of 50–150 ng/mL.

The descriptive statistics were calculated using SPSS (SPSS version 18; PASW Statistics Base 18, Bangkok, Thailand). Since exposures were highly skewed due to the large percentage of values below the limit of detection (LOD), concentrations were reported as the median and range. For comparison of the paired mother and cord blood serum concentrations, only pairs with both measurements above the LOD levels were used with the Wilcoxon signed-rank test. Binary logistic regression was used to evaluate whether various factors were significantly associated with the probability of having glyphosate concentrations over the LOD. Several a priori factors were evaluated for inclusion as potentially significant covariates, such as maternal location/province and educational level of the mother, but these were found not to be significantly associated with the probability of having glyphosate concentrations over the LOD. Thus, occupational factors were evaluated in single-factor models and reported as unadjusted odds ratios (ORs). In models examining secondary exposure factors, maternal occupation was included as a covariate in the model to control for the influence of maternal agricultural work on serum levels.

Results

The average age of the 82 women who gave birth during the study was 26 years old (range: 19–34 years), with most having completed secondary school (41%), although 26% only completed primary school. Of the 82 women, 39% of the women described their occupation as agriculturist/farmer, whereas 21% listed themselves as a housewife, and about 13% were employees or owned their own business (often these are small retail stands with food or other items for sale). Of the 82 pregnant women who gave birth during the study, cord blood was only collected from 75 newborns, all born full term (37–41 weeks).

The percentage of maternal samples of glyphosate that were at or below the LOD was 46.3%, whereas for cord serum 50.7% were ≤LOD. Comparison of the glyphosate concentrations in paired serum

samples of mother and cord blood that were both >LOD (n = 36) found that they were significantly different (P < .001), with the mother's serum levels the higher of the two. With regard to occupational factors predicting glyphosate exposures, the odds of having a detectable level of glyphosate in serum were 11.9 higher (95% confidence interval [CI] 3.6-39.5) than the LOD among women who worked in the fields compared with those who did not. Pregnant women who worked in agricultural fields during the first, second, or third trimester of pregnancy also had significantly elevated ORs of 13.5 (95% CI 2.8-64.3), 7.7 (95% CI 2.0-29.8), and 12.4 (95% CI 1.5-102.7), respectively, of having serum levels >LOD compared with those women who never worked in the fields. Likewise, pregnant women who reported picking crops during pregnancy had a significantly elevated OR (5.4; 95% CI 1.4-20.8)), whereas those who reported the agricultural activity of digging in farm soil or controlling weeds during pregnancy did not have elevated ORs.

With regard to secondary factors, pregnant women who lived near agricultural fields (<0.5 km) were significantly more likely to have serum glyphosate levels >LOD than those women living far from agricultural fields (OR=3.7, 95% CI 1.5-9.2). Note that there was no significant relationship between home location and agricultural occupation; thus, the adjusted OR did not reflect collinearity or confounding. Those pregnant women who reported a family member who was an agriculturist/farmer living in the same house were significantly more likely to have elevated serum glyphosate than those who did not (OR=5.9; 95% CI 2.2-16.1); however, when adjusted for occupation, the OR became non-significant because of the high correlation between having a family member who was a farmer in the same house and maternal occupation as a farmer.

The most striking finding of this study was that glyphosate concentrations over the LOD were significantly more likely to be found in the serum of pregnant women collected at childbirth if their occupation was an agriculturist/farmer or conducted various agricultural tasks or if they lived near agricultural fields sprayed with pesticides (unspecified), when compared with women who had no agricultural exposures. Researchers measured glyphosate levels in urine among farmers in the United States and found that farmers who had skin contact with pesticides or did not use rubber gloves had significantly higher glyphosate concentrations in their urine. Among those who did not wear rubber gloves, appreciable differences were found in the urinary glyphosate levels between those who repaired equipment during applications or spilled during mixing/loading or application. This supports the notion that dermal contact is an important route of exposure for this herbicide. Researchers also reported urinary glyphosate in one of the children who lived 1.5 km away from the farm where their father sprayed glyphosate in France. However, in a US study, very few of the spouses (4%) and children (12%) who lived in a home within 1 mile of the sprayed farm fields had measurable urinary glyphosate on the day of spraying, and an even lower percentage had measurable levels on days 1–3 post application. Among the applicators, 60% had measurable glyphosate on the spraying day, with a decrease to 27% on the third day post application. This raises the question of the half-life of glyphosate and how the Thai agricultural women giving birth had measurable levels of glyphosate in their serum. Although these women had regular, repeated exposures to glyphosate, even during their final trimester of pregnancy, it is unlikely that they were exposed in the field on the day prior to giving birth. It is possible that these serum levels were caused by the drift of glyphosate spray, skin contact with the pesticide contaminated family member working in the fields, contamination of the home through clothing (take home exposure), or through poor storage of pesticides near the home. Alternatively, little is known about the metabolism and storage of pesticides during pregnancy and the impact on pesticide half-life. The median (range) glyphosate concentrations of maternal and umbilical cord serums in this current study were 17.5 (0.2–189.1) and 0.2 (0.2–94.9) ng/mL, respectively. The maternal serums of glyphosate at birth were significantly higher than those in cord blood serums. It is not known why maternal serum levels were higher than cord serum samples. At this time, only one study has looked at serum levels of pesticides in pregnant and non-pregnant Canadian women who were not agriculturists or living with a spouse who worked with pesticides. They found no detectable levels of glyphosate in the pregnant women before birth or in their cord blood samples. However, 5% (2/39) of the non-pregnant women in that study had measurable glyphosate serum levels (including one with a level of 93.6 ng/mL). Potential exposures in that study were assumed to be through consumption of pesticides associated with genetically modified foods. In our study, we found measurable glyphosate levels (>LOD) in 14.7% of the maternal serum samples of women who were not agriculturists/farmers by occupation. This is concerning, because researchers studied the effect of glyphosate exposures on the risk of miscarriage among women living

on farms in Ontario, Canada, and found that women who were exposed to glyphosate before conception (from 3 months before up to conception) had a higher risk of spontaneous abortion.

Conclusion

This study suggests that agricultural activities do increase maternal serum levels of glyphosate even in samples taken on the day of birth. In the case of glyphosate, living near farmland where pesticides are sprayed can also significantly increase the risk of serum levels >LOD at birth. These results suggest that a study evaluating the long-term health of children exposed to herbicides during gestation should be considered. Given that herbicides make up the largest volume of pesticide imports in Thailand, and that imports continue to increase, further regulation of the sale and use of pesticides may help safeguard the health of Thai children.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study suggests that agricultural activities increase maternal serum levels of glyphosate, even in samples taken on the day of birth. Living near farmland where pesticides are sprayed can also significantly increase the risk of serum levels >LOD at birth. Limitations of this study include a small sample size (N=82) and large percentages of maternal samples cord serum that were at or below the LOD for glyphosate (46.3% and 50.7%, respectively).

This publication is considered relevant to the risk assessment of glyphosate but reliable with restrictions because the analytical method used for glyphosate could have been described in more detail.

Reliability criteria of exposure studies

Publication: Kongtip *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.	NA	Subjects of a pilot birth cohort being monitored for glyphosate and paraquat.
Exposure to formulations with glyphosate combined with other a.i.	NA	
Exposure to various formulations of pesticides	NA	
Study		
Study design clearly described	Y	
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	

Analytical method described in detail	Y	Analytical method for glyphosate could be described in more detail (derivatization).
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant to the risk assessment of glyphosate but reliable with restrictions because the analytical method used for glyphosate could have been described in more detail.		

1. Information on the study

Data point:	CA 5.3
Report author	Kumar S. *et al.*
Report year	2014
Report title	Glyphosate-rich air samples induce IL-33, TSLP and generate IL-13 dependent airway inflammation
Document No	Toxicology 325 (2014) 42-51
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of this study was to explore the mechanisms of glyphosate induced pulmonary pathology by utilizing murine models and real environmental samples. C57BL/6, TLR4–/–, and IL-13–/– mice inhaled extracts of glyphosate-rich air samples collected on farms during spraying of herbicides or inhaled different doses of glyphosate and ovalbumin. The cellular response, humoral response, and lung function of exposed mice were evaluated. Exposure to glyphosate-rich air samples as well as glyphosate alone to the lungs increased: eosinophil and neutrophil counts, mast cell degranulation, and production of IL-33, TSLP, IL-13, and IL-5. In contrast, in vivo systemic IL-4 production was not increased. Co-administration of ovalbumin with glyphosate did not substantially change the inflammatory immune response. However, IL-13-deficiency resulted in diminished inflammatory response but did not have a significant effect on airway resistance upon methacholine challenge after 7 or 21 days of glyphosate exposure. Glyphosate-rich farm air samples as well as glyphosate alone were found to induce pulmonary IL-13-dependent inflammation and promote Th2 type cytokines, but not IL-4 for glyphosate alone

Materials and methods

Mice

C57BL/6 female (6–9 weeks) mice were purchased from Jackson Laboratory (Sacramento, CA). TLR4–/– mice (backcrossed 10 generations) were received from Cincinnati Children's Hospital Medical Center (CCHMC). Both strains were subsequently bred in house. Female mice of wild type and IL-13–/– BALB/c background were received from the laboratory of Dr. Fred Finkelman, CCHMC. Mice were housed in individually ventilated cages in a pathogen free facility at the Department of Environmental Health, University of Cincinnati (UC) following the UC Institutional Animal Care and Use Committee (IACUC) guidelines and all experiments were conducted following a UC IACUC – approved protocol.

Antibodies and reagents

The following antibodies for flow cytometry were purchased: Ly-6G (Gr-1) eFluor1 450 (RB6-8C5; Isotype Rat IgG2b) from eBioscience (San Diego, CA). CD16/CD32 (2.4G2; Isotype Rat IgG2b) and SiglecF-PE (E50-2440; Isotype Rat IgG2a) were purchased from BD PharMingen (San Jose, CA). A kit for measuring serum levels of MMCP1 was purchased from eBioscience.

Collection of farm air samples during summer pesticide spray seasons

Air samples were collected by three sets of total inhalable aerosol samplers (Button Inhalable Aerosol Sampler, SKC Inc., Eighty Four, PA) operated in parallel on three farms in Butler County, Ohio during

summer glyphosate spray seasons. Samplers were installed at 1.5 m height at the edge of the field downwind from the spraying locations (sizes: approx. 5000 - 10,000 m^2). The sampling period was approximately 24 h starting from glyphosate spraying and air samples were collected at a flow rate of approximately 4 l/min on glass fiber filters. The filters from one set of samplers containing aerosolized glyphosate were eluted using PBS and the suspensions were filtered. A stock solution was prepared by pooling the samples collected from three farms (from now on referred as 'Real Env.') and used for intranasal treatment of mice. The filters from the other two sets of samplers were analyzed for glyphosate and endotoxin to estimate the levels of glyphosate and endotoxin in 'Real Env.' samples.

Analysis of glyphosate in filter extracts

Glyphosate residues from filters were extracted using KH2PO4 buffer/1 M NaOH in an automatic shaker followed by freeze-drying. The freeze-dried samples were dissolved with deionized water and filtered through 0.45 mM Millipore filter. Glyphosate levels in the suspensions were determined by Abraxis ELISA Kit at 450 nm. The average amount of glyphosate per filter was 17.33 µg, which correspond to average airborne concentration of 22.59 ng/m^3.

Analysis of endotoxin in filter extracts

Endotoxin in filter extracts were analyzed using the Limulus amebocyte lysate assay (Pyrochrome LAL; Associates of Cape Cod Inc., Falmouth, MA), as described previously. The samples were spiked with endotoxin standard of 0.50 EU/ml to assure that there was no inhibition or enhancement between the filter extracts and the reagents. The average amount of endotoxin per filter was 24.49 EU, which correspond to average airborne concentration of 4.87 EU/m^3.

Treatment of mice with farm-derived air samples, glyphosate and sensitization with OVA

PBS suspended farm air sample ('Real Env.'; estimated amount of glyphosate: 8.66 µg/mL) and reagent grade glyphosate (Sigma – Aldrich, St. Louis, MO) (100 ng, 1 µg or 100 µg) were delivered (in 30 µL) to the nose of anesthetized mice which were witnessed to aspirate the solution. Treatments were administered either: daily for 7 days or 3 times a week for 3 weeks. Same exposure schedule was followed for OVA alone (100 µg) and for OVA (100 µg) plus different dose of farm air sample and glyphosate. Mice were sacrificed 24 h after final airway treatment with sodium pentobarbital.

Histological analysis of lung

Formalin-fixed paraffin embedded lung sections (5 µm thick) were prepared for H&E and chloroacetate esterase (CAE) staining. The entire histological slide from each mouse was examined in blinded fashion and given a global categorical severity score based on infiltration of cells into parenchymal, peribronchial, and perivascular regions of lungs.

Immunohistochemical staining

To analyze IL-33 and TSLP expression in the lungs section, the following antibodies were used for immunostaining: mouse IL-33 (0.2 mg/mL, AF3626, R&D Systems, Minneapolis, MN); mouse TSLP biotinylated (0.2 mg/ml, BAF555, R&D Systems) and respective isotype controls (R&D Systems). IL-33 and TSLP antibody–antigen complex were detected using Cy3 donkey anti-goat IgG (1:10,000) (Invitrogen/Molecular probes, Grand Island, NY). Slides were counterstained with DAPI (Vector Labs, Burlingame, CA). Images were obtained using a Nikon A1R Si microscope.

Isolation of lung inflammatory cells

Lungs were perfused with PBS, removed, manually minced into 1–2 mm fragments and then placed in Hank's Balanced Salt Solution (Sigma–Aldrich) containing Liberase TL (50 µg/mL; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/mL; Sigma – Aldrich). Tissue was digested at 37°C in a CO_2

incubator for 30 min. The tissue suspension was then passed through a 40 μm cell strainer. ACK lysis buffer (invitrogen) was used to clear red blood cells.

Flow cytometric analysis

Single cell suspensions from lungs (10^6 per mL) were blocked with anti-mouse CD16/CD32 antibodies before cell-surface staining. Cells were stained with fluorescently-labeled antibodies against SiglecF, Ly-6G/C (Gr-1), in different combinations according to the experiment. Analysis was performed using a FACSCanto II cytometer and FACSDIVA software (BD Biosciences). Eosinophils were defined as being $SiglecF^+Gr\text{-}1^+$ and neutrophils as $SiglecF^-$ $Gr\text{-}1^+$.

Cytokine measurement

IL-4, IL-10, IL-13, and IFN-γ production were measured by the in vivo cytokine capture assay (IVCCA). Briefly, biotinylated cytokine-specific mAbs were injected via tail vein immediately before the last airway treatment, and blood was collected 24 h later; sera or plasma were analyzed with microtiter plates wells coated with corresponding anti-cytokine mAbs. Cytokine levels were also assessed in Bronchoalveolar lavage fluid (BALF) that was obtained 24 h after the last airway treatment. A kit for measuring in vivo IL-4 production by IVCCA, R46A2 and XMG1.2 anti-IFN-γ mAbs was purchased from Becton–Dickinson (Franklin Lakes, NJ); eBio1316H and eBio13A anti-IL-13 mAbs JES5-2A5 and JES5-16E3 anti-IL-10 mAbs ELISA Ready-SET-Go analysis kits for measurement IL-33 and IL-5 were purchased from eBioscience. Assays were performed according to the kit's manufacturer protocols.

Statistical analyses

Data were analyzed with Sigma Plot 12.0 (Systat Software, Inc., San Jose, CA). Statistically significant differences in means were determined by one-way ANOVA followed by Bonferroni multiple comparison tests. Kruskal–Wallis tests were conducted if the data did not have a normal distribution. All the data are presented as mean ± SD for each group. Probability values of <0.05 were considered significant.

Results

Exposure of air samples collected during glyphosate spray on farms stimulates airway inflammation

Wild type C57BL/6 (WT) and TLR4–/– mice were intranasally exposed to 'Real Env.' samples (PBS suspended farm air samples) daily for 7 days. 'Real Env.' exposure was found to substantially increase the cell count in both the lungs and BAL fluid of WT and TLR4–/– mice. Additionally, the increase in pulmonary infiltrate in lungs was found to be higher in TLR4–/– than in WT mice (Fig. 1A and B). Similarly, an increase in eosinophil and neutrophil levels in 'Real Env.' treated mice (Fig. 1C–F) were observed. This inflammation was also confirmed by histological examinations (Fig. 1G) and elevated IgG1 and IgG2a levels. Additional experiments were conducted using reagent grade glyphosate of different doses. Administration of reagent grade glyphosate to the airway of mice produced substantial pulmonary inflammation whether the daily dose given was 100 ng, 1 μg or 100 μg for 7 days.

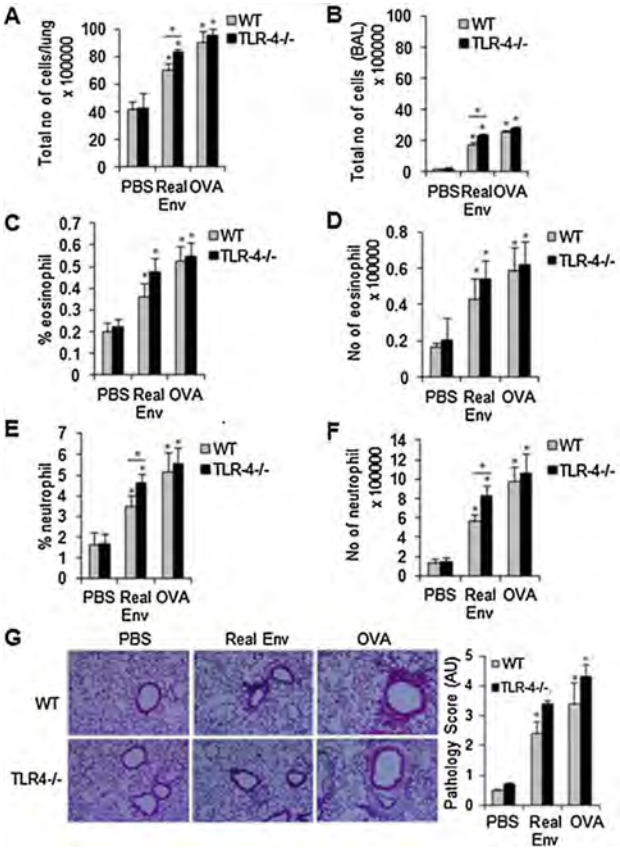


Figure 1: Increase in total number of cells, eosinophils, and neutrophils in lung and BAL fluids upon airway exposure to farm air samples ('Real Env.') and OVA for seven consecutive days (mean ± SD; *n* = 8). Increase in total number of cells in (A) lung and (B) BAL fluids. Increase in percentage (C) and total number (D) of eosinophils and neutrophils (E, F) per lung upon exposure to farm air samples ('Real Env.'). (G) Representative lung sections (H&E staining) and its pathology score from mice treated with PBS, farm air samples ('Real Env.') and OVA intranasally for 7 consecutive days (mean ± SD; *n* = 8); magnification 200x. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4–/– mice group.

In the BALF and lung digests, a significant increase in the total cell count when treated with glyphosate at 1 µg or 100 µg (Fig. 2A and D) was found. Eosinophils (Fig. 2B and C), neutrophils, (Fig. 2E and F), and IgG1 and IgG2a levels) were also increased in glyphosate-treated mice compared to controls. However, No significant changes in the total cell count, eosinophils and neutrophils, IgG1 and IgG2a at glyphosate dose of 100 ng were found. Inflammation was confirmed by histological examination (Fig. 2M). Mice treated with both reagent grade glyphosate and OVA demonstrated significantly higher cell count (Fig. 2G and J), eosinophils (Fig. 2H and K), neutrophils (Fig. 2I and L), IgG1, and IgG2a compared to PBS treated mice.

Because pulmonary mastocytosis is typically observed in protein-allergen-induced experimental asthma, the pulmonary mast cell burden in the mice were assessed.

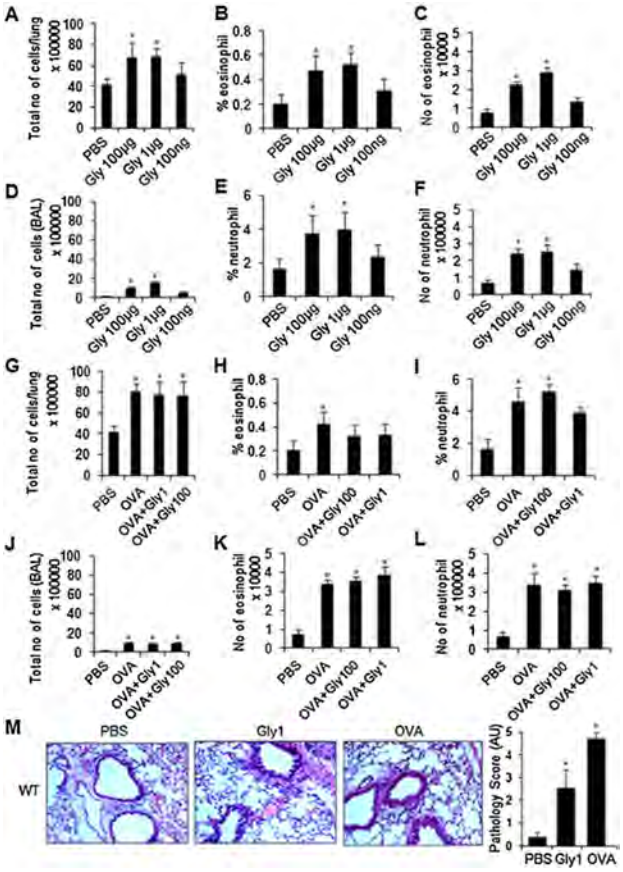


Figure 2: Increase in total number of cells, eosinophils, and neutrophils in lung and BAL fluids of WT mice upon airway exposure to glyphosate and combinations of glyphosate and OVA for seven consecutive days (mean ± SD; $n = 8$). Increase in total number of cells in (A) lungs and (D) BAL fluids upon exposure to different doses of glyphosate (100 ng, 1 µg or 100 µg). Increase in percentage (B) and total number (C) of eosinophils and neutrophils (E and F) per lung upon exposure to two doses of glyphosate. Increase in total number of cells in (G) lungs and (J) BAL fluids upon exposure to combination of glyphosate (1 µg or 100 µg) with OVA (100 µg). Increase in percentage and total number of (H and K) eosinophils and (I and L) neutrophils per lung upon exposure to OVA and combination of glyphosate, respectively. (M) Representative lung sections (H&E staining) and its pathology score from WT mice treated with PBS, glyphosate (1 µg) and OVA (100 µg) intranasally for 7 consecutive days (mean ± SD; $n = 8$); magnification 200 x. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS and treated WT mice group

A significant increase in mast cell number in lungs treated with the substances isolated from the air on active farms ('Real Env.') and reagent grade glyphosate (Fig. 3A and C) was not observed. However, the MCPT-1 levels were found to be substantially higher in both groups indicating increased mast cell degranulation in the treated mice (Fig. 3B and D).

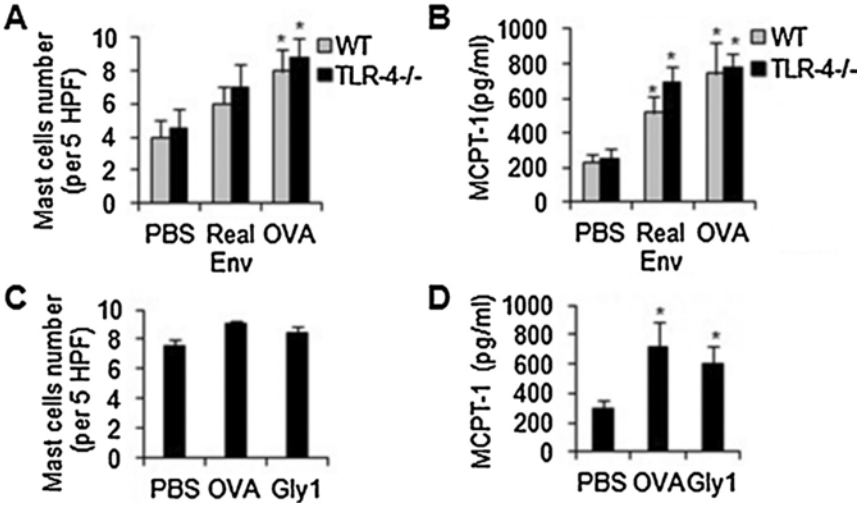


Figure 3: Farm air samples containing glyphosate as well as pure glyphosate alone induce increased mast cell degranulation but no increase in lung mast cell numbers upon airway exposure. (A) Mast cells number in CAE stained lung section and (B) serum MCPT-1 concentration in blood 4 h after last exposure of PBS, farm air samples ('Real Env.'), and ovalbumin (OVA). (C) Mast cells number in CAE stained lung section and (D) serum MCPT-1 concentration from mice treated with PBS, ovalbumin and 1 μg of glyphosate delivered to intranasally for 7 consecutive days (mean ± SD; n = 8). * Indicates statistically significant differences ($p < 0.05$) with respect to PBS and treated mice group.

Glyphosate-rich farm air samples induced airway inflammation and higher production of IL-10, IL-13, IL-5, IFN-γ and IL-4 but glyphosate alone failed to produce IL-4

To evaluate the glyphosate-induced inflammation, the systemic cytokine profile (Fig. 4A–E) of 'Real Env.' and glyphosate exposed mice were measured using IVCCA (Finkelman and Morris, 1999). Significantly higher levels of IL-5, IL-10, IL-13, and IL-4 were found upon treatment with 'Real Env.' alone in WT and TLR4–/– mice (Fig. 4A–D) approaching the levels induced by treating with OVA alone. The production of IL-5, IL-13 and IL-10 following 'Real Env.' exposures was higher in TLR4–/– than in WT mice. No significant difference in IL-4 production between TLR4–/– and WT mice were found (Fig. 4D). Production of these cytokines in mice given two different doses of glyphosate were then tested and found significantly higher levels of IL-5, IL-10, IL-13 and IFN-γ (Fig. 4F) that approached those levels induced by treating with OVA alone. Notably, there was no additional or synergistic effect when OVA was co-administered with glyphosate (Fig. 4G). Another interesting finding is that glyphosate alone was unable to induce significant levels of IL-4 while airway treatment with glyphosate with OVA did so.

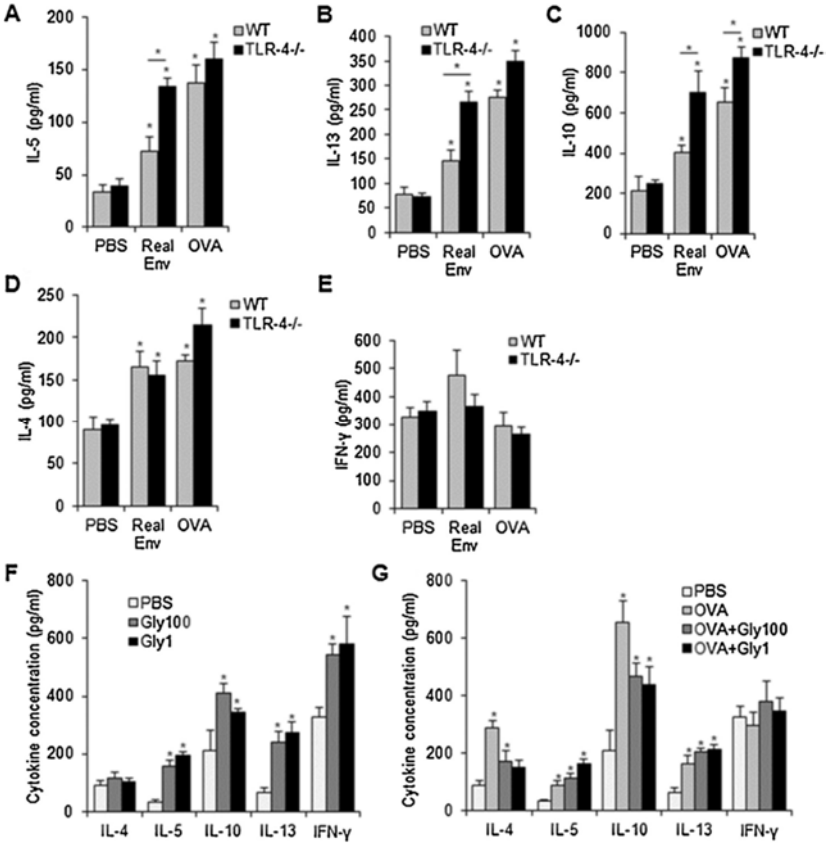


Figure 4: (A–E) Higher production of IL-5, IL-13, IL-10, IL-4 and no change in the IFN-γ levels upon exposure to farm air samples in WT and TLR4–/–mice. (F) The increased level of IL-5, IL-10, IL-13, IFN-γ and no change in the IL-4 levels upon glyphosate (1 or 100 µg) exposure to WT mice. (G) The increased level of IL-4, IL-5, IL-10, IL-13, and no change in IFN-γ levels upon combination of glyphosate (1 or 100 µg) and ovalbumin (100 µg) exposure to WT mice (mean ± SD; $n = 8$). Levels of cytokines were evaluated by IVCCA in serum of mice upon 7 consecutive days of intranasal treatment with farm air samples ('Real Env.') and glyphosate. Blood samples were collected 24 h after the last exposure. IL-5 was measured in the BAL fluids. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4–/– mice group.

IL-33 and TSLP in lungs are increased upon exposure to glyphosate-rich air samples as well as reagent grade glyphosate alone

As the cytokine profile of mice treated with 'Real Env.' and glyphosate approximated those treated with OVA, mediators known to promote type 2 pathology were examined. IL-33 and TSLP appeared to be logical choices because of their well-recognized effector functions, and due to their source – the respiratory epithelium cells which would be the first cells to encounter inhaled glyphosate. The IL-33 and TSLP content of BALF were measured directly and found an abundance of both cytokines in 'Real Env.' – treated WT and TLR4–/– mice (Fig. 5A and B). IL-33 production was observed to be significantly higher in TLR4–/– mice compared to WT mice. An abundance of both cytokines in glyphosate-treated mice were observed (Fig. 5C and D). This finding was confirmed by immunohistochemical staining of IL-33 and TSLP in lung sections of glyphosate-treated mice (Fig. 5E) and 'Real Env.' – treated WT and TLR4–/– mice, which demonstrated substantial production of both cytokines, which was limited to the respiratory epithelium after glyphosate exposure.

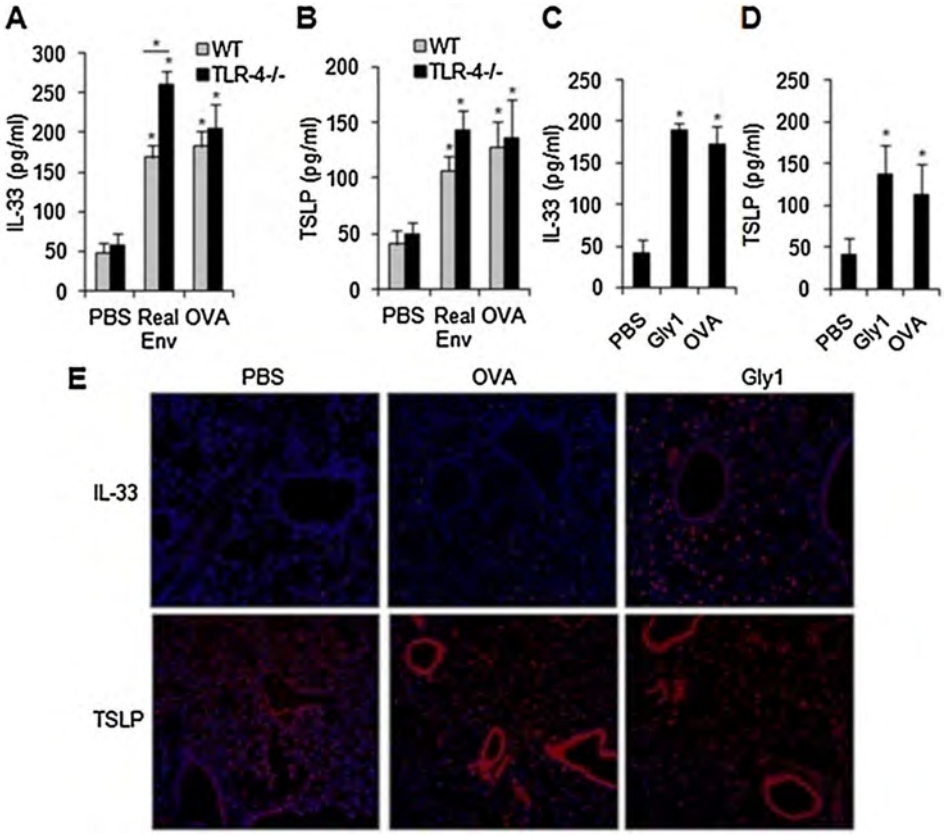


Figure 5: IL-33 and TSLP productions increased in the lung upon exposure to farm air samples and glyphosate. (A and B) ELISA based measurement of IL-33 and TSLP in BAL fluids of PBS, farm air samples and ovalbumin (100 µg) treated WT and TLR4–/– mice, respectively (mean ± SD; *n* = 8). (C and D) ELISA based measurement of IL-33 and TSLP in BAL fluids of PBS, OVA and pure glyphosate (1 µg) treated WT mice, respectively (mean ± SD; *n*=8). (E) Immunofluorescence staining of IL-33 and TSLP in the lung sections of the glyphosate treated WT mice, magnification 200x. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4–/– mice group.

Glyphosate-induced pulmonary inflammation is attenuated in IL-13 –/– mice

Glyphosate as a small molecule may not be efficiently presented to conventional T cells by antigen-presenting cells. The involvement of innate pathways upon glyphosate exposure, as hypothesized, was supported by the absence of an increased production of IL-4. This absence would have been expected if type 2 innate lymphoid cells (ILC2s) were the primary source of the IL-5 and IL-13 detected. IL-33 and TSLP have been well described to induce ILC2s, which in turn causes lung pathology particularly via IL-13-dependent mechanism. To test this hypothesis, IL-13 deficient mice were exposed to glyphosate for 7 and 21 days and assessed lung inflammation. While there was no change in IL-4 levels, the inability to produce IL-13 prevented the rise in IL-5 production, but not the rise in IL-10 production, at both time points during glyphosate treatment. Deficiency in IL-13 also prevented a significant rise in IL-33 and TSLP levels at the early time point but not the latter one (Fig. 6A–D). Lack of IL-13 production was also associated with significantly less ($p < 0.05$) severe cellular infiltration noted on histology (Fig. 6E). Despite significant inflammation, no airway hyper responsiveness was found in glyphosate-treated wild type and IL-13–/– mice.

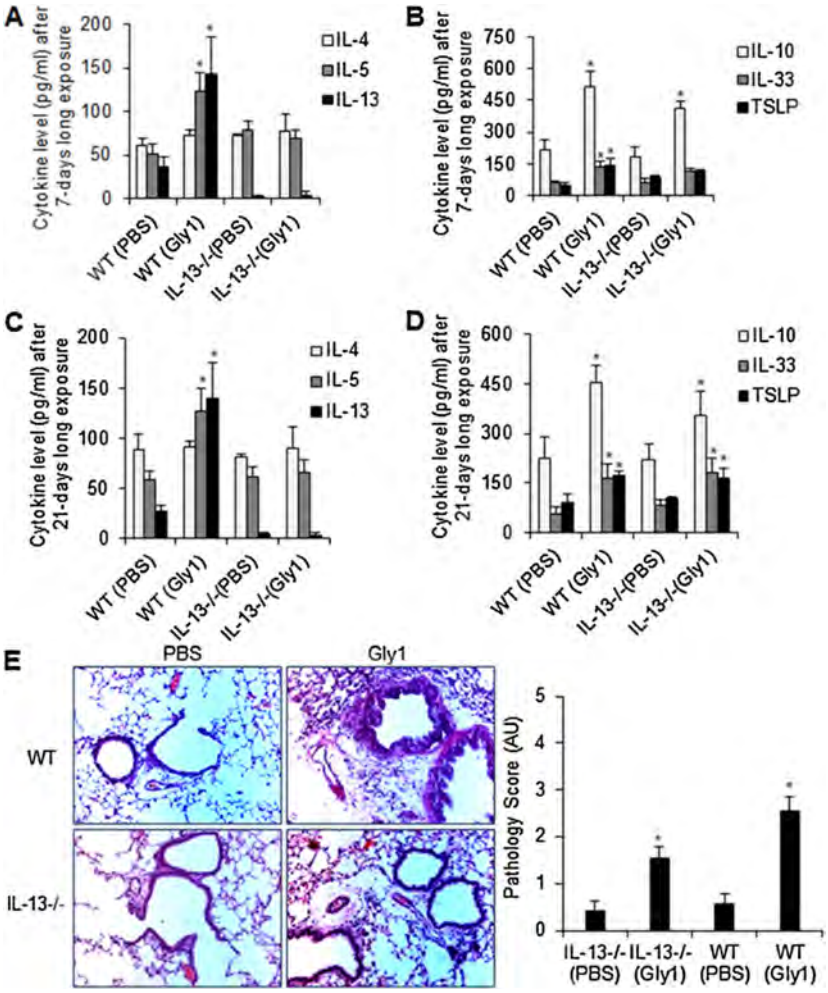


Figure 6: IL-13-deficient mice demonstrated diminished inflammatory response upon glyphosate exposure. (A, C) Diminished production of IL-5 but no change in IL-4 level, and (B, D) diminished production of TSLP, IL-33, IL-10 levels, between IL-13-deficient mice and WT mice upon glyphosate exposure (1 µg) for 7 or 21 days, respectively (mean ± SD; *n*=8). (E) Representative lung sections (H&E staining) from mice treated with PBS and glyphosate (1 µg) intranasally 3 times a week for 21 days; magnification 200 x (left panel). Arbitrary scores were based on inflammatory cells infiltration in lungs parenchyma, peribronchial, and perivascular regions. Analysis was performed in a double-blinded manner (right panel). * Indicates statistically significant differences (p <0.05) with respect to PBS treated control group.

Conclusion

The results demonstrate the capacity of glyphosate-rich air samples from farms as well as pure glyphosate to induce type 2 airway inflammation, over both short and longer time courses. Furthermore, glyphosate induced inflammation was found to be associated with induction of IL-33 and TSLP. This work also highlights the production of IL-13 as well as modulation of innate immune system by glyphosate, which may play an important role in exacerbation of airway inflammation by this low molecular weight chemical.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study evaluated nose-only exposure to glyphosate and collected farm samples containing glyphosate and evaluated the immune response in the lungs. This is not a guideline study nor an endpoint used in risk assessment. This study in not usable for risk assessment in terms of hazard assessment. In terms of exposure, the study determined that average amount of glyphosate per filter

from environmental samples after spray application to fields was 17.33 µg, which correspond to average airborne concentration of 22.59 ng/m^3. The method for the collection and analysis of the air samples was not validated and the assumptions and calculations used in the determination of the average airborne concentration were not provided, therefore the results cannot be verified. While the study itself is acceptable, it is unreliable in terms of usable endpoints for risk assessment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the method for the collection and analysis of the air samples was not validated.

Reliability criteria for *in vivo* toxicology studies

Publication: Kumar *et al*., 2014.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity is reagent grade. Source: Sigma - Aldrich, St. Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Sampling of aerosols from field spraying also contain co-formulants of the GBH applied.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	Female mice of wild type and IL-13-/- BALB/c background.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Intranasal application of air samples taken during glyphosate field application (24 hours) and glyphosate.
Dose levels reported	Y	Field air sample and 100 ng, 1 µg or 100 µg of glyphosate delivered intranasally.
Number of animals used per dose level reported	Y	
Method of analysis described for analysis test media	Y	Glyphosate measured in air sample with ELISA kit.
Positive control	Y	Ovalbumin.
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	

Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	For glyphosate.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the method for the collection and analysis of the air samples was not validated.		

1. Information on the study

Data point:	CA 5.4
Report author	Kwiatkowska M. *et al.*
Report year	2017
Report title	DNA damage and methylation induced by glyphosate in human peripheral blood mononuclear cells (in vitro study)
Document No	Food and Chemical Toxicology (2017) Vol. 105, 93-98
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The purpose of this study was to assess DNA damage (determination of single and double strand-breaks by the comet assay) as well as to evaluate DNA methylation (global DNA methylation and methylation of p16 (CDKN2A) and p53 (TP53) promoter regions) in human peripheral blood mononuclear cells (PBMCs) exposed to glyphosate. PBMCs were incubated with the compound studied at concentrations ranging from 0.1 to 10 mM for 24 h. The study has shown that glyphosate induced DNA lesions, which were effectively repaired. However, PBMCs were unable to repair completely DNA damage induced by glyphosate. A decrease in global DNA methylation level at 0.25 mM of glyphosate was also observed. Glyphosate at 0.25 mM and 0.5 mM increased p53 promoter methylation, while it did not induce statistically significant changes in methylation of p16 promoter. To sum up, it was shown for the first time that glyphosate (at high concentrations from 0.5 to 10 mM) may induce DNA damage in leucocytes such as PBMCs and cause DNA methylation in human cells.

Materials and methods

Chemicals – Glyphosate (95% purity) commercially obtained from Sigma-Aldrich, USA.

Isolation of PBMCs - PBMCs were isolated from leucocyte-buffy coat from blood collected from 9 healthy volunteers (aged 18-55 years) with no symptoms of infectious disease. For each parameter, 3 leucocyte-buffy coats were taken from 3 blood donors. After dilution with PBS (1:4) PBMCs were isolated from the buffy coat by centrifugation using lymphocyte separation medium (LSM). The PBMCs were collected, suspended in erythrocyte lysis buffer and incubated. Afterwards PBS was added and the cells centrifuged. The supernatant was decanted and the cells were washed twice with RPMI with L-glutamine and 10% fetal bovine serum (FBS). The cells were resuspended in RPMI medium with L-glutamine, 10% FBS and penicillin-streptomycin (0.5%) and counted in the hemocytometer. The final PBMCs density used in the experiments after addition of glyphosate was $1x10^6$ cells/mL. After incubation, PBMCs were diluted to a density of 5×10^4 cells/mL for the comet assay and condensed to a density of 5×10^6 cells/mL for the conduct of epigenetic methods. The viability of the cells was over 94%.

Treatment of PBMCs - Glyphosate was dissolved in PBS and the final concentrations of glyphosate in the comet assay were in the range of 0.25 to 10 mM, while epigenetic changes were assessed after exposure to glyphosate at 0.25 and 0.5 mM. For the determination of DNA damage the cells were incubated with glyphosate for 24 hours and repair of the DNA lesions was assessed after a recovery period of 120 minutes following incubation. In the comet assay, 0.25 mM was chosen as the lowest concentration of glyphosate which produced any statistically significant changes in DNA damage. Epigenetic changes were assessed at 0.25 mM which did not induce DNA damage and at 0.5 mM which induced statistically significant DNA damage. For the assessment of cell viability PBMCs were incubated for 24 hours with different concentrations of glyphosate. Afterwards, the cells were

centrifuged and the test solution discarded. The cells were then resuspended in RPMI medium and incubated for an additional 2 hours. Cell viability was 90.0%, 90.2%, 90.4%, 88.5%, and 87.8% for control, 0.5, 5, 7.5 and 10 mM of glyphosate, respectively. Each DNA damage experiment included hydrogen peroxide as the positive control.

Alkaline comet assay - The comet assay was performed under alkaline conditions with some modifications. A freshly prepared cell suspension in 0.75% low melting point agarose dissolved in PBS was layered onto microscope slides pre-coated with 0.5% normal melting point agarose. The cells were lysed for 1 hour at 4 ^{o}C in an alkaline buffer and DNA was allowed to unwind for 20 minutes in an alkaline solution. Electrophoretic separation was performed in an alkaline solution at 4 ^{o}C for 20 minutes at an electric field strength of 0.73 V/cm (28 mA). Then, the slides were washed in water and stained with 2 mg/mL 4',6-diamidino-2-phenylindole (DAPI) and covered with cover slips. The comets were observed at 200 x magnification in an Eclipse fluorescence microscope. Fifty images (comets) were randomly selected from each sample and the mean value of DNA in the comet tail was taken as an index of DNA damage (expressed in percent). For one blood donor, 3 parallel tests with aliquots of the sample of the cells were performed for a total number of 150 comets. A total number of 450 comets from 3 blood donors was recorded to calculate mean ± SEM.

DNA repair - The control samples and the PBMCs treated with glyphosate at 0.5, 5, 7.5 and 10 mM were washed and resuspended in fresh RPMI 1640 medium with L-glutamine preheated to 37 ^{0}C. Aliquots of the suspension were taken immediately after incubation at time "zero" and 120 minutes later. The samples were placed in an ice bath to stop DNA repair. The repair was assessed as a decrease in the extent of DNA damage measured after 120 minutes of post-incubation using the alkaline version of the comet assay.

Methylation of p16 (CDKN2A) and p53 (TP53) promoter regions - Chemical modification of 500 ng of genomic DNA was performed with the Cells-to-CpG™ Bisulfite Conversion Kit. For methylation analysis, a quantitative methylation-specific real-time PCR assay (qMSP) was conducted in 3 independent experiments including 3 blood donors with FastStart SYBR Green Master. All samples were amplified in triplicate. To determine the methylation status of a particular gene expressed as the methylation index (MI) in percentage, the Ct values of the methylated gene of interest were compared with the Ct values of the unmethylated gene of interest.

Global DNA methylation - Global DNA methylation was determined by means of DNA quantification using 5-methyl cytosine (5-mC) monoclonal antibodies in an ELISA-like reaction using the Methylflash Methylated DNA Quantification Kit. DNA (100 ng) isolated from whole blood PBMCs was used for analysis. Each sample was analyzed in duplicate and the determination was repeated whenever there was a failure in detection. The calculation of the amount of 5-mC was done with the use of a standard curve. Methylation levels were calculated relative to the methylated control DNA and expressed as a percentage of methylated DNA.

Statistical analysis - The mean value was calculated for three independent experiments (3 blood donors), whereas for each individual an experimental point was a mean value of at least 2 (methylation analysis) or 3 replications. Statistical analysis was conducted using the Mann-Whitney test, the Student's t-test and one-way analysis of variance (ANOVA) with a post hoc multiple comparisons procedure. The differences were considered to be statistically significant when $p<0.05$.Data analysis was performed using STATISTICA software.

Results

Analysis of DNA strand-breaks and DNA repair - DNA damage (single and double strand-breaks and alkali-labile sites formation) was statistically significantly increased from a glyphosate concentration of 0.5 mM on. At 10 mM the statistically significant increase in DNA damage exceeded 13 times the control value. After 120 minutes of recovery significant repair was observed of the DNA lesions induced by glyphosate (5.61% vs 2.93%, 10.51% vs 5.07%, , 14.91% vs 7.76%, and 27.49% vs 11.05% at 0.5 mM, 5 mM, 7.5 mM, and 10 mM of glyphosate, respectively.

DNA methylation status - As compared to control cells, the percentage of the global DNA methylation level was statistically significantly decreased by glyphosate at 0.25 mM, but not at 0.5 mM. On the contrary, p53 promoter methylation was statistically significantly increased at both concentrations of glyphosate. Methylation of the p16 gene promotor was increased after treatment with glyphosate but this change was not statistically significant.

Discussion and Conclusions

This study revealed that glyphosate decreased global DNA methylation in PBMCs statistically significantly at a concentration of 0.25 mM. Surprisingly, the effect of glyphosate at 0.5 mM on global DNA methylation was not statistically significant although the mean value from three independent experiments was still decreased as compared to control. Along with the decreased global DNA methylation, a statistically significant increase in methylation of the p53 promoter region was observed at 0.25 and 0.5 mM. Altered p53 promoter hypermethylation is an epigenetic pattern frequently observed in human cancers. Thus, the results of this study suggest that glyphosate at high concentrations (≥ 0.25 mM) may cause a down-regulation of p53 gene expression and activate proto-oncogenes or retrotransposable sequences which may induce genomic alterations by insertion and/or homologous recombination. This study showed for the first time that glyphosate may induce DNA damage in human leucocytes and cause epigenetic alterations in animal cells.

3. Assessment and conclusion

Assessment and conclusion by applicant:
It was the objective of this study to investigate the effect of high glyphosate concentrations on DNA integrity and DNA methylation in PBMCs *in vitro*. It was demonstrated that glyphosate increased statistically significantly DNA damage (single and double strand-breaks and alkali-labile sites formation) from 0.5 mM up to 10 mM. Repair of the DNA lesions was significant at all concentrations tested after 120 minutes of recovery. The percentage of the global DNA methylation level was statistically significantly decreased by glyphosate at 0.25 mM but not at 0.5 mM. On the contrary, p53 promoter region methylation was statistically significantly increased as compared to control cells at 0.25 and 0.5 mM.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the lowest concentration at which DNA damage was observed (0.5 mM) is higher than the blood concentrations in rats (0.3 mM) obtained after dosing at the limit dose of 2000 mg/kg bw where no MN effects were seen.

Reliability criteria for *in vitro* toxicology studies

Publication: Kwiatkowska *et al.,* 2017.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95%. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	Salt not mentioned
AMPA is the tested substance	N	
Study		

Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described		
Test concentrations in physiologically acceptable range (< 1 mM)	Y/N	0.25 to 10 mM
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	For DNA damage. The concentration at which DNA damage was observed is higher than the blood concentrations in rats obtained after dosing at the limit dose of 2000 mg/kg bw for the detection of MN in vivo. The results obtained are not corroborated by regulatory in vivo genotoxicity studies.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the lowest concentration at which DNA damage was observed (0.5 mM) is higher than the blood concentrations in rats (0.3 mM) obtained after dosing at the limit dose of 2000 mg/kg bw where no MN effects were seen.		

1. Information on the study

Data point:	CA 5.8.1
Report author	Kwiatkowska M. *et al.*
Report year	2020
Report title	Evaluation of apoptotic potential of glyphosate metabolites and impurities in human peripheral blood mononuclear cells (*in vitro* study)
Document No	Food and chemical toxicology (2020) Vol. 135, pp. 110888
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (Research conducted in an academic laboratory)
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The study aimed to assess the effect of glyphosate, its metabolites: aminomethylphosphonic acid (AMPA), methylphosphonic acid and its impurities: PMIDA, N-methylglyphosate, hydroxymethylphosphonic acid and bis (phosphonomethyl)amine on apoptosis induction in human peripheral blood mononuclear cells (PBMCs). PBMCs were exposed to the compounds studied at concentrations ranging from 0.01 to 5 mM for 4 h. It was observed an increase in reactive oxygen species (including hydroxyl radical) and cytosolic calcium ions levels as well as reduction of transmembrane mitochondrial potential (ΔΨm) in PBMCs exposed to the compounds examined. All substances studied changed PBMCs membrane permeability, activated caspase-8, -9, -3 and caused chromatin condensation, which showed that they were capable of inducing apoptosis both via extrinsic and particularly intrinsic pathway. Generally the study demonstrated that there were no differences between apoptotic changes induced by glyphosate, its metabolites or impurities, and observed changes were provoked by high concentrations of investigated compounds. Since clear changes were only seen at high concentrations, a low apoptotioc potential of these compounds was concluded.

Materials and methods

Chemicals; The investigated compounds i.e., aminomethylphosphonic acid (AMPA) (purity 98%), methylphosphonic acid (purity 98%), N-(phosphonomethyl)iminodiacetic acid (PMIDA) (purity 98%), N-methylglyphosate, hydroxymethylphosphonic acid (purity 98%) and bis-(phosphonomethyl)amine (purity 97%) were synthetized by the Institute of Industrial Organic Chemistry, Warsaw, Poland. Glyphosate [N-(phosphonomethyl)glycine] (purity 95%) in acid form was bought from Sigma-Aldrich, USA. The investigated compounds were dissolved in phosphate-buffered saline (pH 7.4). Other chemicals were purchased from POCh (Poland) and Roth (Germany) and were of analytical grade. Different concentrations of glyphosate have been selected in this study:

• 0.01 mM (low concentration) that is quite similar to the concentration determined in blood of humans who were not directly exposed to this herbicide,

• 0.05 mM to 0.5 mM (moderate to high concentration) that corresponds to the concentration that may penetrate into human blood as a result of glyphosate formulation poisoning,

• 5 mM to 10 mM (very high concentration) that corresponds to the concentration detected in humans after acute poisoning with glyphosate formulation.

Cells isolation; PBMCs were isolated from leucocyte-buffy coat obtained from blood collected in Blood Bank in Lodz, Poland. Blood was taken from healthy, non-smoking volunteers who showed no signs of infection disease symptoms at the time the blood samples were collected. The study was approved by the Bioethics Committee of the University of Lodz No. KBBN-UŁ/I/3/2013. The final PBMCs density

used in the experiments (after addition of glyphosate, its metabolites or impurities) was $2x10^6$ per 1 mL. The viability of the cells was over 95% as determined by flow cytometry.

Quantitative determination of apoptosis (YO-PRO-1/PI staining); Apoptosis is characterized by changes in cell membrane permeability. Apoptotic cells that have altered membrane permeability (membrane integrity is maintained) are permeable for a marker YOPRO-1 (carbocyanine nucleic acid stain), which exhibits green fluorescence, whereas they are not permeable for the dye propidium iodide (PI) that shows red fluorescence. The cells were treated with glyphosate, its metabolites and impurities in the final concentrations ranging from 0.01 to 10 mM and incubated for 4 h at 37 °C in total darkness. Apoptosis was induced with 10 μM of camptothecin. After the incubation, the samples were centrifuged at 300 g for 5 min at 4 °C, the supernatant was removed, and the cells were supplemented with RPMI with L-glutamine and 10% FBS. Then, the mixture of YO-PRO-1 and PI (0.1 μM each) was added to the samples, which were incubated for 20 min on ice in total darkness. The samples were analysed by flow cytometry (LSR II, Becton Dickinson) with excitation maximum at 488 nm to visualize the YO-PRO-1 green fluorescence (520/30 bandpass filter) and PI red fluorescence (610/20 bandpass filter). FMC gate on PBMCs has been established for data acquisition and the data were recorded for a total of 10,000 events per sample.

Determination of biochemical and morphological hallmarks of apoptosis

Cytosolic calcium ion level; Calcium ion accumulated in mitochondria and endoplasmatic reticulum is an important secondary messenger in controlling apoptotic cell death. The level of cytosolic calcium ions was analysed by flow cytometry (LSR II, Becton Dickinson) using a fluorescent probe Fluo-3/AM according to manufacturer's protocol. Fluo-3/AM passes through membrane of living cells, in which it is cleavaged by intracellular esterases to Fluo-3. Inside the cell, Fluo-3 exhibits green fluorescence after complexation with calcium ions. PBMCs treated with glyphosate, its metabolites or impurities in the final concentrations ranging from 0.01 to 10 mM were incubated for 4 h at 37 °C in total darkness. Next, the cells were centrifuged at 300 g for 5 min at 4 °C, suspended in Fluo-3/AM (1 μM) solution and incubated at 37 °C for 20 min in total darkness. Then, Hanks' Balanced Salt Solution (HBSS), composed of inorganic salts and supplemented with glucose (with 1% of BSA) was added to the cells suspension, and PBMCs were incubated for 40 min at 37 °C in total darkness. The cells were centrifuged at 300 g for 5 min at 4 °C and washed twice with HEPES buffer. After centrifugation, PBMCs were suspended in HEPES buffer and incubated for 10 min at 37 °C in total darkness. The samples were analysed using flow cytometer (LSR II, Becton Dickinson) with excitation at 490/500 nm to visualize the Fluo-3 fluorescence. FMC gate on PBMCs has been established for data acquisition and the data was recorded for a total of 10,000 events per sample.

Mitochondrial transmembrane potential ($\Delta\Psi m$); Mitochondrial dysfunction leading to collapse of transmembrane mitochondrial potential has been shown to participate in the induction of apoptosis. Transmembrane mitochondrial potential was shown as red fluorescence intensity of MitoTracker Red CMXRos (excitation/emission maxima – 579/599 nm). This probe is cell permeable and contains mildly thiol-reactive chloromethyl moiety for mitochondrial labeling. Nigericin and valinomycin (1 μM) were used to increase and decrease $\Delta\Psi m$, respectively. PBMCs were exposed to glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. Then, the samples were centrifuged at 300 g for 5 min at 4 °C, the supernatant was decanted, and the cells were suspended in PBS. The cells were stained with MitoTracker CMXRos in the final concentration of 1 μM for 15 min at 37 °C in total darkness, and then analysed in 96-well plates using a microplate reader (Cary Eclipse, Varian).

Caspase-8, -9 and -3 activity; Caspases are critical enzymes of apoptosis. The caspases are able to break down peptide bonds via cysteine residues in various substrates, and therefore they catalyze the apoptotic cell death irreversibly in mammals including human beings. Analysis of caspase-8 and -3 was executed according to the manufacturer's protocols. The assays were based on the hydrolysis of the peptide substrates such as acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETDAMC) by caspase-8 and acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, which resulted in the release of the fluorescent 7-amino-4-methylcoumarin (AMC). The excitation and emission wavelengths of AMC were 360 nm and 460 nm, respectively. Caspase-9 colorimetric assay was based on hydrolysis of the substrate acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-pNA),

which led to the release of p-nitroaniline (pNA) that absorbance was determined at 405 nm. Camptothecin (10 μM) was used to induce apoptosis. Detection of caspase-3 and -8 activities was executed using fluorescent microplate reader (Fluoroskan Ascent FL, Labsystem) and determination of caspase-9 activity was performed using absorbance microplate reader (BioTek ELx808, Bio-Tek).

Hoechst 33342/PI staining; Chromatin condensation paralleled by DNA fragmentation is one of the most important criteria, which are used to identify apoptotic cells. Morphological changes of chromatin in PBMCs were assessed by double staining with Hoechst 33342 and PI. The cells were exposed to glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. After incubation, PBMCs were centrifuged at 200 g for 3 min at 4 °C. The supernatant was removed, and the cells were suspended in PBS (0.5 ml). Then the mixture of 1 μl of Hoechst 33342 and 1 μl of PI (1 mg/ml each) was added. After 1 min incubation at 37 °C in total darkness, the cells were analysed by fluorescence microscope (Olympus IX70, Japan) at 400x magnification. PMBCs were classified on the basis of their morphological staining characteristics: viable (blue fluorescence), early apoptotic (intensive bright blue fluorescence), late apoptotic (blue-violet fluorescence) and necrotic (red fluorescence).

Determination of reactive oxygen species level

Oxidation of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; ROS play a central role in regulation of the main pathways of apoptosis mediated by mitochondria, death receptors and the endoplasmic reticulum (ER). The rate of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) oxidation was assessed by flow cytometry. 6-CarboxyH2DCF-DA is a compound widely used for the detection of intracellular oxidants production. The probe diffuses across cellular membrane where it is hydrolyzed by intracellular esterases to 6-carboxy-2′,7'dichlorodihydrofluorescein (6-carboxy-H2DCF) that after oxidation, yields highly fluorescent 6-carboxy-2′,7′-dichlorofluorescein (DCF). The cells were treated with glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. Next, fluorescent marker was added to PBMCs, which were stained for 15 min at 37 °C in the dark. The final concentration of the fluorescent probe was 10 μM. Positive control consisted of hydrogen peroxide (2 mM). FMC gate on PBMCs has been established for data acquisition, and fluorescence was measured with the excitation and emission maxima of 488 and 530 nm, respectively. The data was recorded for a total 10,000 events per sample.

Oxidation of hydroxyphenyl fluorosceine; Highly reactive oxygen species (mainly hydroxyl radical) were assessed using flow cytometer (Becton Dickinson, LSR II) and 3-(p-hydroxyphenyl)-fluorescein (HPF). HPF is nonfluorescent until it reacts with hydroxyl radical. As a result of oxidation, the probe exhibits bright green fluorescence (excitation/emission maxima – 490/515 nm). Formation of hydroxyl radical was provoked by the addition of the mixture of ferrous perchlorate(II) (0.1 mM) and hydrogen peroxide (1 mM) to PBMCs suspension. Finally, the cells were treated with HPF in the final concentration of 2 μM and incubated for 15 min at 37 °C in total darkness. The data was recorded for a total of 10,000 events per sample.

Statistical analysis; The statistical analysis was performed with STATISTICA 8 data analysis software (2000 StatSoft, Inc., Tulsa, OK, USA). In this study, one-way analysis of variance (ANOVA) (p and F added to the description of the results) with post hoc multiple comparisons procedure (Tukey test) was used to assess statistical differences in case of normal distribution (significance marked on the charts). The difference was considered to be significant for $P < 0.05$. The individual analysis was performed on blood from 4 to 5 donors, while each experiment was repeated trice.

Results

Apoptotic changes; After 4 h incubation, all compounds studied caused an increase in the number of apoptotic cells. Apoptotic changes were observed in PBMCs treated with 0.5 mM and 5 mM of glyphosate, PMIDA and hydroxymethylphosphonic acid. Two glyphosate metabolites: AMPA and methylphosphonic acid as well as two glyphosate impurities: N-methylglyphosate and bis-(phosphonomethyl)amine caused apoptotic changes only at their highest concentration of 5 mM (Fig. 1).

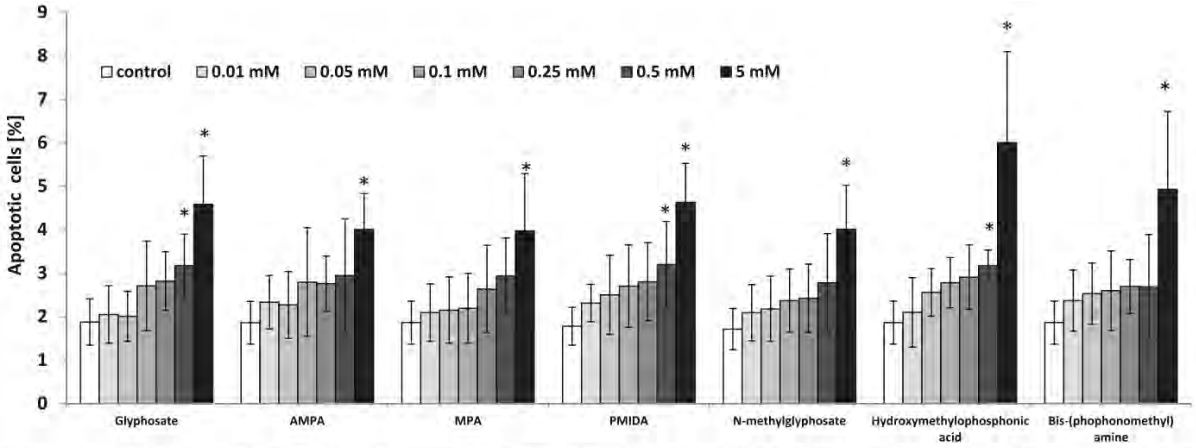


Fig. 1. Changes in the number of apoptotic PBMCs (expressed in per cent) after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to the control ($P < 0.05$).

Morphological changes of chromatin; Cell staining with Hoechst 33342 and PI allowed for the observation of early apoptotic cells (blue color), late apoptotic cells (blue-violet color) and necrotic cells (red color) in the population of PBMCs exposed to the compounds examined for 4 h. Selected photographs showing the presence of individual types of apoptotic and necrotic PMBCs are presented below (Photo 1).

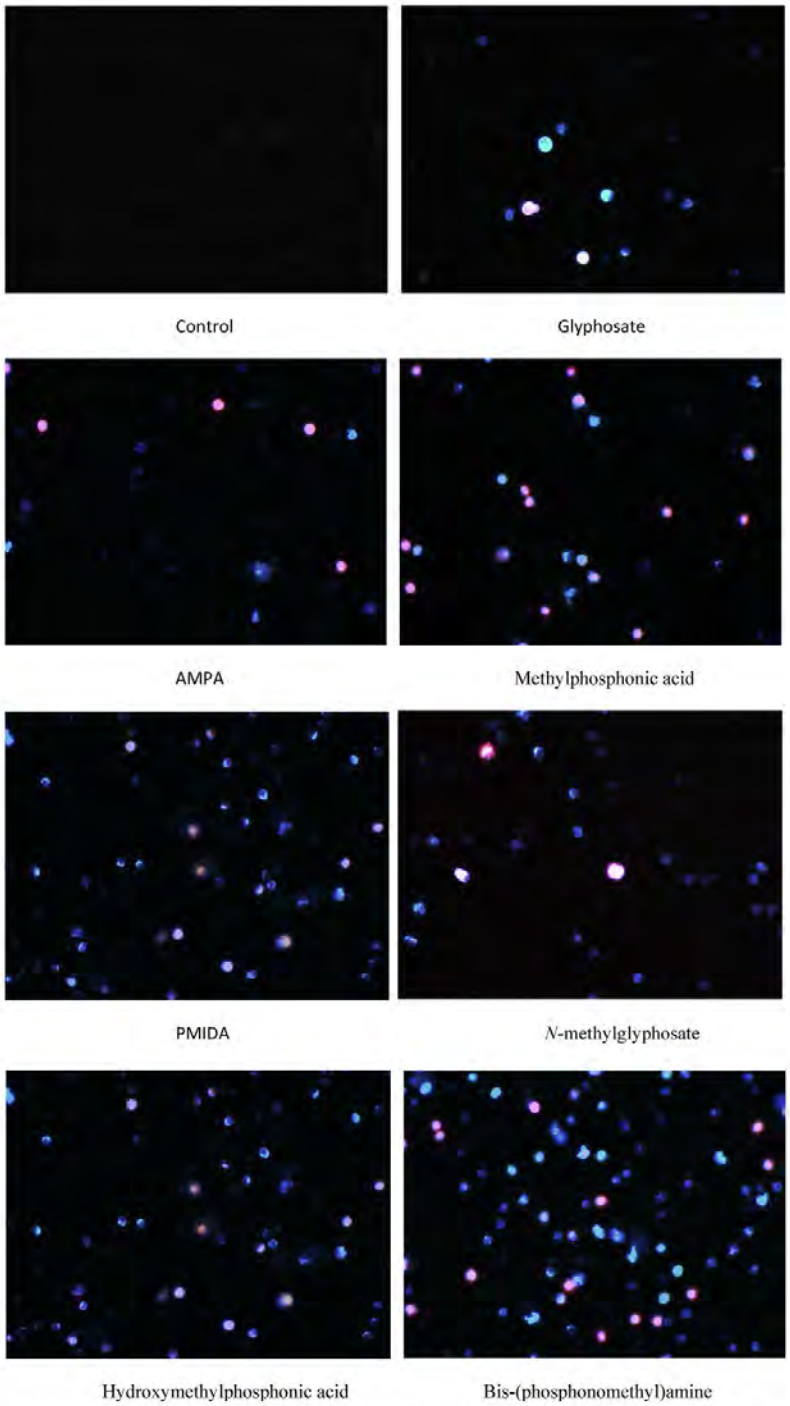


Photo 1. The representative photomicrographs of Hoechst 33324/PI-stained PBMCs pretreated with PBS (control) and with 5 mM of glyphosate, AMPA, methylphosphonic acid, PMIDA, *N*-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine. Viable cells (blue fluorescence), early apoptotic cells (intensive bright blue fluorescence), late apoptotic cells (blue-violet fluorescence) and necrotic cells (red fluorescence).

Cytosolic calcium ion level; A statistically significant increase in cytosolic calcium ion level was observed after incubation of PBMCs with glyphosate and other compounds studied (except for PMIDA). Statistically significant changes were observed for the highest concentration (5 mM) of glyphosate, AMPA and N-methylglyphosate. Glyphosate metabolite - methylphosphonic acid and glyphosate impurity - bis(phosphonomethyl)amine from the concentration of 0.5 mM caused an increase in cytosolic calcium ion level, while hydroxymethylphosphonic acid induced changes in the parameter studied from the concentration of 0.25 mM (Fig. 2).

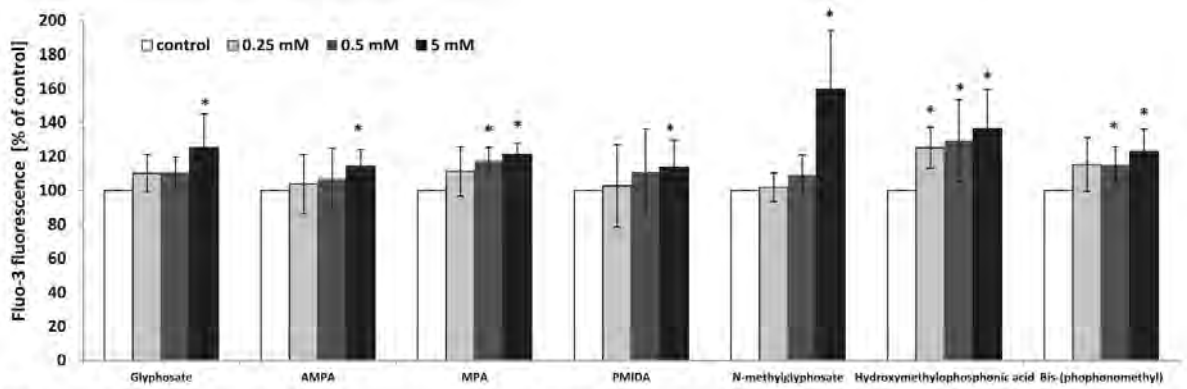


Fig. 2. Changes in cytosolic calcium ion level in control PBMCs and PBMCs incubated with glyphosate, its metabolites and impurities in the concentrations ranging from 0.25 to 5 mM for 4 h (*) Statistically significant changes compared to control ($P < 0.05$).

Transmembrane mitochondrial potential (ΔΨm); Most of the compounds studied caused a decrease in transmembrane mitochondrial potential, while hydroxymethylphosphonic acid (at 0.25 mM) caused statistically significant increase in the parameter examined. Glyphosate and one of its production impurities: bis(phosphonomethyl)amine (from the concentration of 0.05 mM) decreased ΔΨm. Other glyphosate metabolites: AMPA and methylphosphonic acid, and two glyphosate impurities: PMIDA and N-methylglyphosate decreased the parameter studied from the concentration of 0.1 mM (Fig. 3).

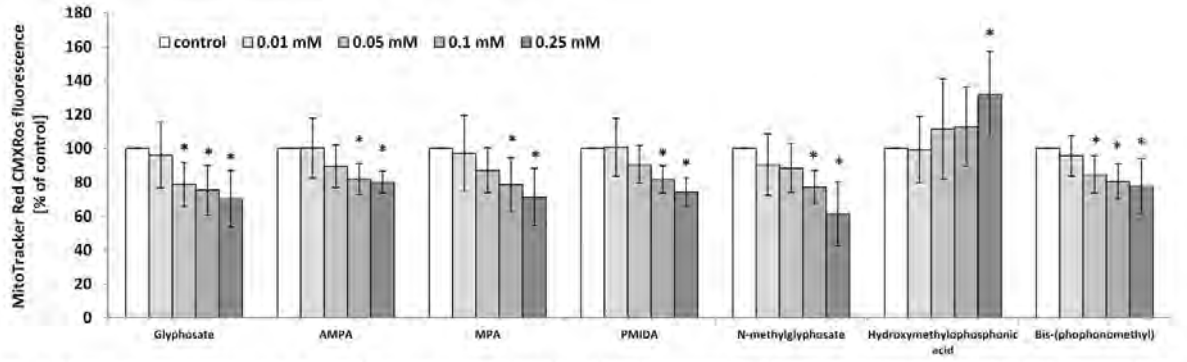


Fig. 3. Changes in transmembrane mitochondrial potential in control PBMCs and PBMCs incubated for 4 h with glyphosate, its metabolites and impurities in the concentrations ranging from 0.01 to 0.25 mM (*) Statistically significant changes compared to control ($P < 0.05$).

Caspase-8 and -9 activity; Glyphosate, AMPA, PMIDA and bis-(phosphonomethyl)amine increased in caspase-8 activity. The changes were observed in PBMCs treated with 0.5 mM of glyphosate and AMPA and with the highest concentration (5 mM) of PMIDA and bis-(phosphonomethyl)amine. Other compounds studied such as methylphosphonic acid, N-methylglyphosate and hydroxymethylphosphonic acid did not cause statistically significant changes in caspase-8 activity (Fig. 4). Glyphosate, its metabolites and impurities caused a substantial increase in caspase-9 activity. Changes in the enzyme activity were observed for the highest concentration (5 mM) of glyphosate, its two metabolites: AMPA and methylphosphonic acid and all glyphosate impurities: PMIDA, N-methylglyphosate, hydroxymethylphosphonic acid and bis(phosphonomethyl)amine (Fig. 5).

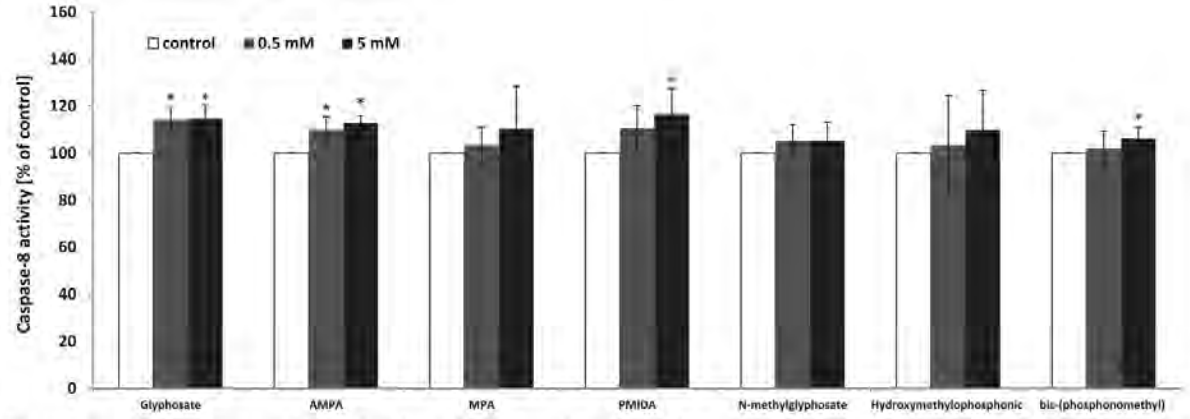


Fig. 4. Changes in caspase-8 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

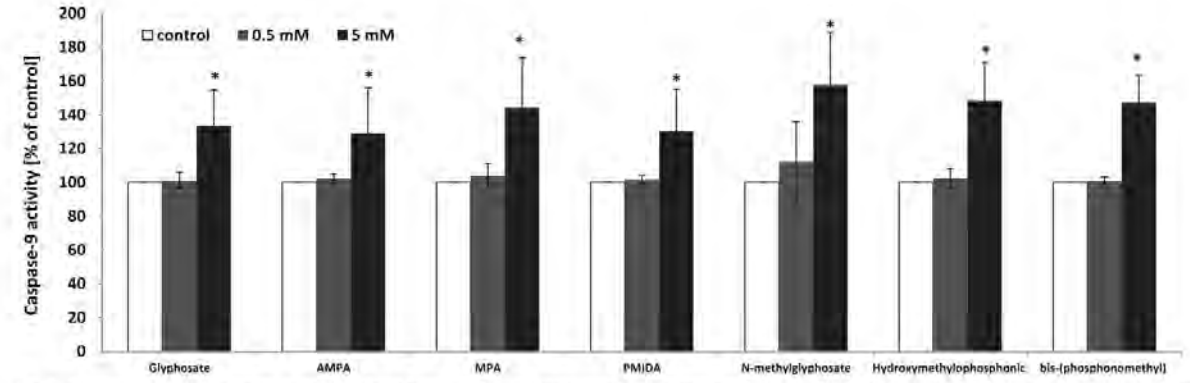


Fig. 5. Changes in caspase-9 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

Caspase-3 activity; An increase in caspase-3 activity was noted in PBMCs treated with 0.25 mM of glyphosate and PMIDA and with 0.5 mM of AMPA, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine. Other compounds tested: N-methylglyphosate and methylphosphonic acid only at the highest concentration of 5 mM caused an increase in caspase-3 activity (Fig. 6).

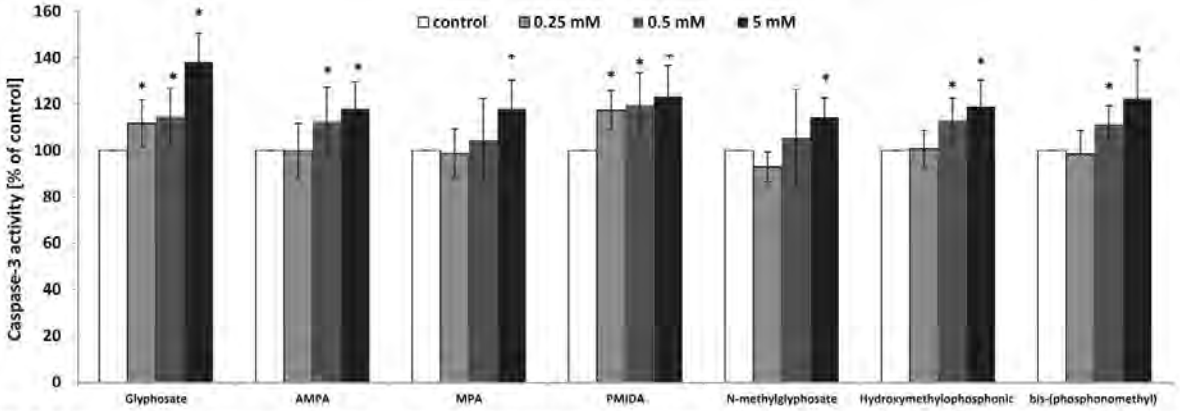


Fig. 6. Changes in caspase-3 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

ROS level; It was observed that glyphosate, its metabolites and impurities induced a statistically significant increase in H2DCFDA oxidation in PBMCs. Changes in ROS level were observed after 4 h of exposure of PBMCs to 0.25 mM of glyphosate, methylphosphonic acid, PMIDA, N-methylglyphosate and hydroxymethylphosphonic acid. Other compounds studied like AMPA and bis-(phosphonomethyl)amine from the concentration of 0.5 mM caused an increase in the H2DCFDA oxidation (Fig. 7).

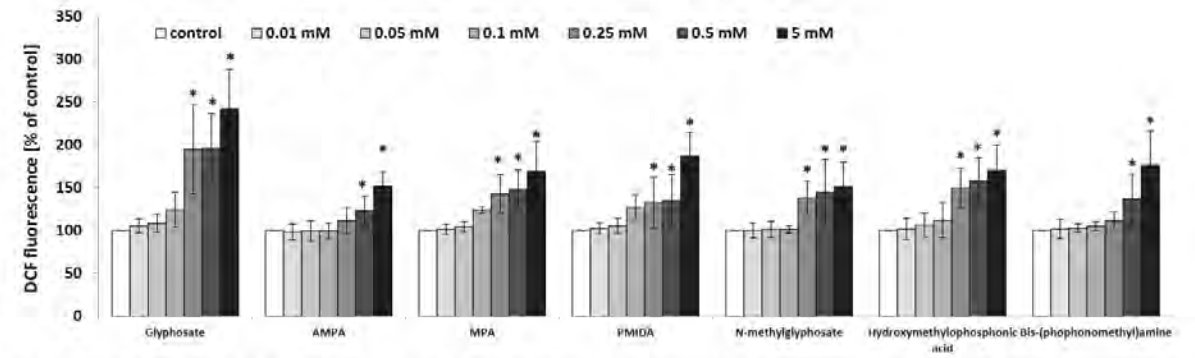


Fig. 7. Changes in total ROS level in PMBCs incubated with glyphosate, its metabolites and impurities for 4 h (*) Statistically significant changes compared to the control ($P < 0.05$).

Hydroxyl radical level; Glyphosate, its metabolites and impurities increased highly reactive oxygen species level, including hydroxyl radicals in PMBCs. Statistically significant changes were observed for most of the compounds studied from the concentration of 5 mM. An increase in HPF fluorescence was noted in cells treated for 4 h with the highest concentration (5 mM) of glyphosate, its impurities: N-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine and metabolites: AMPA and methylphosphonic acid. The strongest changes in HPF oxidation were caused by PMIDA, which changed this parameter from the concentration of 0.5 mM (Fig. 8).

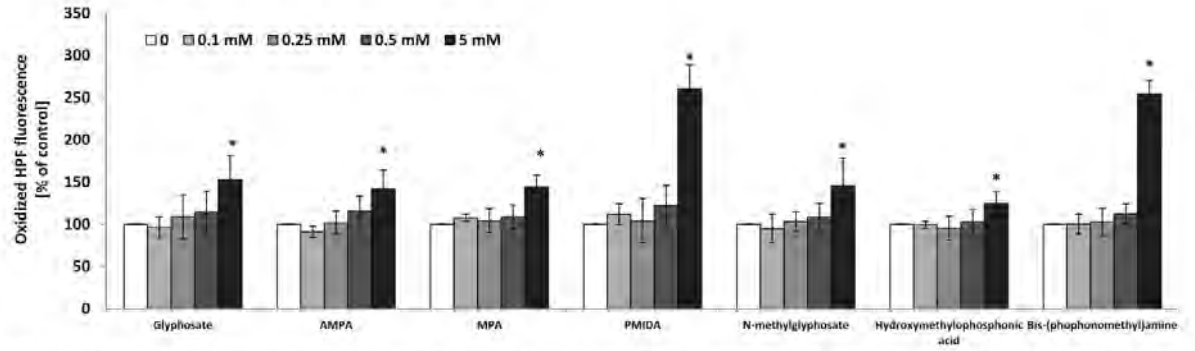


Fig. 8. The level of highly reactive oxygen species (mainly hydroxyl radical) in human PMBCs incubated with glyphosate, its metabolites and impurities for 4 h (*) Statistically significant changes compared to the control ($P < 0.05$).

Discussion

Apoptotic potential of glyphosate has been evaluated in various cell types; however, the effect of glyphosate, its metabolites (including AMPA and methylphosphonic acid) and its impurities on apoptotic changes in human leucocytes has not been assessed. This study has described apoptotic potential of glyphosate, its metabolites and impurities in human PBMCs. Besides a quantitative determination of apoptotic cells (staining with YO-PRO-1/PI fluorescent probes) the analysis concerned evaluation of the mechanism of action of these substances by measurement of a variety of parameters involved in the programmed cell death. The activities of caspases, of both initiator caspase-8 and -9, as well as executor caspase-3 were determined. Alterations in cytosolic calcium ion and ROS levels were also analysed. Moreover, changes in transmembrane mitochondrial potential and chromatin condensation were assessed. PBMCs were exposed to tested compounds for 4 h, the time necessary to observe apoptotic changes. Flow cytometry analysis has demonstrated a statistically significant increase in the number of apoptotic cells exposed to all compounds studied. Apoptotic changes induced by glyphosate, PMIDA and hydroxymethylphosphonic acid were observed from the concentration of 0.5 mM, while those induced by AMPA, methylphosphonic acid, N-methylglyphosate and bis-(phosphonomethyl)amine from the concentration of 5 mM. A slight increase in the number of apoptotic cells (5.76%) treated with glyphosate was observed, compared to the control (1.22%).

It was observed that methylphosphonic acid at lower concentration (0.25 mM) (in comparison to other analysed compounds) caused a statistically significant increase of cytosolic calcium ion level in PBMCs. Other compounds such as methylphosphonic acid and bis(phosphonomethyl)amine induced apoptosis from the concentration of 0.5 mM, while glyphosate, AMPA and N-methylglyphosate from 5 mM. It was also observed that aminomethylphosphonic acid did not cause any statistically significant increase in this parameter.

Our study demonstrated that all compounds analysed caused reduction of transmembrane mitochondrial potential. Glyphosate and its impurity - bis(phosphonomethyl)amine, from the concentration of 0.05 mM, caused reduction of $\Delta\Psi m$, while other compounds analysed induced the same changes from the concentration of 0.1 mM (except for hydroxymethylphosphonic acid, that caused reduction of the discussed parameter at the concentration of 0.25 mM). An increased ROS production was observed in PBMCs exposed with all analysed compounds. The obtained results are consistent with data published, which observed ROS formation in human PBMCs treated with glyphosate, AMPA and particularly glyphosate preparation - Roundup 360 PLUS. It has been shown that the increase in ROS level, and hydroxyl radical in particular may contribute to DNA damage, and thus apoptotic cell death. For that reason, the formation of highly reactive oxygen species (including hydroxyl radical) was assessed in PBMCs treated with glyphosate, its metabolites and impurities. It has been noted that glyphosate, its impurities: N-methylglyphosate, hydroxymethylphosphonic acid and bis(phosphonomethyl)amine as well as its metabolites: AMPA and methylphosphonic acid at the concentration of 5 mM caused an increase in hydroxyl radical level. The highest increase in this parameter was noted in PBMCs exposed to PMIDA (from 0.5 mM Similarly, our study showed that AMPA at lower concentration did not induce hydroxyl radical formation, and the increase in discussing parameter was noted only at its highest level of 5 mM. The proteolytic activity of caspases involves cleavage of their substrates at aspartate residues. In this study we have observed that glyphosate, its metabolites and impurities caused chromatin condensation in human PBMCs. Based on our own research, and also on studies of other authors it may be suggested that glyphosate, its metabolites and impurities induce apoptosis both via the intrinsic pathway (evidenced by the observed increase in total ROS and hydroxyl radical levels, a decrease in transmembrane mitochondrial potential and an increase in caspase-9 activity), and - to a lesser extent via the extrinsic pathway (evidenced by changes in caspase-8 activity) (except for methylphosphonic acid, Nmethylglyphosate and hydroxymethylphosphonic acid).

Conclusion

Toxicity of pure glyphosate versus its metabolites and impurities: The results obtained in this study do not indicate an essential role of metabolites and production impurities of glyphosate in toxic (proapoptotic) action of glyphosate and possibly glyphosate-based preparations. Research presented in this paper indicated that both AMPA and MPA exerted a smaller effect on ROS and hydroxyl radical formation than glyphosate. For other analysed parameters, no significant differences have been demonstrated between glyphosate and its metabolites activities. Studies on toxicity of glyphosate metabolite - MPA - are even more limited, while our results clearly demonstrated a relatively low toxicity of this compound. The study also revealed that some of glyphosate impurities were characterized by a slightly stronger proapoptotic potential than the parent compound. They constitute, however, a minor impurities of glyphosate and should not significantly increase toxicity of N-(phosphonomethyl)glycine-based products (contrary to surfactants).

Obtained results clearly indicate low proapoptotic properties of all analysed compounds. Initial clear apoptotic effects are associated with their highest analysed concentrations, which correspond to the concentrations to which a human organism could be exposed only as a result of acute or subacute poisoning with glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes in-vitro investigations of glyphosate, its metabolites (AMPA and methylphosphonic acid) and impurities (PMIDA, N-methylglyphosate, hydroxymethylphosphonic acid, and bis-(phosphonomethyl)amine) on six intermediate endpoints of apoptosis (membrane permeability, cytosolic calcium concentration, mitochondrial transmembrane potential, caspase activity, chromatin condensation, and ROS quantitation by two methods) in human peripheral blood mononuclear cells. The reason for selection of this model is not stated but is possibly as potential target tissue for Non-Hodgkins Lymphoma. The methodologies used are frequently reported in literature but are not a standardized or validated method by GLP standards; there is no OECD guideline. Positive control results are not presented and it is unclear if positive controls were used for all assays; wording is sufficiently poor that it may be inferred that some positive controls were used, e.g. nigericin and valinomycin in the studies of mitochondrial transmembrane potential, campothecin in the caspase assays, although these may alternatively be reagents for the assay. It is unclear if assays were conducted in duplicate or triplicate (the stated term was "trice" which may be either twice or thrice), which may then also influence statistical evaluation. However, the methodology appears basically sound.

Apoptotic or pre-apoptotic activity was seen generally consistently across the assays. While glyphosate, its metabolites, and impurities were seen to increase apoptotic endpoints in these assays (0.5 mM and higher), clear effects occurred only at high concentrations.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no proper cytotoxicity tests were performed, no positive controls were used and the concentration range at which most of the effects were observed is beyond the acceptable physiological range (> 1 mM). The concentration range at which the glyphosate impurities were tested is the same as that for glyphosate which is not a realistic approach for risk assessment of impurities.

Reliability criteria for *in vitro* toxicology studies

Publication: Kwiatkowska *et al*., 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity (95%). Source: Sigma-Aldrich, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also glyphosate impurities were tested.
AMPA is the tested substance	Y	
Study		
Test system clearly and completely described	Y	PBMCs
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Partly	0.01, 0.05, 0.25, 0.5, 5, 10 mM
Cytotoxicity tests reported	?	

Positive and negative controls	N	No positive controls.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no proper cytotoxicity tests were performed, no positive controls were used and the concentration range at which most of the effects were observed is beyond the acceptable physiological range (> 1 mM). The concentration range at which the glyphosate impurities were tested is the same as that for glyphosate which is not a realistic approach for risk assessment of impurities.		

1. Information on the study

Data point:	CA 5.4
Report author	Mañas F. *et al.*
Report year	2013
Report title	Oxidative stress and comet assay in tissues of mice administered glyphosate and AMPA in drinking water for 14 days
Document No	Journal of Basic & Applied Genetics (2013) Vol. 24(2), Article 7 - research
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study the levels of thiobarbituric acid reactive substances (TBARs); quantified superoxide dismutase (SOD) and catalase (CAT) activity in liver, kidney, lung and heart were determined, and the comet assay in blood and liver of mice administered glyphosate (40 or 400 mg/kg/day) or AMPA (100 mg/kg/day) in drinking water for 14 days was performed. Exposure to glyphosate 400 mg/kg induced a statistically significant ($p < 0.05$) decrease of SOD activity in heart and an increase in CAT activity in kidney. In the comet assay there were statistically significant differences in all the treatments and tissues studied in comparison to control animals ($p \leq 0.01$). The major results of this study were that mice administered glyphosate or AMPA in drinking water for 14 days induced a significant increase in DNA damage in liver and blood but minor effects on oxidative stress parameters. DNA effects on liver and blood indicate that these compounds could be of concern in terms of their potential to damage the genetic material, and that oxidative stress does not seem to be the mechanism causing that effect.

Materials and Methods

Chemicals - Analytical grade glyphosate (96% purity), and analytical grade AMPA, (99% purity) were obtained from Sigma-Aldrich, Argentina.

Animals - Twenty-four Balb C mice of approximately 45 days of age were used in this study. Four groups of six animals each (a control group, 2 groups treated with glyphosate and a group treated with AMPA) were used for the determination of oxidative stress (TBARs, SOD and CAT) and DNA damage in the comet assay. During 7 days prior to the beginning of the study, the volume of water ingested per day was measured in every 2 animals per cage to determine the concentration of glyphosate and AMPA in the drinking water to be supplied to each group. Mice received approximately 40 or 400 mg/kg bw/day of glyphosate and 100 mg/kg bw/day of AMPA. The control group received drinking water without test compound. After 14 days of exposure, before sacrifice, peripheral blood was drawn from the tail vein to perform the comet assay. At necropsy, heart, lungs, liver, and kidneys were removed for the determination of TBARs, SOD and CAT and frozen at -80 ºC pending analysis. A part of the liver was homogenized in phosphate buffer (pH 7.4) immediately after excision for the conduct of the comet assay. All determinations were performed in triplicate.

Single cell gel electrophoresis assay in mouse blood and liver - The protocol followed the general guidelines proposed by Singh et al. (Exp. Cell Res., 175, 184-191, 1988) with minor modifications. The slides were fixed in absolute ethanol, stained with ethidium bromide and scored using fluorescence microscopy. Images of 100 “nucleoids” counted for each animal were captured with a camera attached to the fluorescence microscope and linked to the Comet Score 1.5 software. Tail moment (TM),

percentage of DNA in tail (% of DNA) and tail length (TL) were used to estimate DNA damage (in arbitrary units).

TBARs, SOD and CAT determinations - Tissue homogenates (10%) were prepared in chilled 0.05 M potassium phosphate buffer at pH 7.4. TBARs concentrations, expressed as nmol of malondialdehyde MDA/g of tissue, were measured spectrophotometrically at 532 nm in liver and kidney homogenates. TBARs concentrations were determined using a standard curve at different concentrations of MDA versus optical density, individually prepared for each tissue. Superoxide dismutase (SOD) activity was assessed spectrophotometrically in the supernatant of liver homogenates. One unit of enzymatic activity has been defined as the amount of enzyme capable of causing 50% inhibition of auto-oxidation of epinephrine. Catalase (CAT) activity was measured at 240 nm by the decomposition of the H_2O_2.

Statistical analysis
Statistical analysis was performed using Prism software (PRISM, 1997). The Kolmogorov-Smirnov test was performed to verify if the results follow a normal distribution. ANOVA followed by the Dunnett's test, or the Kruskal-Wallis test followed by the Dunn's test were performed for data with and without normal distribution, respectively.

Results

The volume of water ingested per animal/day did not show any statistically significant differences among the experimental groups throughout treatment. No statistically significant differences were found in liver, kidney, lung and heart for all oxidative stress parameters measured with the exception of a decrease in SOD in the heart and an increase in CAT in the kidney at a daily glyphosate dose of 400 mg/kg bw. A non-statistically significant increase in CAT was observed in the lung. There were no statistically significant changes in the concentrations of MDA/g of tissue at 40 and 400 mg/kg bw/day of glyphosate and at 100 mg/kg bw/day of AMPA. Tail intensity, tail length and tail moment were statistically significantly elevated in blood and liver of all dosed groups with the exception of tail intensity in liver at 40 mg/kg bw/day glyphosate.

Discussion and Conclusion

The presence of glyphosate or AMPA in drinking water did not affect water consumption. Statistically significant differences in oxidative stress (decrease in SOD activity in the heart and increase in CAT activity in the kidney) were only observed in mice treated with glyphosate at 400 mg/kg bw for 14 days. The increase in CAT activity in the lungs was not statistically significant. A non-statistically significant decrease in SOD activity was observed in all tissues of animals treated with 100 mg/kg bw AMPA but no effect of AMPA was found on CAT activity. Liver was the only tissue where no effects were recorded, neither in TBA reactive substances nor in SOD and CAT activity. In the comet assay of blood statistically significant differences from control were seen for glyphosate and AMPA in tail intensity, tail length and tail moment. Tail moment values in the comet assay were similar for both glyphosate and AMPA treatments. In this study, genotoxic changes were observed with glyphosate at 40 and 400 mg/kg bw/day and AMPA at 100 mg/kg bw/day. However, statistically significant changes in the levels of SOD and CAT especially in heart and kidney were only seen with glyphosate at 400 mg/kg bw/day. This suggests that the genotoxic effects of glyphosate and AMPA are much more important than the indicators of oxidative stress.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to investigate the effect of glyphosate and AMPA on indicators of oxidative stress and DNA integrity in mice after oral exposure for 14 days via the drinking water. The results of this study indicate that no statistically significant differences have been found in liver, kidney, lung and heart for all oxidative stress parameters measured with the exception of a decrease in SOD activity in the heart and an increase in CAT activity in the kidney at a daily glyphosate dose

of 400 mg/kg bw. There was an increase in CAT activity in the lung but this was not statistically significant and didn't show a dose-effect relationship. A statistically significant increase in DNA damage parameters was observed for glyphosate and AMPA with the exception of tail intensity in the liver for glyphosate at 40 mg/kg bw/day. No clear dose-effect relationship was evident for DNA damage parameters in blood after treatment with glyphosate. A dose-effect relationship was present for tail length and tail moment in the liver.

This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the increased DNA damage seen (only 2 dose levels tested for glyphosate with too few animals) didn't show a dose-effect relationship in blood and occurred at dose levels (40 and 400 mg/kg bw/day) that are much lower than the 2000 mg/kg bw used in regulatory *in vivo* MN tests in the mouse with negative results.

Reliability criteria for *in vivo* toxicology studies

Publication: Manas et al., 2013	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	2 dose groups for glyphosate, one dose group for AMPA
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	Very old determination methods applied
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 96%. Source: Sigma-Aldrich, Argentina.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	Y	Purity of 99%. Source: Sigma-Aldrich, Argentina.
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Exposure via drinking water
Dose levels reported	Y	
Number of animals used per dose level reported	Y	
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported		Only 2 dose levels used. DNA damage (comet) at 40 and 400 mg/kg bw/day (no dose-effect relationship) not

		confirmed by regulatory genotoxicity (MN) studies up to 2000 mg/kg bw/day
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the increased DNA damage seen (only 2 dose levels tested for glyphosate with too few animals) didn't show a dose-effect relationship in blood and occurred at dose levels (40 and 400 mg/kg bw/day) that are much lower than the 2000 mg/kg bw used in regulatory in vivo MN tests in the mouse with negative results.		

1. Information on the study

Data point:	CA 5.6
Report author	Manservisi F. *et al.*
Report year	2019
Report title	The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system
Document No	Environmental Health (2019) Vol.18, 15
Guidelines followed in study	Pilot study based on OECD test guideline 443: Extended one-generation reproductive toxicity study.
Deviations from current test guideline	Yes, only one dose level for each test item and insufficient number of animals per dose level used.
GLP/Officially recognised testing facilities	No, no GLP statement delivered.
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The present pilot study examines whether exposure to glyphosate-based herbicides (GBHs) at the dose of glyphosate considered to be "safe" (the US Acceptable Daily Intake - ADI - of 1.75 mg/kg bw/day), starting from in utero life, affect the development and endocrine system across different life stages in Sprague Dawley (SD) rats. In pups, anogenital distance (AGD) at PND 4 was statistically significantly increased both in Roundup-treated males and females and in glyphosate-treated males. Age at first estrous (FE) was significantly delayed in the Roundup-exposed group and serum testosterone concentration significantly increased in Roundup-treated female offspring from the 13-week cohort compared to control animals. A statistically significant increase in plasma TSH concentration was observed in glyphosate-treated males compared with control animals as well as a statistically significant decrease in DHT and increase in BDNF in Roundup-treated males. Hormonal status imbalances were more pronounced in Roundup-treated rats after prolonged exposure.

Materials and methods

Chemicals - Glyphosate (purity of > 99.5%), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy). The representative formulated product, Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%), water (42.5%) and surfactant (16%)) was purchased from Consorzio Agrario dell'Emilia, Bologna, Italy.

Animals and experimental design - Each of the 24 virgin female SD rats (17 weeks old, 270–315 g) was mated outbred with one breeder male rat of the same age and strain. Every day, the females were examined for the presence of sperm. After evidence of mating, females were housed separately during gestation and delivery. Newborns were housed with their mothers until weaning. Weaned offspring were co-housed, by sex and treatment group, not more than 3 per cage. Cages were identified by a card indicating study protocol code, experimental and pedigree numbers, and dosage group. The cages were placed inside a single room at 22 °C ± 3 °C and at 50% ± 20% relative humidity. A light/dark cycle of 12 hours was maintained. Two groups of dams and their pups were treated with either glyphosate or MON 52276 diluted in drinking water to achieve the desired glyphosate dose of 1.75 mg/kg bw/day. The F0 female breeders received the test item from gestation day (GD) 6 to the end of lactation, while the offspring (F1) continued to be exposed after weaning for an additional 6 or 13 weeks. Glyphosate or MON 52276 solutions were freshly prepared on a daily basis taking into account body weight and water consumption. During pregnancy and lactation, embryos and offspring (F1) were all retained in the litter and received the test compounds mainly through their dams (F0). The day of birth was designated post-natal day 1 (PND 1) for pups and lactation day 1 (LD 1) for dams. After weaning, the offspring (F1) were treated via the drinking water until sacrifice. On PND 28, offspring were randomly distributed in

two cohorts: 8/sex/group of the 6-week cohort and 10/sex/group of the 13-week cohort. Altogether, 108 rats (54 males and 54 females) were enrolled in the post-weaning treatment phase.

Measurements in F0 dams and litters prior to weaning - Mean gestational length was calculated as the number of days from detection of a positive vaginal smear (GD 0) to birth of a litter. Pregnancy was confirmed by the occurrence of parturition. The body weight of the dams was recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, the body weight of the dams was recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 (last measurement before weaning). Body weight of the pups by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. Feed and water consumption of the dams was recorded twice weekly during gestation on GD 0, 3, 6, 9, 12, 15, 18, and 21, and twice weekly during lactation on LD 1, 4, 7, 10, 13, 16, 19, 21 and 25. Dead pups were removed when found and sexed when possible. Sex was determined on PND 1. The mean litter size was assessed on PND 0 (within 24 hours from delivery), 1, 4, 7, 10, 13, 16, 19, 21, and 25. Litter size included dead as well as live offspring. Dead pups were visually examined by floating the lungs in saline, to distinguish if they were stillborn (died in utero) or died shortly after birth. Live-birth index was determined on PND 0 as (number of pups born alive / total number of pups born) × 100. Survival index, calculated as (total number of live pups at designated time point / number of live pups born) × 100, was measured on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. For all the pups, anogenital distance (AGD) was measured and body weight recorded on PND 4.

Post weaning endpoints up to adulthood - After weaning, body weight was measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifice, the means of individual body weights were calculated for each group and sex. Daily water and feed consumption per cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2. Time to vaginal opening (VO) was determined by daily inspection of all female pups starting on PND 28. The body weight of each female was recorded on the day VO was observed. Time to balano-preputial separation (BPS) was determined by daily inspection of all males beginning on PND 35. The body weight of each male was recorded on the day BPS was observed. The female rats belonging to the developmental (6-week) cohort were also monitored for the time to first estrous (FE).

Estrous cycle characterization - Starting on approximately PND 95 and for the duration of 3 weeks, daily vaginal lavage was performed on female rats of the 13-week cohort. To reduce variability, vaginal cytology samples were collected by vaginal lavage at the same time of the day over the course of the experiment, in the mid-morning, between 10:00 and 13:00 h. Collection, processing and vaginal smear evaluation was performed as described previously.

Necropsy - Five days after weaning, dams were sacrificed and following tissues were collected and fixed in alcohol: mammary glands (4 sites: axillary and inguinal, right and left), adrenal glands, uterus (including cervix), ovaries, and vagina. The adrenal glands, uterus and ovaries were also weighed. For the determination of serum testosterone, blood was collected and serum prepared by centrifugation and stored at -80 °C pending analysis. All male and female pups belonging to both cohorts were sacrificed on PND 73 ± 2 for the 6-week cohort and PND 125 ± 2 for the 13-week cohort. Following tissues were collected for histopathology and fixed in alcohol: mammary gland (4 sites: axillary and inguinal, right and left), thyroid and parathyroid, adrenal glands, bladder and prostate, seminal vesicles and coagulating gland, left and right testis with epididymis (half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation), uterus (including cervix), ovaries and vagina. During necropsy, all tissues with gross lesions were removed for histopathology. Adrenal glands, bladder and prostate, seminal vesicles and coagulating gland, left testis, left epididymis, uterus (including cervix) and ovaries were weighed. In case of paired organs, both organs were preserved. Organ weight was expressed as absolute and relative organ weight. Rats were sacrificed randomly across the 4 stages of the estrous cycle. In order to determine and allow correlation with histopathology in reproductive organs and hormone analysis, the stage of estrous cycle was determined by histological appearance of the various components of the reproductive tract for F1 females of the 6-week cohort or by a vaginal smear examined on the day of necropsy for F1 females of the 13-week cohort.

Sperm analysis - Sperm analyses were performed on each male from both cohorts, at scheduled necropsies on PND 73± 2 and PND 125 ± 2. At necropsy, half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation. Spermatids resistant to homogenization and spermatozoa present in the caput/corpus and cauda epididymis were counted. The tunica albuginea was removed from the (half) testicle, and a sample of the parenchyma was weighed and homogenized in 5 mL saline-TritonX-100 at 0.05%. The samples were then diluted 10-20 times in saline, and the mature spermatids resistant to homogenization (step 17-19 spermatids) were counted using a Thoma chamber. Four fields per animal were recorded, and the numbers of spermatids per gram of testis were calculated. To calculate the daily sperm production (DSP) these values were subsequently divided by 6.1, which is the number of days step 17-19 spermatids are present in the seminiferous epithelium. Similarly, the segments of the epididymis (caput, corpus and cauda) were cut with a scissor, weighed, homogenized, diluted and counted as described for the testes. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for each segment of the epididymis calculated. The epididymal sperm transit time through the epididymal caput/corpus and cauda was calculated by dividing the number of spermatozoa present in each portion of the epididymis by the DSP of the associated testis. To assess the percentage of morphologically abnormal sperm half of left cauda epididymis of each rat was transferred to a Petri dish containing 2.5 mL (for 70 day old animals) or 3.5 mL (for 120 day old animals) of Dulbecco's PBS at 37°C, cut in 2-3 pieces and incubated for approximately 3 minutes at 37°C with gently swirling to facilitate release of sperm cells from the cauda. Dried smears of epididymal spermatozoa were stained with 1% Eosin Y for 30 minutes and evaluated at 400 x magnification. Five hundred spermatozoa per rat were evaluated and scored as morphologically normal or abnormal according to the presence or absence of head or tail defects.

Histopathology - After fixation, samples were trimmed, processed, embedded in paraffin wax, sectioned to a thickness of 4-5 μm and then processed in an alcohol-xylene series and stained with hematoxylin and eosin for microscopic evaluation. Histopathology evaluation was performed blind by at least two pathologists. At least one senior pathologist peer reviewed all lesions of oncological interest as well as any lesion of dubious interpretation. All the pathologists used the same evaluation criteria and the same classification based on international standard criteria (INHAND, NTP) described in the specific Standard Operating Procedures and long adopted at the CMCRC/RI.

Hormone analysis - Serum concentration of free (fT) and total testosterone (TT), 5α-dihydrotestosterone (DHT), 17β-estradiol (E2) and Sex Hormone Binding Globulin (SHBG) were measured in duplicates by solid phase enzyme-linked immunosorbent assays (ELISAs): "Estradiol rat ELISA" (#DEV9999), manufactured by Demeditec Diagnostics GmbH, "Rat Free Testosterone (F-TESTO) ELISA" (#CSB-E0597r), "Rat Testosterone, T ELISA" (#CSB-E05100R); "Rat dihydrotestosterone (DHT) ELISA" (#CSB-E07879r), and "Rat sex hormone-binding globulin (SHBG) ELISA" (#CSB-E12118r), manufactured by Cusabio Biotech Co. Ltd. The detection range and the Lower Limit of Detection (LLD) of each ELISA kit was 2.5-1280 pg/mL and 2.5 pg/mL for E2, 0.3-60 pg/mL and 0.15 pg/mL for fT, 0.13-25.6 ng/mL and 0.06 ng/mL for TT, 10-2000 pg/mL and 5 pg/mL for DHT, 375-6000 ng/mL and 375 ng/mL for SHBG. Each kit was used following the manufacturer's instructions and absorbance was measured at 450 nm using a 96-well plate reader. Plasma pituitary hormones were measured in duplicate using the "Rat Pituitary Magnetic Bead Panel", a Luminex® bead-based immunoassay, following manufacturers' instructions. Seven plasma pituitary hormones were measured in plasma samples from 40 pups (20 females and 20 males) randomly selected from the 6-week cohort (N = 48 total): adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF), follicle stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH), prolactin (PRL) and thyroid stimulating hormone (TSH). FSH and LH were also assessed in 40 pups (20 females and 20 males) randomly selected from the 13-week cohort (N = 60 total). BDNF and TSH results from the 6-week cohort showed marginal differences by exposure groups in male pups, it was therefore attempted to validate these results by measuring BDNF and TSH in all male pups (N = 30) from the 13-week cohort. Plasma TT was measured in duplicates in all dams (N = 24) using an ELISA kit, the "Testosterone Parameter Assay Kit", following manufacturers' instructions.

Statistical methods - Where data on a particular endpoint were collected from both sexes, analyses were conducted separately. All statistical tests were made using a significance level of $\alpha = 0.05$. For

continuous data including body weight, weight gain and organ weights, which are most often normally distributed, one-way ANOVA, followed by a Dunnett's test was used to compare treatment versus control groups. For hormone data, which are usually non-normally distributed and have high inter-individual variability, a screening for outliers was made, based on a Box and Whisker Plot procedure and considering as outliers the values that were outside the box boundaries by more than 3 times the size of the box itself. In the case of hormone ratios, the same outliers of the single evaluation were considered. Nonparametric Kruskal-Wallis' tests, using beta approximation, were used in cases where data were not normally distributed (all hormones). Counting data, not normally distributed, were also analyzed with appropriate regression models. Where the observations were grouped (such as for litter data), fixed and mixed effect models were estimated (litter as random effect) and both reported. For biological parameters related to the body weight (such as the AGD), statistical analyses were always performed including the body weight of each pup in the regression model. The incidence of pathological lesions, reported as the numbers of animals bearing lesions, were compared using a two-tail Fisher exact test. The statistical analysis was performed using Stata/IC 10.1 (for all regressions) and Statisti× 10 (for all the other tests); graphs were obtained using Microsoft Excel and Statisti× 10.

Results

No statistically significant differences were observed between the control and the glyphosate and MON 52276 groups for gestational index, mean gestational length, relative weight gain during pregnancy, relative weight gain during lactation, total number of pups delivered at PND 0, litter size, sex ratio, mean life birth index, number of dams with reported stillbirths, number of still born pups, and survival index on PND 1 and PND 21. Also no treatment related effects were observed for water or feed consumption during gestation or lactation. In pups, AGD on PND 4 was statistically significantly increased both in MON 52276 treated males and females and in glyphosate treated males. Results were still significant after running multilevel linear regression models adjusted for body weight and litter as a random effect. Post-weaning body weights as well as water and feed consumption showed no difference in both female and male offspring. In female offspring, age and body weight at vaginal opening (VO) was similar across treatment groups, however, age at FE was statistically significantly delayed in the MON 52276 exposed group. Female offspring in the control and glyphosate treated groups presented the FE within 6 days from the VO, while in the MON 52276 treated group 2/10 females presented a more than doubled interval (12 and 14 days) between VO and FE. In female pups followed up to 13-weeks (N = 30), the percent of time spent in each stage of the estrous cycle did not differ between glyphosate and MON 52276 treated animals and controls. In male offspring, exposure to glyphosate or MON 52276 did not affect BPS or sperm parameters (number of mature spermatids in the testis, daily sperm production, number and sperm transit time through caput/corpus and cauda epididymis and morphology). There were no treatment-related gross lesions in F0 and F1 reproductive and endocrine organs in either sex and there was no statistically significant effect on absolute and relative organ weight of adrenal glands, uterus and ovaries in the dams, adrenal glands, testis, epididymis, bladder/prostate and seminal vesicles/coagulating glands in male offspring (with the exception of a decrease in absolute epididymal weight in the 13-week cohort), and adrenal glands, uterus and ovaries in female offspring.

Most pituitary hormones were unaffected by exposure to glyphosate or MON 52276 in males, with the exception of a statistically significant increase in plasma TSH in the glyphosate group of the 6-week cohort and the MON 52276 group of the 13-week cohort and a statistically significant increase in BDNF in the MON 52276 group of the 6-week cohort. Apart from a statistically significant decrease in DHT in MON 52276 treated males of the 13-week cohort and a statistically significant increase in total testosterone in MON 52276 treated females of the 13-week cohort no effects were found on sex hormones. Hormone ratios were calculated as indicators of the general balance between hormones and sex steroid hormone bioavailability. The TT/SHBG ratio was statistically significantly increased in MON 52276 treated females of the 13-week cohort. The E2/SHBG ratio was statistically significantly increased in MON 52276 treated males of the 6-week cohort. The fT/TT ratio was statistically significantly decreased in glyphosate treated males of the 6-week cohort and in MON 52276 treated males of the 13-week cohort. Male and female MON 52276 treated rats of the 13-week cohort showed a marked and statistically significant decrease in DHT/TT ratio. No such differences were observed for DHT/TT ratio in males and females of the 6-week cohort and no statistically significant differences were

observed for the E2/TT ratio in males and females of the glyphosate and MON 52276 treated groups of both cohorts.

Discussion

MON 52276, when administered to rats from in utero through adulthood at a dose level corresponding to the glyphosate RfD defined by the US EPA (1.75 mg/kg bw/day), elicited subtle but potentially adverse effects on reproductive development and hormone concentrations. Overall, these effects indicate an impact on pre- and peri-pubertal sexual maturation. The effects of treatment with glyphosate were essentially limited to increased AGD and TSH concentration, and both changes were specific to males. MON 52276 seemed to affect both females and males, resulting in a statistically significant increase of AGD and sexual hormones imbalances in both cohorts. Statistically significant differences in apical endpoints (AGD and FE) together with changes in hormonal activity detected in both treatment groups should be taken into account suggesting evidence for reproductive toxicity via an endocrine disruption mechanism. A longer AGD at birth in both sexes and an increased age at FE, together with the increased TT in female offspring, are considered endpoints for androgen-mediated activity. The significant increase in AGD and the delay in the appearance of the first estrous cycle observed in MON 52276-treated female rats is consistent with increased developmental androgenization. The first ovulation is the true endpoint of a series of morphological and functional changes at different levels of the hypothalamic–pituitary–gonadal (HPG) axis, hence, it constitutes the unequivocal sign that puberty has been achieved. No difference in the achievement of vaginal opening (VO) was observed among the groups tested. In males, a prolonged, albeit of low-intensity, androgenizing effect could eventually evoke a counteracting feed-back response from the HPG axis. As apical endpoint, an increased AGD was observed in both treatment groups. Hormone profiling in males, revealed a decreased serum DHT in the MON 52276 group of the 13-week cohort, suggesting an effect on TT metabolism after a prolonged exposure. The lower conversion of TT to DHT might indicate a possible reduction in 5α-reductase enzyme activity. However, this effect was not observed in females of the 6-week cohort. It is of note that males treated with MON 52276 showed normal seminiferous tubules and sperm production which is consistent with the absence of any effect on testosterone and FSH. A statistically significant increase in TSH was observed in glyphosate treated males of the 6-week cohort and MON 52276 treated males of the 13-week cohort. Since no histological changes were observed in the thyroid gland, the increased plasma concentration of TSH can be considered as not indicative of a thyroid related effect. BDNF is a neurotrophin playing a fundamental role in survival and differentiation of selected neuronal populations during development, and in the maintenance and plasticity of neuronal networks during adulthood. A statistically significant increase in BDNF was observed in MON 52276 treated males of the 6-week cohort but not in males of the 13-week cohort. BDNF is an explorative and new endpoint for neurodevelopment and the utility of neurotrophins as potential biomarkers is not completely understood.

The present study has some limitations. First, this is a pilot study performed on a limited number of animals where only one dose was used. However, the dose was selected specifically for its relevance to human health risk assessment, as it is the chronic current RfD defined by the USEPA (1.75 mg/kg bw/day) and therefore a dose level that is expected to be “safe”. Furthermore, the number and timing of blood sample collection was limited to the final sacrifice of animals, considering that this was a pilot study and that *in vivo* blood sampling could lead to maternal and pups stress. Another source of uncertainty, which is currently difficult to assess, is the timing of blood sampling during the necropsy session (9.00 am – 3.00 pm), a circadian-dependent modulation of circulating hormones cannot be completely ruled out. Standard errors in different hormone concentrations were large, in relation to the relatively small group sizes and the physiological variability of hormone concentrations. In females, the estrous cycle status at the time of necropsy is another important source of variability when analyzing sexual hormone profiles. However, even if sacrificing animals on a specific day of the cycle might improve the ability to observe changes in the baseline hormone concentrations, the issue of sacrificing animals in the same cycling period (e.g estrous) is still controversial. The updated OECD Test Guidelines on reproductive-developmental toxicity do not require the sacrifice of females in the same stage of estrous, only the examination of the estrous cycle on the day of necropsy is recommend to allow correlation with the histopathology in reproductive organs. Finally, the adjuvant(s) present in Roundup Bioflow (corresponding to 16% of the formulation) could not be studied since the nature of the co-

formulants is a trade secret. These are supposed to be surfactants, diluents or adjuvants stabilizing glyphosate and allowing its penetration in plants. The majority of significant changes observed in hormonal status emerged in the 13-week cohort (animals sacrificed at adulthood) compared to the 6-week cohort (animals sacrificed after puberty) suggesting that more prolonged exposures were more effective in producing imbalances in the hormone concentrations. In this experiment, MON 52276 was shown to be definitely more potent than glyphosate alone.

Conclusions

The present study demonstrates that exposure to MON 52276 at a dose level equivalent to 1.75 mg glyphosate acid/kg bw/day, from the prenatal period to adulthood, induced endocrine effects and altered reproductive developmental parameters in male and female SD rats. MON 52276 exposure was associated with androgen-like effects, in particular in females, including a statistically significant increase of ano-genital distance in both males and females, a delay of first estrous and increased testosterone in females. MON 52276 exposure was also associated with altered testosterone metabolism in both males and females, where a statistically significant decrease in DHT/TT ratio was observed in the longest treated group (13-week cohort). Overall, MON 52276 elicited more pronounced effects than glyphosate, which only increased anogenital distance and TSH concentration in male rats in the peripubertal window (6-week cohort).

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this pilot study the effect of glyphosate and its reference formulation Roundup Bioflow (MON 52276) at a dose of 1.75 mg glyphosate acid eq./kg bw/day on endocrine modulation was investigated in female rats during pregnancy and lactation and in male and female rats during lactation, the peripubertal period and adulthood. The endpoints explored were body weight, water and food consumption, gestational parameters, litter parameters, landmarks of sexual development, estrous cyclicity, gross and histopathology of reproductive and endocrine tissues, sperm parameters and serum and plasma hormone levels. MON 52276 exposure was associated with statistically significant increase of ano-genital distance in males and females, a delay of first estrous and increased serum testosterone in females and altered testosterone metabolism in both males and females. MON 52276 elicited more pronounced effects than glyphosate, which only increased statistically significantly anogenital distance during the peripubertal period. The statistically significant increase in TSH levels in glyphosate and MON 52276 treated rats was not associated with histopathological changes in the thyroid and thus of minor toxicological significance. The effect of glyphosate on ano-genital distance is not corroborated by a regulatory reproductive toxicology study where rats were exposed to much higher doses of glyphosate (> 1,000 mg/kg bw/day).

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the limited number of animals used per dose level and only one dose level was tested.

Reliability criteria for *in vivo* toxicology studies

Publication: Manservisi *et al.,* 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Pilot study based on OECD test guideline 443 but with deviations.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	

Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of > 99.5% as Pestanal™ . Source: Sigma-Aldrich, Milan, Italy.
Only glyphosate acid or one of its salts is the tested substance	N	Also representative formulated product tested. Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%), water (42.5%) and surfactant (16%)). Source: Consorzio Agrario dell'Emilia, Bologna, Italy.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral via the drinking water.
Dose levels reported	Y	1.75 mg glyphosate acid eq./bw/day.
Number of animals used per dose level reported	Y	Dams: 8/group. Offspring: 8 M + 8F/group (6-week cohort); 10 M + 10F/group (13-week cohort).
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	Not possible, only comparison between glyphosate and MON 52276.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered as relevant for the risk assessment of glyphosate but reliable with restrictions because of the limited number of animals used per dose level and only one dose level was tested.		

1. Information on the study

Data point:	CA 5.7
Report author	Martinez A. *et al.*
Report year	2019
Report title	Effects of glyphosate and aminomethylphosphonic acid on an isogeneic model of the human blood-brain barrier.
Document No	Toxicology Letters (2019) Vol. 304, 39-49.
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, the effect of acute exposure to glyphosate (GPH) on the blood-brain barrier in vitro based on induced pluripotent stem cells (iPSCs) and compared to two chemical analogs: aminomethylphosphonic acid (AMPA) and glycine (GLY), for concentrations ranging from 0.1 μM to 1000 μM was investigated.

Materials and methods

Chemicals - Glyphosate (EPA 547 1,000 μg/mL solution), aminomethylphosphonic acid (AMPA) and glycine (GLY) were purchased as analytical grade reagents from Sigma-Aldrich, St. Louis, USA. The purity of the test chemicals was not reported.

Cell culture - Induced pluripotent stem cell line IMR90-c4 iPSC (RRID: CVCL_C437) was purchased from WiCell cell repository (WiCell, Madison, WI). iPSC colonies were maintained on hPSC-grade growth factor reduced Matrigel (C-Matrigel, Corning, Corning, MA) in the presence of Essential 8 medium (E8, ThermoFisher, Waltham, MA).

iPSC differentiation - iPSCs were differentiated into brain microvascular endothelial cells (BMEC). iPSCs were seeded as single cells on T-Matrigel at a cell density of 20,000 cells/cm^2 in E8 supplemented with 10 μM Y-27632. 24 hours after seeding, cells were maintained in E8 for 5 days prior to differentiation. Cells were maintained for 6 days in unconditioned medium (UM: DMEM/F12 with 15 mM HEPES, 20% knockout serum replacement, 1% non-essential amino acids, 0.5% Glutamax and 0.1 mM β-mercaptoethanol). After 6 days, cells were incubated for 2 days in the presence of EC+/+ (EC medium supplemented with 1% platelet-poor derived serum, 20 ng/mL human recombinant basic fibroblast growth factor (bFGF) and 10 μM retinoic acid). After such maturation process, cells were dissociated by Accutase® treatment and seeded as single cells on tissue-culture plastic surface (TCPS) coated with a solution of collagen from human placenta and bovine plasma fibronectin at 80 μg/cm^2 and 20 μg/cm^2, respectively. Twenty-four hours after seeding, cells were incubated in presence of EC-/- (EC medium supplemented with 1% platelet poor derived serum (PDS)). Barrier phenotype experiments were performed 48 hours after seeding. Differentiation of iPSCs into neurons was done using an adherent 3-step differentiation method. Co-culture experiments were performed by seeding iPSC-derived BMECs at day 8 of differentiation on inserts juxtaposed over 16-days iPSC-derived neurons. BMECs were maintained in EC medium, whereas neurons were maintained in neuron maturation medium (NMM).

Glyphosate, AMPA and glycine treatment - Dilutions of glyphosate, AMPA and glycine were made immediately before the experiments and maintained in cell medium for 24 or 48 hours. In co-culture experiments, the test compounds were added in the apical chamber at a concentration of 100 μM and incubated for 6 hours. iPSC-derived neuron monocultures exposed to similar concentrations served as controls.

Cell metabolic activity - Following treatment, CellTiter Aqueous® MTS reagent ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) was added to each sample following recommendations by the manufacturer. Cells were maintained for 60 minutes at 37 °C followed by a measurement of absorbance at 490 nm using an ELISA plate reader. Absorbance obtained from the test samples were subtracted from background absorbance and normalized against controls (untreated cells).

iPSC-derived BMECs barrier function - iPSC-derived BMECs were seeded at a seeding density of 10^6 cells/cm^2 on Transwells (polyester, 0.4 μm pore size, Corning) and coated as previously described. Barrier function was assessed 48 hours after seeding of iPSC-derived BMECs monolayers. Barrier tightness was measured by assessing both the transendothelial electrical resistance (TEER) and paracellular diffusion. TEER was measured using an EVOHM STX2 chopstick electrode. For each experiment, three measurements were performed for each insert and the average resistance obtained was used for the determination of the barrier function.

Fluorescein, glyphosate and mannitol permeability assay - To assess changes in paracellular permeability, sodium fluorescein was added in the apical (top) chamber at a final concentration of 10 μM. 100 μL aliquots were sampled from the basolateral (donor) chamber every 15 minutes for up to 60 minutes. Each aliquot sample was replaced with 100 μL of cell medium. Fluorescein content in the samples was assessed using a fluorimeter ELISA plate reader. Glyphosate permeability was assessed by incubating cells in the presence of 100 μM glyphosate dissolved in EC-/- in the apical chamber. Sampling in the basolateral chamber occurred as previously described. For the determination of glyphosate the samples taken were alkalinized with 17 μL of borax solution followed by the addition of 17 μL of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) solution for the derivatization of glyphosate. Samples were allowed to incubate in the dark under gentle shaking for 2 hours. The derivatization process was terminated by adding 137 μL dichloromethane. The sample was homogenized and centrifuged at 2000 rpms for 5 minutes to separate the organic phase and analysis by spectrophotometry at 265 nm. Blank EC-/- medium was used as the blank, whereas glyphosate dissolved in EC-/- at concentrations ranging from 10 nM to 10 μM was used to establish a standard curve. For the measurement of mannitol permeability, [^{14}C] D-mannitol was added in the apical chamber with sampling in the basolateral chamber as described previously. Radioactivity was assessed by adding 100 μL sample to 5 mL liquid scintillation cocktail and counted using a Beckman-Coulter LS6500 liquid scintillation counter. The permeability across BMECs monolayers was obtained by calculating the clearance slope from both samples and blank inserts and by the calculation of the Pe value following the method of Perriere *et al*.

Immunocytochemistry - Cells were stained on tissue culture polystyrene (TCPS) plates and fixed with 4% paraformaldehyde. Cells were blocked for 1 hour at room temperature in PBS supplemented with 10% normal goat serum (PBS-G) with 0.2% Triton-X100 and were then incubated overnight in the presence of claudin-5, occludin, GLUT1 or βIII tubulin. Cells were washed with PBS containing 1% bovine serum album, and incubated in the presence of Alexa Fluor®-488 conjugated secondary antibodies for 1 hour at room temperature. Thereafter the cells were counterstained with DAPI and observed on a Leica inverted epifluorescence microscope. Micrograph pictures were acquired using Leica Acquisition Suite X and processed using ImageJ. Semi-quantitative analysis was done by measuring the average fluorescence intensity of each micrograph picture using the built-in measure tool in ImageJ. Average fluorescence values from negative controls were subtracted from the fluorescence values obtained in the test samples.

Flow cytometry - iPSC-derived BMECs at Day 10 of differentiation were treated with 100 μM glyphosate, AMPA or glycine for 24 hours. Cells were harvested by enzymatic dissociation using Accutase® and fixed with 4% paraformaldehyde. Cells were blocked in PBS-G supplemented with 0.2% Triton-X100 dissolved in PBS for 30 minutes, following by an overnight incubation at 4 °C in primary antibody solution (GLUT-1, SPM498 dissolved in PBS-G). Cells were then washed with PBS containing 1% BSA and incubated in the presence of Alexa Fluor® 555-conjugated antibody. As isotype control, cells were exposed to mouse IgG as primary antibody and analyzed using a BD FACSVerse®, with a fluorescence photomultiplier tube (PMT) adjusted to IgG isotype control. Fluorescence intensity for

each sample was obtained from a count of 10,000 cellular events. Median fluorescence intensity (MFI, geometric mean) was determined for each sample and corrected against IgG isotype.

Glucose and doxorubicin uptake assay - Glucose uptake assays were performed by incubating cells grown on TCPS in presence of cell medium supplemented with [^{14}C] D-glucose. Cells were incubated for 60 minutes at 37 °C. Afterwards the cells were washed with ice-cold PBS and homogenized with PBS + 0.2% Triton-X100 for 10 minutes. Radioactivity was assessed by adding 100 µL sample to 5 mL liquid scintillation cocktail and counted using a Beckman-Coulter LS6500 liquid scintillation counter. The doxorubicin uptake assay was performed by pre-incubating iPSC-derived BMECs in the presence of glyphosate, AMPA or glycine at 100 µM for 2 hours. Doxorubicin was added to obtain 5 µM as a final concentration and allowed to incubate for 1 hour. Cells were homogenized as previously described and total fluorescence assessed by fluorimetry. Total protein content obtained from cell homogenates was determined using a BCA protein assay.

Statistical analysis - Cells were randomly assigned treatment conditions prior each experiment. Data are represented as mean ± S.D. from three or more independent experiments. One-way analysis of the variance (ANOVA) coupled with Dunnett (or Kruskal-Wallis) tests analysis were performed using Prism 7.0 built-in package (GraphPad Software). A p-value < 0.05 was considered as statistically significant.

Results

BMEC cell viability – For the assessment of the effect of glyphosate on the viability of BMECs cell monolayers a range of concentrations was used partially overlapping the levels found in patients reported as asymptomatic, minor and moderate (17, 241, and 428 µM, respectively). Treatment with glyphosate, AMPA or glycine for 24 hours at concentrations ranging from 10 to 1000 µM resulted in no changes in cell metabolic activity. This indicates that glyphosate and AMPA unlikely have toxicity towards the blood-brain barrier.

Fluorescein permeability in BMECs monolayers - Changes in the barrier function in BMECs monolayers were measured using TEER and fluorescein permeability. In addition to the previous concentrations used, 2 concentrations (0.1 and 1 µM) were included to reflect average plasma concentrations reported in occupational exposure. No changes in TEER were noted for any of the concentrations tested of glyphosate, AMPA or glycine. However, a biphasic response was noticed in fluorescein permeability. At 0.1 µM, a slight but not statistically significant decrease in fluorescein permeability was found for glyphosate, AMPA and glycine followed by a statistically significant increase for both glyphosate and AMPA at 1 and 10 µM but not at 100 and 1,000 µM. To confirm the increase in the paracellular profile of glyphosate, changes in paracellular permeability were investigated using [^{14}C]-mannitol, an alternative paracellular flux marker at 1 and 10 µM. A modest but statistically significant increase in mannitol permeability was observed at 10 µM glyphosate. No significant increase was noted following AMPA or glycine treatment.

Tight junction complexes integrity - To better understand the effect of glyphosate and AMPA on the barrier function, changes in tight junction complexes were investigated, in particular changes in claudin-5 and occludin, by immunocytochemistry. No changes in claudin-5 immunolocalization were observed. However, a dose-dependent decrease in claudin-5 relative expression was noted in all groups as quantified by fluorescence intensity. Glyphosate decreased claudin-5 fluorescence intensity at 100 and 1000 µM, but treatment with AMPA already decreased significantly claudin-5 fluorescence intensity at 10 µM. No changes in occludin localization occurred following treatment although a significant decrease in occludin protein levels was noted in all treatment groups with the exception of 10 µM glyphosate. Unlike claudin-5, this effect appeared to be dose-independent.

Diffusion across the BBB - The ability of glyphosate to cross the blood brain barrier (BBB) was investigated following a single exposure at 100 µM in the apical chamber for 2 hours and measurement of the amount of glyphosate present in the basolateral chamber. After 2 hours of diffusion, the amount of glyphosate capable of crossing BMEC monolayers was about 1.67 ± 0.31% of the applied dose. It

was found that the permeability of glyphosate was significantly greater than fluorescein ($18.67 \pm 3.55 \times 10^{-6}$ cm/min versus 10.59×10^{-6} cm/min) or mannitol ($13.10 \pm 2.03 \times 10^{-6}$ cm/min). Also the effect of glyphosate and AMPA on drug efflux transporters was investigated using doxorubicin as a drug efflux substrate. With the exception of AMPA that showed a 2-fold increase over control, exposure to 100 μM glyphosate or glycine for 24 hours showed no differences compared to controls.

Modulation of glucose uptake in BMECs - As previous studies reported changes in glucose levels in certain vertebrates following exposure to glyphosate and AMPA changes in GLUT1 (the main glucose transporter at the BBB) localization and expression in BMECs following treatment by immunocytochemistry were studied. Following exposure at 100 μM glyphosate, but not at 1,000 μM, an apparent increase in GLUT1 immunoreactivity was noted. Similar results were obtained with AMPA although less pronounced. A flow cytometry analysis was performed where changes in mean fluorescence indexes were compared following exposure at 100 μM for 24 hours. Exposure to glyphosate yielded an increase in GLUT1 expression levels compared to control. Although AMPA showed no differences in GLUT1 expression, glycine exposure resulted in a significant decrease compared to control.

Barrier function of neurons co-cultured with BMECs - As glyphosate showed the ability to cross the BBB and produced changes in GLUT1 expression and glucose uptake in BMECs monolayers, the effects of glyphosate on neurovascular coupling using a BMEC/neurons co-culture model was investigated. First, the ability of such co-cultures to yield barrier function was assessed by measuring differences in TEER between BMECs monocultures and BMECs co-cultured with iPSC-derived neurons. A 3-fold increase in TEER in BMECs co-cultured with neurons compared to BMECs maintained in monocultures was observed. These co-cultures were then exposed to 100 μM glyphosate, AMPA or glycine for 24 hours. TEER measurements indicated that there was no statistically significant difference in barrier tightness when compared to controls. A mild increase in fluorescein permeability was noted with glyphosate when compared to control, but not with AMPA or glycine.

Neurovascular coupling - The effect of glyphosate on neurovascular coupling was investigated by measuring changes in neural cell metabolic activity using MTS following exposure to 100 μM for 6 hours. A significant decrease in cell metabolic activity was observed in co-cultured neurons when compared to monocultures. When co-cultured neurons were exposed to glyphosate and AMPA the metabolic activity was statistically significantly increased when compared to controls. Glyphosate and AMPA produced no statistically significant changes in metabolic activity of mono-cultured neurons. When the effect of glyphosate, AMPA and glycine was investigated in iPSC-derived neuron colonies by immunocytochemistry against βIII-tubulin, no changes were observed both in monocultures and co-cultures, suggesting that changes in cell metabolic activity is unlikely due to cell death.

Neuron progenitor cells (NPC) - The effect of glyphosate on differentiating and differentiated neurons was investigated by exposing cells to a concentration considered representative of the amount crossing the BBB i.e. 0.1–1 μM. First, the effect of an exposure of 24 hours on the cellular metabolic activity of undifferentiated NPCs was investigated using the MTS assay. A significant decrease in cell metabolic activity was seen at concentrations of glyphosate and glycine of 1 μM although immunofluorescence analysis of these NPCs showed no major alterations in the relative cell density and nestin (a cellular marker of neural stem cells/progenitor cells) immunoreactivity. Then, differentiating NPCs were treated continuously for 16 days (by replacing cell medium every 48 hours) in the presence of 0.1 μM glyphosate, AMPA or glycine. The concentration tested is representative of plasma concentrations reported in occupational workers and is 20 times higher than values reported in non-occupational population. No significant changes in cell metabolic activity were observed between the different groups compared to controls. After 16 days of exposure to glyphosate or AMPA no changes in gross morphology of iPSC-derived neuron colonies were evident.

Neurites density - The exposure of iPSC-derived neurons seeded at low density (50,000 cells/cm^2) to glyphosate or AMPA for 24 hours at concentrations ranging from 1 μM to 1000 μM decreased statistically significantly cell metabolic activity at 10 μM and beyond. AMPA showed similar results, albeit not statistically significant. Treatment with glycine showed only small effects on cell metabolic

activity. Changes in cell density and neurites formation by immunocytochemistry were also investigated and, with the exception of glycine, no depletion in neurites was observed with glyphosate and AMPA. Upon quantification of cell nuclei and neurites per surface area a progressive decrease in neuron density was noted for both glyphosate and AMPA, with a significant decrease at 1000 µM. However, no differences in neurite density were noted with glyphosate and AMPA, with the exception of glycine at 100 and 1000 µM.

Discussion

The toxicity of acute glyphosate poisoning on blood-brain barrier integrity was investigated by assessing its activity on the different cell types of the neurovascular unit. Cells were exposed to glyphosate and AMPA at concentrations ranging from 10 to 1000 µM. This is the concentration range of glyphosate plasma values in patients with self-inflicted poisoning. An increase in fluorescein permeability was noted for glyphosate and AMPA at 1 µM and 10 µM. A similar outcome was noted for mannitol in cells exposed to 1 µM glyphosate. This suggests a possible detrimental effect of glyphosate and AMPA on blood-brain barrier function. Although no major changes in tight junction complexes localization were observed, a decrease was found in both claudin-5 and occludin protein levels after exposure to glyphosate, AMPA and glycine, suggesting that glyphosate and AMPA may interfere with tight junction complexes integrity. Yet, the interference of such compounds on tight junction proteins remains unclear. Although not statistically significant, a 50% increase in paracellular permeability was noted with glycine at 100 and 1000 µM. The data from this study suggest that high levels of glycine may increase the permeability of the BBB and disrupt tight junction complexes. In addition to changes in barrier function, glyphosate permeability in BMEC monolayers was assessed. It is estimated that about 1% of the applied dose (100 µM) diffused across BMECs monolayers. However, the permeability for glyphosate was significantly higher than that for fluorescein despite its high hydrophilicity (xLogP = -4.63). This indicates that glyphosate crosses the BBB via carrier-mediated diffusion. Amongst the different cell types, neurons displayed the most important changes in metabolic activity following exposure to glyphosate and AMPA. Significant changes in neuronal cell metabolic activity were observed following exposure to glyphosate, AMPA or glycine whereas such changes we not observed in BMECs. Such decrease in cell metabolic activity was unlikely to be considered as neurotoxicity since these effects didn't translate in changes in neuronal cell density and neurites formation. The change in cell metabolic activity observed may be due to changes in glucose metabolism, as changes in glucose uptake in BMECs, as well as some changes in GLUT1 expression levels were noted.

Conclusion

The data from this study demonstrate the relative safety of glyphosate and AMPA with regard to the blood-brain barrier after acute exposure with minimal effects observed at concentrations significantly higher than baseline exposure levels, occupational and non-occupational alike. The presence of an active uptake and diffusion of glyphosate across the blood-brain barrier suggests the need of extensive brain-centered studies to evaluate the pharmacokinetics and pharmacodynamics of glyphosate on the central nervous system during acute exposure and in individuals exposed to high amounts of such pesticides.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effect of glyphosate, AMPA and glycine was investigated on he integrity of the blood-brain barrier *in vitro* using an induced pluripotent stem cell line differentiated into brain microvascular endothelial cells (BMEC) and neurons. The endpoints investigated were BMEC cell viability, fluorescein permeability in BMEC cell monolayers, tight junction complexes integrity, diffusion across the blood-brain barrier, modulation of glucose uptake in BMECs, barrier function of neurons co-cultured with BMECs, neurovascular coupling, differentiation of neuron progenitor cells and neurites density. The results of this study indicate that glyphosate or AMPA unlikely present toxicity towards the blood-brain barrier, glyphosate and AMPA at 1 and 10 µM may increase the barrier permeability in BMECs monolayers, glyphosate may increase paracellular permeability in BMECs

monolayers to fluorescein via partial disruption of tight junction complexes integrity, exposure to high levels of glycine or AMPA (100 µM) may impair glucose uptake and metabolism in BMEC monolayers via an alteration in GLUT1 expression and/or activity, exposure to high concentrations of glyphosate (100 µM) may impair neurovascular coupling, chronic exposure to low levels (0.1 µM) of glyphosate or AMPA failed to show any signs of neurotoxicity and that low concentrations (< 10 µM) of glyphosate and AMPA may not have detrimental effects on iPSC-derived neurons.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not sufficiently characterized and no positive controls were used in any of the assays conducted.

Reliability criteria for *in vitro* toxicology studies

Publication: Martinez *et al.,* 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of glyphosate and AMPA not reported. Source: Sigma-Aldrich, St. Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also glycine and AMPA tested.
AMPA is the tested substance	Y	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Concentration range *in vitro* from 0.1 to 1000 µM for some tests.
Cytotoxicity tests reported	Y	
Biochemical methods described	Y	Some could be better documented.
Analytical method described	Y	The method for the analysis of glyphosate.
Positive and negative controls	N	No positive controls were used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	For some tests
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with		

restrictions because the glyphosate used was not sufficiently characterized and no positive controls were used in any of the assays conducted.

1. Information on the study

Data point:	CA 5.7
Report author	Martínez M. et al.
Report year	2018
Report title	Neurotransmitter changes in rat brain regions following glyphosate exposure
Document No	Environmental Research 161 (2018) 212 - 219
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The effects of glyphosate oral exposure (35, 75, 150 and 800 mg/kg bw, 6 days) on brain region monoamine levels of male Wistar rats were examined. Glyphosate-treated rats (35, 75, 150 and 800 mg/kg bw, 6 days), had no visible injury, i.e., no clinical signs of dysfunction were observed. After last dose of glyphosate, serotonin (5-HT), dopamine (DA) and norepinephrine (NE) and its metabolites levels were determined in the brain regions striatum, hippocampus, prefrontal, cortex, hypothalamus and midbrain, by HPLC. Glyphosate caused statistically significant changes in the 5-HT and its metabolite 5-hydroxy-3-indolacetic acid (5-HIAA), DA and its metabolites 3,4-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and NE and its metabolite 3metoxy-4-hydroxyphenylethyleneglycol (MHPG) levels in a brain regional- and dose-related manner. Moreover, glyphosate, dose-dependent, evoked a statistically significant increase in 5-HT turnover in striatum and hypothalamus and in DA turnover in prefrontal cortex and hippocampus, and a statistically significant decrease in NE turnover in prefrontal cortex and hypothalamus. The present findings indicate that glyphosate significantly altered central nervous system (CNS) monoaminergic neurotransmitters in a brain regional- and dose-related manner, effects that may contribute to the overall spectrum of neurotoxicity caused by this herbicide.

Materials and methods

Chemicals; Glyphosate [N-(phosphonomethyl) glycine], molecular formula $C_3H_8NO_5P$ CAS RN 107-83-6, purity ≥ 98%, serotonin (5-HT) and its metabolite [5-hydroxy-3-indolacetic acid (5-HIAA)], dopamine (DA) and its metabolites [3,4-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] and norepinephrine (NE) and its metabolite [3-metoxy-4-hydroxyphenylethyleneglycol (MHPG)] were purchased from Sigma-Aldrich, St Louis, MO, 63103 USA. All other chemicals were of the highest quality grade and obtained from commercial sources.

Animals and experimental design; All experiments using live animals were undertaken in accordance with the ethics requirements and authorized (protocol number 086) by the official ethical committee of our university. Male Wistar rats of 60 days old each weighing 200–210 g (Charles River Inc., Margate, Kent, UK) were used. The animals were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2 °C and 50 ± 10% relative humidity) with a 12 h light/dark cycle (light from 08.00 to 20.00 h). Food (A04 rodent diet, Scientific Animal Food & Engineering, SAFE, Augy, France) and water were available *ad libitum*. Thirty male rats were assigned randomly to five groups of 6 animals each, a control group and four glyphosate treated groups. Animal treated groups received glyphosate orally at the dose of 35, 75, 150 and 800 mg/kg bw [equivalent to 1/160, 1/75, 1/37 and 1/7 of the acute oral rat LD50 ≈ 5.6 g/kg bw] for 6 consecutive days. The doses were chosen taking into account the LD50 oral value as well as the NOAEL (no observed adverse effect level) described in the literature. The glyphosate treated group rats were deprived of food for 6 h before the oral administration of glyphosate, but were allowed water ad libitum. Glyphosate was

dissolved in water and was administered orally by gavage in a maximum volume of 2 mL/rat. Control animals received the vehicle (water) on the same schedules. The animal body weights were measured during the study and food and water consumption of each animal was also assessed. The animals received the treatment at the same time each day, specifically between 10.0h and 11.0h a.m. Three hours after the last dose, the animals were sacrificed by decapitation. The brain was removed quickly and hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex tissues rapidly dissected out at 4 °C. Tissues were rapidly weighed and stored at -80 °C until analysis.

Determination of monoamine levels; The five brain regions analyzed in the present study were hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex. Following sample collections, 300–800 µL of 0.4 M $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ was added to the tissues, and the mixture was homogenized (1 min) by sonication (Labsonic U-Braun). The homogenates were centrifuged (RC5C, Sorvall Instruments) at 12,000g for 20 min at 4 °C and aliquots of supernatants were taken for analysis of 5-HT and its metabolite 5-HIAA, DA and its metabolites DOPAC and HVA and NE, using a high performance liquid chromatography (HPLC) technique with electrochemical detection. Also, aliquots of supernatants were taken for analysis of the norepinephrine metabolite MHPG by HPLC with fluorimetric detection. An acid-catalyzed procedure was used to hydrolyze MHPG-sulphate in homogenates of brain region tissues. Volumes of 200–300 µL of the supernatants (in 0.4 M $HClO_4$) were treated for 3 min at 100 °C in a water bath. The samples were then cooled and 30 - 45 µL of 2 M NaOH were added (final pH: ca. 1.5) and aliquots were injected into a reverse phase HPLC system. For the analysis of catecholamines NE, DA, DOPAC and HVA, the mobile phase consisted of 0.1 M $Na_2HPO_4 \cdot 2H_2O$, 0.1 M citric acid (pH 3.5), 1.6 mM octane sulphonic acid, 0.9 mM EDTA and 10% (v/v) methanol. For the analysis of the indolalkylamines 5-HT and 5-HIAA, the mobile phase consisted of 0.1 M $Na_2HPO_4 \cdot 2H_2O$, 0.1 M citric acid (pH 3.5) and 10% (v/v) methanol. Elution was performed at a flow rate of 1 mL/min and the working electrode potential was set at 0.8 V for catecholamines and 0.7 V for indolalkylamines. The HPLC system consisted of a Shimadzu liquid chromatograph, model LC-9A equipped with a 5 µm particle size C18-Nucleosil reversed phase column (4 mm i.d. × 125 mm) proceeded by a C18 precolumn, an electrochemical detector (Shimadzu, model L-ECD-6A), a sample injector (20 µL valve) and an integrator (Shimadzu, model C-R6A Chromatopac). For the analysis of the norepinephrine metabolite (MHPG), the mobile phase consisted of 0.06 M $Na_2HPO_4 \cdot 2H_2O$, 0.03 M citric acid and 6% (v/v) methanol. Elution was performed at a flow rate of 1.5 mL/min. The HPLC system consisted of a Shimadzu liquid chromatograph, model LC-10AS, a 25 µm particle size Tracer Extrasil ODS reversed phase column (4 mm i.d. × 125 mm), a fluorescence detector (Shimadzu, model RF-551), a sample injector (20 µL valve) and an integrator (Shimadzu, model C-R6A Chromatopac). Excitation and emission wavelengths of the detector were 275 and 315 nm, respectively. Peak areas from the sample chromatograms were used to quantify the analytes by external standard technique using solutions of catecholamines (NE, DA, DOPAC y HVA), indolalkylamines (5-HT and 5-HIAA) and norepinephrine metabolite (MPHG) reference standards (Sigma Chemical Co., St Louis, MO, USA). For tissue specimens as determined by use of a linear least squares regression procedure, a linear relationship existed in the calibration curve of catecholamines (NE, DA, DOPAC, HVA), indolalkylamines (5-HT, 5-HIAA) and norepinephrine metabolite (MPHG) over the range of 0.002–100 µg/g, which always yielded a correlation coefficient exceeding 0.9998. Overall mean recovery of catecholamines (NE, DA, DOPAC and HVA), indolalkylamines (5-HT and 5-HIAA) and norepinephrine metabolite (MPHG) from tissues was 100% for every analyte. Within- and between-day variation was < 4%. Quantification limit (LOQ) was 2 ng/g for NE, DA, DOPAC, 5-HT and 5-HIAA and 10 ng/g for HVA and MPHG in the different tissue matrices. NE, DA and 5-HT turnover were calculated as ratios of metabolites to neurotransmitter.

Data analysis; Statistical analysis of data was performed using GraphPad Prism 6 for Windows. Results are presented as mean ± S.D. of 6 animals per group. Results significantly different from controls are also presented as percentage change over control. One-way ANOVA was carried out to determine significant dose-dependent effect of glyphosate on 5-HT, DA, NE and metabolite levels and the corresponding turnover values in the brain regions studied, followed by Tukey's post hoc test. Statistical significance was set at $P < 0.05$. ANOVA's F values are presented in the Tables. F distribution was calculated with numerator degrees (DFn) and denominator degrees of freedom (DFd).

Results

The glyphosate-treated rats at oral doses of 35, 75, 150 and 800 mg/kg bw/day for 6 days had no visible injury. These doses were selected based on preliminary experiments where the doses and route of administration did not show any adverse effects, abnormal clinical signs as well as changes in body weight, food and water consumption in the animals (Table 1).

Table 1
Effect of glyphosate on body weight gain and food and water consumption in male rats.

Parameter	Animal groups				
	Control	Glyphosate (35 mg/kg bw, 6 days)	Glyphosate (75 mg/kg bw, 6 days)	Glyphosate (150 mg/kg bw, 6 days)	Glyphosate (800 mg/kg bw, 6 days)
Body weight gain (g)	29.17 ± 2.64	27.67 ± 4.23	29.33 ± 3.08	30.00 ± 4.00	27.33 ± 4.50
Food consumption (g)	96.33 ± 7.45	98.00 ± 7.27	95.66 ± 7.39	93.67 ± 9.24	95.50 ± 9.05
Water consumption (mL)	133.83 ± 20.92	131.50 ± 20.12	129.5 ± 21.50	140.50 ± 24.77	151.00 ± 9.19

Results are presented as means ± SD for six rats.
Results are not significantly different from control group.
Continuous probability distribution (F) for all parameters were lower than 1.140 (DFn = 4, DFd = 25).

All the rat groups exposed to glyphosate by oral route did not show statistically difference on weight of tissues (brain regions) or the ratio weight tissue/body weight (%) compared to control group (data not shown). Glyphosate at a dose of 35 mg/kg bw did not affect the 5-HT, DA, NE and metabolite levels in the brain regions studied. In this study, 35 mg/kg bw might be identified as the NOAEL based on neurotransmitter changes in CNS. Glyphosate at doses of 75, 150 and 800 mg/kg bw produced in a dose-dependent manner a significant decrease of 5-HT content respect to control in striatum. Moreover, glyphosate at doses of 150 and 800 mg/kg bw produced a significant decrease of 5-HT content respect to control in hippocampus and prefrontal cortex and only at a dose of 800 mg/kg bw in hypothalamus and midbrain. In addition, the highest dose (800 mg/kg bw) of glyphosate resulted in a significant decrease in the 5-HIAA levels in hippocampus compared to control group. Also, glyphosate at doses of 150 and 800 mg/kg bw significantly increased the turnover (5-HIAA/5-HT) in striatum and hypothalamus compared to control groups (Table 2).

Table 2
Effect of glyphosate on 5-HT and 5-HIAA levels and turnover (5-HIAA/5-HT) in brain regions of male rats.

Parameter	Animal groups: oral dose of glyphosate during 6 consecutive days	Brain regions				
		Striatum	Hippocampus	Prefrontal cortex	Hypothalamus	Midbrain
5-HT (ng/g)	Control	696.28 ± 14.11	321.60 ± 12.99	710.24 ± 39.86	1698.90 ± 15.31	1551.79 ± 70.09
	35 mg/kg bw	650.05 ± 38.04	319.00 ± 8.17	675.01 ± 49.65	1642.00 ± 74.65	1541.00 ± 62.59
	75 mg/kg bw	557.50 ± 112.42** (−20)[a]	315.16 ± 6.98	603.64 ± 133.99	1575.00 ± 147.39	1515.42 ± 51.99
	150 mg/kg bw	445.57 ± 85.33*** (−36)[a]	293.97 ± 6.23* (−9)[a]	507.20 ± 42.11*** (−29)[a]	1360.75 ± 452.82	1495.03 ± 64.02
	800 mg/kg bw	355.11 ± 16.48*** (−49)[a]	291.06 ± 27.67* (−9)[a]	482.80 ± 69.26*** (−32)[a]	1103.07 ± 111.29*** (−35)[a]	1210.46 ± 134.13*** (−22)[a]
		27.45[b]	5.793[b]	10.51[b]	7.330[b]	18.18[b]
5-HIAA (ng/g)	Control	365.50 ± 75.88	390.31 ± 99.55	299.85 ± 48.05	743.32 ± 78.12	865.34 ± 123.9
	35 mg/kg bw	366.30 ± 55.29	383.04 ± 6.35	297.00 ± 48.98	755.00 ± 33.45	846.00 ± 95.25
	75 mg/kg bw	366.49 ± 68.09	351.54 ± 8.99	295.10 ± 64.32	736.09 ± 55.73	820.32 ± 120.66
	150 mg/kg bw	368.70 ± 31.54	307.93 ± 91.06	270.69 ± 58.58	733.13 ± 116.03	805.00 ± 66.93
	800 mg/kg bw	352.41 ± 60.91	269.81 ± 55.41* (−31)[a]	259.39 ± 37.20	726.33 ± 40.54	708.82 ± 151.74
		0.06677[c]	3.669[c]	0.7277[c]	0.175[c]	1.661[c]
5-HIAA/5-HT	Control	0.53 ± 0.12	1.22 ± 0.30	0.42 ± 0.06	0.45 ± 0.04	0.56 ± 0.09
	35 mg/kg bw	0.56 ± 0.06	1.20 ± 0.04	0.45 ± 0.11	0.47 ± 0.20	0.55 ± 0.08
	75 mg/kg bw	0.66 ± 0.08	1.12 ± 0.04	0.5 ± 0.15	0.52 ± 0.19	0.54 ± 0.09
	150 mg/kg bw	0.86 ± 0.26*** (63)[a]	1.06 ± 0.35	0.54 ± 0.14	0.59 ± 0.18* (30)[a]	0.54 ± 0.04
	800 mg/kg bw	0.99 ± 0.14*** (88)[a]	0.93 ± 0.21	0.54 ± 0.09	0.69 ± 0.15*** (53)[a]	0.59 ± 0.13
		20.8[c]	1.591[c]	1.355[c]	13.31[c]	0.3139[c]

** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.01$.
*** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.001$.
* Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.05$.
[a] Percentage change over control in parenthesis.
[b] F (continuous probability distribution; DFn = 4, DFd = 25).

With respect to DA, DOPAC and HVA levels and turnover in brain regions (Table 3), glyphosate at doses of 75, 150 y 800 mg/kg bw produced, in a dose-dependent manner, a statistically significant decrease of DA levels in prefrontal cortex and midbrain compared to control groups. Likewise, glyphosate at the highest dose (800 mg/kg bw) decreased significantly the DA levels in hypothalamus, striatum and hippocampus compared to control group. Moreover, glyphosate at doses of 150 and 800 mg/kg bw decreased significantly the DOPAC metabolite levels in hypothalamus and glyphosate at highest dose only produced a significant decrease of DOPAC levels in hippocampus respect to control group. In addition, the HVA levels significantly decreased after doses of glyphosate 75, 150 and 800 mg/kg bw in hypothalamus, after doses of glyphosate 150 and 800 mg/kg bw in midbrain; and after dose of 800 mg/kg bw in prefrontal cortex respect to control groups. Glyphosate (75, 150 and 800 mg/kg bw) produced a significant increase of the turnover (DOPAC+HVA/DA) in prefrontal cortex respect to control groups. Glyphosate at highest dose only produced a significant increase of the turnover (DOPAC+HVA/DA) in hippocampus (Table 3).

Table 3
Effect of glyphosate on DA, DOPAC and HVA levels and turnover (DOPAC+HVA/DA) in brain regions of male rats.

Parameter	Animal groups: oral dose of glyphosate during 6 consecutive days	Brain regions				
		Striatum	Hippocampus	Prefrontal cortex	Hypothalamus	Midbrain
DA (ng/g)	Control	6406.35 ± 502.83	248.48 ± 59.36	209.00 ± 45.43	600.20 ± 35.36	776.95 ± 34.09
	35 mg/kg bw	6332.00 ± 296.00	231.00 ± 34.60	186.00 ± 21.33	581.00 ± 51.76	732.00 ± 20.86
	75 mg/kg bw	5614.83 ± 1040.66	213.55 ± 29.46	109.14 ± 16.61*** (−48)[a]	555.48 ± 102.85	676.48 ± 28.33*** (−13)[a]
	150 mg/kg bw	5563.85 ± 223.28	177.84 ± 39.32	97.28 ± 10.78*** (−53)[a]	528.52 ± 39.21	666.07 ± 29.05*** (−14)[a]
	800 mg/kg bw	5009.86 ± 468.29** (−22)[a]	73.07 ± 67.49*** (−71)[a]	34.99 ± 33.65*** (−83)[a]	480.02 ± 23.93* (−20)[a]	642.72 ± 22.98*** (−17)[a]
		6.044[b]	12.49[b]	36.86[b]	4.001[b]	23.71[b]
DOPAC (ng/g)	Control	691.04 ± 64.66	9.51 ± 1.77	21.43 ± 2.68	93.22 ± 21.18	48.75 ± 13.78
	35 mg/kg bw	689.00 ± 74.87	9.44 ± 1.29	21.00 ± 2.23	85.00 ± 18.34	47.00 ± 8.38
	75 mg/kg bw	688.17 ± 221.53	9.00 ± 1.22	19.19 ± 1.23	68.19 ± 21.13	45.78 ± 9.33
	150 mg/kg bw	689.62 ± 37.04	7.66 ± 1.26	18.46 ± 1.01	57.55 ± 6.11** (−38)[a]	44.81 ± 4.09
	800 mg/kg bw	683.38 ± 72.08	6.66 ± 1.95* (−30)[a]	20.04 ± 0.84	57.26 ± 4.70** (−39)[a]	43.78 ± 1.93
		0.00389[b]	4.005[b]	2.958[b]	6.156[b]	0.3051[b]
HVA (ng/g)	Control	907.87 ± 258.95	18.29 ± 1.95	50.75 ± 8.78	68.91 ± 14.12	71.46 ± 3.82
	35 mg/kg bw	906.00 ± 132.32	18.00 ± 3.48	50.60 ± 4.97	64.00 ± 8.34	68.00 ± 5.32
	75 mg/kg bw	901.87 ± 260.40	17.45 ± 4.11	50.33 ± 6.29	44.34 ± 17.40** (−36)[a]	65.10 ± 4.41
	150 mg/kg bw	846.17 ± 48.38	16.17 ± 1.26	46.38 ± 4.27	34.77 ± 5.77*** (−50)[a]	52.91 ± 6.04*** (−26)[a]
	800 mg/kg bw	839.75 ± 32.86	16.40 ± 2.59	30.84 ± 11.55** (−39)[a]	36.82 ± 6.41*** (−47)[a]	38.09 ± 11.10*** (−47)[a]
		0.226[b]	0.6519[b]	7.480[b]	11.50[b]	25.26[b]
(DOPAC+HVA)/DA	Control	0.25 ± 0.05	0.12 ± 0.02	0.36 ± 0.09	0.28 ± 0.25	0.15 ± 0.02
	35 mg/kg bw	0.25 ± 0.04	0.12 ± 0.01	0.39 ± 0.02	0.27 ± 0.06	0.16 ± 0.02
	75 mg/kg bw	0.3 ± 0.11	0.13 ± 0.03	0.64 ± 0.07** (78)[a]	0.22 ± 0.07	0.16 ± 0.02
	150 mg/kg bw	0.28 ± 0.02	0.14 ± 0.03	0.67 ± 0.07** (86)[a]	0.18 ± 0.02	0.15 ± 0.01
	800 mg/kg bw	0.31 ± 0.04	0.98 ± 0.91*** (150)[a]	2.88 ± 2.62*** (153)[a]	0.20 ± 0.02	0.13 ± 0.02
		1.269[b]	5.254[b]	39.28[b]	2.009[b]	2.647[b]

*** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.001$.
** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.01$.
* Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.05$.
[a] Percentage change over control in parenthesis.
[b] F (continuous probability distribution; DFn = 4, DFd = 25).

In relation to NE and MHPG levels and turnover, glyphosate at doses of 75, 150 and 800 mg/kg bw produced a significant decrease of the NE levels in striatum and midbrain compared to control. Moreover, glyphosate at doses of 150 and 800 mg/kg bw produced a significant decrease of NE levels in hippocampus and only at a dose of 800 mg/kg bw in prefrontal cortex respect to control groups. Moreover, MHPG levels significantly decreased after doses of glyphosate 150 and 800 mg/kg bw in hippocampus and after dose of glyphosate 800 mg/kg bw in striatum, prefrontal cortex, hypothalamus and midbrain. Glyphosate at dose of 800 mg/kg bw produced a significant decrease of the turnover (MHPG/NE) in prefrontal cortex and hypothalamus compared to control group (Table 4).

Table 4
Effect of glyphosate on NE and MHPG levels and turnover (MHPG/NE) in brain regions of male rats.

Parameter	Animal groups: oral dose of glyphosate during 6 consecutive days	Brain regions				
		Striatum	Hippocampus	Prefrontal cortex	Hypothalamus	Midbrain
NE (ng/g)	Control	210.64 ± 79.72	190.74 ± 13.01	145.81 ± 3.08	1006.77 ± 195.00	452.04 ± 76.01
	35 mg/kg bw	202.00 ± 37.93	183.00 ± 11.02	145.00 ± 9.34	1005.00 ± 74.85	422.00 ± 39.44
	75 mg/kg bw	121.07 ± 42.09* (−43)[a]	159.24 ± 20.21	138.54 ± 11.18	1000.88 ± 76.64	357.03 ± 55.11* (−21)[a]
	150 mg/kg bw	118.13 ± 24.65* (−44)[a]	150.02 ± 30.98* (−21)[a]	137.29 ± 6.11	901.07 ± 72.69	358.61 ± 31.77* (−21)[a]
	800 mg/kg bw	102.74 ± 27.10** (−51)[a]	137.34 ± 15.13** (−28)[a]	127.64 ± 12.35* (−12)[a]	878.22 ± 71.54	358.92 ± 17.12* (−21)[a]
		7.194[b]	7.981[b]	3.908[b]	2.007[b]	5.083[b]
MHPG (ng/g)	Control	161.88 ± 21.25	67.07 ± 8.13	109.60 ± 28.56	50.29 ± 8.27	71.69 ± 15.56
	35 mg/kg bw	159.00 ± 35.00	61.00 ± 5.85	100.00 ± 12.67	46.00 ± 11.27	70.00 ± 9.32
	75 mg/kg bw	155.46 ± 36.00	54.90 ± 6.67	84.67 ± 12.91	43.40 ± 7.21	69.11 ± 8.54
	150 mg/kg bw	132.94 ± 31.34	51.22 ± 9.28* (−24)[a]	84.46 ± 12.41	43.03 ± 21.58	67.72 ± 10.57
	800 mg/kg bw	108.95 ± 10* (−33)[a]	50.47 ± 9.85* (−25)[a]	71.11 ± 10.32** (−35)[a]	20.22 ± 9.25** (−60)[a]	29.01 ± 4.66*** (−60)[a]
		3.748[b]	4.451[b]	4.810[b]	5.198[b]	18.61[b]
MHPG / NE	Control	0.85 ± 0.29	0.35 ± 0.06	0.77 ± 0.18	0.05 ± 0.01	0.16 ± 0.03
	35 mg/kg bw	0.84 ± 0.33	0.34 ± 0.05	0.69 ± 0.09	0.05 ± 0.02	0.17 ± 0.02
	75 mg/kg bw	1.42 ± 0.59	0.35 ± 0.08	0.61 ± 0.08	0.04 ± 0.01	0.20 ± 0.04
	150 mg/kg bw	1.14 ± 0.23	0.35 ± 0.08	0.62 ± 0.10	0.05 ± 0.03	0.19 ± 0.03
	800 mg/kg bw	1.10 ± 0.19	0.37 ± 0.07	0.56 ± 0.05* (−23)[a]	0.02 ± 0.01** (−55)[a]	0.18 ± 0.01
		2.733[b]	0.1513[b]	2.792[b]	5.423[b]	1.923[b]

* Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.05$.
** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.01$.
*** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.001$.
[a] Percentage change over control in parenthesis.
[b] F (continuous probability distribution; DFn = 4, DFd = 25).

Discussion

Pesticides are widely used in agricultural and other settings, resulting in continuing human exposure. The nervous system represents a prime target for both the acute and chronic effects of pesticides. Among them are the organochlorines, pyrethroids, organophosphates, neonicotinoids, herbicides and some novel agents. Acute symptoms can include headache, nausea, dizziness, and sensory paresthesia. Toxicity often involves neuronal hyper-excitability, and disorders of cognition. Toxicological in vitro and in vivo studies have demonstrated specific neurodegenerative effects from exposure to certain pesticides, and human case reports have suggested a causal relationship between certain pesticide exposures and Parkinson's disease (PD) typically associated with degeneration of the dopaminergic neurons. Evidence is also now accumulating that organophosphate pesticides target serotonin and

noradrenergic systems contributing to adverse outcomes related to emotional and social behaviours. Ingestion of the herbicide glyphosate may cause significant toxicity including nausea, vomiting, diarrhea, oral and abdominal pain, renal and hepatic impairment, and pulmonary oedema. Impaired consciousness and seizures have also been reported as sequelae but there are limited data of glyphosate on central nervous system (CNS) toxicity. This study was designed to investigate the effects of glyphosate on CNS monoaminergic neurotransmitter contents (5-HT, DA and NE) in male Wistar rats in order to generate more data on the glyphosate neurotoxicity. The current study showed that exposure to glyphosate, in a region and dose-dependent manner, was accompanied by a significant decrease in the 5-HT, DA and NE contents in the brain regions studied (striatum, hippocampus, prefrontal cortex, hypothalamus, hypothalamus and midbrain), which indicated that glyphosate transfer across the blood-brain barrier, enters the brain, probably accumulates in significant quantity, and exerts neurotoxicity altering the serotonergic, dopaminergic and noradrenergic systems. Researchers have reported similar changes of these brain neurotransmitters after exposure to the insecticide pyrethroid cyfluthrin. It should be noted that the rats treated with glyphosate at dose of 35 mg/kg bw per day did not exhibit any effects on the 5-HT, DA and NE contents in the brain regions studied. In our study, this NOAEL observed (35 mg/kg bw per day) was lowest to that identified on the maternal and developmental toxicity studies (NOAEL of 50 mg/kg bw per day) and used to establish the ADI. For current regulatory evaluation of risks associated with glyphosate exposure, a NOAEL of 35 mg/kg bw per day could be used instead of a NOAEL of 50 mg/kg bw per day. In this regard, taking into account that glyphosate probably accumulates in the CNS and considering that in the present study the glyphosate exposure was only during only 6 days, further research with a longer period of exposure should be necessary to corroborate the proposed NOAEL of 35 mg/kg bw per day. In the present study, quantitative analysis of 5-HT, DA and NE contents showed that the loss of these neurotransmitters was mainly observed in the striatum [5-HT and NE contents decreased significantly (-49% and -51%, respectively) after the highest dose of 800 mg glyphosate/kg bw] and in prefrontal cortex and hippocampus [DA contents decreased significantly (-83% and -71%, respectively) after the highest dose of 800 mg glyphosate/kg bw]. Previously, Researchers also showed in rats that glyphosate decreased DA but not 5-HT levels in striatum as well as reduced the locomotor activity suggesting that the decrease in striatal DA levels could also explain a behavioral hypoactivity. Moreover, in our study, glyphosate after the highest dose of 800 mg/kg bw produced a significant increase of the (5-HIAA/5-HT) turnover in striatum (88%) and of the (DOPAC+HVA/DA) turnover in prefrontal (153%) and hippocampus (150%), but a significant decrease of the (NE/MHPG) turnover in prefrontal cortex (-23%) and hypothalamus (-55%), critical brain regions that regulate cognitive functions. The cooperation of the hippocampus and the prefrontal cortex is vital in spatial working memory performance and decision making. Disconnection or damage to either of the two brain regions induces impaired cognitive behaviors. Because cognitive functions are quite complex, more details are required for the complete understanding of glyphosate-induced neurotoxicity. It would be of interest to investigate the developmental changes of the hippocampus and prefrontal cortex in prenatal glyphosate.

Conclusion

In conclusion, the results demonstrate that glyphosate leads to loss of 5-HT, DA and NE levels in the CNS. The neurochemical effects observed in the present study are an important public health concern. Although we have not data on humans, glyphosate could exert its neurotoxicity, notably on monoamine systems, by inducing DNA damage, neuronal inflammation and oxidative stress mechanisms. Further investigation is needed to involve the glyphosate herbicide with neurodegenerative diseases. Nevertheless, our results support the hazard of glyphosate herbicide and show the importance of minimizing its herbicide use.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Although the study concludes “loss of 5-HT, DA and NE levels in the CNS”, no historical controls are available to assess and compare the changes in the treatment-groups to ascertain if the effects are within background or if they are biologically relevant.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because there was historical control to determine if changes in the levels of neurotransmitters were within historical controls. No positive control included either. Also no analytical verification of dose levels.

Reliability criteria for *in vivo* toxicology studies

Publication: Martínez M. *et al.*, 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Non-guideline
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Technical glyphosate purity of ≥98%. Source: Sigma-Aldrich. No information on storage,
Only glyphosate acid or one of its salts is the tested substance	Y	Yes
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	Wistar rat (male)
Test conditions clearly and completely described	Y	Yes
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	35, 75, 150, 800 mg/kg bw/day for 6 days
Positive control	N	-
Number of animals used per dose level reported	Y	6/dose group.
Method of analysis described for analysis test media	N	-
Validation of the analytical method	N	-
Analytical verifications of test media	N	-
Complete reporting of effects observed	N	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	No historical control data for comparison, no analytical verification of dose or stability.
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because there was historical control to determine if changes in the levels of neurotransmitters were within historical controls. No positive control was included. Also no analytical verification of dose levels.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	McGuire M. K. *et al.*
Report year	2016
Report title	Glyphosate and aminomethylphosphonic acid are not detectable in human milk
Document No	The American journal of clinical nutrition, (2016) Vol. 103, No. 5, pp. 1285-90.
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

It was sought to determine whether glyphosate and its metabolite aminomethylphosphonic acid (AMPA) could be detected in milk and urine produced by lactating women and, if so, to quantify typical consumption by breastfed infants. Milk (n = 41) and urine (n = 40) samples from healthy lactating women living in and around Moscow, Idaho and Pullman, Washington was collected. Milk and urine samples were analyzed for glyphosate and AMPA with the use of highly sensitive liquid chromatography–tandem mass spectrometry methods validated for and optimized to each sample matrix. The milk assay, which was sensitive down to 1 μg/L for both analytes, detected neither glyphosate nor AMPA in any milk sample. Mean ± SD glyphosate and AMPA concentrations in urine were 0.28 ± 0.38 and 0.30 ± 0.33 mg/L, respectively. Because of the complex nature of milk matrixes, these samples required more dilution before analysis than did urine, thus decreasing the sensitivity of the assay in milk compared with urine. No difference was found in urine glyphosate and AMPA concentrations between subjects consuming organic compared with conventionally grown foods or between women living on or near a farm/ranch and those living in an urban or suburban non-farming area.

Materials and Methods

Human subjects – A total of 41 healthy lactating women living in and around Pullman, Washington, and Moscow, Idaho, were included in the study. To be eligible for participation, women had to be 1–3 months postpartum, breastfeeding and/or pumping milk 5 times/day and more, and aged 18 years and older. All but 1 subject also completed a 5-question survey documenting potential glyphosate exposure from the environment and diet.

Milk and urine collection and preservation - After cleaning the breast, approx. 30 mL milk was collected with an hospital-grade electric breast pump into a single-use sterile collection container. A midstream urine sample was also collected into a single-use sterile collection container. The sample containers were immediately placed in ice, separated into aliquots and frozen at -20^{0}C pending analysis. One subject failed to provide a urine sample.

Glyphosate and AMPA analyses - Milk and urine samples were analyzed for glyphosate and AMPA using liquid chromatography-tandem mass spectrometry in the multiple reaction monitoring mode. Two precursor product ion transitions for each analyte and a stable isotope labelled internal standard for each analyte were used to ensure the selectivity of the analytical method. Although 2 quantitative precursor-product ion transitions were monitored, the results were reported using the most sensitive transition for each analyte. The assay was validated separately for milk and urine. Limits of detection (LOD) and quantification (LOQ) for glyphosate in milk were 1.0 and 10.0 μg/L, respectively. The LOD and LOQ for glyphosate in urine were 0.02 and 0.10 μg/L, respectively. The LOD and LOQ for AMPA in milk

were 1.0 and 10.0 µg/L, respectively and those for AMPA in urine were 0.03 and 0.10 µg/L, respectively. Glyphosate and AMPA concentrations in milk were independently confirmed by another laboratory using the same liquid chromatography-tandem mass spectrometry method with minor modifications. Because of differences in instrumentation, the LODs with the more sensitive quantitative ion transitions were 6.0 and 9.0 µg/L for glyphosate and AMPA in human milk, respectively. The LOQ for human milk was 25.0 µg/L for both analytes. Duplicate aliquots from each milk sample were sent to each laboratory separately.

Statistical analyses - For glyphosate and AMPA concentrations in urine a generalized linear mixed model was used assuming a Poisson distribution with a logarithmic link function. For concentrations less than the respective LOD values, one-half LOD (0.01 and 0.015 µg/L for glyphosate and AMPA, respectively) nominal values were used for assessment. For concentrations between the LOD and LOQ, one-half LOQ (0.05 µg/L for both glyphosate and AMPA) nominal values were used for assessment. All values are presented as means ± SDs.

Results

Description of study population and glyphosate exposure - Women were aged 29 ± 5 years, 67 ± 17 days postpartum, and had a BMI (kg/m^2) of 26.8 ± 8.6. 75% of them lived in an urban or suburban non-farming region of the Palouse, and 58% of them reported that they made no effort to eat foods characterized as organic, although they sometimes included them in their diets for convenience. 15% reported ever having personally mixed or used any type of weed killer. All but one of the women having reported ever doing so had mixed or used a weed killer containing glyphosate.

Glyphosate and AMPA concentrations in milk - Regardless of where the samples were analyzed, none of the milk samples contained detectable amounts of either glyphosate or AMPA.

Glyphosate and AMPA concentrations in urine - Glyphosate was detectable in nearly all (n =37) of the urine samples and was quantifiable in 29 of them. Glyphosate values ranged from below the LOD (<0.02 µg/L) to 1.93 µg/L, with a mean concentration of 0.28 ± 0.38 µg/L. AMPA was also detectable in nearly all (n = 38) of the urine samples and quantifiable in 29 of them. Urine AMPA values ranged from below the LOD (<0.03 mg/L) to 1.33 µg/L, with a mean concentration of 0.30 ± 0.33 µg/L. There were no statistically significant effects of consuming organic compared with conventional foods or living on/near a farm compared with living in an urban/suburban region on concentrations of glyphosate in urine, respectively. Neither were there statistically significant effects of consuming organic compared with conventional foods or living on/near a farm compared with living in an urban/suburban region on concentrations of AMPA in urine. Adjusting for potential covariates (age, time postpartum, BMI, parity) did not alter these conclusions. When raw, untransformed values were used in the analysis, there was a statistically significant positive correlation between urinary glyphosate and AMPA concentrations.

Discussion and Conclusion

The results provide evidence that the concentrations of glyphosate and AMPA in milk produced by healthy women are below the detection limits of available validated analytical assays. In urine, glyphosate and AMPA were detectable in many samples, but the concentrations were very low (<0.02 to 1.93 and <0.03 to 1.33 µg/L, respectively) and well below values reported in other healthy adult populations. The RfD for glyphosate is 1.75 mg/kg bw/day. US EPA considers AMPA to be of similar or lesser toxicity than glyphosate and determined that it should be exempt from regulation regardless of concentrations observed in food or feed. Thus, a woman with a typical weight for the study participants, 75 kg, could consume as much as 131.25 mg glyphosate/day with no expected negative effects. Taking an oral bioavailablity of 20% into account and assuming that all glyphosate absorbed is excreted within 24 hours and all absorbed glyphosate is excreted in urine, the urinary output would be 26,250 µg/day. In the current study, the highest reported glyphosate concentration in urine was 1.93 µg/L. As such, even allowing for a relatively high urine output (3 L/day), the highest glyphosate excretion in our study would be 5.79 µg/day, a value which is more than 4,500 times lower. Applying similar parameters and logic

to a 5-kg infant with a mean milk intake of 0.7 L/day and a milk glyphosate concentration of 1 µg/L (the LOD value), then the maximum daily consumption of glyphosate would be 0.7 µg/day which is more than 12,000 times lower than the RfD. It is important to emphasize that the larger international study from which these samples originate was not designed to detect small differences in urine glyphosate and AMPA concentrations based on dietary choices, location of residence (e.g., urban compared with rural), or occupational glyphosate exposure. Detecting small-effect sizes at statistically significant concentrations and adequate statistical power would require 4–5 times as many observations than used in this study.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study breast milk and urine samples from lactating women were analyzed for glyphosate and AMPA. The results provide evidence that the concentrations of glyphosate and AMPA in milk produced by healthy women are below the detection limits of available validated analytical assays. In urine, glyphosate and AMPA were detectable in many samples, but the concentrations were very low and well below the values reported in other healthy adult populations.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with all the reliability criteria of an exposure study.

Reliability criteria for exposure studies

Publication: McGuire *et al*., 2016	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.		
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Reference material (glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	Exposure mainly via food
Study		
Study design clearly described	Y	Monitoring of glyphosate in urine and breast milk of lactating women
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Overall assessment		
Reliable	Y	
Reliable with restrictions		

Publication: McGuire *et al*., 2016	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with all the reliability criteria of an exposure study.		

1. Information on the study

Data point:	CA 5.3
Report author	Mesnage R. *et al*
Report year	2018
Report title	Comparison of transcriptome responses to glyphosate, isoxaflutole, quizalofop-p-ethyl and mesotrione in the HepaRG cell line
Document No	Toxicology Reports (2018) Vol. 5, 819–826
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Together with 3 other herbicide active ingredients (quizalofop-p-ethyl, isoxaflutole and mesotrione) the effect of glyphosate on the transcriptome and metabolome profile of differentiated HepaRG cells was investigated.

Materials and methods

Chemicals - Glyphosate (purity ≥ 96%) was purchased from Sigma-Aldrich, Gillingham, Dorset, UK.

HepaRG cell culture - Differentiated HepaRG™ cells (HPR 116) were purchased from Biopredic International (Rennes, France). Cells were thawed, suspended and plated in general purpose medium (Williams' E medium + GlutaMAX™) containing the ADD670 supplement. Cells were kept in general purpose medium until day 8, when the culture becomes well organized and includes well-delineated trabeculae and many canaliculi-like structures. At this time, the culture is composed of primitive biliary epithelial cells and mature hepatocytes with basal metabolic activities similar to freshly isolated primary cells. From day 8 to day 14, cells were switched to the test medium composed of Williams' E medium + GlutaMAX™ supplemented with 2% fetal bovine serum and 1% DMSO, as well as different concentrations of glyphosate or solvent as a control. Glyphosate was tested at 0.06 μM (concentration representative of low environmental exposure), 6 μM, and 600 μM.

Library generation and RNA-sequencing - A 100 ng aliquot of total RNA from each sample was used to prepare total RNA libraries using the KAPA Stranded RNA-Seq Kit with RiboErase, and samples were randomised before preparation. Polymerase chain reaction (PCR) was performed for 14 cycles for final library amplification. Resulting libraries were quantified using the Qubit 2.0 spectrophotometer and average fragment size assessed using the Agilent 2200 Tapestation. The transcriptome of HepaRG cells exposed to glyphosate was sequenced employing this strategy, except that the libraries were prepared as previously described. A total of 3 separate sequencing pools were created using equimolar quantities of each sample with compatible indexes: 2 with 17 samples each, and one with 16 samples. Paired-end reads of 75bp were generated for each library using the Illumina NextSeq®500 in con-junction with the NextSeq®500 v2 High-output 150-cycle kit.

Mass spectrometry-based metabolomics - Approximately 5,000,000 HepaRG cells per sample were harvested from the 6 well-plates to obtain a sufficient quantity of material to perform the metabolomics experiment. Cells were detached using 0.05% trypsin EDTA, and centrifuged to eliminate trypsin residues. Finally, cell pellets were frozen at -80 °C pending analysis. Metabolomics analysis of the frozen cell pellets was conducted by Metabolon Inc. The sample extracts were stored overnight under nitrogen before preparation for analysis. The resulting extract was analysed on four independent instrument platforms: two different separate RP/UPLC-MS/MS with positive ion mode electrospray

ionization (ESI), a RP/UPLC-MS/MS with negative ion mode ESI, as well as by hydrophilic-interaction chromatography(HILIC)/UPLC-MS/MS with negative ion mode ESI as previously described. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Biochemical identifications are based on 3 criteria: retention index within a narrow retention time/index (RI) window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The use of all three data points can be utilized to distinguish and differentiate biochemicals. Peaks were quantified using the area-under-the-curve method.

Statistics - Metabolome data analysis was performed using in-house services of Metabolon Inc. Biochemical data were normalized with respect to protein concentration as determined by the Bradford assay. The Welch's two-sample t-test was used to test whether control and treatment group means are different from two independent populations. This version of the two-sample t-test allows for unequal variances. FDR methods and estimated q-values were used to account for the highest number of false positive results caused by the high number of statistical tests. The RNA-seq data analysis was performed using the new version of the Tuxedo protocol with HISAT2, StringTie and Ballgown. A standard linear model-based comparison of transcript abundance was performed without adjusting for other covariates to identify differentially expressed transcripts for each group. Although 3 concentrations were tested for glyphosate, no multigroup comparisons were used because dose spacing was too large to allow reliable conclusions to be drawn from these methods. Instead, pairwise comparisons were used.

Results

Control, untreated cell cultures presented no visible signs of aging after a 6-day exposure. Transcriptome profiles of HepaRG cells were then determined using the Illumina-based RNA sequencing platform. The highest concentration of glyphosate tested caused significant changes in transcriptome profiles. Alterations in gene expression caused by the 2 lowest concentrations (0.06 and 6 μM) failed to pass the statistical threshold that took into account the high number of tests performed. A total of 7 transcripts had their levels altered ($p < 0.05$) with glyphosate at 600 μM. The number of genes disturbed by the exposure to glyphosate was insufficient to use a functional annotation tool for pathway enrichment analysis. It is not clear if glyphosate lacks hepatic toxic effects at these concentrations or if this experimental design lacks sensitivity to detect hepatic effects of weak toxicants. To further explore changes in liver metabolism caused by glyphosate in greater detail, a global metabolome profiling of HepaRG cells exposed to three concentrations of glyphosate was explored. The Metabolon HD4 platform detected 802 named biochemicals in the HepaRG samples. Overall, glyphosate did not cause significant alterations in metabolome composition. However, exposure did cause a significant decrease in long chain fatty acids (LCFAs) and polyunsaturated fatty acids (PUFAs). HepaRG cells exposed to the lowest concentration of glyphosate tested (0.06 μM) showed the most dramatic effects in the levels of these fatty acids as either significant or trends towards significant lower levels. At the higher glyphosate concentrations of 6 μM and 600 μM, lower lipid levels were also observed but these did not reach statistical significance.

Discussion and conclusion

An in-depth investigation was conducted of transcriptome profile alterations in HepaRG human liver cells caused by exposure to pesticide active ingredients. Glyphosate was found to be only weakly toxic inducing little change in transcriptome profiles. Interestingly, a follow-up metabolomics analysis of HepaRG cells treated with the lowest (0.06 μM) concentration of glyphosate revealed a significant decrease in the levels of LCFAs and PUFAs. Although these findings from an *in vitro* tissue culture model system cannot be readily translated to effects *in vivo*, they are nevertheless indicative of differences in toxicity potency between pesticide ingredients. The exact nature of this low dose effect of glyphosate cannot be determined from this single experiment, but it is possible that at higher concentrations, more overtly toxic mechanisms are masking the effects on lipids. Another possibility is that a saturation effect is occurring once the low dose is exceeded bearing in mind that glyphosate levels found in the HepaRG cells during the metabolomics analysis increased by 3.7-and 336.35-fold at the

intermediate and highest concentrations tested compared to the negative controls. Glyphosate was the least toxic of the compounds tested in this study.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Together with 3 other herbicide active ingredients (quizalofop-p-ethyl, isoxaflutole and mesotrione) the effect of glyphosate on the transcriptome and metabolome profile of differentiated HepaRG cells was investigated at 0.06, 6 and 600 µM. Glyphosate was found to be only weakly toxic inducing little change in transcriptome profiles when compared with the other herbicides tested. A follow-up metabolomics analysis of HepaRG cells exposed to glyphosate at 0.06 µM revealed a significant decrease in the levels of long chain fatty acids (LCFAs) and polyunsaturated fatty acids (PUFAs). At the higher glyphosate concentrations of 6 and 600 µM, lower lipid levels were also observed but these did not reach statistical significance. It is not clear, however, how these findings from an *in vitro* tissue culture model can be translated to effects *in vivo*.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no positive control was used and no cytoxicity tests were performed to optimise the concentration range to be explored.

Reliability criteria for *in vitro* toxicology studies

Publication: Mesnage *et al*., 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate (purity ≥ 96%). Source: Sigma-Aldrich, Gillingham, Dorset, UK.
Only glyphosate acid or one of its salts is the tested substance	N	Also three other pesticide active ingredients were tested (quizalofop-p-ethyl, isoxaflutole and mesotrione).
AMPA is the tested substance	N	
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.06, 6 and 600 µM.
Cytotoxicity tests reported	N	
Transcriptomics and metabolomics methods described	Y	
Positive and negative controls	N	No positive controls included.
Complete reporting of effects observed	Y	
Statistical methods described	Y	

Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no positive control was used and no cytoxicity tests were performed to optimise the concentration range to be explored.		

1. Information on the study

Data point:	5.8.2/016
Report author	Mesnage , R. *et al.*
Report year	2018
Report title	Ignoring Adjuvant Toxicity Falsifies the Safety Profile of Commercial Pesticides
Document No	doi.org/10.3389/fpubh.2017.00361
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Currently, the health risk assessment of pesticides in the European Union and in the United States focuses almost exclusively on the stated active principle. Nonetheless, adjuvants can also be toxic in their own right with numerous negative health effects having been reported in humans and on the environment. Despite the known toxicity of adjuvants, they are regulated differently from active principles, with their toxic effects being generally ignored. Adjuvants are not subject to an acceptable daily intake, and they are not included in the health risk assessment of dietary exposures to pesticide residues. Here, this gap in risk assessment by reference to glyphosate, the most used pesticide active ingredient is illustrated. The case of neonicotinoid insecticides, which are strongly suspected to be involved in bee and bumblebee colony collapse disorder is also investigated. Authors of studies sometimes use the name of the active principle (for example glyphosate) when they are testing a commercial formulation containing multiple (active principle plus adjuvant) ingredients. This results in confusion in the scientific literature and within regulatory circles and leads to a misrepresentation of the safety profile of commercial pesticides. Urgent action is needed to lift the veil on the presence of adjuvants in food and human bodily fluids, as well as in the environment (such as in air, water, and soil) and to characterize their toxicological properties. This must be accompanied by regulatory precautionary measures to protect the environment and general human population from some toxic adjuvants that are currently missing from risk assessments.

Materials and methods

Not applicable because this is a review article.

In this review, a focus was done on the co-formulants included in the commercial formulations of glyphosate-based herbicides, in particular the polyethoxylated tallow amines (POEAs), which is considered as a representative good model system because they are the most used pesticides worldwide.

Results

A total of 750 different formulations of glyphosate-based herbicides are marketed worldwide. Indeed, each name of a given formulation represents a different mixture of active principle and co-formulants (Table 1). As a result of the variability in co-formulants, and since most of them are not compulsorily declared, the effects of pesticides are complex and combinatorial. The literature is quite heterogeneous because these co-formulants vary between commercial pesticide formulations and thus have different and/or additive side effects between themselves and with glyphosate. In fact, this causes confusion in the scientific community, with authors not always declaring the formulation that they tested. Authors even sometimes confuse commercial formulations with the active ingredient; they state that “glyphosate” was used when in reality they employed a formulation in their studies.

The problem of reproducibility and consistency in the results of toxicological studies could be partly due to the fact that comparisons are performed between different formulations. Some studies even compare formulations and glyphosate alone treating the two as equivalent and therefore ignoring the effects of adjuvants in the former. This adds yet further confusion and questions over the reliability of the data obtained. For example, a recent study investigating effects of a Roundup formulation on damselfly larvae concluded that "the toxicity of Roundup cannot be fully attributed to the surfactant POEA" and that "Roundup® […] contains POEA as surfactant". This is not accurate as not all Roundup formulations contains a POEA surfactant and that the manufacturer company Monsanto has moved away from the use of POEA-based surfactants in their latest generation of Roundup formulations. The authors of this study do not indicate which commercial formulation of Roundup they have tested on damselfly larvae, and thus it is unknown if POEA was present. Therefore, although they present interesting results on the toxicity of Roundup on an environmental toxicity indicator organism, at the same time they bring confusion to the field by concluding that their study "confirms the toxicity of the surfactant POEA".

Séralini and colleagues have conducted the most extensive study on the composition and toxicity of the different ingredients that constitute glyphosate-based herbicides. They have compared the toxicity of different brands of glyphosate-based herbicides in tissue culture cell assays and showed that several commercial formulations were up to 1,000 times more toxic than glyphosate alone, the regulated active ingredient. In addition, adjuvant mixtures generally contain several ingredients, and these can sometimes be mixtures themselves. For example, POEAs are mixtures of diethoxylates of tallow amines with different toxicological properties, which are characterized by their oxide/tallow amine ratio. The toxicity of POEA increases as the tallow amine chain is shortened. The most commonly used POEA is POE tallow amine (POE-15), which was used in the first formulations of glyphosate commercialized under the trade name "Roundup." By using cell culture model systems, Séralini and colleagues demonstrated that the toxicity of glyphosate-based formulations was proportional to their concentration of POE-15 or other ethoxylated surfactants. The formulations that did not contain ethoxylated surfactants were approximately 100 times less toxic to human cells. It was quite a surprising finding to see that the toxicity of two formulations of the same active ingredient could differ by a factor of 100. Thus, the consumer could buy one or another glyphosate-based herbicide formulation without being aware of this difference in toxicity.

The study by Séralini and colleagues based on tissue culture cell lines clearly has its limitations, including exacerbating the observed differential toxicity profiles of the formulations tested. However, the findings of greater toxicity of commercial formulations over glyphosate alone *in vitro* have been replicated *in vivo* in laboratory animals, other animal model systems such as sea urchins, microorganisms (bacteria, microalgae, protozoa), and crustaceans. A more recent study on two life stages of the Pacific oyster shows that POEA-based adjuvants can be very toxic to embryonal and larval development (EC50, 262 µg/L). Metamorphosis tests revealed that although EC_{50} values exceeded 100,000 µg/L for glyphosate and its metabolite ami-nomethylphosphonic acid, they were as low as ~6,000 µg/L for some commercial formulations. Studies have also revealed that some ethoxylated adjuvants can be endocrine disruptors at lower non-toxic concentrations. Recently, it has been reported that POEA-based adjuvants promote triglyceride accumulation in 3T3-L1 adipocytes at concentrations from 0.1 to 10 µM. This is in contrast to glyphosate alone, which did not promote lipid accumulation in this same adipocyte cell line (Mesnage and Antoniou, unpublished results). Another study has shown that ethoxylated adjuvants can inhibit aromatase activity disrupting estrogen–androgen balance.

Table 1. Overview of adjuvants used in pesticide formulations.

Adjuvant type	Example
Penetration agents	Petroleum or mineral oils, vegetable oils, organosilicon
Oder masking agent	1-octanal
Dyes	fd&c blue no. 1, fd&c red no. 40
Preservatives	Hexamethylenetetramine, potassium benzoate, sorbic acid
Stabilizer	Diisopropanolamine, hydroxyethylidene diphosphonic acid, silver nitrate
Diluents	Aluminum hydroxide
Surfactants	Anionic: alkylbenzene sulfonates, sodium laureth sulfate, soap Cationic: dioctadecyldimethylammonium chloride Amphoteric: cocamidopropyl betaine, cocamidopropyl hydroxysultaine Non-ionic: alkoxylated alcohol, ethoxylated alcohol, nonylphenol ethoxylate, tallow amine ethoxylate, alkyl polyoxyethylene ether
Emulsifers	Alkanoic and alkenoic acids, monoesters and diesters of α-hydro-ω-hydroxypoly (oxyethylene), glyceryl monostearate, sodium metasilicate
Propellant	1,1-Difluoroethane, butane, propane
Solvents	*N*-methyl-2-pyrrolidone, polychloromethanes, chlorinated volatile organic compounds, xylene, isopropanol
Antifoaming agent	silicones (e.g., dimethylpolysiloxane), fatty acids
Carriers	Biochar, cyanobacteria, clay minerals, siliceous minerals, zinc-layered hydroxide, polymeric materials such as chitosan, lignin, and poly(ethylene) glycol

This non-exhaustive list presents compounds grouped by category that are classically used as adjuvants in commercial pesticide formulations. Some of these molecules can have dual roles. For instance, surfactants (wetters) are also used as plant penetration agents.

A comparison of the effects of a glyphosate-based herbicide and glyphosate at an equivalent concentration of 25 mg/kg/day on the composition and metabolism of the gut microbiome in Sprague-Dawley rats found that the commercial formulation but not glyphosate alone affected the numbers of observed species in both the cecum and the colon. Although glyphosate has been patented as an antiparasitic agent and suggested to be a bacterial antibiotic (US patent number: US7771736 B2), it is likely that the effects of glyphosate-based herbicides on the gut microbiome could be due to the damaging properties of surfactants present in the adjuvant mixture on the integrity of the intestinal epithelium. In support of this possibility is the observation that emulsifiers have been shown to alter gut microbiome composition in mice by sweeping the lining of the gut, which consequently gave rise to colitis and metabolic syndrome.

More recently, it was shown that the chronic (2 years) administration of a glyphosate-based herbicide (Roundup) induced liver toxic effects in rats at an environmental concentration and daily intake of active ingredient was declared safe by regulatory agencies worldwide. However, further research is required to elucidate whether the glyphosate, the adjuvants, or the combination of the two is at the basis of the observed kidney and especially liver toxicity seen in these animals. It is difficult to attribute the toxicity of a commercial formulation to a given ingredient if they are not tested in parallel in an experiment. Glyphosate-based herbicides can not only contain POEA but also contain multiple adjuvants having intrinsic toxicological properties. These formulations can also include methylchloroisothiazolinone having allergenic properties, light aromatic petroleum distillates having liver toxic effects, or sodium o-phenylphenate considered as possibly carcinogenic to humans.

This and other work has led the European Commission to recommend a ban on the use of POEA-type adjuvants in glyphosate-based herbicide products. Although this can be seen as a positive step forward for public health, this does not exclude the use of POEA in other non-glyphosate-based products. For example, in France, 126 formulations of glyphosate were removed from the market in July 2016, but other POEA-containing pesticides can still be bought. In addition, French farmers can still source POEA as a separate adjuvant mixture (product name Regain, authorization 9300433) to mix with a glyphosate formulation in the spray tank (https://ephy.anses.fr/adjuvant/regain). POEA is also still authorized as a co-formulant in pesticide formulations containing other ingredients such as 2,4-D. Thus, farmers and the general public can still readily be exposed to POEA despite it being banned in glyphosate-based herbicides.

Furthermore, the finding that POEA is widely found in fields in the United States where glyphosate-based herbicides are applied raises concerns that this and other classes of pesticide adjuvants may be entering the food and feed chain undetected, with as yet unknown health consequences.

Discussion and conclusion

The study of the effects of chemical mixtures on health indicators is frequently aired as a priority for the field of toxicology in the twenty-first century. However, within this framework, ignoring the toxicity of the combination of each active ingredient with its adjuvants could lead to misrepresentations of the safety profile of commercial pesticides. Therefore, it is recommended the following actions to protect the public from toxicity that may arise from ingestion of adjuvants:

1. Biomonitoring of different human population groups to identify the true body burden of adjuvant classes of chemicals.
2. Surveying of food products to accurately identify sources of exposure.
3. Long-term laboratory animal toxicity studies comparing commercial formulations with their active principle to measure adverse outcomes stemming from the adjuvants.
4. The gaps in knowledge and consequent uncertainties in risk assessment concerning the toxicity of chemical mixtures, including adjuvants, need to be acknowledged by regulators. Thus, an additional safety factor needs to be added when calculating MRL and ADI values.
5. All ingredients used in the manufacture of commercial formulations of pesticides should be subjected to the same risk assessment. The classification as inert or active has no scientific basis.

Given the all-pervasive nature of adjuvants in products used in both an agricultural and urban/domestic environment, potential toxicity arising from exposure to these chemical mixtures can be greater than from any pesticide active principle. Although considering that all chemicals have intrinsic toxicological properties and that hazardous chemical properties do not necessarily translate into a risk for human health, it is scientifically not sound to argue that adjuvants are so safe that they can be ignored. The implementation of the above recommendations will allow major progress to be made in protecting the environment and general human population from these toxicants. Current practices in risk assessment and regulation fall far short of providing such protection.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This review article is presenting information on unverified effects of a small subset of co-formulants in some Glyphosate-based formulations, with an emphasis on POEA. This co-formulant is a surfactant banned in the EU glyphosate-based products.

Literature review section reaffirms low toxicity of the active ingredient compared with formulated products. No new data presented.

1. Information on the study

Data point:	CA 5.8.3
Report author	Mesnage R. *et al.*
Report year	2017
Report title	Evaluation of estrogen receptor alpha activation by glyphosate-based herbicide constituents
Document No	Food and Chemical Toxicology (2017) Vol. 108, 30-42
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

In this study, the estrogenic potential of glyphosate, commercial GBHs and polyethoxylated tallowamine adjuvants present as co-formulants in GBHs was evaluated. Glyphosate (≥10,000 μg/L or 59 μM) promoted proliferation of estrogen-dependent MCF-7 human breast cancer cells. Glyphosate also increased the expression of an estrogen response element-luciferase reporter gene (ERE-luc) in T47DKBluc cells, which was blocked by the estrogen antagonist ICI 182,780. Commercial GBH formulations or their adjuvants alone did not exhibit estrogenic effects in either assay. Transcriptomics analysis of MCF-7 cells treated with glyphosate revealed changes in gene expression reflective of hormone-induced cell proliferation but did not overlap with an ERα gene expression biomarker. Calculation of glyphosate binding energy to ERα predicts a weak and unstable interaction (-4.10 kcal mol-1) compared to estradiol (-25.79 kcal mol-1), which suggests that activation of this receptor by glyphosate is via a ligan- independent mechanism. Induction of ERE-luc expression by the PKA signaling activator IBMX shows that ERE-luc is responsive to ligand-independent activation, suggesting a possible mechanism of glyphosate-mediated activation.

Materials and Methods

Chemicals - Glyphosate used was the PESTANAL® analytical standard (≥98.0%) obtained from Sigma-Aldrich (UK). The batch of glyphosate (≥98.0%) purchased from AccuStandard (New Haven, CT, USA) was tested exclusively in the ERE transcription luciferase reporter gene assay. Glyphosate-based formulations available on the market were Glyphogan (France, 39-43% iso-propylamine salt of glyphosate, 13-18% of POEA), Roundup Grand Travaux Plus (France, 450 g/L of glyphosate, 90 g/L of ethoxylated etheralkylamine), Roundup Original DI (Brazil, 445 g/L of glyphosate diammonium salt, 751 g/L of other ingredients) and Roundup Probio (UK, 441 g/L of the potassium salt of glyphosate, and other ingredients). POEA was purchased from ChemService (West Chester, PA, USA). The agricultural spray adjuvant was Gen-amin T200 (France, 60-80% of POE-15).

Cell culture - MCF-7, MDA-MB-231 and T47D cell lines were obtained from Prof Joy Burchell (Research Oncology Department, King's College London). T47D-KBluc cells were purchased from the American Type Culture Collection (ATCC, Teddington, UK) and harbour a stably integrated copy of a luciferase reporter gene under control of a promoter containing ERE. All cells were grown in a maintenance medium. Stock solutions of glyphosate, glyphosate-based herbicide formulations, POEA and Genamin T200 surfactant formulation were prepared in serum-free medium and adjusted to pH 7.2. Stock solutions of with estradiol, 3-isobutyl-1-methylxanthine (IBMX) and BPA were prepared by dissolution in ethanol. The solutions for testing were prepared by dilution of the stock solutions in test medium taking care that the solvent concentrations were always kept below 0.5% for the cell assays and below 0.0005% for transcriptome profiling. Cells were released from the flask substrate with trypsin and counted with a hemocytometer prior to seeding. A 24-hour recovery period was allowed for cell adherence in DMEM maintenance medium before cultures were subjected to the desired tests.

E-screen assay - The E-screen allows the determination of estrogenic effects by measuring ER-mediated cell proliferation in hormone responsive cells (MCF-7, MDA-MB-231, T47D). Cells were seeded into 48-well plates at a density of 20,000 cells per well in 250 µL maintenance medium. Following a 24-hour incubation to allow cell attachment, the medium was changed to the medium containing the test compounds. The test medium was refreshed after 3 days. Following another 3-day period of incubation, an MTT assay was performed. The MTT test allows the measurement of cytotoxic effects since the activity of mitochondrial dehydrogenase enzymes indirectly reflects cellular mitochondrial respiration. Cells were incubated with 250 µL of MTT solution for 2 hours. The test was terminated by lysing the cells with dimethyl sulfoxide (DMSO) and optical density was measured at 570 nm using the SPECTROstar Nano plate reader. The proliferative effect was expressed as a percentage of the control cell culture receiving no treatment.

ERE-luciferase reporter gene assay - The ERE-mediated transcription of a luciferase reporter gene was determined in T47D-KBluc cells using the Steady-Glo® luciferase assay system following the manufacturer's instructions. T47D-KBluc cells were seeded in 96-well plates at a density of 20,000 cells per well in 50 µL of maintenance medium and allowed to attach overnight. Prior to adding the test substance an initial 24-hour incubation was performed in the absence of test substance to improve residual estrogen clearance and assay sensitivity. After incubation, Steady-Glo® luciferase reagent was added. The plates were left to stand for 10 minutes in the dark at room temperature to allow cell lysis. Bioluminescence was measured using the Orion II microplate luminometer. ER-mediated gene activation was confirmed by ascertaining if the observed effects were subject to inhibition by addition of the estrogen antagonist ICI 182,780.

Microarray gene expression profiling - MCF-7 cells were seeded into 96-well plates with maintenance medium at a density of 20,000 cells per well. After steroid deprivation in hormone free medium, the cells were treated with test substance in triplicate in three independent experiments. RNA extraction was performed using the Agencourt RNAdvance Cell V2 kit according to the manufacturer's instructions. The samples were checked for RNA quality and quantified. Subsequently, technical replicates of samples, were pooled appropriately such that the final input amount of each biological replicate was 3 ng. Transcriptome gene expression profiles were determined using the Affymetrix Human Transcriptome 2.0 Array. Data were imported and normalized together in Omics Explorer 3.0 using the Robust Multi-array Average (RMA) sketch algorithm. These microarray data were submitted to Gene Expression Omnibus (NCBI) and are accessible through accession number GSE86472.

RNA-sequencing gene expression profiling – Gene expression profiling was performed by applying Illumina sequencing by synthesis technology. The RNA-seq data were submitted to Gene Expression Omnibus (NCBI) and are accessible through accession number GSE8770.

Statistical analysis - The ERE transcription luciferase reporter gene assay and the E-Screen assay were performed 4 and 6 times in triplicate, respectively. The concentrations required to elicit a 50% response (AC_{50}) were determined using a nonlinear regression fit. For the transcriptome analysis, pair-wise comparisons were performed using a t-test controlling for batch effects in Omics Explorer 3.0. Affymetrix microarray and RNA-seq gene expression profiling were performed 3 times in triplicate. Data used for the functional analysis were selected at cut off p-values of <0.05 with fold change >1.2 to evaluate the ER activation signature. Gene and disease ontology were analyzed using the Thomson Reuters MetaCore Analytical Suite recognizing network objects (proteins, protein complexes or groups, peptides, RNA species, compounds among others). The p-values are determined by hypergeometric calculation and adjusted using the method of Benjamini and Hochberg.

Results

E-screen assay - The AC_{50} of 17β-estradiol and BPA in MCF-7 human breast cancer cells was 0.0013 µg/L and 46 µg/L, respectively. Glyphosate induced cell proliferation starting between 1,000 and 10,000 µg/L and peaking around 1,000,000 µg/L. Similar but less pronounced results were observed with the T47D cell line with cells retaining a response to glyphosate but not to Roundup Probio. No proliferative

effects were observed in the ER-negative, hormone-independent MDA-MB-231 cell line, suggesting that the proliferative effects were mediated via the ER. Roundup Probio, tested in MCF-7 cells induced a non-statistically significant trend of cell proliferation .

ERE-luciferase reporter gene assay – Glyphosate stimulated ERE-mediated transcription of the luciferase reporter gene starting at a concentration of 1,000 µg/L. Roundup Probio did not show ER activity at a glyphosate equivalent concentration, which induced a cell proliferative effect when glyphosate is tested alone. This may be explained by the potentially higher toxicity of the glyphosate-based formulation, which could have resulted in cell death at the higher concentrations tested. Two other commercial formulations, Roundup Original DI and Roundup Grand Travaux Plus, also gave negative results. To evaluate whether the luciferase reporter gene stimulatory effects observed with glyphosate were mediated through the ER, experiments were performed with the addition of the potent ER antagonist ICI 182,780. This antagonist was effective at suppressing ER activation induced by 0.001 and 0.01 µg/L but not at 0.1 µg/L of 17β-estradiol. The addition of ICI 182,780 effectively blocked the stimulatory effects of glyphosate at 2,000-20,000 µg/L, confirming its agonist-like mode of action.

Microarray gene expression profiling - MCF-7 cells treated for 48 hours with glyphosate, Roundup Probio, POEA, or bisphenol A, and 17β-estradiol were subjected to full transcriptome profiling. The analysis of gene ontology confirms that genes having their expression altered by treatment of MCF-7 cells with 10,000 µg/L glyphosate were involved in cell cycle regulation, as well as in stimulation by steroid hormones. The transcriptome of glyphosate-treated cells was also reflective of cell death through apoptosis. Roundup ProBio was assessed at the glyphosate equivalent concentrations of 1 µg/L (environmental level), 100 µg/L and 1000 µg/L (showing a cell proliferative trend). The statistical analysis of differential expression showed that genes having their function altered by Roundup or POEA had low fold changes). No genes whose expression was increased or repressed by POEA showed fold changes higher than 2. The study of transcriptome profiles shows that POEA alone is unlikely to have estrogenic effects. This was confirmed in that none of the treatments exhibited statistically significant correlations to the ER biomarker

RNA-sequencing gene expression profiling - In order to confirm endocrine disturbances provoked by glyphosate, RNA extracted from MCF-7 cells treated with glyphosate (10,000 µg/L), estradiol (0.27 µg /L) or bisphenol A (80 µg/L) for 48 hours were subjected to a full RNA-Seq analysis using the Illumina sequencing platform. Although their gene expression profile were different, MCF-7 cells treated by these 3 chemicals presented alterations reflecting a response to steroid hormones and a modulation of cell proliferation, although the significance of the overlapping genes and those in these pathways was lower for glyphosate than the other compounds. Overall, the RNA-seq method was more sensitive and identified 2-3 times more genes whose expression was significantly altered compared to the microarray approach. A total of 5102, 2939 and 1083 genes had their expression significantly disturbed by estradiol, BPA and glyphosate, respectively. Afterwards the ER gene expression biomarker was applied to see if ERα agonist effects could be detected after transcriptome profiling using RNA-Seq platforms. The results were similar to those obtained using the microarray data. Glyphosate failed to pass the threshold of significance for ERα activation. Although the RNA-seq platform was able to identify more statistically significant genes than microarrays, the genes altered differed between the two methods.

Molecular dynamics simulation and ONIOM binding energy calculations - The ability of glyphosate to bind to ERα was evaluated by molecular dynamics simulations and ONIOM calculations. Results from the molecular dynamics simulations of glyphosate-ER interactions reveal that glyphosate enters the active site with a large number of water molecules. The glyphosate phosphonate group interacts with ARG 394 by creating hydrogen bonds. It was noted that glyphosate is unlikely to interact with HIS 524, a residue having a pivotal role in maintaining protein structure in the biologically active agonist conformation. The results of the ONIOM binding energy assessment strongly imply that the binding of glyphosate at the active site of the receptor is weak and unstable, suggesting that glyphosate is unlikely to bind to ERα.

Discussion and Conclusions

The results of this study suggest that glyphosate is a weak activator of ERα in hormone-dependent human breast cancer cells. The glyphosate intake necessary to reach a systemic concentration representative of the estrogenic effects shown in this study would only be encountered in cases of extreme exposures (incidental ingestion, mishandling). An evaluation of the glyphosate binding energy confirmed that this compound is unlikely to activate the ER. The presence of glyphosate-associated cytotoxic effects could explain the discrepancies between the results we obtained with the ERα biomarker and those from the cellular assays. It is thus plausible that glyphosate is activating ERα through a ligand-independent mechanism albeit at high concentrations. To determine any estrogenic potential of adjuvant co-formulants, a number of glyphosate-based formulations were tested and no estrogenic effects could be demonstrated. However, cytotoxicity was observed at glyphosate-equivalent concentrations lower than those required to elicit a proliferative response to glyphosate alone.

Conclusion
This study has demonstrated that glyphosate activates ERα in breast cancer cells but only at relatively high concentrations, and that this activation happens through a ligand-independent pathway. These results suggest that humans exposed to glyphosate would not exhibit ER activation at typical exposure levels.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to evaluate the possible estrogenicity of glyphosate and glyphosate-based formulations and their adjuvants. The tests performed were the E-screen using different cell lines, the ERE-luciferase reporter gene assay, microarray gene expression profiling and RNA-sequencing gene expression profiling. An increase in cell proliferation was observed in human breast cancer cells (MCF-7) at 10,000 µg/L and reached a maximum response at 1,000,000 µg/L. Similar but less pronounced results were observed with the T47D cell line. Glyphosate stimulated ERE-mediated transcription of the luciferase reporter gene starting at a concentration of 1,000 µg/L. The analysis of gene ontology confirms that genes having their expression altered by treatment of MCF-7 cells with glyphosate were involved in cell cycle regulation, stimulation by steroid hormones and cell death through apoptosis. ONIOM binding energy assessment strongly implies that the binding of glyphosate at the active site of the estrogen receptor is weak and unstable, suggesting that glyphosate is unlikely to bind to ERα.

This study has demonstrated that glyphosate activates ERα through a ligand-independent pathway only at high concentrations that are not encountered at typical exposure levels. This publication is considered relevant for glyphosate risk assessment and reliable without restrictions.

Reliability criteria for *in vitro* toxicology studies

Publication: Mesnage *et al*., 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 98.0%. Source: Sigma-Aldrich (UK).
Only glyphosate acid or one of its salts is the tested substance	Y	Also glyphosate based formulations and surfactants

		were tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This study has demonstrated that glyphosate activates ERα through a ligand-independent pathway only at high concentrations that are not encountered at typical exposure levels. This publication is considered relevant for glyphosate risk assessment and reliable without restrictions.		

1. Information on the study

Data point:	CA 5.3
Report author	Milic M. *et al.*
Report year	2018
Report title	Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate
Document No	Arh Hig Rada Toksikol (2018) Vol. 69, 154-168
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this 28 day-study, the effects of herbicide glyphosate administered by gavage to Wistar rats at daily doses equivalent to 0.1 of the acceptable operator exposure level (AOEL), 0.5 of the consumer acceptable daily intake (ADI), 1.75 (corresponding to the chronic population-adjusted dose, cPAD), and 10 mg kg-1 body weight (bw) (corresponding to 100 times the AOEL) were evaluated. At the end of each treatment, the body and liver weights were measured and compared with their baseline values. DNA damage in leukocytes and liver tissue was estimated with the alkaline comet assay. Oxidative stress was evaluated using a battery of endpoints to establish lipid peroxidation via thiobarbituric reactive substances (TBARS) level, level of reactive oxygen species (ROS), glutathione (GSH) level, and the activity of glutathione peroxidase (GSH-Px). Total cholinesterase activity and the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were also measured. The exposed animals gained less weight than control. Treatment resulted in significantly higher primary DNA damage in the liver cells and leukocytes. Glyphosate exposure significantly lowered TBARS in the liver of the AOEL, ADI, and cPAD groups, and in plasma in the AOEL and cPAD group. AChE was inhibited with all treatments, but the AOEL and ADI groups significantly differed from control. Total ChE and plasma/liver ROS/GSH levels did not significantly differ from control, except for the 35 % decrease in ChE in the AOEL and ADI groups and a significant drop in liver GSH in the cPAD and 100xAOEL groups. AOEL and ADI blood GSH-Px activity dropped significantly, but in the liver it significantly increased in the ADI, cPAD, and 100xAOEL groups vs. control.

Materials and methods

Chemicals - Glyphosate (purity of ≤100 %), purchased under the brand name PESTANAL®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany.

Animals - Thirty male Wistar rats were obtained from the breeding unit at IMROH and were kept under pathogen-free, steady-state microenvironmental conditions in polycarbonate cages with 40-60 % humidity at 22 °C and normal 12-hour light/dark cycle. The animals had free access to standard Good Laboratory Practice-certified food and tap water. At 3 months of age, the rats were weighted, inspected, and judged to be healthy and fit for the experiment by a licensed veterinarian at IMROH.

Experimental design - Rats were randomly divided into 6 dose groups of 5 animals each and treated orally by gavage for 28 days. Glyphosate was tested at 0.1, 0.5, 1.75 and 10 mg/kg bw/day, doses corresponding respectively with the AOEL from EFSA, the ADI, the chronic population-adjusted dose (cPAD) from EPA and 100 times the AOEL. Ethyl methanesulfonate (EMS) dissolved in PBS served as the positive control and was administered at 300 mg/kg bw over the last 3 days of the experiment. PBS served as the negative control. Body weight was monitored once a week and the glyphosate doses

adjusted accordingly. Survival and clinical signs of toxicity were monitored on a daily basis. All animals were humanely sacrificed and dissected on day 29, 24 hours after the last dose. All animals were subjected to necropsy and examined for gross pathological changes.

Sample collection and preparation - Liver was excised, rinsed with cold PBS (pH 7.4) and weighed. Livers were then washed in cold TBS buffer (pH 7.5) to remove as much blood as possible, homogenized in a 50 mM potassium PBS (pH 7.4) with 1 mM EDTA, and centrifuged at 20,000 x g at 4°C for 30 minutes to obtain the supernatant. In a separate procedure, a small portion of the liver tissue was minced in a chilled mincing solution to obtain a cellular suspension. The cell suspension was left a few seconds for large clumps to settle, and the supernatant was used to prepare comet slides. This was performed within 60 minutes from sacrifice to avoid confounding necrotic changes. Blood samples were collected directly from the heart into heparinized vacutainers with an extra addition of Li-heparin and mixed vigorously. Samples were then kept at 4°C pending further processing. Plasma for the biochemical assays was prepared by centrifugation of heparinized blood at 976 x g at 4°C for 10 minutes and kept at -20°C pending analysis.

Alkaline comet assay in leucocytes and liver cells - Two microgels were prepared per tissue per animal. Slides were marked with a randomly generated code. For each slide, an aliquot of 10 µL of the cell suspension was mixed with low melting point agarose (LMPA, 0.5 %) dissolved in TBS buffer. "Sandwich" agarose microgels made of four layers were prepared on microscopic slides. Slides were pre-coated with 1 % normal melting point agarose (NMPA) and air-dried. The second gel layer of 0.6 % NMPA was then applied. The third layer consisted of 0.5 % LMPA mixed with heparinized whole blood (10 µL per slide) or 10 µL of liver cell suspension per slide. Finally, 0.5 % LMPA was applied as the top layer over the gel-embedded cells. After solidification of the gel on ice-cold metal tray, the slides were submerged in freshly prepared cold lysing solution (pH 10) at 4°C overnight. The slides were quickly washed with distilled water and left in a vertical Coplin jar with chilled electrophoresis buffer (pH >13) at 4°C for 10 minutes. The slides were then transferred into a horizontal electrophoresis unit. After electrophoresis, the slides were washed 3 times with neutralization buffer (pH 7.5). All gels were dehydrated with 70 % and 96 % ethanol, respectively, air dried, and stored at room temperature. Before scoring, the slides were stained with ethidium bromide (20 µg/mL) and analyzed with a fluorescent microscope at 200x magnification using the Comet Assay IV image analysis system equipped with appropriate filters. Three hundred cells (2 x 150 nucleoids) were scored in total for each animal and sample. Medium-sized cells (parenchymal cells or hepatocytes, between 30 and 40 µm of head length) and small-sized cells (non-parenchymal cells, <30 µm of head length) were recorded separately. Areas near slide margins were not scored. DNA damage was measured as comet DNA tail intensity (% of DNA in tail) and tail length (TL, expressed in µm, measured from the estimated edge of the comet head). Comets with small or non-existing head and large, diffuse tails (cells with >80 % DNA in the tail) were excluded from analysis. The frequency of such comets ("hedgehogs" or "clouds") was determined based on visual scoring among 100 nucleoids per sample.

Determination of ROS levels in plasma and liver - ROS levels in plasma and liver homogenates were measured using 2′,7′-dichlorofluorescein diacetate (DCF-DA). The acetate group of DCFH-DA gets DCFH-DA into the cells or organelles, and once inside, it is removed by cellular esterases, producing reduced DCFH which then can be oxidized by peroxides to form fluorescent oxidized DCF that can be measured spectrophotometrically. Plasma samples and 1 % liver tissue homogenate were prepared by dilution with ice-cold PBS (pH 7.4). Black 96-well plates were filled with 0.07 mL of PBS, and 0.03 mL of 1 % liver tissue homogenate or with 0.1 mL of 10 % plasma in quintuplicate for each glyphosate concentration and sample type. 20 µL of 0.12 mM DCFH-DA dye in PBS was then added to each well, and the plates were incubated at 37°C for 30 minutes. Control for dye autofluorescence was prepared without the addition of dye. Control samples were included in each experiment. Samples were analyzed using a Victor3™ multilabel plate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The readings were expressed as relative fluorescence units (RFU).

Determination of lipid peroxidation in plasma and liver - The concentration of thiobarbituric reactive substances (TBARS), as a measure of lipid peroxidation, was determined. 5 µL of butylated hydroxytoluene (BHT, 0.2 % w/v) and 750 µL of phosphoric acid (1 % v/v) were added to 50 µL of

sample. After mixing, 250 µL of thiobarbituric acid (TBA, 0.6 % w/w) and 445 µL of water were added, and the reaction mixture was incubated at 90°C for 30 minutes. The mixture was cooled, and absorbance measured at 532 nm using a Shimadzu UV probe spectrophotometer. TBARS concentrations were calculated using the standard curves for 1,1,3,3-tetramethoxypropane, obtained by increasing its concentrations, and expressed as µM.

Determination of GSH in plasma and liver - GSH levels were analyzed with a fluorogenic bimane probe using monochlorobimane (MBCl), which reacts specifically with GSH to form a fluorescent adduct. Plasma samples and liver tissue homogenates were prepared as previously described for ROS determination. 20 µL of 0.24 mM MBCl dye in PBS was then added to react at 37 °C for 20 minutes. The amount of GSH in the samples was analyzed using a Victor3™ multilabel plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Control samples were included in each experiment. The readings were expressed as relative fluorescence units (RFU). Each sample analysis was performed in quintuplicate.

Determination of GSH-Px activity in whole blood and liver - GSH-Px activity in whole blood and in the supernatant of liver homogenate were determined in accordance with the European standardized method. The amount of GSH oxidized by t-butyl hydroperoxide was determined based on the decrease in β-NADPH absorbance at 340 nm, measured by spectrophotometry. One unit of GSH-Px was the number of micromoles of β-NADPH oxidized per minute. Its activity in whole blood was expressed per gram of haemoglobin (U g_{Hb}^{-1}), and in liver homogenate per gram of total protein (U $g_{protein}^{-1}$).

Protein quantification - Proteins were quantified according to the method of Bradford, using bovine serum albumin as the standard.

Cholinesterase activity in plasma - Plasma samples were analyzed for total ChE, AChE, and BChE activities in a 0.1 M sodium phosphate buffer (pH 7.4) at 25°C using ATCh (1.0 mM) and DTNB (0.3 mM) as described by Ellman *et al*. To distinguish between AChE and BChE activities the BChE-selective inhibitor ethopropazine (20 µM) was used. Increase in absorbance was monitored at 412 nm over 4 minutes by means of a Cecil 9000 spectrophotometer. Enzyme activities were expressed as IU g/protein.

Statistical analysis - Statistical analysis was run on Dell Statistica software STATISTICA, version 13.2. The data were first evaluated with descriptive statistics. The results were expressed as means ± standard deviation, and for the comet assay also medians and ranges (min-max) were used. Relative liver weights were logarithmically transformed and analyzed with one-way ANOVA. For pairwise organ comparison the *post-hoc* Tukey's HSD test was used. Normality was tested with the Levene's test. Since the results of the alkaline comet assay were not normally distributed even after logarithmic transformation, the non-parametric Mann-Whitney U test was used. For multiple comparisons of cholinesterase activities, TBARS, and GSH-Px activities between the glyphosate and control groups the Kruskal-Wallis test was used. ROS and GSH levels were compared between the groups using the non-parametric Mann-Whitney U test. P values of ≤ 0.05 were considered statistically significant.

Results and Discussion

Changes in body and liver weight - No premature death or any clinical signs of systemic toxicity in male adult Wistar rats was observed in any of the groups dosed with glyphosate for 28 days. Gross necropsy did not reveal any treatment-related findings. All glyphosate-treated animals showed a similar weight gain throughout the 28-day treatment period, with significant difference between the day before treatment and at the end of treatment. There were no statistically significant changes in relative liver weight.

Alkaline comet assay in leucocytes and liver cells - Glyphosate-treated rats had higher primary DNA damage in leukocytes when compared to control in tail length and tail intensity. While tail length was significantly greater at all dose levels, only the lowest dose tested resulted in significantly higher mean tail intensity. One reason for that could be high standard deviations. It is worth noting that glyphosate

caused greater DNA damage in liver cells than in the leukocytes. The greatest liver cell DNA damage (as tail intensity) occurred at the lowest and the highest dose both in parenchymal and non-parenchymal liver cells. In fact, the group exposed at 10 mg/kg bw/day suffered even greater damage in medium-sized liver cells than the positive control.

ROS levels in plasma and liver - No statistically significant difference was found in ROS levels in plasma between the glyphosate dose groups and control. A small decrease in ROS of around 7 % was seen in the groups with the highest glyphosate doses. Liver tissue showed a similar pattern with a decrease in ROS at the highest dose levels which was statistically significant at 10 mg/kg bw/day. ROS levels in the liver were 100 times greater than in the plasma.

Lipid peroxidation in plasma and liver - TBARS concentrations in plasma and liver were decreased in all glyphosate-dosed groups when compared to control.

GSH levels in plasma and liver – In plasma, no statistically significant difference was evident between the glyphosate-dosed groups and the control group. In the liver, however, a statistically significant decrease in GSH was seen in the two highest dose groups.

GSH-Px activity in whole blood and liver - GSH-Px activity in whole blood was statistically significantly decreased at 0.5 and 1.75 mg/kg bw/day but not at 10 mg/kg bw/day. In contrast, GSH-Px activity in the liver was statistically significantly increased at 0.5, 1.75 and 10 mg/kg bw/day.

Cholinesterase activity in plasma - Glyphosate did not significantly affect total cholinesterase (ChE) activity, even though there was a decrease about 35 % at 0.1 and 0.5 mg/kg bw/day when compared to control. Acetyl cholinesterase (AChE) activity in turn, was statistically significantly decreased at 0.1 and 0.5 mg/kg bw but not at the higher dose levels. There was no statistically significant change in activity of butyl cholinesterase in the glyphosate dose groups when compared to the control group.

Discussion and conclusion

This study suggests that sub-chronic exposure to glyphosate mostly affects DNA in the liver and white blood cells. General oxidative stress was not confirmed, while total cholinesterase activity showed some, but inconsistent, changes from control. Exposure to environmentally relevant levels of glyphosate, presumably not harmful to humans, seems to have different effects from exposure to much higher doses, especially where oxidative stress is concerned. In this study it has been demonstrated that even without evidence of oxidative stress, small doses allowed for human exposure can produce significant primary DNA damage and inhibit AChE, which may both be related to indirect action through glycine substitution.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Glyphosate was orally administered to male rats at 0.1, 0.5, 1.75 and 10 mg/kg bw for 28 days to investigate its effect on DNA damage, oxidative stress and cholinesterase activity. The endpoints of this study were DNA damage as measured in the alkaline comet assay, ROS in plasma and liver, lipid peroxidation in plasma and liver, GSH in plasma and liver, GSH-Px activity in whole blood and liver and total cholinesterase, acetyl cholinesterase and butyl cholinesterase activity in plasma. The results of the alkaline comet assays revealed a statistically significant increase in tail length and tail intensity in leucocytes and small and medium sized liver nuclei. With the exception of tail length of small sized liver nuclei no dose effect relationship was evident. Tail intensity of the leucocytes could not be assessed because of the very high variability of the results. From the results of the oxidative stress markers in plasma and liver and cholinesterase activity in plasma it can be concluded that there was no dose related effect.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the high variability of the results of the comet assay, although the conduct of this assay was in general compliant with OECD test guideline 489.

Reliability criteria for *in vivo* toxicology studies

Publication: Milic *et al*., 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	*In vivo* Comet assay was compliant with OECD TG 489.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of ≤100 %). Source: PESTANAL®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	0.1, 0.5, 1.75 and 10 mg/kg bw/day, positive and negative control included.
Number of animals used per dose level reported	Y	5 males per dose group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	Limited to body weight, organ weight, DNA damage, oxidative stress and ChE activity.
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the high variability of the results of the comet assay, although the conduct of this assay was in general compliant with OECD test guideline 489.		

1. Information on the study

Data point:	CA 5.4
Report author	Nagy K. *et al.*
Report year	2019
Report title	Comparative cyto- and genotoxicity assessment of glyphosate and glyphosate-based herbicides in human peripheral white blood cells
Document No	Environmental Health (2018) Vol. 17, pp. 52/1-52/13
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study investigates the cyto- and genotoxic potential of the active ingredient glyphosate and GBHs in human mononuclear white blood (HMWB) cells. HMWB cells were treated for 4 h at 37 °C with increasing concentrations (1–1000 μM) of glyphosate alone and in three GBHs (Roundup Mega, Fozat 480 and Glyfos) to test cytotoxic effect with fluorescent colabelling and genotoxic effect with comet assay. In addition, each concentration was tested with and without metabolic activation using human liver S9 fraction.

Materials and methods

Chemicals; Analytical-grade glyphosate (N-(phosphonomethyl) glycine, CAS No: 1071-83-6) was purchased from VWR International Kft (Debrecen, Hungary) and samples of three GBHs, i.e.

• Roundup Mega containing 551 g/L or 42% (w/w) potassium salt of glyphosate (CAS No: 70901-12-1; equivalent to 450 g/L glyphosate) and 7% (w/w) ethoxylated etheralkylamine (CAS No: 68478-96-6);

• Fozat 480 containing 480 g/L or 41% (w/w) isopropylammonium salt of glyphosate (CAS No: 38641-94-0; equivalent to 360 g/L glyphosate) and < 5% (w/w) hygroscopic substances;

• Glyfos containing 480 g/L or 42% (w/w) isopropylammonium salt of glyphosate (equivalent to 360 g/L glyphosate) and 9% (w/w) polyethoxylated tallow amine (CAS No: 61791-26-2);

were kindly provided by pesticide applicators. Composition data for each formulation were retrieved from the material safety data sheets (MSDS). Chemicals used for the assays and human liver-derived metabolic activation system (S9 fraction) were obtained from Sigma-Aldrich Chemie GmbH (Heidelberg, Germany). Cell culture medium and its supplements were obtained from Biowest (Nuaillè, France). The acetomethoxy derivative of calcein (Calcein AM) and propodium iodide (PI) fluorescent dyes were purchased from Biotium (Hayward, CA, USA). Heparin-containing vacutainers were purchased from BD Vacutainer Systems (Plymouth, UK).

Cell cultures; Human peripheral whole blood samples were obtained by venipuncture and collected into heparin-containing vacutainer tubes from four non-smoking, healthy volunteers (three males and one female, aged 20–40 years) without known previous contact with pesticides, mutagens or carcinogens. Cultures were prepared within 1 h of phlebotomy. Human mononuclear white blood (HMWB) cells were prepared from erythrocytes by density-gradient centrifugation over Histopaque-1077 gradient. The buffy coat was aspirated and re-suspended in RPMI 1640 medium containing 10% foetal calf serum (FCS).

Cell treatment; HMWB cells were treated in the cell-culture medium with increasing concentrations (1 μM, 10 μM, 100 μM, 250 μM, 500 μM, 750 μM and 1 mM) of glyphosate alone and in three GBHs in

a way that the concentrations of glyphosate in GBHs were equivalent. Concentrations were chosen based on the results from previous in vitro studies performed on human lymphocytes. The stock solutions and the dilution series were made in phosphate-buffered saline (PBS) and adjusted with 1 M NaOH to pH 7.2. Aliquots of different concentrations of glyphosate and GBH solutions, as well as PBS as negative control, were added to the cell cultures and incubated for 4 h at 37 °C. The PBS content was always < 10% (v/v) in the cell culture medium. The experiments were conducted in the presence and absence of S9 fraction. 100 μL of the working S9 mix containing 10% (v/v) of S9 fraction was composed of 8 mM MgCl2, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate, and 4 mM NADP was added to the S9+ samples. 100 μM hydrogen peroxide was used as a positive control.

Cytotoxicity assay; After treatment, aliquots of samples were immediately subjected to cytotoxicity test. Calcein AM and propidium iodide (PI) fluorescent dyes were used to colabel the cells. Calcein AM is a non-polar compound that passively crosses the plasma membrane of living cells, where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm. PI is a DNA intercalating dye, which is able to permeate membranes of dead and dying cells but cannot penetrate plasma membranes of live healthy cells. Both fluorescent dyes were dissolved in PBS (pH 7.2) to a final concentration of 2 μM each. 200 μL of this working solution added to the cell pellets (1 × 105 cells), and incubated for 30 min at 4 °C, protected from light. The labelled cells were washed and re-suspended in ice-cold PBS buffer. 40 μL of the cell suspension was put on a microscope slide for immediate microscopic examination at 100x magnification using a Zeiss Axioplan epifluorescent microscope. FITC filter for Calcein AM and TRITC filter for PI was applied to excite the colabelled cells. Survival rate was determined by visual examination of 10 randomly selected non-overlapping fields per slide. Each field contained 10 to 30 images. Cell viability was expressed as the mean of the percentages of living cells from repeated experiments. The proportions of living cells observed in technical replicates were subjected to statistical analysis.

Genotoxicity assay; The alkaline version of the comet assay was performed according to the methodology of Collins (2004). Following treatment, samples were centrifuged and HMWB cells were resuspended in serum-free medium at a cell density of 2000 cells/μL. Degreased frosted slides were coated with two layers: 1% normal melting point agarose (NMA) covered with 0.75%, low melting point agarose (LMA) containing the cells (~2 × 105 per slide). After solidification, the embedded cells were lysed (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris base, pH 10, 1% sodium N-lauroyl sarcosinate and 1% Triton X-100 added fresh) at 4 °C for 18–20 h, shielded from light. After lysis, the DNA was allowed to unwind for 20 min in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA, pH 13) and subjected to electrophoresis in the same buffer for 20 min at 0.8 V/cm and 300 mA in a horizontal electrophoresis tank (Cleaver Scientific, Rugby, UK). Finally, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris base-HCl, pH 7.5) to remove excess alkali and detergent. After drying, each slide was stained with ethidium bromide (20 μg/ml) and stored in a humidified container at 4 °C until analysis. The fluorescence signal was detected at 400x magnification using a Zeiss Axioplan epifluorescent microscope equipped with a CCD camera connected to an image analysis system. The Comet Imager v.2.2.1. Software (MetaSystems GmbH, Germany) was used to analyse 2 × 50 randomly captured comets from duplicate slides and compute the DNA damage parameters. Percentage of DNA in the tail (tail DNA%) and tail length in μm (TL) were measured to quantify DNA damage. The results are presented as mean of the median values of DNA damage parameters from repeated experiments. The medians of technical replicates were subjected to statistical analysis.

Data analysis; Experiments were independently performed three times from three different donors. The rate of cell viability and the central values of DNA damage parameters in the comet assay induced by various concentrations of the xenobiotics in repeated experiments were statistically compared to that of untreated cells using ANOVA with Dunnett's post hoc test. To statistically analyse the effect of metabolic activation, DNA damage values were pooled from three repeated experiments to compare results from S9-treated and S9-untreated samples at each exposure concentration by using Mann-Whitney test because pooled data sets followed non-normal distribution. Statistically significant difference was accepted at 5% significance level.

Results

Cytotoxicity; The viability of HMWB cells treated with glyphosate alone was found to be over 86% in the absence and presence of S9 over the entire concentration range (Fig. 1). Regardless of metabolic activation, GBHs induced a significant decrease in the proportion of living cells from 250 μM of Round Mega and Glyfos whereas from 500 μM of Fozat 480.

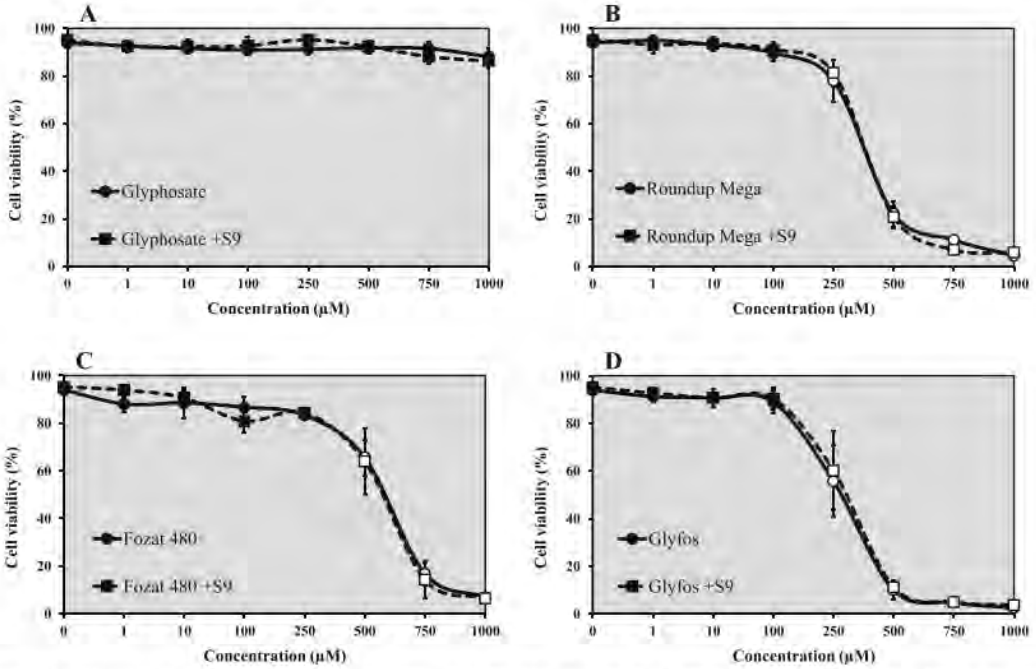


Fig. 1. Effect of 4-h exposure to increasing concentrations of glyphosate (A), Roundup Mega (B), Fozat 480 (C) and Glyfos (D) on cell viability in the absence and presence (+S9) of metabolic activation system. The data points indicate the means ± stand error of the mean (SEM) of three repeated experiments. Statistically significant decrease of cell viability, indicated by empty data points, was determined by comparing the values induced by various doses of glyphosate or GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.

DNA damage; Exposure of HMWB cells to glyphosate in the 0–1000 μM concentration range did not result in dose-dependent increase of DNA damage measured by the comet assay parameters. Unlike the active principle, GBHs induced statistically significant increase of both DNA damage parameters from 500 μM (Roundup Mega and Glyfos) and 750 μM (Fozat 480) compared to untreated cells. S9 treatment did not influence the effect of active ingredient glyphosate over the whole concentration range. On the contrary, addition of S9 to the assay modified the effects of GBHs in a non-consistent manner. The presence of metabolic enzymes significantly decreased the DNA damage induced by Roundup Mega and Fozat 480 at 1000 μM and from 10 μM, respectively. Metabolic activation could be observed only in samples exposed to 250 μM or higher concentrations of Glyfos indicated by the statistically significant differences in the tail DNA% and TL values between the S9-treated and S9-untreated cells (Tables 1 and 2).

Table 1
DNA damage induced by 4-h exposure to glyphosate and GBHs with and without metabolic activation system (S9) in HMWB cells measured as tail DNA%.

Concentration (μM)	Tail DNA% ± SEM							
	- S9				+ S9			
	Glyphosate	Roundup Mega	Fozat 480	Glyfos	Glyphosate	Roundup Mega	Fozat 480	Glyfos
0	1.51 ± 0.05				1.39 ± 0.18			
1	2.81 ± 0.30	2.61 ± 0.39	2.27 ± 0.60	1.51 ± 0.41	2.90 ± 0.49	1.91 ± 0.60	1.60 ± 0.05	3.64 ± 2.33††
10	2.86 ± 0.28	2.97 ± 0.87	8.39 ± 5.37	2.08 ± 0.69	2.03 ± 0.38	1.77 ± 0.82	2.19 ± 1.10†††	0.99 ± 0.10†††
100	2.51 ± 0.52	2.49 ± 0.94	13.09 ± 9.67	1.87 ± 0.80	2.06 ± 0.57	2.23 ± 0.77	1.99 ± 0.76†††	1.24 ± 0.33
250	2.42 ± 0.18	(3.47 ± 0.55)	6.59 ± 1.52	(6.11 ± 2.58)	3.01 ± 0.45	(3.58 ± 0.07)	2.97 ± 0.47†††	(22.84 ± 14.24†††)
500	2.60 ± 0.84	(16.88 ± 3.54***)	(7.47 ± 0.69)	(17.69 ± 2.06*)	2.98 ± 0.24	(13.71 ± 3.09)	(4.59 ± 0.65†††)	(36.81 ± 15.40†††
750	3.64 ± 1.00	(24.67 ± 2.56***)	(28.58 ± 1.93*)	(37.81 ± 5.92***)	3.46 ± 0.67	(25.64 ± 6.53**)	(24.20 ± 1.20***†	(50.47 ± 10.64*†††)
1000	1.57 ± 0.24	(44.75 ± 0.46***)	(45.94 ± 3.74***)	(54.58 ± 2.74***)	1.51 ± 0.16	(31.45 ± 4.14***†††)	(22.28 ± 2.40***†††)	(62.38 ± 2.99**†††)

Data are means of median values of three repeated experiments.
Data in parentheses refer to samples with significant cytotoxic response.
Statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) increase in DNA damage was determined by comparing the values of DNA damage induced by various doses of glyphosate and GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.
Statistically significant ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$) difference in DNA damage levels between S9-treated and S9-untreated cells induced by the same concentration of glyphosate and GBHs was determined by Mann-Whitney test.

Table 2
DNA damage induced by 4-h exposure to glyphosate and GBHs with and without metabolic activation system (S9) in HMWB cells measured as tail length (μm).

Concentration (μM)	Tail length (μm) ± SEM							
	- S9				+ S9			
	Glyphosate	Roundup Mega	Fozat 480	Glyfos	Glyphosate	Roundup Mega	Fozat 480	Glyfos
0	0.47 ± 0.19				0.35 ± 0.06			
1	0.66 ± 0.06	0.52 ± 0.08	0.39 ± 0.08	0.11 ± 0.08	0.60 ± 0.08	0.34 ± 0.14	0.18 ± 0.04	0.56 ± 0.45††
10	0.67 ± 0.07	0.77 ± 0.27	1.77 ± 1.09	0.29 ± 0.15	0.65 ± 0.12	0.40 ± 0.17	0.57 ± 0.26†††	0.05 ± 0.04†††
100	0.64 ± 0.13	0.56 ± 0.27	2.70 ± 1.92	0.27 ± 0.15	0.68 ± 0.15	0.42 ± 0.18	0.41 ± 0.17†††	0.17 ± 0.10
250	0.99 ± 0.22	(1.09 ± 0.14)	1.51 ± 0.12	(1.27 ± 0.38)	0.90 ± 0.25	(1.03 ± 0.25)	1.00 ± 0.32†††	(4.41 ± 2.42†††)
500	0.93 ± 0.35	(3.93 ± 1.41*)	(1.62 ± 0.12)	(5.26 ± 1.37**)	0.81 ± 0.20	(2.87 ± 0.37*)	(1.19 ± 0.23†††)	(7.31 ± 2.27*†††)
750	1.05 ± 0.22	(5.40 ± 0.76**)	(6.57 ± 0.11**)	(8.91 ± 0.97***)	0.99 ± 0.21	(4.51 ± 1.07***)	(4.61 ± 0.19***†††)	(9.55 ± 0.50**†)
1000	0.31 ± 0.08	(6.77 ± 0.41***)	(8.13 ± 0.61**)	(10.09 ± 0.39***)	0.28 ± 0.07	(5.62 ± 0.32***†††)	(3.90 ± 0.31***†††)	(10.41 ± 0.22***†)

Data are means of median values of three repeated experiments.
Data in parentheses refer to samples with significant cytotoxic response.
Statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) increase in DNA damage was determined by comparing the values of DNA damage induced by various doses of glyphosate and GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.
Statistically significant ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$) difference in DNA damage levels between S9-treated and S9-untreated cells induced by the same concentration of glyphosate and GBHs was determined by Mann-Whitney test.

Discussion

The use of glyphosate containing products both for agricultural and residential purposes continues to rise since their first introduction to the market in 1974. Today, uncountable GBHs are registered in more than 130 countries worldwide. The unrestricted application of GBHs in the past few decades has resulted in the accumulation of glyphosate residues in environmental media exposing – and also impairing the health of – aquatic and terrestrial animals, as well as humans. In this study, we measured potential differences in the cyto- and genotoxicity between the declared active ingredient glyphosate and three marketed GBHs in HMWB cells in vitro with the use of the comet assay. To examine the toxicity of potential metabolites of the selected herbicides, human liver-derived metabolic enzyme system (S9) was also applied. The cytogenetic side effects of glyphosate and GBHs in humans have received pronounced scientific coverage in the last decade, resulting in numerous in vivo and in vitro human studies, which demonstrated that glyphosate alone or GBHs have detectable genotoxic potential, while others have reached opposite conclusions. Our results indicate that glyphosate alone could not induce DNA strand breaks in HMWB cells up to 1000 μM measured with the comet assay even in the presence of metabolic activation. This finding contrasts with previous studies employing isolated human blood mononuclear cells treated with glyphosate concentrations corresponding to ours, but using different experimental settings. A study showed with comet assay that glyphosate at 0.7 μM (in vitro) caused a statistically significant increase of DNA damage in human peripheral blood lymphocytes; however, they applied longer (20 h) exposure time than used in this study, and, unlike in our experiment, cells were incubated on microscope slides embedded in agarose gel at 25 °C. A further study also observed DNA damage in the above cell type from 20.7 μM (3.5 μg/ml) and from 250 μM, respectively. Although they incubated the cell cultures under the same conditions as used in our study (for 4 h at 37 °C), they applied LMA at lower concentration (0.5%), as well as lower electrophoretic voltage (0.7 V/cm) and time (15 min). The execution of comet assay by them applying 24 h exposure time and lower electrophoretic voltage was also different from the method we used. These discrepancies in the experimental design along with the possible inter individual variability in response to genotoxic insults may explain the contradictory findings. The cytotoxic potential of the active ingredient glyphosate in the 0–1000 μM concentration range was found to be minimal, whereas formulations showed to have pronounced cell-killing activity to HMWB cells in our study. All the three GBHs resulted in substantially decreased cell viability (< 23%) from 500 μM (Roundup Mega and Glyfos) and from 750 μM (Fozat 480) concentrations. Exposure to GBHs also caused statistically significant ($p < 0.05$) increase of DNA damage from 500 μM (Roundup Mega and Glyfos) and 750 μM (Fozat 480). However, this observation cannot be explained by the direct genotoxic potential of GBHs, rather due to the high level of cytotoxic activity of the formulations, because cell death mechanisms, both apoptosis and necrosis, can produce spontaneous DNA fragmentation that can act as a confounder in the assessment of primary DNA damage in the comet assay. The high cytotoxic potential of GBHs may be attributed to the presence of other ingredients in the formulations, which has been reported in previous publications. POEA, the declared co-formulant in Glyfos, is more than 1000 times more cytotoxic than glyphosate alone, and concerns were also raised for its genotoxic potential at concentrations not causing cytotoxicity. Thus, EFSA concluded that POEA is clearly more toxic than glyphosate when tested in GBHs, and in response to this, Glyfos was withdraw from the Hungarian market in 2017. The ethoxylated etheralkylamine added to Roundup Mega has very similar toxicological properties as POEA, which is supported by the identical dose-response relationships of Roundup Mega and Glyfos, both of them containing surfactants at roughly the same concentration. The adjuvant content of Fozat 480 (< 5% hygroscopic substances) could not be determined exactly from the MSDS; however, we can suspect from the path of the cell viability dose-response that it may also contain ethoxylated surfactants similarly to the other two GBHs. It is proven that ethoxylated adjuvants can be embedded into the cell membrane disrupting its integrity and permeability and therefore increasing the bioavailability of glyphosate. In this sense, surfactants in GBHs have an indirect synergistic effect with glyphosate. Researchers concluded that DNA damage observed in HMWB cells after exposure to glyphosate, Roundup 360 PLUS or the metabolite of glyphosate (aminomethylphosphonic acid, AMPA) was not due to direct interaction of these compounds with DNA as no DNA adducts formation has been observed, but rather ROS-mediated effects induced by the chemicals leading to cell death and indirect DNA damage. Previous studies have not been able to clearly demonstrate the oxidative DNA damaging potential of glyphosate; however, GBHs produced

an increase in reactive oxygen species in both in vitro and in vivo test systems highlighting the role of adjuvants in the cytotoxic effects observed in our experiments. Despite the definite cytotoxic effect, Roundup Mega and Fozat 480 are still commercially available not only in Hungary, but also in many other European countries under various brand names. In our study, the presence of metabolic activation did not alter the cytotoxic potential of either the active ingredient glyphosate or the GBHs. By contrast, we noted decreased DNA damages in the comet assay with Roundup Mega and Fozat 480, but increased damage with Glyfos as a result of S9 treatment. In humans, the only metabolite of glyphosate is the AMPA. It has been shown that AMPA is not able to induce DNA damage below a relatively high (4.5 mM) concentration, which is in agreement with our data. The diverse response of GBHs to metabolic activation observed in the comet assay may be explained by the presence of variable adjuvants in the formulations that underwent different metabolic modifications. Our results may indicate that the metabolite(s) of POEA in Glyfos can be more toxic than the parent compound in the comet assay, but not in the cell viability assay. There is no evidence in the published literature that POEA or other surfactants, or even products which contain these adjuvants, might undergo metabolic activation, and afterwards, become able to cause cytogenetic effects. The potential metabolic transformation of adjuvants can be hypothesized only from animal experiments; however, these studies have provided contradictory findings so far. This can still be considered as a critical knowledge gap to clarify the genotoxic potential of adjuvants in common commercial formulations of glyphosate under in vivo circumstances. A complex long-term experimental animal study has recently been initiated to assess possible risks resulting from the ubiquitous exposure to GBHs, and has already provided preliminary data on Roundup-induced endocrine effects and altered reproductive developmental parameters in male and female Sprague Dawley rats at a dose level considered as “safe”.

Conclusion

This is the first study that compared toxic effects of various glyphosate-based herbicide formulations to each other and with the declared active ingredient glyphosate in isolated human mononuclear white blood cells. GBHs caused much stronger cytotoxic effect on HMWB cells in comparison to glyphosate that may be attributed to the effect of various surfactants added to the formulations or their interaction with the active ingredient glyphosate and/or with other components of GBHs. Therefore, the GBHs-induced DNA damage observed in the comet assay could be most likely explained by non-genotoxic mechanisms and cannot indicate direct DNA damaging effects of glyphosate-based herbicide formulations. Nevertheless, by applying extended exposure durations and/or other test systems, such as the cytokinesis-block micronucleus cytome assay would allow for the discrimination between the cyto- and genotoxic effects and for the determination of the possible permanent genotoxic effect of these herbicides. Furthermore, this study, for the first time, pointed out the possibility that POEA containing formulation can undergo metabolic activation which draws attention to the need for comprehensive investigation of the toxicity of formulations to confirm our results and to assess the true health risks of environmental and occupational exposures.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes a well conducted comet assay using human lymphocytes as the test system to examine the genotoxicity and cytotoxicity of glyphosate and 3 commercial products containing glyphosate. No DNA damage was induced by analytical grade glyphosate. The 3 glyphosate products induced an increase in tail intensity in the comet assay only at highly cytotoxic concentrations, non-toxic concentrations induced no DNA damage.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and no positive control was used.

Reliability criteria for *in vitro* toxicology studies

Publication: Nagy *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity, content and storage conditions are not reported. Source: VWR International Kft, Debrecen, Hungary.
Only glyphosate acid or one of its salts is the tested substance	N	Also 3 GBH tested: Roundup Mega, Fozat 480, Glyfos.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Human mononuclear white blood cells.
Test conditions clearly and completely described	Y	Comet selection criteria were not stated. It is not stated if the slides were coded prior to scoring
Metabolic activation system clearly and completely described	Y	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	1, 10, 100, 250, 500, 750 and 1000 μM.
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	No HCD so it is unknown what degree of background variation is apparent in this test system. This is exacerbated by the use of a single set of control cultures
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and no positive control was used.		

1. Information on the study

Data point:	CA 5.5
Report author	Pahwa M. *et al.*
Report year	2019
Report title	Glyphosate use and associations with non-Hodgkin lymphoma major histological sub-types: findings from the North American Pooled Project
Document No	Scandinavian Journal of Work, Environment & Health. 2019;45(6):600–609
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Not applicable for epidemiologic studies
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this paper a pooled reanalysis of the data from 2 published non-Hodgkin's lymphoma (NHL) case control studies was conducted: McDuffie et al. 2001 and DeRoos et al. 2003. The reanalysis sought to evaluate associations for glyphosate use and NHL overall and by histological sub-type. In addition, the pooled analysis implemented more extensive control of confounding factors than in the original publications and considered the impact of excluding pesticide information provided by next-of-kin or proxy respondents. The OR for NHL overall for ever using glyphosate was 1.4 (95% CI 1.1, 1.8). After adjustment for other pesticides, the OR was reduced to 1.1 (95% CI 0.8, 1.5). ORs for ever using glyphosate, adjusted for other pesticides, by NHL sub-type were 0.7 (95% CI 0.4, 1.2) for FL, 1.2 (95% CI 0.8, 1.9) for DLBCL, 1.8 (0.9, 3.7) for SLL, and 1.5 (95% CI 0.9, 2.6) for other NHL sub-types. Adjusted ORs excluding data from proxy respondents [supplemental table 1 in the article] were: 0.95 (95% CI 0.7, 1.3) for NHL overall, 0.7 (95% CI 0.4, 1.1) for FL, 1.1 (95% CI 0.7, 1.8) for DLBCL, 1.8 (0.0, 3.8) for SLL, and 0.96 (95% CI 0.5, 1.9) for other NHL sub-types. ORs for NHL overall were not elevated in the higher categories for years of use (> 3.5 years OR 0.9, 95% CI 0.6, 1.4) or lifetime days of use (> 7 days OR 1.1, 95% CI 0.7, 1.8) though there was an elevated OR for the category of more than 2 days of use/year (OR 1.7, 95% CI 1.0, 2.9). For NHL subtypes, consistent patterns of association across exposure metrics were not seen, with the possible exception of SLL, though SLL findings were not statistically significant. In general, exclusion of proxy respondents reduced ORs to a minor extent with the exception of the analyses for other NHL subtypes. For NHL overall, ever use and analyses that considered duration of use per se or lifetime days of use did not show a relationship with glyphosate. There was a moderate association seen for glyphosate use for the metric > 2 days/year. There was some limited evidence of an association between glyphosate use and SLL across exposure metrics, but the small number of SLL cases resulted in very imprecise OR estimates as evidenced by wide 95% CIs. The major limitation of the study is possible case recall bias in the reporting of prior pesticide use.

Materials and methods

Study population and exposure assessment

Pahwa et al. pooled data from case control studies in the US and Canada. For NHL specifically, this study is essentially a reanalysis of the published studies by McDuffie et al. (2001) in Canada and DeRoos et al. (2003) in the US. Case identification in the US was through cancer registries and hospitals during the 1980s in four US states (Iowa/Minnesota, Kansas, and Nebraska) and between 1991 and 1994 in six Canadian provinces (Quebec, Ontario, Manitoba, Saskatchewan, Alberta, and British Columbia). Methods for each study have been previously described. For this pooled analysis, the original histology

codes were revisited and classified according to a single scheme [International Classification of Diseases for Oncology version 1 (ICD-O-1)].

Participants, or their proxies, provided information about demographic characteristics, pesticide use, agricultural exposures, and exposure to other known or suspected NHL risk factors, including lifestyle and medical and occupational history. Self-reported glyphosate use was examined using several exposure metrics: ever/never, duration (years used), frequency (days/year handled), and lifetime-days (number of years used multiplied by number of days/year handled). Categories were created for duration, frequency, and lifetime-days analyses based on the median of glyphosate used/handled among controls. Some participants had missing data for duration and frequency of glyphosate use despite reporting that they had ever used glyphosate. In duration and frequency analyses, values for missing data were assigned to cases and controls based on the median duration or frequency of reported glyphosate use among controls by state/province and 10-year age group (simple imputation) and were used for the main analyses. Ordinal analyses and associated trend tests were conducted to determine possible changes in association for increasing increments of every five years, five days/year, and ten lifetime-days of glyphosate use.

Statistical analyses

Unconditional multiple logistic regression was performed using the LOGISTIC procedure of the SAS 9.4 statistical software package (SAS Institute, Cary, NC, USA) to calculate ORs and 95% CIs for associations between glyphosate exposure metrics (ever/never, duration, frequency, lifetime-days, and as ordinal variables) and NHL overall and by histological sub-type [diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), small lymphocytic lymphoma (SLL), and other]. Initial logistic regression models (OR) contained the following variables: age [age at diagnosis (cases); age at interview or death (controls)], state/province, sex, lymphatic or hematopoietic cancer in a first-degree relative, response by a proxy, and use of any personal protective equipment (PPE). Pesticides that were correlated with glyphosate use in the pooled data and that had previously been associated with NHL based on the individual case-control studies, specifically 2,4-dichlorophenoxyacetic acid, dicamba and malathion, were included in the more fully adjusted logistic regression models (OR). The former models will be referred to as crude and the latter models as adjusted.

Trends for duration, frequency, and lifetime-days of glyphosate use and NHL ORs were assessed by the asymptotic Cochran-Armitage trend test. Subjects who never used glyphosate were the reference group for all analyses. There was a small proportion of subjects (N=175, 2.6% of all participants) with missing age values. These were imputed using simple imputation based on state/province- and case/control-specific means of age rounded to the nearest whole number. Sensitivity analyses were conducted by excluding proxy respondents from the main analyses.

Ethics approval and consent to participate

Ethics approval for the pooled analysis was obtained from the University of Toronto Health Sciences Research Ethics Board (#25166) and an exemption was obtained from the US National Institutes of Health Office of Human Subjects Research (#11351). Investigators of individual studies received human subjects' approval from their institutions for each study prior to collection of data.

Results

Characteristics of NHL cases and controls

A total of 1690 NHL cases and 5131 controls was available for analysis – 69.6% of the cases and 70.6% of the controls were from the US studies. All NHL cases and controls, including those with proxy respondents, were included in analyses of ever/never glyphosate use. For assessments involving duration of use, 1520 cases and 4183 controls were included. For frequency and lifetime-days analyses, 898 cases and 2938 controls were included. The numbers of cases and controls available for the sensitivity analysis excluding proxy respondents were smaller (Figure 1). Characteristics of NHL cases and controls, including histological sub-types, are presented in Table 1.

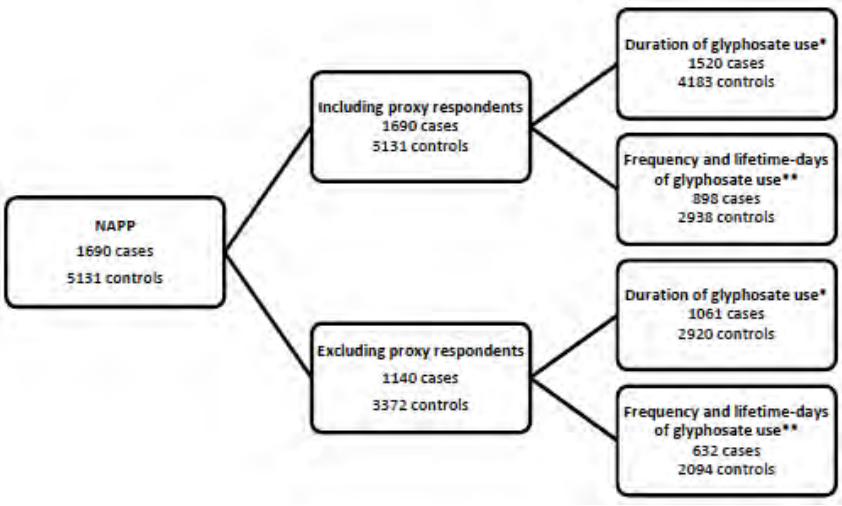


Figure 1. Subjects in main and proxy respondent analyses of glyphosate use and NHL in the North American Pooled Project (NAPP). * Duration (years) information was not collected in Kansas, ** Frequency (days/year) information was not collected in Iowa, Minnesota, and Kansas.

Table 1. Characteristics of non-Hodgkin lymphoma (NHL) cases and controls in the North American Pooled Project (NAPP). [OR=odds ratio; CI=confidence interval].

Characteristics	Cases (N=1690)		Controls (N=5131)			
	N	%	N	%	OR [a]	95% CI
Histological sub-type						
Diffuse large B-cell lymphoma (DLBCL)	647	38				
Follicular lymphoma (FL)	468	28				
Small lymphocytic lymphoma (SLL)	171	10				
Other	404	24				
State/Province U.S						
Nebraska	385	22	1432	28		
Minnesota	329	19	642	13		
Iowa	293	17	603	12		
Kansas	170	11	948	18		
Canada						
Ontario	142	8	585	11		
British Columbia	126	7	230	4		
Quebec	117	7	291	6		
Alberta	65	4	196	4		
Manitoba	34	2	113	2		
Saskatchewan	29	2	91	2		
Age (years) [b]						
≥19–≤29	26	2	277	5		
≥30–≤39	97	6	445	9		
≥40–≤49	159	9	514	10		
≥50–≤59	288	17	726	14		
≥60–≤69	564	33	1264	25		
≥70–≤79	402	24	1189	23		
≥80–≤89	137	8	610	12		
≥90	17	1	106	2		
Sex						
Male	1506	89	4424	86	1.00	ref
Female	184	11	707	14	0.94	0.75–1.17
Respondent type						
Self	1140	67	3372	66	1.00	ref
Proxy	533	32	1692	33	1.03	0.90–1.17
Unknown/missing	17	1	67	1		
Lymphatic or hematopoietic cancer in a first-degree relative						
No	1493	88	4790	93	1.00	ref
Yes	139	8	202	4	2.13	1.69–2.67
Unknown/missing	58	3	139	3		
Ever diagnosed with selected medical conditions [c]						
No	1011	60	3346	65	1.00	ref
Yes	545	32	1389	27	1.12	0.92–1.37
Unknown/missing	134	8	396	8		
Ever used any type of personal protective equipment						
No	374	22	1127	22	1.00	ref
Yes	105	6	310	6	1.12	0.86–1.45
Unknown/missing	1211	72	3694	72		

[a] Adjusted for age and state/province.

[b] Cases - mean 62.72 (SD 13.78) years; Controls - mean 61.66 (SD 17.13) years.

[c] Ever diagnosed with ≥1 of the following select medical conditions: allergies (any, food, or drug), asthma, hay fever, infectious mononucleosis, rheumatoid arthritis, tuberculosis, or received chemotherapy or radiation therapy.

Glyphosate use and associations with NHL overall and by major histological sub-type

Overall, 113/1690 cases (7%) and 244/5131 (5%) controls reported having used glyphosate at any point in their lifetime. In crude analyses, there was a significant association between ever use of glyphosate and NHL overall (OR1.4, 95% CI 1.1–1.8) that was attenuated appreciably when adjusted for ever use of the pesticides 2,4-D, dicamba, and malathion (OR1.1, 95% CI 0.8–1.5) (Table 2).[1] Adjusted ORs by for NHL subtypes were: 0.6 (95% CI 0.4 – 1.2) for FL, 1.2 (95% CI 0.8–1.9 for DLBCL, 1.8 (95%CI 0.9 – 3.7) for SLL, and 1.5 (95% CI 0.9 – 2.6) for other NHL subtypes.

When ORs for NHL and glyphosate use were examined by duration, there were lower ORs with longer use for NHL overall and for subtypes except SLL (see table 2). Conversely, there were higher ORs for those who reported use for more than 2 days per year verses those who reported use ≤ 2 days per year. Analyses by lifetime days of use – the metric used in most studies – showed near null results for all subtypes in the higher lifetime days category, except for SLL. It bears noting that the analyses by duration included 90% of cases and controls (Kansas subjects did not have the required data), while the analyses of days/year and lifetime days included only 53% of subjects (cases and controls from Kansas, Iowa, and Minnesota did not have the required data and were excluded). As such, these latter analyses may not be representative of results for the entire pooled study population.

Table 2. Adjusted Odds Ratios (95% confidence intervals) by Various Glyphosate Exposure Metrics

metric	NHL overall	FL	DLBCL	SLL	Other NHL
ever use	1.1 (0.8, 1.5)	0.6 (0.4, 1.2)	1.2 (0.8, 1.9)	1.8 (0.9, 3.7)	1.5 (0.9, 2.6)
duration					
≤ 3.5 years	1.3 (0.9, 1.8)	0.7 (0.3, 1.3)	1.6 (0.97, 2.7)	1.4 (0.6, 3.7)	1.8 (0.95, 3.5)
> 3.5 years	0.9 (0.6, 1.4)	0.6 (0.3, 1.3)	0.9 (0.5, 1.7)	1.9 (0.8, 4.8)	1.1 (0.5, 2.5)
days/year					
≤ 2	0.7 (0.5, 1.2)	0.4 (0.2, 1.2)	0.7 (0.4, 1.4)	1.3 (0.4, 4.3)	1.1 (0.5. 2.7)
≥ 2	1.7 (1.0, 2.9)	1.3 (0.6, 3.2)	2.1 (1.1, 4.3)	2.3 (0.6, 8.8)	1.6 (0.6, 4.5)
lifetime days					
≤ 7	0.9 (0.5, 1.5)	0.6 (0.3, 1.6)	0.8 (0.4, 1.7)	1.0 (0.2, 4.8)	1.4 (0.6, 3.5)
> 7	1.1 (0.7, 1.8)	0.8 (0.3, 1.8)	1.1 (0.6, 2.2)	2.2 (0.7, 6.9)	1.3 (0.5, 3.3)

Sensitivity analyses excluding proxy respondents

A sensitivity analysis was performed by excluding cases and controls whose data were provided by proxy respondents. The overall pattern of OR estimates were generally similar to the main analyses. However, for SLL, ORs were slightly higher in the > 2 days/year subgroup OR 2.3 (95% CI 0.6, 8.8) with proxies compared to OR 2.6 (95% CI 0.7, 10.1)) without proxies. For other NHL subtypes, ORs were slightly to appreciably lower for ever use with proxies OR 1.5 (95% CI 0.9, 2.6) compared to OR without proxies of 1.0 (95% CI 0.5, 1.9); OR for > 3.5 years of use 1.1 (95% CI 0.5, 2.5) compared to OR without proxies 0.5 (95% CI 0.2, 1.6)); and OR for > 7 lifetime days with proxies 1.4 (95% CI 0.6, 3.5) compared to OR without proxies of 1.1 (95% CI 0.4, 3.3)).

[1] Adjusting for other pesticides appreciably changed the NHL OR for glyphosate. Therefore, it seems most appropriate to focus on the ORs from the adjusted analyses.

Discussion & Conclusion

The objective of this study was to evaluate potential associations between glyphosate use and NHL in the NAPP, a pooled dataset that allowed for a more comprehensive analysis than previously possible in the individual studies. For NHL overall, the OR for ever use of glyphosate and NHL was near null when adjusted for reported use of 2,4-D, dicamba, and malathion (OR1.1, 95% CI 0.8, 1.5). Analyses by years of use and lifetime days of use showed near null results for NHL overall (OR 0.9 and 1.1, respectively), while the OR for > 2 days/year was elevated (OR 1.7, 95% CI 1.0, 2.9). It bears noting, however, that the analyses by days per year and lifetime days of use included only 50% of the pooled population – essentially the Canadian subjects and 1 of the 4 US case-control studies. It is uncertain, therefore, how representative these results are for the entire pooled population. The results of those analyses should be interpreted accordingly.

In analyses of NHL sub-types, there tended to be moderate positive associations between the various glyphosate exposure metrics and SLL. However, as there were only 15 SLL cases overall who ever used glyphosate and 14, 7, and 7 exposed SLL cases in the analyses by years of use, days per year, and lifetime days of use, respectively, the results were not statistically significant and the 95% CIs were imprecise. For other cell types, there were moderate positive relationships for glyphosate use of more than 2 days per year, though ORs were near null for these cell types for the higher category of years of use and lifetime days of use.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The main advantage of this pooled analysis compared with the previously published individual studies was to enable a more comprehensive analysis for glyphosate with regard to confounding factors and proxy respondents. In general, adjusting for use of 2,4-D, dicamba, and malathion reduced ORs for glyphosate. Analyses that excluded proxy respondents were generally similar to analyses that included them, though there were some instances, specifically for other NHL subtypes, where excluding proxies appreciably reduced the adjusted OR..

Left unaddressed in this pooled analysis is the intractable issue of case-recall bias in case control studies. Crump has shown in an analysis of all the case control studies that have reported ORs for glyphosate, including the studies in this pooled analysis, that results for all pesticides were markedly skewed toward positive associations (Crump K, Risk Analysis DOI: 10.1111/risa.13440). Crump noted particularly that the ORs for individual pesticides in the McDuffie et al. study (and 2 other studies not included in this pooled analysis) were nearly all greater than 1.0. He considered this evidence of case recall bias. Fundamentally, using self-reported exposure recollections from cases and controls violates the basic principle that data should be collected under equivalent circumstances for the groups to be compared (viz., cases and controls). That is impossible when pesticide recall is likely to be affected by their grievous illness for cases and not for controls. Accordingly, while this pooled analysis is an advance in understanding confounding by other pesticides and in assessing the impact of reporting by proxies (except in analysis where 50% of the subjects were excluded due to data limitations) in the 2 included studies, systematic error related to case recall bias remains an outstanding issue for interpreting the results for glyphosate.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because it concerns a pooled case control study which is subject to recall and selection bias. Notably, potential case-recall bias remains an unresolved issue in this pooled reanalysis.

Reliability Criteria: Epidemiology studies

Publication: Pahwa M. *et al.*, 2019	**Criteria met? Y/N/?**	**Comments**
Study Design		
Adequate study design given study objectives	Yes	Pooled reanalysis of 2 previously published studies.
Appropriate study population to address potential glyphosate-related health outcomes	Uncertain	Populations had very limited glyphosate exposure frequency.
Exposure studied		
Exposure to formulations with glyphosate as a.i.	Yes	
Exposure to formulations with other a.i.	Yes	
Exposure to other farm exposures	Uncertain	
Study Conduct/analysis		
Adequate description of study population	Yes	
Adequate description of exposure circumstances	Yes	
Comparable participation by groups being compared	No	Much less participation by controls: example: McDuffie study – participation 67% for cases, 48% for controls.
Information provided by proxy respondents	Yes, substantial	31% for cases, 40% for controls in DeRoos study; 21% for cases, 15% for controls McDuffie study (per Chang & Delzell 2016)
Adequate statistical analysis	Yes	More comprehensive than the original publications regarding confounding & proxy responses. Data for 47% of subjects were missing for analyses by days of use per year and

Publication: Pahwa M. *et al.*, 2019	**Criteria met? Y/N/?**	**Comments**
		lifetime cumulative days of use.
Adequate consideration of personal confounding factors	Yes	Better than original studies.
Adequate consideration of potentially confounding exposures	Yes	Better than original studies
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Yes	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because illustrates bias toward positive findings for glyphosate in the original publications due to confounding and, in part, due to proxy responses. Recall bias unresolved. Missing data for 47% of subjects for analyses by days/year of use and lifetime cumulative days of use hinders interpretation of related results.		

1. Information on the study

Data point:	CA 5.6
Report author	Panzacchi S. *et al.*
Report year	2018
Report title	The Ramazzini Institute 13-week study on glyphosate-based herbicides at human equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation
Document No	Environmental Health (2018) Vol. 17, 52
Guidelines followed in study	Based on the National Toxicology Program's (NTP) Modified One-Generation Reproduction Study 2011.
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The current study represents the first phase of a long-term investigation of glyphosate-based herbicides (GBHs) that we are conducting over the next 5 years. In this paper, the study design, the first evaluation of in vivo parameters and the determination of glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) in urine is presented.

Materials and methods

Chemicals - Glyphosate (purity of > 99.5%), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy). The representative formulated product, Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%), water (42.5%) and surfactant (16%)) was purchased from Consorzio Agrario dell'Emilia, Bologna, Italy.

Animals - Male and female SD rats were obtained from the colony used at the Cesare Maltoni Cancer Research Center laboratories of the Ramazzini Institute (CMCRC/RI). The animal room conditions were 22 °C ± 3 °C and 50 ± 20% relative humidity and a light/dark cycle of 12 hours. During the experiment the animals received standard pellet feed and tap water ad libitum.

Experimental design - Each of 8 virgin female SD rats (17 weeks old, 270-315 g) per dose group was placed individually in a polycarbonate cage with a single male rat of the same age and strain until evidence of copulation. Gestational day (GD) 0 was defined as the day on which sperm was found in vaginal smears. After mating, matched females were housed separately during gestation and delivery and pups were housed with the dams until weaning. The day on which parturition was completed was designated as lactating day (LD) 0 for the dam and postnatal day (PND) 0 for the offspring. On PND 28, the offspring were weaned and identified by ear punch. Sequentially, they were allocated in the same treatment group of their mother to obtain 18 animals (8 for the 6-week cohort and 10 for the 13-week cohort) per sex and for each dose group. No more than 2 males and 2 females from the same litter were included in the same cohort/treatment group. Altogether, 54 males and 54 females were enrolled in the post-weaning treatment phase. Rats were treated with glyphosate and MON 52276 at 1.75 mg glyphosate acid eq./kg bw/day in the drinking water. One group received only tap water as control. After weaning, until the end of the experiment (PND 73 ± 2 or 125 ± 2), glyphosate and MON 52276 were administered in the drinking water to F1 animals on the basis of the average body weight and average water consumption per sex and per experimental group. Males and females were considered separately because of their difference in weight gain, body weight and water consumption. Animals were checked 3 times daily on working days and 2 times daily on Sundays and non-working days. Clinical signs were checked before the start of the treatment, and at least every two days until the end of the experiment. The body weight of the dams was recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, the body weight of the dams was recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 and the

body weight of the pups by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. After weaning, body weight was measured twice a week until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifice. The mean individual body weights were calculated for each group and sex. Feed and water consumption of the dams were recorded twice weekly on GD 0, 3, 6, 9, 12, 15, 18, 21, and during lactation on LD 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28. After weaning the daily feed and water consumption per cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2. The mean individual feed and water consumption were calculated for each group and sex. The day before terminal sacrifice, all the animals were placed individually in metabolic cages and starved for around 16 hours during which the animals had free access to water alone or to the test compound solutions. In the morning of the following day, samples of at least 5 mL of urine collected from each animal were transferred to labelled tubes for analysis of glyphosate and AMPA. Samples from 3 dams/group and from 5 rats/sex/group belonging to the 6-week and 13-week cohorts were used for analysis.

Analysis of glyphosate and AMPA in urine - Analysis of glyphosate and AMPA in drinking water, feed and urine were performed by Neotron Laboratories. The specification and results are maintained in the experimental documentation. Analysis was performed using LC-MS/MS. The limit of quantification (LOQ) for glyphosate and AMPA was 0.10 µg/L in water, 50 µg/kg in feed, and 1 µg/L in urine.

Statistical analysis - Means ± standard deviations (SD), were calculated for continuous variables. For body weight, water and feed consumption over time multilevel mixed-effect linear regression models were used, to control for within subject correlation across time also considering the litter effect during the lactation period. Analysis of variance and Dunnett's test (when applicable) were also performed to compare body weight gain in different periods and consumption of food and water as mean consumption in several periods. All tests were two tailed with results considered as statistically significant if $p < 0.05$. Statistical analyses were performed by using STATA version10.

Results

Mortality, body weight, water and food consumption, clinical signs and litter data - All 24 dams and 108 rats from the 6-week and 13-week cohorts survived until sacrifice. Body weight and body weight gain of the dams during gestation and lactation were not statistically different among groups. Post-weaning body weight of female and male offspring was homogeneous and no statistically significant differences in body weight gain were observed among groups. Water and feed consumption during gestation and lactation were not different across groups. Litter sizes were fully comparable among groups, with mean number of live pups of 13.6 (range 10–16) in the control group, 13.3 (range 11–17) in the glyphosate group and 13.9 (range 11–16) in the MON 52276 group. Post weaning water and feed consumption were not affected by treatment. There was no clinical evidence of alterations in activity or behavior, reflexes, eyes or skin, respiratory, gastrointestinal, genitourinary and cardiovascular systems.

Analysis of glyphosate and AMPA in urine – The results of the analysis are presented in the Table below.

Table: Glyphosate and AMPA concentration in urine. Results are reported as mean ± standard deviations

		Dams		Offspring (6-week cohort)		Offspring (13-week cohort)	
	Treatment	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)
Male	Control			0.012 ± 0.010	0.003 ± 0.003	0.011 ± 0.010	0.006 ± 0.004
	Glyphosate	–	–	0.938 ± 0.414	0.014 ± 0.007	1.684 ± 0.768	0.023 ± 0.012
	Roundup			1.174 ± 0.439	0.011 ± 0.005	2.280 ± 1.520	0.027 ± 0.016
Female	Control	0.009 ± 0.001	0.006 ± 0.002	0.013 ± 0.007	0.005 ± 0.001	0.008 ± 0.005	0.003 ± 0.005
	Glyphosate	0.480 ± 0.010	0.024 ± 0.002	0.938 ± 0.377	0.016 ± 0.010	1.354 ± 0.359	0.013 ± 0.006
	Roundup	0.700 ± 0.106	0.024 ± 0.001	0.910 ± 0.383	0.018 ± 0.007	1.524 ± 0.585	0.021 ± 0.007

The urinary concentrations of glyphosate and AMPA of rats treated with glyphosate at 1.75 mg/kg bw/day were comparable to those observed in rats treated with MON52276 at 1.75 mg glyphosate acid

eq. /kg bw/day, despite the limited sample size and the large standard deviations. Glyphosate and AMPA urinary levels were all below or close to the LOQ of 0.001 mg/kg in the control group. In the treated rats, the majority of glyphosate was excreted unchanged, with urinary levels about 100-fold higher than that of AMPA. For example, the mean urinary levels of glyphosate were 1.354 and 1.524 mg/kg bw in glyphosate and MON 52276 treated females in the 13-week cohort, respectively, while the corresponding AMPA levels were 0.013 and 0.021 mg/kg bw. In glyphosate and MON 52276 treated rats, a time-dependent increase in the mean urinary concentration of glyphosate was observed. In glyphosate and MON 52276 treated males, an increase of approximately 2-fold was observed of the mean urinary concentration of glyphosate in the 13-week cohort when compared to the 6-week cohort. In glyphosate treated females, the 6-week cohort showed a 2-fold higher mean urinary concentration of glyphosate than the dams after weaning, while the 13-week cohort showed a 1.5-fold increase compared to the 6-week cohort. In the MON 52276 group, the increase was less steep, but the time-dependent pattern was still evident. In glyphosate and MON 52276 treated rats, the levels of AMPA were comparable at the different time points in both males and females. Large variations were observed of the AMPA concentrations in urine, in particular those close to the LOQ as in the control groups.

Discussion

Survival, body weights, food and water consumption of rats were not affected by the treatment with glyphosate or MON 52276. No clinical changes were observed in the animals of the dosed groups. Overall, oral treatment of glyphosate and MON 52276 via the drinking water seemed to be well tolerated. Exposure to glyphosate and MON 52276 led to comparable concentrations of glyphosate and AMPA in urine, indicating that systemic exposure does occur at the selected exposure level of 1.75 mg/kg bw/day, corresponding to the US ADI. The bioavailability of glyphosate in this study is also supported by the evident increase of glyphosate concentration in urine in relation to the length of treatment. The adjuvants and the other substances present in MON 52276 did not seem to exert a major effect on the absorption and excretion of glyphosate, even though mean values of glyphosate seem to be somewhat higher in the formulation treated group. The levels in urine were also comparable between the two sexes, although a consistent inter-individual variability was observed. In rats, glyphosate in urine appears to be the most accurate biomarker of exposure to glyphosate based herbicides (GBHs). The results from this study confirm previous evidence that in rodents most of the administered dose of glyphosate (98%) is excreted as unchanged parent compound, whereas the metabolite AMPA in urine is at around 0.2–0.3% of the administered dose. Furthermore, with the level of exposure to glyphosate used in this pilot study, AMPA urinary values of treated animals (0.011–0.027 mg/kg) were already close to the LOQ (0.001 mg/kg) which might limit the reliability of the data. Glyphosate concentrations in urine of treated animals (0.480–2.280 mg/kg) were found to be 100-fold higher than the AMPA concentrations and at least 500-fold higher than the LOQ. Therefore, in order to assess exposure to glyphosate in rats, in particular at doses that are equal or lower than 1.75 mg/kg bw/day, glyphosate appears to be the biomarker of choice. The presence of negligible levels of glyphosate (0.003–0.013 mg/kg) in some of the urine samples of the control groups might reflect an ubiquitous environmental contamination at ultra-low doses of glyphosate, which is consistent with previous reports from other authors. As the current LOQ of glyphosate in HPLC for pelleted animal feed is 0.050 mg/kg, this represents a technical limiting factor for the testing ultra-low doses of glyphosate.

Conclusion

A pilot study was performed on the health effects of glyphosate and its formulation Roundup Bioflow (MON 52276) administered orally to rats at the US ADI of 1.75 mg/kg bw/day. Treatment with either glyphosate or MON 52276 seemed to be overall well tolerated, consistent with previous experiments performed by the US NTP. Both glyphosate and MON 52276 exposure led to comparable urinary concentrations of glyphosate and AMPA with an increasing excretion of glyphosate in urine with duration of treatment. This indicates the systemic bioavailability of glyphosate and a possible mechanism of bioaccumulaton. The adjuvants and the other substances present in the formulation did not seem to exert a major effect on the absorption and excretion of glyphosate when administered orally. The results of this study confirm that, in rodents, glyphosate is a much more relevant biomarker in urine than AMPA, in particular at doses that are equal or lower than 1.75 mg/kg bw/day.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study the general toxicity of glyphosate was compared against that of its reference formulation MON 52276 in pregnant rats and their progeny. Also the urinary excretion of glyphosate and AMPA was investigated. The test compounds were administered via the drinking water resulting in a daily dose of 1.75 mg glyphosate acid eq./ kg bw. The endpoints investigated were mortality, body weight, water and food consumption, and clinical signs in dams and offspring and litter data. There was no mortality and no statistically significant differences were observed among control, glyphosate, and MON 52276 groups in any of the endpoints investigated. Urinary concentrations of glyphosate and AMPA of rats treated with glyphosate at 1.75 mg/kg bw/day were comparable to those observed in rats treated with MON 52276 at 1.75 mg glyphosate acid eq. /kg bw/day. This indicates that the co-formulants in this glyphosate formulation have little influence on the oral bioavailability of glyphosate. In the treated rats, the majority of glyphosate was excreted in urine unchanged at levels of about 100-fold higher than that of AMPA and the mean urinary concentration of glyphosate increased with the duration of treatment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only one dose level for glyphosate and MON 52276 was considered, only 8 animals were used per dose and per sex and the method of analysis of glyphosate and AMPA in urine and its validation were not fully reported.

Reliability criteria for *in vivo* toxicology studies

Publication: Panzacchi *et al*., 2018	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Only a part of the reproductive toxicology study was reported, one dose level of glyphosate or MON 52276 was considered and 8 females per dose group were used.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	For the part that was reported.
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate (purity of > 99,5%), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy).
Only glyphosate acid or one of its salts is the tested substance	N	The representative formulated product, Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%),

		water (42.5%) and surfactant (16%) was purchased from Consorzio Agrario dell'Emilia, Bologna, Italy.
AMPA is the tested substance	Y	Determined in urine of rats treated with glyphosate and MON 52276.
Study		
Test species clearly and completely described	Y	Male and female SD rats.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	1.75 mg glyphosate acid eq./kg bw/day administered as glyphosate and as MON 52276.
Number of animals used per dose level reported	Y	8 virgin female SD rats per dose group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Method of analysis described for analysis of urine	N	No details on the conduct of the method of analysis and no complete validation data set.
Complete reporting of effects observed	Y	Limited to body weight,
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	Not possible with one dose level of each test item.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only one dose level for glyphosate and MON 52276 was considered, only 8 animals were used per dose and per sex and the method of analysis of glyphosate and AMPA in urine and its validation were not fully reported.		

1. Information on the study

Data point:	CA 5.6
Report author	Perego M.C. *et al.*
Report year	2017
Report title	Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells *in vitro*
Document No	J. Appl. Toxicol. 2017; 37: 692–698
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restriction

2. Full summary of the study according to OECD format

The aim of this study was to determine the potential endocrine disruptor effects of glyphosate (GLY) on ovarian function evaluating cell proliferation, steroidogenesis and gene expression using bovine granulosa cells (GC) and theca cells as *in vitro* models. GC proliferation was impaired ($P< 0.05$) after exposure to GLY at 0.5, 1.7 and 5 μg ml^{-1}. GC progesterone production was not affected ($P\geq 0.05$) at all doses tested, while estradiol production was inhibited ($P< 0.05$) by GLY at 5 μg ml^{-1}. At the same concentration, GLY showed no effect ($P\geq 0.05$) on theca cell proliferation and steroidogenesis. At higher concentrations (0.01 and 0.3 mg ml^{-1}), GLY had no significant effect ($P\geq 0.05$) on GC proliferation and steroidogenesis. These studies, for the first time, suggest that GLY may affect the reproductive system in cattle via direct action on ovarian function; however, further studies will be required to understand better the mechanism of action and to determine the *in vivo* reproductive effects of GLY.

Materials and methods

Cell culture

Ovaries from non-pregnant beef heifers were collected from a local slaughterhouse and were treated as previously described (Lagaly *et al*., 2008; Langhout *et al*., 1991; Spicer & Aad, 2007). Thecal cells (TC) were collected from large (8–22 mm) follicles as previously described (Lagaly *et al*., 2008; Spicer & Chamberlain, 1998; Stewart *et al*., 1995). Follicular fluid was collected and final cell preparations were prepared in serum-free medium (Dulbecco's modified Eagle medium and Ham's F12) as previously described (Lagaly *et al*., 2008; Schreiber & Spicer, 2012). Trypan blue exclusion method was performed to determine viable cells (Langhout *et al*., 1991; Spicer *et al*., 1993; Tiemann *et al*., 2003a,b). Cells were then plated (2.5×10^5 in 20–80 μl of medium) on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1 ml of basal medium composed of a mixture of 1:1 Dulbecco's modified Eagle medium and Ham's F-12 containing glutamine, gentamicin and sodium bicarbonate (Sigma-Aldrich Co., St. Louis, MO, USA) as previously described (Schreiber & Spicer, 2012). Plates were maintained in a humidified 95% air and 5% CO_2 environment at 38.5 °C changing medium every 24 h. Cells were also kept in the presence of 10% fetal calf serum (FCS; Equitech-Bio, Inc., Kerrville, TX, USA) for the first 48 h of culture to procure an optimal attachment. After 48 h, cells were washed twice with serum-free medium and the different treatments were applied in serum-free medium containing 500 ng ml^{-1} of testosterone (as an estradiol [E_2] precursor; from Steraloids, Wilton, NH, USA) for 48 h. Ovine follicle-stimulating hormone (FSH; NIDDK-oFSH-20, activity: 175 × NIH-FSH-S1 U mg^{-1}, from the National Hormone and Pituitary Program, Torrance, CA, USA) was added to all treatments, because insulin-like growth factor (IGF) 1 alone does not have an effect on steroid production (Ranzenigo *et al*., 2008; Spicer *et al*., 2002).

After aspiration of follicular fluid, large follicles were bisected and GC were separated from the TC via blunt dissection and the theca interna was enzymatically digested as previously described (Aad *et al*., 2006; Spicer & Chamberlain, 1998; Stewart *et al*., 1995). The non-digested tissue was eliminated by using sterile syringe filter holders with metal screens of 149 μm mesh (Gelman, Ann Arbor, MI, USA) and filtered TC were then centrifuged at 50 g for 5 min. As described for granulosa cells (GC), TC were washed with serum-free medium and resuspended in serum-free medium containing collagenase and DNase. TC (2.0×10^5 viable cells per well) were plated and cultured as described for GC. Culture medium was also supplemented with 30 ng ml^{-1} of ovine luteinizing hormone (LH; LH activity; 2.3 × NIH-LH-S1 U mg^{-1}; from the National Hormone and Pituitary Program) because progesterone (P_4) and androstenedione (A_4) production are not induced by IGF1 in the absence of LH (Spicer & Stewart, 1996; Stewart *et al*., 1995).

Assays

Medium collected from individual wells was frozen at -20°C for subsequent steroid analyses. Radioimmunoassays (RIA) were performed to determine concentrations of P_4, E_2 and A_4 as previously described (Lagaly *et al*., 2008; Langhout *et al*., 1991; Spicer *et al*., 1993; Stewart *et al*., 1995). The intra- and inter-assay coefficients of variation were 7% and 13% for the P_4 RIA, and 8% and 17% for the E_2 RIA, respectively.

Determination of granulosa cell and theca cell numbers

The numbers of GC and TC, in the same wells from which medium was collected, were determined via a Coulter counter (model Z2; Beckman Coulter, Inc., Hialeah, FL, USA), and used to calculate steroid production on ng or pg per 10^5 cell basis. Cells were washed twice using 0.9% saline solution (500 μl), exposed to 500 μl of trypsin (0.25% wt/vol= 2.5 mg ml^{-1}) for 20 min at 37°C and then scraped from each well and enumerated as previously described (Lagaly *et al*., 2008; Ranzenigo *et al*., 2008).

RNA extraction and quantitative reverse transcription–polymerase chain reaction

At the end of the treatment period, medium was either aspirated or collected from each well depending on the experiment and cells from two replicate wells were lysed in 0.5 ml of TRI reagent solution (Life Technologies, Inc., Grand Island, NY) as previously described (Lagaly *et al*., 2008; Spicer & Aad, 2007). RNA samples were solubilized in DEPC-treated water (Life Technologies, Inc., Grand Island, NY), quantitated by spectrophotometry at 260 nm using a NanoDrop ND-1000 spectrophotometer, and stored at -80°C. Cholesterol side chain cleavage enzyme (*CYP11A1*) and aromatase (*CYP19A1*) primers and probes for quantitative reverse transcription–polymerase chain reaction were designed using Primer Express™ software (Foster City, CA, USA) as previously reported (Lagaly *et al*., 2008; Spicer & Aad, 2007)[11]. The bovine *CYP11A1* and *CYP19A1* primer and probe sequences and information are described by Lagaly *et al.* (2008). Relative quantification of target gene mRNAs were expressed using the comparative threshold cycle method as previously described (Lagaly *et al*., 2008; Spicer & Aad, 2007).

Experimental design

Experiment 1 was performed to evaluate the effects of glyphosate (GLY) on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng ml^{-1}), FSH (30 ng ml^{-1}) and IGF1 (30 ng ml^{-1}; recombinant human IGF1 from R&D Systems, Minneapolis, MN, USA) with or without various doses of GLY (i.e., 0, 0.5, 5 μg ml^{-1}; Sigma-Aldrich Co.). Only the 0 and 5 μg ml^{-1} doses of GLY were tested in the absence of IGF1 to determine any possible effect of GLY on FSH-stimulated steroidogenesis. After 48 h of treatment, cells were counted and medium was collected for E_2 and P_4 determinations. In a separate set of cells, the effect of 2-day treatment with 5 μg ml^{-1} GLY on GC viability was evaluated using the trypan blue exclusion method as previously described (Adashi *et al*., 1987; Spicer & Alpizar, 1994).

Experiment 2 was designed to evaluate the effects of higher doses of GLY on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng ml^{-1}), FSH (30 ng ml^{-1}) and IGF1 (30 ng ml^{-1}) with or without the various doses of GLY (i.e., 0, 0.01, 0.3 mg ml^{-1}). After 48 h of treatment, cells were counted and medium was collected for E2 and P_4 determinations.

Experiment 3 was designed to test the effect of GLY on *CYP11A1* and *CYP19A1* mRNA abundance in GC. Cells were cultured as previously described for experiment 1 except that no testosterone was included in the medium and treatments were only applied for 24 h: no additions, FSH (30 ng ml^{-1}) plus IGF1 (30 ng ml^{-1}), and FSH plus IGF1 plus GLY (5 μg ml^{-1}). After 24 h of treatment, cells were lysed for RNA extraction as described earlier. A combined treatment of FSH and IGF1 was selected to test the GLY effect because the inhibitory effect of GLY was seen with this treatment in experiment 1.

Experiment 4 was performed to study the effect of GLY on TC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing LH (30 ng ml^{-1}) and IGF1 (0 or 30 ng ml^{-1}) with or without GLY (*i.e.*, 0 and 5 μg ml^{-1}). After 48 h of treatment, cells were counted and medium was collected for P_4 and A_4 determinations.

Experiment 5 was designed to determine the effect of GLY on serum-stimulated GC proliferation. Cells were cultured for 4 days in 10% FCS. During the last 2 days of culture, cells were treated as follows: control (no additions) or GLY (1.7 μg ml^{-1}). At the end of treatment, cells were counted.

Statistical analysis

Experimental data are presented as the least squares means ± SEM of measurements from replicated culture wells. Each experiment was performed three times with different pools of GC collected from 10 to 20 ovaries for each pool and each treatment replicated three times within each experiment. Treatment effects and interactions were assessed using the GLM procedure of the Statistical Analysis System for Windows (version 9.2; SAS Inst. Inc., Cary, NY, USA). Main effects were treatment, experimental replicate (i.e., pool of cells) and their interaction. Steroid production was expressed as ng or pg per 10^5 cells per 24 h, and cell numbers determined at the end of the experiment were used for this calculation. Mean differences in cell growth and steroid production between treatments were determined using the Fisher's protected least significant difference procedure (Ott, 1977) $P< 0.05$ was considered statistically significant.

Results

Experiment 1: Dose–response of glyphosate on granulosa cell proliferation and estradiol and progesterone production in the presence of follicle-stimulating hormone with or without insulin-like growth factor 1

GLY at 0.5 and 5 μg ml^{-1} was found to decrease significantly the GC proliferation in the presence of FSH plus IGF1 (Fig. 1). Regarding steroid production, GLY at all tested concentrations (0.5 and 5 μg ml^{-1}) had no effect on GC P4 production (Fig. 2A). GLY had no effect on GC E2 production in the presence of FSH whereas GLY at 5 μg ml^{-1} decreased ($P< 0.05$) E2 production in the presence of FSH plus IGF1 (Fig. 2B). Cell viability was not significantly affected by 2-day treatment with 5.0 μg ml^{-1} GLY (91.6 vs. 88.6±5.0% for control and GLY-treated GC, respectively).

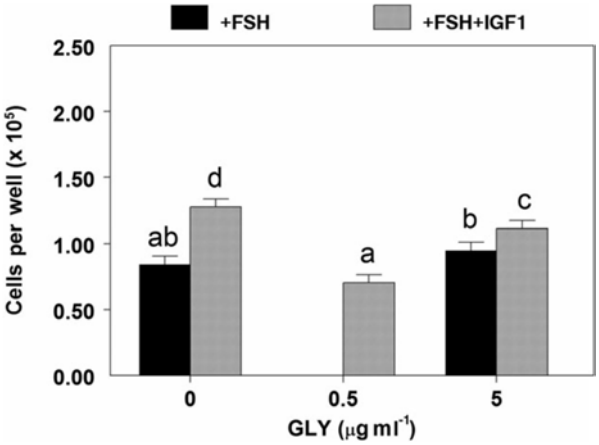


Figure 1. Effect of GLY on numbers of granulosa cells from bovine follicles (experiment 1). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h either with 30 ng ml^{-1} FSH alone and GLY at 0 or 5 μg ml^{-1}, or with 30ng ml-1 FSH and IGF1 (30 ng ml^{-1}) with GLY at 0, 0.5 or 5.0 μg ml^{-1}. All cells were treated concomitantly with 500 ng ml^{-1} of testosterone. Values are means ± SEM from three separate experiments (n = 9). Means without a common letter (a-d) differ (P< 0.05). FSH, follicle-stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.

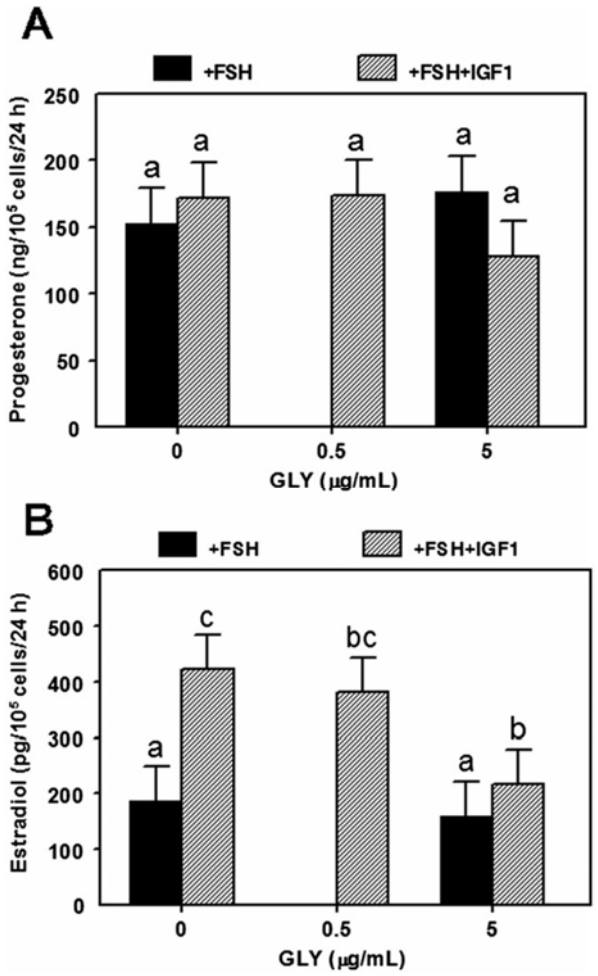


Figure 2. Effect of GLY on progesterone (A) and estradiol (B) production by granulosa cells from bovine follicles (experiment 1). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h either with 30 ng ml^{-1} FSH alone and GLY at 0 or 5 μg ml^{-1}, or with 30 ng ml^{-1} FSH and IGF1 (30 ng ml-1) with GLY at 0, 0.5 or 5.0 μg ml^{-1}. All cells were treated concomitantly with 500 ng ml^{-1} testosterone. Values are means +/-SEM from three separate experiments (n = 9). Within a panel, means without a common letter (a–c) differ (P< 0.05). FSH, follicle stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.

Experiment 2: Dose–response of glyphosate on granulosa cell proliferation and estradiol and progesterone production in the presence of follicle-stimulating hormone plus insulin-like growth factor 1

GLY at all tested concentrations (0.01 and 0.3 mg ml^{-1}) had no significant effect either on GC proliferation or steroid production. Cell numbers averaged 1.25, 1.47 and 1.00 ± 0.2 (×105 cells per well) for 0, 0.01 and 0.3 mg ml^{-1} GLY, respectively. P4 production averaged 99, 154 and 91 ± 23 ng 10-5 cells per 24 h for 0, 0.01 and 0.3 mg ml^{-1} GLY, respectively. E2 production averaged 130, 157 and 187 ± 28 pg 10-5 cells per 24 h for 0, 0.01 and 0.3 mg ml^{-1} GLY, respectively.

Experiment 3: Effect of glyphosate treatment on CYP19A1 and CYP11A1 mRNA in granulosa cells

The combined IGF1 plus FSH treatment increased (P< 0.05) CYP19A1 and CYP11A1 mRNA abundance by threefold and twofold, respectively, above untreated control GC (Fig. 3A,B). GLY (5µg ml^{-1}) had no significant effect on CYP19A1 or CYP11A1 mRNA in GC co-treated with FSH and IGF1 (Fig. 3A,B).

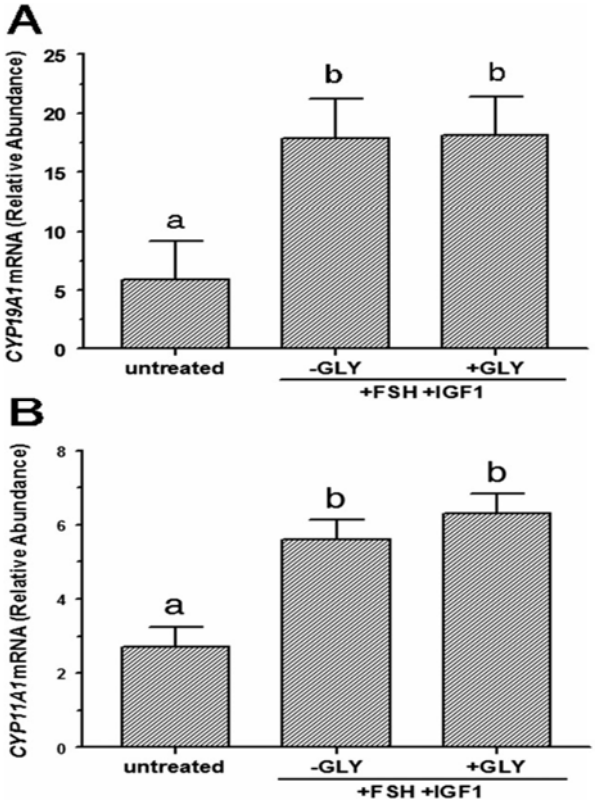


Figure 3. Effect of GLY on abundance of CYP19A1 (A) and CYP11A1 (B) mRNA in granulosa cells frombovine follicles (experiment 3). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 24 h either with either no additions (controls), or with FSH (30 ng ml^{-1}) and IGF1 (30 ng ml^{-1}) with GLY at 0 or 5.0 µg ml^{-1}. Values are means ± SEM from three separate experiments (n = 6). Within a panel, means without a common letter (a–b) differ (P< 0.05). FSH, follicle stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.

Experiment 4: Dose–response of glyphosate on theca cell proliferation and androstenedione and progesterone production in the presence of luteinizing hormone with or without insulin-like growth factor 1

GLY at the tested concentration of 5 µg ml^{-1} had no significant effect on TC proliferation and P4 or A4 production in the presence of LH either with or without IGF1. IGF1 significantly increased cell numbers

and steroid production. Cell numbers averaged 0.46, 0.53, 0.99 and 1.06 ± 0.05 for: LH alone; LH plus 5 µg ml^{-1} GLY; LH plus IGF1; and LH plus IGF1 plus 5 µg ml^{-1} GLY, respectively. P4 production averaged 40.5, 40.4, 70.3 and 78.3 ± 3.7 pg 10-5 cells per 24 h for: LH alone; LH plus 5 µg ml^{-1} GLY; LH plus IGF1 alone; and LH plus IGF1 plus 5 µg ml-1 GLY, respectively. A4 production averaged 1.45, 1.37, 2.75 and 2.47 ± 0.12 ng 10-5 cells per 24 h for: LH alone; LH plus 5 µg ml^{-1} GLY; LH plus IGF1 alone; and LH plus IGF1 plus 5 µg ml^{-1} GLY, respectively.

Experiment 5: Effect of glyphosate on serum-induced granulosa cell proliferation

Alone, GLY (1.7 µg ml^{-1}) increased (P< 0.05) GC proliferation (Fig. 4). Cell numbers were increased by 11% after 1 day and by 8% after 2 days of GLY treatment (Fig. 4).

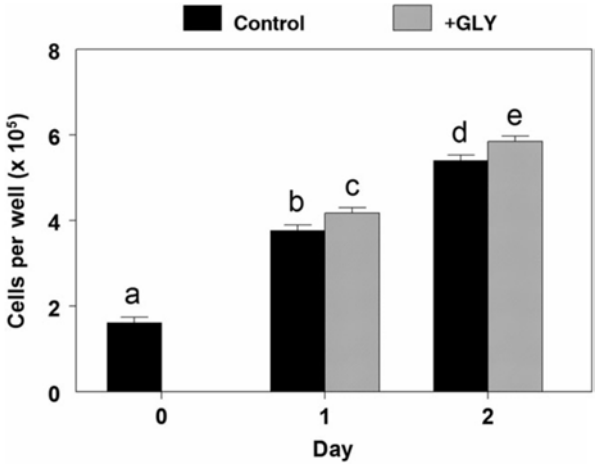


Figure 4. Effect of GLY on serum-induced proliferation of bovine granulosa cells (experiment 5). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 0, 1 or 2 days with 10% fetal calf serum and GLY at 0 or 1.7 µg ml^{-1}. Values are means ± SEM from three separate experiments (n = 9).Means without a common letter (a–e) differ (P< 0.05). GLY, glyphosate.

Discussion

In the present study, both cell proliferation and the production of P4, A4 and E2, hormones that are connected to normal ovarian cyclicity and establishment and maintenance of pregnancy (Wood & Strauss, 2002), were evaluated to determine if GLY has effects on bovine GC and TC. GLY was tested at low doses, from 0.5 µg ml^{-1}, very far below agriculture use and authorized residues in food or feed (approximately 7000 and 800 times lower, respectively) (Gasnier et al., 2009). No effects were observed on GC P4 production in the presence of FSH either with or without IGF1, whereas GLY at 5 µg ml^{-1} had inhibitory effects on E2 production in the presence of FSH plus IGF1, showing a potential impairment on GC function that is essential for oocyte survival (Petro et al., 2012). Results of our study also showed that GLY at 5 µg ml^{-1} had an inhibitory effect on GC proliferation in the presence of FSH plus IGF1, and this inhibitory effect of 5 µg ml^{-1} GLY on cell numbers was not associated with a change in cell viability.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this *in vitro* study, glyphosate had minimal effects on granulosa cells (GC). In the presence of FSH only, glyphosate had no effect on GC cell viability or on progesterone or estradiol production. In the presence of FSH and IGF1, glyphosate reduced GC proliferation without a dose-response at 0.5 and 5 µg ml^{-1} but not at lower test concentrations (0.01 and 0.3 µg ml^{-1}) and did not affect progesterone

production or CYP19A1 and CYP11A1 mRNA expression; estradiol production was reduced at 5 µg ml^{-1} only (not at lower test concentrations). Without FSH or IGF1, 1.7 µg ml^{-1} of glyphosate slightly increased GC proliferation in response to serum (≤11%).

Glyphosate at 5 µg ml^{-1} had no effect on the theca cell (TC) proliferation or the production of progesterone or androstenedione.

Overall, with the exception of slight, non-dose-related alterations in GC proliferation under different test conditions, this study showed no effects of glyphosate on GC at physiologically relevant test concentrations. Glyphosate had no effect on TH.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized, no positive controls were used and the tests were conducted with only one or 2 test concentrations of glyphosate.

Reliability criteria for *in vitro* toxicology studies

Publication: Perego *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma Aldrich.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Test system clearly and completely described	Y	Bovine granulosa and theca cells.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y (partly)	0, 0.5, 5, or 0, 10, 300 µg/mL (1.77 mM), or 0, 5 µg/mL.
Cytotoxicity tests reported	Y?	Viability tested at only one concentration of glyphosate.
Biochemical methods described	Y?	Some could be more detailed.
Positive and negative controls	N	No positive controls.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Limited since max. 2 test concentrations.

Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized, no positive controls were used and the tests were conducted with only one or 2 test concentrations of glyphosate.		

1. Information on the study

Data point:	CA 5.6
Report author	Pham T. H. *et al.*
Report year	2019
Report title	Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice
Document No	Toxicological Sciences (2019) Vol. 169(1), 260–271
Guidelines followed in study	None
Deviations from current test guideline	NA
Previous evaluation	No
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, it was analyzed the effects of glyphosate, alone or in formula, on the male reproductive system. Pregnant mice were treated from E10.5 to 20 days postpartum by adding glyphosate or a GBH (Roundup 3 Plus) to their drinking water at 0.5 (the acceptable daily intake, ADI dose), 5 and 50mg/kg/day. Male offspring derived from treated mice were sacrificed at 5, 20, and 35 days old (d.o.) and 8 months old (m.o.) for analysis.

Materials and methods

Chemicals - Glyphosate (purity 99.2%) was purchased from Sigma-Aldrich, St Louis, USA.

Animals – Four-month-old outbred Swiss adult mice were exposed to glyphosate via the drinking water at concentrations corresponding with 0.5, 5 and 50 mg/kg bw/day from the day of vaginal plug detection (embryonic day 0.5) to 20 days post-partum (dpp). The control group received water alone. Control and treated mice of 5, 20, 35 days old and 8 months old were euthanized and the reproductive organs excised. At least 5 animals derived from at least 3 to 4 different litters in each group were used for this test.

Testosterone quantification - Serum was collected from ketamine/xylazine-anaesthetized adult animals by terminal cardiac exsanguination and aliquots were stored at -20°C. Testosterone levels in the serum were assayed in duplicate using a commercial radioimmunoassay based on competitive binding with I125-labeled testosterone according to the manufacturer's recommendations.

Epididymal sperm count - The mice were euthanized and the epididymis excised, rapidly frozen in liquid nitrogen and stored at -80°C pending sperm count. The tissue was first cut in pieces and homogenized in 6 mL of 0.15 M NaCl containing 0.005% (v/v) Triton X-100. After homogenization by sonication, an aliquot of the cell suspension was loaded onto a Malassez hemocytometer, and spermatozoa heads were counted. The average sperm count was calculated from at least 6 controls or treated animals.

Histology and numbers of germ cells and Sertoli cells - Testis samples were fixed in Bouin's solution and embedded in paraffin. Histological sections (5 µm thick) were stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC), only the adult animals were perfused and the testes were fixed for 24 hours in 4% (w/v) paraformaldehyde and then embedded in paraffin. Testis sections (5 µm thick) were incubated overnight at 4°C with a primary rabbit polyclonal antibody DDX4 and then with secondary Alexa-Chicken anti-rabbit 488 antibody during 1 hour to check the number of undifferentiated spermatogonia in testes of mice of 5 days old. For IHC on testes of mice of 35 days old, testis sections (5 µm thick) were incubated overnight at 4°C with goat anti-ZBTB16 and rat anti-GATA1 and then with secondary Alexa-chicken anti-goat 488 antibody and secondary Alexa-donkey anti-rat 594 during 1 hour. The sections were all counterstained with 0.001% (v/v) 4,6-diamidino-2-phenylindole dihydrochloride and mounted in Vectashield before microscopic analysis. To quantify the number of Sertoli cells (GATA1-positive cells) and undifferentiated spermatogonia (ZBTB16-positive

cells), the cells were manually counted in 30 sections on average of seminiferous epithelium at stage VII in controls and glyphosate treated groups. Cells in 3 different areas of the testis were analysed for each biological replicate

RNA extraction and quantitative PCR - Total RNA was extracted from testes of animals of 5 days old using the RNeasy plus mini kit according the protocol of the manufacturer and reverse transcription was performed with 1 mg of RNA using the iScript cDNA Synthesis Kit according to the manufacturer's instructions. The resulting cDNA was diluted 5 times and used for quantitative PCR. QPCR was performed using the iTaq Universal SYBR Green Supermix according to the manufacturer's instructions on a CFX384 Touch Real-Time PCR Detection system. The PCR amplification of the coding regions of Actb and Rplp0 was used for normalization. The data from at least 6 samples were analysed, compared, plotted, and expressed as a fold change in treated samples compared with controls.

Statistical analysis - Statistical tests were carried out using R software. For each experiment, the results were separated and compared by modality. First, the data were tested for normality by the Shapiro test and homoscedasticity by the Bartlett test. If the data distribution for each modality followed a normal distribution and if the variances were equal, the ANOVA test was performed, a parametric comparison test. If the data of at least 1 of the groups were not normally distributed or if the data of the groups were distributed normally but the variances were not equal, the Kruskal-Wallis test was carried out, a nonparametric comparison test of average. The differences were considered statistically significant when $p < 0.05$. In this case, the Tukey test was performed after the ANOVA test and the Mann-Whitney test after the Kruskal-Wallis test. In animals of 5 and 20 days old and 8 months old, data were collected by randomising in view of origin litter and pups were used as the experimental unit for statistical analysis. In animals of 35 days old, corresponding to the first complete wave of spermatogenesis, litters were used as the experimental unit for statistical analysis.

Results

Male reproductive parameters of prepubertal mice of 5 days old – No statistically significant change has been found in the number of spermatogonia of mice exposed to glyphosate at 0.5, 5 and 50 mg/kg bw/day. No statistically significant change has been found in RNA expression of Cyp11a1, Cyp19a1 and Ret at all dose levels tested. Statistically significant changes were observed in RNA expression of Bax (increase at 0.5 mg/kg bw only), Bcl2 (increase at all dose levels), Dazl (increase at 0.5 mg/kg bw only), Kit (decrease at 50 mg/kg bw only), Sall4 (decrease at all dose levels), Nanos3 (increase at 0.5 and 50 mg/kg bw but not at 5 mg/kg bw) and Foxo1 (increase at 0.5 mg/kg bw only).

Male reproductive parameters of mice of 20 days old - The histopathological analysis of testis sections revealed that glyphosate causes an adverse effect on testis morphology when compared with the control. An increase in vacuoles in the seminiferous epithelium was observed at all dose levels. Empty seminiferous tubules were seen at 5 mg/kg bw but not at 0.5 and 50 mg/kg bw glyphosate.

Male reproductive parameters of mice of 35 days old – No statistically significant change has been found in relative epididymis weight, relative seminal vesicles weight, epididymal sperm count and GATA1 positive cells. A decrease in relative testes weight was recorded at 0.5 mg/kg bw glyphosate but not at the other dose levels. Serum testosterone was found to be statistically significantly decreased at 0.5 and 50 mg/kg bw but not at 5 mg/kg bw glyphosate. The number of ZBTB16 positive cells were statistically significantly decreased at 5 mg/kg bw glyphosate but not at 0.5 and 50 mg/kg bw.

Male reproductive parameters of mice of 8 months old – No statistically significant changes were noted for relative epididymis weight, relative seminal vesicles weight and serum testosterone. A statistically significant decrease has been observed for relative testes weight of mice exposed to glyphosate at 0.5 mg/kg bw but not at the higher dose levels.

Discussion

Glyphosate did not have any effect on the body weight of animals tested in this study. The decrease in relative testicular weight observed at 0.5 (statistically significant) and 5 mg/kg bw (not statistically significant) in mice of at 35 days old and at 0.5 mg/kg bw (statistically significant) in mice of 8 months

old indicates that glyphosate, even at low doses, can significantly impair spermatogenesis, as testicular weight is a very reliable indicator of the normal function of male reproductive system. This fact was confirmed by measuring the level of testosterone in plasma after glyphosate exposure. In this study, serum testosterone levels were statistically significantly decreased in mice of 35 days old when exposed to glyphosate at 0.5 and 50 mg/kg bw and in mice of 8 months old when exposed to glyphosate at 0.5 mg/kg bw but not at higher dose levels. The treatment with different doses of glyphosate did not produce monotonic dose-responses. This is the case for relative weight of the epididymis and seminal vehicles, testosterone levels and sperm counts in mice of 35 days old. Glyphosate exposure led to a non-statistically significant decrease in epididymal sperm count in mice of 35 days old at 0.5 and 5 mg/kg bw leading to a non-statistically significant decrease in relative epididymis weight. This decrease in sperm count suggests that exposure to glyphosate could reduce male fertility.

The increase in the expression of key apoptosis genes (Bax, Bcl2) in mice of 5 days old exposed to glyphosate could influence the balance between Sertoli cells and spermatogonia. At 5 mg/kg bw a statistically significant decrease in spermatogonia (ZBTB16 positive cells) number was observed in animals of 35 days old. As the cell count of the pool of undifferentiated spermatogonia in the testes of mice of 5 days old confirmed that there were no changes in the number of germ cells in the glyphosate treatment groups, the decrease in spermatogonia number in mice of 35 days old may be the result of a modification of key genes involved in germ cell differentiation. In this study, there was a decrease in Sall4 and Kit mRNA expression in 5-day old mice exposed to glyphosate. Both factors are part of the retinoic acid (RA)-dependent signalling pathway involved in the differentiation of Aal spermatogonia into A1 which are irreversibly committed in the differentiation process. This result suggests that glyphosate is able to alter spermatogonial differentiation and maturation and this explains, at least in part, the molecular mechanism responsible for the morphological changes observed in 20-day old animals and the decrease in sperm count observed in adults.

Conclusion

This study shows that glyphosate at the ADI dose of 0.5 mg/kg bw/day could have endocrine disrupting effects which could impair the male reproductive system in mice.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effect of glyphosate exposure from the day of vaginal plug detection to 20 days post-partum via the drinking water at concentrations corresponding with 0.5, 5 and 50 mg/kg bw/day on male reproductive parameters in mice of 5, 20 and 35 days old and 8 months old was investigated. The parameters measured were the number of spermatogonia and expression of genes important to testicular function in 5-day old mice, testicular histopathology in 20-day old mice, relative weight of testes, epididymis and seminal vesicles, epididymal sperm count, serum testosterone levels, GATA1 positive cell count and ZBTB16 positive cell count in 35-day old mice, and relative weight of testes, epididymis and seminal vesicles and serum testosterone levels in 8-month old mice. No statistically significant change was found for the number of spermatogonia in 5-day old mice. The only genes of which the expression was statistically significantly changed in a dose-related fashion were Bcl2 and Kit. In 20-day old mice, sperm depleted seminiferous tubules were noted at 5 mg/kg bw but not at 0.5 and 50 mg/kg bw glyphosate. In 35-day old mice there was no statistically significant change in the relative weight of the epididymis and the seminal vesicles, epididymal sperm count and GATA1 positive cell count. No dose-effect relationship could be established for relative weight of testes, serum testosterone levels and ZBTB16 positive cell count. In 8-month old mice no statistically significant change could be observed for relative weight of epididymis and seminal vesicles and serum testosterone levels. No dose effect relationship could be established for the decrease in relative testes weight. From these data it can be concluded that there is no evidence that glyphosate dosed orally to mice up to 50 mg/kg bw/day during the perinatal period is an endocrine disruptor and has an adverse effect on testicular function and development. This has been corroborated by regulatory reproduction toxicology studies with rats at much higher dose levels.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test conditions were not clearly described and the number of animals tested per dose level is too limited.

Reliability criteria for *in vivo* toxicology studies

Publication: Pham *et al*., 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity ≥ 99.2%. Source: Sigma-Aldrich.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup 3 plus.
AMPA is the tested substance		
Study		
Test species clearly and completely described	Y?	
Test conditions clearly and completely described	Y?	Not completely described. Control and treated young prepubertal or adult mice were euthanized, and reproductive organs were dissected in 5, 20, 35 days old (d.o.), and in 8 months old (m.o.) mice. Only male mice were analysed.
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	0.5, 5, 50 mg/kg bw/day.
Number of animals used per dose level reported	Y?	5 animals derived from at least 3 to 4 different litters in each group
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y?	Not always presented in tables.
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	

Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test conditions were not clearly described and the number of animals tested per dose level is too limited.		

1. Information on the study

Data point:	CA 5.5
Report author	Presutti R. et al.
Report year	2016
Report title	Pesticide exposures and the risk of multiple myeloma in men: An analysis of the North American Pooled Project
Document No	International Journal of Cancer (2016) Vol. 139, 1703–1714
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Three case-control studies in the United States and Canada were pooled to create the North American Pooled Project (NAPP) to investigate associations between pesticide use and haematological cancer risk. This analysis used data from 547 MM cases and 2700 controls. Pesticide use was evaluated as follows: ever/never use; duration of use (years); and cumulative lifetime- days (LD) (days/year handled 3 years of use). Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using logistic regression adjusted for age, province/state of residence, use of proxy respondents and selected medical conditions.

Materials and Methods

Study population - The North American Pooled Project (NAPP) is comprised of three population-based incident case-control studies conducted by the U.S. National Cancer Institute in Kansas, Iowa/Minnesota and Nebraska in the 1980s, and the Cross Canada Study of Pesticides and Health (CCSPH), a population-based incident case-control study that was conducted in Quebec, Ontario, Manitoba, Saskatchewan, Alberta and British Columbia in the early 1990s. All 4 studies aimed to investigate the effects of pesticides and other agricultural exposures on the risk of lymphatic and hematopoietic cancers. The present analysis is restricted to a subset of three NAPP studies conducted in Iowa, Nebraska and Canada (CCSPH) where multiple myeloma (MM) cases were recruited. The study design and data collection in the CCSPH were modeled after the U.S. studies, making the data amenable to pooling. Eligible participants included white men aged 30 years or older in Iowa, white men and women aged 21 years or older in Nebraska, and men aged 19 years or older in Canada. Deceased participants were considered eligible in Iowa and Nebraska, but not in the Canadian study. Proxy respondents were used on behalf of deceased subjects in Iowa and Nebraska, and were permitted in Iowa, Nebraska, and Canada for participants requiring assistance due to illness or disability. Incident MM cases were identified using state and provincial cancer registry records, with the exception of Nebraska and Quebec, where cases were recruited from hospitals. Population-based controls were identified using random digit dialing (all studies), Medicare records and state mortality files (Iowa and Nebraska), health insurance records (Alberta, Saskatchewan, Manitoba and Quebec), telephone listings (Ontario) and voter lists (British Columbia). Cases and controls were frequency-matched to the overall case distribution by age (62 years in Nebraska and Canada, 65 years in Iowa), vital status and year of death (if applicable), sex (Nebraska) and province of residence (Canada). The participation rates in the MM subset of NAPP studies were modest for Canadian controls (48%) and higher in Iowa (78%) and Nebraska (85%). Participation rates were higher for cases in Canada (58%), Iowa (84%) and Nebraska (88%).

Exposure assessment - A set of *a priori* pesticides to be investigated in this analysis included agents that showed positive (significant or nonsignificant) associations in the earlier U.S. and Canadian studies. Pesticides that met these criteria were 2,4-D, captan, carbaryl, chlordane, DDT, glyphosate, lindane, malathion, methoxychlor, permethrin and the pyrethrins. Self-reported information on pesticide use,

farming activities and demographic characteristics were obtained through standardized interviews with participants. Individuals who provided an affirmative answer to general questions about pesticide use or exposure to substances within broad groups (i.e. insecticides, herbicides, fungicides) were subsequently asked more detailed follow-up questions regarding specific agents, including the frequency and duration of exposure. Participants who did not report any pesticide use were classified as unexposed. Among individuals reporting pesticide use, missing information for duration or frequency of exposure was treated as missing or unknown. Information on duration of pesticide use (years) was collected in all studies, whereas frequency information (days per year) was only collected in Canada and Nebraska. The data from Nebraska were excluded from the analysis since the number of exposed cases for pesticides of interest was low and the proportion of missing data was prohibitively high (>40%).

Exposure metrics - Associations were examined for dichotomous exposure (ever/never pesticide use) and by major chemical classes i.e. phenoxy herbicides, and organochlorine, organophosphate and carbamate insecticides. The duration of exposure was evaluated for each individual pesticide using years of self-reported use. Cumulative exposure was investigated using a composite lifetime days (LD) metric, defined as: LD = years of pesticide use x days/year of pesticide use. Analyses of cumulative exposure were restricted to the Canadian subset of the NAPP data, where sufficient information about both years and days/year was available. For subjects with missing information for the duration of pesticide use, simple conditional imputation was carried out. Age- and state/province-specific median values for years and days/year were assigned to participants classified as exposed based on the ever/never metric. Imputed values were only assigned if <35% of exposure duration data were missing among cases, and if the proportions of missing data differed by <20% between cases and controls.

Statistical analysis - Descriptive analyses were performed on potential confounders identified from the literature including age, province/state of residence, use of a proxy respondent, farming history (ever lived or worked on a farm) and personal medical history. Covariates that were significantly ($p < 0.05$) associated with MM or those that produced meaningful changes (≥5%) in the OR estimates were retained in the final models. Unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CI) for pesticide exposure variables with adjustment for age, province/state of residence, use of proxy respondent, and ever being diagnosed with any allergy, hay fever, or rheumatoid arthritis. For all analyses of individual pesticides, the referent population consisted of subjects who did not report any pesticide use, or those who indicated that they had not used that specific agent. Duration of pesticide exposure and cumulative LD were modeled as ordinal variables and linear trends were examined (*p*-trend). Cut-offs for categories were based on the median duration and LD among cases and controls for each pesticide. The use of proxy respondents was also considered as an effect modifier and sensitivity analyses were conducted excluding information provided by proxy respondents All analyses were performed using SAS version 9.3 .

Results

The analysis included a total of 587 MM cases and 3,588 controls from Iowa, Nebraska and six Canadian provinces. Female cases ($N = 40$) and female matched controls ($N = 707$) contributed by the Nebraska study were excluded due to the very low prevalence of pesticide use among females. The youngest MM case in the NAPP dataset was 31 years old and, therefore, the controls of an age of 30 years and younger were excluded ($N = 181$) to maintain a comparable distribution of age between cases and controls. The final analysis included 547 male cases and 2,700 male controls. Among the participants, cases were older than controls. This was expected since a common age-matched control group was used for all cancer sites in the NAPP and the majority of MM cases are typically diagnosed at slightly older ages (>65 years) than non-Hodgkin lymphoma and Hodgkin lymphoma cases. Proxy respondents were used for 35% of the cases and 28% of the controls overall. Associations of demographic characteristics and medical history covariates with MM were modeled using logistic regression, with adjustment for age (in years) and province or state of residence. A history of any type of cancer among first-degree relatives was significantly associated with MM risk. However, having a first-degree relative with any lymphatic or haematopoietic cancer, including MM, was not associated with MM. A number of conditions associated with stimulation of the immune system showed statistically significant inverse associations with MM risk. In this summary only data on glyphosate are reported.

Pesticide exposure (ever/never in the NAPP) - For the "never" use of glyphosate 502 (91.8%) cases and 2,504 (92.7%) controls and for the "ever" use of glyphosate 45 (8.2%) cases and 196 (7.3%) controls were identified. The adjusted OR was 1.29 (95% CI 0.9-1.85). When proxy respondents were excluded then the adjusted OR was 1.07 (95% CI 0.69-1.66).

Pesticide exposure relative to the years of exposure (Iowa and Canada subset of the NAPP) – For no exposure to glyphosate 471 (91.4%) cases and 1,832 (91.7%) controls, for up to 3 years of exposure to glyphosate 22 (4.3%) cases and 87 (4.3%) controls and for more than 3 years 22 (4.3%) cases and 80 (4.0%) controls were identified. The adjusted OR was 1.30 (95% CI 0.79-2.16) for up to 3 years of exposure and 1.34 (95% CI 0.80-2.23) for more than 3 years of exposure. The trend was not statistically significant ($p = 0.16$). When the adjusted OR was taken with exclusion of proxy respondents it was 1.19 (95% CI 0.67-2.11) for up to 3 years of exposure and 0.95 (95% CI 0.50-1.83) for more than 3 years exposure. The trend was not statistically significant ($p = 0.90$).

Pesticide exposure relative to the number of lifetime days (LD) (Canadian subset of the NAPP) - For no exposure to glyphosate 310 (90.6%) cases and 1,228 (91.0%) controls, for up to 6 LD of exposure to glyphosate 18 (5.3%) cases and 65 (4.8%) controls and for more than 6 LD 14 (4.1%) cases and 56 (4.2%) controls were identified. The adjusted OR was 1.35 (95% CI 0.76-2.40) for up to 6 LD of exposure and 1.11 (95% CI 0.59-2.07) for more than 6 LD of exposure. The trend was not statistically significant ($p = 0.48$). When the adjusted OR was taken with exclusion of proxy respondents it was 1.43 (95% CI 0.76-2.70) for up to 6 LD of exposure and 0.94 (95% CI 0.44-1.99) for more than 6 LD of exposure. The trend was not statistically significant ($p = 0.74$).

Discussion and Conclusions

Despite the increase in the overall sample size resulting from pooling data from the CCSPH and U.S. NCI studies, the numbers of exposed participants were still low for some pesticides, and information for duration or frequency was sparse and not collected in all MM studies. Our ability to investigate the effects of high levels of exposure was further limited, since few participants reported frequent and long-term pesticide use. Exposure misclassification due to the use of proxy respondents may also influence results. Studies have shown that farmers may be able to recall pesticide use better compared to nonfarmers, and certain types of proxy respondents, such as friends and family members who also work in agriculture, may be more likely to recall the use of certain specific pesticides. It should be recognized that a large number of comparisons were made and some of the effect estimates were based on small numbers of exposed cases and controls. Therefore, it cannot be excluded that some of the observed associations may represent chance findings. Despite these limitations, this study has several important strengths. The NAPP is one of the largest pooled case-control studies of agricultural exposures and haematopoietic cancers. This analysis was the first to investigate the association between pesticide exposure and MM risk in a pooled sample of Canadian and American participants. Since similar pesticides were used in both Canada and the United States, it was possible to investigate the effects of these exposures in the NAPP overall. Furthermore, similarities in the design of the case-control studies facilitated successful pooling of these datasets, which afforded a larger sample size for more comprehensive and powerful analyses. Specifically, the investigation of different aspects of pesticide exposure, such as duration of use and cumulative lifetime exposure provided an informative and novel addition to this analysis of pesticide use alone. A further advantage of this study was the extensive medical history information that was collected in the Canadian and U.S. studies. This allowed us to take into account the influence of several conditions that result in sustained stimulation of the immune system, such as rheumatoid arthritis, systemic lupus erythematosus, certain viral infections and allergies, which were inversely associated with MM in our data.

No statistically significant increases in risk of multiple myeloma (MM) associated with self-reported exposure to glyphosate were observed.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Four population-based incident case-control studies (3 US studies and one Canadian study) pooled in the North American Pooled Project (NAPP) aimed to investigate the effects of pesticides and other agricultural exposures on the risk of lymphatic and hematopoietic cancers. The present analysis is restricted to a subset of three NAPP studies (Iowa, Nebraska and Canada) where multiple myeloma (MM) cases were recruited. Self-reported information on pesticide use, farming activities and demographic characteristics was collected and the odds ratios (OR) were calculated for "ever/never" exposure, years of exposure and cumulated lifetime days of exposure to glyphosate with and without exclusion of proxy respondents. The result is that no statistically significant increases in risk of multiple myeloma (MM) associated with self-reported exposure to glyphosate were observed.

This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because it concerns pooled case control studies which are subject to recall bias and in which confounding factors could not be ruled out.

Reliability criteria for epidemiology studies

Publication: Presutti *et al*., 2016	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	Y	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	
Study		
Study design – epidemiological method followed	Y	Pooled case control studies
Description of population investigated	Y	
Description of exposure circumstances	Y	May be subject to recall bias
Description of results	Y	
Have confounding factors been considered	N	Confounding factors cannot be ruled out
Statistical analysis	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because it concerns pooled case control studies which are subject to recall bias and in which confounding factors could not be ruled out.		

1. Information on the study

Data point:	CA 5.6
Report author	Ren X. *et al*.
Report year	2019
Report title	Effects of chronic glyphosate exposure to pregnant mice on hepatic lipid metabolism in offspring
Document No	Environmental pollution (2019) Vol. 254, pp. 112906.
Guidelines followed in study	None
Deviations from current test guideline	Not applicable.
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The present study aims to investigate the toxic effects of prenatal exposure to pure glyphosate or Roundup on lipid metabolism in offspring. During gestational days (GDs), ICR mice (from Institute of Cancer Research) were given distilled water, 0.5% glyphosate solution (w/v, 0.5 g/100 ml) or 0.5%-glyphosate Roundup solution orally. The livers and serum samples of the offspring were collected on gestational day 19 (GD19), postnatal day 7 (PND7) and PND21. The results showed a significant decrease in the body weight and obvious hepatic steatosis with excessive lipid droplet formation in offspring. Moreover, the concentrations of lipids such as triglycerides (TGs), total cholesterol (T-CHO), and low-density lipoprotein cholesterols (LDL-C) increased to a significant extent in both the serum and livers. Furthermore, there were significant differences in the expression levels of the genes SREBP1C, SREBP2, Fasn, Hmgcr, Hmgcs and PPARa, which are related to lipid biosynthesis or catabolism in the liver.

Materials and methods

Animals; Ten-week-old female and male ICR mice were purchased from Nanjing Qinglongshan Experimental Animal Center (Nanjing, China). After one week of adaptation, one male and two female mice were housed in each cage from 5.00 p.m. to 8.00 a.m. daily to obtain pregnant mice. Pregnant mice were placed into separate cages once the pregnancy was confirmed by a vaginal smear the following morning. This day was defined as the first day of gestation. Animals were fed with water and feed ad libitum. The temperature and relative humidity in the animal house were controlled at 23 ± 2 C and 50 ± 10%, respectively, and the animals were kept on a 12-h light/dark cycle. The animal experiments were approved by the Animal Welfare Committee of Nanjing Agricultural University (Nanjing, China) and implemented in accordance with the National Institutes of Health Guidelines for Animal Care and the Committee of Animal Research Institute.

Chemicals and treatment; Pure glyphosate (N-(phosphonomethyl)glycine) and Roundup (as the isopropylamine salt) were provided by Shanghai Ryon Biological Technology Co., Ltd. (Shanghai, China) and Sinochem Crop Protection Products Co., Ltd. (Shanghai, China), respectively. Glyphosate and Roundup were diluted with distilled water to obtain 0.5% active ingredient solutions (w/v, 5 g glyphosate/1 L solution). Then, the subjects were administered the 0.5% glyphosate or Roundup solution through the drinking water (pH was controlled at 7.2 ± 0.2).

Animal treatment and sampling; A total of 30 pregnant mice were randomly divided into three groups: CON (control, n = 10), GLP (0.5% glyphosate treated, n = 10), and RU (0.5% Roundup treated, n = 10). Half of the pregnant mice (five from each group) were exposed throughout the first 19 days of pregnancy and were sacrificed on GD19. The other half of the pregnant mice were exposed throughout the pregnancy period and given distilled water after giving birth. Weekly body weights of the offspring were recorded, and their anogenital distances were measured separately to identify their sexes. Seven and 21 days after birth, the prenatally exposed offspring were sacrificed (preferably two females and two males

per mother) for blood and tissue analysis. The water consumption of the pregnant mice was measured, and the real exposure dose of glyphosate in both GLP and RU groups was approximately 7 ml (Table 1). The serum was extracted through centrifugation (3500 rpm, 15 min, 4 C) and was used to assay the biochemical indexes. Parts of the livers were stored at 80°C for lipid concentration determination and reverse transcriptionpolymerase chain reaction (RT-PCR). Liver samples were either fixed in a 4% paraformaldehyde solution or embedded in optimal cutting temperature compound (O.C.T. compound) provided by Sakura Finetek Japan Co., Ltd. (Tokyo, Japan) prior to frozen sectioning for the histological observation of tissue sections.

Histological preparation; Some parts of the liver tissue were fixed in 4% paraformaldehyde solution for 24 h and then dehydrated, clarified, embedded with paraffin and sectioned. Tissue sections (5 mm) were used for hematoxylineeosin (H&E) staining. The remaining liver tissue was embedded with O.C.T. compound and sectioned using a microtome cryostat manufactured by Thermo Fisher Scientific Instrument Co., Ltd. (Shanghai, China) for Oil Red O staining.

Serum biochemical and liver lipid concentration assays; To preliminarily diagnose the liver injury and lipid content of the organisms, the following serum biochemical indexes were determined: aspartate transaminase (AST), alanine transaminase (ALT), triglyceride (TG), total cholesterol (T-CHO), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). Additionally, liver homogenate was centrifuged to obtain the supernatant (3500 rpm, 15 min, 4 °C) to measure the TG, T-CHO, LDL-C and HDL-C content. Both the serum biochemical indexes and hepatic lipid content were assayed with commercial reagent kits purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China).

Analysis of gene expression; Total RNA was extracted from liver tissue with the ISOGEN 2 reagent kit (from NIPPON GENE CO., LTD.) (Tokyo, Japan) according to the manufacturer's instructions. The concentration of the obtained RNA was determined by a spectrophotometer, and the purity was measured using a NanoDrop® 8000. Then, PrimeScriptTM RT Master Mix (from Takara Bio Inc.) was used to reverse transcribe RNA to cDNA, which acted as a template for the SYBR® Premix Ex Taq™ PCR kit (from Takara Bio Inc.) for real-time PCR. The expression levels of the genes SREBP1C (Sterol Regulatory Element Binding Protein 1C), SREBP2 (Sterol Regulatory Element Binding Protein 2), Fasn (Fatty acid synthase, which catalyzes fatty acid synthesis), Scd (Stearoyl-CoA Desaturase 1), Acc (Acetyl-CoA Carboxylase), Hmgcr (3-hydroxy-3-methyl-glutaryl-CoA reductase), Hmgcs1 (3-hydroxy-3-methylglutaryl-CoA synthase 1), Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2) and PPARa (Peroxisome proliferator-activated receptor alpha) were determined. The relative expression levels of the above genes were normalized to b-actin expression. All primers were designed and supplied by GenScript Bio-Tech Co., Ltd. (Nanjing, China).

Data analysis; The software packages SPSS Statistics 20.0 and GraphPad Prism (GraphPad Software, San Diego, CA, USA) were utilized to analyse the data. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed. Values are expressed as the mean ± standard error of the mean (SEM), and statistical significance was set as $p < 0.05$.

Results

Table 1: Effects of chronic glyphosate exposure on the performance of pregnant mice.

Items	CON	GLP	RU	*P*
Water consumption (ml)	9.69 ± 0.76^{a}	7.88 ± 0.46^{b}	7.45 ± 0.34^{b}	0.023
Feed intake (g)	9.00 ± 0.16	8.32 ± 0.55	9.63 ± 0.72	0.261
Body weight gain (g)	32.60 ± 3.30	35.96 ± 2.92	29.84 ± 0.70	0.281
Number of fetuses (n)	10.80 ± 1.50	14.40 ± 1.57	12.60 ± 2.11	0.376
Average birth weight (g)	1.73 ± 0.13	1.65 ± 0.08	2.03 ± 0.72	0.220

Each value represents the mean ± SEM of the group (n = 5).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

Physical and organ development; From GD19 to PND21, the body weight in both GLP and RU groups decreased and finally saw a statistically significant reduction on PND21 ($p < 0.05$) (Fig. 1). When separated according to sex, offspring showed no significant differences in either body weight or weight gain among the three groups (Table 2).

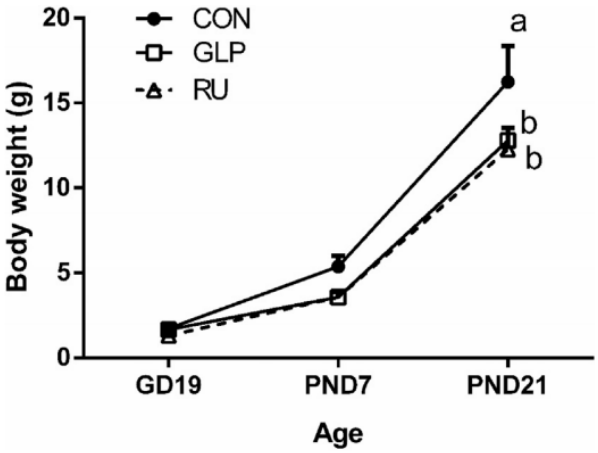


Figure 1: Effects of chronic glyphosate exposure to pregnant mice on the body weights of offspring at the ages of GD19, PND7 and PND21 (mean ± SEM). Different letters indicate statistically significant differences, $p < 0.05$.

Table 2: Effects of chronic glyphosate exposure to pregnant mice on the physical development of the offspring (g).

Items	Female				Male			
	CON	GLP	RU	*P*	CON	GLP	RU	*P*
PND7								
Body weight	5.72 ± 0.61	4.40 ± 0.28	4.29 ± 0.59	0.429	5.24 ± 1.00	4.80 ± 0.30	4.89 ± 0.88	0.918
Body weight gain	3.19 ± 0.80	2.74 ± 0.25	3.08 ± 0.50	0.222	3.51 ± 0.88	3.15 ± 0.29	2.87 ± 0.45	0.115
PND21								
Body weight	14.69 ± 2.44	13.63 ± 0.78	13.26 ± 1.06	0.786	15.79 ± 2.21	15.10 ± 0.85	13.86 ± 1.82	0.732
Body weight gain	12.04 ± 2.01	11.98 ± 0.76	11.24 ± 0.66	0.704	14.06 ± 2.09	13.45 ± 0.82	11.84 ± 1.33	0.243

Each value represents the mean ± SEM of the group (n = 7–10).

Liver histological observation; In both GLP and RU groups, relatively elevated numbers of vacuoles exhibiting hepatic lipid droplets were observed within the hepatocytes of both female and male offspring, when compared with the CON group. Additionally, the red areas observed in the Oil Red O stained sections represent lipid substances. In females, there tended to be more lipid droplets in the GLP group than in the other two groups. In contrast, in males, both the GLP and RU groups showed excessive lipid deposits. In addition, there were several clusters of monocytes in both the GLP and RU groups of PND7 females. It appears that glyphosate could cause inflammation in early-aged female mice.

Serum biochemical index; Compared with the CON group, TG levels showed a significant increase in the GLP group in both GD19 fetuses ($p < 0.01$) (Table 3) and PND21 female mice ($p < 0.05$) (Table 4). With respect to T-CHO levels, GLP mice showed a remarkable increase in both PND7 males ($p < 0.01$) and PND21 females ($p < 0.05$) compared with CON mice. LDL-C levels also increased in PND7 mice in both the GLP and RU groups ($p < 0.05$) (Table 4). The increased lipid content reflects the adverse effects of glyphosate on lipid metabolism, although this disturbed effect was not detected in every individual. Furthermore, significantly elevated AST levels in PND7 females in the RU group ($p < 0.01$) are theoretically considered to be a result of an injured liver.

Table 3: Effects of chronic glyphosate exposure to pregnant mice on the blood biochemical indexes in fetuses.

Items	CON	GLP	RU	*P*
TG (mmol/L)	0.15 ± 0.10^{b}	0.79 ± 0.21^{a}	0.30 ± 0.10^{b}	0.002
T-CHO(mmol/L)	2.15 ± 0.49	2.56 ± 0.52	1.22 ± 0.06	0.165
LDL-C (mmol/L)	1.03 ± 0.09	1.08 ± 0.04	0.91 ± 0.05	0.185
HDL-C (mmol/L)	0.14 ± 0.01	0.17 ± 0.02	0.17 ± 0.01	0.433
AST (IU/L)	67.88 ± 14.17	103.20 ± 10.30	76.21 ± 10.55	0.134
ALT (IU/L)	20.71 ± 1.02	47.54 ± 10.20	32.09 ± 2.35	0.091

Each value represents the mean ± SEM of the group (n = 20).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

Table 4: Effects of chronic glyphosate exposure to pregnant mice on the blood biochemical indexes of PND7 and PND21 offspring.

Items	Female				Male			
	CON	GLP	RU	*P*	CON	GLP	RU	*P*
PND7								
TG (mmol/L)	1.46 ± 0.07	1.79 ± 0.26	1.60 ± 0.08	0.324	1.11 ± 0.14	1.45 ± 0.14	1.45 ± 0.20	0.240
T-CHO (mmol/L)	1.46 ± 0.07	1.60 ± 0.27	1.42 ± 0.19	0.792	2.01 ± 0.23^{b}	2.92 ± 0.19^{a}	1.97 ± 0.11^{b}	0.007
LDL-C (mmol/L)	1.30 ± 0.18^{b}	1.73 ± 0.07^{a}	1.42 ± 0.07^{ab}	0.047	0.58 ± 0.07^{c}	0.99 ± 0.12^{b}	1.75 ± 0.11^{a}	0.000
HDL-C (mmol/L)	1.12 ± 0.11	1.18 ± 0.09	0.86 ± 0.04	0.074	0.86 ± 0.12	0.88 ± 0.04	1.13 ± 0.11	0.189
AST (IU/L)	28.03 ± 6.14^{b}	29.09 ± 6.22^{b}	63.84 ± 8.03^{a}	0.008	65.17 ± 7.65	72.54 ± 10.41	81.71 ± 10.52	0.503
ALT (IU/L)	30.93 ± 3.77	40.51 ± 4.86	39.89 ± 4.08	0.245	29.22 ± 2.39	32.01 ± 2.89	39.27 ± 2.29	0.058
PND21								
TG (mmol/L)	1.30 ± 0.18^{b}	2.41 ± 0.37^{a}	1.36 ± 0.15^{ab}	0.022	1.01 ± 0.11	1.10 ± 0.37	1.19 ± 0.38	0.741
T-CHO (mmol/L)	1.30 ± 0.18^{b}	2.06 ± 0.16^{a}	1.37 ± 0.15^{b}	0.028	0.92 ± 0.07	1.01 ± 0.06	1.05 ± 0.21	0.778
LDL-C (mmol/L)	2.26 ± 0.26	2.23 ± 0.12	2.65 ± 0.18	0.293	1.24 ± 0.19	1.08 ± 0.27	0.64 ± 0.13	0.115
HDL-C (mmol/L)	1.41 ± 0.28	1.65 ± 0.31	1.29 ± 0.10	0.570	1.56 ± 0.31	1.49 ± 0.17	1.22 ± 0.29	0.713
AST (IU/L)	41.14 ± 12.38	70.64 ± 9.87	50.04 ± 12.01	0.286	39.37 ± 9.28	54.06 ± 8.10	22.56 ± 8.90	0.091
ALT (IU/L)	19.02 ± 0.43	22.50 ± 4.61	19.48 ± 3.20	0.722	14.27 ± 4.06	16.01 ± 2.34	16.34 ± 3.04	0.890

Each value represents the mean ± SEM of the group (n = 7–10).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

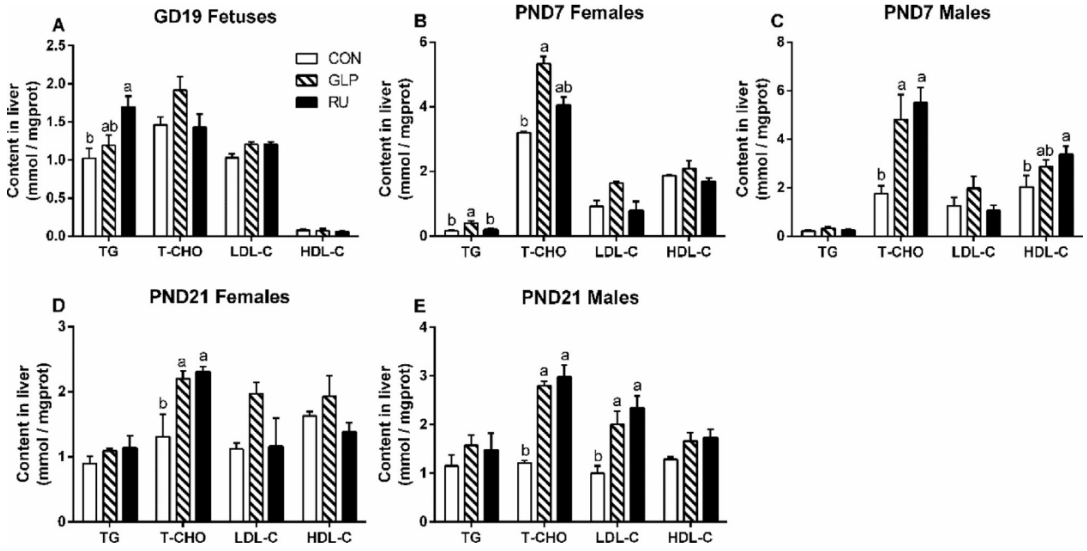


Figure 2: Effects of chronic prenatal glyphosate exposure on the lipid content in the livers of the offspring (mean ± SEM). (A) shows the TG, T-CHO, LDL-C and HDL-C content in livers of GD19 fetuses, and (B), (C), (D) and (E) show these parameters in PND7 and PND21 females and males, respectively. Different letters indicate statistically significant differences. a, b $p < 0.05$.

Lipid concentration in the liver; Compared with that in CON mice, TG levels in the RU group significantly increased in GD19 fetuses and PND7 female offspring ($p < 0.05$) (Fig. 2A and B). Moreover, T-CHO levels of both PND7 and PND21 offspring increased in the GLP or RU groups ($p < 0.05$) (Fig. 2B-E). Elevated TG and T-CHO levels in the liver can probably cause lipid deposits. The LDL-C levels of PND21 males showed a noticeable increase in both the GLP and RU groups ($p < 0.05$) (Fig. 2E), and the HDL-C levels in PND7 males were elevated in the RU group ($p < 0.05$) (Fig. 2C).

Both low-density and high-density lipoproteins can transport cholesterol in the extracellular environment. The elevated level of these proteins in serum is considered to be the result of increased cholesterol levels.

Expression levels of genes related to lipid metabolism in the liver; The relative expression levels of the genes SREBP1C, SREBP2, Fasn, Acc, Scd, Hmgcr, Hmgcs1 and Hmgcs2 in the GLP and RU groups showed a significant increase in GD19 fetuses and PND7 and PND21 offspring ($p < 0.05$) (Fig. 3). These genes are closely related to hepatic lipid production, so their elevation contributes to increased fat storage. However, this kind of increase does not match well to the trend in serum lipid content alteration. The levels of PPARa in PND7 males and PND21 females increased remarkably in both the GLP and RU groups, which is likely due to the growing demand for lipid catabolism caused by the increased lipid content.

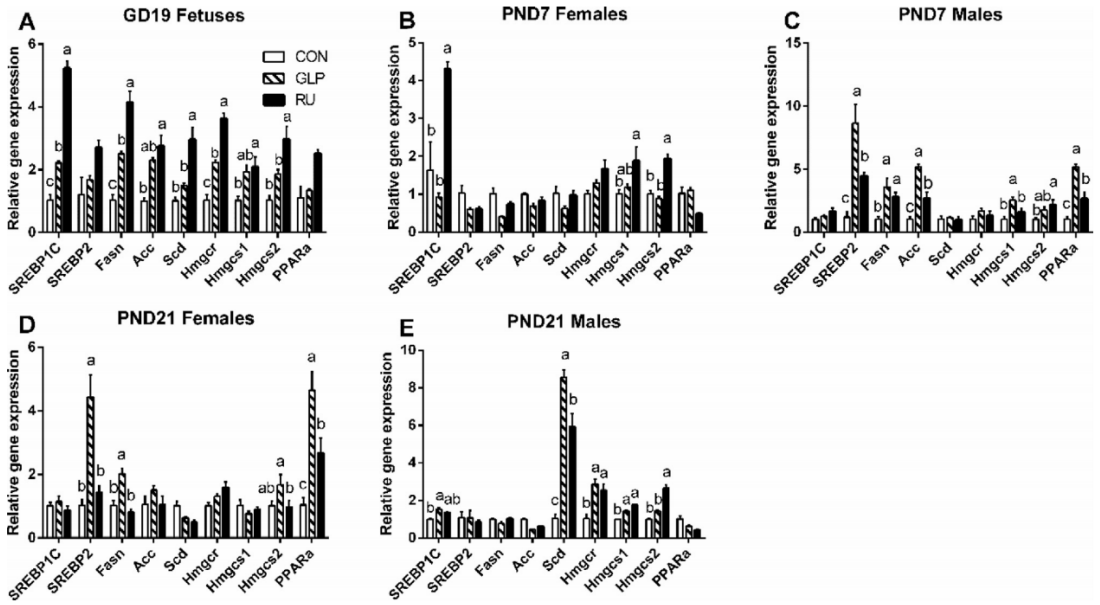


Figure 3: Effects of chronic prenatal glyphosate exposure on relative mRNA expression levels in the livers of the offspring (mean ± SEM). The relative expression levels of the genes SREBP1C, SREBP2, Fasn, Acc, Scd, Hmgcr, Hmgcs1, Hmgcs2 and PPARα genes in the livers of (A) fetuses, (B) PND7 female offspring, (C) PND7 male offspring, (D) PND21 female offspring, and (E) PND21 male offspring are shown. Different letters indicate statistically significant differences. a, b $p < 0.05$.

Discussion

Previous studies have found that glyphosate could cause fatty liver disease at the level of transcriptome and proteome. The transgenerational potential of glyphosate between dams and their litter successfully raises concerns about the hepatotoxicity of glyphosate in the progeny. The present study was designed to study the toxic effects of chronic prenatal glyphosate exposure on lipid metabolism in the livers of offspring. The results suggested that chronic maternal exposure to glyphosate can lead to the disruption of lipid metabolism in the next generation. In the present study, 19 or 21 gestational days was typically chosen as the exposure period, and offspring were collected on gestational day 19 and after birth on PND7 and PND21. The average water consumption in the GLP and RU groups was approximately 7 ml, which means that the pregnant mice took an average of 35 mg glyphosate per day. Thus, the real glyphosate exposure dose in fetuses was probably less than 35 mg per day. To our knowledge, the median lethal dose (LD50) of glyphosate in mice is 5000 mg/kg body weight (bw), and the non-observed adverse effect level (NOAEL) is 500 mg/kg bw. Although the true administered dose of glyphosate that the offspring received in the present study is far lower than the NOAEL, several phenomena still revealed excessive lipid production. Additionally, according to the classification criteria of the EU and the OECD Globally Harmonized System, glyphosate is not listed as an acute oral toxin based on 145 studies. In chronic exposure research, the “overall NOAEL” was assessed to be 100 mg/kg bw per day based on human beings, which is also higher than the present study's dose. Taken together, a dose lower

than overall NOAEL can still cause hepatic lipid metabolism disruption, although the consistency is not perfect among the tested parameters and groups in this study. Physical development is used as an important indicator of the health state of organisms. The body weights of the prenatal glyphosate-treated offspring showed a reduction compared to that of the CON group, which is in accordance with the previous results. The adverse effects on the physical development of offspring could be explained by the decreased body weight of the pregnant mice, while the feed intake of the pregnant mice showed no statistically significant changes. Therefore, the reduced body weight might be the result of energy consumption for the purpose of detoxification instead of for physical development. The liver is the most important detoxification organ that works to metabolize xenobiotics and can reflect the risk of the xenobiotics to the body system to some extent. To observe the liver state of the prenatally exposed offspring at the level of histology, H&E and Oil Red O staining were implemented in the present study. A relative increase in the number of fat vacuoles can be seen in several photomicrographs in the GLP or RU groups compared with the CON group, which is probably due to the elevated TG and T-CHO levels determined in both the serum and liver. Lipid production and storage in organisms are mainly controlled by liver lipogenesis and catabolism. We learned from the related gene expression level data that the genes related to these two biological processes showed increased expression levels in the GLP and RU groups compared with the CON group. SREBPs are essential activators for the synthesis of fatty acids and cholesterol once combined with a lipid synthesis promoter. SREBP1c, one of the isoforms of SREBP1, regulates the process of lipogenesis that increases from the early age of GD19, while SREBP2 is responsible for cholesterol biosynthesis. Furthermore, the increasing expression levels of downstream genes, such as Fasn, Scd, Acc, Hmgcr and Hmgcrs, contribute to higher fatty acid and cholesterol biosynthesis, which could result in inevitable hepatic fat storage. For lipid catabolism, PPARa plays a crucial role in mitochondrial b-oxidation and peroxisomal fatty acid oxidation, both of which act as important biological reactions in the degradation of liver lipids. Increased PPARa levels in the prenatally exposed offspring might be due to the growing demand for lipid catabolism caused by the rising TG or T-CHO levels. However, the alterations in gene expression are not completely consistent with TG or T-CHO synthesis proteins level or fat storage in the liver sections. This finding might be due to the limited number of subjects, and we will try to explore more detailed reasons and explain these reasons in our future studies. Taken together, we believe that glyphosate could affect lipid production by disturbing lipid metabolism-related gene expression. Previous research had detected a time-dependent enhancement in triglyceride and cholesterol levels as glyphosate-treated subjects aged, whereas there were contradictory results shown in bullfrog tadpoles. To explore more mechanistic insights, a lipidomic profiling experiment was conducted, and 62 distinct lipid species were identified as altered, including triglycerides and cholesteryl esters. Additionally, glyphosate is found to be metabolized into glyoxylate, which inhibits fatty acid oxidation enzymes, all of which are considered to be tightly associated with hepatic lipid dysregulation. Together with the reported impairment of mitochondrial oxidative phosphorylation and enzymatic activity after glyphosate treatment, the efficiency of the respiratory rate and tricarboxylic acid cycle will inevitably decline, which could further induce the diversion of redundant fatty acids into other lipid metabolism pathways. There have been many studies on glyphosate-induced chromosome and DNA aberrations spanning more than 20 years. A great variety of species as well as different doses of glyphosate were used in these studies. The reactive oxygen species and oxidative injury caused by glyphosate or its metabolites are commonly thought to be the reason for the genotoxicity. In the present study, glyphosate's genotoxicity was highly likely reflected in the disruption of lipogenesis. Glyphosate also caused obvious liver damage that appeared in the hematological parameters and histopathological alterations. The increased serum ALT and AST levels in prenatally exposed offspring demonstrated severe liver damage, similar to a reported study. The leakage of liver enzymes is a remarkable indicator of hepatic injury owing to xenobiotics. Additionally, the discovery of clusters of monocytes suggested the presence of inflammatory infiltration and immune responses, but it was not widely observed in all treatment groups. Apart from the liver lesions presented in our study, leukocyte infiltration, necrosis, blood congestion, hydropic degeneration and sinusoid dilation, which could enhance the risk of glyphosate-induced steatosis progression into fatty liver disease, has been observed in hepatocytes in other experiments. Therefore, it is probable that glyphosate-induced lipid metabolism disruption could progress into steatosis if no recovery or interference therapy was performed. Although significant differences were observed between some groups, the changes in the histopathological alterations, blood biochemical indexes and expression levels of lipid metabolism related genes among the groups showed imperfect consistency. This result is highly likely caused by the

limited quantity and individual variation of the subjects. Additionally, there are other factors that could have affected the results, such as sex, age and glyphosate source. Considering that the main aim of the present study was to explore the hepatotoxicity of glyphosate exposure, especially during pregnancy, on the lipid metabolism of offspring, these other factors will be future projects and will be studied in future research.

Conclusion

Chronic prenatal glyphosate exposure can probably cause lipid metabolism disruption in offspring, accompanied by an elevated lipid content in both serum and liver tissue. These alterations in hepatic lipid metabolism might result from rising lipogenesis in hepatocytes through increasing related gene expression.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The current study set out to examine any effects on lipid metabolism in fetuses and pups following prenatal exposure to glyphosate or the glyphosate formulation, Roundup™. Ten pregnant female rats per group were exposed from gestation day 1 through 19 to drinking water containing either 0.5% glyphosate, prepared using "pure" glyphosate (N-(phosphonomethyl) glycine), or 0.5% glyphosate using an appropriate dilution of Roundup™. A similar group of animals were given distilled water and served as the control group. Five females per group were terminated on gestation day 19 for examination of fetuses, while the remaining dams were allowed to litter and maintain their litters to postnatal day 21. Offspring (2/sex/litter where possible) were selected on postnatal days 7 and 21 for evaluation. Fetal and offspring evaluations included liver histology, serum biochemistry, liver lipid concentration and gene expression analysis of genes related to lipid metabolism in the liver.

The study is non-GLP and does not report the following information;

- Purity of test items
- Body weight and clinical signs for pregnant animals
- Clinical observations of offspring
- Achieved dose of glyphosate in treated animals in mg/kg bw/day.
- Measures to control inter animal and intergroup variability such as
    - Time of necropsy distributed equally across groups
    - Standardization of litter size on day 4 of lactation to mitigate variability caused by differences in liter size.
- More than a single dose level of glyphosate (0.5% solution); thus, preventing dose-response characterisation
- Liver weight of fetuses or offspring
- Normal physiological ranges for serum and liver biochemistry in this strain of rat at this laboratory
- Clear reporting of statistical evaluation and differences
- Thorough histological evaluations of the liver with incidence and severity of any recorded findings.

Although the authors concluded that there were treatment related effects on fetal and offspring body weight, there is no evidence from this study to suggest that glyphosate exposure has had any impact on fetal development or pup development postnatally. There was no effect on average birth weight of pups, the slight difference observed in the glyphosate treated group should be attributed to the slightly larger mean litter size observed (14.4 pups compared to 10.8 in the control group). Although figure 1 shows a reduction in mean pup weight in the glyphosate and Roundup™ treated groups (male and females combined). Group mean body weight of pups by sex showed no statistical differences from control.

Given the small group size, the large inter animal variability observed, the lack of consistency between the same parameter across the sexes, timepoints or sampling matrices, and the deficiencies

listed above, it not possible to clearly attribute any of the observed differences to glyphosate exposure. Therefore, the current study provides no evidence that glyphosate exposure causes lipid metabolism disruption in offspring following prenatal (in utero) exposure.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterized, only one dose level was tested, there was large inter animal variability observed and too few animals per dose level were analysed.

Reliability criteria for *in vivo* toxicology studies

Publication: Ren *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Shanghai Ryon Biological Technology Co, Shanghai, China.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup from Sinochem Crop Protection Products, Shanghai, China.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	?	Mice.
Test conditions clearly and completely described	?	
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	One dose level: 0.5% in drinking water.
Number of animals used per dose level reported	Y	10/dose.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterized, only one dose level was tested, there was large inter animal variability observed and too few animals per dose level were analysed.

1. Information on the study

Data point:	CA 5.4
Report author	Roustan A. *et al.*
Report year	2014
Report title	Genotoxicity of mixtures of glyphosate and atrazine and their environmental transformation products before and after photoactivation
Document No	Chemosphere (2014) Vol. 108, 93–100
Guidelines followed in study	OECD Test Guideline 487
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Abstract

The photo-inducible cytogenetic toxicity of glyphosate, aminomethyl phosphoric acid (AMPA), and their various mixtures was assessed by the in vitro micronucleus assay on CHO-K1 cells. Results demonstrated that the cytogenetic potentials of pesticides greatly depended on their physico-chemical environment. The mixture made with the four pesticides exhibited the most potent cytogenetic toxicity, which was 20-fold higher than those of the most active compound AMPA, and 100-fold increased after light-irradiation. Intracellular ROS assessment suggested the involvement of oxidative stress in the genotoxic impact of pesticides and pesticide mixtures.

Materials and methods

Chemicals - Glyphosate and AMPA were purchased from Sigma-Aldrich Chemical Company, St Quentin-Fallavier, France.

Cell culture - Experiments were performed in CHO-K1 cells (ATCC-LGC Standards Sarl, Molsheim, France). CHO-K1 cells were maintained in McCoy's 5A medium supplemented with 10% bovine calf serum, 1 mM glutamine, and 100 U/mL-10 µg/mL penicillin-streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Metabolic activation mixture (S9-mix) - The metabolic activation system used was a 9000g centrifuged supernatant (S9) of a 10% liver homogenate prepared from male Sprague-Dawley rats treated with a single injection of Aroclor 1254 (500 mg/kg bw), 5 days before sacrifice. The protein concentration in the S9 homogenate was 26 mg/mL. For the micronucleus assay, the S9-mix contained 10% S9, 5 mM glucose-6-phosphate, 4 mM NADP, 33 mM KCl and 8 mM $MgCl_2$ diluted in PBS.

Photoactivation - To reproduce photoinduction during exposure to glyphosate and AMPA in the environment, the micronucleus assay was performed with a photoactivation procedure. Irradiation of cell cultures was carried out 1 hour after the addition of test compound using a solar simulator Suntest CPS+ apparatus equipped with a xenon arc lamp (1,100 W), a special glass filter restricting transmission of light below 290 nm and a near IR-blocking filter. The irradiance for the photoactivation was fixed at 750 W/m^2 throughout testing. The combined light dose was 4.5 J/cm^2 for one minute irradiation (0.03 J/cm^2 of UVB, 0.41 J/cm^2 of UVA and 4.06 J/cm^2 of visible light). This irradiation dose is representative of a 1-3 minute period of solar exposure during a clear summer day in the United Kingdom. The temperature of the samples was kept at 4 °C. UVA-visible light (320-800 nm) was obtained using the solar ID65 filter plus a window glass filter.

Micronucleus assay - A total of 50,000 CHO-K1 cells was plated in chamber slides and incubated for 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2. Various concentrations of glyphosate (5, 10, 50, and 100 µg/mL) and AMPA (0.005, 0.01, 0.05, and 0.1 µg/mL without S9-mix; 0.1, 0.5, 1, and 5 µg/mL with S9-mix; 0.00005, 0.0001, 0.0005, and 0.001 µg/mL with light irradiation) were incorporated into duplicate CHO-K1 cell cultures. To determine the background DNA-damage levels in CHO cells in the dark and with light irradiation 2 negative controls were added for glyphosate and AMPA: culture medium and PBS. Mitomycine C (0.06 µg/mL) without S9-mix and benzo[a]pyrene (5 µg/mL) with S9-mix were selected as positive controls. After 3 hours of exposure, cells were rinsed with PBS and incubated in fresh medium containing cytochalasin B (3 µg/mL) for an additional 24 hours to stop cytokinesis. At the end of the incubation period, cells were rinsed twice with PBS and fixed with methanol. The slides were air dried and stained with 5% Giemsa stain in Milli-Q water for 15 minutes. The Cytokinesis Blocked Proliferation Index (CBPI) was used to select adequate concentrations for the assessment of micronuclei. CBPI was determined by scoring the number of mononucleated (M1), binucleated (M2), and trinucleated (M3) cells among 500 Giemsa-stained cells with well-preserved cytoplasm: CBPI = [(M1) + (2 x M2) + (3 x M3)]/500. When a cytotoxic effect was observed, micronucleated cell rates were determined for concentrations inducing less than a 50% decrease of CBPI. When no cytotoxic effect was observed, the maximal concentration assessed was 100 µg/mL. When a clastogenic/aneugenic activity was observed, 4 relevant concentrations were selected to obtain a dose-response relationship. A total of 2000 binucleated cells were examined for each concentration, and micronuclei were identified according to the morphological criteria previously defined.

Intracellular ROS analysis - Intracellular ROS was determined with the cell-based OxiSelect™ Intracellular ROS Assay Kit using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). After diffusion into the cell, this fluorescent probe is deacetylated by cellular esterases to non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by ROS. A total of 50,000 cells were seeded in a black 96-well cell culture plate and incubated at 37 °C for 24 hours. Cells were rinsed 3 times with PBS and incubated in 100 µL of DCFH-DA/media solution at 37 °C for 60 minutes. Cells were subsequently rinsed with PBS and treated with glyphosate or AMPA. Experiments were performed in triplicate. The concentrations tested were the same as those assessed for the micronucleus assay. H_2O_2 (100 µM) was used as the positive control. After a 30-minute incubation period, fluorescence was measured with a fluorometric plate reader at 480 nm and 530 nm.

Statistical analysis – Statistically significant differences between negative controls and treated samples were determined using the ^{2}test. The standard micronucleus assay was considered positive when a dose-response relationship could be established between the numbers of micronucleated cells and the concentrations of pesticide solutions, and when at least one concentration induced a significant increase of micronucleated cells as compared to the medium-only control culture. The dose-response relationships were calculated by nonlinear regression analysis with TableCurve2D®. Model significance was based on three criteria: (i) correlation coefficient r^2 being > 0.62; (ii) model probability (P) being < 0.05; (iii) error probability (PE) being > 0.05. The Minimal Clastogenic Concentration (MCC) was defined as the lowest concentration of test item (µg/mL) that induced a significant increase of micronucleated cells. The Cytogenetic Potency (CP) was defined as the slope of the dose–response curves. It was calculated by non-linear regression analysis with TableCurve2D®.
For the determination of ROS, the dose-response relationships were calculated by linear regression analysis with TableCurve2D®. Model significance was based on three criteria: (i) correlation coefficient r^2 being > 0.62; (ii) model probability (P) being < 0.05; (iii) error probability (PE) being > 0.05. The Oxidative Potency was defined as the slope of the dose-response curves and was calculated according to a standard curve obtained with various concentrations of fluorescent DCF.

Results

No statistically significant increase in the incidence of bi-micronucleated cells (BMC) was observed with glyphosate in the dark and without S9-mix at concentrations up to 100 ug/mL. In the presence of S9-mix a statistically significant and dose-related increase was noted from 10 µg/mL. The calculated

minimal clastogenic concentration (MCC) was 5.8 µg/mL. With light irradiation a statistically significant increase in BMC was noted at the highest concentration of glyphosate tested (100 µg/mL) with a MCC of 93.4 µg/mL. Without S9-mix a statistically significant and dose-related increase in BMC was produced by AMPA from a concentration of 0.01 µg/mL with a MCC of 0.006 ug/mL. In the presence of S9-mix a statistically significant increase in BMC was seen with AMPA from 1 µg/mL with a MCC of 0.78 µg/mL. With light irradiation, the lowest test concentration of AMPA with a statistically significant increase in BMC was 0.0005 µg/mL with a MCC of 0.0004 µg/mL. The oxidative potency of H_2O_2, used as a positive control, was 333.3 nM DCF/μM^{-1} (data not shown). Only AMPA exerted an elevated oxidative effect (5.9 nM DCF/$\mu g.mL^{-1}$), whereas the oxidative potency of glyphosate was very low.

Discussion

The results obtained in the present study confirmed the cytogenetic toxicity of glyphosate and AMPA *in vitro*. In the dark, glyphosate was not directly active in the absence of S9-mix, but it induced micronuclei in the presence of S9-mix. On the contrary, without S9-mix, AMPA displayed a direct cytogenetic effect which was 1,000 fold higher than that of its parent compound. The weak mutagenic activity of glyphosate with S9-mix could be explained by its weak metabolization yield *in vivo*. After sunlight irradiation, glyphosate was weakly active, whereas the cytogenetic effect of AMPA was about 20-fold increased. Since the photodegradation of glyphosate by UV light has been shown to produce AMPA, the photoinducible genotoxic potential of glyphosate could be partially explained by the formation of its metabolite AMPA in the intracellular environment. In the present study, cells were subjected to a very low irradiation that did not induce intrinsic DNA damage. The results of the present study implied more probably the involvement of a photoinduced oxidative stress, which transformed glyphosate and AMPA into photoactivated intermediates favoring interactions with cellular targets.

Conclusion

The results of this study demonstrated that light-irradiation, corresponding to a few minutes of solar exposure, greatly potentiated the cytogenetic impact of AMPA. *In vitro* experiments showed that the genotoxic impact of pesticides greatly depend on their physico-chemical environment,

3. Assessment and conclusion

Assessment and conclusion by applicant:

The cytogenetic effect of two herbicides (glyphosate and atrazine), their metabolites (AMPA and DEA), and mixtures thereof was investigated in CHO-K1 cells in the *in vitro* micronucleus test. Only the results of glyphosate and AMPA tested alone are reported and discussed in the summary. Glyphosate and AMPA were tested with and without metabolic activation and with light irradiation. Also the potency of glyphosate and AMPA to produce ROS was investigated. The concentrations tested ranged from 5 to 100 µg/mL for glyphosate and from 0.00005 to 5 µg/mL for AMPA. No statistically significant increase in the incidence of bi-micronucleated cells (BMC) was observed with glyphosate at concentrations up to 100 ug/mL in the dark and without metabolic activation. However, a statistically significant and dose-related increase in BMC was noted from 10 µg/mL in the presence of metabolic activation. With light irradiation a statistically significant increase in BMC was noted for glyphosate at a concentration of 100 µg/mL. AMPA produced a statistically significant and dose-related increase in BMC from a concentration of 0.01 µg/mL in the dark and without metabolic activation. With metabolic activation a statistically significant increase in BMC was seen with AMPA from 1 µg/mL. With light irradiation the lowest test concentration of AMPA with a statistically significant increase in BMC was 0.0005 µg/mL. Only AMPA was found to produce an elevated oxidative effect, whereas the oxidative potency of glyphosate was very low. The results of glyphosate in the *in vitro* micronucleus test with metabolic activation reported in this study are surprising since glyphosate is essentially unmetabolized *in vitro* in the presence of a rat liver S9 homogenate. Moreover, these results are not corroborated by regulatory *in vivo* micronucleus tests in the mouse dosed up to more than 2,000 mg/kg bw.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate and AMPA tested were not sufficiently characterized and no positive and control historical data were reported. The *in vitro* micronucleus test carried out was in compliance with OECD TG 487.

Reliability criteria for *in vitro* toxicology studies

Publication: Roustan *et al*., 2014	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	In vitro MN test compliant with OECD TG 487.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity not reported. Source: Sigma-Aldrich Chemical Company, St Quentin-Fallavier, France.
Only glyphosate acid or one of its salts is the tested substance	N	Other pesticides (atrazine, desethyl atrazine (DEA)) were tested and mixtures thereof.
AMPA is the tested substance	Y	Tested alone and as mixtures with glyphosate, atrazine and DEA.
Test system clearly and completely described	Y	CHO-K1 cells.
Test conditions clearly and completely described	Y	CHO-K1 cell MN test.
Metabolic activation system clearly and completely described	Y	S9-mix.
Test concentrations in physiologically acceptable range (< 1 mM)	Y	From 5 to 100 µg/mL for glyphosate and from 0.00005 to 5 µg/mL for AMPA.
Cytotoxicity tests reported	Y	Cytokinesis Blocked Proliferation Index (CBPI) and incidence of bi-micronucleated cells (BMC).
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate and AMPA tested were not sufficiently characterized and no positive and control historical data were reported. The *in vitro* micronucleus test carried out was in compliance with OECD TG 487.		

1. Information on the study

Data point:	CA 5.4
Report author	Santovino A. *et al.*
Report year	2018
Report title	In vitro evaluation of genomic damage induced by glyphosate on human lymphocytes
Document No	Environmental Science and Pollution Research (2018) 25:34693-34700s
Guidelines followed in study	Some compliance with OECD 473 and OECD 487
Deviations from current test guideline	Only continuous treatments in the absence of S9 were performed and these exceeded the 1.5 cell cycles recommended by both guidelines No historical control data are reported. Treatment commenced within 24 hours of PHA stimulation rather than 44-48 hours
Previous evaluation	No
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, its was analyzed the *in vitro* clastogenic and/or aneugenic effects of glyphosate by chromosomal aberrations and micronuclei assays. Human lymphocytes were exposed to five glyphosate concentrations: 0.500, 0.100, 0.050, 0.025, and 0.0125 μg/mL, where 0.500 μg/mL represents the established acceptable daily intake value, and the other concentrations were tested in order to establish the genotoxicity threshold for this compound.

Materials and Methods

Study population; Peripheral venous blood was collected from six healthy Italian subjects (two males and four females, mean age ± SD, 27.50 ± 12.55), non-smoking, not alcoholics, not under drug therapy, and with no recent history of exposure to mutagens. All subjects signed the informed consent. The study was approved by the University of Turin ethics committee and was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki.

Blood sample collection and lymphocyte cultures; Blood samples were obtained by venipuncture, collected in heparinized tubes, cooled (4 °C), and processed within 2 h after collection. Lymphocyte cultures, fixation, and staining procedures were performed as previously described. Total time of lymphocyte cultures was 52 and 72 h for CA and MNi assays, respectively. After 24 h of incubation, 8.6 μL of glyphosate stock solution (Sigma-Aldrich, Saint Louis, MO, USA, CAS n. 1071-83-6) at the final concentration of 0.5 mg/mL in dimethyl sulfoxide (DMSO) was added to the lymphocyte culture in order to reach a final glyphosate concentration of 0.500 μg/mL. Similarly, 8.6 μL of glyphosate stock solution diluted 5, 10, 20, and 40 times with DMSO were added to the lymphocyte cultures in order to reach the final glyphosate concentrations of 0.100 μg/mL, 0.050 μg/mL, 0.025 μg/mL, and 0.0125 μg/mL, respectively. In particular, 0.500 μg/mL represents the ADI concentration established by EFSA for this compound, whereas 0.100, 0.050, 0.025, and 0.0125 μg/mL concentrations were tested in order to evaluate the genotoxicity threshold. Three control cultures were assessed: (1) positive control, by adding only MMC (final concentration 0.1 μg/mL culture); (2) 0.1% DMSO solvent control, obtained by adding 8.6 μL of DMSO to the lymphocyte culture; and (3) negative control culture without both glyphosate and DMSO, obtained adding 8.6 μL of RPMI medium to the lymphocyte culture. Only for MNi assay, after 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 μg/mL to block cytokinesis. Similarly, only for CA assay, to arrest cells in mitosis, colchicine was added at the concentration of 0.06 μg/mL during the last 2 h of culture. After 52 h (for CAs assay) and 72 h

(for MNi assay) of incubation at 37°C, the cells were collected by centrifugation, treated for 10 min with a pre-warmed hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a solution of methanol/acetic acid (3:1 v/v). The treatment with the fixative was repeated three times. Finally, the supernatant was discarded, and the pellet, dissolved in a minimal volume of fixative, was seeded on the slides to detect CAs and MNi by conventional staining with 5% Giemsa (pH 6.8) prepared in Sörensen buffer.

Cytokinesis-block micronucleus assays; Microscope analysis was performed at ×400 magnification on a light microscope (Dialux 20, Leica, Germany). MNi, nucleoplasmic bridges (NPB), and nuclear buds (NBUD) were scored in 2000 binucleated lymphocytes with well-preserved cytoplasm per subject (total 12,000 binucleated cells per concentration). Cells containing one of more MNi were scored as "micronucleated cell" (MNC). A total of 2000 lymphocytes per donor per concentration were scored to evaluate the cytokinesis-block proliferation index (CBPI), according to the following formula: [1 × N1] + [2 × N2] + [3 × (N3 + N4)] / N, where N1–N4 represents the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

Chromosomal aberration assay; Microscope analysis was performed at ×1000 magnification on a light microscope (Dialux 20, Leica, Germany). For each subject and glyphosate concentration, 200 complete complete metaphases (for a total of 1200 metaphases for each dose) were analyzed. Cells containing one of more types of CAs were scored as "aberrant cell" (Ab.C). In order to determine cytotoxicity, the mitotic index (MI) was calculated from the number of metaphases in 1000 cells analyzed per subject per concentration (a total of 6000 cells per concentration).

Statistical analysis; Comparison of mean values of the percentage of cells with MNi, MNC, CBPI, NPBs, NBUDs, CAs, Ab.C, and MI between exposure levels and controls was assessed by the nonparametric Mann-Whitney test. Statistical calculations were carried out using the SPSS software package program (version 24.0, Inc., Chicago, IL, USA). All P values were two-tailed, and P values of 5% or less were considered statistically significant for all tests carried out.

Results

Effect of glyphosate on CA formation; Table 1 shows values of CAs found in the human peripheral lymphocytes cultured in the presence of different glyphosate concentrations. Glyphosate was found to induce the following structural CAs: gaps, chromatid and chromosome breaks, dicentric chromosomes, rings, tri-tetraradials, and acentric fragments. This last, together to chromatid breaks, represent the most frequent observed aberrations (Table 1Gaps were excluded from statistical analysis. Glyphosate was found to significantly increase the CA and Ab.C frequencies at all tested concentrations when compared with the solvent control, including the concentration of 0.025 μg/mL, but with the exception of 0.0125 μg/mL. A dose effect was also observed, since the regression analysis revealed a significant correlation between glyphosate concentrations and the CA and Ab.C frequencies (Table 2). Vice versa, no significant differences were found between the DMSO solvent-control and the negative control, whereas the cultures treated with the MMC showed a significant increase of the cytogenetic damage with respect to all concentrations of glyphosate. Finally, no significant differences were found in the MI values between solvent control and all tested concentrations of glyphosate, although at 0.500 μg/mL, the P value resulted to be borderline.

Table 1 Induction of chromosomal aberrations by Glyphosate in human lymphocytes in vitro Number of scored metaphases for each concentration = 1200

Test substance (μg/mL)	Structural CAs							Total CAs	Total CAs + Gaps	Total Ab.C	Total Ab.C + Gaps	(%) CAs/Cell ± S.E.	(%) Ab.C/Cell ± S.E.	(%) MI ± S.E.
	Gaps	B'	B"	DC	R	TR	AF							
NC	8	8	2	0	0	0	7	17	25	17	25	1.417±0.154	1.417±0.154	5.567±0.042
0.1% DMSO	10	9	4	1	0	6	7	27	37	27	37	2.250±0.335	2.250±0.250	5.433±0.056
MMC (0.100)	41	46	30	9	10	14	36	145	186	127	168	12.083±0.300[a]	10.583±0.473	4.200±0.058[a]
Gly (0.500)	17	41	12	12	3	0	28	96	113	95	112	8.000±0.428[a]	7.917±0.375	5.300±0.026
Gly (0.100)	23	31	10	7	2	2	23	75	98	75	98	6.250±0.359[a]	6.250±0.359	5.333±0.080
Gly (0.050)	9	21	6	7	0	0	16	50	59	50	59	4.167±0.167[a]	4.167±0.167	5.367±0.095
Gly (0.025)	10	15	4	4	3	0	20	46	56	46	56	3.833±0.211[b]	3.833±0.211[b]	5.383±0.040
Gly (0.0125)	8	14	5	1	0	0	14	34	42	34	42	2.833±0.211	2.833±0.211	5.400±0.037

CAs, chromosomal aberrations; *Ab.C*, aberrant cells (cells with 1 ore more aberrations); *MI*, Mitotic Index; *NC*, Negative Control; *MMC*, Mitomycin-C; *B'*, chromatid break; *B"*, chromosome break; *DC*, dicentric; *R*, ring; *TR*, tri-tetraradials; *AF*, acentric fragments; *S.E.*, standard error; *Gly*, Gliphosate

[a] $P = 0.004$; [b] $P < 0.006$ (significantly differs from the DMSO solvent control, Mann-Whitney test)

Table 2 Multiple regression analysis between Glyphosate concentrations

Biomarker	β-co	95% CI (Lower) – (Upper)	*P*-value
CAs	0.914	(2.112) – (2.988)	<0.001
Cells with CAs	0.919	(2.099) – (2.935)	<0.001
MI	-0.275	(-0.666) – (0.099)	0.141
MNi	0.908	(4.025) – (5.075)	<0.001
Cells with MNi	0.935	(3.639) – (4.527)	<0.001
CBPI	0.269	(-28.171) – (4.571)	0.151
NPBs	0.674	(0.268) – (0.665)	<0.001
NBUDs	0.395	(0.023) – (0.444)	0.031

CAs, Chromosomal Aberrations; *MI*, Mitotic Index; *MNi*, Micronuclei; *CBPI*, Cytokinesis-block Proliferation Index; *NPBs*, nucleoplasmic bridges; *NBUDs*, nuclear buds

Effect of glyphosate on MNi formation; Table 3 shows the frequencies of MNi found in the human peripheral lymphocytes cultured in the presence of different glyphosate concentrations. Glyphosate significantly increased the MNi frequency at all tested concentrations when compared to the solvent control, with exception of 0.0125 μg/mL (Table 3). Vice versa, no effect has been observed on the frequencies of NBUD and NPB, with the only exception of 0.500 μg/mL of glyphosate that was found to increase in a significant manner the frequency of NPBs with respect to the solvent control. Also in this case, a relationship between the frequency of MNi and the concentrations of glyphosate was observed (Table 2), as well as the DMSO solvent-control cultures did not show significant differences with respect to the negative controls. MMC was found to significantly increase the MNi, NPB, and NBUD formation compared with the negative control solvent controls and all tested concentrations of glyphosate, with exception of 0.500 μg/mL. After 48-h exposure, a significant reduction of the CBPI value in cultures treated with glyphosate was not observed, indicating that, at the tested concentrations, glyphosate does not seem to produce effects on the proliferation index. Finally, at 0.500 μg/mL, glyphosate significantly induced the NPB formation, whereas no differences were found in the frequency of NBUD between DMSO solvent control and all glyphosate concentrations.

Table 3 Induction of micronuclei by Glyphosate in human lymphocytes in vitro. Number of scored binucleated cells for each concentration of the test substance = 12,000

Test substance (μg/mL)	Distribution of BNCs according to the number of MNi				MNi	MNC	MNi/BNCs ± S.E. (%)	MNC/BNCs ± S.E. (%)	CBPI ± S.E	Frequency of BNCs with NPBs (‰)	Frequency of BNCs with NBUDs (‰)
	1	2	3	4							
NC	27	0	0	0	27	27	0.225±0.021	0.225±0.021	1.713±0.003	0.417±0.083	0.833±0.105
0.1% DMSO	33	1	0	0	35	34	0.292±0.024	0.283±0.025	1.589±0.076	0.500±0.129	1.083±0.154
MMC (0.100)	129	9	3	2	164	143	1.367±0.067	1.192±0.015[a]	1.366±0.019	2.333±0.357	3.083±0.473 [a]
Gly (0.500)	132	6	2	1	154	141	1.283±0.017	1.175±0.021[a]	1.545±0.054	1.667±0.211	1.666±0.247
Gly (0.100)	107	7	0	0	121	114	1.008±0.030	0.950±0.029[a]	1.556±0.017	0.883±0.105	1.333±0.167
Gly (0.050)	93	6	1	0	108	100	0.900±0.053	0.833±0.046[a]	1.576±0.015	0.750±0.111	1.250±0.1112
Gly (0.025)	68	5	0	0	78	73	0.650±0.048	0.608±0.035[a]	1.585±0.010	0.667±0.105	1.167±0.105
Gly (0.0125)	39	0	0	0	39	39	0.325±0.021	0.325±0.021	1.589±0.008	0.583±0.083	1.167±0.167

BNCs, Binucleated cells; *MNi*, micronuclei; *MNC*, cells with 1 or more micronuclei; *CBPI*, Cytokinesis-Block Proliferation Index; *NPBs*, nucleoplasmic bridges; *NBUDs*, nuclear buds; *S.E.*, Standard Error; *NC*, Negative Control; *MMC*, Mitomycin-C; *Gly*, Gliphosate

[a] $P = 0.004$ (significantly differs from the DMSO solvent control, Mann-Whitney test)

Discussion

Glyphosate is an active ingredient of most widely used herbicides. Although it is believed to be less toxic than other herbicides, data about its possible genotoxicity are controversial and IARC classified this compound as probably carcinogenic to human (IARC 2015). The genotoxic effects of high concentrations of glyphosate have been documented, although with contradictory results, in a great number of scientific papers, as well as in evaluation reports of different international agencies. On the other hand, the effects of low concentrations of this compound, likely to be encountered in everyday life, were poorly investigated. Results of our study provided information about in vitro clastogenic effects of glyphosate on human lymphocytes at the low ADI concentration of 0.500 μg/mL and its submultiples. Based on the obtained data, it can be concluded that glyphosate significantly increased the CA and MNi levels in human lymphocytes at the ADI concentration of 0.500 μg/mL established by EFSA and at its submultiple concentrations, up to 0.025 μg/mL. The mechanisms underlying genotoxic potential of glyphosate alone or in complex with other compounds are unknown, although the exposure to glyphosate was found to trigger oxidative processes involved in the increase of the genomic damage. NPB frequency was found to increase with increasing glyphosate concentrations, although a statistical significance was found only at the higher glyphosate concentration (Table 3). However, we obtained a significant linear regression (Table 2) due to a steady increase with the dose, indicating a possible effect of the compound inducing this kind of damage, which is consistent with the increased dicentric frequency observed in the chromosomal aberration test. Also, other authors analyzed in vitro the genotoxic potential of glyphosate in lymphocytes, but at exposure levels of higher magnitude orders. For example, in human lymphocytes cultured without S9 and in the presence of glyphosate at concentrations of 3.5, 92.8, and 580 μg/mL, the authors observed a slightly increased frequency of MNi and a significant tail length increase after a comet assay. Other authors evaluated the induction of CAs and MNi in blood cells of other animal models. Positive clastogenic and genotoxic effects of glyphosate on bovine peripheral lymphocytes cultured in vitro with herbicide concentrations ranging from 17 μM (2.874 μg/mL) to 170 μM (28.740 μg/mL) were reported, whereas another study reported no CAs effect of glyphosate at concentrations ranging from 28 (4.734 μg/mL) to 1120 μM (189 μg/mL). Contradictory results were obtained by a further study, in which observed, after 48 h of treatment without S9, a statistically significant increase in MNi frequency at 280 μM (47.34 μg/mL) but not at 560 μM (94.68 μg/mL) of glyphosate in one donor, and the opposite in a second donor (positive at 560 μM but not at 280 μM). Finally, another study in in vitro experiments based on comet assay, showed that 7 mM of glyphosate (1183 μg/mL) caused DNA damage in blood cells of Nile tilapia (Oreochromis niloticus). Concentrations of glyphosate similar to those evaluated in the present paper were tested in HepG2 cells by the MNi assay. Similarly to what we observed in human lymphocytes, these authors found a significantly higher number of MNi at the ADI value of 0.500 μg/mL, as well as at the residential exposure level of 2.91 μg/mL, after 4 h of treatment. Vice versa, negative results on Hep-2 cells were obtained with CA assay at glyphosate concentrations of 0.20 mM (33.8 μg/mL), 1.20 mM (203 μg/mL), and 6.00 mM (1014 μg/mL). Significant levels of DNA damage were also observed in human buccal epithelial cells exposed to glyphosate concentrations ranging between 10 and 20 mg/L, whereas another

study showed that, in peripheral blood mononuclear cells, glyphosate induces DNA damage in the concentration range from 0.5 mM (84.54 μg/mL) to 10 mM (1690 μg/mL), and a significant decrease of global DNA methylation at concentration of 0.25 mM (42.27 μg/mL). Interestingly, the same authors also observed a significantly increased methylation of p53 promoter at concentrations of 0.25 mM and 0.5 mM (42.27 and 84.54 μg/mL). This hypermethylation was found to be able to downregulate the p53 gene expression and to activate proto-oncogenes, with consequent genomic alterations and cancer risk. The possibility of glyphosate causing cancer promotion in skin cells and proliferation in breast cells has been also observed in vivo and in vitro studies by mouse and human models, respectively. In this scenario, the results obtained in the present study require attention. Indeed, increased CA and MNi frequencies in peripheral blood lymphocytes have been positively associated with increased cancer risk and early events in carcinogenesis, respectively. Moreover, it should be emphasized that, beyond the cases of intoxication where glyphosate content in blood was found to range from 0.6 to 150 μg/mL, in subjects who were indirectly exposed to this substance, glyphosate was found in blood at concentrations of 0.074 ± 0.028 μg/mL, a value about seven times lower with respect to the established ADI value, but in the range of concentrations we tested (from 0.5 to 0.0125 μg/mL). At the same time, the genotoxicity of a compound should not be evaluated only after single administrations in in vitro or in vivo systems, but also, and especially, after chronic administration of the same compound, even at lower quantities than those established by the competent agencies. In this sense, the clastogenicity we observed at concentrations of 0.100, 0.050, and 0.025 μg/mL represents an important signal, especially in view of a chronic exposure to these glyphosate concentration levels. Finally, no significant differences in CBPI and MI values were found between all tested concentrations and the solvent control, indicating that, at these concentrations, glyphosate does not influence in a significant manner the replicative capacity of the cells. These data differ from a study which observed a reduction of mitotic and proliferation indices in bovine lymphocytes, but at higher glyphosate concentrations (94.68 μg/mL and 189 μg/mL). Similarly, other authors described decreased levels of MI for other herbicides or insecticides, also in this case, at concentrations much higher than those tested in the present work.

Conclusion

In the present work, there was evidence for cytogenetic effects of glyphosate on cultured human lymphocytes. Despite the limitations of an in vitro study due to the reduced sample size, it is our opinion that the increased cytogenetic damage observed by our group at glyphosate concentrations equal and lower than the established ADI value requires further investigations in order to establish the effective genotoxicity threshold of this extensively used compound. Indeed, the glyphosate concentrations tested in the present work represent more realistic concentrations, likely to be encountered in everyday life, with respect to the higher doses evaluated in other published papers. In this scenario, in order to draw conclusions about the effects associated to the chronic exposure to low doses, in vitro studies are useful tools to investigate the dose response effects, the molecular mechanisms of action of different environmental xenobiotics, and their genotoxicity. This last, compared to other types of toxicity, may result in severe consequences that can be also inherited after long periods following exposure. The same DNA damage that occurs in a single cell, caused by low but chronic exposure to genotoxic compounds, can cause unexpected severe consequences in the long run.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes human lymphocyte chromosome aberration and micronucleus tests with glyphosate. Although broadly compliant with OECD 473 and 487 there are some critical deficiencies which will have adversely influenced the reliability of the findings. Treatment with glyphosate was initiated 24 hours after lymphocyte cultures were stimulated to divide, instead of the recommended 48 hours, consequently the cultures would not have been asynchronous. This could mean cells in some stages of the cell cycle may have been under-represented, whilst others over-represented. Exposure to glyphosate was continuous for 28 hours in the chromosome aberration assay or 48 hours in the micronucleus assay. In contrast OECD test guidelines recommend maximum exposure of 1.5 cell cycles, equivalent to approximately 24 hours for lymphocyte cultures. For both endpoints the

paper does not confirm if the slides were coded prior to analysis. The positive control has been compared statistically to the glyphosate treated cultures rather than the solvent controls.

The authors consider that glyphosate induces tri-tetraradial aberrations (amongst other aberration types) but fails to comment that the frequency of these aberrations observed at a single glyphosate concentration is 3-fold lower than the frequency observed in the solvent control cultures. Furthermore, it is unusual that the only multi-aberrant metaphases observed were a small number of positive control metaphases and gaps did not appear to increase with treatment but chromatid and chromosome breaks did.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and although the genotoxicity tests conducted were in general in accordance with the OECD test guidelines, significant deficiencies were noted.

Reliability criteria for *in vitro* toxicology studies

Publication: Santovito *et al*., 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	In accordance with OECD TG.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity is not reported. Source: Sigma-Aldrich, Saint Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Lymphocytes.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.500, 0.100, 0.050, 0.025 and 0.0125 µg/mL.
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and although the genotoxicity tests conducted were in general in accordance with the OECD test guidelines, significant deficiencies were noted.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Sierra-Diaz E. *et al.*
Report year	2019
Report title	Urinary pesticide levels in children and adolescents residing in two agricultural communities in Mexico
Document No	International Journal of Environmental Research and Public Health (2019), Volume 16, Number 4, 562 p.
Guidelines followed in study	None.
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The objective of this study was to measure the concentration and prevalence of pesticides in a cross-sectional study involving children and adolescents under 15 years of age in two small farming communities located in in State of Jalisco in Mexico, with both communities having very similar characteristics.

Urine samples (first-morning urine) were taken from children under 15 years of age in Agua Caliente located near the largest lake in Mexico (n = 192) and in Ahuacapán located in the south coast of the state (n = 89). A total of 281 urine samples obtained in both communities and processed for the determination of pesticides with high-performance liquid chromatography together with tandem mass spectrometry.
In 100% of the samples, at least two pesticides of the 17 reported in the total samples were detected. The presence of glyphosate was detected in more than 70% of the cases. The mean urinary level of glyphosate 0.363 ± 0.3210 ng/mL in Agua Caliente and 0.606 ± 0.5435 ng/mL in Ahuacapán, detected in 72.91% and 100% of their respective total samples. Substantial differences were detected regarding the other compounds.

Materials and methods

A cross-sectional study was carried out simultaneously in two communities in State of Jalisco, which is one of the three states in Mexico with the greatest index of people poisoned due to the application of pesticides. The first of these was, Agua Caliente, near Lake Chapala, the largest lake in Mexico, and the second was a community in the region of the south coast of the state (Ahuacapán).

Beginning hundreds of years ago, multiple autochthonous communities of people of Nahua native origin who were dedicated to fishing and agriculture settled on the bank of Lake Chapala. Since 2016, the Department of Public Health of the University of Guadalajara has carried out studies in the zone, specifically in the community of Agua Caliente, Poncitlán Municipality, State of Jalisco. In this community, health problems have been detected such as malnutrition and albuminuria, specifically in children and adolescents under the age of 17 years.

The community is inhabited by 998 persons, whose main activities are farming (37.9%), construction work (29.3%), and laboring 7.2% and who alternate with fishing as a means of subsistence. The most common local crops are corn, seasonal beans, and chayote (*Sechium edule*), which the inhabitants irrigate with lake water. The weekly average family income is approximately 52.63 USD.

The community of Ahuacapán is found toward the coast of the Pacific Ocean, in a 180 km straight line from Agua Caliente. A total of 950 inhabitants live there and their principal economic activity is

agriculture, producing sugar cane, corn, tomatoes, citrus, and horticultural products. The water necessary for agriculture derives from springs, deep wells, and the Ayuquila River. The weekly family income in this community is approximately 75 USD. In both localities, there is a total of 550 children and adolescents (58%) aged between less than 1 year and 15 years.

For the urine sampling, communities were invited to participate voluntarily, with the approval of the Department of Public Health of the University of Guadalajara and the local authorities of the two communities. The sample included only children and adolescents aged under 17 years in Agua Caliente and those under 12 years of age in Ahuacapán. Their parents were informed concerning the objective of the study and, after obtaining the parents' signed consent, the minors were asked for a urine sample (first-morning urine). In both communities, anthropometric measurements (weight and height) were performed on the minors.

Urine samples were transported to the laboratory and were processed for the determination of pesticides with the HPLC/MS/MS (high-performance liquid chromatography coupled with tandem mass spectrometry) method with Agilent Technologies® Model 1200 equipment for HPLC and Model 6430B for MS/MS spectrometry. The method for HPLC used a column Zorbax Eclipse XDB C18, Rapid Resolution, 2.1 × 50 mm, 3.5 µm. Mobile phases: A, 0.1% formic acid in water; B, acetonitrile (ACN); gradient of 40% to 100% B; injection volume, 5 µL; flow, 0.5 min; curve range for each pesticide, 0.01 to 1000 µg/mL. The latter was performed at the Laboratory of Applied Pharmacokinetics of the University Center of Exact and Engineering Sciences of the University of Guadalajara. With this method, it was possible to determine the presence of 16 pesticides, as presented in Table 1.

Table 1. Category of pesticides analyzed in urine samples from the two communities.

Name	IUPAC ID	PubChem CID	Agrochemical Category
Acetochlor	2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide	1988	Herbicide
Atrazine	6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine	2256	Herbicide
Carbendazim	methyl N-(1H-benzimidazol-2-yl)carbamate	25429	Fungicide
Carbofuran	(2,2-dimethyl-3H-1-benzofuran-7-yl) N-methylcarbamate	2566	Insecticide, Nematicide, Acaricide
Cyhalothrin	[cyano-(3-phenoxyphenyl)methyl] 3-[(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate	5281873	Insecticide
Diazinon	*O,O*-Diethyl O-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate	3017	Insecticide, Acaricide
Dimethoate	2-dimethoxyphosphinothioylsulfanyl-N-methylacetamide	3082	Insecticide, Acaricide
Emamectin	4″-Deoxy-4″-epi-methylamino-avermectin B1; Epi-methylamino-4″-deoxy-avermectin	11549937	Insecticide
Enilconazole (imazalil)	1-[2-(2,4-dichlorophenyl)-2-prop-2-enoxyethyl]imidazole	37175	Fungicide
Glyphosate	2-(phosphonomethylamino)acetic acid	3496	Herbicide
Malathion	diethyl 2-dimethoxyphosphinothioylsulfanylbutanedioate	4004	Insecticide, Acaricide
Methomyl	methyl (1E)-N-(methylcarbamoyloxy)ethanimidothioate	5353758	Insecticide
Metoxuron	3-(3-chloro-4-methoxyphenyl)-1,1-dimethylurea	29863	Herbicide
Molinate	S-ethyl azepane-1-carbothioate	16653	Herbicide
Pyraclostrobin	Methyl N-[2-[[1-(4-chlorophenyl) pyrazol-3-yl]oxymethyl]phenyl]-N-methoxycarbamate	6422843	Fungicide, plant growth regulator
Thiabendazole	4-(1H-benzimidazol-2-yl)-1,3-thiazole	5430	Fungicide

Conditions for MS/MS spectrometry are described in Table 2 and Figure 1 shows a chromatogram urine sample.

Table 2. Mass spectrometer conditions for pesticide determination.

Mass spectrometer conditions				
Electrospray Interface Condition				
Gas emperature	350 °C			
Gas flow	12 L/min			
Nebulizer	25 psi			
Capillary	+4000	−4000		
Compound name	**Precursor Ion**	**Product Ion**	**Fragmentor**	**Polarity**
L-Cyhalotrin (225.1)	467.1	225.1	80	Positive
Meclizina (201.1)	391.2	201.1	90	Positive
Pyraclostrobin (163)	388	163	120	Positive
Malation (99)	331	99	80	Positive
Clorpyrifos (200)	325	200	30	Positive
Oxandrolona (289.2)	307.2	289.2	100	Positive
Oxandrolona (271.2)	307.2	271.2	100	Positive
Oxandrolona (229.1)	307.2	229.1	100	Positive
Diazinon (153)	305	153	160	Positive
Imazalil (159)	297	159	160	Positive
Paration (264)	292	264	90	Positive
Paration (236)	292	236	90	Positive
Acetoclor (224.2)	270.1	224.2	60	Positive
Acetoclor (148.4)	270.1	148.4	60	Positive
Picloram (222.9)	240.9	222.9	90	Positive
Picloram (194.9)	240.9	194.9	90	Positive
Dimethoate (171)	230	171	80	Positive
Metoxuron (72.1)	229.1	72.1	93	Positive
Ametryn (186)	228.1	186	120	Positive
Ametryn (96)	228.1	96	120	Positive
Carbofuran (123)	222	123	120	Positive
Atrazine (132)	216	132	120	Positive
Thiabendazole (131)	202	131	120	Positive
Carbendazim (160)	192.1	160	110	Positive
Molinate (55.1)	188.1	55.1	78	Positive
Methomyl (106)	163.1	106	30	Positive
Methomyl (88.1)	163.1	88.1	30	Positive
Methomyl (65)	163.1	65	30	Positive
Emamectina (158.1)	887.1	158.1	60	Positive
Glyphosate (149.9)	168	149.9	80	Negative
Glyphosate (124.2)	168	124.2	80	Negative
2,4-D (161.1)	219	161.1	50	Negative

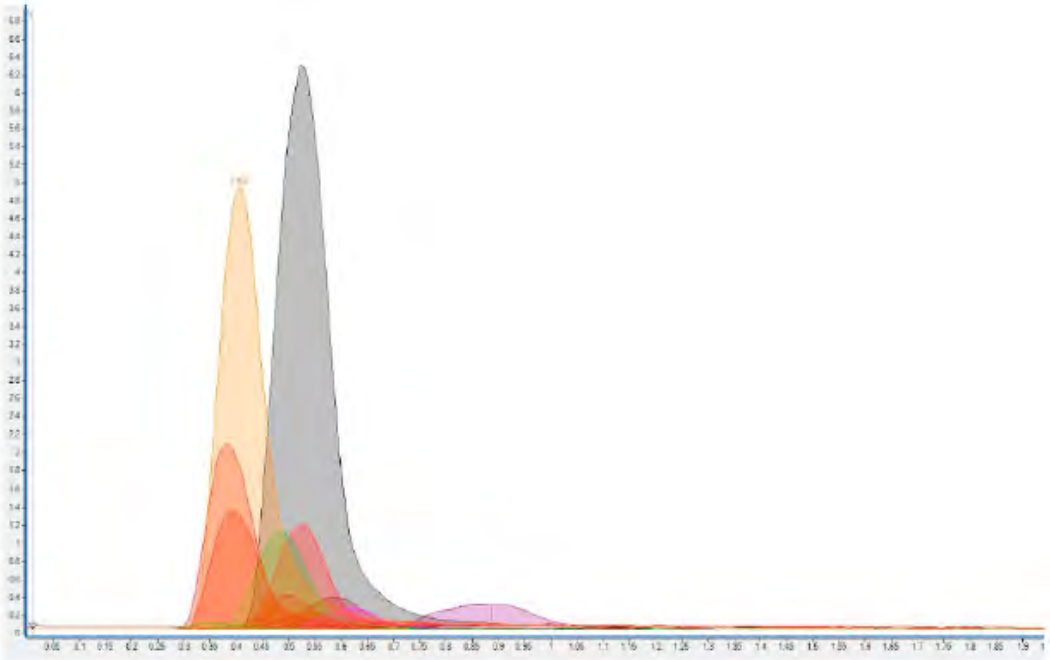


Figure 1. Shows a chromatogram of a urine sample.

Statistical analysis

For the statistical description, absolute frequencies, percentages, means, and standard deviations (SD) were used. Statistical significance was evaluated by means of the Mann–Whitney U, the Chi-squared, and the Fisher exact tests. To compare the two populations and evaluate the differences in the urine pesticide levels, the Mann–Whitney U test was used. Similarly, to compare the frequency of detection rate, the Fisher test was used. Statistical significance was considered with a p of ≤ 0.05. For data processing, Excel® (Microsoft, Redmond, WA, USA) and Epi Info ver. 7.2 (Centers for Disease Control and Prevention (CDC) Atlanta, GA, USA) statistical software were used.

This research was carried out with the authorization of the ethics committee of the Department of Public Health of the University of Guadalajara (registration number DCSP/CEI/2016/260618/038).

Results

A total of 281 children participated, of whom 192 (68.3%) corresponded to the community of Agua Caliente with an average age of 9.4 years (range, 5–15 years). In the community of Ahuacapán, 89 (31.7%) samples were collected, with an average age of 8.31 years (range, 5–13 years; Table 3).

Table 3. Demographic and anthropometric data of the children in both communities.

Variable	Agua Caliente (n = 192)	Ahuacapán (n = 89)
Gender		
Female	84 (43.8%)	40 (44.9%)
Male	108 (56.3%)	49 (55.1%)
Age (years)	9.40 (SD 2.52)	9.31 (SD 2.05)
Age groups		
5–8 y	78 (40.6%)	49 (55.1%)
9–11 y	67 (34.9%)	34 (38.21%)
12–15 y	47 (24.5%)	6 (6.7%)
Weight (kilograms)	29.39 (SD 10.06)	32.27 (SD 11.73)
Height (centimeters)	131.58 (SD 14.12)	132.94 (SD12.97)
Body mass index (k/m^2)	16.46 (SD 2.44)	17.72 (SD 3.66)

Detection of the pesticides was frequent in both communities. Substantial differences in exposure and detection rate were also identified (Table 4). Glyphosate was detected with a higher frequency in Ahuacapán than in Agua Caliente, which presented in 100% of the minors studied in Ahuacapán vs. 73% in Agua Caliente ($p > 0.001$), with minimal values of 0.0020 ng/mL and maximal values of 2.63 ng/mL. Glyphosate is currently one of the most utilised herbicides in Mexico. The differences between the two localities were considered to be associated with the agro-industrial activity and the practices that have become generalised among the small producers of basic crops.

In general terms, 100% of the study subjects were exposed to at least two of the compounds identified in urine. Positive results are noteworthy of six of the compounds in more than 70% of the subjects studied in both communities: malathion, metoxuron, glyphosate, dimethoate, enilconazole, and acetochlor. The greatest prevalence was for the herbicides (60.49%), in second place, fungicides (39.05%), and lastly, the insecticides (20.92%).

The urinary samples in this study was collected during winter, during which there is a significant diminution in the agricultural use of pesticides. However, the authors emphasised that consumption of a conventional diet with seasonal products could play a very important role regarding the presence of these pesticides in urine.

Table 4. Frequencies, percentages, means, and standard deviations (SD) of pesticides in urine.

Pesticide	Agua Caliente	Ahuacapán	
	n (%)	*n* (%)	*p* (Fisher Test)
	Mean ng/mL (SD)	Mean ng/mL (SD)	*p* (Mann–Whitney)
Acetochlor	161 (83.85) 0.008 (0.0867)	44 (49.43) 0.001 (0.0017)	<0.01 0.04
Atrazine	22 (11.45) 0.016 (0.0486)	22 (24.71) 0.043 (0.0930)	<0.01 0.06
Carbendazim	29 (15.10) 0.141 (0.4192)	52 (41.57) 0.330 (0.5040)	<0.01 <0.01
Carbofuran	1 (0.52) 0.246	0	NA NA
Cyhalothrin	138 (71.87) 0.083 (0.0823)	45 (50.56) 0.080 (0.0855)	<0.01 0.52
Diazinon	29 (15.10) 0.007 (0.0199)	20 (22.47) 0.008 ± 0.0180	0.09 0.41
Dimethoate	179 (93.22) 0.146 (0.1834)	44 (49.43) 0.169 (0.2299)	<0.01 0.03
Emamectin	6 (3.12) 0.006 (0.0339)	9 (10.11) 0.019 (0.0582)	0.02 0.34
Enilconazole	177 (92.18) 1.582 (5.6623)	29 (32.58) 0.069 (0.1023)	<0.01 <0.01
Glyphosate	140 (72.91) 0.363 (0.3210)	89 (100) 0.6060 (0.5435)	<0.01 <0.01
Malathion	191 (99.47) 0.681 (0.6431)	55 (61.79) 0.177 (0.1730)	<0.01 <0.01
Methomyl	46 (23.95) 0.016 (0.0292)	0	<0.01 <0.01
Metoxuron	188 (97.91) 0.038 (0.0403)	50 (56.17) 0.037 (0.0414)	<0.01 0.10
Molinate	79 (41.14) 0.191 (0.3698)	55 (61.79) 0.273 (0.4240)	<0.01 <0.01
Pyraclostrobin	62 (32.29) 0.049 (0.2331)	37 (41.57) 0.042 (0.0509)	0.08 0.18
Thiabendazole	29 (15.10) 0.007 (0.0511)	24 (26.96) 0.002 (0.0046)	<0.01 0.12

Conclusion

In a comparative cross-sectional study in two agricultural communities with very similar characteristics in Mexico (a total of 281 children participated in the study), glyphosate was detected in more than 70% of the cases in both communities, having a higher prevalence rate along with malathion, metoxuron, compared to other pesticides analysed in the study. The mean urinary levels of glyphosate were 0.363 ± 0.3210 ng/mL in Agua Caliente and 0.606 ± 0.5435 ng/mL in Ahuacapán. In general, substantial differences in exposure and detection rate were also identified between the communities.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In a comparative cross-sectional study using the urine of children living in two agricultural communities with very similar characteristics in Mexico (a total of 281 children participated in the study), glyphosate was detected in more than 70% of the cases in both communities. The mean urinary levels of glyphosate were 0.363 ± 0.3210 ng/mL in Agua Caliente and 0.606 ± 0.5435 ng/mL in Ahuacapán.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no validation data were presented for the analytical method employed.

Reliability criteria of exposure studies

Publication: Sierra-Diaz *et al*., 2019.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	
Study		
Study design clearly described	Y	Survey of glyphosate concentrations in children.
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	?	No pesticide exposures reported.
Sampling scheme sufficiently documented	Y	Production of one early morning urine sample.
Analytical method described in detail	Y	
Validation of analytical method reported	N	
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no validation data were presented for the analytical method employed.		

1. Information on the study

Data point:	CA 5.5
Report author	Sorahan T.
Report year	2015
Report title	Multiple Myeloma and Glyphosate Use: A Re-Analysis of US Agricultural Health Study (AHS) Data
Document No	International Journal of Environmental Research and Public Health (2015) Vol. 12, 1548-1559
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

RRs for exposed and non-exposed subjects were calculated using Poisson regression; subjects with missing data were not excluded from the main analyses. Using the full dataset adjusted for age and gender the analysis produced a RR of 1.12 (95% CI 0.50 to 2.49) for ever-use of glyphosate. Additional adjustment for lifestyle factors and use of ten other pesticides had little effect (RR 1.24, 95% CI 0.52 to 2.94). There were no statistically significant trends for multiple myeloma risks in relation to reported cumulative days (or intensity weighted days) of glyphosate use. The doubling of risk reported previously arose from the use of an unrepresentative restricted dataset and analyses of the full dataset provides no convincing evidence in the AHS for a link between multiple myeloma risk and glyphosate use.

Materials and Methods

The secondary data file which served as a basis for this study was provided by researchers of the Agricultural Health Study (AHS) taking care of the privacy of the participants. AHS researchers supplied an informative description of the file and the file was found to be internally consistent as well as consistent with data descriptions supplied earlier. All subjects gave their informed consent for inclusion before they participated in the AHS study and ethics approval for the original data collection by AHS researchers was obtained from the Institutional Review Board of the National Institutes of Health. This secondary analysis was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the University of Birmingham Science, Technology, Engineering and Mathematics Ethical Review Committee.

Data on lifestyle factors and use of pesticides were collected from 57,311 private and commercial pesticide applicators from Iowa and North Carolina. Previous analyses were carried out on three subsets of data: Set 1 comprised 54,315 applicators excluding those with any cancers diagnosed before enrolment, applicators lost to follow-up, who had missing data for age at enrolment, or who provided no information on whether they had ever used glyphosate. Set 2 comprised 49,211 applicators and further excludes applicators with missing data on level of education, smoking history, or use of alcohol. Set 3 comprised 40,719 applicators and further excludes applicators with missing data on either use or estimated cumulative exposure days for 2,4-D (2,4-dichlorophenoxy acetic acid), alachlor, atrazine, metolachlor, or trifluralin, and missing data on ever-use of benomyl, maneb, paraquat, carbaryl, or diazinon. The objective of this analysis was to examine findings in an as full a dataset as possible and some analyses have also been carried out on a larger fourth set of 55,934 applicators, a category that does not exclude applicators with missing data on ever-use of glyphosate but only applicators with cancers diagnosed before enrolment, applicators lost to follow-up, or who had missing data for age at enrolment.

Poisson regression was used to estimate RRs and 95% CIs associated with glyphosate exposure metrics, with and without adjustment for other variables. Each variable under analysis was classified into levels

or categories. The analytical approach for the full dataset was to have a “not known/missing” category for each variable so that analyses of all available cases could be maintained. However, it was necessary to ensure that there was at least one case of multiple myeloma in each level of each variable for the regression to successfully calculate RRs. There were no cases of multiple myeloma in those applicators with “unknown use of 2,4-D”, such applicators were combined with those reporting “no use” to create a new category of “no claim of use”. There were no cases of multiple myeloma in applicators with an “unknown level of education”. These applicators were combined with those reporting no education beyond high school. All significance tests were two-tailed and tests for trend (where applicable) were calculated by scoring the levels of a variable and treating the variable as unfactored. All analyses were performed with the EPICURE statistical software, using the double precision DOS version 2.12 of DATAB and AMFIT, dated March 2002.

Results

There were 32 cases of multiple myeloma in Set 1, 26 cases in Set 2, and 22 in Set 3. For the calculation of the RRs there was no adjustment for gender. None of the RRs calculated (RR including statistical adjustment for age at enrolment and RR with additional adjustments) for the three sets is statistically significant and in Set 1, the largest data set, the RR for ever-use of glyphosate is close to unity (RR 1.08, 95% CI 0.48 to 2.41). The point estimates of risk for the smaller datasets show an approximate doubling of risk irrespective of whether adjustment for other variables is carried out. The largest RR is shown for the fully adjusted model of the smallest dataset (RR 2.79, 95% CI 0.78 to 9.96).

Estimated risks of multiple myeloma for reported ever-use for the 54,315 applicators in Set 1 (total of 32 cases) were calculated in relation to use of pesticides and other variables (smoking, alcohol consumption, family history of cancer and education). The RR for ever-use of glyphosate, with adjustment for age at enrolment and gender only, is close to unity (RR 1.12, 95% CI 0.50 to 2.49). The RR for ever-use of glyphosate is little changed with additional adjustment for all 14 other variables (RR 1.24, 95% CI 0.52 to 2.94).

RR estimates were calculated for multiple myeloma in terms of levels of reported cumulative days of glyphosate use and levels of estimated intensity-weighted exposure days for the 54,315 applicators in Set 1. For each exposure metric three sets of RRs have been calculated: firstly, adjusting for age at enrolment and gender; secondly, with additional adjustment for cigarette smoking, use of alcohol, family history of cancer, and level of education; and thirdly, with additional adjustment for use of ten other pesticides. Two tests for trend were applied to each of these analyses, the first scored the levels of cumulative exposure as 1-4, the second scored the level by mean values. There were no statistically significant trends, but a not statistically significantly elevated RR was shown for the highest category of intensity-weighted exposure days in the fully adjusted model (RR 1.87, 95% CI 0.67 to 5.27). There were no cases of multiple myeloma in glyphosate users with unknown extent of use.

RR estimates were calculated for multiple myeloma in terms of levels of ever-use of glyphosate, reported cumulative days of glyphosate use, and estimated intensity-weighted exposure days for the 55,934 applicators in Set 4 with a total of 34 cases of multiple myeloma. The risk of multiple myeloma in ever-users of glyphosate was close to unity (RR 1.18, 95% CI 0.36 to 8.20) and there were no significant trends with either of the two cumulative exposure metrics.

Discussion

This study found no significant trends of multiple myeloma risk with reported cumulative days of glyphosate use and unexceptional point estimates of risk for ever-use of glyphosate. This was irrespective of whether the analyses had adjustment for a few basic variables (age and gender) or adjustment for many other lifestyle factors or pesticide exposures, as long as data on all available pesticide applicators were used. The suspiciously elevated RRs reported previously arose from the use of restricted data sets that, probably by chance, turned out to be unrepresentative. These restrictions would seem to be unnecessary because there is no technical problem in dealing with missing data in Poisson regression. The practice of restricting analyses to subjects with complete data for all variables is, perhaps, a procedure to be carried out with caution as it is clear from this example that such restrictions can lead to misleading findings. It also ignores the fact that findings for missing categories

can often be interesting in their own right.

Conclusion
This secondary analysis of AHS data does not support the hypothesis that glyphosate use is a risk factor for multiple myeloma, and suggests that the practice of restricting analyses to subjects with complete data for all variables is perhaps not to be recommended.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study the relative risk estimates for exposed and non-exposed applicators were calculated using Poisson regression and subjects with missing data were not excluded from the main analyses. When using the full dataset adjusted for age and gender the analysis produced a RR close to unity for ever-use of glyphosate. Additional adjustment for lifestyle factors and use of ten other pesticides had little effect. This study found no statistically significant trends of multiple myeloma risk with reported cumulative days of glyphosate use and unexceptional point estimates of risk for ever-use of glyphosate. This was irrespective of whether the analyses had adjustment for a few basic variables (age and gender) or adjustment for many other lifestyle factors or pesticide exposures, as long as data on all available pesticide applicators were used. The suspiciously elevated RRs reported previously arose from the use of restricted data sets that, probably by chance, turned out to be unrepresentative.

This publication concerns a secondary analysis of the data from the Agricultural Health Study (AHS) and is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the reliability criteria of a well conducted epidemiology study.

Reliability criteria for epidemiology studies

Publication: Sorahan T., 2015	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.		
Study completely described and conducted following scientifically acceptable standards	Y	Secondary analysis of the AHS data
Test substance		
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	
Study		
Study design – epidemiological method followed	Y	
Description of population investigated	Y	
Description of exposure circumstances	Y	
Description of results	Y	
Statistical analysis	Y	
Overall assessment		
Reliable withoiut restrictions	Y	
Reliable with restrictions		
Not reliable		

Publication: Sorahan T., 2015	**Criteria met? Y/N/?**	**Comments**
This publication concerns a secondary analysis of the data from the agricultural health study and is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the reliability criteria of a well conducted epidemiology study.		

1. Information on the study

Data point	CA 5.9 Exposure study
Report author	Steinborn A. *et al.*
Report year	2016
Report title	Determination of Glyphosate Levels in Breast Milk Samples from Germany by LC-MS/MS and GC-MS/MS
Document No.	J Agric Food Chem (2016), Vol. 64, 1414−1421
Guidelines followed in study	Guidance document on analytical quality control and validation procedures for pesticide residues in food and feed, SANCO/12571/2013
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No/Not stated
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study describes the validation and application of two independent analytical methods for the determination of glyphosate in breast milk. They are based on liquid chromatography − tandem mass spectrometry (LC-MS/MS) and gas chromatography − tandem mass spectrometry (GC-MS/MS), respectively. For LC-MS/MS, sample preparation involved an ultra filtration followed by chromatography on an anion exchange column. The analysis by GC-MS/MS involved an extraction step, clean-up on a cation exchange column, and derivatization with heptafluorobutanol and trifluoroacetic acid anhydride. Both methods were newly developed for breast milk and are able to quantify glyphosate residues at concentrations as low as 1 ng/mL. The methods were applied to quantify glyphosate levels in 114 breast milk samples, which had been collected from August to September of 2015 in Germany. The mothers participated at their own request and thus do not form a representative sample. In none of the investigated samples were glyphosate residues above the limit of detection found.

Materials and Methods

Chemicals – Glyphosate standard solution (10 μg/mL), reference glyphosate and internal standard ($^{13}C_2{}^{15}N$ labeled glyphosate) were purchased from LGC Standards, Wesel, Germany and from Dr. Ehrenstorfer, Augsburg, Germany.

Collection of breast milk samples - Breast milk samples were collected in August and September 2015 by the Governmental Institute of Public Health of Lower Saxony, Germany and by the Bavarian Authority for Health and Food Safety Germany for the analysis of glyphosate. All participants signed a declaration of consent concerning the use of their samples for scientific purposes. Participating mothers had not been selected by random sampling and thus do not form a representative sample. There were no restrictions relating to, for example, age and point of sampling during the lactation period for participating in the monitoring program. The milk samples for this study were collected and stored in polypropylene tubes which remained frozen during storage and shipment. In total, 114 milk samples were analyzed and the participants completed a self-administered questionnaire. Information on sample collection, biometric data and self-reported pesticide exposure of the participants is given in Table 1.

Table 1: Biometric data of study participants

parameter	samples from Bavaria, Germany	samples from Lower Saxony, Germany
number of samples	17	97
age of mother (years)		
median	32.1	32.0
range	26–39	22–39
body weight of mother (kg)		
median	63.0	67.0
range	54–90	48–102
duration of lactating period (weeks)		
median	11.0	18
range	3–80	4–52
self-reported exposure to pesticides	6 participants	32 participants

The questionnaire also asked for the place of residence and the jobs practiced in the last 10 years. Thirty-eight participants declared the use of chemical insecticides, herbicides or wood preservatives. At least one participant has worked in a residue analytical laboratory and used pesticide standards regularly. Twenty of the 114 breast milk samples were divided each into two subsamples to allow the parallel analysis by LC-MS/MS and GC-MS/MS.

Fortification of breast milk samples for performance tests - A homogeneous sample of breast milk was prepared and spiked with different volumes of a glyphosate standard solution in water at 10 µg/mL. Twenty-eight stored breast milk samples from a previous study of the Governmental Institute of Public Health of Lower Saxony were pooled and 4 aliquots of 100 mL were spiked with glyphosate resulting in concentrations of 0.5, 1, 3, and 5 ng/mL. An additional aliquot of the pooled sample served as the control. All performance samples were divided into two subsamples and analyzed in parallel with LC-MS/MS and GC-MS/MS. These samples served as independent quality control samples.

Sample Preparation for LC-MS/MS analysis - Removal of fat by centrifugation and removal of proteins by ultra-filtration in one step through centrifugal filtration using a molecular weight cutoff filter of 30 kDa was found to be suitable. To 3 mL of sample, 30 µL of internal standard solution containing 1000 ng $^{13}C_2{}^{15}N$ glyphosate/mL was added to obtain a concentration of 10 ng/mL. After mixing, the sample was transferred to the top part of the cutoff filter tube. The filter was centrifuged at 3500 rpm for 20 minutes and 500 µL of the filtrate was then transferred to the LC filter vial, the solution was filtered and the vial used for measurement. After this procedure, one mL of final extract contained the glyphosate residue of one mL breast milk.

LC-MS/MS analysis - The LC-MS/MS system used consisted of a Nexera UHPLC system from Shimadzu equipped with a 5500 Qtrap system (Sciex) in triple-quad mode. The LC system consisted of an anion exchange LC column (Dionex Ionpac AS 11 (2 × 250 mm) and a AG-11 guard column (2 × 50 mm) from Thermo Fischer Scientific). Twenty-five µL of standard solution or filtrate were injected into the LC system and glyphosate was eluted from the column using a gradient of water (A) and 1 mM citric acid solution brought to a pH of 11 by addition of a dimethyl amine solution (B). Gradient elution consisted of following steps: 100% A from 0 to 2 minutes, linear to 25% B in 5.5 minutes, and linear to 50% B in 2.5 minutes which was held for 4 minutes. After returning to 100% A in 0.5 minutes the system was re-equilibrated for 7.5 minutes before the next injection. The total run time (including injection) was 22.5 minutes with a flow rate of 0.4 mL/min. A column temperature of 40°C was maintained while the temperature of the samples in the autosampler was 12°C. All transitions were measured using a declustering potential of -75 V, an entrance potential of -10 V and a dwell time of 50 ms. The Turbospray source was used in negative electrospray mode using the following parameters: Curtain gas 20 arbitrary units, collision gas medium, ion spray - 4000 V, temperature 400 °C, ion spray gas 1: 40 and ion spray gas 2: 50 arbitrary units. The mass transitions for evaluation [m/z] were 168.2 → 62.8 for quantification, 168.2 → 79.0 for confirmation and, 171.2 → 62.8 for $^{13}C_2{}^{15}N$ glyphosate.

Sample Preparation for GC-MS/MS analysis - A 2 mL milk sample was extracted with 3.75 mL of 0.6% acetic acid and centrifuged for 5 minutes at 4000 rpm. Two mL of the supernatant liquid was extracted with 2 mL dichloromethane for 2 minutes and the two phases separated by centrifugation at 4000 rpm for 5 minutes. One mL of the supernatant liquid was filtered using a 0.45 µm Nylon filter and a cation exchange clean-up was performed using disposable Bio-Rad Poly-Prep columns filled with 1.72 g (equivalent to 2 mL filling volume) of AG 50W-X8 resin (H^+-form). A 0.55 mL aliquot of the filtered extract (corresponding to 0.2 mL breast milk) and 0.100 mL of internal standard solution (20 ng/mL) were added onto the cation exchange column and were eluted followed by 2.0 mL of CAX solution (800 mL HPLC grade water, 13.5 mL 10 N HCl solution and 200 mL methanol). Both eluates were discarded. Glyphosate residues were eluted from the column with 12.5 mL of CAX solution and evaporated to dryness. The residues were dissolved in 1.0 mL of CAX solution. Derivatization reagent (2,2,3,3,4,4,4-hepta fluoro-1-butanol and trifluoroacetic acid anhydride 1:1) was cooled to a temperature of - 20°C. 0.05 mL of the dissolved eluate (corresponding to 0.01 mL breast milk) was added to 1.5 mL of the chilled reagent and after 5 minutes derivatization is started by heating to 92-97°C for 1 hour. After cooling, the excess of derivatization reagent was removed by evaporation. The dry residue was dissolved in 0.2 mL of ethyl acetate containing 0.2 mL/L citral and then concentrated to a final volume of 20 µL. Citral was used to prevent adsorption of the analytes in the inlet and the GC column. Following this procedure, one mL of final extract contained the glyphosate residue of 0.5 mL breast milk.

GC-MS/MS analysis - The GC-MS/MS system consisted of a Thermo Trace GC Ultra equipped with a TriPlus liquid autosampler, split/splitless injector and MS detector TSQ Quantum with triple quadrupole (Thermo Fisher Scientific). The GC column was a Optima 5HT of 30 m length and 0.25 mm internal diameter coated with a 0.25 µm film (Macherey-Nagel). Four µL of the extracts were injected splitless with the injector temperature at 280 °C. The oven temperature was held at 80°C for 1.5 minutes, ramped up at 10 °C/minute to 180 °C, then ramped up at 30°C/minute to 300 °C and held at 300 °C for 2.8 minutes. The carrier gas was helium and the flow rate 1 mL/minute. The expected retention time for the glyphosate derivative was 9.1 minutes. The temperature of the ion source was 280 °C and the electron impact (EI) energy was 70 eV with an emission current of 50 µA. Mass transition for evaluation [m/z] was 612 → 213 for quantification, 611 → 261 for confirmation, and 615 → 213 for $^{13}C_2\ ^{15}N$ glyphosate. Calculations were performed using the ratio of the peak areas of the quantifier transition of glyphosate derivative and the internal standard derivative. Calibration solutions were prepared by volumetric dilution of a glyphosate stock solution in a solution containing 20 ng/mL internal standard. The dilutions were made in CAX solution. Aliquots of 0.05 mL of these calibration solutions were derivatized as described for the breast milk extracts. The concentration of the derivatized calibration solutions ranged from 0.01 to 10 ng/mL. The concentration of the internal standard in the final extract was always 5 ng/mL.

Results and Discussion

Since a very low transfer was observed of glyphosate into muscle, milk and fat in farm animal metabolism studies the LOQ should be as low as possible. The LC-MS/MS method was validated for glyphosate in accordance with the requirements of the EU guidance document for quality control and validation procedure. Recovery and precision of glyphosate were determined for 6 or 7 replicates at two fortification levels. The linearity of the system was tested by injecting 8 standards in water in a concentration range from 0 to 50 ng/mL. A linear relationship between concentration and the ratio of the peak area of glyphosate and its internal standard was observed with a coefficient of determination greater than 0.99. All calibration points were within 20% of the theoretical value. Quantification was performed using single-point calibration which is acceptable if the response of the analyte in the samples is close to the response in the standard. The average recovery of the LC-MS/MS method was 99% at 1 ng/mL and 91% at 5 ng/mL with a relative standard deviation of 16% and 7%, respectively. The LOQ of the LC-MS/MS method of 1 ng/mL demonstrated sufficient recovery and precision. Possible matrix effects were corrected using the stable isotope labeled internal standard $^{13}C_2{}^{15}N$ glyphosate. At a concentration of 0.5 ng/mL, a signal-to-noise ratio of approximately 4 was obtained. This concentration is considered the LOD of the LC-MS/MS method.

For the analysis of glyphosate by GC-MS/MS, extraction with acidified water was combined with clean-up on a cation exchange column to remove interfering substances present in breast milk. To enable analysis by gas chromatography glyphosate was derivatized using heptafluoro-1-butanol and trifluoroacetic acid anhydride. Calibration was performed with freshly prepared derivatives of 8 glyphosate standard solutions in the concentration range from 0.01 to 10 ng/mL. All standard solutions contained the internal standard at 5 ng/mL and the coefficient of determination was always equal to or greater than 0.9980. The average recovery of the GC-MS/MS method was 84% at 1 ng/mL and 83% at 10 ng/mL with a relative standard deviation of 13% and 8%, respectively. All results obtained by GC-MS/MS had to be corrected for (derivatization reagent) blank interferences. A set of at least 4 reagent blanks were analyzed within each set of breast milk samples. The average measured blank values ranged from 0.2 to 0.6 ng/mL. The relative standard deviations of blank values in the sample sets ranged from 19% to 33%. Considering the blank values from the derivatization reagent, the LOQ of the GC-MS/MS method was 1 ng/mL. Notwithstanding the interference problem of the GC-MS/MS method, both analytical methods were able to measure glyphosate residues in breast milk with an LOQ of 1 ng/mL.

In total, 114 different breast milk samples were analyzed for glyphosate. Seventy-five samples were analyzed by LC-MS/MS only. Because of the lower performance, only 19 samples were analyzed exclusively by GC-MS/MS. Twenty milk samples were analyzed by both methods. In addition to the 114 samples, 5 samples for performance testing were analyzed by both LC-MS/MS and GC-MS/MS, 4 breast milk samples which were spiked in advance with glyphosate and one control sample. Glyphosate was identified by LC-MS/MS in all samples spiked with glyphosate. The recoveries for the LC-MS/MS method were 110%, 97% and 102% for the spiking levels of 1, 3, and 5 ng/mL, respectively. In the sample spiked at 0.5 ng/mL, glyphosate could still be detected by the LC-MS/MS method. Due to the interference problem in GC-MS/MS method, no clear detection of glyphosate was possible at this level. The recoveries for the GC-MS/MS method were 70%, 70%, and 54% for the spiking levels of 1, 3, and 5 ng/mL, respectively. Generally, the GC-MS/MS method tended to result in lower concentrations, probably due to the correction for derivatization reagent blank values. The higher bias of the GC-MS/MS method might be due to dilution steps using very small volumes. The concentration step to yield the final volume might have resulted in a partial loss of the glyphosate derivative. Nevertheless, both methods are able to quantify glyphosate residues in breast milk at or above a concentration of 1 ng/mL. Because of the lack of significant blank values in the LC-MS/MS method, residues of glyphosate higher than 0.5 ng/mL are still detectable by this method. In none of the 114 breast milk samples obtained from German women glyphosate was detected.

Conclusion

A LC-MS/MS and a GC-MS/MS method were developed for the detection and quantification of glyphosate in human breast milk. Both methods have been fully validated and are suitable for the determination of glyphosate with an LOQ of 1 ng/mL. The LC-MS/MS method allows the detection of glyphosate at or above a level of 0.5 ng/mL. The LC-MS/MS method is much faster than the GC-MS/MS method, thus making it suitable for higher sample throughput. The positive findings of glyphosate in breast milk of American women could not be confirmed by the results of this study. In none of the 114 breast milk samples collected from German women in August and September 2015 glyphosate was found within the detection limitations of the analytical methods. Available data from farm animal studies on glyphosate with non-labeled material support these results. They provide no indication of a significant carry-over into fatty tissues or milk even at high dosing levels.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Two analytical methods were developed for the determination of glyphosate in human breast milk. In the first method fat was removed by centrifugation and the proteins by ultra-filtration using a molecular weight cutoff filter of 30 kDa. The final extract was then analyzed by LC-MS/MS. In the second method the milk sample was acidified with acetic acid, centrifuged and the supernatant extracted with dichloromethane. The aqueous phase was filtered and cleaned-up using a cation exchange resin. The final extract was then analyzed by GC-MS/MS after derivatization with

heptafluoro-1-butanol and trifluoroacetic acid anhydride. $^{13}C_2{}^{15}N$ glyphosate was used as the internal standard in both methods. Both analytical methods were validated according to the EU guidance document on analytical quality control and validation procedures for pesticide residues in food and feed (SANCO/12571/2013) and were found suitable for the determination of glyphosate in human breast milk with an LOQ of 1 ng/mL. In August and September 2015, 114 breast milk samples were collected from German women and were analyzed for glyphosate. In none of the samples analyzed glyphosate concentrations were found at or beyond the LOQ.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the breast milk samples analyzed were collected on a voluntary basis and there were no restrictions for participating in the monitoring program. As a consequence the samples cannot be considered representative of the German population. Both analytical methods developed were validated in accordance with the EU guidance on the procedures for the analysis of pesticide residues in food and feed.

Reliability criteria of exposure studies

Publication: Steinborn et al., 2016.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	Y	Guidance document on analytical quality control and validation procedures for pesticide residues in food and feed, SANCO/12571/2013.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.		Self-reported exposure to pesticides.
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides		
Study		
Study design clearly described	Y?	Mainly the development of methods for the analysis of glyphosate in human breast milk and applied to a set of 114 samples from German women.
Population investigated sufficiently described	Y?	Biometric data of the study population were rather limited. The samples were collected on a voluntary basis and without restrictions.
Exposure circumstances sufficiently described	Y?	No detail was provided on self-reported exposure to pesticides.
Sampling scheme sufficiently documented	Y?	More detail could have been provided.
Analytical method described in detail	Y	

Publication: Steinborn et al., 2016.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Validation of analytical method reported	Y	
Monitoring results reported	Y	None of the 114 samples analyzed was positive for glyphosate.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the breast milk samples analyzed were collected on a voluntary basis and there were no restrictions for participating in the monitoring program. As a consequence the samples cannot be considered representative of the German population. Both analytical methods developed were validated in accordance with the EU guidance on the procedures for the analysis of pesticide residues in food and feed.		

1. Information on the study

Data point:	CA 5.4
Report author	Suárez-Larios K. *et al.*
Report year	2017
Report title	Screening of Pesticides with the Potential of Inducing DSB and Successive Recombinational Repair
Document No	Journal of Toxicology (2017), Article ID 3574840,
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

A study was realized to ascertain whether glyphosate and other selected pesticides would induce double strand breaks (DSB) in lymphocyte cultures and whether this damage would induce greater levels of proteins Rad51 participating in homologous recombination or of p-Ku80 participating in nonhomologous end joining. Only five pesticides were found to induce DSB of which only glyphosate and another one pesticide induced a significant increase of p-Ku80 protein, indicating that nonhomologous end joining recombinational DNA repair system would be activated. The type of gamma-H2AX foci observed was comparable to that induced by etoposide at similar concentrations. These results are of importance since these effects occurred at low concentrations in the micromolar range, in acute treatments to the cells. Effects over longer exposures in actual environmental settings are expected to produce cumulative damage if repeated events of recombination take place over time.

Materials and Methods

Chemicals - Endosulfan, glyphosate, pentachlorophenol, permethrin, propoxur, paraoxon, AMPA (glyphosate metabolite), endosulfan lactone (endosulfan metabolite), and etoposide (positive control) were obtained from Sigma-Aldrich, Mexico. Purity was not reported.

Evaluation of DNA double strand breaks (DSB)- Three mL of blood were obtained from fully informed healthy young male donors of 21 to 35 years old. The volunteers were nonsmokers, did not consume alcohol and had not taken medication or were not subjected to radiation for medical purposes. The concentrations of glyphosate tested were 0, 0.4, 2, 10, and 50 μM, and those of AMPA were 0, 40, 200, 1,000 and 5,000 μM. Whole blood was diluted in RPMI-1640 medium and treated with the test compound 1.5 hours at 37 ºC, after which 0.075M KCl was added and the incubation continued for 30 minutes. Lymphocytes were recovered by centrifugation and formaldehyde added to reach a final concentration of 4%. Then PBS and Triton X-100 were added followed by an incubation of 30 minutes. Thereafter the cells were washed, supplemented with 4% fetal bovine serum and isolated by centrifugation and 1mL of cold 50% methanol added. After one night at −20 ºC the samples were centrifuged at 0 ºC, and cold methanol added to the cells which were kept at −20ºC until analysis. Treatments were done in duplicate. DNA double strand breaks were detected by means of immunofluorescence of phosphorylated histone H2AX foci. Staining of lymphocyte nuclei was done as follows: the slides were washed and blocked with KCMT buffer for 1 hour at room temperature. Primary antiphospho-histone H2AX (Ser139) in blocking solution was added and left to incubate overnight followed by washes with KCMT buffer. Then the cells were incubated with the secondary antibody Alexa-Fluor 555 goat anti-mouse for 1 hour at room temperature. The slides rinsed in deionized water before mounting in DAPI mounting medium and subsequently analyzed for γ-H2AX foci under a fluorescence microscope. Evaluation of foci was done in 2 slides per concentration and 50 cells were

evaluated in 3 different regions per slide. In total, 300 cells were evaluated per treatment. When a nucleus presented one or more foci, it was considered positive. The extent of DNA damage was classified in 3 categories: percentage of cells without γ-H2AX foci (no DNA damage), with less than 10 γ-H2AX foci (moderate DNA damage) and with more than 10 gamma-H2AX foci (severe DNA damage). Additionally, the damage was expressed as mean percentage of γ-H2AX positive nuclei.

Cytotoxicity - Cytotoxicity was tested using The CellTiter 96 AQueous One Solution Reagent from PROMEGA and following the manufacturer's instructions. Mononuclear cells were isolated from blood using Histopaque-1077. The cells were plated in well plates at 100,000 cells per well, and treated with the test compounds in triplicate for each concentration. Absorbance at 490 nm was recorded using a 96-well plate reader. The percentage of survival was calculated as (Absorbance at 490 nm of treatment/Absorbance at 490 nm of the negative control) × 100.

Western blot analysis of proteins participating in DNA recombination - Mononuclear cells were isolated with Histopaque-1077 and treatments were applied to cells resuspended in RPMI-1640 at 500,000 cells per tube. Glyphosate was tested in duplicate at 1.25, 2.5, and 5 μM. The cells were then centrifuged, the supernatant discarded and 0.5 M sodium azide added. After centrifugation, the cell pellets were kept at −70 °C until used for Western blot analysis. Two separate experiments were performed per test compound, with two donors each time. RIPA lysis buffer solution containing phosphatase and protease inhibitors was added to each cell pellet. The samples were then sonicated, incubated in ice, and centrifuged. The supernatant was recovered and 5 μL of each sample was placed in a 96-well plate for protein quantification. The Lowry assay was performed with the DC Protein Assay kit and the concentration determined in a plate reader at 750 nm. The samples were then stored at −70∘C until use. For the determination of phosphorylated Ku80 (phospho-T714) and Rad51 by Western blotting 35 μg of total protein was separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane with a Trans-Blot SD Semi-Dry Transfer Cell. After incubation with blocking solution and rinsing the appropriate membrane zones were isolated and incubated with rabbit anti-Rad51 polyclonal antibody or rabbit anti- phosphorylated Ku80 (phosphoT714) polyclonal antibody (p-Ku80). Incubation with primary goat anti-Actin polyclonal antibody was done for the determination of β-actin as the internal control. The membranes were then rinsed and incubated with secondary antibody goat anti-rabbit IgG-HRP for Rad51 and p-Ku80 detection and donkey anti-goat IgG-HRP for actin detection. After rinsing the protein fractions were quantified with a luminescence kit. Optical densities were measured with Quantity One software, version 4.1.1. The values obtained for each protein (Rad51 or p-Ku80) were normalized with respect to β-actin and the mean of the normalized negative controls. Results are presented as the % with respect to normalized negative controls. Two membranes were prepared per separate experiment with each test compound .

Statistical Analysis - Statistical calculations were made with the GraphPad Prism 6 software package and the results for γ-H2AX foci and optical density from Western blot analysis were evaluated with the Kruskal-Wallis test and Dunn's multiple comparison as a post hoc test. The value of etoposide as a positive control at the concentration of 50 μM was analyzed with the Mann-Whitney *U* test with respect to the negative control. Cytotoxicity was analyzed with linear regressions. The results were considered statistically significant if $p<0.05$.

Results

Identification of DNA double strand breaks(DSB) - For glyphosate, the mean of the % cells with more than 10 γ-H2AX foci was 0.33, 1.67, 9.33, 8.83, and 3.17 for 0, 0.4, 2, 10, and 50 μM (linear regression, $R^2 = 0.2$, $p = 0.02$). No effect was seen with AMPA. The positive control, etoposide, showed a significant correlation with dose (, $R^2 = 0.82$, $p < 0.0001$) for cells with more than 10 foci.

Cytotoxicity - The concentrations used for the determination of DSB were also used to assess the cytotoxicity of the test compounds that produced DSB. The survival range for glyphosate was 100% to 70% viability for concentrations ranging from 1.25 to 5 μM.

Quantification of p-Ku80 and Rad51 Proteins
The test compounds that showed positive for DSB were further tested to determine whether DNA recombination would be induced. Glyphosate was found to induce statistically significantly p-Ku80 protein in a dose-dependent manner, whereas Rad51 was not significantly affected. Etoposide consistently induced p-Ku80, although with a wide variation between tests at 10 µM.

Discussion and Conclusions

Pesticides amongst which glyphosate were evaluated for their capacity to induce DNA double strand breaks, a lesion related to the formation of chromosomal rearrangements and leukemia risk. Glyphosate exhibited an ability to induce this kind of DNA damage in the form of phosphorylated γ-H2AX foci in the nuclei of human lymphocytes in at least two of the concentrations tested. AMPA tested negative. It is noticeable that the positive control, etoposide, induced more than 85% of cells with more than 10 foci at 50 μM although it induced foci in a manner comparable to glyphosate at lower concentrations. This extent of DNA damage seems to be of relevance to the increase of repair proteins like p-Ku80, since glyphosate also produced a significant increase in this protein, whereas the rest of the test compounds that induced a lower percentage of cells with γ-H2AX foci did not. To evaluate proteins participating in DNA recombination, the highest concentration of glyphosate used was 5 µM to avoid cytotoxicity. Exposure of cells in a non-proliferative state to glyphosate not only induced the breakage of DNA, but also the phosphorylation of Ku80, a protein that participates in the c-NHEJ repair pathway. This pathway is known for being prone to error, introducing micro deletions or micro insertions, which could be mutagenic and alter cell behavior if they occur in coding or regulatory sequences. This is one possible outcome of the DSB in non-proliferating cells. Etoposide, the positive control, is well known as a topo II inhibitor capable of producing complex DSB. The DNA damage induced by etoposide in this study was comparable to the damage induced by paraoxon and glyphosate (DSB and pKu80 induction), so the question emerging from these results is whether the outcome for cells damaged by glyphosate would be similar to the outcome of cells damaged by etoposide and whether they would also induce chromosomal rearrangements.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to assess whether glyphosate and its metabolite AMPA produced DNA double strand breaks in human peripheral lymphocytes and whether proteins involved in DNA repair were induced. The results show that glyphosate, but not AMPA, increased the mean of the percent cells with more than 10 γ-H2AX foci, however, without a clear dose-effect relationship. Glyphosate was found to induce statistically significantly a protein involved in DNA repair, p-Ku80, at 5 µM without a dose-effect relationship (when measured as median OD).

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because glyphosate as a test chemical was not sufficiently characterized and the effect found on an indicator of DNA double strand breaks was not concentration related and occurred at concentrations that were much lower than the systemic concentrations (approx. 300 µM) of regulatory *in vivo* MN tests at 2000 mg/kg bw which were negative.

Reliability criteria for *in vitro* toxicology studies

Publication: Suarez-Larios *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	No guideline study for genotoxicity
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Only source reported: Sigma-Aldrich Mexico.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	Y	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	Not applied
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Up to 50 µM
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	SS increase at 2 concentrations but no dose effect relationship for γ-H2AX foci. SS increase of P-Ku80 at 5 µM but no dose-effect relationship in treated cultures (measured as median OD)
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	Results of this study are not corroborated by in vivo MN studies with much higher blood concentrations (approx. 300µM).
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because glyphosate as a test chemical was not sufficiently characterized and the effect found on an indicator of DNA double strand breaks was not concentration related and occurred at concentrations that were much lower than the systemic concentrations (approx. 300 µM) of regulatory *in vivo* MN tests at 2000 mg/kg bw which were negative.		

1. Information on the study

Data point:	CA 5.3
Report author	Tang J. *et al.*
Report year	2017
Report title	Ion imbalance Is Involved in the Mechanisms of Liver Oxidative Damage in Rats Exposed to Glyphosate
Document No	Frontiers in Physiology (2017) Vol. 8, Article 1083
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study aimed to investigate the effects of glyphosate on rats' liver function and induction of pathological changes in ion levels and oxidative stress in hepatic tissue. Sprague-Dawley rats were treated orally with 0, 5, 50, and 500 mg/kg body weight of the GLP. After 5 weeks of treatment, blood and liver samples were analyzed for biochemical and histomorphological parameters. The various mineral elements content in the organs of the rats were also measured. Significant decreases were shown in the weights of body, liver, kidney and spleen between the control and treatment groups. Changes also happened in the histomorphology of the liver and kidney tissue of GLP-treated rats. The GLP resulted in an elevated level of glutamic-oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and IL-1b in the serum. Besides, decreased total superoxide dismutase (T-SOD) activity and increased malondialdehyde (MDA) contents in the serum, liver, and kidney indicated the presence of oxidative stress. Moreover, increase of hydrogen peroxide (H_2O_2) level and catalase (CAT) activity in the serum and liver and decrease of glutathione (GSH) and lutathione peroxidase (GSH-Px) activity in the kidney tissue further confirmed the occurrence of oxidative stress. The results of RT-PCR showed that the mRNA expressions of IL-1α, IL-1β, IL-6, MAPK3, NF-κB, SIRT1, TNF-α, Keap1, GPX2, and Caspase-3 were significantly increased in the GLP-treated groups compared to the control group. Furthermore, PPARα, DGAT, SREBP1c, and SCD1 mRNA expressions were also remarkably increased in the GLP-treated groups compared to the control group

Materials and methods

Chemicals - Glyphosate was obtained from Shanghai Ryon Biological Technology Co. Ltd (Shanghai, China). Purity was not reported.

Animals - Male Sprague-Dawley rats of 8 weeks old and weighing 180–220 g were purchased and were allowed to acclimate for at least one week prior to testing. All rats were housed in separate cages and had unrestricted access to food and water throughout the study.

Animal treatment and sample collection - Rats were randomly assigned to 4 groups (n = 8/group) and were orally administered glyphosate by gavage at 5, 50, and 500 mg/kg bw/day for 35 days. Distilled water was used as the negative control. Twenty-four hours after the last gavage, rats were weighed and sacrificed. Blood samples were collected from the jugular vein and placed at 37^{o}C for one hour before being centrifuged for biochemical assays. Liver, kidney, spleen, heart, lungs, brain, adrenal glands, muscle and fat tissue were collected, rinsed with PBS, dried and weighed. A piece of liver and right kidney was used for morphometric analysis and another piece was used to prepare homogenates for the analysis of parameters of oxidative stress or frozen in liquid nitrogen for subsequent qualitative reverse transcription polymerase chain reaction (RT-PCR).

Histological preparation - Samples of liver and kidney were fixed in 4% formaldehyde solution for 24 hours, dehydrated in alcohol, clarified with xylene, and embedded in paraffin. Paraffin blocks were sectioned into 5μm slices and stained with hematoxylin-eosin (HE) for microscopic examination.

Biochemical evaluation - Liver and kidney homogenate and serum were used for the assessment of liver function (serum GOT and GPT) and oxidative stress (total SOD, MDA, H_2O_2, CAT, GSH, and GSH-peroxidase or GSH-px). The activity of serum GOT and GPT was assayed according to the method that is normally applied in clinical biology. The analysis of total SOD activity was based on SOD-mediated inhibition of nitrite formation from hydroxyammonium in the presence of O^{2-}generators (xanthine/xanthine oxidase). Total SOD activity was expressed in U/mg protein. MDA was evaluated by the thiobarbituric acid reactive substances method (TBARS) and the results were expressed in nmol/mg protein. GSH-px activity was estimated by the determination of reduced GSH in the enzymatic reaction. GSH-px activity was expressed in U/mg protein. CAT activity was assayed by the method developed by Aebi, and calculated as nM H_2O_2 consumed/min/mg of protein. Protein concentrations in the supernatant were measured according to the Coomassie Brilliant Blue method.

Serum Cytokine Measures - Serum levels of IL-1β and IL-6 were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit.

Quantitative RT-PCR (qRT-PCR) analysis - Total RNA was extracted from tissue using the reagent box of Total RNA Kit, according to the manufacturer's instructions. The concentration of RNA was measured by spectrophotometry and the purity was ascertained by the A 260/A 280 ratio. Total RNA from each sample was reverse transcribed to cDNA with an Omniscript® Reverse Transcription kit with Oligo-dT primers according to the manufacturer's instructions and used for RT-PCR. The target fragments were quantified by real-time PCR with 100 ng of the cDNA template. Each sample was tested in duplicate. The gene expression data were normalized to β-actin expression. For each real-time PCR assay, the threshold cycle Ct was determined for each reaction. Ct values for each gene of interest were normalized to the housekeeping gene, β-actin and PCR amplification efficiencies were taken into account by amplifying various amounts of target cDNA for each reaction. The fold differences in mRNA expression of samples were relative to the internal control sample, which was included in all runs.

Ion Concentration - The concentrations of Al, Fe, Cu, Zn, and Mg in liver, kidney, spleen, lung, heart, muscle, brain, and fat tissue were determined by inductively coupled plasma optical emission spectrometry using nitric acid–perchloric acid–based wet digestion. Approximately 200 μL or 0.5 g of each sample was digested with nitric acid (75%) and perchloric acid (25%) in a microwave digester. The same part of the organ was used from the control and treated animals.

Statistical Analysis - The data were expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test, which was performed with GraphPad Prismsoftware. Differences were considered to be statistically significant when $p< 0.05$.

Results

Body weight and body weight gain were statistically significantly decreased at 500 mg/kg bw/day and at 50 and 500 mg/kg bw/day, respectively. Absolute and relative organ weight was statistically significantly reduced for liver, spleen and kidney at 500 mg/kg bw/day.
Liver sections of rats exposed to glyphosate showed apoptosis of some hepatocytes, focal necrosis and mononuclear cell infiltration. At 5 mg/kg bw/day rats showed mild periportal expansion and apoptosis of some hepatocytes. At 50 and 500 mg/kg bw/day apoptosis of some hepatocytes and monocyte infiltration was observed in liver tissue. In the kidney, marked histological changes were observed, including proximal and distal tubular necrosis and glomerular toxicity. The histologic score of hepatic and renal damage was statistically significantly increased at all dose levels. Serum activity of GOT and GPT was statistically significantly increased at 500 mg/kg bw/day.

Assessment of oxidative stress - In serum, total SOD activity was statistically significantly decreased at

500 mg/kg bw/day. MDA content was significantly increased at 50 mg/kg bw/day but not at 500 mg/kg bw/day and CAT activity was significantly increased at 500 mg/kg bw/day. In liver, total SOD activity was statistically significantly decreased and H_2O_2 levels increased at 500 mg/kg bw/day. In kidney, total SOD and GSH-px activities were significantly decreased at 50 and 500 mg/kg bw/day. A statistically significant decrease in GSH levels was observed at 50 mg/kg bw/day but not at 500 mg/kg bw/day.

Serum IL-1β and IL-6 levels - In serum, the level of IL-1β was statistically significantly increased at 500 mg/kg bw/day. There was no statistically significant change for IL-6.

Expression of genes related to inflammation in the Liver - Hepatic IL-1α and IL-1β mRNA expression were statistically significantly increased at all dose levels. IL-6, MAPK3, SIRT1, TNF-α, GPX2, and *Caspase*-3 mRNA expression was significantly increased at 50 and 500 mg/kg bw/day. NF-κB mRNA expression showed only a significant increase 50 mg/kg bw/day and *Keap*1 mRNA expression was only increased at 5 mg/kg bw/day.

Expression of genes related to lipid metabolism in the liver - PPARα, *SREBP1c*, and *SCD1* mRNA expression were significantly increased at 50 and 500 mg/kg bw/day and *DGAT* mRNA expression was significantly increased at 500 mg/kg bw/day.

Concentration of Al, Fe, Cu, Zn and Mg in tissues – The Al concentration was statistically significantly increased in liver at 50 and 500 mg/kg bw/day but significantly decreased in lung at 50 mg/kg bw/day and in muscle at 500 mg/kg bw/day. Fe was significantly increased at 5 mg/kg bw/day only in liver, at 50 mg/kg bw/day only in kidney and spleen and at 500 mg/kg bw/day in lung. Cu was significantly increased in brain and fat tissue at 500 mg/kg bw/day and Zn was only significantly increased in liver at 50 and 500 mg/kg bw/day. Mg levels were only increased in brain tissue at 500 mg/kg bw/day.

Discussion and Conclusions

The results of this study showed that exposure to glyphosate for 35 days at doses up to 500 mg/kg bw/day led to a statistically significant reduction in body weight and body weight gain and in absolute and relative weight of liver, kidney and spleen. Histopathological examination of the tissues also revealed effects in liver and kidneys. Liver effects were corroborated by a significant increase in GOT and GPT. The results of this study showed that SOD activity was significantly decreased in serum, liver and kidney of rats treated with glyphosate when compared with the control group. MDA content was significantly increased in serum and kidney. CAT activity was also significantly elevated in serum and H_2O_2 levels were increased in liver tissue, suggesting oxidative stress. Taken together, the data demonstrated that glyphosate exposure could result in liver and kidney damage due to oxidative stress. The level of IL-1β was significantly increased at 500 mg/kg bw/day. Therefore, the relationship was investigated between oxidative stress and the transcription of genes related to inflammation. In this study, mRNA expression of *IL-1α, IL-1β, IL-6, MAPK3, NF-κB, SIRT1, TNF-α, Keap1, GPX2* and *Caspase-3* were all increased upon exposure to glyphosate. Genes related to lipid metabolism such as *PPARα, SREBP1c, DGAT*, and *SCD1* were significantly upregulated in rats exposed to glyphosate. The results from this study show that the liver toxicity induced by glyphosate is mediated by inflammation, oxidative stress and lipid related pathways. Tissue concentrations of Al, Fe and Zn were significantly increased in liver tissue of rats exposed to glyphosate. Concentrations of Fe were also increased in kidney, spleen, and lung tissue although not always in a dose-related manner. Al concentration was decreased in lung and muscle tissue whereas Cu concentrations were increased in brain and fat tissue and Mg in brain tissue of rats exposed to glyphosate. Combined, these results suggest that glyphosate exposure impaired the ion-balance of Al, Fe, Mg, Cu, and Zn.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to investigate the toxicity, oxidative stress and metal ion concentrations in tissues of rats after oral exposure to glyphosate for 35 days at doses up to 500 mg/kg bw/day. Oxidative stress was studied by the determination of markers of oxidative stress such as SOD, CAT, H_2O_2, MDA, GSH and GSH-px and the transcription of genes related to inflammation and lipid metabolism. Statistically significant effects were found on body weight, body weight gain, organ weight, serum indicators of liver toxicity and histopathology of the liver and the kidney. Significant changes were also reported on markers of oxidative stress and transcription of genes related to inflammation and lipid metabolism. Many of the effects reported were mild in nature and/or didn't show a clear dose-effect relationship. Also the effects on metal ion concentrations in organ tissues were not always consistent and often didn't show a dose-effect relationship.

This publication is considered relevant but reliable with restrictions because the test material was not sufficiently characterized, the number of animals used for this study duration is not sufficient, the results were not always accurately reported and are not corroborated by regulatory toxicology studies of similar test durations and dose ranges.

Reliability criteria for *in vivo* toxicology studies

Publication: Tang *et al*., 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Number of animals per dose level lower than minimum required for 4-week testing
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	?	Not described in sufficient detail
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Only source reported: Shanghai Ryon Biological Technology Co. Ltd., China.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	
Dose levels reported	Y	
Number of animals used per dose level reported	Y	
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	Sometimes inaccurate reporting of data in tables. Results not concordant with short term regulatory toxicology studies
Statistical methods described	Y	

Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant but reliable with restrictions because the test material was not sufficiently characterized, the number of animals used for this study duration is not sufficient, the results were not always accurately reported and are not corroborated by regulatory toxicology studies of similar test durations and dose ranges.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Thongprakaisang S. *et al.*
Report year	2013
Report title	Glyphosate induces human breast cancer cells growth via estrogen receptors
Document No	Food and Chemical Toxicology (2013) Vol. 59, 129–136
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10-12 to 10-6 M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity.

Materials and Methods

Chemicals - Glyphosate (purity >98%) was obtained from AccuStandard (New Haven, CT, USA).

Cell lines and culture conditions - A hormone-dependent human breast cancer cell line (T47D), a stably ERE-luc construct transfected hormone-dependent breast cancer cell line (T47D-KBluc) and a hormone-independent human breast cancer cell line (MDA-MB231) were obtained from the American Type Culture Collection (ATCC).

In vitro estrogen receptor activation-reporter assay - To study the estrogenicity and/or anti-estrogenicity of glyphosate, the T47D-KBluc cell line, stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct was used in this study. To minimize the effect of estrogen in the medium, 5 days prior to the start of the assay, cells were incubated in a non-phenol red RPMI modified medium with replacement of 10% FBS by 10% dextran-charcoal treated FBS (CSS). One day prior to the assay, cells were seeded at 10^4 cells/100 µL/well and were allowed to attach overnight. The dosing medium was further modified by reduction to 5% CSS and then replaced with 100 µL/well of dosing medium containing glyphosate at concentrations ranging from 10^{-12} to 10^{-6} M. Estradiol (E2) in the same range of concentrations was used as the positive control. Dosing medium without glyphosate was used as the negative control and wells without cells were used as the blank. After 24 hours of incubation, cells were washed with 100 µL PBS and harvested in 25 µL lysis buffer. The luciferase assay was performed by injecting 50 µL of reaction buffer and 50 µL of 1 mM D-luciferin and fluorescent intensity was measured by means of the microplate luminometer. Luciferase activity was quantified as relative light units (RLU).

Cell viability MTT assay - Cell growth and cell viability were tested using the 3-(4,5-dimetylthiazol, 2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent assay. Cells were seeded at 10^4 cells/100 µL/well

in 96-well microtiter plates. For the E2 withdrawal condition, cells were cultured in 10% CSS and a non-phenol red RPMI medium for 4 days before seeding. After 24 hours the cells were treated with E2 or glyphosate at concentrations ranging from 10^{-12} to 10^{-6} M. For E2 receptor antagonist conditions, E2- or glyphosate-treated cells were co-incubated with ICI 182780 at 1 and 10 nM. Cell sensitivity to a chemical was expressed as the % cell viability compared to control cells.

Cell number counting - T47D Cells were prepared in E2 withdrawal conditions 4 days before the start of the assay. Cells were placed in a 24 well culture plate with 10^4 cells/mL/well and incubated overnight. The medium was then replaced with 1 mL of treatment solution and incubated for 72 hours. Afterwards, the cells were washed with 1 mL PBS and then 100 µL of a trypsin–EDTA solution was added to detach. An aliquot of the cells was taken for counting using a counter analyzer.

Western blot analysis - Whole-cell extracts were prepared from cells treated for 6 and 24 hours with 10^{-12}, 10^{-9}, and 10^{-7} M glyphosate and non-treated control. The cells were lysed, incubated on ice and centrifuged. The supernatants were collected and either processed or stored at -80 ^{o}C until use. The protein concentration was measured using Bradford reagent and each lysate was aliquot for an equal amount of protein, 30 µg, before mixing with Laemmli loading buffer and then boiled at 95 °C for 5 minutes. The samples were resolved over 7.5% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell. The membrane was treated with blocking solution for one hour at room temperature and subsequently probed overnight with primary antibody (ERα, ERβ or β-actin) and then rinsed. HRP-conjugated secondary antibodies were added to the membrane for 2 hours and the membranes rinsed with TBS-T. Protein visualization was achieved by using enhanced chemiluminescence and the emitted light was captured on film. The signals on the films were quantified using densitometry.

Statistical analysis - Data are presented as the means ± SE. Statistical significance was determined using the Student's t-test. A two-tailed $p<0.05$ was evaluated as a statistically significant difference.

Results

T47D, hormone-dependent breast cancer cell growth - The hormone-dependent T47D and hormone-independent MDA-MB231 cell lines were studied both in completed medium and estrogen withdrawal medium to differentiate the effect of glyphosate from that of endogenous estrogen. Estrogen in a concentration range of 10^{-12} to 10^{-6} M was used as the positive control. Cell growth was assessed using the MTT cell viability assay. The results showed that T47D and MDA-MB231 cells exhibited different patterns of responses to glyphosate. In the absence of E2, glyphosate produced cell proliferation in T47D cells of approximately 15–30%. This effect was about half of the E2 response which is the most potent agonist in hormone dependent ER-positive breast cancer cells. No effect was observed on cell proliferation in MDA-MB231 cells both in the absence or presence of E2.

Cell proliferation via estrogen receptors - Due to the fact that the proliferative effect of glyphosate occurred only in T47D cells in the absence of E2, it was hypothesized that ER signaling may be involved in glyphosate-induced cell proliferation. Therefore, the effect of glyphosate on T47D cells was investigated in the presence of an ER antagonist, ICI 182780, to inhibit the estrogen receptor mediated response. The effective concentration of 1 nM of ICI 182780 was added to varying concentrations of glyphosate and E2 to observe its antagonistic activity. The results showed that ICI 182780 at 1 nM mitigated the proliferative effects of both glyphosate and E2. A higher concentration of ICI 182780 (10 nM) completely inhibited the growth promoting effects of glyphosate. These results suggest that glyphosate may produce the proliferative effect via the ER.

ERE-transcription activity via estrogen receptors - T47D-KBluc cells, stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct, were treated with the concentrations of glyphosate that produced cell proliferation. The results showed that glyphosate in a concentration range of 10^{-12} to 10^{-6} M induced ERE activation 5- to 13-fold of control and these effects were less than about half of that induced by E2. Glyphosate co-incubation with the ER antagonist, ICI

182780, exhibited a significant reduction in responses. ICI 182780 at 10 nM completely inhibited the ERE transcriptional activity of glyphosate. Since glyphosate was shown to induce cell proliferation and ERE activation via the ER, the potential effects of glyphosate on endogenous E2 signaling was investigated. Cells were co-incubated with glyphosate and E2 and the results revealed that glyphosate suppressed E2-induced ERE activation suggesting that glyphosate behaves as an ER antagonist in the presence of E2.

Expression of ERα and ERβ in human breast cancer cells - The expression of proteins involved in ERs including ERα and ERβ, was studied with the Western blot technique. After 6 hours of exposure, glyphosate increased the levels of both ERα and ERβ in a concentration-dependent manner while after 24 hours only ERα showed a significant induction at the highest glyphosate concentration tested (10^{-7} M). This result suggests that glyphosate alters the expression of both ERα and ERβ in human breast cancer cells.

Discussion

In this study, glyphosate was found to increase cell proliferation of a hormone dependent breast cancer T47D cell line at concentrations ranging from 10^{-12} to 10^{-6} M while this effect was not observed in a hormone independent breast cancer MDA-MB231 cell line. The results from the ERE luciferase assay confirmed ER activation because the responses seen could be blocked by the ER antagonist ICI 182780. Glyphosate induced rapid activation of ERβ while activation of ERα was slower but prolonged. It is suggested that glyphosate may behave like a weak xenoestrogen which can activate both subtypes of ER but with a different time course. Although the nature of the binding of glyphosate to the ER is still unknown, the ability of glyphosate to stimulate the ERE-gene transcription activity and upregulation of ERα protein expression suggests that glyphosate may exert the stimulatory effects via an ER-dependent mechanism.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to investigate the possible estrogenic effect of glyphosate and its mode of action. The endpoints explored were the cell proliferation of hormone-dependent and hormone-independent cell lines with and without an ER antagonist, ERE-transcription activity with and without an ER antagonist, and expression of ERs. Glyphosate was found to produce cell proliferation in a hormone-dependent cancer cell line but not in a hormone-independent cancer cell line in the absence of E2. In the presence of a potent ER antagonist the cell proliferation caused by glyphosate in a hormone-dependent cancer cell line was reduced. The interaction of glyphosate with the ER was confirmed by ERE activation with and without an ER antagonist. When cells were co-incubated with glyphosate and E2, glyphosate suppressed the E2-induced ERE activation suggesting that glyphosate behaves as an antagonist in the presence of an endogenous agonist. It was demonstrated that glyphosate alters the expression of both ERα and ERβ in human breast cancer cells.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test results are not corroborated by *in vivo* regulatory ED toxicology studies such as the uterotrophic assay and the female pubertal assay (U.S. EPA Endocrine Disruptor Screening Program).

Reliability criteria for *in vitro* toxicology studies

Publication: Thongprakaisang *et al*., 2013	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of >98%. Source: AccuStandard, New Haven, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	The slight estrogenic effect of glyphosate reported was not confirmed in *in vivo* studies such as the uterotrophic assay and the female pubertal assay.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	Results not consistent with other publications on ED.
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test results are not corroborated by *in vivo* regulatory ED toxicology studies such as the uterotrophic assay and the female pubertal assay.		

1. Information on the study

Data point:	CA 5.4
Report author	Townsend M. *et al.*
Report year	2017
Report title	Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity
Document No	Regulatory Toxicology and Pharmacology (2017) Vol. 85 79-85
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

The resulting DNA damage and cytotoxicity of various glyphosate concentrations on human cells was studies to evaluate DNA damaging potential. Utilizing human Raji cells, DNA damage was quantified using the comet assay, while cytotoxicity was further analyzed using MTT viability assays. Several glyphosate concentrations were assessed, ranging from 15 mM to 0.1 µM. It was found that glyphosate treatment is lethal to Raji cells at concentrations above 10 mM, yet has no cytotoxic effects at concentrations at or below 100 µM. Treatment concentrations of 1 mM and 5 mM induce statistically significant DNA damage to Raji cells following 30-60 min of treatment, however, cells show a slow recovery from initial damage and cell viability is unaffected after 2 h. At these same concentrations, cells treated with additional compound did not recover and maintained high levels of DNA damage. While the cytotoxicity of glyphosate appears to be minimal for physiologically relevant concentrations, the compound has a definitive cytotoxic nature in human cells at high concentrations.

Materials and Methods

Chemicals and reagents - Glyphosate (95% w/w purity) was obtained from Sigma-Aldrich, Inc. (Milwaukee, WI).

Cell culture - Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Cells were cultured in RPMI 1640 and supplemented with 10% FBS and 2 mM L-glutamine. Media were replaced every 48 hours and the cells used for testing were placed in exponential growth with a minimum viability of 95%. Raji cells were selected for this study because the replication time is 18 hours allowing the assays to cover the entire cell cycle.

Cell viability assay - For use in the MTT viability assay, glyphosate was diluted in cell culture RPMI media to the final test concentrations and stored at 4 oC. Glyphosate stock solutions were diluted in RPMI media to their final test concentrations. Raji cells were incubated for 24 hours in a 96-well plate at 37 oC and 5% CO2. Afterwards, MTT reagent was added to each well. Following 3 hours of incubation, 100 µL of DMSO detergent was added to each well and incubated for 2 hours at 4 oC, and cytotoxicity evaluated at 570 nm absorbance.

Alkaline comet assay - Raji cells were incubated with either hydrogen peroxide as the positive control, PBS as the negative control, or glyphosate. The concentration and time points varied depending on the experimental run. The time intervals tested included 10, 20, 30, 40, 50, 60, 75, 90, 105, and 120 minutes. The concentrations of glyphosate used at each of the time points were 0.1, 1, 10, and 100 µM, and 1, 5, 10, and 15 mM. 200,000 cells per 100 µL were incubated with positive and negative controls and several concentrations of glyphosate at 37 ºC. Once treated, the cells were washed with PBS and suspended at 200,000 cells per 100 µL of PBS for the conduct of the comet assay. The test samples were mixed with

low melting point agarose and layered on double frosted microscope slides and placed in alkaline lysis buffer for 60 minutes, rinsed with purified water and then placed in alkaline electrophoresis buffer for 20 minutes and submitted to electrophoresis. After fixation and drying, the slides were stained with propidium iodide and imaged using a Zeiss Axioscope fluorescence microscope. All comets were scored using TriTek CometScore Freeware v1.5. Every experimental run tested a single concentration for multiple time points. Each time point contained a minimum of two slides as replicas. Approximately 50 comets were analyzed per slide, totaling 100 comets per time point and per treatment concentration. Each concentration was replicated multiple times to ensure consistency. Comet assay results are reported as tail moment which is defined as the product of the tail length and the percentage of DNA in the tail. A similar protocol was followed to test the effects of secondary glyphosate exposure at 1 mM and 5 mM by adding 200 µL glyphosate solution to the cells after 60 minutes of initial treatment, while 200 µL of PBS was added to the negative control.

Statistical analysis - Relationships between exposure time and tail moment were modeled statistically using a natural spline to account for nonlinearity. The number of knots was selected based on Akaike Information Criteria (AIC) and parameters were estimated using least squares. P-values of <0.05 were considered statistically significant.

Results

MTT analysis of Raji cells exposed to various glyphosate concentrations for 24 hours indicated a significant loss of cell viability at 10 and 15 mM. Glyphosate concentrations of 5 mM and lower did not have a significant effect on viability when compared to the negative control.

Comet assay analysis of Raji cells exposed to 10 and 15 mM glyphosate indicated severe DNA damage and cell death soon after exposure. Within 30 min of treatment, all cells had adopted an apoptotic profile which is characterized by a loss of a defined comet head and a large, fragmented DNA tail. Tail moments were significant after just 10 minutes of glyphosate exposure. Raji cells exposed to glyphosate concentrations of 1 and 5 mM produced statistically significant DNA damage after 40 minutes of treatment. Tail moments reached a maximum after 60 and 80 minutes of treatment at 5 mM and 1 mM, respectively. A steady decrease in tail moment was observed in later time points and the cells were able to recover to full viability after 120 minutes of treatment. Cells exposed to 10 and 100 µM of glyphosate did not show statistically significant DNA damage, and the cells retained full viability throughout 120 minutes of treatment. Raji cells exposed to physiologically relevant concentrations of glyphosate for 120 minutes did not experience any significant DNA damage. Following treatment with 10 µM glyphosate, Raji cells showed no signs of DNA damage and the ‘head’ of the cells stayed intact throughout the 2-hour exposure time. To further investigate the comet results at 1 and 5 mM, cells were treated again with glyphosate at these concentrations one hour after initial treatment. A significant difference could be observed between cells receiving only primary treatment and cells receiving the additional treatment. Raji cells exposed twice to glyphosate did not show the same pattern of recovery, with tail moments reaching levels above 20 for 1 mM and 25 for 5 mM. Cells with only primary exposure to glyphosate showed a decrease in DNA damage, with tail moments dropping from 15 to 5.8 for 1 mM and 23.67 to 6.74 for 5 mM.

Discussion

The results show that the DNA damaging and cytotoxic potential of glyphosate is related to exposure length and treatment concentration. Glyphosate only induced significant DNA damage at concentrations several orders of magnitude larger than those attainable *in vivo*. The data support the established evidence that glyphosate is not genotoxic in human cells at physiologically relevant concentrations. While these data ultimately support glyphosate's classification as a potential carcinogen, they suggest that its effects are negligible when exposure is minimal. Furthermore, study results obtained at 1 mM and 5 mM suggest that cells initially damaged by glyphosate may have the ability to repair and regain viability after single exposure. The results show also that the extent of DNA damage changed drastically across different incubation time points. For example, incubation at 1 mM for one hour produced severe DNA damage whereas no DNA damage was evident after 2 hours at the same concentration. If cells had

only been evaluated at this time point, results would suggest that there was no cytotoxic activity and the initial DNA damaging event would be missed. Cytotoxic activity might also be underestimated by standard viability assays in which the DNA damage is insufficient to induce cell death. Considering multiple time points made it possible to observe both DNA damage as well as the ensuing recovery.

Conclusion

Exposure of human cells to glyphosate produces minimal cytotoxicity and DNA damage at concentrations at or below 100 µM.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The purpose of this study was to investigate the concentration and time dependent DNA damaging potential of glyphosate in Burkitt's B Cell Lymphoma (Raji) cells using the comet assay and MTT viability assay. The cells were exposed to glyphosate concentrations ranging from 0.1 µM to 15 mM and resulting DNA damage and loss of cell viability were measured after various lengths of exposure. DNA damage could only be observed at 1mM and higher which are concentrations that cannot be attained in vivo. The DNA damage seen at 1 and 5 mM reached its maximum between 60 and 80 minutes of incubation which returned to control values thereafter. To reach 1 mM of systemic concentration *in vivo* experimental animals have to be treated orally with glyphosate at dose levels that are much higher than those used in long term carcinogenicity studies which showed no carcinogenic effect of glyphosate.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria for *in vitro* testing although no historical control data were reported. The significance for the risk assessment of glyphosate is limited because DNA damage has only been demonstrated at concentrations of glyphosate that cannot be attained in *in vivo* test systems.

Reliability criteria for *in vitro* toxicology studies

Publication: Townsend *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95%. Source: not reported.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y/N	0.1 µM to 15 mM for alkaline comet assay
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	

Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Human cell exposure to glyphosate has minimal cytotoxicity and DNA damage at concentrations at or below 100 μM. Only effects found beyond 1 mM.
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria for in vitro testing although no historical control data were reported.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Trasande L. *et al.*
Report year	2020
Report title	Glyphosate exposures and kidney injury biomarkers in infants and young children
Document No	Environ Pollution (2020), 256, 113334
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Yes, all research was performed in accordance with relevant guidelines/regulations.
Acceptability/Reliability:	Yes/Reliable without restriction

2. Full summary of the study according to OECD format

The goal of this study was to assess biomarkers of exposure to glyphosate and assess potential associations with renal function in children. While previous studies have indicated that glyphosate may have nephrotoxic effects, few have examined potential effects on kidney function in children. In this study, three cohorts across different phases of child development and measured urinary levels of glyphosate. Associations of glyphosate with three biomarkers of kidney injury was evaluated: albuminuria (ACR), neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury marker 1 (KIM-1). Multivariable regression analyses examined associations of glyphosate with kidney injury biomarkers controlling for covariates. Glyphosate was identified in 11.1% of the total participants. The herbicide was detected more frequently in the neonate population (30%). Multivariable regression models failed to identify significant associations of log-transformed glyphosate with any of the kidney injury biomarkers, controlling for covariates age, sex, and maternal education. While detectability of glyphosate in children's urine at various ages and stages of life was confirmed, there was no evidence for renal injury in children exposed to low levels of glyphosate.

Materials and methods

Study populations; We briefly describe a longitudinal birth cohort of children (Starting Early), in whom urine samples were measured at 10-19 months of age, as well as two cross-sectional studies Preventing Environmental Exposures in Pregnancy (PEEPS), and Bright Start which represent older children (ages 3-8) and newborns (<30 days), respectively. The three cohorts are comprised of chronologically ordered categories of age in children as follows: Bright Start (newborns), Starting Early, and PEEPS. Inclusion in the present study was determined if sufficient urine was available for the experimental testing. All research was performed in accordance with relevant guidelines/regulations. In particular, informed consent was obtained from participants' parents or their legal guardians for use of collected samples for use in future studies such as this one of glyphosate exposure.

Urine collection and storage; Urine samples for each cohort were obtained at study visits using age-appropriate methods (urine bags and cotton balls in untrained babies and infants, freshly voided urine in trained children) and immediately transferred to sterile polyethylene cups. Within 2 h after collection from the infants, urine was transferred to cryovials for storage at -80 °C prior to laboratory analysis. We measured urinary markers of kidney injury NGAL, and KIM1 using Luminex xMAP technology (see below). Urine albumin and creatinine were measured using standard methods of quantitative spectrophotometry at the ARUP National Reference Laboratory (Salt Lake City, Utah) and the results were used to calculate ACR (Albumin-to-Creatinine Ratio [mg:mg]).

Analysis of urine samples for glyphosate; 200 μL of urine sample was transferred into a 15 mL polypropylene (PP) tube and spiked with the labeled internal standard mixture (2-^{13}C, 99%; ^{15}N, 98+% Glyphosate), allowed to stand at room temperature for 30 min and then diluted to 1.0 mL (5-fold dilution) with 1% formic acid in water. The diluted sample was vortexed for 1 min, centrifuged and

filtered through a nonsterile regenerated cellulose (RC) membrane filters (0.2 mm; Phenomenex, Inc., Torrance, CA, USA). The filtrate then transferred into an auto sampler vial for LC-MS/MS analysis. An Agilent 1260 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with an ABSCIEX 4500 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) running under negative mode electrospray ionization was used for the analysis. An anion-exchange column, Dionex IonPac AS 21 (2 mm x 250 mm, 7 μm) was employed for the separation of target chemicals under isocratic elution condition with a mobile phase consist of 1% formic acid in water and acetonitrile (95:5) mixture. Isotopic dilution mass spectrometry with MRM mode of analysis was used for selective quantification of the target chemicals. The QA/QC protocols included matrix spike (mean spike recoveries (n=5) for glyphosate was 109.5%) and procedural blanks (non-detected or < LOQ). Midpoint calibration standard and HPLC grade water were injected between every 20 samples analyzed to check the instrument detection linearity and carry-over effects, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as three and ten times of signal to noise ratio, respectively. LOD for glyphosate was 0.1 ng/mL, and LOQ was 0.33 ng/mL.

Analysis of kidney injury biomarkers; NGAL was measured in human urine using the Luminex performance human kidney Biomarker kit (FCSTM16-01, R&D Systems). Urine samples were thawed and diluted 1:10 in calibrator diluent and protocol followed manufacturer's instructions. Data were captured on a Luminex MAGPIX instrument with xPonent 4.2 software. Standard curve R^2 values were 0.98 and high- and low-quality controls were within expected range. KIM-1 was measured in human urine using the Milliplex MAP Human Kidney Injury Magnetic Bead Panel Kit (HKI1MAG-99K, EMD Millipore). Urine samples were thawed and diluted 1:2 in assay buffer and protocol followed manufacturer's instructions. Data were captured on a Luminex MAGPIX instrument with xPonent 4.2 software. Standard curve R2 values were 0.97 and high- and low-quality controls were within the expected range.

Statistical analysis; We first described the demographics of our study population (N = 108), including age, sex, maternal education, race/ethnicity, BMI category, and intervention arm in the case of the Starting Early group. Body mass index (BMI), calculated as weight in kilograms divided by height in meters squared, was used to measure adiposity in the Starting Early group. We used standardized BMI *z* scores given that BMI varies widely by age and sex, following the Centers for Disease Control and Prevention year 2000 norms. Overweight and obese were categorized as BMI *z*-score of 1.036 or greater (85th percentile for age and sex) and 1.64 or greater (95th percentile), respectively. We calculated median glyphosate, albuminuria, KIM-1, and NGAL levels, as well as interquartile ranges for all biomarkers and percent detected for glyphosate levels by study group. We also described mean and standard deviation in each one of the mentioned biomarkers of interest in the total population. Univariate regressions examined log-transformed glyphosate concentrations and their detectability (using logistic regression) as dependent variables with covariates examined singly. We created dummy variables for maternal education with one category for those children whose mothers did not complete a high school education and another for those whose mothers at least completed high school, and created a dummy variable for missing values (N = 34) that we used as reference. We also created an ordinal variable reflecting the age of the children in increasing order from youngest to oldest type as follows: Healthy Start, Starting Early, and PEEPS (N ¼ 108). We also performed univariate analyses by study arm in the Starting Early sample (N = 66). Multivariable analyses of continuous log-transformed glyphosate exposure examined age, sex, and maternal education as predictors (N ¼ 108). We assessed differences in detectability of glyphosate using multivariable logistic regressions with age, sex, and maternal education as predictors. All regressions examining predictors of log-transformed glyphosate were retransformed from the logarithmic scale. We then examined associations of glyphosate with kidney injury. First, we calculated Pearson correlation coefficients for all log-transformed kidney injury markers KIM-1, NGAL, and ACR, as well as log-transformed glyphosate levels. For glyphosate levels below the limit of detection (LOD), we imputed values equal to LOD/square root of 2. We also performed univariate and multivariable regressions of urinary log-transformed glyphosate as predictor of log-transformed kidney injury markers KIM-1, NGAL, and ACR, controlling for age, sex and maternal education. Sensitivity analyses were also included; we performed univariate and multivariable regressions of creatinine-adjusted urinary log-transformed glyphosate as predictor of log-transformed kidney injury markers KIM-1, NGAL, and ACR. Statistical analysis was performed with Stata/IC Version 14 (StataCorp: College Station, TX).

Results

The population was largely Hispanic, and comprised roughly equal numbers of males and females (Table 1). Only 4% of our participants had a mother with a college degree but the majority of parents in our study population graduated from high school (62.1%). The mean urinary glyphosate concentration was 0.278 ± 0.228 µg/mL, with a range of 0.105-2.125 µg/mL. We identified glyphosate in 11.1% of the participants. The herbicide was detected in 7.6-30% of the three cohorts: 30% of neonates had glyphosate exposure above the LOD, followed by PEEPS cohort (12.5%) and Starting Early (7.6%). We also observed a wide detection range in NGAL biomarkers. We also noted that 40% of children in the Starting Early cohort were overweight or obese. Younger children had higher urinary levels of glyphosate in both univariate and multivariable regressions. Children with mothers without a high school diploma had slightly lower levels of urinary glyphosate relative to those with mothers who attained a high school education in our multivariable regression. However, we found no other significant associations of demographic covariates with glyphosate in univariate or multivariable models. Similarly, we found no statistically significant associations of demographic factors in relationship to detectability of urinary glyphosate in children. No univariate regressions with urinary glyphosate identified a statistically significant association with increased renal biomarker injury (Fig. 1A-C respectively and Table 2). Multivariable regressions with urinary glyphosate failed to identify statistically significant associations of log-transformed glyphosate with KIM-1, NGAL, or ACR (Table 2). In sensitivity analyses of univariate and multivariable regressions with creatinine-adjusted log-transformed glyphosate, there was also no statistically significant evidence for increased levels of markers for renal injury (Table 2). In additional sensitivity analyses without the newborns similar results were reported with no statistically significant associations of log-transformed glyphosate with kidney injury biomarkers (Table 2).

Table 1: Study population (n=108)[a]

Demographics			N	%
Male (Sex)			56	51.9
Study	Bright Start (Neonates)		10	9.3
	Starting Early		66	61.1
		Intervention Arm	39	59.1
		Control Arm	27	40.9
	PEEPS		32	29.6
Marernal Education	None		7	9.5
	Elementary/Middle		21	28.4
	High School/General Equivalency Diploma		35	47.3
	Some college		7	9.5
	College grad		3	4.1
	Other		1	1.4
Race/Ethnicity	Hispanic		67	90.5
	Non-Hispanic White		5	6.8
	Non-Hispanic Black		1	1.4
	Non-Hispanic Asian		1	1.4
BMI Categories[b]	Underweight		2	3.0
	Normal		40	60.6
	Overweight		12	18.2
	Obese		12	18.2

Distribution of Glyphosate and Kidney Injury Biomarkers	Mean (SD)	Detection Range (Min. & Max. Concentrations)
Glyphosare (ng/mL)	0.278 (0.228)	0.105–2.125
KIM-1 (ng/mL)	80.17 (76.92)	12.38–560.6
NGAL (pg/mL)	12,486 (21,934)	530.7–131,196
ACR[c] (mg/g)	26.23 (25.43)	1.414–119.0

Distribution of Glyphosate and Kidney Injury Biomarkers by Study Groups (N = 108)			
Study	Healthy Start (Neonates)	Starting Early	PEEPS
Glyphosate (ng/mL), Median (IQR)	<LOD (<LOD-1.06)	<LOD (<LOD-LOD)	<LOD (<LOD-LOD)
Percent Detected	30.0%	7.58%	12.5%
KIM-1 (ng/mL), Median (IQR)	88.9 (71.0–114)	47.1 (33.8–71.7)	76.5 (40.3–140.2)
NGAL (pg/mL), Median (IQR)	3770 (2520–7200)	9080 (3880–18,700)	1920 (1240–5770)
ACR[c] (mg/g), Median (IQR)	9.45 (6.00–34.3)	28.0 (19.1–42.4)	5.66 (3.54–9.00)

[a] Maternal Education 34 missing, Race/Ethnicity 34 missing, BMI 42 missing, KIM-1 9 missing, NGAL 13 missing, and ACR 12 missing.
[b] Underweight (Below − 1.036 SD); Normal (Between −1.036 and + 1.036 SD); Overweight (Between + 1.036 and + 1.64 SD); Obese (Above + 1.64 SD).
[c] Albumin-to-creatinine ratio.

Table 2: Multivariable regressions of creatinine-adjusted urinary log-transformed glyphosate as predictor of log-transformed KIM-1, NGAL, ACR biomarkers[a]

All ages N = 108 [a]			
Increment per one log unit increase	KIM-1	NGAL	ACR
Glyphosate Univariate	0.974 (−17.92, 27.78)	−663.8 (−2462, 2641)	−0.287 (−6.773, 10.91)
Glyphosate Multivariable	−1.55 (−14.93, 17.62)	−887.8 (−1800, 662.2)	−5.05 (−14.14, 9.85)
Creatinine-adjusted models[b]			
Glyphosate Univariate	−1,648 (−15.22, 17.47)	−640.5 (−3242, 4079)	7.603 (−9.522, 32.88)
Glyphosate Multivariable	−3.70 (−13.47, 10.26)	−819.8 (−1688, 657.8)	0.858 (−13.80, 22.43)
Non-neonates N = 98[c]			
Increment per one log unit increase	KIM-1	NGAL	ACR
Glyphosate Univariate	−5.351 (−24.13, 27.39)	1489 (−4383, 17944)	3.002 (−10.66, 35.99)
Glyphosate Multivariable	−3.568 (−14.90, 15.96)	395.3 (−5128, 12782)	12.05 (−37.98, 106.6)
Creatinine-adjusted models[d]			
Glyphosate Univariate	−4.89 (−20.66, 22.43)	2083 (−5679, 23234)	8.24 (−16.60, 54.17)
Glyphosate Multivariable	−3.73 (−14.45, 14.88)	132.8 (−5165, 11668)	10.37 (−25.33, 72.33)

[a] KIM-1 N = 99; NGAL N = 95; ACR N = 96. Multivariable regression models controlling for demographic covariates age, sex, and maternal education, where maternal education missing observations were imputed as the majority category "Equal or Greater Than High School".
[b] Creatinine-adjusted regression models KIM-1 N = 95; NGAL N = 92; ACR N = 96.
[c] KIM-1 N = 89; NGAL N = 86; ACR N = 86. Multivariable regression models controlling for demographic covariates age, sex, and maternal education, where maternal education missing observations were imputed as the majority category "Equal or Greater Than High School".
[d] Creatinine-adjusted regression models KIM-1 N = 85; NGAL N = 83; ACR N = 86.

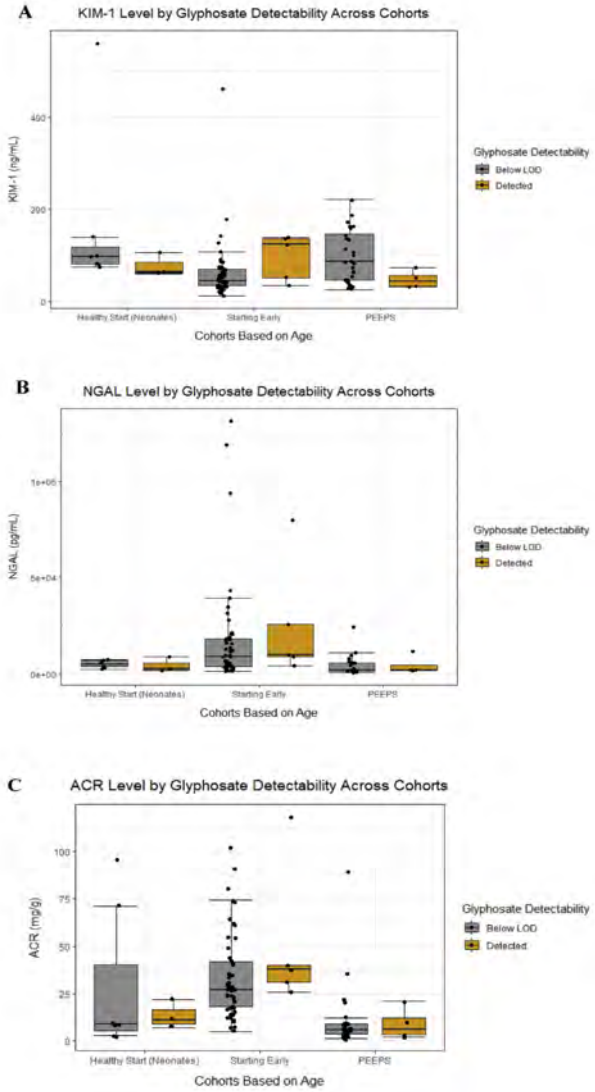


Figure 1: A. The dot plot illustrates the urinary KIM-1 level in participants enrolled in the different studies, 1B. The dot plot illustrates the urinary NGAL level in participants enrolled in the different studies, 1C. The dot plot illustrates the urinary albumin:creatine ratio (ACR) in participants enrolled in the different studies.

Discussion

The main findings of this study are the substantial and frequent prevalence of glyphosate in children across the first decade of life in three independently assembled samples. We identified glyphosate in 11.1% of the total participants, and the herbicide was detected more frequently in the neonate population (30%). However, urinary concentrations detected in the neonates, toddlers, and young school age children was significantly lower than levels documented in adults with occupational or environmental exposure. No association of urinary glyphosate was found with any of the three renal biomarkers (KIM-1, NGAL or ACR). Urinary excretion of albumin and serum creatinine concentration are conventional biomarkers for renal dysfunction. Novel biomarkers such as urinary excretion of KIM-1 and NGAL have been developed and it has been that they have diagnostic utility for earlier detection of acute kidney injury (AKI) prior to significant loss of function. KIM-1 also predicted renal damage from acute exposures in animal studies. This study has a number of limitations.

We used maternal education as a proxy for family educational attainment or socioeconomic status (SES) which are well-known factors for different levels of chemical exposures in children or renal injury outcome. We emphasize that low sample size also limited us to perform analyses in the three cohorts in conjunction, in lieu of stratified analysis. We acknowledge that the strength of any conclusion about the frequency of detecting glyphosate in newborns is limited by the sample size. The source of the glyphosate in the newborns, which was detected more frequently than in the other groups but not at high levels, could be via lactation or infant formula. It is possible that immature or different metabolic pathways for glyphosate in neonates, kidney injury during delivery, or lactation may have affected the findings in this age group. However, the addition of neonates in our study evaluating the association of glyphosate and kidney injury biomarkers may have biased findings towards the null. Our results also suggest that glyphosate levels declined with increasing age. The differences by age may represent differences in dietary behavior and access to foods free of contamination or improved elimination of the herbicide, though further research is needed. Previous larger scale case-control studies have shown in the past changes in renal function, kidney injury, or chronic kidney disease of unknown etiology (CKDu) upon glyphosate exposure, especially of adult occupational exposures. Several strengths in this study are notable. Our study is the first to document the prevalence of glyphosate exposures in young children through age 8 years. Our frequent detection of glyphosate suggests the need for inclusion and biomonitoring of glyphosate exposures in national surveys or screenings in populations of interest, which could provide more information in regards to these exposures and outcomes. Glyphosate biomonitoring could be added into studies such as the National Health and Nutrition Examination Survey (NHANES). Our study is novel in that we examine specific renal biomarkers in relationship to glyphosate in a young population. The lack of evident renal toxicity in association with glyphosate exposure in young children does not exclude a potential adverse impact of longer term exposure to the pesticide. Problems during labor and delivery can compromise renal perfusion leading to increased excretion of tubular injury biomarkers in the neonatal period. This could interfere with the ability to detect an independent effect of glyphosate in this age group. In addition, the regenerative capacity of the renal tubular epithelium in infants and young children may mask an adverse effect of the pesticide on tubule integrity. Urinary biomarkers such as NGAL and KIM-1 have been demonstrated to be useful biomarkers of tubular injury even without reduced kidney function, highlighting their utility in detecting subclinical insults to the kidney. Further research could explore more in detail the association between glyphosate and kidney injury biomarker types across different age groups in larger cohorts, and assess whether more prolonged exposure could have progressive deleterious effects.

Conclusion

In this study, we identify frequent prevalence of levels of glyphosate (11.1%), present across all three age ranges, and particularly present in the youngest population of neonates. There is no evidence for renal injury in young children exposed to low levels of glyphosate. Further studies of larger sample size are indicated to better understand the potential deleterious effects of the herbicide after different levels, routes, and duration of exposure.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study evaluated three cohorts across different phases of child development and measured urinary levels of glyphosate. They evaluated associations of glyphosate with three biomarkers of kidney injury: ACR, NGAL, and KIM-1. Sample collection and analysis as well as statistical evaluation of data have been conducted using well described methodologies. Multivariable regression models failed to identify significant associations of log-transformed glyphosate with any of the kidney injury biomarkers, controlling for covariates age, sex, and maternal education. The authors confirm detectability of glyphosate in children's urine at various ages and stages of life, there is no evidence in this study for renal injury in children exposed to low levels of glyphosate.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good monitoring study.

Reliability criteria of exposure studies

Publication: Trasande et al., 2020.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	Y	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.	Y	
Exposure to formulations with glyphosate combined with other a.i.	Y	
Exposure to various formulations of pesticides	Y	Environmental exposure.
Study		
Study design clearly described	Y	Longitudinal birth cohort and 2 cross sectional studies. Study of the association between renal biomarkers and glyphosate in urine.
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	Environmental exposure.
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Statistical analysis	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good monitoring study.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Vanlaeys A. *et al.*
Report year	2018
Report title	Formulants of glyphosate-based herbicides have more deleterious impact than glyphosate on TM4 Sertoli cells
Document No	Toxicology in Vitro (2018) Vol. 52, 14–22
Guidelines followed in study	None
Deviations from current test guideline	NA
Previous evaluation	No
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Roundup and Glyphogan are glyphosate-based herbicides containing the same concentration of glyphosate and confidential formulants. Formulants are declared as inert diluents but some are more toxic than glyphosate, such as the family of polyethoxylated alkylamines (POEA). In this study glyphosate alone, glyphosate-based herbicide formulations and POEA on the immature mouse Sertoli cell line (TM4) was tested, at concentrations ranging from environmental to agricultural-use levels.

Materials and methods

Chemicals - Glyphosate was purchased from Sigma-Aldrich, St Louis, USA. The purity was not reported.

Culture of TM4 Sertoli cells and treatments - The murine TM4 Sertoli cell line was obtained from the American Type Culture Collection (ATCC Manassas, USA). Cells were maintained in DMEM/HamF12 medium containing 0.2% glutamine, 1.2 g/L $NaHCO_3$, 15mM Hepes, 5% horse serum and 2.5% fetal calf serum, 100 U/mL of antibiotics and fungizone (complemented DMEM/Ham F12 medium) at 37 °C (5% CO2, 95% air) during 24 hours to 80% confluence in 24-well plates or in 6-well plates (for measurement of GST activity). Cells were then exposed to various concentrations of glyphosate.

Crystal violet cell viability assay - After incubation with glyphosate at different concentrations, culture supernatants were discarded and cells incubated in medium containing crystal violet solution (0.1% w/v in PBS 0.01 M, pH 7.4) for 30 minutes at 20 °C with gentle rocking. Excess dye and non-adherent dead cells were removed through 3 washing steps with PBS. Diluted acetic acid solution (10%) was then added to release the crystal violet taken up by cells, and the optical density reflecting living adherent cells was determined by absorption at 600 nm using a plate reader.

MTT cytotoxicity assay - This enzymatic test is based on the cleavage of MTT (tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a blue-colored product (formazan) by the mitochondrial enzyme succinate dehydrogenase. Activity of mitochondrial dehydrogenase enzymes indirectly measures activity of mitochondrial respiration and antioxidant defense systems. Culture medium was removed, and cells were washed once with PBS and then incubated with 500 µL MTT solution per well after each treatment. The plates were incubated for 3 hours at 37 °C. The reaction was stopped by placing the plates for 10 minutes at 4 °C followed by adding to each well 500 µL 0.04 N hydrochloric acid-containing isopropanol. The plates were then vigorously shaken for 40 minutes to solubilize the blue formazan crystals formed. Absorbance was measured by spectrophotometry at 570 nm.

Glutathione-S-transferase (GST) activity - For preparation of S9 fractions enriched in GST, the medium was removed, and cells were detached by treatment with trypsin-EDTA and washed twice with PBS at room temperature. Cells were then resuspended in 500 µL of 50 mM phosphate buffer at pH 7.2 containing 0.25 M sucrose and 1mM DTT. Then, they were homogenized and centrifuged at 9000g at

4 °C for 30 minutes. Supernatants corresponding to the S9 fraction were collected and stored at -80 °C. The protein concentration of each S9 fraction was determined using the Bradford assay. For the determination of GST activity 250 µg of S9 cell fraction was mixed with 10 µL of 100 mM reduced L-glutathione and 990 µL phosphate buffer (pH 6.5). The reaction was initiated by the addition of 10 µL of 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution in 95% ethanol. After incubation for 90 seconds at 37 °C absorbance was measured at 340 nm every 60 seconds for 3 minutes.

Lipid Sudan Black B staining - Sudan Black B stains lipids, including phospholipids and sterols. Culture medium was removed and cells were washed once with PBS. Cells were then incubated for 5 minutes at room temperature with 500 µL of Sudan Black solution with gentle shaking. This solution was removed, and cells were washed 3 times or more with 70% ethanol to remove excess stain and then at least 3 times with PBS. Then, intracellular Sudan Black B was extracted by incubation with DMSO for 30 minutes with gentle rocking and absorbance measured at 600 nm using a microplate reader.

Statistical analysis - The experiments were repeated at least in triplicate in different weeks on three independent cultures on each occasion (n=9). All data are presented as the mean ± standard error (SEM). Statistical differences from controls were determined by an ANOVA test. Results were statistically significantly different from controls when $p < 0.05$.

Results

Viability of TM4 cells - Glyphosate was found to have no impact on cell viability of TM4 cells after 24 hours of exposure at concentrations ranging from 10 to 10,000 ppm.

Mitochondrial succinate dehydrogenase activity - The measurement of succinate dehydrogenase (SD) activity was used to assess the effect of glyphosate on mitochondrial function and viability after 24 hours of exposure. Reduced SD activity was seen for glyphosate over the entire concentration range from 0.001% (approx. 85%) to 1% (approx. 75%) as derived from the graph.

Inhibition of glutathione-S-transferase (GST) activity – The effect of glyphosate on GST activity involved in the anti-xenobiotic defense system was evaluated. At an LC_{50} concentration glyphosate was found to have no impact on GST activity.

Lipid droplet accumulation - After staining with Sudan Black B exposure of TM4 cells to glyphosate for 24 hours at 2,500 or 5,000 ppm induces an increase in cytoplasmic lipid droplets as assessed microscopically.

Discussion and conclusions

Measurements of cell viability, respiratory chain activity, detoxification system and lipid accumulation were undertaken in immature murine TM4 Sertoli cell line following 24 hours of exposure to glyphosate at concentrations ranging from environmental levels to agricultural use concentrations (1%, 10,000 ppm). This study demonstrated that at sub-agricultural use levels (10-10,000 ppm) TM4 cell viability is not affected by glyphosate. Exposure of TM4 cells to glyphosate reduces mitochondrial succinate dehydrogenase (SD) activity at the lowest concentrations tested in TM4 cells, as compared with other cell types. The formulants present in commercial herbicides are known to increase glyphosate penetration into cells by membrane disruption and thus probably potentiate perturbation of mitochondrial permeability induced by glyphosate. This mechanism may explain the higher toxicity of formulations on mitochondrial activity. In this study, we also demonstrated that 24 hours exposure of TM4 cells to glyphosate induced lipid droplet accumulation.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study the effect of glyphosate on murine TM4 Sertoli cells was investigated *in vitro*. The endpoints were cytotoxicity, glutathione transferase activity and lipid accumulation. In contrast to the glyphosate-based formulations and co-formulants tested glyphosate was found to have no impact on cell viability after 24 hours of exposure at concentrations ranging from 10 ppm to 10,000 ppm.

Glyphosate reduced succinate dehydrogenase to some extent over the entire concentration range from 10 (approx. 85% of control) to 10,000 ppm (approx.75% of control) with no dose-effect relationship and was found to have no impact on glutathione transferase activity. Exposure of TM4 cells to glyphosate for 24 hours at 2,500 or 5,000 ppm induces an increase in cytoplasmic lipid droplets. These concentrations are far beyond what is physiologically feasible *in vivo*.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterized and no positive controls were used in any of the assays conducted.

Reliability criteria for *in vitro* toxicology studies

Publication: Vanlaeys *et al*., 2018	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also co-formulants and formulations were tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y (partly)	
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive controls used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterized and no positive controls were used in any of the assays conducted.		

1. Information on the study

Data point:	CA 5.5
Report author	Wang L. *et al.*
Report year	2019
Report title	Glyphosate induces benign monoclonal gammopathy and promotes multiple myeloma progression in mice
Document No	Hematol Oncol 12, 70 (2019)
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate is the most widely used herbicide in the USA and worldwide. There has been considerable debate about its carcinogenicity. Epidemiological studies suggest that multiple myeloma (MM) and non-odgkin lymphoma (NHL) have a positive and statistically significant association with glyphosate exposure. As a B cell genome mutator, activation-induced cytidine deaminase (AID) is a key pathogenic player in both MM and B cell NHL. Vk*MYC is a mouse line with sporadic MYC activation in germinal center B cells and considered as the best available MM animal model. Vk*MYC mice and wild-type mice were treated with drinking water containing 1000 mg/L of glyphosate and examined animals after 72 weeks. Vk*MYC mice under glyphosate exposure developed progressive hematological abnormalities and plasma cell neoplasms such as splenomegaly, anemia, and high serum IgG. Moreover, glyphosate caused multiple organ dysfunction, including lytic bone lesions and renal damage in Vk*MYC mice. Glyphosate-treated wild-type mice developed benign monoclonal gammopathy with increased serum IgG, anemia, and plasma cell presence in the spleen and bone marrow. Finally, glyphosate upregulated AID in the spleen and bone marrow of both wild-type and Vk*MYC mice. Conclusions: These data support glyphosate as an environmental risk factor for MM and potentially NHL and implicate a mechanism underlying the B cell-specificity of glyphosate-induced carcinogenesis observed epidemiologically.

Materials and methods

Mouse model and treatments; All chronic and acute animal experiments were performed in accordance with NIH guidelines and under protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Wild-type (WT) C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Vk*MYC mice in the C57Bl/6 genetic background were obtained from Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ). Vk*MYC and WT mice were intercrossed to obtain WT and Vk*MYC littermates. Sex-matched WT and Vk*MYC mice (8 weeks old) were assigned to treatment or control groups based on body weight. For chronic study of glyphosate effects, treatment groups were provided 1.0 g/L glyphosate (Sigma-Aldrich, St. Louis, MO) in their drinking water for 72 weeks. Regular drinking water was provided for the control groups (Fig. 1a). Every 6 weeks, blood was collected from the tail vein of mice, and the serum IgG level was measured. At week 72, the remaining 3 surviving Vk*MYC mice reached humane endpoints. These 3 treated Vk*MYC mice were used for M-spike detection and pathologic analyses, along with mice from other groups. Other Vk*MYC mice that were sacrificed before week 72 were analysed for total serum IgG levels, complete blood cell count, and total serum creatinine. For comparison, mice from other groups were euthanized at week 72 and their tissues and blood analyzed. For acute treatment, 8-week old mice (n = 5 per group) were given 0, 1.0, 5.0, 10.0, or 30.0 g/L of glyphosate for 7 days before sacrifice. The same variables were analyzed in the acute study.

Blood and post-mortem assays; Whole-blood complete blood count (CBC), IgG enzymelinked immunosorbent assay (ELISA), serum protein electrophoresis, flow cytometry, and histological examinations of relevant tissues were performed as described previously. Serum creatinine was measured by ELISA using a creatinine assay kit (#ab65340, Abcam, Cambridge, MA) according to the manufacturer's protocol.

Western blotting analyses; Mouse tissues were processed for Western blotting. The antibodies were from Cell Signaling Technology (Danvers, MA, USA): AID (L7E7) (#4975) and β-actin (#3700). Blotting was run with 3 technical replicates. Horseradish peroxidase- conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody.

Statistics; Statistical analysis was carried out using GraphPad InStat 3 software (GraphPad Software, Inc., San Diego, CA, USA). The statistical significance between the groups was determined by one-way or two-way analysis of variance (ANOVA) with the appropriate post hoc testing using Tukey's test. Statistical significance was accepted at $P \leq 0.05$. All data are shown as mean ± SEM unless otherwise indicated.

Results

*Chronic glyphosate exposure reduces survival and induces splenomegaly in Vk*MYC mice*; Eight-week-old Vk*MYC mice and their WT littermates were provided 1.0 g/L glyphosate in drinking water for 72 weeks, and animals were monitored at regular intervals before sacrifice (Fig. 1a). Glyphosate significantly affected the health of Vk*MYC mice, all of which had to be euthanized by week 72 (Fig. 1b). Surviving mice in other groups were sacrificed at week 72 (at age 80 weeks) for necropsy. Inspection of organs revealed a marked increase in spleen weight and size in Vk*MYC mice treated with glyphosate compared to the other 3 groups (Fig. 1c, e). Glyphosate significantly augmented the splenocyte number in Vk*MYC mice (Fig. 1d). Findings indicate that glyphosate induces splenomegaly in both WT and Vk*MYC mice.

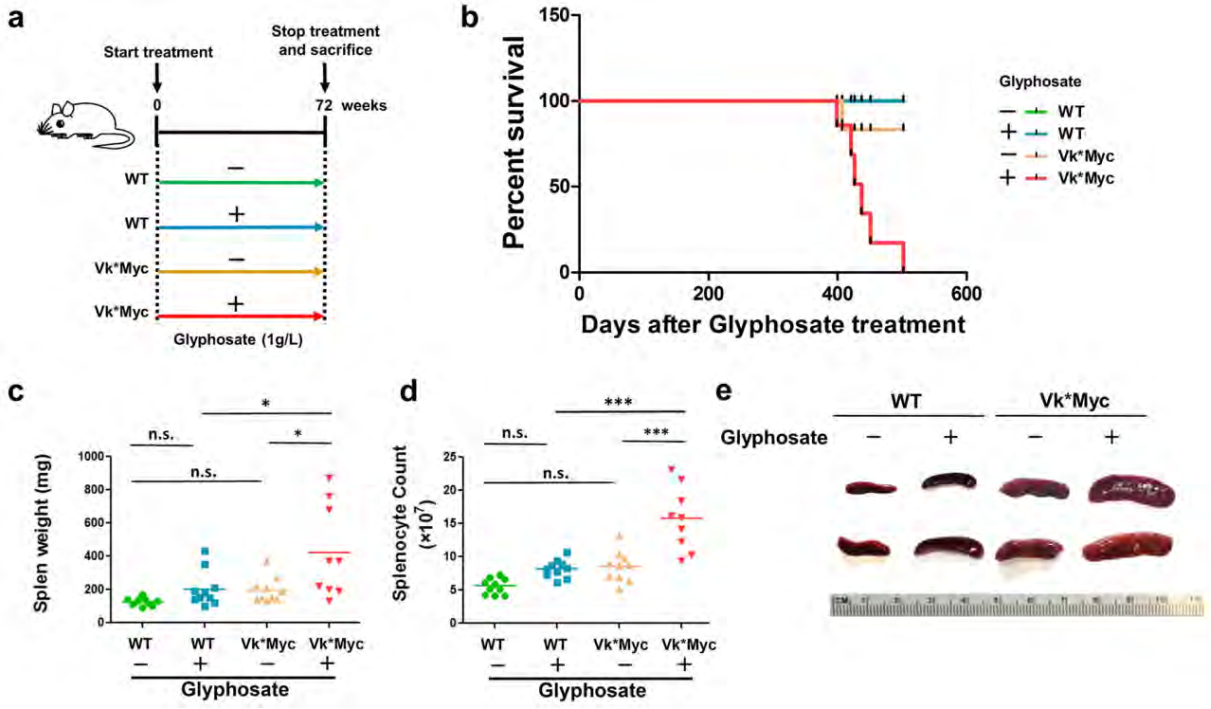


Fig. 1: Glyphosate reduced survival and induced splenomegaly in Vk*MYC mice. a Schematic diagram of the chronic glyphosate exposure regimen in 4 groups of mice. b The percentage of mice surviving under glyphosate exposure. The line (blue) to indicate untreated WT mice aligned directly with that for WT treated mice and so was not visible. c Mouse spleen weight at sacrifice. d The total number of splenocytes per spleen from mice at sacrifice. e Representative images of spleens from 4 groups (2 per group).

*Hematological abnormalities occur in Vk*MYC mice with chronic glyphosate exposure*; As illustrated in Fig. 2a, untreated Vk*MYC mice exhibited higher IgG levels than untreated WT mice. Upon glyphosate exposure, WT mice showed moderate yet steady increasing in IgG levels, suggesting that

glyphosate induces benign monoclonal gammopathy, a mouse equivalent to human MGUS. Vk*MYC mice receiving glyphosate had greater IgG elevation, and by week 30, IgG levels jumped to 11.78 g/L, more than 5-fold the 2.07 g/L observed in untreated Vk*MYC mice. From week 36 to week 72, the mean IgG level was significantly higher in treated WT and Vk*MYC mice compared to the untreated control groups, and Vk*MYC mice, treated or untreated, had higher IgG levels than their WT counterparts. Overt MM diagnosis was determined by serum protein electrophoresis (SPEP) analysis to detect the M-spike, which is a significant IgG monoclonal peak. SPEP results showed that Vk*MYC mice treated with glyphosate had a clear M-spike, whereas weaker M-spike was observed in glyphosate-treated WT mice. No clear M-spike was present in the untreated WT mice or Vk*MYC mice (Fig. 2b). This is the direct in vivo evidence that glyphosate exposure leads to M-spike, a cardinal hematological abnormality consistent with MM. Hematological abnormalities were present in glyphosate treated mice as compared to untreated control mice (Fig. 2c–i). Data support the notion that glyphosate induces multiple hematological abnormalities characteristic of MM in mice.

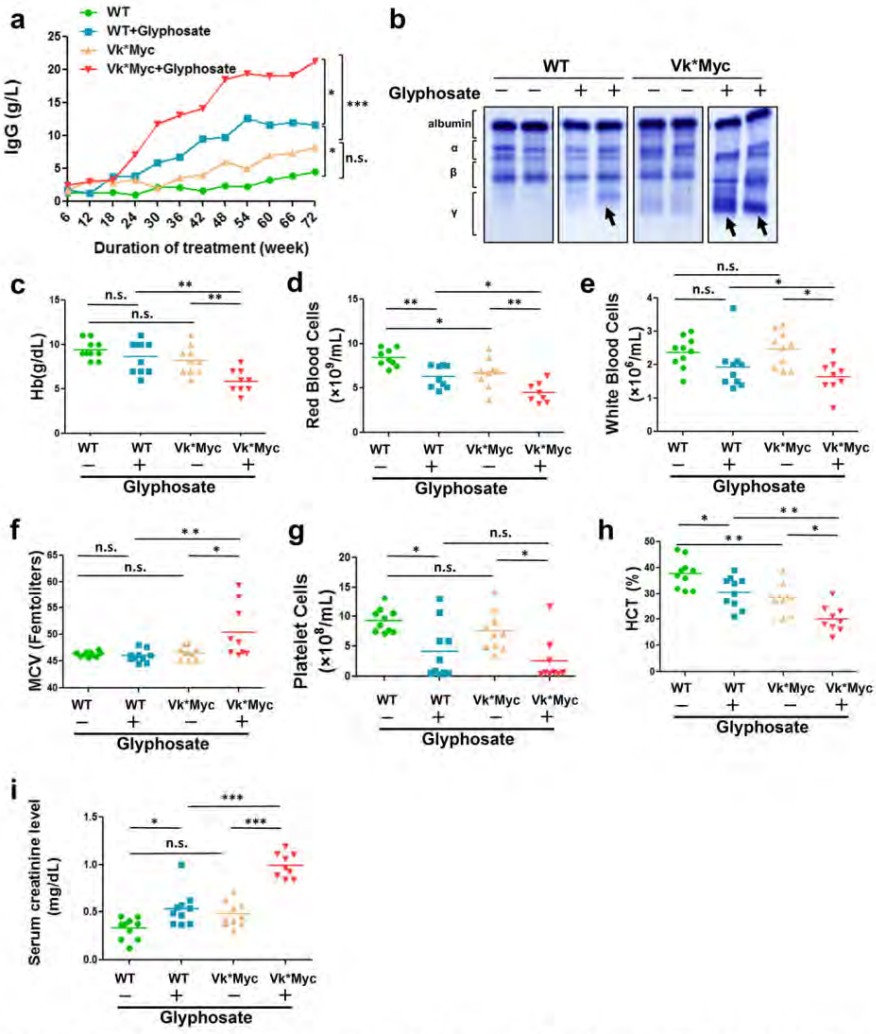


Fig. 2. Hematological abnormalities found in Vk*MYC mice treated with glyphosate. **a** Total serum IgG in mice during 72 weeks of glyphosate treatment. Mouse blood samples were collected and assayed for IgG every 6 weeks. **b** Immunoglobins from mice as determined by SPEP at week 72. Arrows indicate IgG clonal peaks (M-spike; γ-globulin peak). SPEP was performed for all mice in each group, and representative results of 2 mice per group are shown. **c**–**h** Complete blood cell counts in mice. Hemoglobin concentration (Hb, **c**), red blood cell count (**d**), white blood cell count (**e**), mean red cell volume (MCV, **f**), platelet cell count (**g**), and hematocrit (HCT, **h**) are shown. **i** Total serum creatinine in mice at week 72. The horizontal lines indicated the mean value. Data were analyzed by two-way ANOVA (**b**) or one-way ANOVA (**a**, **d**, **e**). $n = 10$ mice per group

*Vk*MYC mice chronically exposed to glyphosate develop progressive plasma cell neoplasms;* Plasma cells exhibit CD138hi B220–(high CD138 expression without B220 expression). Flow cytometric analyses of cells harvested from the spleens and bone marrow showed expansion of plasma cells in mice under glyphosate exposure. A marked increase in the numbers of CD138hi B220– cells was detected in both WT and Vk*MYC mice treated with glyphosate (Fig. 3a). These data demonstrate that glyphosate treatment expands the plasma cell population in the spleen and bone marrow in both WT and Vk*MYC mice.

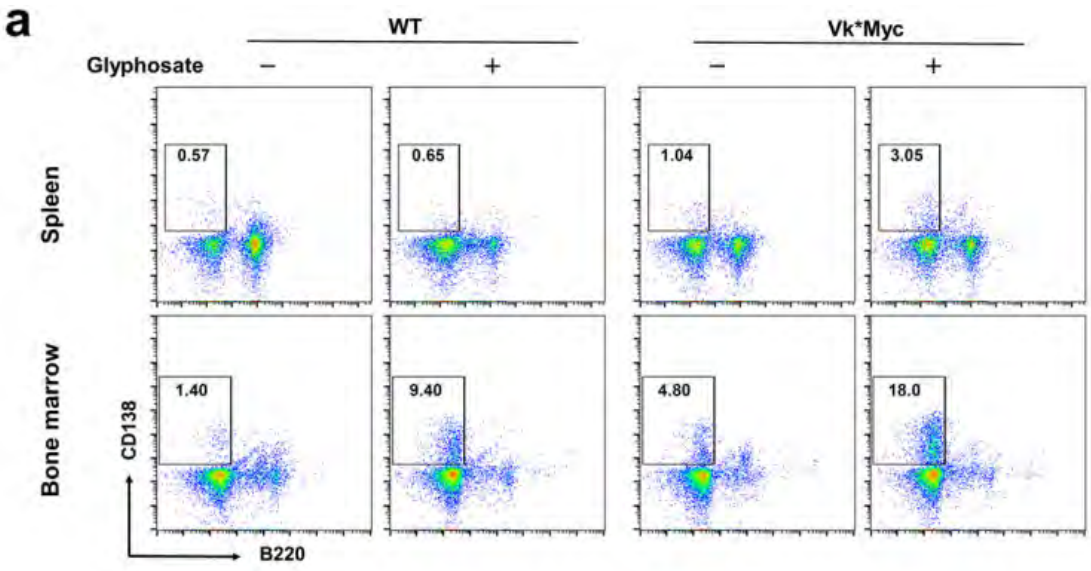


Fig. 3 Glyphosate-treated Vk*MYC mice developed progressive plasma cell neoplasms. **a** Representative flow cytometry plots detecting cell surface markers CD138 (*Y*-axis) and B220 (*X*-axis) in splenocytes (upper panel) and bone marrow cells (lower panel). The numbers on the axes denoted the $\log_{10}$ values of fluorescence. The numbers in the inserts show the percentage of $CD138^{high}B220^{-}$ cells in the entire cell population.

Chronic glyphosate exposure triggers multiple organ dysfunction; To determine whether target organ damage occurred in glyphosate-treated mice, the femoral shaft, spleen, liver, lung, and kidney were sectioned and stained with hematoxylin and eosin (H&E). Severe destructive osteolytic bone lesions in the femoral shaft were readily detectable in glyphosate-treated Vk*MYC mice. Treated WT mice showed smaller bone lesions. No lesions were observed in the control groups (Fig. 4a). Plasma cells with a perinuclear clear zone and eccentric round nucleus were observed in glyphosate-treated WT and Vk*MYC mice (Fig. 4b, c). Next, the histopathologic changes in the liver, lung, and kidney were analyzed. In glyphosate-treated mice, hepatic fibrosis and collagen deposition were observed in Vk*MYC mice, whereas WT mice showed less severe liver damage; the 2 control groups had normal hepatic tissue architectures (Fig. 4d). The lungs in treated Vk*MYC mice were severely damaged, with large distal air spaces filled by lymphocytes, neutrophils, cell debris, and hyperplastic pneumocytes; those from untreated WT mice had normal alveolar spaces and alveolar septa lined with normal pneumocytes. The lungs from treated WT mice and untreated Vk*MYC mice showed an intermediate phenotype (Fig. 4e). Renal tubular obstruction by large casts, indicative of necrotic tubular cells, were detected in glyphosate-treated WT and Vk*MYC mice, but not in the untreated groups; there were more and larger casts in treated Vk*MYC kidneys than in WT kidneys (Fig. 4f). Taken together, these data indicate that glyphosate treatment damages multiple organs in both WT and Vk*MYC mice with more severe damage occurring in Vk*MYC mice.

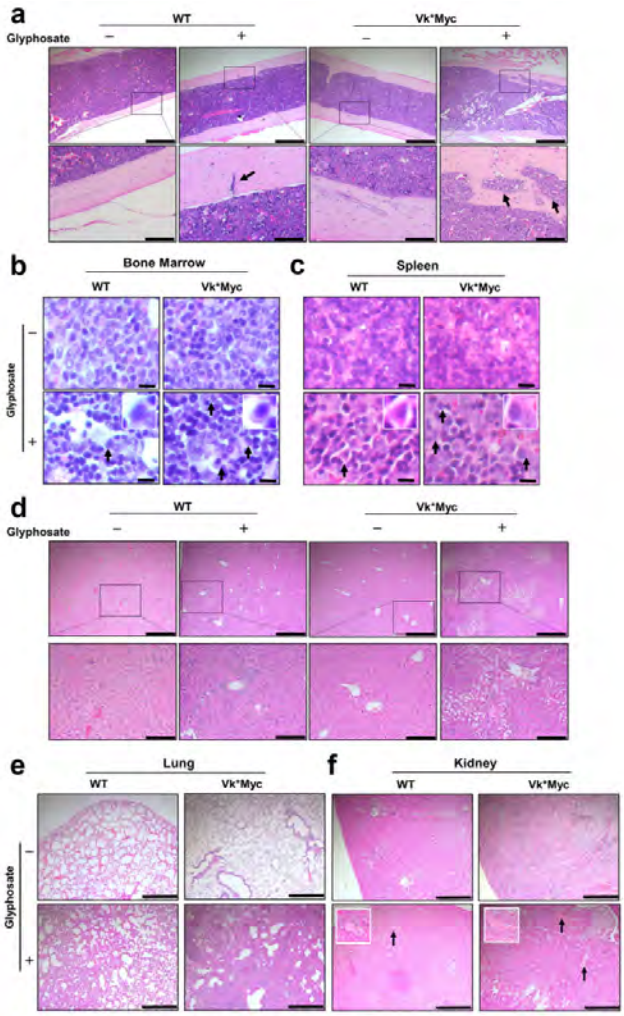


Fig. 4 Glyphosate led to multiple organ dysfunction. **a** Histological evaluation of bone morphology from 4 groups of mice. Bone lytic lesions (indicated by arrows) were detected in the femoral shaft of Vk*MYC mice treated with glyphosate. Scale bar = 500 μm (top) or 100 μm (bottom). **b** Infiltrating plasma cells in the bone marrow of glyphosate-treated mice. Scale bar = 20 μm. Arrows pointed to plasma cells. **c** Infiltrating plasma cells in the spleen of glyphosate-treated mice. Scale bar = 20 μm. Arrows point to plasma cells. **d** Collagen deposition in the liver was observed in glyphosate-treated Vk*MYC mice. *n* = 10 mice per group. Scale bar = 500 μm (top) or 200 μm (bottom). **e** Destruction of lung morphology was observed in glyphosate-treated Vk*MYC mice. *n* = 10 mice per group. Scale bar = 500 μm. **f** Protein deposition (indicated by arrows) in the kidney was observed in glyphosate-treated Vk*MYC mice. *n* = 10 mice per group. Scale bar = 500 μm. All panels show 1 representative image each from 4 groups of mice unless otherwise indicated

Chronic glyphosate exposure induces AID upregulation; To investigate the underlying mechanisms of glyphosate-mediated MGUS induction and MM progression, expression of activation-induced cytidine deaminase (AICDA, also known as AID) in mice treated with 1.0 g/L glyphosate for 72 weeks was examined. It was found that AID was upregulated in both the bone marrow and the spleen of WT and Vk*MYC mice (Fig. 5a).

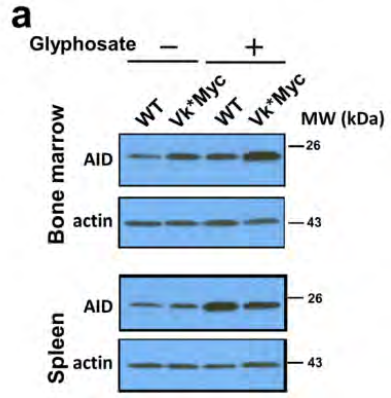


Fig. 5 Glyphosate-induced AID upregulation. **a** Western blotting analysis of mice treated with 1.0 g/L of glyphosate for 72 weeks.

Acute glyphosate exposure induces AID upregulation; To determine the acute effect of glyphosate, 8-week-old WT and Vk*MYC mice were treated with increasing doses of glyphosate (1, 5, 10, and 30 g/L) in drinking water for 7 days. This acute treatment neither increased spleen weight nor affected body weight significantly. Next, expression of AID in the spleen, bone marrow, and lymph nodes were analysed. It was found that AID was upregulated in a glyphosate dose-dependent manner in the spleen and bone marrow of WT and Vk*MYC mice treated with 10 and 30 g/L of glyphosate (Fig. 5c). Given the role of AID in MM pathogenesis in the context of its capacity to induce mutations and chromosome translocations, these results from mice with chronic and acute glyphosate treatment support an AID mediated mutational mechanism in the etiology of MGUS and MM under glyphosate exposure.

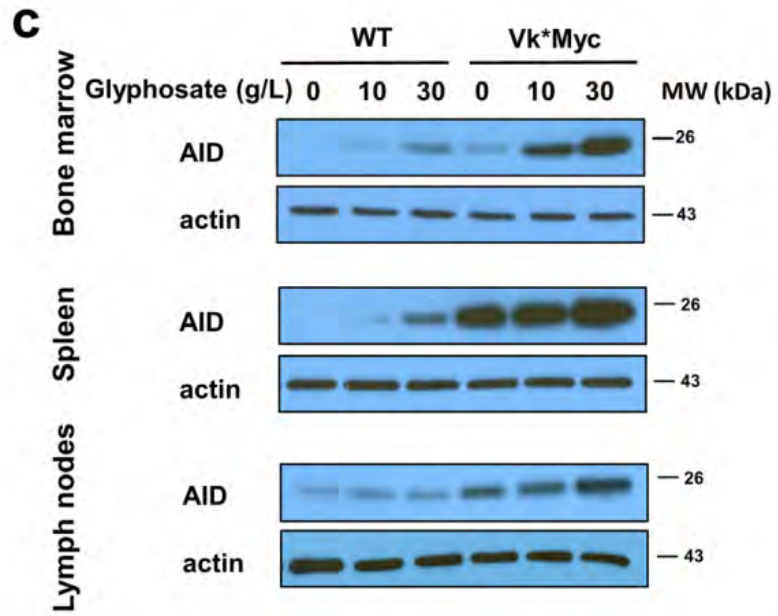


Fig. 5 c Western blotting analysis of mice treated with glyphosate for 7 days. One representative mouse

Discussion

In this study, it was demonstrated that glyphosate induces benign monoclonal gammopathy (mouse equivalent to MGUS in human) in WT mice and promotes MM progression in Vk*MYC mice. In Vk*MYC mice, glyphosate causes hematological abnormalities like anemia and multiple organ dysfunction like lytic bone lesions and renal damage, which are hallmarks of human MM. Beyond epidemiology and animal models, the mechanism of action is the third pillar required to define a compound as a carcinogen. Numerous studies have revealed that glyphosate may induce DNA damage, oxidative stress, inflammation, and immunosuppression, as well as modulate cell proliferation and death and disrupt sex hormone pathways. However, these mechanistic studies have failed to explain why glyphosate exposure is only positively associated with MM and NHL. Our results demonstrate that glyphosate treatment, either at a chronic low dose or acute high doses, upregulates the expression of AID in the bone marrow and spleen of both WT and Vk*MYC mice. The data disclose, for the first time, that glyphosate elicits a B cell-specific mutational mechanism of action in promoting carcinogenesis, as well as offering experimental evidence to support the epidemiologic finding regarding its tissue specificity in carcinogenesis (i.e., only increasing the risk for MM and NHL). The “acceptable daily intake (ADI)” of glyphosate currently allowed in the USA, defined as the chronic reference dose as determined by EPA, is 1.75 mg/kg body weight/day; an average adult male or female in the USA who weighs 88.8 or 76.4 kg and drinks 2 L (8 glasses) water daily containing 77.7 (for male) or 66.9 (for female) mg/L glyphosate would reach the ADI. A dose of 1,000 mg/L glyphosate in drinking water (~ 15-fold the ADI) was chosen in this study, which caused significant adverse effects and accelerated MM progression in Vk*MYC mice, i.e., animals predisposed to MM.

Conclusion

The data provide in vivo evidence to support that glyphosate induces MGUS and promotes disease progression to MM. A B cell-specific mutational mechanism for glyphosate exposure that increases MM and NHL risk was uncovered, providing a molecular basis for human epidemiological findings.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to investigate the pathogenic role of glyphosate in multiple myeloma using Vk*MYC mice. The study did demonstrate the ability of glyphosate to impact measured parameters in the tested models. However, this study is not appropriate for human health risk assessment. The number or animals per group was below the recommended number for guideline toxicity studies and to perform sufficient statistical analysis. Only one dose level was used in the chronic study. It was not possible to correlate effects with a glyphosate dose-response as the water consumption of animals was not provided and is therefore impossible to calculate a dose on a mg/kg bw basis for risk assessment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not characterized, only one dose was considered for the chronic study and the number of animals used per group was either too low (acute study) or not reported (chronic study).

Reliability criteria for *in vivo* toxicology studies

Publication: Wang *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance		
AMPA is the tested substance		
Study		
Test species clearly and completely described	Y	Wild-type (WT) C57Bl/6 mice and Vk*MYC mice.
Test conditions clearly and completely described		
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	For the chronic study: 1 g/L in drinking water for 72 weeks.

		For the acute study: 1, 5, 10, 30 g/L for 7 days.
Number of animals used per dose level reported	Y	5/group for the acute study, no/group not reported for the chronic study.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	Not possible. Only one dose level used.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not characterized, only one dose was considered for the chronic study and the number of animals used per group was either too low (acute study) or not reported (chronic study).		

1. Information on the study

Data point:	CA 5.5
Report author	Wozniak E. *et al.*
Report year	2020
Report title	Glyphosate affects methylation in the promoter regions of selected tumor supressors as well as expression of major cell cycle and apoptosis drivers in PBMCs (in vitro study)
Document No	Toxicology in Vitro 63 (2020)
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

The effect of glyphosate on selected epigenetic parameters and major cell cycle drivers in human peripheral blood mononuclear cells (PBMCs) was determined. The cells were incubated with glyphosate at 0.5, 10 and 100 μM. The analysis included: global DNA methylation, methylation in the promoter regions of tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1) by the Real-Time PCR and the expression profile of the indicated genes by Real-Time PCR. The obtained results have revealed significant reduction of global DNA methylation level in PBMCs exposed to glyphosate. Tested compound changed methylation pattern of the P21 and TP53 suppressor gene promoters, but in case of other analyzed genes: P16, BCL2 and CCND1 we did not identify any statistically significant changes. Gene profiling showed that glyphosate changed the expression of genes involved in the regulation of cell cycle and apoptosis. Glyphosate decreased expression of P16 and TP53 as well as an increase in the expression of BCl2, CCND1 and P21. Summing up, our results have shown a potential disturbance in methylation processes and gene expression in human PBMCs exposed to glyphosate, but the observed changes do not prejudge about the final metabolic effects, which are depended on many other factors.

Materials and methods

Chemicals; N-(phosphonomethyl)glycine (glyphosate) (purity 95%), fetal bovine serum (FBS), penicillin - streptomycin, TRIzol™ and using primers were bought from Sigma-Aldrich, (USA). RPMI 1640 medium with L-glutamine and lymphocyte separation medium (LSM) (1.077 g/cm^3) were purchased in Cytogen (Germany). Invisorb Spin Tissue Mini Kit was bought in Stratec (Germany). Methylated DNA Quantification Kit was bought in Abcam (United Kingdom). Transcriptor First Strand cDNA Synthesis Kit and FastStart Essential DNA Green Master was purchased from Roche (Basel, Switzerland). EZ DNA Methylation™ Kit was bought from Zymo Research (USA). Methyl Primer Express®, v.1.0 was obtained from Life Technologies. TRIzol™ Reagent was purchased from Thermo Fischer Scientific, Waltham, MA, USA. Other chemicals were from Roth (Germany) and POCh (Poland) and were of analytical grade.

Cells isolation; PBMCs were isolated from leucocyte-buffy coat obtained from blood purchased in Blood Bank in Lodz, Poland. Blood was obtained from four healthy volunteers (aged 18–55), who showed no signs of infection disease symptoms at the time the blood samples were collected. The investigation was approved by the Bioethics Committee of the University of Lodz No. 1/KBBN-UŁ/II/2017. Cells isolation was determined. The final PBMCs density used in the experiments (after addition of glyphosate) was 1×10^6 cells/mL.

Cells treatment; Glyphosate was dissolved in PBS, pH 7.4. The concentrations of glyphosate were from 0.5 to 100 μM (0.085–17 mg/L). In a previous study, no changes in cell viability after treatment of PBMCs with glyphosate were observed at the above mentioned concentrations. The cells were incubated

with investigated xenobiotic for 24 h in four independent experiments (four blood donors). During incubation, the cells were resuspended in RPMI supplemented with 10% FBS and penicillin/streptomycin solution (50 U/mL and 50 μg/mL, respectively) at 37°C, 5% CO_2. After incubation, the cells were centrifuged, glyphosate was discarded, and the cells were resuspended in RPMI medium.

Methylation levels

Global DNA methylation; Genomic DNA from human PBMCs was isolated using Invisorb Spin Tissue Mini Kit (Stratec Molecular GmbH, Berlin, Germany). Global DNA methylation was determined by colorimetric measurement of 5-methylcytosine in DNA using Methylated DNA Quantification Kit (Abcam). For global DNA methylation analysis, 100 ng of genomic DNA was used, following the protocol provided by the manufacturer. Methylation levels were calculated relatively to the methylated control DNA (included in the kit) and expressed as a percentage of total methylated DNA using the following formula:

$$5-\mathrm{mC}\% = \frac{(\text{Sample OD} - \text{Negative Control OD}) \div S)}{\text{Positive Control OD} - \text{Negative Control OD}) \times 2 \div P} \times 100\%$$

where:
S - the amount of input sample DNA in ng; P - the amount of input positive control in ng; 2 - a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC.

Methylation at promoter regions of tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1); Chemical modification of 500 ng of genomic DNA was analysed using EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, US), according to manufacturer's instruction. For methylation analysis, methylation-specific real-time PCR assay (MSP-PCR) was conducted with FastStart SYBR Green Master (Roche, Basel Switzerland). Bioinformatic analysis of the potential methylation sites within the promoter regions of the proto-oncogens BCL2 and CCND1 as well as methylated and unmethylated primers were designed by utilizing of Methyl Primer Express®, v.1.0 (Life Technologies, Carlsbad, CA, US). The DNA sequences around the transcription start sites (from -1000 to 300 bp) of both genes, were obtained from the DBTSS. The primer list is presented in Supplementary Table 1. All samples were amplified in duplicate and in the presence of positive control (CpG methylated Jurkat genomic DNA, fully methylated), negative control (5-Azadc-treated Jurkat genomic DNA) (NEB, Ipswich, MA, US) and blank control (water). The methylation status of a particular gene is expressed as methylation index (MI):

$$\Delta Ct = CtU - CtM$$

$$MI = \frac{1}{1 + 2^{-\Delta Ct}} \times 100\%$$

where: MI - methylation index [%]; Ct values of the methylated gene (M) were compared with the Ct values of the unmethylated gene (U).

Gene expression: RNA was extracted with TRIzol™ Reagent (Thermo Fischer Scientific, Waltham, MA, USA). RNA samples with a 260/280 nm ratio in the range of 1.8–2.0 were used for further analysis. cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel Switzerland). The cDNA was quantified by real-time PCR using FastStart SYBR Green Master (Roche, Basel Switzerland). Gene expression was normalized to the mean expression of all three housekeeping genes (GAPDH, RPL13, RPLP0) and was presented as a relative gene expression. The 2ΔCt (Ctgene–Ct mean from GAPDH, RPL13, RPLP0) method was used to calculate the expression levels of studied genes. The $2^{-\Delta Ct} \times 100$ values were re-calculated into relative copy number values.

Statistical analysis; The statistical analysis was performed with STATISTICA 13.1 data analysis software (2000 Stat-Soft, Inc., Tulsa, USA). Statistical analysis was conducted using the one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparisons procedure. The

difference was considered to be significant for $P < .05$. The individual analysis was performed on blood from four donors, while each experiment (conducted for blood from one donor) was repeated twice or three times depending on the method.

Results

Analysis of global DNA methylation level; Statistically significant changes in 5-mC level were observed in PBMCs treated with glyphosate (Fig. 1). As compared to control cells, the level of global DNA methylation was significantly decreased after glyphosate treatment at all concentrations tested: 0.5 μM, 10 μM and 100 μM.

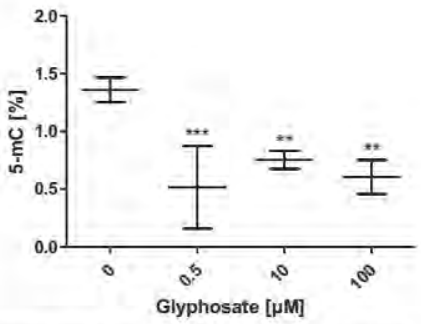


Fig. 1. 5-methylcytosine (%) in human PBMCs incubated with glyphosate (0.5–100 μM) for 24 h. Mean ± SD was calculated for four individual experiments. Statistically different from control at $^{**}p < .01$; $^{***}p < .001$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Analysis of methylation at promoter regions of the selected tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1); Statistically significant ($p < .05$, one-way ANOVA) decrease of methylation within P21 gene promoter was found in PMBCs treated with glyphosate from its lowest concentration of 0.5 μM (Fig. 2A). The opposite response to the investigated xenobiotic (0.5 μM) such as hypermethylation of gene promoter, was observed on TP53 tumor suppressor gene (Fig. 2A). In the case of other analyzed genes: P16, BCL2 and CCND1 we did not determine statistically significant changes in gene promoter methylation level (Fig. 2A, Fig. 3A).

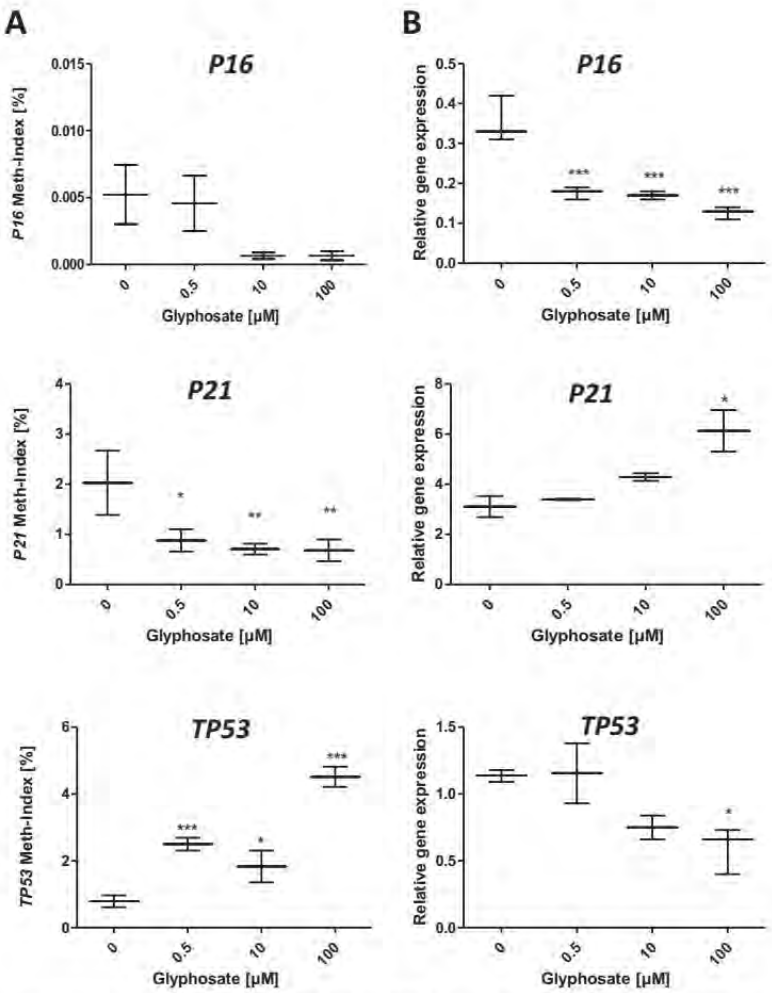


Fig. 2. Methylation (A) and expression (B) of suppressor genes (*P16*, *P21* and *TP53*) in human PBMCs incubated with and glyphosate (0.5–100 µM) for 24 h. Mean ± SD was calculated for four individual experiments. Statistically different from control at *p < .05; **p < .01; ***p < .001. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Analysis of gene expression of the selected tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1); The analysis of methylation of the selected gene promoters using Real-Time PCR let us assess their expression at the transcript level. A statistically significant decrease of P16 expression in PBMCs treated with all concentrations of glyphosate were identified (Fig. 2B). Glyphosate induced significant decrease in TP53 expression only at the highest concentration of 100 µM ($p < .05$, one-way ANOVA). Expression of other cell cycle drivers like P21 demonstrated a significant increase only at the highest concentration of glyphosate ($p < .05$, one-way ANOVA; Fig. 2B), which was also effective in increasing of BCL2 expression (Fig. 3B). Expression of the cyclin was significantly increased by two tested concentrations (10 µM and 100 µM) of glyphosate ($p < .05$, oneway ANOVA) (Fig. 3B).

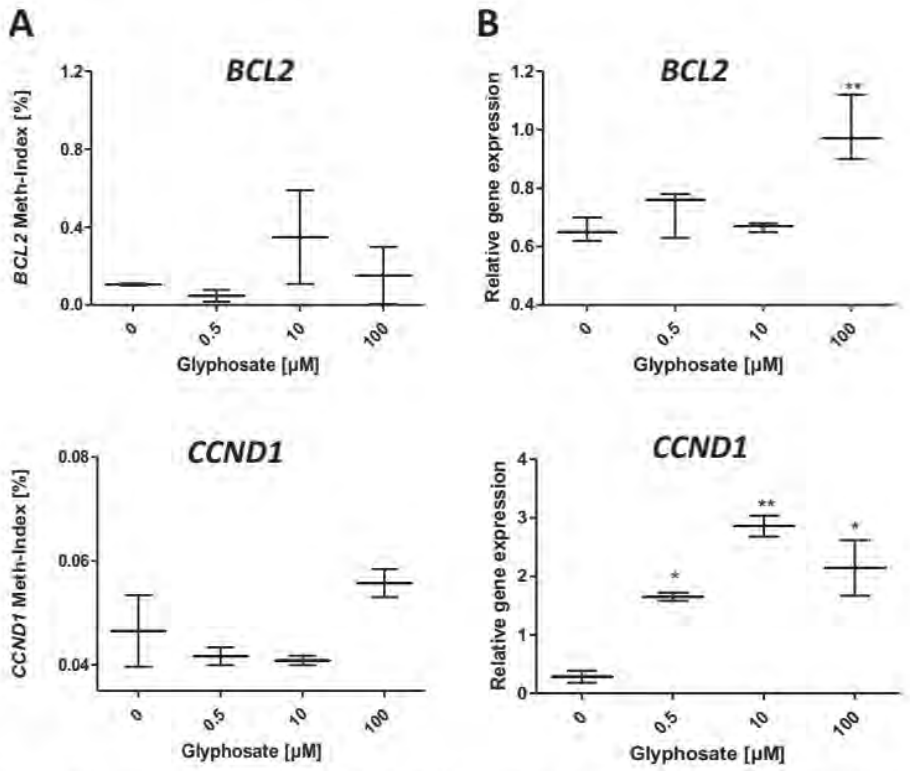


Fig. 3. Methylation (A) and expression (B) of proto-oncogenes (*BCL2* and *CCND1*) in human PBMCs incubated with and glyphosate (0.5–100 μM) for 24 h. Mean ± SD was calculated for four individual experiments. Statistically different from control at *p < .05; **p < .01. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Discussion

The knowledge that pollutants may influence the epigenome raises grave concerns concerning their long-term effects on chronic diseases development. A study showed that glyphosate can predispose breast cells to tumorigenesis via epigenetic reprogramming, which occur through TET3-mediated global and local DNA hypomethylation. Just recently, changes in the DNA methylation machinery due to glyphosate treatment were also identified in the fish model of Japanese medaka (Oryzias latipes), where upregulation of DNMT1, DNMT3a, Tet1 and Tet3 gene expression was shown. The current results have shown that treatment of PBMCs with glyphosate changed global DNA methylation profile. Decreased 5-mC level in PBMCs treated with low concentration of glyphosate that is comparable to that determined in human body after environmental exposure (0.5 μM) were found. Moreover, the obtained results are in agreement with previous findings, showing that glyphosate at high concentrations (determined in blood during glyphosate acute poisoning at 250–500 μM), reduces total DNA methylation in PBMCs. Recently, a study observed that glyphosate causes global DNA hypomethylation in non-neoplastic mammary epithelial MCF10A cells and triggers tumorigenesis in a "two-hit oncogenic model". Also a specific DNA hypomethylation signature of genes (i.e., local DNA hypomethylation), which was linked to the TET3 pathway that potentially can be used as an epimark to glyphosate exposure, was identified. Similar findings i.e. global hypomethylation of DNA were found in blood cells of tobacco farmers (n = 40) using pesticides and other xenobiotics during plant cultivation. However, literature data regarding potential epigenetic effects exerted by herbicides became contradictory. Recent epidemiological studies point to an increase of DNA methylation level under the combined effects of various pesticides, including glyphosate. It was found that long-term exposure of farmers (n = 137), employed at growing soybeans cultivation, to complex mixtures of pesticides, resulted in DNA hypermethylation and micronuclei formation in their blood cells probably due to impairment of DNA repair mechanisms. Nevertheless, the farmers were exposed to the mixture of different pesticides and heavy metals that are known to induce DNA hypermethylation and to disturb the cell cycle progression. These findings were confirmed in a study on children living in an industrial areas. In blood samples of young subjects exposed to increased heavy metals and polycyclic aromatic hydrocarbons concentrations, DNA hypermethylation was observed. Detailed analysis of selected genes promoters involved in cellular

metabolism regulation including cell cycle, i.e. TP53, P21, P16, CCND1 or BCL2, revealed significant changes in their methylation profiles. Beside global DNA hypomethylation we have found an increased methylation within the CpG islands of the TP53 gene promoter in PBMCs treated with glyphosate in all range of concentrations. The lowest concentration (0.5 μM) of glyphosate induced 5-mC methylation of the TP53 gene promoter. This result is in agreement with previous studies, where observed statistically significant increase of CpG islands methylation within the TP53 gene promoter after treatment of PBMCs with 250 μM and 500 μM glyphosate were identified. TP53 is known to be genome guard and tumor suppressor gene. It has been shown that hypermethylation of CpG islands within its promoter results in the silencing of gene transcription, but it is not clear-cut relationship in this case. Glyphosate reduced TP53 expression only from the concentration of 100 μM. Changes in TP53 expression and identified functions of p53 protein in the regulation of cell cycle directed us towards the findings of an author, who have shown that only Roundup but not glyphosate changed cell cycle of sea urchin embryonic cells due to delayed activation of the CDK1/cyclin B complex, acting downstream from p53. The effect of glyphosate on cell cycle was also excluded in the experiments on human lymphocytes; however the tested concentrations range of this compound was between 0.0125 and 0.5 μg/mL (0.07–2.9 μM), while the current study showed that glyphosate at 100 μM caused downregulation of TP53 expression in PBMCs. Beside hypermethylation of TP53 gene promoter and down regulation of its transcript level in PMBCs treated with glyphosate (100 μM), hypomethylation within P21 gene promoter was detected for all glyphosate concentrations. Changes in methylation of the P21 gene promoter were correlated with increased expression of the cell cycle regulator at 100 μM of glyphosate, that would suggest inhibition of PBMCs cycling in G0/G1 phase. Further analysis of molecular drivers of the cell cycle revealed a decreased expression of the P16 gene encoding the cell cycle inhibitor in glyphosate-treated cells, staring from its lowest concentration comparable to that occurring during environmental exposure. However, the analysis carried out did not show any changes in the methylation level within the P16 gene promoter region in PBMCs due to glyphosate treatment. These results correlate with previous findings, in which higher concentrations of glyphosate (250 μM and 500 μM) also did not cause any changes in methylation of P16 gene promoter. The protein product of P16 gene, p16, is an inhibitor of cyclin D. Literature data suggests that low expression of P16 may result in overexpression of cyclin D1 (CCND1), activation of its complexes with CDK4 and CDK6 complexes (cyclin D1/CDK4, cyclinD1/CDK6) and overcoming the G0/G1 checkpoint of the cell cycle. In the tested experimental conditions, an increased expression of the CCND1 due to glyphosate treatment at its whole tested range of the concentrations (0.5–100 μM) was identified, but no statistically significant changes in the methylation level of the gene promoter has been shown. Beside of changes in the expression level of the major cell cycle regulators and/or methylation pattern within their gene promoters, significant upregulation of the BCL2 expression at the mRNA level at the highest tested concentration of glyphosate (100 μM) was also identified. Therefore, this data may suggest a disturbance of apoptosis induction, due to sustained antiapoptotic abilities of BCL2. In contrast, in vitro studies on the effect of glyphosate on apoptosis in mature rat testicular cells and PBMCs showed that glyphosate only at very high concentrations (29.57 mM/0.5 mM, in rat testicular cells and PBMCs; respectively) induced apoptosis. Thus, the obtained data may not result in specific metabolic effects until the exposure to high doses or cumulative long-term exposure of humans to glyphosate will be preceded.

Conclusion

This study has attempted to assess epigenetic mechanisms of action of glyphosate in human PBMCs, which has been poorly studied in cellular models including blood cells. The conducted analysis have shown that glyphosate significantly affected global DNA methylation of PBMCs as well as methylation in the promoter regions of selected tumor suppressors (P21 and TP53) as well as expression of major cell cycle and apoptosis drivers (P16, TP53, BCl2, CCND1 and P21). Changes in the DNA methylation profile were minimally correlated with gene expression level, however, regulation of transcription process is performed at many different levels and further and more global analysis (genome-wide based on) are necessary to give clear answer about epigenetic-transcriptomic changes induced by glyphosate. It should be noted that glyphosate induced changes in concentrations corresponding to environmental or occupational exposure. Conducting of further in vitro studies on various epigenetic modifications caused by glyphosate with different target cell cultures is still warranted.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective was to assess epigenetic mechanisms of action of glyphosate in human PBMCs, which has been poorly studied in cellular models including blood cells. The study was conducted using an *in vitro* test system. The ability of glyphosate to impact the measured parameters was demonstrated (global DNA methylation of PBMCs, methylation in the promoter regions of selected tumor suppressors (P21 and TP53), and expression of major cell cycle and apoptosis drivers (P16, TP53, BCl2, CCND1 and P21). However, a positive control was not used, and a clear dose-response was not established for all of the measured parameters. Additionally, the measured effects in vitro are not clearly linked to an adverse outcome in vivo. While it is stated that the concentrations used are comparable to environmental exposure, external exposure was not linked to a corresponding internal concentration. Therefore, it is not possible to calculate a dose for risk assessment purposes. The study is useful for supplemental information on *in vitro* effects resulting from glyphosate exposure, but, is not appropriate for derivation of an endpoint in human health risk assessment.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because the assays conducted comply in general with the quality criteria for *in vitro* testing.

Reliability criteria for *in vitro* toxicology studies

Publication: Wozniak *et al*., 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95%. Source: Sigma-Aldrich, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	PBMC.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.5 to 100 µM
Cytotoxicity tests reported	Y	Ref. to earlier paper.
Positive and negative controls	Y	Methylated control DNA as positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because the assays conducted comply in general with the quality criteria for in vitro testing.

1. Information on the study

Data point:	CA 5.6
Report author	Zhang, J-W. *et al.*
Report year	2019
Report title	The toxic effects and possible mechanisms of glyphosate on mouse oocytes
Document No	Chemosphere 237 (2019) 124435
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities.
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The effects of glyphosate on oocyte maturation, as well as its possible mechanisms remain unclear. The present study revealed that mouse oocytes had reduced rates of germinal vesicle breakdown (GVBD) and first polar body extrusion (PBE) after treatment with 500 mM glyphosate. Reactive oxygen species (ROS) were found in mouse oocytes exposed to glyphosate, as shown by changes in the mRNA expression of related antioxidant enzyme genes (*cat, sod2, gpx*). After 14 h of exposure to glyphosate, metaphase II (MII) mouse oocytes displayed an abnormal spindle morphology and DNA double-strand breaks (DNA-DSBs). Simultaneously, mitochondria showed an aggregated distribution and decreased membrane potential in mouse oocytes exposed to glyphosate. The protein expression levels of apoptosis factors (Bax, Bcl-2) and the mRNA expression levels of apoptosis-related genes (*bax, bcl-2, caspase3*) were measured by Western blot and qRT-PCR, respectively. Meanwhile, the expression levels of autophagy-related genes (*lc3, atg14, mtor*) and proteins (LC3, Atg12) were significantly decreased in the glyphosate treatment group compared with the control group.

Materials and methods

Antibodies and chemicals: Rabbit polyclonal anti-LC3A/B (light chain 3, LC3), anti-p-MAPK, anti-g-H2AX and anti-b-actin antibodies were purchased from Cell Signaling Technology. Mouse monoclonal anti-Bax, anti-Bcl-2, antiAtg12, anti-Annexin V and anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology. The Reactive Oxygen Species Assay Kit was purchased from Beyotime Biotechnology (S0033). The Mitochondrial Membrane Potential Assay Kit with JC-1 was obtained from Beyotime (C2005). Mito Tracker Red CMXRos was purchased from Cell Signaling Technology (#9082).

Mice: Female Kunming mice (25-30 g) were purchased from the Institute of Zoology, Chinese Academy of Sciences. They were housed in a temperature-controlled room with 12D:12L (dark vs. light) and had unrestricted access to food and water under conditions of constant temperature (23 ± 2° C).

Oocyte collection and treatment; To collect fully grown GV oocytes, the mice were superovulated by injecting them intraperitoneally with 10 IU Pregnant Mares Serum Gonadotropin (PMSG) 48 h earlier. The mice were sacrificed by cervical dislocation and the ovaries were placed in M2 medium. Oocytes were released from the ovaries by puncturing the follicles with a fine needle, and denuded oocytes were collected by gentle pipetting. GV oocytes were cultured in an incubator in 50 μL droplets of culture medium under liquid paraffin oil at 37° C in 5% CO_2. Glyphosate was dissolved in M16 medium and diluted to a final concentration of 50, 100, 200 or 500 μM. After culturing for 2 h or 14 h, GVBD and MII oocytes were used for the subsequent experiments.

Immunofluorescent staining: To determine the levels of intracellular ROS production, denuded MII oocytes from 6 mice were incubated with M16 medium that contained 10 μM dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37° C in the dark. After washing three times with 1% Bovine Serum Albumin/Phosphate-buffered saline (BSA/PBS), the oocytes were placed in 50 μL of M16 medium droplets and the fluorescence was observed with a confocal laser-scanning microscope (Zeiss LSM 710

META, Germany) with the same scanning parameters. The fluorescence intensity of each oocyte was analysed using ImageJ software. For mitochondrial staining, MII oocytes from 6 mice were fixed in 4% paraformaldehyde (PFA) for 1 h and then placed in membrane permeabilization solution containing 0.5% Triton X-100 for 20 min at room temperature. After washing three times with 1% BSA/PBS, the oocytes were incubated in 200 nM Mito Tracker Red CMXRos in M16 medium for 30 min in the dark. After several washes, the oocytes were costained with DAPI (4', 6-diamidino-2-phenylindole) for 5 min. Images were captured by the confocal laser-scanning microscope. To measure mitochondrial membrane potential (MMP), MII oocytes from 6 mice were incubated at 37° C for 20 min with the 1 x JC-1 probe, then washed twice with JC-1 buffer (5 min each) to remove surface fluorescence. Images of fluorescence were captured using confocal microscopy as above. MMP was quantified as the ratio of red to green fluorescence using ImageJ software. To measure the spindle, apoptosis, autophagy and DNA damage, MII oocytes from 24 mice were stained with anti-α-tubulin, antiAnnexin-V, anti-LC3and anti-γ-H2AX antibodies, respectively. The denuded MII oocytes were fixed with 4% PFA for 30 min, permeabilized in 0.5% Triton-X 100 for 20 min at room temperature, and blocked with 1% BSA/PBS for 1 h at 4 C. The oocytes were incubated with anti-α-tubulin (1:200), anti-LC3 A/B (1:200), antiAnnexin-V (1:200) and anti-γ-H2AX (1:200) at room temperature for 2 h followed by three washes with 1% BSA/PBS. The oocytes were then incubated with an appropriate secondary antibody for 2 h at room temperature and then costained with DAPI for 5 min in the dark. After three washes, oocytes were mounted on a glass slide and representative images were captured by the confocal laser-scanning microscope. ImageJ software was used to quantify the fluorescence intensity in the images.

Quantitative real-time PCR: Total RNA was extracted from 100 MII oocytes from 12 mice using the TRNpure Total RNA Kit (Nobelab, Beijing, China) according to the manufacturer's instructions. The first strand cDNA was generated with the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, China). All gene expression was determined using the FS Universal SYBR green PCR master mix (Roche, Canada) under the following conditions: 50°C for 2 min, 95° C for 10 min, 40 cycles of 95° C for 15 s and 60° C for 1 min. Each sample was tested in triplicate and *gapdh* was used as an internal control gene. Relative expression levels were analysed by the $2^{-\Delta\Delta Ct}$ method.

Western blot: A total of 200 MII oocytes from 24 mice were lysed in 1 x SDS sample buffer containing 1 x protease inhibitor Cocktail (cwbiotech) on ice for 20 min. They were boiled for 5 min at 95° C, then subjected to 12% SDS-PAGE and the separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked for 2 h with 5% nonfat milk in TBST and then probed with primary antibodies for 2 h at room temperature (anti-p-MAPK antibody, 1:500; anti-Bcl-2 antibody, 1:1000; anti-Bax antibody, 1:1000; anti-LC3 antibody, 1:1000; anti-Atg12 antibody, 1:500; anti-α-tubulin antibody, 1:1000; and anti-β-actin antibody, 1:1000). After washing three times with TBST (10 min each), membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibodies (1:1000). Three washes later, the protein bands were visualized using BeyoECL Plus (Thermo Fisher Scientific), and the signals were acquired by Tanon 5500. The images were quantified using ImageJ software.

Statistical analysis; At least 50 oocytes were analysed for each experiment. For each treatment, at least three biological replicates were performed and data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using the GraphPad Prism software followed by Student's unpaired two-tailed t-test and two-way ANOVA. Statistical significance was set at P value: *<0.05, **<0.001, ***<0.0005, ****<0.0001.

Results

Glyphosate composition analysis: The chemical composition of glyphosate was analysed before the start of the experiment by Ultra Performance Liquid Chromatography/Quadrupole-Time-of-Flight-Mass Spectrometry (UPLC/Q-TOF-MS). Specific results from this analysis were not reported.

Glyphosate treatment reduced the GVBD and PBE in mouse oocytes: To test the effect of glyphosate on the mouse oocytes maturation, the oocytes were cultured in culture medium supplemented with increasing concentrations of glyphosate (50, 100, 200 or 500 μM). Typical images of exposure to glyphosate on GVBD and PBE are shown in Fig. 2. After treatment with glyphosate, GVBD (Fig. 2A

and B) and PBE (Fig. 2C and D) were significantly decreased in the 200 mM and 500 mM groups, while there was no significant variation in the 50 μM and 100 μM treatment groups. Hence, the 500 μM glyphosate treatment was adopted for subsequent experiments. These observations were considered to show that glyphosate exposure decreased mouse oocyte developmental competence.

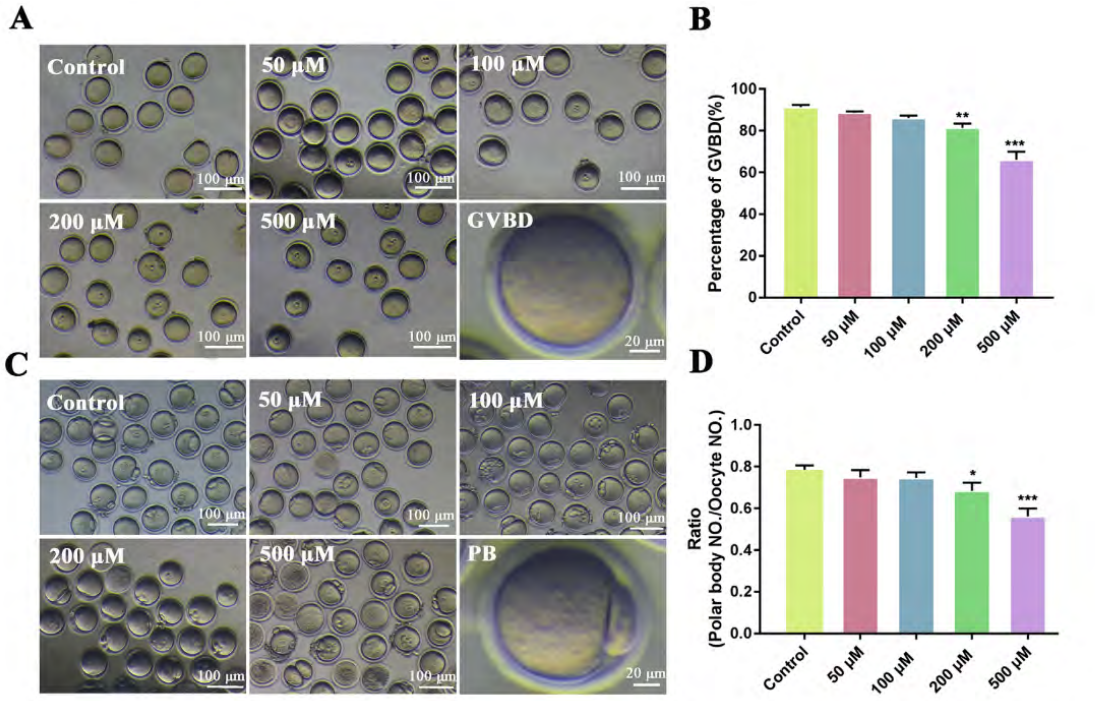


Fig. 2. Effects of glyphosate on GVBD and PBE in mouse oocytes. (A) Representative images of mouse oocytes after exposure to different concentrations of glyphosate for 2 h. (B) GVBD rates of different groups after 2 h of treatment. The data are expressed as the mean ± SEM. (C) Representative images of mouse oocytes after exposure to different concentrations of glyphosate for 14 h. (D) Quantification of the presence of PBE in control and treatment oocytes. The data are expressed as the mean ± SEM.

Glyphosate treatment increased ROS generation and DNA damage in mouse oocytes: It was tested whether defects observed in glyphosate-exposed mouse oocytes were mediated by oxidative stress. As shown in Fig. 3A and B, the fluorescence intensity of DCFH-DA was significantly higher in the 500 μM glyphosate treatment group than in the control oocytes. The levels of *sod2* and *gpx* mRNA expression were significantly increased in glyphosate-exposed oocytes when compared with the control group (Control: sod2: 1.005 ± 0.066, gpx: 1.003 ± 0.052; 500 μM: sod2: 1.702 ± 0.148, gpx: 1.545 ± 0.181); the expression of *cat* mRNA was also increased (Control: 1.001 ± 0.036; 500 μM: 1.241 ± 0.111). These results suggested that glyphosate administration enhanced ROS production.As described above, glyphosate is cytotoxic to oocytes. We further glyphosate treatment would cause DNA damage in mouse oocytes using g-H2AX staining, which indicates the presence of DNA-DSBs. As shown in Fig. 3D, there were no significant DSB foci in the control oocytes; however, there was a dense mass of γ-H2AX foci associated with chromatin in glyphosate-exposed oocytes. As shown in Fig. 3E, the fluorescence intensity of γ-H2AX also significantly increased after glyphosate treatment compared to that in the control group. These results were considered to indicate that glyphosate treatment results in DNA damage in mouse oocytes.

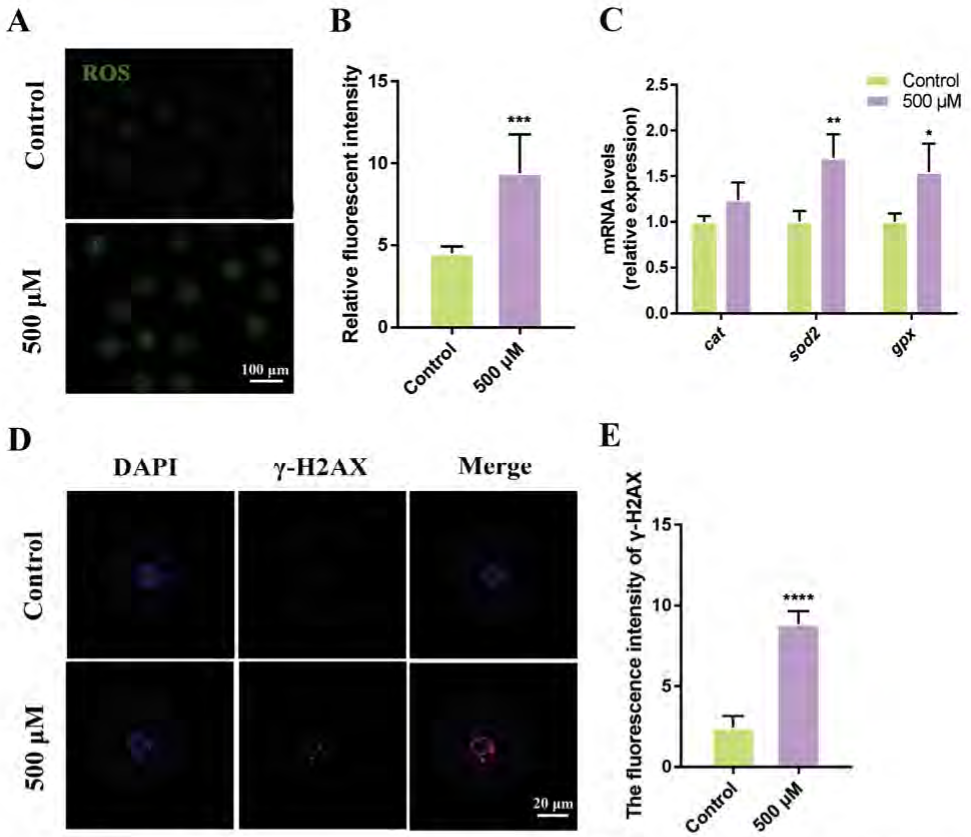


Fig. 3. Effects of glyphosate on ROS generation and DNA damage in mouse oocytes. (A) Representative images of DCFH-DA fluorescence in control and glyphosate-exposed mouse oocytes. (B) Quantitative analysis of ROS fluorescence intensity in control and glyphosate-exposed groups. The data are expressed as the mean ± SEM. (C) Relative expression levels of oxidative stress-related genes were examined by qRT-PCR. The data are expressed as the mean ± SEM. (D) Representative images of control and glyphosate-treated oocytes exhibiting γ-H2AX immunostaining. (E) The γ-H2AX fluorescence intensity obviously increased after glyphosate treatment. The data are presented as the mean ± SEM.

Glyphosate treatment disturbs spindle morphology in mouse oocytes: To investigate whether the oocytes with glyphosate treatment showed defects in spindle positioning and chromosome scattering, MII oocytes were assessed by immunocytochemical staining with anti-α-tubulin antibody and DAPI. In contrast to oocytes from the control group, which presented a normal spindle appearance and well-aligned chromosomes at the equatorial plate, the glyphosate-exposed group showed misaligned chromosomes and abnormal spindle morphology(Fig. 4A). The rate of abnormal spindles in the glyphosate treatment group was also increased (Control: 9.333 ± 0.491%; 500 μM: 15.767 ± 1.369%). as shown in Fig. 4BThe effect of glyphosate on the expression levels of spindle assembly regulatory protein was also assessed. In Fig. 4C and D, Western blots revealed that the level of p-MAPK protein was significantly reduced after 500 μM glyphosate treatment (Control: 0.856 ± 0.053; 500 μM: 0.517 ± 0.070). These results were considered to suggest that glyphosate treatment disturbs spindle morphology in mouse oocytes.

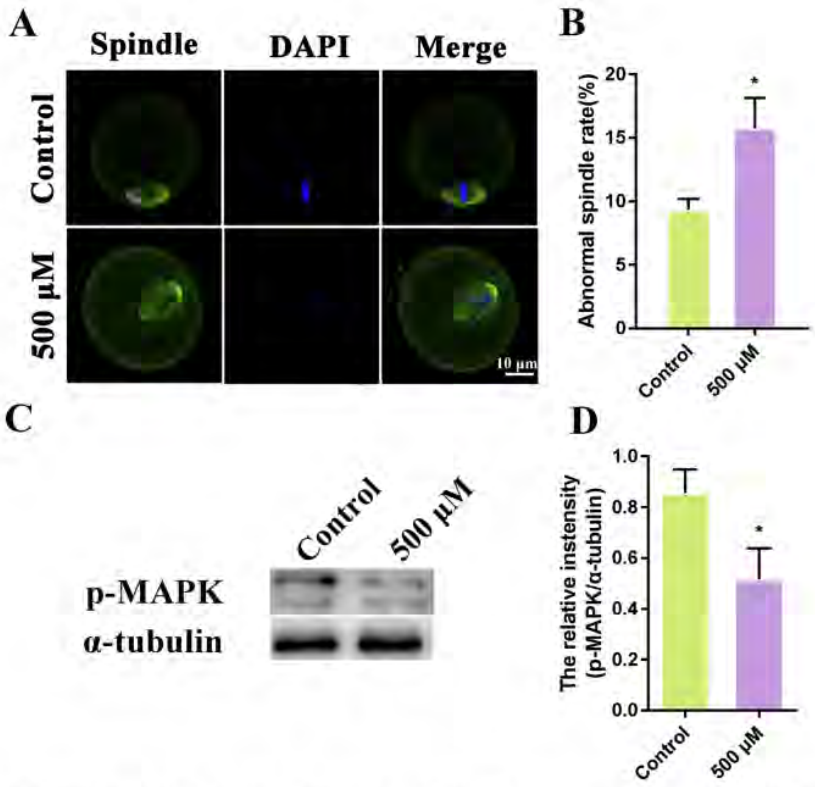


Fig. 4. Effects of glyphosate on spindle morphology and chromosome alignment in mouse oocytes. (A) Representative confocal images of spindle morphology in control and glyphosate exposed groups. (B) Percentage of cells exhibiting abnormal spindle/chromosome morphology. The data are expressed as the mean ± SEM. (C) The expression level of p-MAPK protein in oocytes from different groups was analysed by Western blot analysis. (D) The relative intensity of p-MAPK protein expression (p-MAPK/α-tubulin) was significantly reduced after treatment with 500 μM glyphosate. The data are expressed as the mean ± SEM.

Glyphosate treatment resulted in mitochondrial injury in mouse oocytes: A homogeneous distribution of mitochondria was present in the control group,but an aggregated distribution was seen in the glyphosate-exposed group by staining with Mito Tracker Red(Fig. 5A). Alterations in MMP of mouse oocytes from different groups were evaluated by staining with JC-1 (Fig. 5B). As shown in Fig. 5C, the result illustrated that the MMP was significantly lower in the glyphosate-exposed group compared with the control group (Control: 1.871 ± 0.082; 500 μM: 4.517 ± 0.155). Based on these data, glyphosate treatment was considered to interfere with mitochondrial function in mouse oocytes.

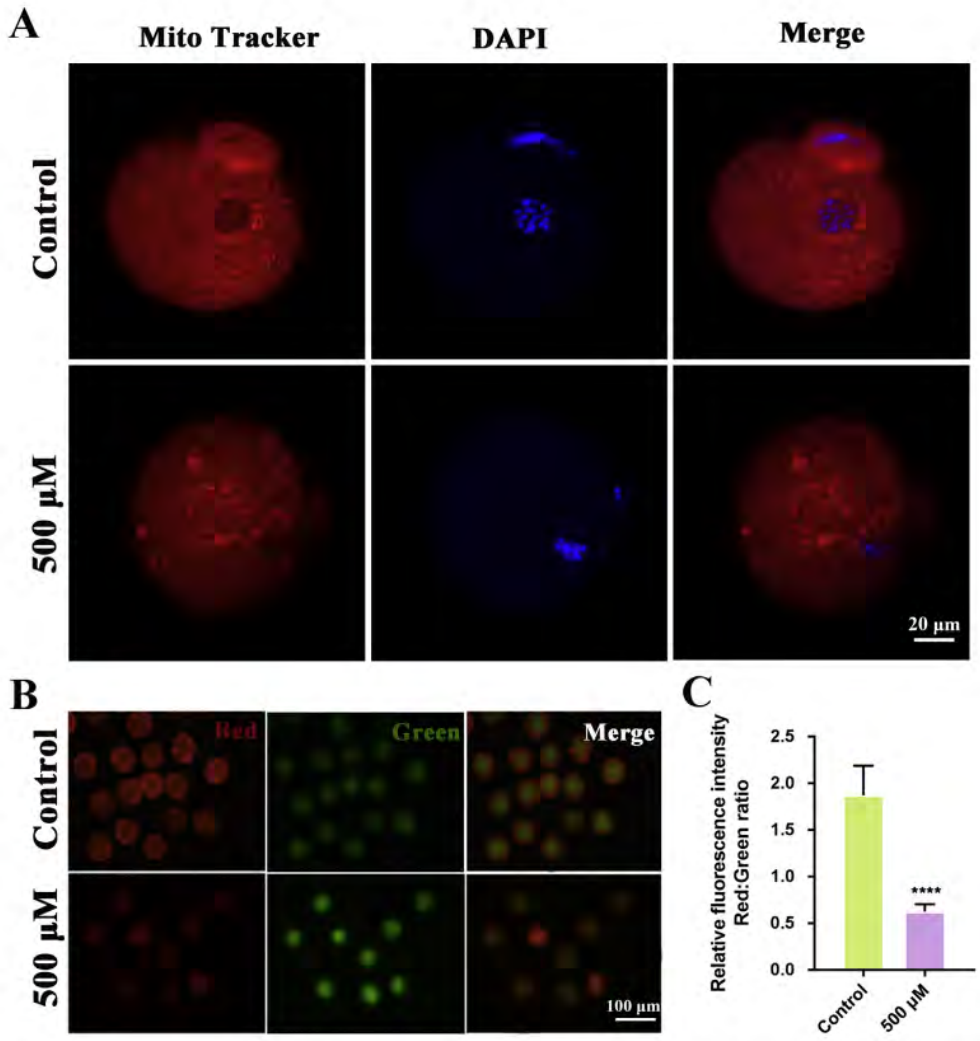


Fig. 5. Glyphosate treatment results in mitochondrial injury in mouse oocytes. (A) Representative images of mitochondrial distribution in control and glyphosate-treated oocytes by staining with MitoTracker Red. (B) Representative images of MMP in mouse oocytes from different groups stained with JC-1. (C) MMP levels (red/green fluorescence intensity) in glyphosate-exposed oocytes was significantly lower than that in the control group. The data are presented as the mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Glyphosate treatment induced early apoptosis and autophagy in mouse oocytes: Annexin-V staining was conducted to identify whether early apoptosis occurred in glyphosate-treated oocytes. In the control group, Annexin-V signals were detected only in the zona pellucida, whereas the treatment group had a clear green signal in the membrane and zona pellucida Fig. 6A). In Fig. 6B, the apoptotic fluorescence intensity of oocytes was notably higher in the treatment group than in the controls. Apoptosis-related protein expression levels were also assayed via As shown in Fig. 6G and H, Western blot analysis, which showed that the expression of Bcl-2 protein decreased and the expression of Bax protein increased after exposure to 500 μM glyphosate (Control: Bcl-2: 0.645 ± 0.009, Bax: 0.427 ± 0.020; 500 μM: Bcl-2: 0.549 ± 0.011, Bax: 0.556 ± 0.018). To confirm this result, the mRNA expression levels of apoptosis-related genes were tested by qRT-PCR. The qRT-PCR results were consistent with the Western blot results. Together, these results were considered to indicate that glyphosate induced early apoptosis in mouse oocytes. Whether autophagy had occurred in glyphosate-treated oocytes was assessed by LC3 immunofluorescent staining; As shown in Fig. 6D and E, the fluorescence intensity was significantly higher in the 500 μM glyphosate treatment group than in the oocytes from the control group. The mRNA expression levels of autophagy-related genes were also assessed by qRT-PCR and showed an increasing trend (Control: *lc3*: 0.860 ± 0.013, *atg14*: 1.024 ± 0.149, *mtor*: 0.847 ± 0.171; 500 μM: *lc3*: 1.591 ± 0.109, *atg14*: 1.800 ± 0.139, *mtor*: 1.268 ± 0.242 (Fig. 6F). Western blot analysis showed that the expression of LC3 and Atg12 protein increased after exposure to 500 μM glyphosate (Control: LC3: 0.280 ± 0.021, Atg12: 0.435 ± 0.007; 500 μM: LC3:0.504 ± 0.030, Atg12: 0.580 ± 0.016). Together, these results were considered to indicate that glyphosate induced autophagy in mouse oocytes.

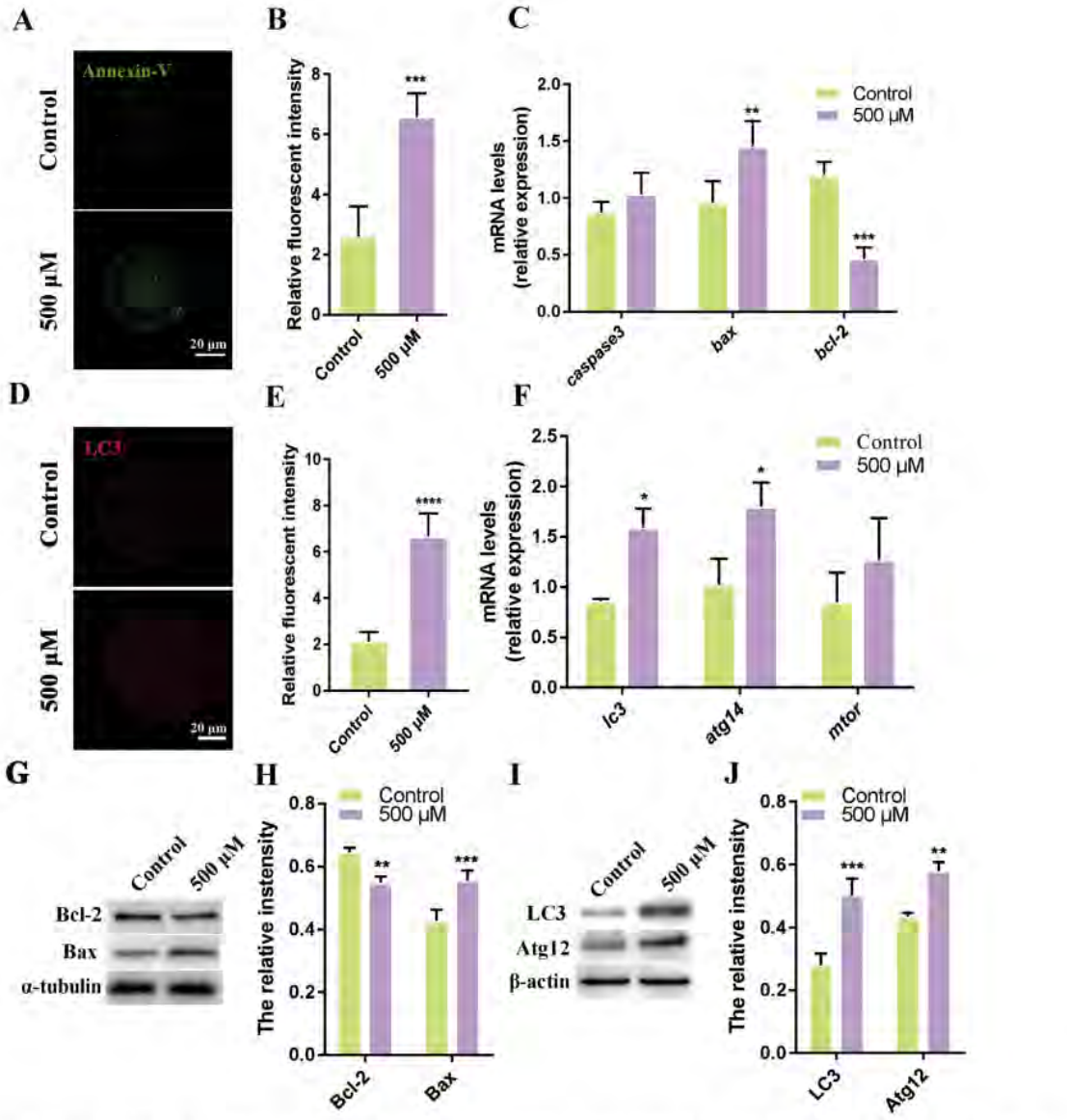


Fig. 6. Glyphosate treatment induces early apoptosis and autophagy in mouse oocytes. (A) Representative images of apoptosis (Annexin-V staining) in mouse oocytes. (B) Quantification of Annexin-V signal was recorded in control and glyphosate-exposed oocytes. (C) Relative mRNA levels of *caspase3*, *bax* and *bcl-2* in different groups of oocytes. (D) Representative images of autophagy (LC3 staining) in mouse oocytes. (E) Quantification of the fluorescence signal was recorded for the control and glyphosate-exposed oocytes. (F) The relative mRNA level of *lc3*, *atg14* and *mtor* in different groups oocytes. (G) The expression levels of apoptosis-related protein in oocytes from different groups were analysed by Western blot analysis. (H) The relative intensities of Bcl-2 and Bax protein expression were calculated. (I) The expression levels of autophagy-related proteins in oocytes from different groups were analysed by Western blot analysis. (J) The relative intensities of LC3 and Atg12 protein expression were calculated.

Discussion

In recent years, numerous studies have demonstrated the damaging effects caused by glyphosate in various organisms using different test systems. However, few studies evaluated the reproductive effects of glyphosate on female mammals. Therefore, we investigated the toxic effects of glyphosate on mouse oocyte maturation in vitro. Based on the experimental results, we believed that glyphosate contributed to the destruction of oocyte development . Germinal vesicle breakdown and the first polar body extrusion are markers of oocyte nuclear maturation. Our experimental results indicated that mouse oocytes exposed to glyphosate in vitro experienced a decrease in the proportion of GVBD and PBE. The decrease in the ratio of GVBD and PBE indicates that the growth and developmental ability of the oocyte is destroyed. Reactive oxygen species are excessively generated when cells are subjected to various noxious stimuli, and the oxidative system and the antioxidant system are out of balance, resulting in cell damage. Therefore, we examined the level of ROS in mouse oocytes after glyphosate exposure, and our results showed that ROS levels were increased. To survive, the expression of the antioxidant enzyme genes is increases increased. The mRNA expression levels of sod2, cat and gpx were significantly increased in mouse oocytes exposed to glyphosate. Mouse oocytes exposed to glyphosate produced

excessive ROS, which in turn affected their growth and development. Oocytes are rich in mitochondria. These mitochondria play a leading role in cell metabolism. The changes in their morphology, quantity, distribution and function are closely related to cell metabolism, proliferation and differentiation. Mitochondria are mostly uniformly distributed in the cytoplasm in oocytes with higher developmental potential, while they are aggregated in oocytes with lower developmental potential. A lack of mitochondrial rearrangement in the cytoplasm is a marker of immature cytoplasm. Our results showed that mouse oocytes exposed to glyphosate had aggregated mitochondria and a decrease in membrane potential. These findings indicated that glyphosate destroyed the function of the mitochondria in oocytes. The cytoskeleton plays an important supporting role in the oocyte maturation process. The morphology and kinetic changes of the spindle may reflect the quality of oocytes, and its integrity determines the correctness of chromosome segregation. Abnormalities in spindle morphology will lead to meiosis and fertilization failure. MAPK is a regulated protein involved in microtubule assembly and mitotic spindle assembly. The experimental results showed that after glyphosate exposure, the expression level of p-MAPK protein in mouse oocytes was decreased. So, we proposed that glyphosate affected formation of the spindle by altering the level of p-MAPK, which in turn resulted in a reduction in the rate of mouse oocyte maturation. Excessive accumulation of ROS products has an adverse effect on lipids, proteins, and DNA. In addition, ROS damages the sugarphosphate back-bone of DNA, resulting in single- and double-strand breaks. DNA damages in cells primarily originate from oxidative stress. g-H2AX produced by phosphorylation of H2AX can be used as a biomarker to clearly reflect the extent of DNA damage and repair. Oocytes exposed to glyphosate showed an increase in g-H2AX foci, showing that glyphosate could destroy DNA integrity and produce cytotoxicity in oocytes. Oxidative stress is often associated with apoptosis and autophagy. Therefore, we first detected apoptosis in oocytes by Annexin-V staining. Our experimental results showed that the apoptotic fluorescence intensity of oocytes was increased after glyphosate exposure. The Bcl-2 gene is an inhibitor of apoptosis, and Bax is a pro-apoptotic gene that plays an important role in regulating apoptosis. In the mitochondrial mediated apoptosis pathway, abnormal intracellular signals lead to the activation of Bax, inhibit the expression of Bcl-2, induce mitochondria release of cytochrome c, form apoptotic bodies or initiate caspase cascade activation, and then lead to the occurrence of apoptosis. Western blot analysis and qRT-PCR showed that the expression of Bcl-2 was decreased and the expression of Bax was increased in glyphosate-treated oocytes. Although there was no significant difference in caspase3 mRNA expression, there was an increasing trend after exposure to glyphosate. Autophagy mainly includes phagophore, autophagy formation, autophagosome formation and degradation. The combination of PI3K complex and Atg14 is involved in the formation of phagophore. Atg gene regulates the formation of autophagosomes by forming Atg12-Atg5 and LC3-II complexes. Microtubule associated light chain protein (LC3) is present in autophagosomes; therefore, the LC3 protein is widely used as a marker for autophagosomes. Mammalian target of rapamycin, mTOR, also plays a role in the autophagy pathway. Our results showed that the fluorescence intensity of LC3 was significantly increased, and the expression levels of autophagy-related proteins and genes were increased in the glyphosate treatment group. We considered that oocytes exposed to glyphosate-induced ROS, which could further activate autophagy and early apoptosis, experience a decrease in oocyte quality.

Conclusion

In summary, glyphosate exposure was considered to have caused a block in mouse oocyte development, spindle assembly disruption and chromosome scattered distribution, mitochondrial aggregation and membrane potential reduction, DNA damage, and increased oxidative stress levels, which then led to cellular apoptosis and autophagy. These Our results were considered to provided evidence for the toxic effects of glyphosate on reproductive systems.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In vitro intracellular changes in Kunming mice oocytes were evaluated after being cultured in medium supplemented with 500 μM glyphosate. Findings included: decreased germinal vesicle breakdown, decreased first polar body extrusion, increased mRNA expression of anti-oxidant enzyme related

genes, abnormal spindle morphology, increased DNA double strand breaks, aggregated mitochondria, decreased mitochondrial membrane potential, increased protein expression of apoptosis factors, increased mRNA expression of apoptosis related genes and decreased autophagy-related genes.

No dose-response could be determined seeing as only one concentration was tested, far in excess of that considered biologically relevant. Whilst some evaluations were conducted on oocytes harvested from a wider data set of 24 mice (protein expression levels of apoptosis factors by Western blot analysis), a number of the assessments were conducted on oocytes from just 12 mice (mRNA expression of oxidative stress-related, apoptosis-related and autophagy-related genes) or 6 mice (mitochondrial staining, measurement of mitochondrial membrane potential). This narrow source of oocytes limits the robustness of certain conclusions. Furthermore, there are insufficient details reported in the methods to establish whether mice were of the same age before oocyte harvesting or the purity of the glyphosate tested.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the poor characterization of the glyphosate tested, no cytotoxicity testing, the lack of a positive control and insufficient dose-response characterization at biologically relevant doses.

Reliability criteria for *in vitro* toxicology studies

Publication: Zhang JW *et al*., 2019.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity and source not reported. Chemical analysis was performed but the results were not clear.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	50, 100, 200, 500 µM. Most of the assays were carried out at 500 µM which is a concentration that cannot be reached systemically in the rat at 2000 mg/kg bw after oral intake.
Cytotoxicity tests reported	N	

Positive and negative controls	N	No positive control used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the poor characterisation of the glyphosate tested, no cytotoxicity testing and the lack of a positive control.		

グリホサートカリウム塩

要旨及び評価結果

（環境動態）

検索期間：2010 年 1 月 1 日～2019 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Albers, Ch., et al.
Report year	2018
Report title	Soil Domain and Liquid Manure Affect Pesticide Sorption in Macroporous Clay Till
Document No	Journal of Environmental Quality
Guidelines followed in study	OECD 106
Deviations from current test guideline	1 mM $CaCl_2$ solution (standard: 10 mM solution), at 10°C (standard: 20 – 25°C); 4 concentrations (standard: 5), no explicit measurements of concentrations in the solid phase, i.e. no real mass balances available
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Reliable with restrictions (the study does not meet the validity criteria as required according the OECD 106 guideline)

2. Full summary of the study according to OECD format

In this study, it was observed that sorption of strongly sorbing pesticide, glyphosate, varied by more than an order of magnitude across soil domains in 5-m-deep clay till profiles with biopores and fractures. Eight soil domains were identified in each of the profiles: five matrix soils and three in the macropores. Glyphosate showed high variation in sorption between fractures and matrix soil from the same depths. The domain-specific sorption of both tebuconazole and glyphosate was, however, overruled by dilute liquid manure. Liquid manure unexpectedly had a greater effect on glyphosate sorption, which was strongly decreased by dissolved organic matter and phosphate in the manure. The variation in sorption across domains, as well as the effects of liquid manure, should be taken into account when assessing leaching risks.

Materials and methods

Soil sampling

Soil was sampled at two locations, Gjorslev (55°20.988′N, 12°23.672′E) and Lund (55°14.698′N, 12°17.418′E) in the Stevns area of southeastern Denmark. At both sites, soil profiles were excavated to a depth of ~5 m. We sampled composite soil samples from eight domains that were clearly separated on the basis of different soil horizons and the presence or absence of biopores and fractures (Fig. 1). The surface of wormholes (Domain 3) was sampled by scraping off the outer 1 to 2 mm of the pore walls. Deeper soil pores surrounding decayed roots were dissected out, and the outer Fe oxides were scraped off with a knife to sample only the 5- to 10-mm wide, inner, greyish part (Domain 5). The surface of even deeper larger fractures with Mn and Fe oxide coatings was sampled by scraping off the outer 1 to 2 mm (Domain 7). At least 240 g soil was sampled from each fracture domain to have sufficient material for sorption experiments and analysis of sediment parameters. The matrix soil samples (bulk soil in the case of the plow layer, Domain 1) were also compositely sampled, comprising ~1 kg from 20 to 50 subsamples. All soil samples were sieved twice through a 2-mm sieve and stored at 2°C. Soil samples from the reduced zone were packed in airtight aluminum tape on location and sieved in a glove box under a reducing N_2/H_2 atmosphere. The fraction <2 mm was stored in anoxic jars at 2°C.

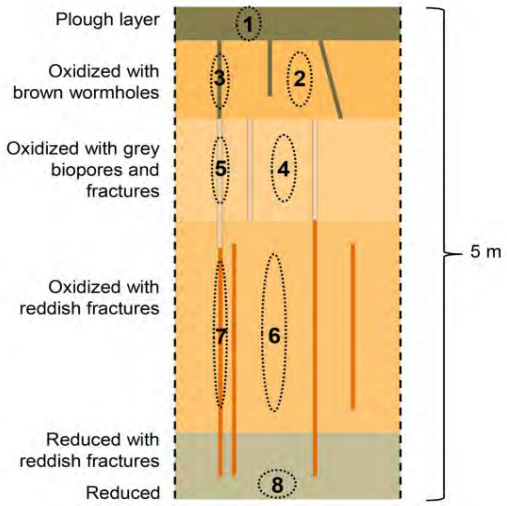


Fig. 1. A schematic representation of the soil profiles in the Gjorslev and Lund sites and their associated soil domains (Domains 1-8). The approximate depth of the lower boundary of each matrix soil domain was (Gjorslev/Lund): Domain 1 (35/35 cm), Domain 2 (105/130 cm), Domain 4 (200/260 cm), and Domain 6 (390/420 cm).

Characterization of the Soil Domains

Soil texture was determined by sieving (0.063-2 mm) and by laser diffraction (<0.063 mm, Mastersizer 3000, Malvern). Water content was determined by drying at 105°C for 24 h. Total carbon and total organic carbon (TOC) were determined on an elemental analyzer (Leco CS-200) on dried (50°C) and crushed samples as they were (total C) or after acid treatment to remove carbonates (TOC). Total inorganic C was calculated as the difference between total C and TOC. The pH was determined in a 1:2.5 soil/liquid slurry with Milli-Q water or 10 mM $CaCl_2$. The $pH_{sorption}$ (i.e., the pH measured at conditions similar to those during the sorption experiments) was also determined with $CaCl_2$, pesticide, and NaN_3 concentrations similar to those used in the sorption experiments. Soil-specific surface area was measured using a Coulter SA 3100 BET analyzer (Coulter Corporation) and calculated using the Brunauer-Emmett-Teller equation. Total Fe and ferrous Fe^{2+} were measured as described by Komadel and Stucki (1988). Iron and manganese oxides were extracted using the citrate-bicarbonate-dithionite (CBD) method and quantified by atomic absorption spectroscopy (PerkinElmer AANALYST 400). Amorphous Fe and Al oxides were extracted using ammonium oxalate solution. Cation exchange capacity was determined by standard method (Chapman, 1965). All analyses of soil parameters were single measurements.

Characterization of Liquid Pig Manure and Soil Extract

Liquid pig manure was sampled from a conventional farm that raised sows and offspring (weaner production) and was stored at 2°C for 4 wk. Topsoil extract was obtained by horizontal rolling of plow layer soil and Milli-Q water (1:1) for 24 h at 22°C. The liquid manure and the topsoil suspension were centrifuged (15 min, 3500 g), and the extracts were stored as frozen subsamples to be used in the subsequent sorption experiments. After thawing, the extracts were sonicated for 30 min before use in sorption experiments. Total organic C in soil and manure extracts and in the aqueous phase of selected sorption experiments was analysed on a TOC analyzer (TOC-Vcph, Shimadzu) after filtration (5 µm polyvinyl difluoride [PVDF], Millipore). Conductivity was determined using a conductivity probe (LE703, Mettler Toledo). The concentrations of major inorganic cations and anions were determined by ion chromatography (Metrohm 819 with a Metrosep A 150/4.0 column). Total Cu, Zn, Al, Ba, Fe, Mn, and S contents were measured on an inductively coupled plasma mass spectrometer (Elan 6100DRC, PerkinElmer) using a multielement scanning method (TotalQuant, PerkinElmer).

Chemicals

(P-methylene-^{14}C)-glyphosate (radiochemical purity = 99 %, specific activity = 122 MBq/mmol) were purchased from Izotop. Glyphosate (purity 97 %) was purchased from Dr. Ehrenstorfer, Germany.

Sorption of Glyphosate

Sorption experiments were performed using a batch-equilibrium method inspired by the Organization for Economic Cooperation and Development (OECD) guideline (OECD, 2000). Eleven-milliliter Pyrex glass vials with 15-mL polypropylene centrifuge vials were used for glyphosate. The final soil/liquid ratio was in all vials 1:10, which in general resulted in between 20 and 95 % sorption of the added pesticide. In each vial, 1 g of soil (wet weight) was mixed with 1 mM $CaCl_2$ solution (8.0 - 9.7 mL) and NaN_3 (20 µL of a 100 g/L solution) was added to repress biodegradation of the pesticides during incubation. One millimolar $CaCl_2$ was used, since it better represented the concentrations in the local soil water than the 10 mM $CaCl_2$ suggested in the OECD guideline. The soil-liquid slurries were then equilibrated at 10°C for 24 h by vertical rotation (7 revolutions/min) before addition of ^{14}C-labeled pesticide and, for the two highest pesticide concentrations, nonradioactive pesticide (both dissolved in 1 mM $CaCl_2$). Initial total concentrations of glyphosate were 30, 120, 1200 (thereof 120 µg/L radioactive glyphosate) and 12,000 µg/L (thereof 120 µg/L radioactive glyphosate). After addition of the pesticides, the vials were rotated at 10°C for another 24 h. The vials were then centrifuged at 1250 g (glass vials) or 3000 g (plastic vials) for 15 min. The pesticide concentration of the aqueous phase was determined by liquid scintillation counting (Tri-Carb 2810 TR, PerkinElmer) of the ^{14}C activity in duplicate 1-mL samples. The ^{14}C activity was counted for 30 min or until 1 % uncertainty (2S, 95 % confidence limit). Sorption to the vials was tested by including reagent blanks without soil, but no such sorption was found. The pesticide concentration in the solid phase (soil) was calculated based on pesticide missing in the aqueous phase.

The sorption experiments were all performed in duplicate. The difference in distribution coefficients between duplicates was <15%, and in most cases, it was <5% . All sorption experiments with soil from reduced zones wer prepared under a N_2 /H $_2$ atmosphere in a glove box with solutions that had been flushed with N_2.

Freundlich Sorption Models

Glyphosate sorption was described by an extended Freundlich equation, as suggested by de Jonge et al. (2001):

$$C_s = K_{Fex} C_w^{n_{ex}} C_w^{-D}$$

where K_{Fex} is the extended Freundlich coefficient, n_{ex} is the extended Freundlich exponent, and D is a parameter that adds extra curvature to the line in a double-logarithmic plot (i.e., increases the concentration sensitivity compared with the simple Freundlich model). The extended Freundlich model was fitted to the experimental data by nonlinear optimization.

Results

Soil Domains

Both soil profiles had a characteristic depth zonation with eight visually different domains based on different layers and the presence or absence of macropores (Fig. 1). At the Gjorslev site, the upper 35 cm was a relatively homogenous dark brown (10YR 3/2) plow layer (Ap horizon, Domain 1) rich in organic matter (Table 1). The plow layer was followed by an oxidized layer of variable color with a predominantly yellow-brown (10YR 4/4) matrix (Domain 2) perforated by brown (10YR 4/3), vertical wormholes where the soil was enriched in organic matter (Domain 3). Many of the wormholes were present within fractures (geological and desiccation) and extended to a depth of ~ 110 cm. The following layer (105–200 cm) was oxidized with a light brown (10YR 5/3) matrix (Domain 4) and numerous small biopores from decayed plant roots with a diameter of ~ 1 mm. The pores were surrounded by gray (10YR 8/1) pore soil with a diameter of 5 to 10 mm (Domain 5) and a thin, outer layer of Fe oxides. This layer also had gray fractures that extended into the next layer where they changed to reddish. The next layer (200–390 cm) was also oxidized with a light brown (10YR 5/3) matrix (Domain 6) and many parallel fractures (Domain 7). The surface of the larger fractures was coated with Fe and Mn oxides of variable reddish to almost black colors (10YR 4/6). This domain was devoid of visible biopores. The matrix was

reduced at the bottom of the profile (Domain 8), as visible by its gray color (5Y 5/1). The oxidized reddish fracture surfaces extended ~ 50 cm into the reduced zone. Similar horizons and domains were present in the Lund profile, although at slightly different depths (Table 1). Soil parameters for both profiles are available in Table 1 and Table 2.

Table 1. Main soil parameters from the soil profiles.

Domain	Sample depth		TOC†		$pH_{Sorption}$‡		Fe_{CBD}§		Mn_{CBD}¶		Surface area	
	Gjorslev	Lund	Gjorslev	Lund	Gjorslev	Lund	Gjorslev	Lund	Gjorslev	Lund	Gjorslev	Lund
	m		%				g kg^{-1}		mg kg^{-1}		m^2 g^{-1}	
1. Plough layer	0.1–0.3	0.2–0.3	0.89	0.67	7.33	7.38	5.6	4.2	286	204	4.1	4.2
2. Matrix	0.6–0.8	0.7–1.2	0.14	0.12	7.30	7.16	9.0	6.4	333	261	16.7	18.5
3. Wormholes	0.4–0.8	0.7–1.2	0.40	0.37	7.88	7.30	7.6	5.9	269	202	12.1	11.3
4. Matrix	1.5–1.6	2.0–2.6	0.05	0.05	8.20	8.26	4.7	3.9	170	153	12.4	13.0
5. Gray macropores	1.1–2.0	2.0–2.6	0.05	0.06	8.19	8.29	2.1	1.1	24	31	11.0	11.6
6. Matrix	2.5–3.5	3.0–3.5	0.05	0.05	8.16	8.31	4.5	2.9	97	97	11.8	12.2
7. Reddish fractures	2.5–3.5	3.0–3.6	0.05	0.06	8.09	8.28	18.8	12.6	798	910	16.0	21.2
8. Reduced zone	4.3–4.5	4.5–4.8	0.18	0.17	8.24	8.53	3.6	0.74	88	44	8.4	11.9

† TOC, total organic C.

‡ $pH_{Sorption}$, the pH measured at conditions similar to those during the sorption experiments.

§ Fe_{CBD}, total Fe oxides (extractable with citrate–bicarbonate–dithionite).

¶ Mn_{CBD}, total Mn oxides (extractable with citrate–bicarbonate–dithionite).

Table 2. Major soil paramters

Domain no.	Description	Sample depth (m) Gjorslev / Lund	$pH_{Sorption}$ Gjorslev / Lund	pH_{CaCl2} Gjorslev / Lund	pH_{H2O} Gjorslev / Lund
1	Plough layer	0.1-0.3 / 0.2-0.3	7.33 / 7.38	6.49 / 6.86	7.64 / 7.77
2	Matrix	0.6-0.8 / 0.7-1.2	7.30 / 7.16	6.81 / 6.86	8.09 / 7.92
3	Wormholes	0.4-0.8 / 0.7-1.2	7.88 / 7.30	7.21 / 6.80	8.35 / 7.80
4	Matrix	1.5-1.6 / 2.0-2.6	8.20 / 8.26	7.67 / 7.65	8.67 / 8.63
5	Grey macropores	1.1-2.0 / 2.0-2.6	8.19 / 8.29	7.64 / 7.79	8.77 / 8.77
6	Matrix	2.5-3.5 / 3.0-3.5	8.16 / 8.31	7.62 / 7.47	8.73 / 8.68
7	Reddish fractures	2.5-3.5 / 3.0-3.6	8.09 / 8.28	7.58 / 7.56	8.52 / 8.59
8	Reduced zone	4.3-4.5 / 4.5-4.8	8.24 / 8.53	7.54 / 7.59	N.D. / 8.27

Domain no.	Description	TOC (%) Gjorslev / Lund	TIC (%) Gjorslev / Lund	Surface (m^2/g) Gjorslev / Lund	CEC (cmol/kg) Gjorslev / Lund
1	Plough layer	0.89 / 0.67	0.32 / 0.20	4.1 / 4.2	11.0 / 9.25
2	Matrix	0.14 / 0.12	0.08 / 0.11	16.7 / 18.5	12.5 / 12.2
3	Wormholes	0.40 / 0.37	0.19 / 0.17	12.1 / 11.3	12.0 / 11.9
4	Matrix	0.05 / 0.05	1.95 / 2.41	12.4 / 13.0	8.77 / 7.52
5	Grey macropores	0.05 / 0.06	2.58 / 3.17	11.0 / 11.6	8.80 / 7.75
6	Matrix	0.05 / 0.05	1.88 / 2.98	11.8 / 12.2	8.53 / 7.15
7	Reddish fractures	0.05 / 0.06	1.83 / 2.67	16.0 / 21.2	9.46 / 8.26
8	Reduced zone	0.18 / 0.17	2.21 / 2.85	8.4 / 11.9	7.05 / 4.79

Domain no.	Description	Clay (%) Gjorslev / Lund	Silt (%) Gjorslev / Lund	Fine sand (%) Gjorslev / Lund	Med. sand (%) Gjorslev / Lund	Coarse sand (%) Gjorslev / Lund
1	Plough layer	6.5 / 6.3	41.1 / 39.4	35.9 / 35.7	10.6 / 12.3	6.0 / 6.3
2	Matrix	8.5 / 7.8	46.1 / 43.2	30.0 / 32.3	9.2 / 11.2	6.2 / 5.5
3	Wormholes	8.6 / nd	42.4 / nd	33.2 / nd	9.1 / nd	6.6 / nd
4	Matrix	13.1 / 13.4	42.7 / 43.2	28.8 / 28.5	8.7 / 8.9	6.7 / 6.0
5	Grey macropores	12.9 / 12.5	43.1 / 41.0	27.6/ 25.5	8.8 / 8.8	7.5 / 12.2
6	Matrix	12.9 / 13.6	45.0 / 43.5	27.1 / 26.4	9.0 / 7.4	6.0 / 9.1
7	Reddish fractures	10.6 / 14.1	40.8 / 45.5	24.8 / 26.0	8.8 / 9.9	15.1 / 12.0
8	Reduced zone	10.7 / 14.3	51.3 / 45.5	24.7 / 28.3	8.0 / 7.6	5.3 / 4.3

Sorption Is Domain Specific

Glyphosate sorption followed the Freundlich model with a high concentration dependence ($0.87 < n < 1.32$, Table 3).

Table 3. Extended Freundlich parameters (glyphosate) for sorption to eight soil domains in the Gjorslev and Lund profiles.

Domain	Glyphosate‡					
	K_{Fex}		*n*		*D*	
	Gjorslev	Lund	Gjorslev	Lund	Gjorslev	Lund
1	130	72.5	1.06	1.06	0.040	0.036
2	443	947	1.04	0.98	0.041	0.043
3	239	496	1.07	0.97	0.045	0.040
4	536	125	0.88	1.12	0.039	0.042
5	3849	353	1.01	1.01	0.053	0.053
6	424	359	0.87	0.91	0.037	0.037
7	124	58.1	0.92	0.93	0.011	0.000
8	217	251	1.08	1.32	0.049	0.071

‡ K_{Fex}, the extended Freundlich coefficient; *D*, a parameter that adds extra curvature to the line in a double-logarithmic plot. K_{Fex} equals the predicted distribution coefficient at 1 μg L^{-1} glyphosate.

The extended Freundlich model fitted the sorption data of glyphosate very well, though with a tendency to slightly underestimate sorption at the lowest concentration (Fig. 2). Glyphosate sorption was very concentration dependent (Fig. 2), which is why the extended Freundlich model fitted the sorption data better.

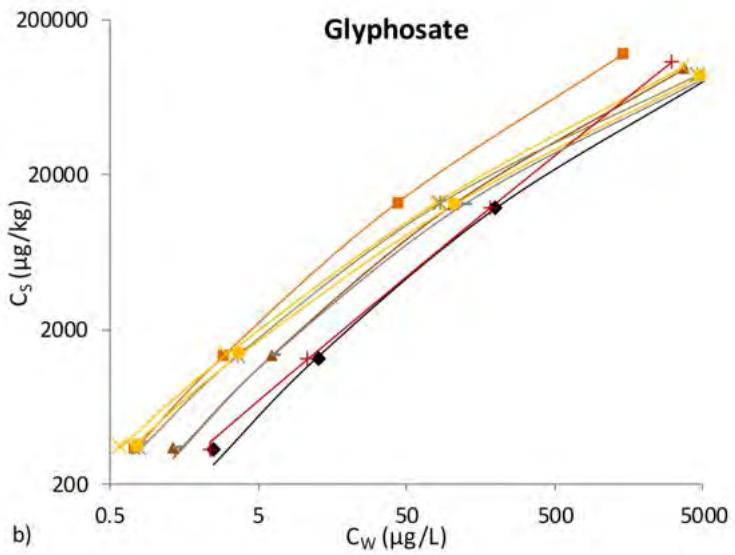


Fig. 2. Sorption isotherms for glyphosate (extended Freundlich model) in the eight soil domains from the Gjorslev profile. Note: Cs is the pesticide concentration in the soil phase, and Cw is the pesticide concentration in the aqueous phase.

The concentration dependence can be exemplified by Domain 6 (matrix soil), where the Kd at the lowest glyphosate equilibrium concentrations (0.8–0.9 mg/L) was 377 for Lund and 453 for Gjorslev, whereas at the highest equilibrium concentrations (4.2–4.8 mg/L), the Kd was only 18 for Gjorslev and 22 for Lund. Domain 7 (reddish macropores from same depth as Domain 6) was an exception with low sorption and little concentration dependence, with a Kd of 55 (Lund) to 137 (Gjorslev) at the lowest concentration and 33 to 34 at the highest. Hence, two very different sorption strengths and concentration dependencies were observed from the same soil depth. Also, at the 0.4- to 1.2-m depth, sorption of glyphosate varied in the two domains at both study sites, being twice as high in matrix soil (Domain 2) than in soil from the wormholes (Domain 3). This fits well with the much lower sorption of glyphosate to the plow layer, which shows some similarities with the wormholes.

There was no correlation between Fe oxide content (expressed either as total Fe oxides or amorphous Fe oxides) and glyphosate sorption (expressed as K_{Fex}) (Fig. 3). There was also no correlation when K_{Fex} was plotted against any other measured soil parameter.

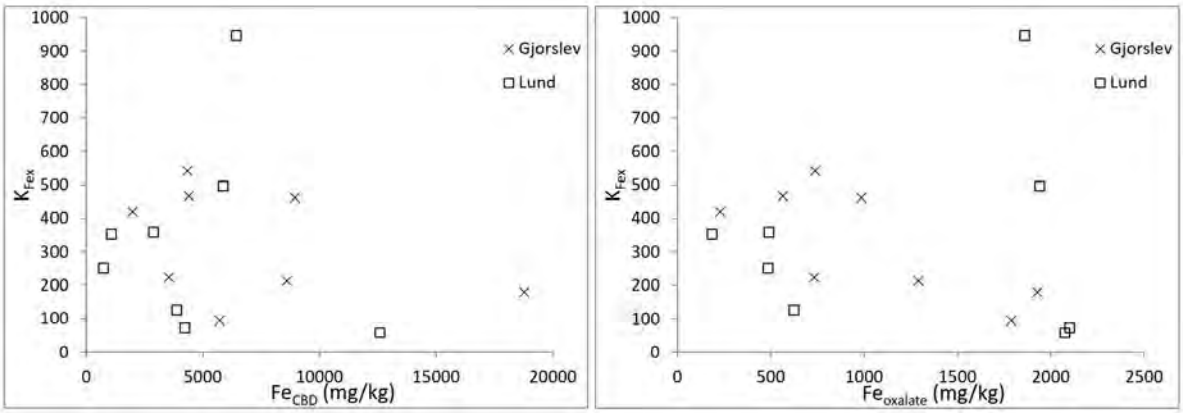


Fig. 3. Relationship between total iron oxide concentration (Fe_{CBD}) or amorphous iron oxides ($Fe_{oxalate}$) and sorption of glyphosate (K_{Fex}) in the eight soil domains at the two study sites. K_{Fex} was determined at a µg/L basis and therefore denotes the calculated partitioning coefficient at 1 µg/L.

It may be important to consider differences in pesticide sorption between soil domains from the same depth when modeling the risk of pesticide leaching. In clayey tills, most water transport takes place in the macropores; sorption studies, on the other hand, would normally be conducted on bulk soil samples, resembling the matrix samples in the present study. Most sorption studies are furthermore performed only with soil from the plow layer, and leaching in the actual fields may therefore be different from the leaching calculated from such sorption studies. For glyphosate, the leaching would most likely be higher than expected, since sorption to the soil of the upper biopores and especially to the surfaces of the metal oxide coated fractures is lower than in their corresponding matrix domains.

Topsoil Extract and Liquid Manure Extract Reduce Pesticide Sorption

The addition of topsoil extract had an effect on glyphosate sorption, decreasing sorption (K_{Fex}) by 3 to 37 % depending on the domain (Table 4), and the addition of liquid manure had an even larger effect. Ten percent liquid pig manure changed sorption (K_{Fex}) dramatically, with a decrease of 83 to 95 % in the Gjorslev Domains 1 to 6 and 8, and 76 to 83 % in the corresponding Lund domains (Fig. 4). Manure additionally changed the other extended Freundlich parameters (n and D), as the sorption of glyphosate was less concentration dependent when manure was present.

Table 4. Sorption parameters for glyphosate with different liquid treatments. Control is without any additions.

	Control			Topsoil extract (50%)			Manure (0.1%)			Manure (1%)			Manure (10%)		
Domain	K_{Fex}	n_{ex}	D	K_{Fex}	n_{ex}	D	K_{Fex}	n_{ex}	D	K_{Fex}	n_{ex}	D	K_{Fex}	n_{ex}	D
Gjor-1	130	1.06	0.040	86	1.16	0.044	96	1.17	0.046	76	1.09	0.037	16	1.14	0.028
Gjor -2	443	1.04	0.041	338	0.98	0.032	454	0.99	0.036	358	1.07	0.045	41	1.28	0.038
Gjor -3	239	1.07	0.045	193	1.07	0.042	240	1.09	0.048	239	1.08	0.045	37	1.13	0.028
Gjor -4	536	0.88	0.039	354	0.97	0.042	543	0.89	0.039	369	1.03	0.049	29	1.33	0.042
Gjor -5	384	1.01	0.053	295	1.02	0.049	408	0.98	0.049	249	1.14	0.057	28	1.28	0.039
Gjor -6	424	0.87	0.037	283	1.00	0.046	386	0.93	0.042	249	1.08	0.049	27	1.31	0.042
Gjor -7	124	0.92	0.011	142	0.99	0.023	201	0.86	0.012	262	0.98	0.030	67	1.14	0.032
Gjor -8	217	1.08	0.049	169	1.12	0.050	223	1.09	0.051	160	1.17	0.052	19	1.36	0.043
Lund-1	72	1.06	0.036	-	-	-	72	1.01	0.030	35	1.11	0.033	12	1.02	0.019
Lund-2	947	0.98	0.043	-	-	-	1026	0.96	0.044	657	1.00	0.043	47	1.20	0.035
Lund-3	496	0.97	0.040	-	-	-	472	0.96	0.038	262	1.07	0.045	23	1.25	0.036
Lund-4	125	1.12	0.042	-	-	-	244	0.98	0.039	253	1.09	0.050	30	1.27	0.040
Lund-5	353	1.01	0.053	-	-	-	350	1.04	0.055	258	1.09	0.053	18	1.39	0.044
Lund-6	359	0.91	0.037	-	-	-	354	0.97	0.045	241	1.07	0.047	30	1.27	0.040
Lund-7	58	0.93	0.000	-	-	-	116	0.85	0.000	259	0.88	0.016	88	1.12	0.031
Lund-8	251	1.32	0.071	-	-	-	264	1.33	0.073	265	1.33	0.073	14	1.29	0.035

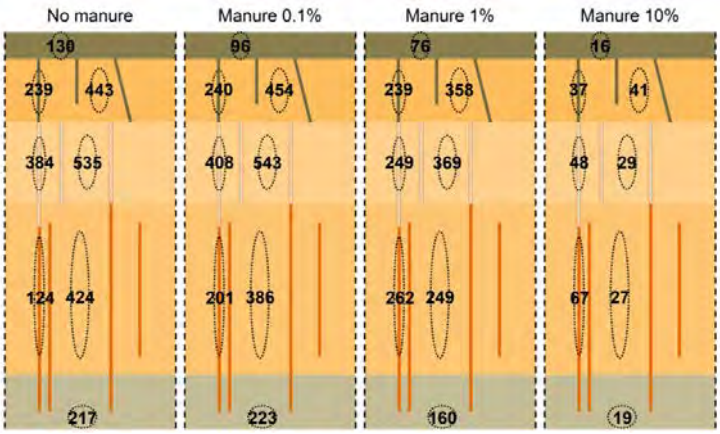


Fig. 4. Effect of liquid pig manure extract (%, v/v) on the sorption (K_{Fex}) of glyphosate in the Gjorslev soil domains. The K_{Fex} equals the predicted distribution coefficient at a glyphosate concentration of 1 µg L^{-1}

Why Do Topsoil Extract and Manure Reduce Glyphosate Sorption

Several soil water parameters have been suggested to influence glyphosate sorption. These include pH, phosphate, divalent metal ions like Cu^{2+} and Zn^{2+} and dissolved organic matter.

Change in pH cannot explain the general decrease in sorption when topsoil extract or pig manure was added.

The manure had a high conductivity (21,900 µS/cm, Table 5). In parallel experiments, it was observed an increase in sorption at increased ionic strengths (data not shown), which has also been reported previously in the literature. The high ionic strength in the manure therefore cannot explain the decreased sorption.

Table 5: Major analyzed parameters for the liquid manure and topsoil extracts. ND = not determined.

Parameter	Unit	Liquid pig manure	Topsoil extract
DOC	mg/L	2648	32
Dry matter	%	1.5	ND
Conductivity	µS/cm	21900	214
PO_4^{3-}	mg/L	182	<0.1
Cu	mg/L	4.9	0.03
Zn	mg/L	7.5	0.12
Fe	mg/L	10.2	22.7
Al	mg/L	0.28	32.0
Na^+	mg/L	623	0.35
K^+	mg/L	2500	0.57
Ca^{2+}	mg/L	<13	3.8
Mg^{2+}	mg/L	<8	0.15
F^-	mg/L	<0.5	1.2
Cl^-	mg/L	1481	4.5
Br^-	mg/L	30.7	0.86
NO_3^-	mg/L	0.64	33.1
SO_4^{2-}	mg/L	10.4	10.3
Ba	mg/L	0.2	0.16
Mn	mg/L	0.4	0.18
S	mg/L	668	11.2
Density	-	1.02	ND

Both the humic and fulvic acid fractions of soil organic matter decreased glyphosate sorption, when added to soil (Fig. 5).

Divalent metal ions and phosphate would be relevant only with manure addition. Divalent metal ions (Cu^{2+} and Zn^{2+}) at concentrations corresponding to 1 and 10% pig manure had no effect on sorption in Domain 1 and increased sorption in Domain 6 (Fig. 5). The Cu^{2+} and Zn^{2+} ions are therefore not likely to have caused the manure effect. Phosphate, on the other hand, reduced glyphosate sorption at concentrations corresponding to those in the pig manure (Fig.5). Both phosphate and dissolved organic matter are therefore likely candidates to explain the manure effect on glyphosate sorption.

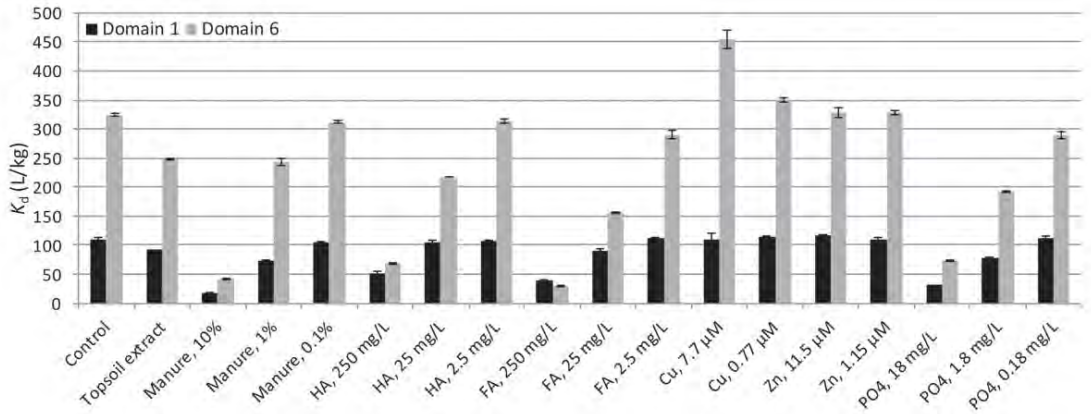


Fig. 5. Effect of topsoil extract, liquid pig manure extract, organic matter (humic acids [HA] and fulvic acids [FA]), divalent metals (Cu and Zn), and phosphate on glyphosate sorption (expressed as the distribution coefficient, K_d) in Domains 1 and 6 from the Gjorslev site. Concentrations correspond to the tested manure concentrations. Error bars are minimums and maximums of duplicate samples. Results from experiments with topsoil extract and liquid manure are included for comparison. Controls are without any additions.

Conclusion

The study has demonstrated that the sorption of glyphosate varies by an order of magnitude across eight identified soil domains in macroporous clayey till. It was expected that glyphosate would show the strongest sorption in domains with high Fe oxide content. This turned out to be wrong, since there was no correlation between glyphosate sorption and any measured soil parameter, including extractable Fe oxides. The domain-specific sorption of glyphosate was by far overruled by addition of liquid manure that strongly decreased glyphosate sorption due to its content of dissolved organic matter and phosphate. The variation across domains and the effects of solutes like the liquid fraction of manure should be taken into account when using sorption data in assessment of leaching risks. Our results suggest that hydrological modeling should focus more on sorption to fracture surfaces and pay less attention to traditional bulk sorption data when predicting pesticide transport through clay macropores. How much sorption influences leaching will, after all, also depend on general hydrological parameters such as pore size, connectivity, and climatic conditions.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes the sorption behavior of glyphosate to different soil domains from two agricultural soils in Denmark. The set-up of the experiment was based on the OECD 106 guideline but with some deviations: The study was conducted with 1 mM $CaCl_2$ solution (standard: 10 mM solution), at 10°C (standard: 20 – 25°C); with 4 concentrations (standard: 5), no concentrations in the solid phase were explicitly measured, i.e. no real mass balances available.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.1.1
Report author	Alexa, E. et al.
Report year	2010
Report title	Studies on the biodegradation capacity ^{14}C–labelled glyphosate in vine plantation soils
Document No	Journal of Food, Agriculture & Environment (2010) Vol.8 (3&4): 1193–1
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Experimental conditions not sufficiently described to assess validity, relevant endpoint not reported (DT_{50}))

2. Full summary of the study according to OECD format

Glyphosate is among the most widely used broad spectrum herbicides in the world because they are highly efficacious, cost effective, practically non–toxic and degrade readily in the environment. The herbicide is inactivated and biodegraded by soil microbes, degradation rate depends on soil microbial activity and factors that affect this activity. Glyphosate degradation rates vary considerably across a wide variety of soil types and microflora population types. The aim of this paper was to study the biodegradation capacity of glyphosate in soil samples collected from vine plantation from Timis county, Romania, belonging to Banat's University of Agricultural Science, Timisoara, in presence of organic and inorganic supplement, at different concentration levels. After addition of glyphosate–phosphonomethyl–^{14}C–labeled, the accumulated $^{14}CO_2$ (as % of total ^{14}C) was monitored during 44 days. Investigated soil shows a high degradation capacity of over 85 % of total radioactivity after 44 days from the treatment application. Addition of inorganic supplement causes a decrease of glyphosate biodegradation capacity to 10.77–12.87 % of total radioactivity, while in presence of straw the accumulated $^{14}CO_2$ (as % of total ^{14}C) during the 44 days ranged between 59.97 and 87.58 %. The amount of $^{14}CO_2$ released reached the highest level in the first 4 days after herbicide application, both in control and experimental variants with organic and inorganic supplement (from 2.61 to 30.27 % of total radioactivity). By glyphosate addition the growth and multiplication of soil microorganisms, whose biomass is digested in the range of 9–12 days of treatment, according to the daily mineralization rate (DMR) values, is stimulated. Our results on the activity of microorganisms showed that glyphosate degradation in soil is mainly performed by micromyces.

Materials and methods

Chemicals and soil samples

Glyphosate-phosphonomethyl–^{14}C– labeled (Sigma) lot number 012K9428/29, specific activity 2.2 mCi $mmol^{-1}$ and commercially formulated glyphosate of isopropylamine ammonium salt (Roundup) were purchased from Monsanto, Romania. Liquid Scintillation Cocktail (Quicksafe A cocktail) was used in Triathler Liquid Scintillation Counting. All other reagents were of analytical reagent grade.

The soil characterized as cambic moderately gleyed chernozem were sampled in March 2010 from the vine plantation (Burgundy grape variety) of Banat's University of Agricultural Science in Timisoara

(Western part of Romania). Sampling depth was between 0 and 10 cm. The glyphosate treatments and both organic and inorganic fertilizers are usually applied in grape–vine plantation. The soil samples were dried at room temperature for 48 h and crushed to pass a 2 mm sieve.

The basic physico–chemical soil characteristics and chemical composition of added inorganic and organic supplements were as follows:

- soil: clay 42.1 %; sand 29.2 %; silt 28.7 %; pH in H_2O 7.93; organic matter 3.95 %; N total 0.266 %; P 30 ppm; Fe 20,340 ppm; Cu 10 ppm; Mn 300 ppm; Zn 8 ppm

- organic supplement: pH 6.5; N_{total} 14 %; organic matter 7.5 %; P 30 ppm; Zn 35.89 ppm; Cr 42.60 ppm; Ni 25.61 ppm; Cu: 31.51 ppm; Cd 2.01 ppm; Fe 487.7 ppm

- inorganic supplement: N_{total} 15 %; P_2O_5 5 %; K_2O 20 %; CaO 2 %; MgO 1 %; S 9 %; Cu 0.1 %; Fe 0.1 %; Mn 0.5 %; Zn 0.1 %

- wheat straw: cellulose 35 %; lignin 18 %; ash 8 %; hemicellulose 35 %

^{14}C–labelled glyphosate biodegradation radio-assay

Evaluation of ^{14}C–labelled glyphosate biodegradation was done according to Getenga et al. using liquid scintillation counter Triathler (Finland) for radio–assay. In the incubation experiment, 25 g soil samples in duplicates were placed in biometer flasks. The soil was conditioned by being moistened to 85 % of the field water capacity. The biometer flask content is a plastic vial with soil treated with glyphosate, a vial containing 10 ml distillated water, which assures atmosphere saturation with water vapor and a plastic vial filled with 10 ml 0.2 M NaOH , to trap the $^{14}CO_2$ released during mineralization by soil microorganisms. Non–labelled glyphosate solution in distilled water in concentration of 1.5 ppm was added to each soil sample and the initial radioactivity was done by glyphosate–phosphonomethyl–^{14}C–labeled with specific activity 0.5 mCi. The soils were incubated at 20°C, in the dark for 44 days. In order to evaluate the biodegradation of ^{14}C–labeled glyphosate during the incubation period, samples were taken every 4 days. The NaOH solution was mixed with 5 ml of Quicksafe A cocktail in a 20 ml scintillation vial before it was radio–assayed. After every sampling the vial was refilled with fresh 0.2 M NaOH. The amount of $^{14}CO_2$ released during mineralization was quantified on the base of ^{14}C disintegration number.

By adding the percentages at each sampling, the total amount of mineralized glyphosate depending on time is obtained. The mineralization curves of $^{14}CO_2$ accumulated were compared during 44 days.

The experimental treatments were: Control – soil with glyphosate in concentration of 1.5 ppm; OSI – soil with glyphosate and addition of organic supplement 3.2 %; OSII - soil with glyphosate and addition of organic supplement 6.4 %; ISI – soil with glyphosate and addition of inorganic supplement 8 %; ISII – soil with glyphosate and addition of inorganic supplement 16 %; WSI – soil with glyphosate and addition of wheat straw 1 %; WSII – soil with glyphosate and addition of wheat straw 2 %.

Evaluation of microbial response parameters

Microbial communities in soils treated with glyphosate were evaluated using the method described by Seeley *et al*. 20. A soil sample (about 20 g) was treated with 1.5 ppm glyphosate unlabeled solution (Roundup) and incubated at 22 ± 3°C in an Erlenmeyer flask. Daily humidity was corrected so that it does not to fall below 75–80 % of the wet field capacity. After 3 and 10 days we determined the number of culturable microorganisms using the count plate method. For the quantitative determination of eubacterias we used Topping medium: yeast extract 0.25 %, peptone powder 0.25 %, agar 1.8 % and distillated water, pH 7.6. To quantify the number of actinomycetes we used Gause medium: KNO_3 0.1 %, K_2HPO_4 0.05 %, $MgSO_4$ 0.05 %, NaCl 0.05 %, $FeSO_4$ 1 %, corn starch 2 %, agar 2 %, distillated water, pH 7, and for estimation of the micromycetes number we used Czapek Dox medium: $NaNO_3$ 0.3 %, K_2HPO_4 0.1 %, $MgSO_4$ 0.05 %, KCl 0.05 %, $FeSO_4$ 0.001 %, sucrose 0.3 %, agar 1.5 %, pH 5.5. To secure a microbial count the samples were diluted (in 0.1 % sodium pyrophosphate) and plated, and after incubation the colonies that develop were counted. The microbial count of the original samples

was then determined by multiplying the average number of colonies that develop by the degree of dilution (dilution factor of the samples in the plate). Dilutions, expressed as negative exponents, were 10^{-5} for micromycetes and 10^{-7} for eubacterias and actinomycetes determinations. The results were expressed in colony forming units (CFU) per g soil (dry matter).

Results

^{14}C–glyphosate calibration

^{14}C–glyphosate calibration was done on the basis of quench curve method. The curve establishes the relationship between a quench parameter (QP) and the counting efficiency. Quench parameter indicates a relative light production from the sample. In the Triathler the quench parameter (QP) is, in mathematical terms, the center of spectrum gravity in the counting window. The collective effect of quench is a reduction in the number of photons produced and, therefore, detected CPM (counts per minute). The Triathler uses parabolic regression to form the curve. First the quench curve was made by counting a set of standard samples with the same activity but variable quench (Table 1). The Triathler prints the quench parameters and the corresponding efficiencies of the standards. When unknown samples are counted, the quench parameter is measured for each sample. Corresponding efficiency for the measured quench parameter is obtained from the curve and the DPM (desintegrations per minute or absolute radioactivity) corresponding value is calculated ($DPM = CPM \cdot Eff^{-1}$). The efficiency taken from the curve and an error percentage (err %), which is the difference of efficiencies (difference between measured eff. and the one taken from the quench curve, are indicated in Table 2.

Table 1. The data recorded for Triathler calibration.

Sample	Time (s)	Counts	CPM	QP
1	300	1,094,190	218,838	44.144
2	300	550,900	110,180	42.995
3	300	489,535	97,907	34.861
4	300	108,650	21,730	28.142
5	300	27,095	5,419	23.558

Std DPM: 220,000.

Eff.= - 0.0006·qp2 + 0.0743·qp - 1.2895.

Table 2. The efficiencies obtained on the basis of data analysis.

Eff	QP	Eff	err (%)
0.99472	44.14	0.99472	0.00
0.50082	43.00	0.50082	0.00
0.44503	34.86	0.44503	0.00
0.09877	28.14	0.09877	0.00
0.02463	23.56	0.02463	0.00

Results regarding ^{14}C–glyphosate biodegradation

Experimental results regarding the amount of $^{14}CO_2$ (%) released reported to total initial radioactivity, in accordance with prelevation chart are represented in Table 3. The biodegradation degree of glyphosate in soil was estimated as ratio between the number of ^{14}C–glyphosate disintegration in the sample and the number of disintegration in the standard. From these data it can be observed that in both control and experimental variants with organic and inorganic supplement addition, the amount of $^{14}CO_2$ released recorded the maximum value in the first 4 days after herbicide application, ranging from 2.61 % to 30.27 % of total radioactivity. The biodegraded glyphosate amount decreases for all analyzed samples, being less than 1 % after 44 days. The experimental results are in accordance with previous data obtained, which show that the glyphosate biodegradation has only two phases, the initial rapid phase for about

20 days due to microorganisms action on free glyphosate in soil followed by a slow final phase when the microorganisms act on glyphosate adsorbed on the soil compounds. From Table 3 it can be observed that, for control, $^{14}CO_2$ resulting from the glyphosate decomposition reached maximum value after 4 days (30.27 %) and decreases with time advancing: 20.27 % after 8 days, 11.86 % after 12 days, 10.94 % after 16 days and only 3.94 after 20 days reaching 0.35 mg $^{14}CO_2$ after 44 days. In Figure 1a–c the glyphosate mineralization curves expressed as accumulated $^{14}CO_2$ as % from total radioactivity are represented. Accumulated $^{14}CO_2$ in the case of control sample, without fertilizers, increased from 30.24 % after 4 days, to 50.54 % after 8 days from herbicide application, respectively, 80 % after first 24 days and slow growing to 85.96 % of total radioactivity after 44 days (Figure 1a). The soil characteristics influence the degradation capacity of glyphosate in the presence of microorganisms. In the literature there are several papers describing that the adsorption of glyphosate by soils depends on cationic exchange capacity, clay content, pH and organic matter. Studies regarding the effect of pH on the adsorption of glyphosate by soils and clays agreed that an increase of pH decreased the adsorption of glyphosate. It was due to an increase in negative charge of glyphosate and mineral surface with an increase in pH value resulting in a decrease in the adsorption. The analyzed soil has a high content of clay (42.1 %), iron and pH in H_2O (7.93). The experimental results show a high glyphosate biodegradation capacity in control sample (85.96 % after 44 days) and availability of glyphosate to microorganisms, due to low level of glyphosate adsorption on soil particles, according to other studies.

Table 3. Impact of added supplement on $^{14}CO_2$ release (% of total radioactivity).

Variant	Accumulated $^{14}CO_2$ (% of total ^{14}C) in the prelevation time (A-K)										
	A	B	C	D	E	F	G	H	I	J	K
Control	30.27	20.27	11.86	10.94	3.9	2.82	2.27	1.47	1.12	0.69	0.35
OSI	30.23	19.13	12.76	9.54	3.86	2.8	2.27	1.47	1.12	0.91	0.49
OSII	33.66	16.38	13.24	4.13	3.9	2.53	1.9	1.62	0.7	0.7	0.49
ISI	2.61	1.5	0.69	0.62	0.54	0.48	0.46	0.36	0.3	0.26	0.25
ISII	2.73	1.94	1.63	1.49	1.28	1.1	0.91	0.76	0.41	0.42	0.2
WSI	25.51	15.27	4.86	4.08	2.78	2.62	1.94	1.58	1.13	0.81	0.73
WSII	21.96	13.71	7.13	6.58	2.64	2.17	1.65	1.24	1.19	0.96	0.74

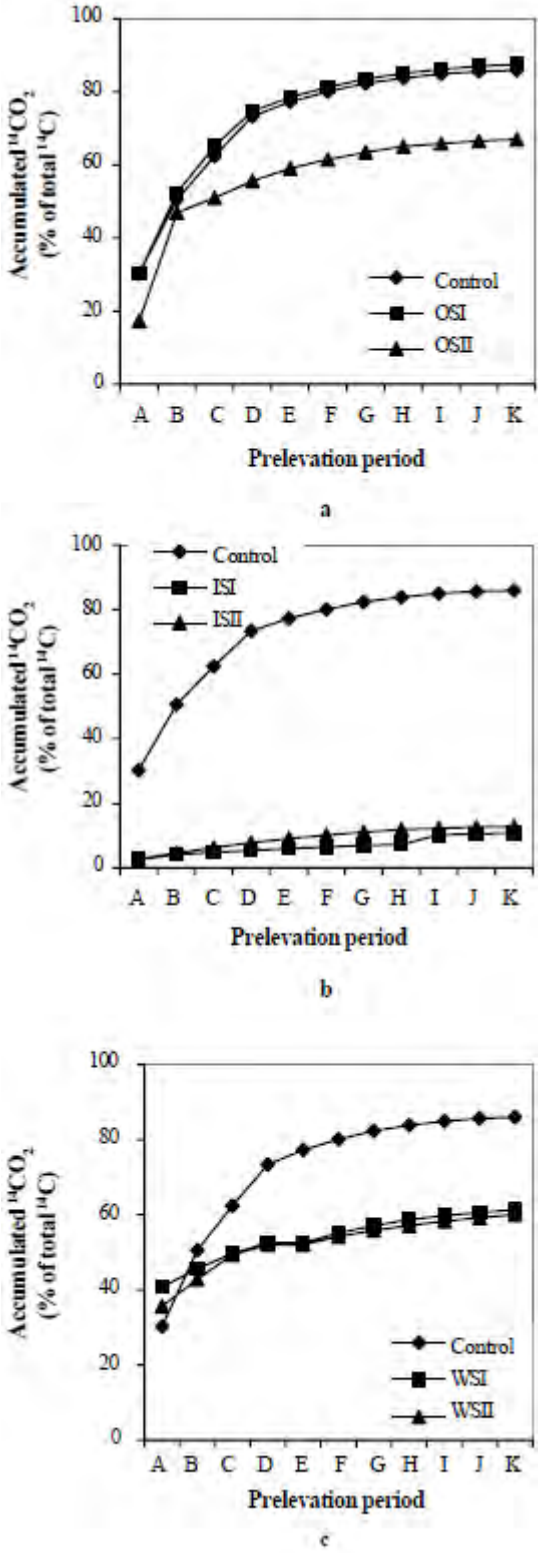
In Figure 1a–c the glyphosate mineralization curves expressed as accumulated $^{14}CO_2$ as % from total radioactivity, in the case of organic and inorganic supplement addition are represented. The experimental results obtained show significant differences between the amount of biodegradable glyphosate according to the type and amount of organic or inorganic fertilizer added. Addition of organic fertilizer at a rate of 3.2 % does not lead to significant changes in curve shape of glyphosate mineralization (Figure 1b). Increasing the amount of organic fertilizer to 6.4 % leads to decrease in the amount of released $^{14}CO_2$. Total accumulated $^{14}CO_2$ after 44 days from the glyphosate application was 87.58 % for organic substrate addition OSI, respectively, 67 % in case of organic substrate addition of OSII. Our results are in accordance with those of Getenga and Kengara, showing that compost addition does not stimulate intense mineralization of glyphosate by microbes.

Mineralization curves in Figure 1b show the reduced availability of glyphosate to biodegradation in the presence of inorganic fertilizers. In case of mineral fertilizers addition in a proportion of 8 % of inorganic supplement, the amount of $^{14}CO_2$ 4 days after the herbicide administration was 2.61 % of the total radioactivity and decreased slowly reaching 0.25 % between 40 and 44 days. Biodegradation capacity of glyphosate in the presence of mineral fertilizers was much reduced compared to the control sample (Table 3, Figure1b). The total amount of $^{14}CO_2$ released after 44 days was only 10.77 % in case of ISI and 12.87 % in case of ISII.

From Table 3 it can be observed that the biodegraded glyphosate percentage was between 2.61 to 2.73 % after the first 4 days and decreased to 0.2 % after 44 days of experimentation. Glyphosate contains functional groups of amine, carboxylate and phosphonate that can form strong coordination bonds with metal ions to give bidentate and tridentate complexes. Addition of inorganic fertilizers rich in metal ions leads to decrease in glyphosate biodegradation ability and reduces the amount of $^{14}CO_2$ released. Cruz

et al. studied the competitive adsorption between glyphosate and phosphate in different Brazilian soils. The results showed that on the clays glyphosate was not easily displaced by phosphate even in the ratio of 10.0 of phosphate/glyphosate. Our results are in accordance with those because in analyzed soil with high content of clays (42.1 %) the glyphosate is not displaced by phosphate ions. On the other hand, the addition of inorganic fertilizer rich in phosphate and nitrate led to micro–organisms orientation on nitrogen and phosphate source easily accessible, respectively, reduced availability of glyphosate for biodegradation. Thus, the amount of $^{14}CO_2$ released is 10 times lower in variants fertilized with inorganic supplement (ISI, ISII). The increased content of mineral fertilizer, in the case of ISII, did not lead to significant changes regarding the release of $^{14}CO_2$ from the glyphosate biodegradation. In WSI and WSII where wheat straw at a rate of 1 % and 2 % was added, there was a noticeable decrease in the amount of $^{14}CO_2$ pursued as a result of glyphosate biodegradation compared with the control. Thus, after 4 days the percentage of released $^{14}CO_2$ was 25.51 % in WSI and 21.96 % in WSII (Table 3). After 8 days from the glyphosate application, the biodegradation capacity decreased to 4.86 and 7.13 %. $^{14}CO_2$ total amount accumulated as a result of glyphosate biodegradation was 61.31 %, in the case of 1 % straw addition and 59.97 % to 2 % straw addition (Figure 1c).

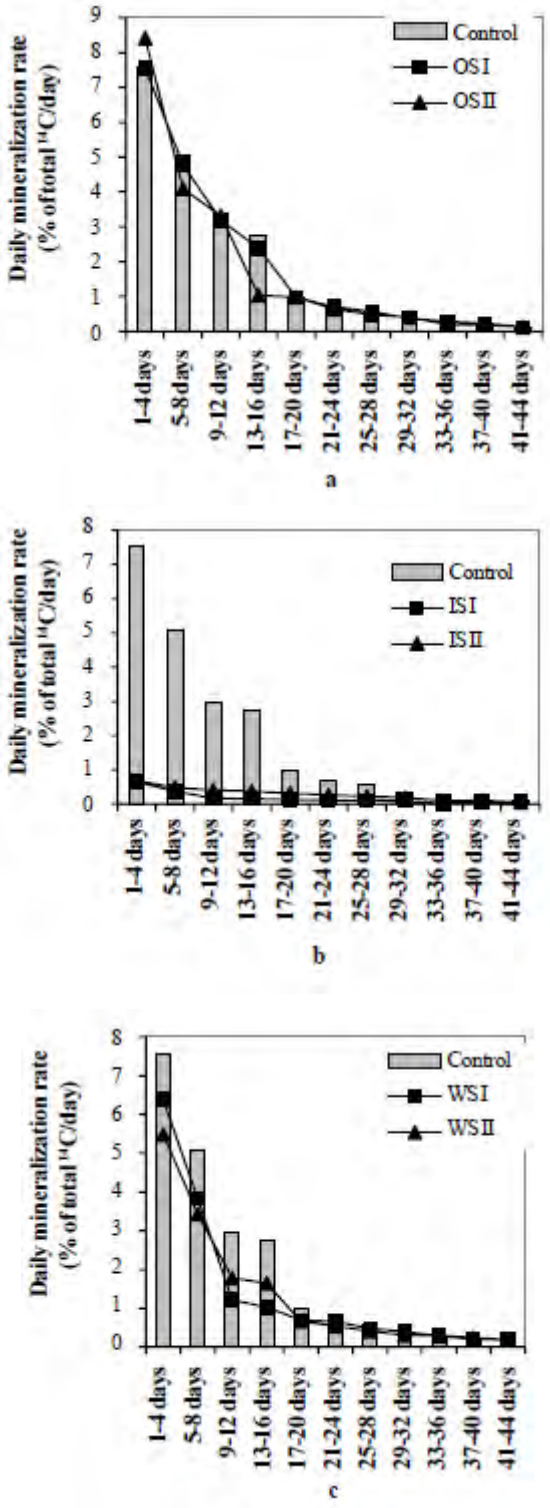
Figure 1. Mineralization of ^{14}C–glyphosate in soil with different supplements (a– control versus OS, b– control versus IS, c– control versus WS).



The daily mineralization rate (DMR) of glyphosate (Figure 2a–c) in the case of different supplement addition was highest for all variants in the first 4 days of experiment, decreasing during incubation. If mineral fertilizers were added (ISI, ISII), the DMR value was much lower than in other cases. The explication is due to existing mineral compounds intake in inorganic fertilizers, compounds with which

glyphosate forms complexes hard accessible for microbial metabolism but also, due to lack of energy substrate supporting the respiratory activity of microorganisms.

Figure 2. Daily mineralization rate of glyphosate (a– control versus OS, b– control versus IS, c– control versus WS).



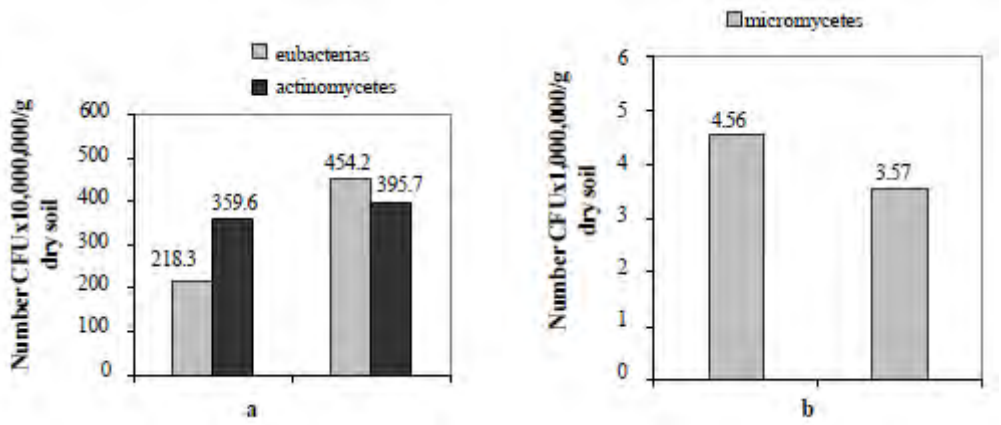
Besides, in the organic material addition, OSI, OSII, WSI and WSII, DMR value was highest in the first 4 days of experimentation. The highest values of $^{14}CO_2$, corresponding to DMR, were determined in OS even exceeding the control (7.567 mg $^{14}CO_2$), DMR value was higher than control value also for range

of 9–12 days. This could be due to labelled carbon release from the microbial protoplasm which assimilates the labelled glyphosate, respectively of fungal biomass.

Results regarding microorganism activity in soil

Glyphosate remains unchanged in the soil for varying lengths of time, depending on soil texture and organic matter content. Soil microorganisms break down glyphosate and many can use glyphosate as a sole source of phosphorus. On the base of results regarding the number of culturable microorganisms existing in the soil with glyphosate (Figure 3a, b) it can be observed that at 10 days after the treatment application, the eubacteria number increases from $218.3x10^5$ to $454.2x10^5$ CFU g^{-1} dry soil.

Figure 3. The variations of microorganisms number after 3 and 10 days since glyphosate addition in control a) eubacterias and actinomycetes, b) micromycetes.



Conclusion

The soil characteristics influence the degradation capacity of glyphosate in the presence of microorganisms. The soil sampled from the vine plantation (Burgundy grape variety) of Banat's University of Agricultural Science in Timisoara shows a high degradation capacity, over 85 % of total radioactivity after 44 days from the treatment application. Addition of inorganic substratum causes a decrease in glyphosate biodegradation capacity to 10.77 – 12.87 % of total radioactivity, while in presence of straw the accumulated $^{14}CO_2$ (as % of total ^{14}C) during the 44 days ranged between 59.97 – 87.58 %. The amount of $^{14}CO_2$ released reached the highest level in the first 4 days after herbicide application both in the control and experimental variants with organic and inorganic substratum (from 2.61 to 30.27 % of total radioactivity). The growth and multiplication of soil microorganisms whose biomass is digested in the range of 9 – 12 days of treatment, according to the daily mineralization rate (DMR) values is stimulated by glyphosate addition. Our results on the activity of microorganisms have shown that glyphosate degradation in soil is mainly performed by micromyces.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a degradation experiment with glyphosate on an European agricultural soil in the laboratory. Only the mineralization of the substance is reported. Further data like mass balances, residues in soil and a half–life are not reported .The validity of the study cannot be evaluated due to missing information.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.1.1
Report author	Al–Rajab, A., Schiavon, M.
Report year	2010
Report title	Degradation of ^{14}C–glyphosate and aminomethylphosphonic acid (AMPA) in three agricultural soils
Document No	Journal of environmental sciences (China), (2010) Vol. 22, No. 9, pp. 1374-80
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Reliable with restrictions (some deficiencies in the test design, not all information reported to check validity of the study)

2. Full summary of the study according to OECD format

Glyphosate (N–phosphonomethyl glycine) is the most used herbicide worldwide. The degradation of ^{14}C–labeled glyphosate was studied under controlled laboratory conditions in three different agricultural soils: a silt clay loam, a clay loam and a sandy loam soil. The kinetic and intensity of glyphosate degradation varied considerably over time within the same soil and among different types of soil. Our results demonstrated that the mineralization rate of glyphosate was high at the beginning of incubation and then decreased with time until the end of the experiment. The same kinetic was observed for the water extractable residues. The degradation of glyphosate was rapid in the soil with low adsorption capacity (clay loam soil) with a short half–life of 4 days. However, the persistence of glyphosate in high adsorption capacity soils increased, with half-live of 19 days for silt clay loam soil and 14.5 days for sandy loam soil. HPLC analyses showed that the main metabolite of glyphosate, aminomethylphosphonic acid (AMPA) was detected after three days of incubation in the extracts of all three soils. Our results suggested that the possibility of contamination of groundwater by glyphosate was high on a long–term period in soils with high adsorption capacity and low degrading activities and/or acid similar to sandy loam soil. This risk might be faster but less sustainable in soil with low adsorption capacity and high degrading activity like the clay loam soil. However, the release of non–extractable residues may increase the risk of contamination of groundwater regardless of the type of soil.

Materials and methods

Chemicals

[Phosphonomethyl–^{14}C]–glyphosate was obtained from ARC–ISOBIO (Belgium) diluted in water. Its specific radioactivity was 385 GBq/mmol and its radiochemical purity 99 %. Non–radioactive glyphosate (purity 98.5 %) was obtained from CIL Cluzeau (France). AMPA, 10 ng/µL in water, was obtained from Dr. Ehrenstorfer GmbH (Germany). Sarcosine (N–methylglycine) $C_3H_7NO_2$, purity 99 %, was obtained from Fluka (Germany). FMOC–chloride (purity 99 %), sodium tetraborate decahydrate (purity 99.5 %), potassium hydroxyde (purity 86 %), potassium dihydrogen phosphate (purity 99.5 %) were also obtained from Fluka (Germany). Acetonitrile was obtained from (SDS, France). All solvents were of high performance liquid chromatography (HPLC) grade.

Selected soils and treatments

Three cultivated soils from the Lorraine region in eastern France were selected on the basis of their texture and pH (Table 1). None of these soils had ever been exposed to glyphosate. Soil types were classified as rendzic leptosol, fluvic cambisol, and stagnic luvisol, hereafter referred to as: clay loam soil, sandy loam soil and silt clay loam soil, respectively. The surface layers (0–25 cm) of all three soils were sampled on the same day.

Soils were air dried and sieved to 2 mm maximum particle size. Soil samples (25 g) were placed in glass jars of 60 mm diameter by 40 mm high. Samples were prepared in triplicates for each soil and each sampling time. An aqueous solution of 0.51 mg glyphosate and 45.1 kBq (equivalent to 1800 g/ha) was added to each soil sample. The volume of aqueous solution was calculated for each soil to obtain samples with moisture content of 80 % of soil retention capacity.

Table 1 Principal characteristics of the soils (surface layers, 0-25 cm) used in this study

Soil	Clay (%)	pH (water)	OC[a] (%)	K_f[b]	Fe oxides[c] (g/kg)	Fe amorphous[d] (g/kg)	Total Cu[e] (mg/kg)	Total P_2O_5 (g/kg)
Sandy loam	10.5	5.1	0.82	34.5	9.73	2.89	7.89	1.24
Silt clay loam	30.6	6.3	1.45	33.6	40.05	8.52	29.80	3.24
Clay loam	34.9	7.9	1.91	16.6	33.16	2.51	14.11	2.74

[a] Organic carbon content; [b] K_f values obtained from Al-Rajab et al., 2008; [c] subtraction of extracted iron by sodium dithionite-citrate and by acid ammonium oxalate; [d] extracted iron by acid ammonium oxalate in darkness; [e] dissolved by HF.

Laboratory degradation studies

Each soil sample was placed in an individual airtight jar (1.5 L). A scintillation vial containing 10 mL water was placed in each jar to maintain a humid atmosphere and prevent desiccation of the soil. A second scintillation vial with 10 mL of 0.5 mol/L NaOH solution was also placed into each jar to trap any CO_2, which evolved from the soil due to mineralization of organic matter and $^{14}CO_2$. The jars were incubated in the dark at 20°C for 80 days. Analyses were performed in triplicates and one control of unspiked soil per type of soil was considered.

Evaluation of soil micro-organism activity

The total CO_2 fixed by the NaOH was evaluated by titrating an aliquot (8 mL) with 0.2 mol/L HCl, in the presence of 3 mL of 20 % $BaCl_2$ and thymolphtalein at 4 % in ethanol, on day 0, 1, 2, 3, 5, 8, 12, 17, 22, 30, 40, 65, and 80. On each sampling date, the replacement of the CO_2 trapping solution by fresh solution allowed air renewal in the jars.

Estimation of mineralization of glyphosate

The amount of $^{14}CO_2$ trapped by NaOH as a result of the mineralization of ^{14}C–glyphosate was determined by liquid scintillation counting. NaOH (1 mL, in duplicates) of each sample received 10 mL Ultima Gold scintillation cocktail (LSC–cocktail) from Packard (USA) in a plastic scintillation vial. Radioactivity was measured during 10 min using a Packard Tri–Carb 1900 CA liquid scintillation counter (Packard, USA).

Residues in soil

Extractable residues of glyphosate were evaluated and analysis as follow. Soils samples in triplicates were removed from incubation for each soil on day 0, 1, 2, 3, 5, 8, 12, 17, 22, 30, 40, 65 and 80 after treatment. The soil of each sample (25 g) was transferred into a 250–mL PPCO (Nalgene, VWR, USA) centrifuge flask. The soil was extracted thrice with 100 mL distilled water (easily available residues) then 3 times with 100 mL of 0.1 mol/L KH_2PO_4. The samples were rotary shaken at (20 ± 2)°C for 2 hr, and then centrifuged at 5000 ×g for 20 min. The supernatants were combined, the volumes adjusted and radioactivity was determined using liquid scintillation as described above. The supernatants of each sample were filtered through Whatman 40 filter papers, and transferred into a round bottom glass bottle (1000 mL), and then frozen at –30°C for 48 hr before being freeze dried (Edwards–Modulyo–RUA). The freeze–dried extracts were dissolved in 7 mL distilled water and filtered through 0.2 µm using Minisart RC–25 filters (Sartorius, France), then the extracts were stored in freezer at –30°C till derivatization and analysis by HPLC.

Analysis

Derivatization of residues

This analysis was carried out only on the aqueous soil extracts. A 0.5 mL of 0.05 mol/L buffer borate was added to 3 mL of the aqueous solution to be analysed, then left to settle for 15 min. Then 3 mL ethyl ether were added and the solution was agitated vigorously for 2 min. The mixture was left to settle. After 15 min, 1.5 mL of the aqueous phase was removed and 0.25 mL acetonitrile added, followed by 0.25 mL of a solution of FMOC–Chloride in acetonitrile (1 g/L). The mixture was left to react for 60 min at

ambient temperature. Two milliliter of ether ethyl was added and the solution was agitated vigorously for 2 min. The solution was left to settle for 1 hr and then the aqueous phase was recovered in a 2–mL vial for high performance liquid chromatography (HPLC) analysis.

Analysis of residues

The residues were analyzed by HPLC in a Varian chromatograph equipped with a fluorescence detector and a β–radioactivity detector (Flo–one β, Packard, USA) in the following operating conditions: Lichrosorb–NH2 column (5 µm, 4 mm × 250 mm) (CIL–Cluzeau, France) thermostated at 30°C, injection volume 50 µL, analysis time 22 min, flow rate 0.8 mL/min, elution KH_2PO_4 0.05 mol/L, pH 5.7, acetonitrile (70/30) (V/V). Detection was performed in the following conditions: (1) β–radioactivity detector: Scintillator Ultima–Flo, flow rate 1.2 mL/min, counting cell 500 µL, and (2) fluorescence detector: λ excitation 260 nm; λ emission 310 nm. Standards of the glyphosate (purity >98.5 %), AMPA (purity >98.5 %, CIL–Cluzeau, France) and sarcosine (N–methylglycine, purity >99 %, Fluka) were used for calibration (0, 10, 20, 50 and 100 µg/L). The retention time was 4.2 min for sarcosine, 6.6 min for AMPA, and 13.3 min for glyphosate.

Non–extractable radioactivity

After extraction by water and KH_2PO_4, all soil samples were air dried. Remaining non–extractable ^{14}C–radioactivity was determined by combustion. An aliquot of 0.3 g was mixed with 0.15 mg cellulose powder and the sample was burnt at 900°C with a 307 Packard Oxidizer (Packard, USA). The released $^{14}CO_2$ was trapped with 10 mL Carbosorb (Packard, USA) and the radioactivity was counted after the addition of 10 mL of Permafluor (Packard, USA).

Statistics

Statistical analyses were performed using Stat Box computer software (Grimmer Software version 6.4). Comparison of the means was done using the Newman–Keuls test at levels of 0.05, 0.01 and 0.001. Curves were plotted using SigmaPlot (Version 10, Systat Software Inc., USA). Data in figures represent the mean and standard deviation of triplicate samples.

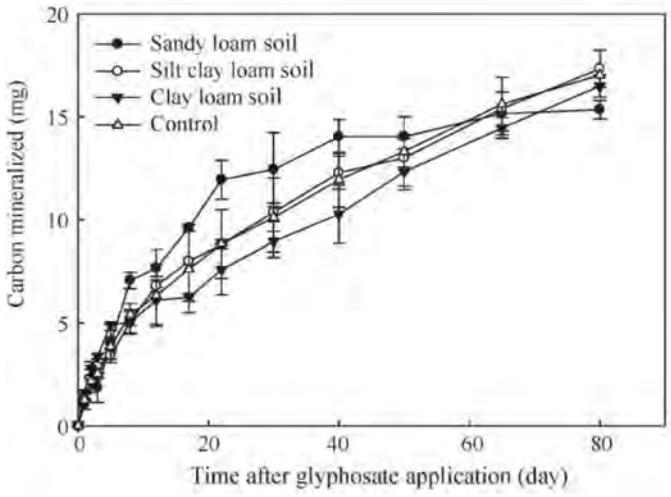
Results

Microbial activity

Total carbon mineralization of treated or untreated soils during the incubation was used as an indicator of the total microbial activity in the soils (Figure 1). Endogenous carbon was steadily mineralized in each soil during incubation and the intensity of mineralization differed slightly among soils between day 5 and day 50. During this period, mineralization was slightly faster in the sandy loam soil (14.4 mg carbon) than in the other two soils (13.73 mg for silt clay loam soil and 11.8 mg for clay loam soil). After 50 days, the slowdown in mineralization activity was more rapid for sandy loam soil than for the other two soils.

At the end of experiment (after 80 days of incubation), the total amount of carbon mineralized was similar for all three soils indicating that each soil presented significant microbial activity and that glyphosate had no toxic effect on soil micro–organisms.

Figure 1 Mineralization activity of microflora of three soils (clay loam, sandy loam and silt clay loam soils). The control is the average of mineralization activity for the three untreated soils.



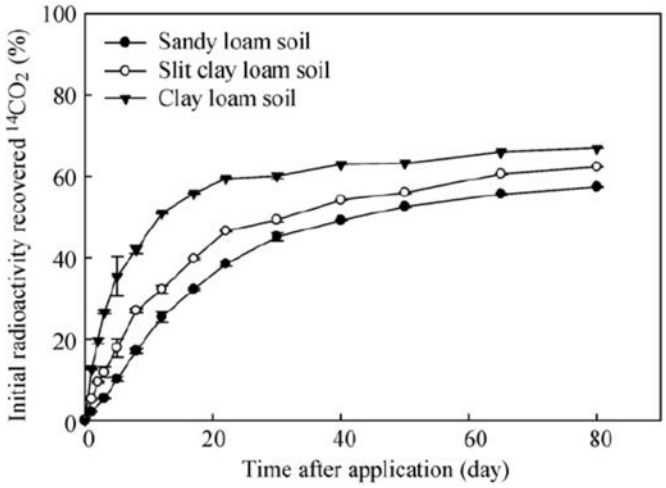
Mineralization of glyphosate

We observed an immediate and high rate of glyphosate degradation after its application on soil (Figure 2). The absence of lag phase indicates that the microflora of soil already had an enzymatic system capable of degrading glyphosate and as such did not need an adaptation period.

Mineralization of glyphosate after 17 days of incubation reached 32.2 % to 39.7 % of the initial amount applied to the two soils (sandy loam (pH 5.1) or silt clay loam (pH 6.3)). However, the mineralization rate was more rapid and intense for the clay loam soil (pH 7.9) with 48.4 % reached by 12 days of incubation. Thereafter, the mineralization of glyphosate declined gradually for all three soils. The endogenous activity of mineralization was comparable for the three investigated soils. The fast mineralization of glyphosate in clay loam soil appears due exclusively to a bioavailability more important than in other two soils.

We have previously shown that the adsorption of glyphosate in clay loam soil (K_f = 17) is lower than the other two soils (K_f = 34) (Al–Rajab et al., 2008). The half–lives of glyphosate derived from the mineralization rates were significantly different for the three soils, and were 42, 31, and 12 days for sandy loam, silt clay loam, and clay loam soils respectively. These results show that the degradation of glyphosate in biologically active agricultural soils could be influenced by the adsorption of glyphosate. Otherwise, the effect of organic matter content in the soil on mineralization of glyphosate was not clear under the conditions of this study.

Figure 2 Mineralization of ^{14}C–glyphosate to $^{14}CO_2$ in three soils incubated at 20°C.



Glyphosate degradation products – Extractable residues

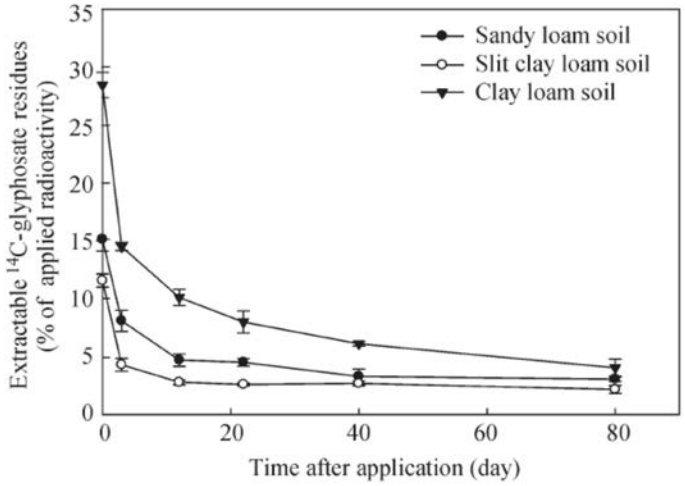
The soil was extracted separately three times with distilled water, then three times with 0.1 mol/L KH_2PO_4. The extraction rate of glyphosate residues with H_2O is influenced by: (1) the degradation,

which produces new products (metabolites) that differ in their water solubility and their reactivity with soil constituents; (2) by the process of adsorption–desorption, and (3) the formation of non–extractable residues over time; these sequestered residues are not available to be extracted by H_2O.

The extraction rate of glyphosate with water is an indication of the accessibility of the residues for microbial degradation and/or their transfer to groundwater under natural conditions. The extraction of glyphosate residues with water is directly related to the K_f measured for these soils (Figure 3). The observed difference of glyphosate extractable residues with water between the sandy loam soil and silt clay loam soil (which have the same K_f value) is certainly related to their texture. For the sandy loam soil, the sandy texture and unstable structure results in a better accessibility to the extraction solution, which in turn leads to a greater extraction efficiency when compared to clay loam soil.

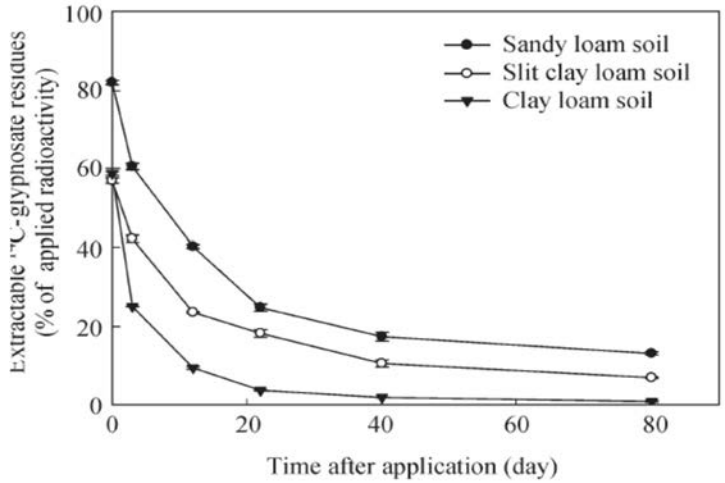
The extraction curves are opposite to those of the mineralization, with the same ranking of soils. These results indicate that the degrading activity of the microflora of soil is linked to the rate of glyphosate available for passage in the aqueous phase.

Figure 3 Evolution of extractable ^{14}C–glyphosate residues with H_2O from the three soils during incubation at 20°C.



On the other hand, the extraction of glyphosate from soil with 0.1 mol/L KH_2PO_4 was more efficient than extraction with H_2O. It did not seem affected by the level of bonds energy between the soil and residues of herbicide (Figure 4). In fact, in the sandy loam soil of Kf = 34, the percentage of glyphosate ^{14}C–phosphonomethyl extracted at T0, immediately after treatment, was (81.9 ± 0.55) % of the initial amount applied (Figure 4). Thereafter, this value decreased slowly to reach (13.0 ± 0.41) % of the initial amount applied at the end of incubation. In contrast, in the silt clay loam soil, with similar value of Kf = 34, the percentage of extracted residues at day 0 was only (56.9 ± 0.7) %, which is similar to that obtained for the clay loam soil which has a different Kf value of 17. This difference may be due to the high clay content in these two soils (silt clay loam and clay loam) and their structures, which reduces the performance of extraction of KH_2PO_4. We can assume that the treatment in a dry soil may cause an entry of glyphosate into the microporisity of aggregates during the capillary invasion by the aqueous solution of treatment (Guimont et al., 2005). The size of this compartment would be defined at the time of treatment and may depend on the physicochemical and physical properties (size of microporal compartment), and the moisture rate of soil at application time. This availability to extraction decreased overtime, more quickly in the sandy loam soil than in the other two brown soils, and at the end of experiment it reached 13.0 %, 6.9 %, and 0.8 % of the initial amount for sandy loam, silt clay loam, and clay loam soils, respectively. The evolution of extraction rate with KH_2PO_4 over time in the three soils is related to the mineralization of residues and the rate that non–extractable residues become available for mineralization and extraction.

Figure 4 Evolution of extractable ^{14}C–glyphosate residues with KH_2PO_4 from the three soils during incubation at 20°C.



Glyphosate degradation products – Degradation products

The analysis of water extracts by HPLC showed the appearance of two degradation products of glyphosate AMPA and/or sarcosine. However, this analysis of glyphosate residues by HPLC did not allow us to measure the sarcosine because its retention time was too short and equal that of co–eluted and unlabelled organic compounds. This analysis showed only the very rapid onset of AMPA in the extracts and its predominance compared to glyphosate as of the day 12 of application for the clay loam soil.

The appearance of AMPA during incubation varied significantly depending on the speed of mineralization of glyphosate in each soil (Table 2). In sandy loam soil, there was only 12.7 % of AMPA present on day 3 after treatment, whereas 87.3 % of the initial radioactive glyphosate was present on the same day. Thereafter, the percentage of AMPA increased gradually overtime, reaching 58.9 % of residues after 22 days of incubation, and 91.1 % at the end of the experiment.

Table 2 Mass balance of ^{14}C–glyphosate residues during incubation over 80 days as percentage of initial radioactivity (%)

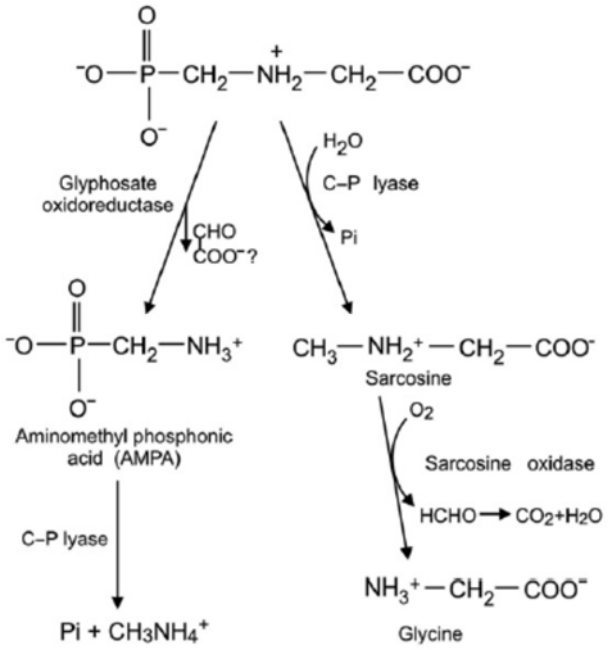
Incubation time (day)	Sandy loam soil		Silt clay loam soil		Clay loam soil	
	Glyphosate	AMPA	Glyphosate	AMPA	Glyphosate	AMPA
0	100	nd	100	nd	100	nd
3	87.3	12.7	79.7	20.3	51.5	48.5
12	71.0	29.0	58.5	41.5	40.2	59.8
22	41.1	58.9	25.6	74.4	12.0	88.0
40	22.3	77.7	22.5	77.5	5.6	94.4
80	8.9	91.1	14.9	85.1	0.9	99.1

AMPA: aminomethylphosphonic acid. nd: not detected.

The extractable residues of glyphosate with water are easily available to the degradation or transfer by water in soil. The half–life of glyphosate extractable with water was estimated and was found to vary depending on the biological activity of soil. It was 19 days for the sandy loam soil, 14.5 days for the silt clay loam soil and 4 days for the clay loam soil.

Together, our results suggest that the rupture of the $–CH_2–NH–$ bond giving rise to AMPA is easier than breaking the $–CH_2–PO_3H_2$ bond that results in either sarcosine and phosphorus, or methylamine and phosphorus (Figure 5). The break of the $–CH_2–NH–$ bond may depend on the overall activity of the microflora and the retention of glyphosate by the soil; while the rupture of the $–CH_2–PO_3H_2$ bond could be related to a more specific bacterial population. This difference in the rupture speed of these two links leads to some accumulation of AMPA in the soil (Figure 5).

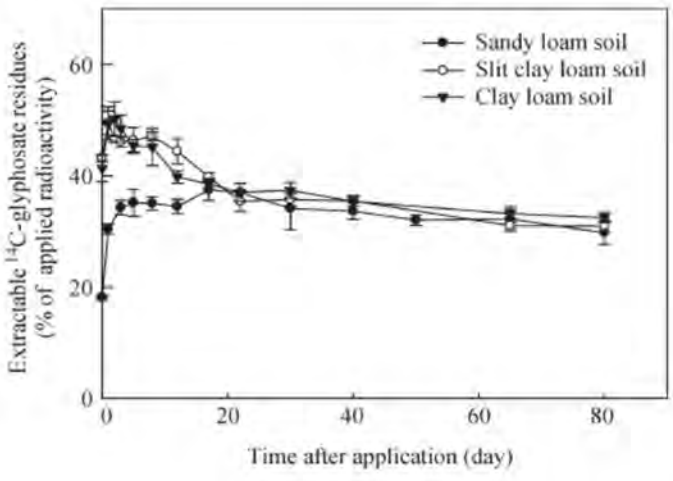
Figure 5 Microbial degradation of glyphosate in soil through sarcosine or AMPA (Liu et al., 1991)



Non–extractable glyphosate residues

The non–extractable residues represent the fraction, which cannot be extracted from the soil by the series of KH_2PO_4 extractions (exhaustive extraction) (Figure 6). Upon application of glyphosate on a sandy loam soil, we observed the formation of non–extractable residues at 18.1 % of the initial applied amount of herbicide. Subsequently, it progressed during 3 days to 35 %, staying stable until day 22, and then decreased very gradually over time until 30 % of initial applied amount of glyphosate was present at the end of experiment. In contrast, the formation of non–extractable residues for the clay loam and the silt clay loam soils was more intense and rapid than in the sandy loam soil. It reached 41.3 % and 43 % of the initial applied amount for the clay loam and silt clay loam soils respectively at day 0, and 49.4 % for both soils at day 1. For both soils, the rate stayed stable after day 2 until which decreased to 32.4 % and 30.9 %, respectively by the end of experiment. The rates of non–extractable residues seems specific for each soil, but are defined by day 3 after treatment.

Figure 6 Evolution of non–extractable residues in three soils during incubation time at 20°C



The rate of non–extractable residues is probably dependent on the physico–chemical properties and physical aspects of the soils including the size of the microporal compartment. This rapid formation of non–extractable residues immediately after treatment with a maximum reached within 2 to 8 days after application is very specific for glyphosate and could probably be due to: (1) the high solubility of glyphosate in water (10.5 g/L) (Agri–tox, 2009), (2) the physico–chemical properties that allow glyphosate to immediately establish high energy bonds with the constituents of soil, (3) the physico–

chemical properties of soils (texture, meso and microporisity), and/or (4) the treatment conditions.

The treatment of herbicide on a dry soil promotes the capillary invasion and the rapid transport of the solution of treatment in the microporisity intra aggregate (Guimont et al., 2005) subsequently making the glyphosate inaccessible to KH_2PO_4. Furthermore, the clayey texture promotes the importance of the microporosity. This explains the similar behaviour of clay loam and silt clay loam soils in the formation of non–extractable residues of glyphosate. In fact, these two soils have very different K_f values (17 and 34 respectively) but they have the same texture. These two soils, particularly the silt clay loam soil, differs strongly from the sandy loam soil which forms relatively a low rate of non–extractable residues and whose texture is sandy although having the same K_f (34) as the silt clay loam soil. We also noted that the initiation of the degradation of glyphosate did not affect the evolution of extractable residues rate. This implies that AMPA was not playing different role comparing to glyphosate. The very slow decrease of non–extractable residues showed that these residues can return by diffusion, and under the effect of a concentration gradient, to areas accessible to microorganisms to subsequently undergo mineralization. We note that from day 22 until the end of incubation the rates of non–extractable residues of glyphosate were similar for the three soils. The mineralization of glyphosate in three soils affects only the extractable fractions with water and KH_2PO_4 influenced by the forces adsorption defined by K_f.

The ^{14}C mass balance for each sample revealed a deficit (loss) that fluctuated from (4 ± 2) % at day 0 (application of glyphosate) to (6.0 ± 3.4) % after 80 days of incubation independent of soil type and different sampling dates over time. These losses were probably partially caused by the handling of samples during analyses (extraction and concentration). Because of these low losses, results were corrected and returned to 100 % by distributing the deficit on the various compartments assessed in proportion to their respective importance.

Conclusion

We simultaneously monitored in controlled conditions the principal processes involved in ^{14}C–glyphosate dissipation and their interactions in three agricultural soils over a period of 80 days. The results of this experiment showed that for agricultural soils with a significant and comparable biological activity, the fate of glyphosate and its potential in polluting water is closely related to the adsorption and the formation of non–extractable residues, which are dependent on soil texture and its moisture condition at the time of treatment. Our results showed that for a clay soil at basic pH, the glyphosate could be available to reach the groundwater in few days after treatment if the conditions are favourable for precipitation. Conversely, in the case of an acid sandy soil, the potential pollution of groundwater by glyphosate is greatly reduced by the strong adsorption of its residues in the soil. In case of rain following treatment, the risk of groundwater pollution by glyphosate will be low but may continue to be present for long time since the mineralization is slow. In this system, the silt clay loam soil is apparently less favourable for water pollution since it showed a strong adsorption of glyphosate and the formation of large amount of non–extractable residues. In the three investigated soils, a low level of water pollution (background) could be occurred over a long time by the sequestered residues of glyphosate, which are either gradually released into the soil solution, or circulated by the water through the soil.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a soil degradation experiment with glyphosate in three agricultural soils from the EU. The study conditions are sufficiently described. However some deficiencies were identified: the test vessels were air–tight and did not allow for air exchange; no information whether the applied test solution was mixed with the soil; $^{14}CO_2$ was passively (and potentially not quantitatively) collected; soil moisture was too high (80 % of soil retention capacity); for analytical method no LoD/LoQ provided; no radioactive material balance can be established; identification of glyphosate and AMPA was done only in aqueous extracts. Only few quantitative results are reported, mainly graphical plots; calculation method of DT_{50} not reported.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.1.1 and KCA 7.1.4.1.1
Report author	Al-Rajab, A. et al.
Report year	2014
Report title	Behavior of the non-selective herbicide glyphosate in agricultural soil
Document No	American Journal of Environmental Science 10 (2): 94-101, 2014
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (some deviations from study guidelines, not all necessary data reported to derive comprehensive DT_{50} values, preferential flow in the soil column)

2. Full summary of the study according to OECD format

Glyphosate [*N*-phosphonomethyl]glycine is a systematic, non-selective, organophosphorus herbicide used worldwide in agriculture and industrial zones. Following its application, residues of glyphosate can threaten soil or aquatic organisms in adjacent water. In this study, we followed the degradation, stabilization, remobilization and leaching of ^{14}C-glyphosate in three agricultural soils in laboratory incubations and in lysimeters under field conditions. Glyphosate degradation was relatively rapid with a half-life of 14.5 days in the silt clay loam soil incubated at 20°C. Glyphosate's degradation product, Aminomethylphosphonic Acid (AMPA), represented more than 85 % of residues after 80 days of laboratory incubation. Leaching of glyphosate in lysimeters of three different investigated soils under outdoor conditions was very slow, less than 1 % of the initial applied amount has been detected in the leachates after 100 days of experimentation. Glyphosate rapidly formed non-extractable residues after treatment. In summary, glyphosate was removed from soil very rapidly and its leaching seems to be very slow regardless the type of treated soil. On the other hand, the contamination risk of groundwater with its metabolite AMPA at long term is probably due to the release of the non-extractable residues.

Materials & Methods

Chemicals

[Phosphonomethyl-^{14}C]-glyphosate diluted, purity 99 % was purchased from ARC-ISOBIO (Belgium). Glyphosate [*N*-phosphonomethyl]glycine, purity 99 % was purchased from Cluzeau (CIL, Paris). Aminomethylphosphonic Acid (AMPA), 10 ng µ/L in water, was purchased from Dr. Ehrenstorfer GmbH (Germany). Sarcosine [*N*-methylglycine], purity 99 % was purchased from Fluka (Germany). H_2PO_4, FMOC-chloride, Potassium hydroxide and Sodium tetraborate decahydrate were purchased from Fluka (Germany). Methanol and acetonitrile (HPLC grade) were purchased from SDS (France).

Sampling

Soils used in this study were obtained from three different agricultural lands in Lorraine region (France). Therefore, based on information provided by the landowners, these soils were never exposed to direct agricultural application of glyphosate and their properties were as following: Sandy loam soil (Sand:Silt:Clay (59:30:11), pH 5.1; % organic matter 0.82); silt clay loam soil (Sand:Silt:Clay (16:53:31), pH 6.3; % organic matter 1.45); and clay loam soil (Sand:Silt:Clay (35:30:35), pH 7.9; % organic matter 1.91).

In the laboratory studies, soils were air dried then sieved at 2 mm and stored in fridge at 4°C until treatment. Otherwise, in the outdoor leaching study, lysimeters were prepared in site using an undisturbed soil for each type of soil separately, a total of 7 columns of each soil were used in this study.

Lysimeters were polyvinyl chloride pipes of 10 cm wide and 35 cm long. Therefore, the 21 lysimeters of the three selected soils were placed in the experimental field of ENSAIA (54500 Vandoeuvre-lès-Nancy, France) for 100 days.

Extraction of Glyphosate

The efficacy of different solvents for extraction of glyphosate from soil was evaluated as follows. A 5 g portion of each soil (in triplicate) was treated with a 0.5 mL solution of H_2O (concentration of 19.4 Bq/g) of [Phosphonomethyl-^{14}C]-glyphosate and 0.1 µg/g of unlabelled glyphosate. Treated soil was placed into a 250 mL PPCO (Nalgene®, VWR, USA) centrifuge bottle and 25 mL of selected solvent were added. Five different solvents were tested separately for the glyphosate extraction efficacy: Ammonium oxalate monohydrate 0.1 M; potassium dihydrogen phosphate (KH_2PO_4) 0.1 M; a mixture of (NH_4OH 0.5 M + KH_2PO_4 0.1 M + H_3PO_4 0.5 %); $CaCl_2$ 0.1 M and distilled water. Bottles were rotary shaken for 2 h, then centrifuged at 5000 g for 20 min, the supernatant of each sample was recovered. Extraction of each sample has been repeated twice, the supernatants of the same sample were combined and a portion of 1 mL counted by Liquid Scintillation Counter (LSC). Thereafter, extraction of glyphosate from soil samples was effectuated with (KH_2PO_4) 0.1 M.

Laboratory Degradation Study

About 25-g soil samples were placed in glass jars (60 mm diameter, 40 mm high). Samples of silt clay loam soil were prepared in triplicates for each sampling time. Each sample was amended by 0.51 mg of glyphosate and 45.1 kBq in water. Final soil moisture was 80 % of the soil retention capacity. After treatment, each sample was added to a Mason jars (1.5 L). At the same time, a plastic vial of 10 mL H_2O was added to each jar in order to maintain the humidity of soil (Al-Rajab *et al*., 2009). Another plastic scintillation vial with 10 mL of 0.5 N NaOH was placed into each jar for trapping $^{14}CO_2$. Jars were incubated at 20°C in the dark for 80 days. The radioactivity trapped in NaOH was counted at each sampling time using a Liquid Scintillation Counter LSC Packard Tri-Carb 1900 CA (Packard, USA). 1 mL of NaOH was added to 10 mL of scintillation cocktail in a plastic scintillation vial to measure the radioactivity in the LSC during 10 min. At each sampling date, the 25-g soil samples were extracted separately using KH_2PO_4 as described previously. Then, after the 3rd and last extraction, soil samples were air-dried at the lab ambient temperature for 3 days. The remaining ^{14}C-radioactivity in the samples after extraction was referred as (non-extractable residues) which was determined by combustion at 900°C using a 307 Packard Oxidiser (Packard, USA).

Leaching Study

Lysimeters were prepared and placed in the experimental field of Lorraine University (France) 3 months before the treatment. During the experimentation of 100 days, the average temperature was 10°C; total precipitation was 235 mm; in total 8 leachates samples were collected. Leached radioactivity from each lysimeter was determined directly after collection, Therefore, water samples were stored at -18°C until analysis.

Analytical Methods

^{14}C-Radioactivity has been determined using a Liquid Scintillation Counter LSC. Glyphosate residues were determined using a Varian HPLC (USA) equipped with two detectors: A fluorescence detector and a β-radioactivity detector. A Lichrosorb (NH_2) column (4×250 mm, 5 µm) purchased from (CIL-Cluzeau, France) was used and thermostated at 30°C. Fluorescence detector was set at (λ 260 and 310 nm), while the flow rate of 1.2 mL/min was adopted in the β-radioactivity detector with a counting cell of 500 µL. The mobile phase was a mixture of (KH_2PO_4 0.05 mol^{-1}, pH 5.7)/acetonitrile (70/30: V/V) at flow rate of 0.8 mL/min. The injected volume was 50 µL. Within these conditions, the retention times were 4.2, 6.6 and 13.3 for sarcosine, AMPA and glyphosate respectively. Determination of the non-extractable residues in soil has been effectuated by combustion of 0.5 g portions at 900°C using an oxidizer (Packard, USA). Statistical analyses were conducted using Stat Box(Version 6.4, Grimmer Software, France).

Results

Extraction of Glyphosate

Extraction recovery of glyphosate varied from 4 to 74 % of the initial applied amount (Table 1). $CaCl_2$ (0.1 M) and water were the less effective solvents in glyphosate extraction in the three investigated soils. However, ammonium oxalate (0.1 M) was the most efficient solvent with a recovery rate ranged from 60 to 74 %. The only issue with the extraction with ammonium oxalate was that the extracts were very dark and need an intensive clean up. On the other hand, potassium dihydrogen phosphate (KH_2PO_4 0.1 M) was adopted as a suitable solvent since the extracts were clear and it showed an acceptable recovery rate varied from 45 to 49 % in investigated soils (Table 1). Recovery rate with citric acid (20 %) was not high enough (less than 37 %) for the three investigated soils.

Dissipation of Glyphosate

Results showed an immediate and high degradation rate of glyphosate after its application on the soil (Figure 1). Mineralization of glyphosate after 17 days of incubation reached 39.7 % of the initial amount applied. Thereafter, the mineralization of glyphosate declined gradually. The half-life of glyphosate derived from the mineralization rates was 31 days for silt clay loam soil. However, the extraction curves are opposite to those of the mineralization (Figure 2). The percentage of extracted residues from the silt clay loam soil at T0 was only 56.9±0.7 %. This availability to extraction decreased overtime, it reached 6.9 % of the initial amount for silt clay loam soil. HPLC analysis showed the appearance of two degradation products of glyphosate AMPA and sarcosine. However, this analysis of glyphosate residues by HPLC did not allow us to measure the sarcosine because its retention time was too short and equal that of co-eluted and unlabeled organic compounds. The half-life of glyphosate extractable was 14.5.

Table 1. Extraction efficiency of glyphosate from the selected soils using different solvents

Extraction efficiency (%: Mean±standard deviation: n = 3)			
Solvent	Sandy loam	Silt clay loam	Clay loam
KH_2PO_4 (0.1M)	44.9 (±0.3)	48.8 (±0.7)	48 (±0.5)
Ammonium oxalate (0.1 M)	59.9 (±0.7)	73.5 (±0.2)	61.1 (±0.1)
Citric acid (20%)	34.2 (±0.1)	36.4 (±0.2)	28.9 (±0.2)
$CaCl_2$ (0.1 M)	5.7 (±0.5)	3.6 (±0.9)	10.3 (±0.6)
H_2O	14.3 (±0.2)	23.5 (±0.1)	31.7 (±0.1)

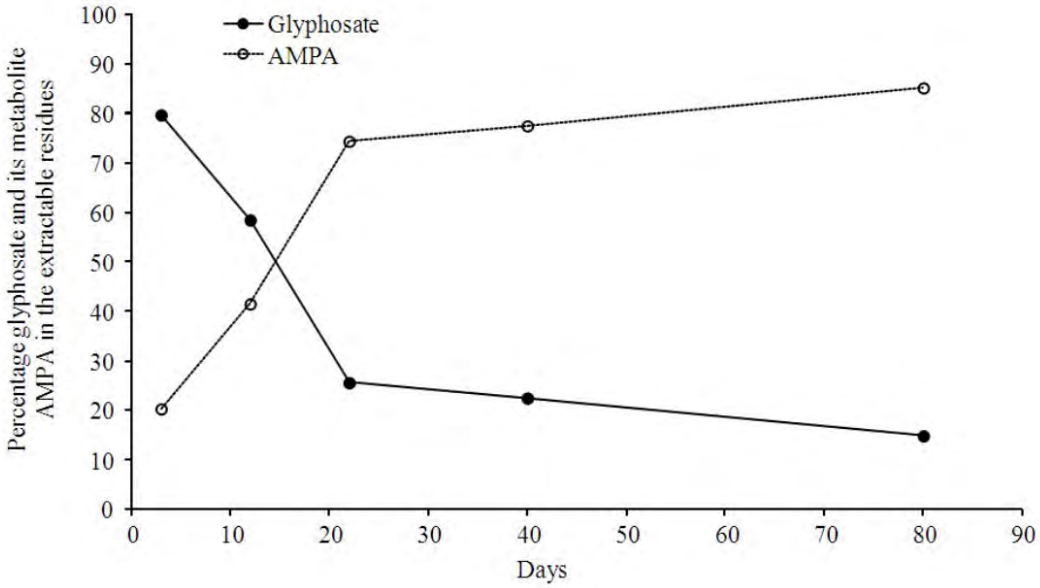


Figure 1. Residues evolution of glyphosate and AMPA in the extractable residues in silt clay loam soil during incubation at 20°C

Discussion

Extraction of Glyphosate

Extraction and determination of glyphosate in agricultural soil is problematic due to its high solubility and its physic-chemical properties (Botero-Coy *et al*., 2013). In the present study, extraction recovery of glyphosate varied from 4 to 74 % of the initial applied amount (Table 1). $CaCl_2$ (0.1 M) and water were the less effective solvents in glyphosate extraction in the three investigated soils. However, ammonium oxalate (0.1 M) was the most efficient solvent with a recovery rate ranged from 60 to 74 %. The only issue with the extraction with ammonium oxalate was that the extracts were very dark and need an intensive clean up.

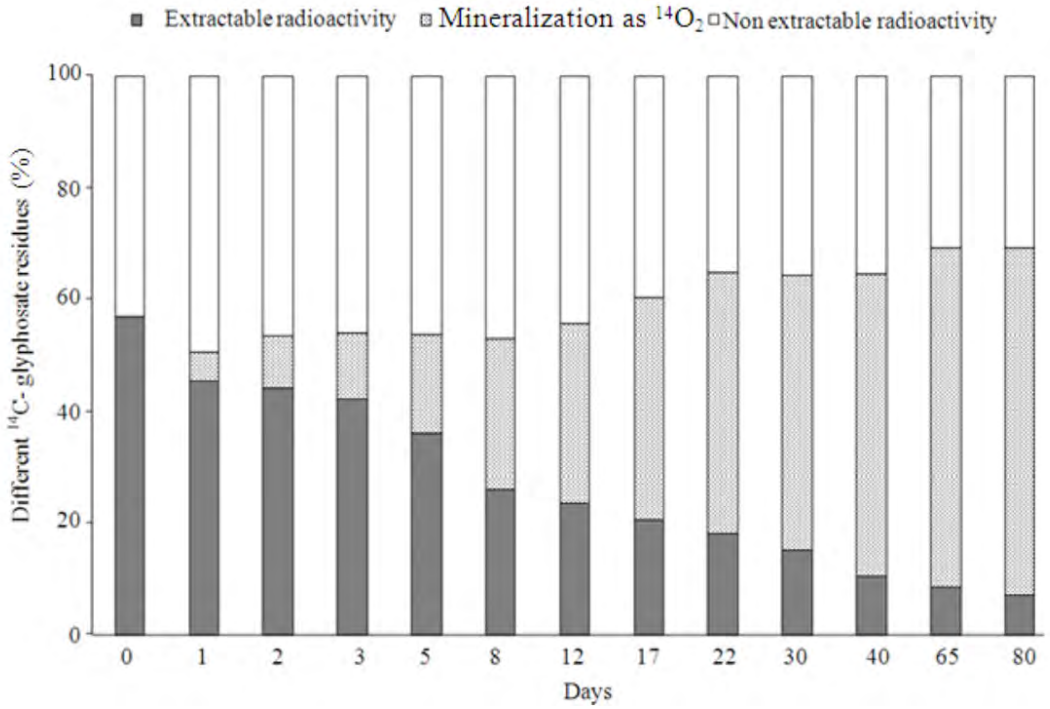


Figure 2. Evolution of different portions of ^{14}C-glyphosate residues (extractable, mineralization as $^{14}CO_2$ and Non-extractable) in silt clay loam soil during incubation at 20°C

On the other hand, potassium dihydrogen phosphate (KH_2PO_4 0.1 M) was adopted as a suitable solvent since the extracts were clear and it showed an acceptable recovery rate varied from 45 to 49 % in investigated soils (Table 1), this rate was similar to that one reported by other studies (Cheah and Lum, 1998; Landry *et al*., 2005). Recovery rate with citric acid (20 %) was not high enough (less than 37 %) for the three investigated soils. Non-extractable residues of glyphosate in soil increase with the time; consequently, glyphosate will be less available for extraction or degradation.

Dissipation of Glyphosate

Monitoring of mineralization of glyphosate labelled on the phosphonomethyl group allows assessing both the loss of glyphosate and AMPA. We observed an immediate and high degradation rate of glyphosate after its application on the soil (Figure 1). The absence of lag phase indicates that the microflora of soil already had an enzymatic system capable of degrading glyphosate and as such did not need an adaptation period. Mineralization of glyphosate after 17 days of incubation reached 39.7 % of the initial amount applied. Thereafter, the mineralization of glyphosate declined gradually. The fast mineralization of glyphosate in the soil appears due to its bioavailability. The half-life of glyphosate derived from the mineralization rates was 31 days for silt clay loam soil. On the other hand, the effect of organic matter content in the soil on mineralization of glyphosate was not clear under the conditions of this study. The extraction rate of glyphosate is an indication of the accessibility of the residues for microbial degradation and/or their transfer to groundwater under natural conditions. The extraction

curves are opposite to those of the mineralization (Figure 2). The percentage of extracted residues from the silt clay loam soil at T0 was only 56.9±0.7 %. We can assume that the treatment in a dry soil may cause an entry of glyphosate into the microporisity of aggregates during the capillary invasion by the aqueous solution of treatment (Guimont et al., 2005; Al-Rajab et al., 2010b). The size of this compartment would be defined at the time of treatment and may depend on the physicochemical and physical properties and the moisture rate of soil at the application moment. This availability to extraction decreased overtime, it reached 6.9 % of the initial amount for silt clay loam soil. The evolution of extraction rate with KH_2PO_4 over time in the soil is related to the mineralization of residues and the availability of non-extractable residues for mineralization or extraction. A similar behaviour of extractable residues of glyphosate over time was reported by (Getenga, 2004; Miles, 1998). HPLC analysis showed the appearance of two degradation products of glyphosate AMPA and sarcosine. However, this analysis of glyphosate residues by HPLC did not allow us to measure the sarcosine because its retention time was too short and equal that of co-eluted and unlabelled organic compounds.

The appearance of AMPA during the first days of incubation is due the fast mineralization of glyphosate in soil, reaching about 85.1 % of residues after 80 days of treatment (Figure 1). Our results are consistent, to some extent, with those obtained by (Cheah and Lum, 1998) who reported the rate of AMPA in the extracts of a sandy loam soil increased gradually over incubation time and reached 50 % of residues after 45 days of treatment. The half-life of glyphosate extractable was 14.5, this value is in accord with the half-lives of 6 to 9 days reported in other study for glyphosate in four agricultural soils incubated at 25°C (Eberbach, 1998) as well as the 19.2 days half-life observed in a sandy loam soil by (Cheah and Lum, 1998). However, much longer half-lives have also been reported by (Getenga, 2004) whereby the half-life of glyphosate was 85.6 days in a clay soil. The fraction of non-extractable residues represent the residues which cannot be extracted from the soil by the series of KH_2PO_4 extractions (exhaustive extraction) (Figure 2). The formation of the non-extractable residues NER in the silt clay loam soil reached 43 % of the initial applied amount at T0 and 49.4 % at T1. The rate stayed stable until T2 after which it decreased to 30.9 % by the end of experiment. The rate of non-extractable residues decreased over time unlike other pesticides such as atrazine where the rate of non-extractable residues increases gradually over dozens of days (Winkelmann, 1991). The rate of non-extractable residues is probably dependent on the properties and physical aspects of the soils including the size of the microporal compartment. This rapid formation of non-extractable residues immediately after treatment is very specific for glyphosate. The treatment of herbicide on a dry soil promotes the capillary invasion and the rapid transport of the solution of treatment in the microporisity intra aggregate, subsequently making the herbicide inaccessible for extraction (Guimont *et al*., 2005). We also reported that the initiation of the degradation of glyphosate did not affect the evolution of extractable residues rate. The very slow decrease of non-extractable residues showed that these residues can return by diffusion and under the effect of a concentration gradient, to areas accessible to microorganisms to subsequently undergo mineralization.

Leaching of Glyphosate

This study showed that water circulation in the soil might has an important role in contamination of groundwater with glyphosate. The diminution of soil macroporosity on the surface layer (where most residues usually present) with the time slows the water infiltration and might encourage the desorption of glyphosate residues. The circulation of glyphosate residues in soil could be due to a preferential water flow regarding the presence of its residues in the 1st collected leachates (Figure 3). In disaccording with results reported by (Dousset et al., 2004), our study showed that the residues of glyphosate were detected in the first leachates samples of three soils, the cumulated precipitation was 85 mm. Detection of glyphosate residues in the 1st leachates was due to the preferential flow (Laitinen et al., 2006). In the case of silt clay loam soil, the maximum residues concentration of 9.5±7 µg/L has been reached after 2 months of application. However, (De Jonge and Jacobsen, 2000) have reported residues concentration of glyphosate much higher than what was obtained from the current study. Concentration of leached residues decreased dramatically after 2 months until the end of experiment (Figure 3). Our findings were in accord with results reported by (De Jonge and Jacobsen, 2000; Landry et al., 2005) who detected the glyphosate residues in the soil leachates after 3 months of application. Overall, the total residues

(extractable and non-extractable) of glyphosate in the soil should be considered to evaluate its persistence in the soil, not only the extractable residues.

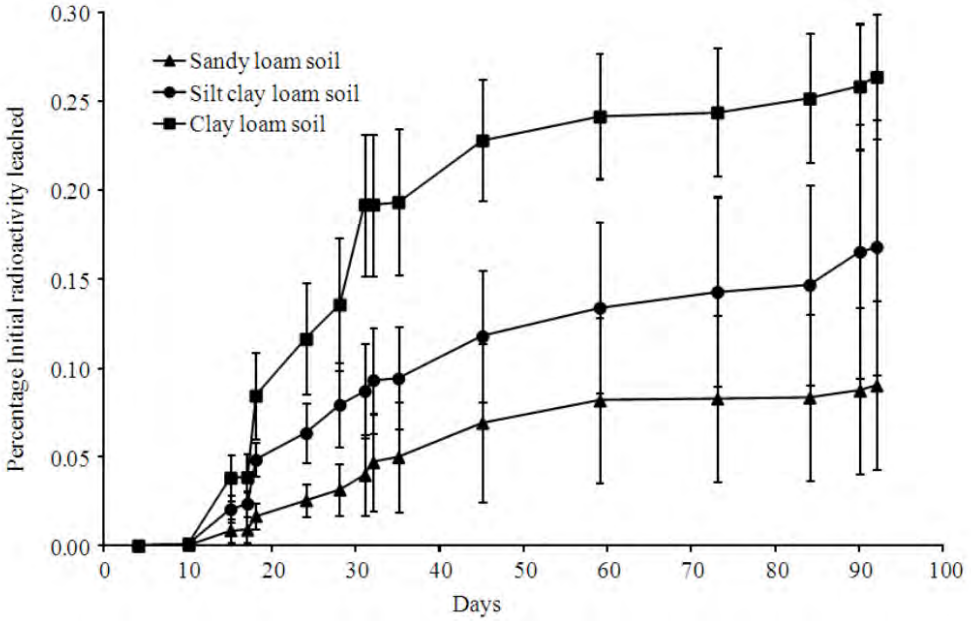


Fig. 3. Radioactivity leached from lysimeters of the investigated soils treated with ^{14}C-glyphosate under outdoor conditions

Conclusion

The present study monitored the residue dynamics of glyphosate in agricultural soil in controlled and outdoor conditions. Results obtained for the fate study suggest that the water pollution with this herbicide is closely related to the adsorption and the formation of non-extractable residues, which are themselves dependent on soil texture and its moisture condition at the time of treatment. In case of rain following treatment, the risk of groundwater pollution by glyphosate will be low but may continue to be present for long time since the mineralization is slow. The silt clay loam soil could be less favourable for water pollution since it showed a formation of large amount of non-extractable residues. In the semi-field lysimeters study, leaching of glyphosate was limited, but its metabolite AMPA seems to be the main potential pollutant of the groundwater. The water circulation mode in the soil was preferential flow which facilitate a fast leaching of residues to reach the groundwater.

In summary, these results suggest that the organophosphorus herbicide glyphosate is rapidly degradable in the agricultural soil. Leaching of glyphosate seems to be very slow regardless the type of the soil. Release of the non-extractable residues of glyphosate probably increases the risk of groundwater pollution with its metabolite AMPA at long term. More investigations are requested for a better understanding of the effect of soil content of organic carbon and soil microflora on environmental behavior of glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes the dissipation and leaching behavior of glyphosate and its metabolite AMPA in three French soils. The soil dissipation experiments were conducted with ^{14}C-labelled glyphosate and show few deviations from the relevant OECD guideline. However, no detailed values on the measurements per time point are reported for all soils.

The outdoor leaching experiments were conducted with 35-cm long undisturbed columns from the three different soils. ^{14}C-labelled glyphosate was applied. Preferential flow was identified as the main reason for the fast detection of glyphosate in the leachate.

The article is therefore classified as reliable with restrictions (Category 2).

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
General criteria for reliability considered for all data requirements indicated by the corresponding EU data points as specified in EC Regulation (EU) No 283/2013	1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines met.	No
	2. Previous exposure to other chemicals is documented (where relevant).	Yes
	3. The test substance is dissolved in water or non-toxic solvent	Yes
	4. Glyphosate, when the test substance, is sufficiently documented - identity of the test material reported (i.e. purity, source, content, storage conditions)	Yes
	5. Only glyphosate is the tested substance (excluding mixture), and information on application of glyphosate is described	Yes
	6. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite)	Yes
	7. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes
	8. Analytical verifications performed in test media (concentration)/ collected samples, stability of glyphosate in test media documented	No
	9. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information	Yes
	10. Assessment of the statistical power of the assay is possible with reported data.	Yes
	11. If statistical methodology was applied for findings reported, then the data analysis applied is clearly reported (e.g., checking the plots and confidence intervals)	Yes
	12. Field locations relevant/comparable to European conditions. Soils not completely matching the OECD criteria but from Europe or to some extent representative for the European Agriculture.	Yes
	13. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5 %), bulk density, water retention, microbial biomass (~1 % of organic carbon)	Yes
	14. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass	Yes
	15. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year	Yes
	16. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max	No

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
	3 months at 4 +/- 2°C).	
	17. Data on precipitation is recorded	Yes
	18. The temperature was in the range between 20-25°C and the moisture was reported	Yes
	19. The presence of glyphosate identified in samples collected from groundwater, soil, surface waters, sediments or air from European areas	Yes
	20. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate	Yes
	21. Analytical methods clearly described and adequate Statement of specificity and sensitivity of the analytical methods is included	No
	22. Radiolabel characterization: purity, specific activity, location of label	Yes
	23. If degradation kinetics are included: expect to see data tables provided, model description. Statistical parameters for kinetic fit.	No
	24. Glyphosate monitoring data: description of matrix analysed, and analytical methods fully described as above.	No
	25. For environmental fate studies: clear description of application rate and relevance to approved uses.	Yes

1. Information on the study

Data point:	KCA 7.1.4.3
Report author	Aronsson, H. et al.
Report year	2010
Report title	Leaching of N, P and glyphosate from two soils after herbicide treatment and incorporation of a ryegrass catch crop
Document No	Soil use and management (2011), Volume 27, Number 1, pp. 54–68
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (experiment not sufficiently described to evaluate the validity of the results)

2. Full summary of the study according to OECD format

During 2005–2007, studies were carried out in two field experiments in southwest Sweden with separately tile–drained plots on a sandy soil (three replicates) and on a clay soil (two replicates). The overall aim was to determine the effects of different cropping systems with catch crops on losses of N, P and glyphosate. Different times of glyphosate treatment of undersown ryegrass catch crops were examined in combination with soil tillage in November or spring. Drainage water was sampled continuously in proportion to water flow and analysed for N, P and glyphosate. Catch crops were sampled in late autumn and spring and soil was analysed for mineral N content. The yields of following cereal crops were determined. The importance of keeping the catch crop growing as long as possible in the autumn is demonstrated to decrease the risk of N leaching. During a year with high drainage on the sandy soil, annual N leaching was 26 kg⁄ha higher for plots with a catch crop killed with glyphosate in late September than for plots with a catch crop, while the difference was very small during 1 yr with less drainage. Having the catch crop in place during October was the most important factor, whereas the time of incorporation of a dead catch crop did not influence N leaching from either of the two soils. However, incorporation of a growing catch crop in spring resulted in decreased crop yields, especially on the clay soil. Soil type affected glyphosate leaching to a larger extent than the experimental treatments. Glyphosate was not leached from the sand at all, while it was found at average concentrations of 0.25 µg⁄L in drainage water from the clay soil on all sampling occasions. Phosphorus leaching also varied (on average 0.2 and 0.5 kg⁄(ha yr) from the sand and clay, respectively), but was not significantly affected by the different catch crop treatments.

Materials and methods

Experimental fields

The study was conducted over 2 years (2005–2007) in field experiments with a similar treatment design, but located at different sites, Lanna and Lilla Böslid in southwest Sweden. In both experiments, leaching was measured in separately tile–drained experimental plots where drainage flow was measured continuously and water was sampled in proportion to flow. Precipitation and air temperature were recorded at both sites.

Lanna site, clay soil.

Lanna research station (58°20′N, 13°07′E) is situated in a region which has a mean annual temperature of 6.1°C and mean annual precipitation of 558 mm (Lanna, 1961–1990). The experimental field, which was established in 2001, consists of 10 plots (790 m^2). Each plot was separately tile–drained at ca. 1 m depth and the drains were backfilled with 10–cm gravel at the bottom and then with the soil. The soil at Lanna consists of 47 % clay (<2 µm) in the topsoil (0–0.3 m depth) and 55–60 % clay in the subsoil

(0.3–0.9 m depth). During the study, the topsoil had an organic matter content of 4.4 % and a mean pH of 6.6. The mean amount of ammonium lactate soluble P was 3.4 mg⁄100 g dry soil which is considered as low P status. The soil contains numerous cracks and macropores in the upper 1.0 m of the profile. More details on this soil are given by Bergström *et al*. (1994). At Lanna, the same plots were used during the two experimental years, with the same treatment being applied on each plot during the two consecutive years (with two replicates).

Lilla Böslid site, sandy soil.
Lilla Böslid experimental farm (56°35′N, 12°56′E) is located ca. 240 km south of Lanna. The mean annual temperature is 7.2°C and the mean annual precipitation is 803 mm (Halmstad, 1961–1990). The sandy soils in this region are commonly drained as the groundwater levels are often high because of a clay layer under the sand deposits. This experimental field was constructed in 2002, and consists of 36 separately tile–drained plots, each 320 m^2. The tile drains are at 0.9–m depth. The soil is an unstructured sand with 9 % clay in the topsoil (0–0.3 m depth) and 1–2 %in the subsoil (0.3–0.9 m depth). At the time of study, the topsoil had a mean organic matter content of 4.9 % and a pH value of 6.1. The mean amount of ammonium lactate soluble P was 12.8 mg⁄100 g dry soil. This value indicates that this soil is rich in P and that reduced P application rates are recommended for spring cereals. At Lilla Böslid, the experimental lay–out allowed two experimental years on different plots by dividing the field into two sections and using one section each year (with three replicates).

Experimental design and management practices
During the year before the experiment started, a spring cereal was grown at Lilla Böslid and winter wheat at Lanna. The experiments started in 2005 by undersowing a catch crop of ryegrass (*Lolium perenne L.*) in a cereal crop in three of five treatments at Lanna and in all treatments at Lilla Böslid (Table 1). At Lanna, glyphosate was applied in four treatments at the beginning of October, and in one in spring. Glyphosate treatment in October was combined with tillage in November (mouldboard ploughing, 25–cm depth) or in April (stubble cultivation, 6–cm depth). At Lilla Böslid, different times of glyphosate treatment in autumn were tested in combination with mouldboard ploughing (25–cm depth) in November or April. There was also one treatment without use of herbicide and spring ploughing, which was considered as a control treatment representing the best scenario for low N leaching. At Lilla Böslid, the soil was tine–cultivated to ca. 10 cm depth just before ploughing. Dates of tillage and glyphosate treatment are shown in Table 1. At Lanna, glyphosate was applied as Glyphomax Bio at a dose of 3.5 or 4.0 L⁄ha and at Lilla Böslid as Round–up Bio, 3.5 L⁄ha. The crop following incorporation of the catch crop was a spring cereal (oats or barley). It was fertilized with 100–110 kg N⁄ha at Lanna and with 90 kg N⁄ha at Lilla Böslid. A dose of 10 kg⁄ha of mineral P was applied at Lilla Böslid in 2006 and the same amount at Lanna in 2007.

Sampling and analyses of water, soil and crops
Drainage water from the plots at both sites was led to an underground monitoring station with temperatures never >15°C and <10°C during the main drainage periods when discharge rates were recorded using tipping buckets connected to a data logger which stored accumulated daily drainage volumes from each plot. Flow–proportional water samples of 15 mL were taken using a peristaltic pump after every 0.2 mm discharge. The samples for each plot were collected in individual polyethylene bottles which were emptied every 2 weeks during drainage periods for analysis of total–N, NO_3–N, total–P and PO_4–P. During sampling, the bottles were prepared with sulphuric acid for conservation of glyphosate. Glyphosate and the degradation product of aminomethylphosphonic acid (AMPA) were analysed for the same samples on 5–6 occasions during each of the two drainage seasons. At Lanna, glyphosate was analysed in samples from treatments A–D and at Lilla Böslid, in treatments F–J (Table 1). These events were primarily chosen to represent periods when drainage started in autumn with high flow periods. The first samples were taken before glyphosate treatment to ensure that any leaching detected originated from the experimental treatments. During the first year, the samples from replicates were pooled for analyses of glyphosate because of the high cost of analyses, but during the second year, all samples were analysed individually. Prior to analysis, the water samples were pretreated with a C18 ion exchange column for removal of non–polar substances, which also caused some filtration of particles (unknown size). Then glyphosate was derived with trifluoroacetic acid⁄trifluorethanol before combined gas chromatograph⁄mass spectrometer (GC⁄MS) analyses. The partitioning between particle–bound and

dissolved glyphosate was not examined and some particles were also filtered before analysis. Thus, the analysis mainly covered the amount of dissolved glyphosate, but it is also likely that some particle–bound glyphosate was included as water samples were acidified during storage, which may have resulted in some dissolution of particle–bound glyphosate.

Table 1. The different experimental treatments at the two sites during the 2 years, with planned and actual time of glyphosate treatment and catch crop incorporation

		Time of glyphosate treatment			Time of incorporation		
	Catch crop	Plan	Act yr 1	Act yr 2	Plan	Act yr 1	Act yr 2
Lanna, clay soil							
A	Per. ryegr.	1 Oct	4 Oct 2005	4 Oct 2006	10 Nov	11 Nov 2005	10 Nov 2006
B	Per. ryegr.	1 Oct	4 Oct 2005	4 Oct 2006	1 Apr	28 Apr 2006	12 Apr 2007
C	Per. ryegr.	1 Mar	14 Apr 2006	19 Mar 2007	1 Apr	28 Apr 2006	12 Apr 2007
D	–	1 Oct	4 Oct 2005	4 Oct 2006	10 Nov	11 Nov 2005	10 Nov 2006
E	–	1 Oct	4 Oct 2005	4 Oct 2006	1 Apr	28 Apr 2006	12 Apr 2007
Lilla Böslid, sandy soil							
F	Per. ryegr.	20 Sep	26 Sep 2005	26 Sep 2006	10 Nov	24 Nov 2005	24 Nov 2006
G	Per. ryegr.	20 Sep	26 Sep 2005	26 Sep 2006	1 Apr	12 Apr 2006	2 Apr 2007
H	Per. ryegr.	5 Oct	4 Oct 2005	10 Oct 2006	10 Nov	24 Nov 2005	24 Nov 2006
I	Per. ryegr.	5 Oct	4 Oct 2005	10 Oct 2006	1 Apr	12 Apr 2006	2 Apr 2007
J	Per. ryegr.	20 Oct	31 Oct 2005	22 Nov 2006	1 Apr	12 Apr 2006	2 Apr 2007
K	Per. ryegr.	–	–	–	1 Apr	12 Apr 2006	2 Apr 2007

Per. ryegr., perennial ryegrass.

Calculations and statistical analysis

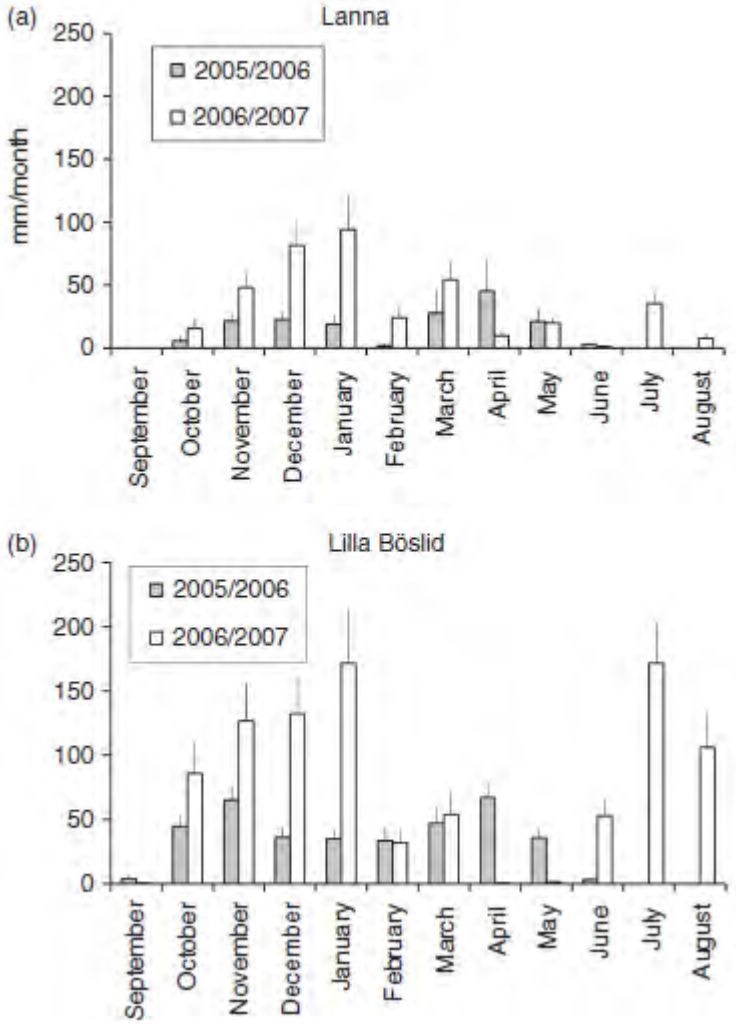
Analysis of variance was carried out by the Mixed procedure in SAS 9.1 (SAS Institute Inc. 2003: SAS/Stat 9.1 Users' Guide. Cary, NC, USA) for the statistical analysis of differences in yields, catch crop biomass and N and P contents, soil mineral N, leaching of N and P and concentrations of glyphosate between treatments. The t–test at P = 0.05 was used for pairwise comparisons by the PDIFF statement. Block was used as the random variable in analysis of a single year. For the Lanna site, the average for the 2 years was analysed by calculating an average per plot and by using block as random variable. For the Lilla Böslid site, where the experiment was carried out in separate plots during the 2 years, year was used as random variable when analysing the average for the 2 years.

Results

Drainage and climate conditions

The two experimental years represented varying climate and drainage conditions, 1 yr with a cold winter with relatively small drainage amounts, and one mild winter with high drainage. At Lanna, the mean temperature during December 2005–March 2006 was –3.5°C, while it was +2.1°C during the same period 2006–2007. For Lilla Böslid, corresponding values were –2.6°C and +4.3°C. At Lanna, the measured precipitation was 480 mm during 2005–2006 and 759 mm during 2006–2007 (1 September–31 August). At Lilla Böslid, the corresponding figures were 542 mm and 950 mm, respectively. During the first year, the precipitation was considerably lower than the long–term mean value for both sites, but higher during the second year. Different precipitation and temperature conditions during winter clearly affected drainage and the N and P leaching during the two experimental years. The high rainfall resulted in much drainage during the autumn and winter of 2006–2007. During summer 2007, southwest Sweden was exposed to several large low pressure cells, which resulted in extremely rainy conditions and major drainage events. There was some variation in drainage water totals between individual plots as shown in Figure 1 where standard deviations for all plots are included. These differences could not be attributed to different experimental treatments except for 2005–2006 at Lilla Böslid, when treatment K had higher drainage than most of the other treatments (P = 0.03).

Figure 1. Mean monthly drainage (mm) from all plots during the two experimental years at the two sites Lanna (a) and Lilla Böslid (b). Standard deviations are shown with narrow bars.



Management practices, catch crop growth and crop yields

The planned time of glyphosate treatment in September and beginning of October corresponded quite well to the actual time at both sites (Table 1). From field observations, the catch crop was still intact 1 week after treatment, but after 3 weeks, it was totally killed in both years. However, glyphosate treatment in late October at Lilla Böslid was delayed by up to 4 weeks because of bad weather conditions, especially in 2006 (Table 1) when the catch crop was treated in late November. This resulted in a poor effect of the glyphosate, and only 50 % of the catch crop was killed 3 weeks after treatment. Glyphosate treatment in spring (treatment C at Lanna) resulted in problems with the timing. Obtaining an effect of the herbicide, while simultaneously being able to cultivate this heavy clay soil, was a challenge. In spring 2007, when there was a very dense catch crop, it was particularly difficult to incorporate the catch crop material in this treatment, and about 20–40 % of the catch crop was estimated to be still growing at harvest of the following crop. Shallow cultivation in spring worked much better after glyphosate treatment in autumn (treatments B and E) with respect to incorporation of plant material, although this tillage practice is not common for this type of soil.

Leaching of glyphosate

At the sandy soil at Lilla Böslid, drainage water was analysed for glyphosate on eight occasions during the experimental period (November 2005, December 2005, April 2006, October 2006, November 2006, December 2006, January 2007 and March 2007). Glyphosate was only detected twice and occurred at trace levels, that is at concentrations above the detection limit (ca. 0.01 µg/L), but under the limit for determination of the concentration (ca. 0.05 µg/L). These occasions were in treatments F and I at sampling on 20 December 2006 and in treatment J on 8 January 2007.

AMPA was not found at all. As a result of bad weather conditions during October–November 2006, glyphosate application in treatment J was not possible until 22 November. If there had been a risk of glyphosate transport, it would probably have arisen during conditions like these, but the risk seemed to be very small for this soil. The adsorption of glyphosate in the sandy soil was probably very efficient, probably because of Al/Fe–oxides, the same as for P. At the Lanna clay soil, glyphosate was found at concentrations above the determination limit in all samples except two during the experimental period (Table 2). Thus, application of glyphosate both in autumn and in spring resulted in some transport to drainage water, but with this experimental design, it was possible that application of glyphosate during 2005–2006 also affected to some extent the results from 2006 to 2007. Even at sampling in spring 2005, before the start of the experiment at Lanna, traces of glyphosate were found in drainage water. This probably originated from autumn 2004 when glyphosate was applied to borders between the experimental plots. Concentrations were low, on average 0.25 µg/L, and only exceeded 1 µg/L on one occasion (January 2007 in treatment D). The concentrations of glyphosate measured at Lanna were similar to those found in monitoring of streams in agricultural catchments in southern Sweden (Adielsson *et al.*, 2007).

Table 2. Measured concentrations of glyphosate and its metabolite AMPA

	A		B		C		D	
	Glyphosate	AMPA	Glyphosate	AMPA	Glyphosate	AMPA	Glyphosate	AMPA
Glyphosate treatment	4 Oct 2005 4 Oct 2006		4 Oct 2005 4 Oct 2006		14 Apr 2005 20 Mar 2006		4 Oct 2005 4 Oct 2006	
24 Apr 2005	Trace*	nd*	Trace*		–	–	Trace*	nd*
15 Nov 2005	0.39	nd	0.86	Trace	–		0.85	0.40
27 Nov 2005	nd	nd	0.19	nd	–		0.01	ed
5 Apr 2006	0.23	nd	0.48	Trace	Trace*	Trace*	0.16	Trace
2 May 2006	–	–	–	–	0.29	Trace	–	–
1 Jun 2006	Trace	nd	0.08 (0.05)	nd	–	–	0.04 (0.01)	nd
1 Nov 2006	0.53[a] (0.09)	Trace	0.66[a] (0.48)	Trace	0.19[a] (0.04)	Trace	1.04[a] (0.37)	Trace
15 Nov 2006	0.21[a] (0.11)	Trace	0.44[b] (0.02)	0.10	0.10[a] (0.06)	Trace	0.51[a] (0.07)	0.10
8 Jan 2006	0.18[a] (0.01)	Trace	0.66[b] (0.12)	Trace	0.12[a] (0.06)	Trace	0.18[a] (0.06)	Trace
15 Jan 2006	0.13[a] (0.01)	Trace	0.50[b] (0.01)	Trace	0.10[a] (0.02)	Trace	0.20[a] (0.09)	Trace
13 Mar 2007	0.14[a] (0.04)	Trace	0.31[b] (0.02)	Trace	0.07[a] (0.02)	nd	0.13[a] (0.03)	Trace
21 May 2007	Trace	Trace	0.06[a] (0.04)	Trace	0.14[a] (0.07)	Trace	0.05[a] (0.03)	Trace

Standard deviations are shown in brackets. Different superscript letters indicate significant differences between treatments ($P = 0.01$). *Samples taken before treatment with glyphosate. nd, not detected. Trace, between detection and determination limit, ca. between 0.02 and 0.05 µg/L.

Discussion

Results from this study indicate that soil texture was the dominant factor in influencing both P and glyphosate losses, whereas different treatments had small or no effects. For glyphosate, this was not surprising, as soil structure and transport pathways have been shown to be of major importance for glyphosate leaching (Vereecken, 2005; Borggaard & Gimsing, 2008). The immediate detection of glyphosate in drainage water from the clay soil at Lanna clearly shows that there are rapid pathways for water and solutes in this soil, as reported previously by Larsson & Jarvis (1999). The glyphosate analyses did not distinguish between dissolved and particle–bound glyphosate; however, as 70–80 % of the P losses were in particle–bound form, this might also be an important transport form for glyphosate. In studies on two soils in Denmark, the contribution of colloid–facilitated transport was up to 27 % and 52 % for a sandy loam and a sandy soil, respectively (de Jonge *et al.*, 2000). It is probable that total leaching of glyphosate, especially from the clay soil, was underestimated in this study as it is uncertain of the extent to which particle–bound glyphosate was included in the analyses. Soil tillage practices affect transport pathways through the soil. For example, conservation tillage has been shown to increase the amount of macropores and related preferential flow paths (Shipitalo *et al.*, 2000), but time of ploughing may also affect the partitioning between different types of losses. Spring ploughing instead of autumn ploughing protects the surface against destruction of soil aggregates over winter and is highly relevant in minimizing particle–bound P losses by erosion (Kronvang *et al.*, 2005), especially in combination with a catch crop (Ule´ n, 1997). In contrast, losses of dissolved compounds may increase

when the soil is not cultivated in autumn. This was reported in studies of glyphosate losses in Norway (Stenrød *et al.*, 2007) and Denmark (Lærke Baun *et al.*, 2007) where tillage in autumn increased the leaching of particulate–bound glyphosate, while there was increased leaching of dissolved glyphosate when the soil was not tilled in autumn. These findings are supported by the results from Lanna, where there are indications of higher losses of total–P after ploughing in autumn, but differences in concentrations or yearly transport are ns. Spring tillage at Lanna (treatment B) gave significantly higher concentrations of glyphosate in drainage water than the other treatments on four occasions ($P = 0.01$) in 2006–2007, which may indicate that spring tillage conserved transport pathways through the topsoil during winter. However, it is not possible to draw conclusions about the partitioning between dissolved and particle–bound glyphosate. Another study on the Lanna soil in lysimeters shows that losses of particle–bound glyphosate were negligible and that almost all leached glyphosate was in dissolved form (Bergström *et al.*, 2010). There were no indications of increased transport of dissolved P in spring–ploughed plots, with or without a catch crop over winter. However, catch crop plant material may constitute a risk of dissolved P leaching if exposed to freezing, as shown by Bechmann *et al.* (2005).

In the sandy soil at Lilla Böslid, glyphosate was efficiently sorbed, which was also true for P. The high P status of this soil did not seem to increase the risk of P losses, although studies have shown a relationship between high P content of the soil and P leaching (Heckrath *et al.*, 1995). The larger proportion of dissolved P at Lilla Böslid, compared with Lanna, could be an indication of enhanced P desorption because of high soil P content, but this is probably not the case as P concentrations in drainage water were consistently low and stable. There is also considered to be an increased risk of glyphosate transport in soils with high P content, as PO_4–P and glyphosate may compete for the same surface binding sites on soil mineral particles (Gimsing & Borggaard, 2002). However, the P and glyphosate sorption capacity of the subsoil and the degree of saturation of sorption sites have a large impact on actual P losses, and there was no indication of saturated conditions in the sandy soil at Lilla Böslid.

The results from the sandy soil at Lilla Böslid show that the time available for catch crop growth and N uptake during autumn significantly affected the accumulation of N in the soil and the risk of N leaching during the following winter, although it is somewhat surprising that there is no clear correlation between soil mineral N in autumn and N leaching. The results also show that glyphosate treatment in September or early October resulted in fast release of N available for leaching. This confirms the findings by Snapp & Borden (2005) that N mineralization increases when the catch crop is treated with glyphosate 8 days before incorporation, compared with no treatment before incorporation. The time of catch crop incorporation after chemical kill–off in autumn seems to be of minor importance according to the results from both sites. This is somewhat surprising for the sandy soil, as several studies have shown that time of tillage in autumn clearly influences N mineralization and N leaching from this type of soil (e.g. Wallgren & Lindén, 1994; Djurhuus & Olsen, 1997; Stenberg *et al.*, 1999). In the present study, glyphosate treatment obviously had a similar effect to incorporation on N release in the soil, at least during the second year. For the clay soil at Lanna, the results are similar to those found in a study in an adjacent field (Aronsson & Stenberg, 2010), where time of tillage in autumn or spring did not affect N leaching to any large extent.

Growing a catch crop may affect the yield of the main crop because of inter–plant competition, although this effect is often small or negligible (Ohlander *et al.*, 1996). The catch crop may also affect the following crop after incorporation.

This effect can be positive as a result of the fast remineralization of catch crop N (Lyngstad & Borresen, 1996). It may also be negative as a result of the immobilization of catch crop N or depletion of soil mineral N content in spring as a result of N uptake by the catch crop. This pre–emptive effect (Thorup–Kristensen, 1993) probably contributed to decreased yields at Lanna together with regrowth of the catch crop and at Lilla Böslid. Incorporation of a living catch crop in November–December or in February–March instead of April would probably have been more suitable for remineralization of catch crop N, as suggested by Torstensson & Aronsson (2000). To improve synchronization with the N requirements of the following crop, the N mineralization dynamics must be considered rather than increasing N fertilization rates after incorporation of catch crops.

Conclusion
To develop recommendations to achieve decreased nutrient leaching and pesticide contamination of water, the leaching and contamination risks need to be considered in relation to each other, and crop production aspects also need to be considered. It is clear from this study that N leaching was considerably lower from the clay soil (2–22 kg N/ha/yr) than from the sandy soil (15–53 kg N/ha/yr). It was also clear that spring incorporation of a catch crop could not be recommended on the clay soil as it negatively affected crop yields. Glyphosate treatment causes some risk of contamination of percolating water on the clay soil, irrespective of time of application, while time of incorporation does not affect leaching of N and P on either clay soil or sandy soil. This suggests that clay soil should not be given special priority for the use of catch crops with chemical treatment or for the use of excluded tillage in autumn. The reasons are that the overall risk of N leaching is relatively low and that the beneficial effects on N leaching may be counteracted by some risk of glyphosate leaching. Moreover, there is no reduction in P losses.

For the sandy soil, N leaching was much higher than from the clay soil and keeping the soil covered with a catch crop until November or until spring considerably reduced N leaching during high–flow conditions compared with chemical kill–off in September or mid-October. It was difficult to achieve a good herbicide effect with delayed chemical treatment, indicating that there always has to be a compromise between N leaching and successful weed control. Despite the low risk of glyphosate and P leaching, the results suggest that for a sandy soil, glyphosate treatment should be excluded during autumn when growing catch crops to maximize the reduction in N leaching. Incorporation of the catch crop as late as possible in autumn or in very early spring probably reduces the risk of decreased crop yields of the following crop as a result of N uptake by the catch crop.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a long–term leaching experiment in Sweden on agricultural soils with glyphosate. The method is not sufficiently described to evaluate the validity of the results.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.3
Report author	Bento, C. P.M. et al.
Report year	2017
Report title	Glyphosate and AMPA distribution in wind-eroded sediment derived from loess soil
Document No	Environmental Pollution 220 (2017) 1079 -1089
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable

2. Full summary of the study according to OECD format

Glyphosate is one of the most used herbicides in agricultural lands worldwide. Wind-eroded sediment and dust, as an environmental transport pathway of glyphosate and of its main metabolite aminomethylphosphonic acid (AMPA), can result in environmental- and human exposure far beyond the agricultural areas where it has been applied. Therefore, special attention is required to the airborne transport of glyphosate and AMPA. In this study, we investigated the behavior of glyphosate and AMPA in wind-eroded sediment by measuring their content in different size fractions (median diameters between 715 and 8 μm) of a loess soil, during a period of 28 days after glyphosate application. Granulometrical extraction was done using a wind tunnel and a Soil Fine Particle Extractor. Extractions were conducted on days 0, 3, 7, 14, 21 and 28 after glyphosate application. Results indicated that glyphosate and AMPA contents were significantly higher in the finest particle fractions (median diameters between 8 and 18 μm), and lowered significantly with the increase in particle size. However, their content remained constant when aggregates were present in the sample. Glyphosate and AMPA contents correlated positively with clay, organic matter, and silt content. The dissipation of glyphosate over time was very low, which was most probably due to the low soil moisture content of the sediment. Consequently, the formation of AMPA was also very low. The low dissipation of glyphosate in our study indicates that the risk of glyphosate transport in dry sediment to off-target areas by wind can be very high. The highest glyphosate and AMPA contents were found in the smallest soil fractions (PM10 and less), which are easily inhaled and, therefore, contribute to human exposure.

Materials & Methods

Soil

We used the topsoil of a silty loam loess soil from Huldenberg, Belgium. The soil was air-dried and then sieved through a 1-mm sieve. It was tested for glyphosate and AMPA residues and found free of glyphosate and AMPA. The main soil properties of the sieved soil are shown in Table 1. Figure 1 shows the grain size distribution of the soil after disintegration of all aggregates.

Table 1. Soil properties of the loess soil used in this study

Parameters	Value
Particle size distribution:	
<2 µm (clay) (%)	10
2–50 µm (silt) (%)	79
>50 µm (sand) (%)	11
pH $CaCl_2$	5.8
Organic matter (OM) (%)	3.2
Particle density (g cm^{-3})	2.5
N total (g kg^{-1})	1.7
P available (mg kg^{-1})	0.4
K available (mg kg^{-1})	209
Mg available (mg kg^{-1})	121
Na available (mg kg^{-1})	10
C/N ratio	9

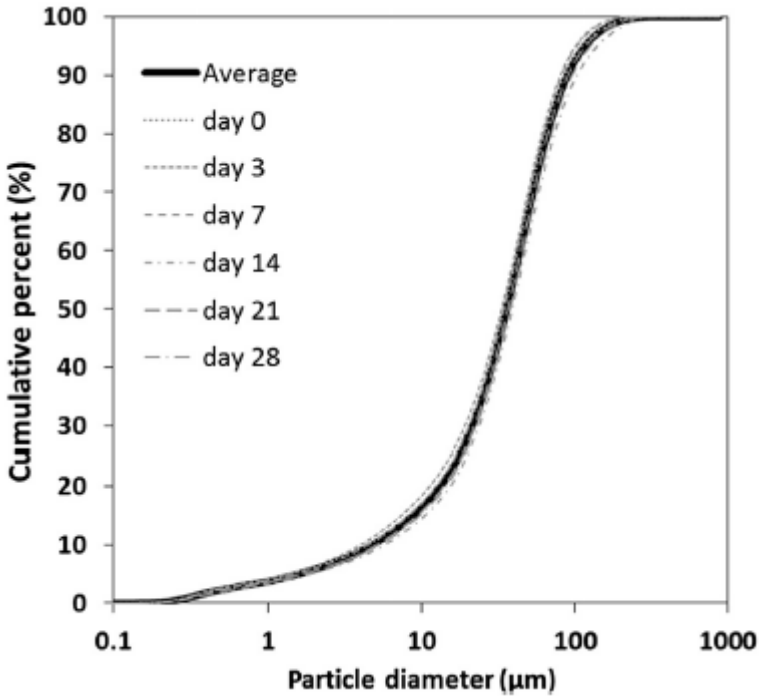


Figure 1. Particle size distribution of the start soil after disintegration of all aggregates.

Glyphosate preparation and application in the soil

Preparation of glyphosate solution

Glyphosate solution was prepared by diluting 980 mL of CLINIC®, a glyphosate-based herbicide that contains 360 g/L of glyphosate, in Millipore water to achieve a final stock solution of 0.42 g/L. A concentration of glyphosate in soil of 8.4 mg/kg was used in this study, which corresponds to an application rate of 1.26 kg a.i./ha (typically applied in agricultural fields), assuming a soil depth of 1 cm and a bulk density of 1.5 g/cm^3.

Application in soil

A plastic sheet was put on the ground and an approximately 5-cm thin layer of the air-dried and sieved soil (42 kg) was spread on it. The soil was then sprayed with the prepared glyphosate solution (see Section 2.2.1). During the application, the soil was thoroughly mixed with a rake. The soil was then stored in a plastic bag at room temperature (22ºC) and dark conditions. A small portion of the soil was collected after glyphosate application and oven-dried (105ºC) for 24 h to determine the initial soil moisture content, which was found to be 5.4 % (w/w).

Facilities and instrumentation

The experiment was carried out in the facilities of the Geography and Tourism Research Group of the Katholieke Universiteit Leuven, Belgium. A closed-return wind tunnel was used. The tunnel has two test sections, both of which were used in this study. The dimensions of the large test section are 760 cm (length) x 120 cm (width) x 60 cm (height), and those of the small test section are 150 cm (length) x 35 cm (width) x 30 cm (height). A detailed description of the wind tunnel can be found in the technical report by Goossens and Offer (1988).

Apart from the wind tunnel, we also used a modified version of the Soil Fine Particle Extractor developed in a previous study by Goossens (2012). This instrument draws up the sediment, previously spread on a table, with a plastic hose attached to a BASE 440 three-engine vacuum cleaner connected to a cyclone dust separator (RIBO, Villanova, Italy). The hose is 300 cm long and 4 cm in diameter; the separator is 70 cm high and 40 cm in diameter. Coarse particles settle in the separator and are thus removed from the sample. Separation is accomplished by the circular motion of the particles and enhanced by selective gravitational settling. Some of the smallest particles remain suspended in the separator. After initial separation in the separator, the dust enters a tube 139 cm long and 16 cm in diameter, which operates as an elutriator. Dust is then accelerated through a small pipe 36 cm long and 7.6 cm in diameter and hits an impactor (diameter: 8.7 cm) installed near the bottom of a settling chamber. Only the finest particles will suspend in the chamber. These particles then enter a 200-cm long plastic tube. Further granulometrical separation is performed in this tube, which operates as a second elutriator. Particles then enter the vacuum cleaner and settle in a 50-L deposition chamber, where they can be collected. Three 1200-W engines that generate a suction rate up to 510 m^3/h and create an under pressure of 2200-mm H_2O power the instrument. For this study, only one engine (170 m^3/h) was used.

Experimental design

To perform each experimental run, a total of 8 kg of pre-treated soil (enough to fill the sediment tray in the wind tunnel) was taken one day before each experimental run. The soil was then oven-dried at 37.5°C for 24 h to ensure a soil moisture 2 % (the highest soil moisture allowed to guarantee wind erosion; see Nourzadeh et al. (2013)). Soil samples (in duplicate) were always taken before and after the drying process to control for any effect on glyphosate decay and AMPA formation/decay. The oven-dried soil was then subjected to wind erosion, which was carried out in the wind tunnel. In the small test section, a tray 150 cm long x 35 cm wide x 2 cm deep was installed. The upwind 75 cm were filled with a piece of wood; the downwind 75 cm were covered with a thin sheet of plastic (to avoid direct contact between the glyphosate-treated soil and the metal of the tray). The oven-dried soil was then put into the tray. Its surface was carefully flattened using a slat. The wind tunnel was then closed and turned on to allow the soil sediment to erode until the entire tray was empty. We used a free-stream wind speed of 10.0 m/s, which was well above the deflation threshold of the sediment used (6.5 m/s according to visual observations made before the test). It took approximately 1 h until the tray was empty. After each run, sediment samples (in triplicate) were collected (≥2 g for most of the samples; and always ≥1 g) at 4 different places in the wind tunnel using a clean brush. The distances from the trailing edge of the tray were as follows: sample 1: 10 cm; sample 2: 480 cm, sample 3: 1290 cm, and sample 4: 1865 cm. Due to aeolian selection, the samples become finer as they are taken further from the source. Because of the restricted length of the wind tunnel, sample 4 was the finest sample that could be obtained with the wind tunnel technique. To collect even finer samples we used the Soil Fine Particle Extractor and 3 more samples were collected. After each wind tunnel run, the tunnel was first thoroughly cleaned with the vacuum cleaner. A sample (sample 7) was then taken from the deposition chamber of the vacuum cleaner, which at this stage was directly connected to the cyclone separator. The sediment in the separator was

then mixed with the remaining dust in the deposition chamber and put on a clean table. After assembling the entire Soil Fine Particle Extractor, the sediment on the table was sucked up and samples 5 and 6 were collected just downwind from the cyclone separator (sample 5) and in the deposition chamber of the vacuum cleaner (sample 6). All experimental runs (wind tunnel + Soil Fine Particle Extractor) and collection of samples were conducted on days 0, 3, 7, 14, 21 and 28 after glyphosate application. All samples were stored in plastic tubes and frozen at -18°C until glyphosate and AMPA analysis.

Particle size distribution and organic matter content

To analyze the particle size distribution of samples 2 to 7 we used a Malvern Mastersizer S laser particle size analyzer (Malvern Ltd, Malvern, UK). Sample 1, which exclusively consisted of large aggregates, was analyzed optically with a microscope. For the latter sample, we collected a subsample from the main sample and measured the nominal diameter of all aggregates. Using these data, the aggregate size distribution of the sample could be determined. To get an idea of the internal particle size distribution of the large aggregates themselves, we also collected several of these aggregates, carefully crushed and dispersed them, and then analyzed them with the Mastersizer instrument. The OM content was estimated by oxidation at 600ºC and detected by close infra-red using a SC-144DR equipment (LECO Corporation, St Joseph, MI, USA). When there was insufficient sample for analysis, the triplicates were mixed together.

Glyphosate and AMPA content

Glyphosate and AMPA contents in the samples were analyzed as described by Bento et al. (2016). Briefly, glyphosate and AMPA were extracted from 1 g of soil or wind-eroded sediment with 5 mL of 0.6 M KOH (potassium hydroxide, p.a. 85 %). After shaking and centrifuging the samples, 1 mL of the supernatant was transferred to a 10-mL plastic tube. Isotopically labelled glyphosate and AMPA were added at this point and then a derivatisation step was carried out with FMOC to improve retention and MS/MS detection as described by Bento et al. (2016). Solvent standards with isotopically labelled internal standards were prepared together with all the samples for each batch of samples, and derivatized the same way. Glyphosate and AMPA contents were then determined by liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) using an XBridge™ Shield RP C18 column 100 mm x 2.1 mm i.d. (Aquity UPLC I-Class coupled to a Micromass Ultima triple-quadrople MS, Waters, The Netherlands). Chemicals used, mobile phases and instrumentation conditions of the HPLC-MS/MS were as described by Yang et al. (2015b) and Bento et al. (2016). With each batch of samples, two blank soil samples of the loess soil used in this study were fortified at 0.5 mg/kg and added as quality control (QC) samples. To ensure the quality of the analysis when processing real samples, the fortified samples were analysed twice, at the beginning and at the end of each batch. The quantification of the sample batch was considered satisfactory when the QC recoveries were between 70 and 120 %. A detailed description of the method validation and quality control can be found in Bento et al. (2016).

Statistical analysis

All statistical analyses were performed in SPSS 22, and the graphs in Figure 4 were produced in SigmaPlot 10.0. A one-way ANOVA to ln-transformed data followed by Dunett T3 post-hoc tests was performed to test for significant ($p < 0.05$) differences in clay, silt or organic matter (OM) content between extracted size fractions of the wind-eroded sediment. Besides, a power function was applied to the non-aggregated samples (sample 3-7) to test the correlation between the clay or OM content and the particle size of the samples. To test for significant differences of glyphosate or AMPA residues between extracted size fractions of the wind-eroded sediment, an analysis of covariance (ANCOVA) to ln-transformed data followed by Bonferroni tests was performed ($p < 0.05$). The assumption of homogeneity of regression slopes was not violated. Moreover, a categorical principal components analysis (non-linear PCA) was performed to determine the relationship between sediment properties (clay, silt, OM) and glyphosate or AMPA content in the wind-eroded sediment. The loading of a given variable was considered meaningful if its absolute value was ≥ 0.40 for a given component. Besides, a Pearson correlation was computed to assess the relationship between glyphosate or AMPA contents and clay, silt or OM. A reconstruction of the distribution of glyphosate in the original soil in the sediment

tray before the start of each wind tunnel experiment was also performed. This was done by considering the glyphosate content for a large number of narrow grain size classes, which could be estimated by applying an exponential regression analysis to the data (only the samples without aggregates, i.e., samples 3-7).

Results & Discussion

Physicochemical composition of the wind-eroded sediment

Particle size distribution

The particle size distribution of the different extracted fractions of the wind-eroded sediment is shown in Figure 2a. Sample 1 was composed of large, macroscopic aggregates only. Sample 2 consisted of individual grains and micro-aggregates, mixed with a few macroscopic aggregates. Samples 3-7 only contained individual grains with some small micro-aggregates (as verified under the microscope) and were mostly composed of particles ≤100 µm in diameter. More than 96 % of the particles of samples 5-7 were ≤50 µm in diameter. The median diameters of the samples were: 715 ± 69 µm (sample 1), 58 ± 2 µm (sample 2), 33 ± 1 µm (sample 3), 29 ± 1 µm (sample 4), 18 ± 1 µm (sample 5), 8 ± 1 µm (sample 6) and 11 ± 3 µm (sample 7). These median diameters are further used as reference codes in the data analysis presented here.

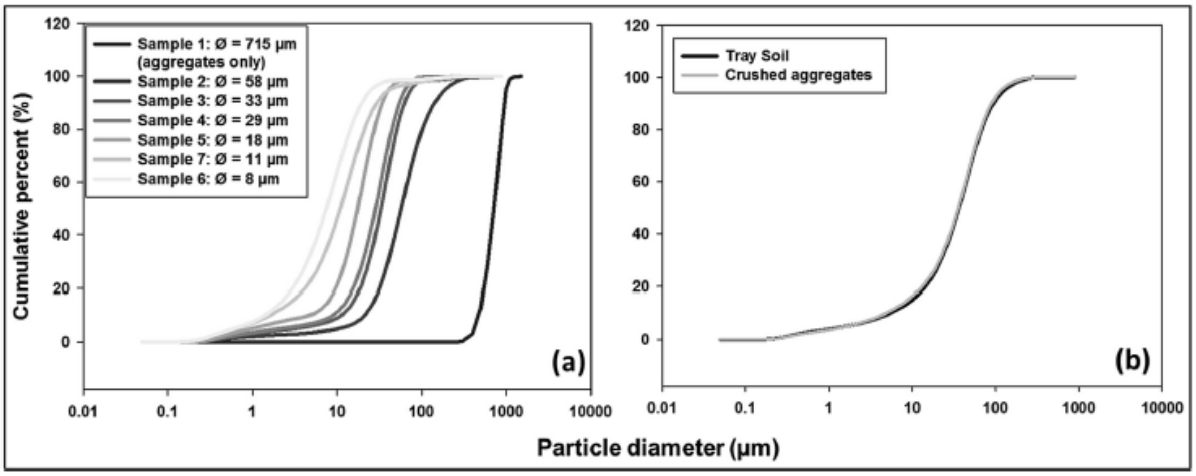


Figure 2. **Particle size distribution of (a) the different extracted fractions of wind-eroded sediment; (b) the crushed aggregates and the original sediment in the sample tray. Ø = median diameter.**

Crushing of the macroscopic aggregates (sample 1) and analyzing their grain size distribution showed that the aggregates are perfect compositions of the original tray sediment (Figure 2b), with a median particle diameter of 36 ± 2 µm for both the aggregates and the original tray soil.

Clay, silt and OM content

The clay (<2 µm), silt (2-50 µm) and OM content of the different extracted fractions of the wind-eroded sediment are shown in Figure 3. The clay content was significantly higher for the finest extracted size fraction (median diameter of 8 µm) and lowered significantly with increasing particle size (Figure 3), except for the samples with a 715-µm median diameter which consisted exclusively of macroscopic aggregates. A strong negative correlation was also observed between the clay content and the particle size of the non-aggregated samples (median diameters between 8 and 33 µm; Clay (%) = 67.7 $MDES^{-0.78}$, R^2 = 0.99; MDES = median diameter of the extracted sample). Likewise, the OM content was highest for the finest extracted fractions (samples with median diameter of 8 and 11 µm) and lowered significantly with increasing particle size (Figure 3). Nevertheless, this decrease in OM was no longer significant after a particle size ≥33 µm. A strong negative correlation was also observed between the

OM content and the particle size of the non-aggregated samples (OM (%) = 13.1 $MDES^{-0.61}$, R^2 = 0.90). All samples were mostly composed of silt (Figure 3). The silt content decreased as the samples became coarser, but to a lower extent compared to clay and OM. In the aggregated samples (median diameters of 58 and 715 µm), the silt content was significantly lower than in the non-aggregated samples.

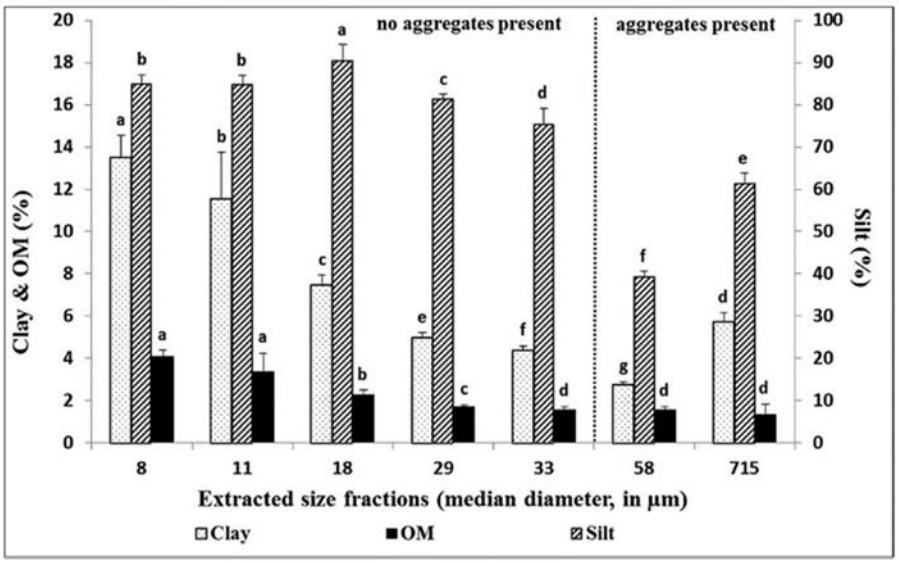


Figure 3. **Clay, silt and organic matter (OM) content of the extracted size fractions. The 715-µm samples consist exclusively of large aggregates. Different lowercase letters within the same type of bars mean significant differences in silt or clay or OM between extracted size fractions (p <0.05).**

Glyphosate and AMPA content in the wind-eroded sediment

Relationship between glyphosate or AMPA and particle size

Glyphosate content (Figure 4a) varied between 5.5 and 16 µg/g, with a significantly higher content in the finest extracted fractions (median diameters from 8 to 18 µm). AMPA content (Figure 4b), on the other hand, was rather low, varying between 0.07 and 0.7 µg/g. Here too, AMPA content was significantly higher in the finest extracted fractions. In Figure 5, the relationship between glyphosate (or AMPA) content and particle size of the wind-eroded sediment is better shown. Here, it is clearly visible that glyphosate and AMPA contents were highest in the finest samples (median diameter: 8 µm) and became lower with increasing particle size until ≈33 µm (Figure 5a and b). Note that this does not necessarily mean that the highest amounts of glyphosate and AMPA in a sample occur in the finest fractions of that sample: the mass of coarse grains is much higher than that of fine grains, so even when the concentration is higher in the fine fractions it is possible that the coarse fractions contain more glyphosate and AMPA in weight. A larger spread was observed for AMPA (Figure 5b) than for glyphosate (Figure 5a). However, this larger spread is not meaningful since it just reflects the increase of AMPA content in the course of time (see Figure 4b). For the individual days, the lower AMPA content with increasing particle size became better visible. It also became stronger over time. The effect of the presence of macroscopic aggregates in a sample was also very prominent (Figure 5). Once macroscopic aggregates were present (samples with median diameters of 58 and 715 µm), glyphosate and AMPA contents remained constant regardless of how numerous or how large the aggregates were. This seems to be related with the fact that the aggregates are perfect compositions of the original soil in the sediment tray (Figure 2b) regardless of their size. Because, in an aggregate, the largest mass is represented by the coarsest grains, glyphosate and AMPA contents will be rather low, approaching the concentration in the coarsest individual grains, albeit a little higher because of the presence of a higher percentage of fine particles in the aggregates. When comparing the glyphosate content in the different sediment fractions with its content in the parent soil, it was, on average, 1.4 times higher in the finest fractions of the wind-eroded sediment (median diameters between 8 and 18 µm) than in the parent soil. In contrast, the

coarsest fractions (median diameters between 29 and 58 µm) had glyphosate contents that were, on average, 1.2 times lower than that in the parent soil. Only the samples entirely composed of macroscopic aggregates (median diameter of 715 µm) matched the glyphosate content of the parent soil, confirming once again that the large aggregates are perfect compositions of the original soil in the sediment tray. Clymo et al. (2005) also reported a much higher concentration of the herbicide pendimethalin in the PM2.5 fraction when compared to their field soil, but not for the herbicide metolachlor. According to these authors, pendimethalin is less volatile than metolachlor and therefore, the former has a higher affinity to the particle phase while the latter has a higher affinity to the gas phase. Glyphosate is also on-volatile and tends to strongly adsorb to soil particles; therefore its preference to the particle phase is also expected.

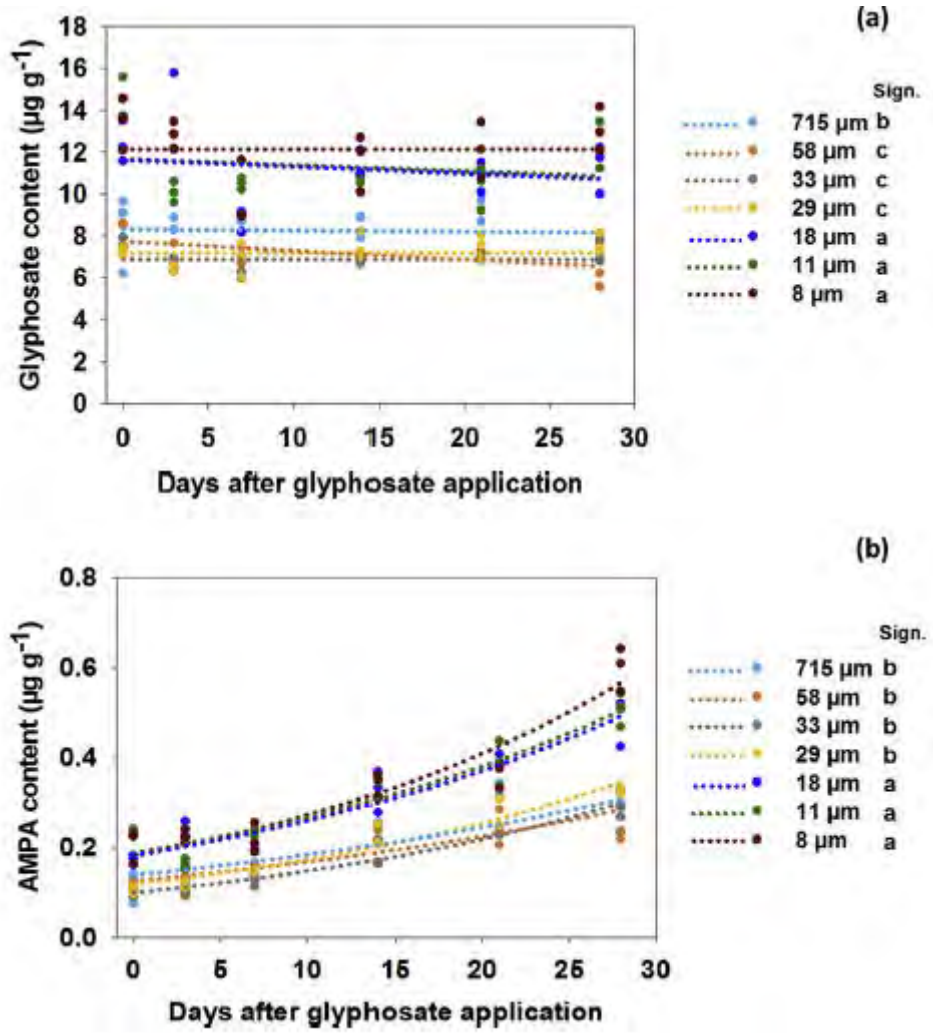


Figure 4. **Glyphosate (a) and AMPA (b) content in the different extracted size fractions of the wind-eroded sediment during the 28 days after glyphosate application, and respective trendlines. Note the different vertical scales between (a) and (b). To the right of the legends, different lowercase letters mean significant differences in glyphosate (a) and AMPA (b) content between extracted size fractions, using an ANCOVA followed by Bonferroni tests ($p < 0.05$).**

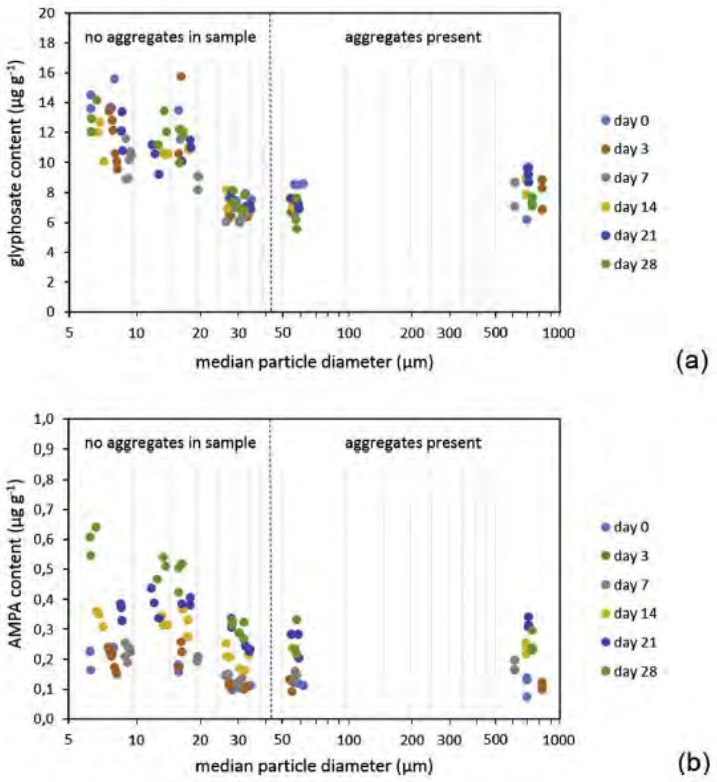


Figure 5. **Relationship between (a) glyphosate content and particle size, (b) AMPA content and particle size.**

Relationship between glyphosate or AMPA and clay, silt and OM

Figure 6 shows the results of the categorical principal components analysis performed to determine the relationship between the studied sediment properties (clay, silt and OM) and glyphosate and AMPA content. The proportion of variance-accounted-for by the first component is 61.1 %, whereas the second component accounts for 28.1 %. Thus, the two components together account for a considerable proportion (89.2 %) of the variance. All sediment properties analyzed in this study loaded in the first component together with glyphosate and AMPA, whereas only the duration of the experiment (days) loaded in the second component together with AMPA (Figure 6). The studied sediment properties do, therefore, play a major role in adsorbing glyphosate and AMPA. The duration of the experiment, on the other hand, was only meaningful for AMPA.

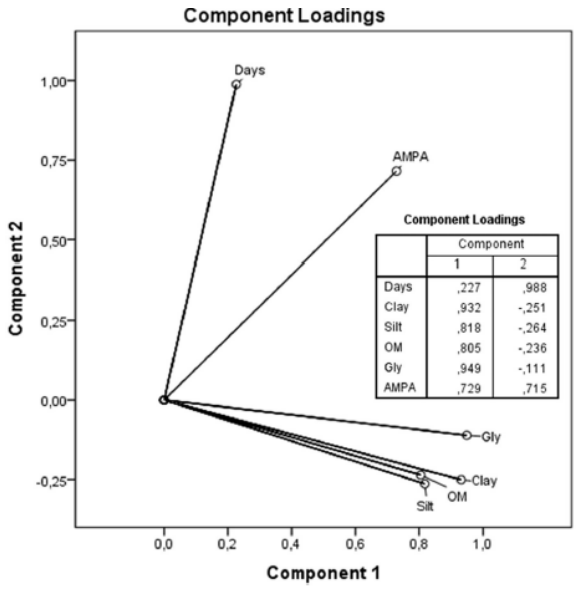


Figure 6. **Categorical principal components analysis (non-linear PCA). Gly - glyphosate; OM - organic matter.**

The order to which glyphosate and AMPA contents in the wind-eroded sediment are influenced by the studied sediment properties is as follows: clay >OM >silt (Figure 6). Glyphosate content correlates significantly and positively to the clay content (R^2 = 0.63, p <0.01). For coarser soil fractions, such as silt, the relationship with glyphosate content is considerably less expressed (R^2 = 0.27) but still significant (p <0.01). Significantly positive correlations were also observed between AMPA content and clay (R^2 = 0.16, p <0.01), and AMPA content and silt (R^2 = 0.10, p <0.01) (Figure 6). Organic matter also appears as a strong factor influencing glyphosate adsorption to wind-eroded sediment (Figure 6): glyphosate content correlates significantly and positively to the OM content (R^2 = 0.49, p <0.01). However, one should realize that a positive correlation between glyphosate content and OM would be observed anyway because both are a function of particle size (both are higher for smaller particles, see Figure 3). Therefore, the effect of OM on glyphosate adsorption cannot be confirmed with certainty. In summary, these results show that the highest concentrations of glyphosate and AMPA in the finest fractions are related to the higher clay and OM content in these same fractions, although the role of silt cannot be ignored. Sprankle et al. (1975) also reported that glyphosate was readily adsorbed to clay and OM, and that less glyphosate was adsorbed by a sandy loam soil than by a clayey loam soil.

Glyphosate and AMPA content through time and consequences for their airborne off-site transport with dust

The fact that glyphosate and AMPA contents are highest in the fine fractions of the soil has important consequences for the airborne off-site transport of these compounds, because particles <20 µm have the capacity of being transported in long-term suspension. This can easily be shown by calculating the aeolian threshold for long-term suspension, which, according to the model of Pye and Tsoar (1990), is $u_\infty/u^* < 0.1$, where u_∞ is the terminal fall velocity and u* the friction velocity. Using this criterion, 20-µm particles are already transported in long-term suspension when u* <0.3 m/s. Assuming a roughness length z_0 of 3-10 cm (typical value for agricultural areas, depending on the type of crop, see Ramli et al. (2009)), this corresponds to a 10-m height wind speed of 3.5-4.4 m/s, which are very typical values for many inland agricultural areas. For 10-µm particles, the critical wind speed is much lower: only 1.2-1.4 m/s (at 10 m height). At these wind speeds, particles are able to travel tens to even several hundreds of km before they settle back to the Earth's surface. During the 4-week experiment, nearly no glyphosate decay took place (Figure 4a). Consequently, the formation of AMPA (Figure 4b) was very slow and remained low during the experimental period. Glyphosate and AMPA decay mostly by microbial activity (Bento et al., 2016; Gimsing et al., 2004; Nomura and Hilton, 1977), and for the latter a minimum soil moisture is required (Bento et al., 2016; Schroll et al., 2006). In our study, the soil

moisture content during storage, after applying glyphosate but before the 24-h drying process prior to each wind tunnel test, was 5.4 %. This soil moisture content revealed to be very low to allow for soil microbial activity and consequent glyphosate decay. According to Bento et al. (2016), the decay of glyphosate and AMPA in a silty loam loess soil is already significantly slower at a soil moisture content of 8 % (w/w; 20 % of water holding capacity). Schroll et al. (2006) also showed that pesticide mineralization (using glyphosate) was nearly inexistent for soil moisture contents ≤2.6 % (w/w; equivalent to the ≤-20 MPa reported by these authors). Very important in this context is that wind erosion of fine, dusty particles only occurs when the topsoil (and, therefore, also the particles themselves) is sufficiently dry. Nourzadeh et al. (2013) tested several types of loamy soils using a field wind tunnel and found that the maximum moisture content to allow wind erosion of these soils was only 2 %, well below the limit for a substantial decay of glyphosate. Besides wind erosion, for many silty soils tillage erosion is a second (and in many cases even more important) mechanism for emission of fine particulates.

For tillage-emitted particles the probability for off-site transport is also highest when the particles are dry. Since the decay of glyphosate in our study occurred already extremely slowly for a soil moisture content of 5.4 %, its decay would be nearly inexistent for such dry wind-eroded sediment. Therefore, if glyphosate is applied during a dry period and emission of fine particles happens thereafter (either by wind erosion if the soil cover is still small, or by tillage activities if there is already some cover), then the potential for airborne glyphosate transport to off-site areas is considerable. In fact, Farenhorst et al. (2015), in a 2-year study where bulk deposition samples were collected and analyzed for several pesticides and some metabolites, showed that glyphosate and AMPA were the compounds detected at the highest concentrations. These authors also reported that glyphosate "accounted for 65 % of the total pesticide deposition over the 2 years" and that its deposition was >5 times larger in the dryer year. Other studies also reported the occurrence of glyphosate and/or AMPA in the atmosphere (Humphries et al., 2005; Messing et al., 2011; Quaghebeur et al., 2004).

Potential contribution of glyphosate and/or AMPA contaminated airborne dust to human exposure

Figure 7 shows the reconstruction of the distribution of glyphosate in the original non-aggregated soil in the sediment tray before the start of each wind tunnel experiment. As expected, the glyphosate distribution was nearly identical for the six experimental runs, and it was predominantly concentrated in the finest fractions. On average for the six experimental runs, 13 % of the glyphosate in the original soil was concentrated in the PM2.5 fraction (particles <2.5 μm), 15 % in the PM4 fraction, and 28 % in the PM10 fraction. It is currently unknown whether the distribution of glyphosate in Figure 7 also applies to the macroscopic aggregates, but because the aggregates are almost perfect compositions of the original soil in the sediment tray (see Figure 2b) the distribution of glyphosate within the aggregates is probably not far off from that shown in Figure 7. For AMPA, 14 % was concentrated in the PM2.5 fraction, 15 % in the PM4 fraction, and 29 % in the PM10 fraction. These results reconfirm that glyphosate and AMPA are considerably susceptible to be transported with airborne dust. After having accomplished their airborne transport trajectory, the glyphosate and/or AMPA containing soil particles will settle to the ground, thereby contaminating the deposition area. When the deposition is induced by rainfall and the particles and the soil become wet, glyphosate and/or AMPA will most probably further decay. When dry deposition occurs and the conditions remain dry for a while, glyphosate may remain in the deposited sediment until the soil becomes wet and the soil microorganisms active. But during their airborne transport the particles may also become inhaled, especially the finest fractions (PM10 and smaller), which can penetrate deeply into the human respiratory system. Some of the smallest particles (PM2.5) may even remain fixed in the lungs.

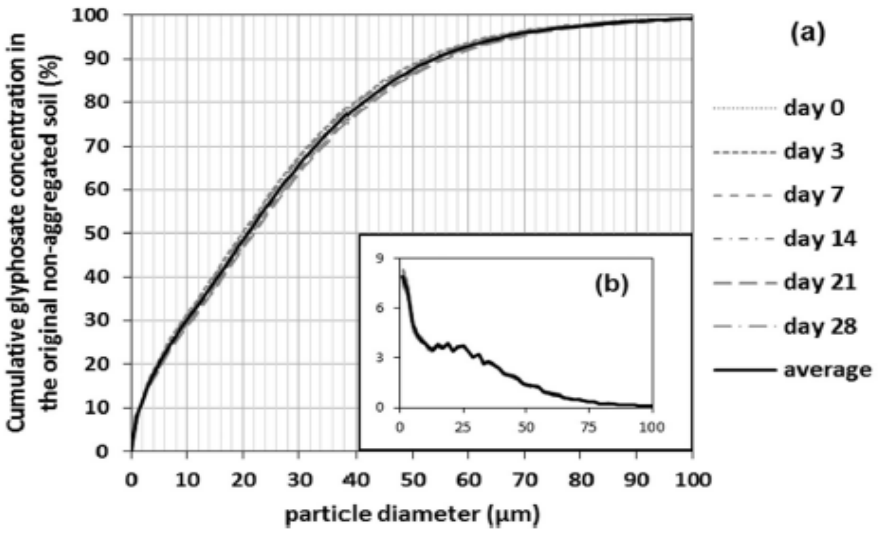


Figure 7. **Calculated cumulative (a) and non-cumulative (b) distribution of glyphosate in the original soil (after destruction of the aggregates) for the six experimental days.**

Inhalation of such fine particulates has already been associated to various health problems and around 2.1 million people world-wide are estimated to die every year from lung and respiratory system diseases caused by fine particulate matter (Kim et al., 2015; Shah et al., 2013). The risks to human health are expected to increase even more when these fine particulates are polluted with adsorbing chemicals. Bartos et al. (2009), who assessed the airborne exposure health risks associated to several toxic compounds, reported that 83-94 % of the cumulative cancer risk was due to particle-bound chemicals. To our knowledge, a human health risk assessment focusing on exposure to glyphosate and AMPA contaminated airborne particulates has not been done so far. As shown by our study, airborne particulates can be contaminated with very high concentrations of glyphosate and AMPA. According to Williams et al. (2000), the oral and dermal absorption of glyphosate and AMPA is low to very low, and they don't bio-accumulate. Any ingested glyphosate or AMPA is, therefore, expected to be eliminated unmetabolized through the urine or feces (Brewster et al., 1991; Williams et al., 2000). Nevertheless, in the review article of Williams et al. (2000), values of absorbed glyphosate in the body system of rats between 15 % and 36 % were reported, and absorbed percentages were dependent on the applied dose levels and on single or repetitive oral exposures. Brewster et al. (1991), who studied the tissue distribution of glyphosate in rats after a single oral dose, reported that significant (>1 %) doses of administered glyphosate were detected in the small intestine, bone, colon and kidney. These authors also reported that >94 % of the body burden was unmetabolized glyphosate and that only 1.2 % of the administered dose of glyphosate was still remaining in some tissues after one week, mostly in the bone. Glyphosate is reported as being harmless to humans or mammals mostly due to the absence of the shikimate pathway in animals (the mode of action of glyphosate in plants is the disruption of this shikimate pathway) (Brewster et al., 1991; Helander et al, 2012). However, studies have shown that glyphosate induced DNA damage (Mañas et al., 2009b), inhibited the activity of human serum enzymes (El-Demerdash et al., 2001), and that glyphosate exposure was related to Parkinson's disease (Barbosa et al., 2001; Gui et al., 2012; Wang et al., 2011). AMPA, on the other hand, has been shown to induce DNA damage and to produce chromosomal aberrations in human lymphocites (Mañas et al., 2009a). According to the IARC (2015), there are few studies on the effects of AMPA, but the existing ones all gave positive results as regards its genotoxicity. The IARC (2015) also reported that there is strong evidence that glyphosate and AMPA can induce oxidative stress. Inhalation of glyphosate is normally disregarded and considered a minor route of exposure in humans due to its low vapor pressure (IARC, 2015). Nevertheless, as shown in our study, very high concentrations of glyphosate may occur in airborne particulate matter that can be inhaled by humans and, thus, exposure through this route might have been underestimated in assessments done so far. The considerations above show that there is sufficient reason to take off-site airborne transport of glyphosate and/or AMPA contaminated soil fractions seriously, especially during sufficiently long periods of draught.

Conclusion

Our study indicates that glyphosate and AMPA contents are highest in sediment particles <10 µm (PM10), and that their content diminishes with increasing particle size. The risk of off-site airborne transport of glyphosate and AMPA with dust is, therefore, very high. Because glyphosate and AMPA hardly decay under dry conditions of the soil, this risk is intensified if glyphosate is applied in arid and semi-arid areas or during long periods of draught. If glyphosate and AMPA contaminated PM10 fractions of soil are emitted to the atmosphere, they may be inhaled by humans and animals. This contributes to the risk of human and animal exposure and, therefore, more attention should be paid to this route of exposure in environmental and human health risk assessment studies. Moreover, glyphosate applications during dry periods in regions susceptible to wind erosion should be avoided.

Assessment and conclusion by applicant:

The article describes the glyphosate and AMPA distribution in wind-eroded sediment derived from a Belgian loess soil. The distribution of the substances as dust via air and their dissipation and formation behavior is evaluated. Correlations to different soil parameters are presented.

The article was seen as reliable (Category 1).

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
General criteria for reliability considered for all data requirements indicated by the corresponding EU data points as specified in EC Regulation (EU) No 283/2013	1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines met.	No
	2. Previous exposure to other chemicals is documented (where relevant).	No
	3. The test substance is dissolved in water or non-toxic solvent	Yes
	4. Glyphosate, when the test substance, is sufficiently documented - identity of the test material reported (i.e. purity, source, content, storage conditions)	Yes
	5. Only glyphosate is the tested substance (excluding mixture), and information on application of glyphosate is described	Yes
	6. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite)	Yes
	7. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes
	8. Analytical verifications performed in test media (concentration)/ collected samples, stability of glyphosate in test media documented	Yes
	9. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information	Yes
	10. Assessment of the statistical power of the assay is possible with reported data.	Yes
	11. If statistical methodology was applied for findings reported, then the data analysis applied is clearly reported (e.g., checking the plots and confidence intervals)	Yes
	12. Field locations relevant/comparable to European conditions. Soils not completely matching the OECD criteria but from Europe or to some extent representative for the European Agriculture.	Yes

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
	13. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5 %), bulk density, water retention, microbial biomass (~1 % of organic carbon)	Yes
	14. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass	Yes
	15. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year	No
	16. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	No
	17. Data on precipitation is recorded	No
	18. The temperature was in the range between 20-25°C and the moisture was reported	No
	19. The presence of glyphosate identified in samples collected from groundwater, soil, surface waters, sediments or air from European areas	Yes
	20. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate	Yes
	21. Analytical methods clearly described and adequate Statement of specificity and sensitivity of the analytical methods is included	Yes
	22. Radiolabel characterization: purity, specific activity, location of label	No
	23. If degradation kinetics are included: expect to see data tables provided, model description. Statistical parameters for kinetic fit.	No
	24. Glyphosate monitoring data: description of matrix analysed, and analytical methods fully described as above.	No
	25. For environmental fate studies: clear description of application rate and relevance to approved uses.	Yes

1. Information on the study

Data point:	KCA 7.1.2.1.1, KCA 7.1.2.1.2, KCA 7.1.3.1, KCA 7.1.4.2
Report author	Bergstrom, L. et al.
Report year	2011
Report title	Laboratory and Lysimeter Studies of Glyphosate and Aminomethylphosphonic Acid in a Sand and a Clay Soil
Document No	Journal of environmental quality (2011), Vol 40, No 1, pp. 98–108
Guidelines followed in study	OECD 106 Guideline
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Not all validity criteria of the studies were met)

2. Full summary of the study according to OECD format

Due to the increasing concern about the appearance of glyphosate [N– (phosphonomethyl) glycine] and its major metabolite aminomethylphosphonic acid (AMPA) in natural waters, batch laboratory and lysimeter transport studies were performed to assess the potential for leaching of the compounds in two agricultural soils. Unlabelled and ^{14}C–labelled glyphosate were added at a rate corresponding to 1.54 kg a.s./ha on undisturbed sand and clay columns. Leachate was sampled weekly during a period of 748 d for analyses of glyphosate, AMPA, total ^{14}C, and particle–bound residues. Topsoil and subsoil samples were used for determination of glyphosate adsorption, glyphosate degradation, and formation of AMPA and its degradation. The influence of adsorption on glyphosate degradation was confirmed, giving very slow degradation rate in the clay soil (half–life 110–151 d). The kinetics of AMPA residues suggest that although AMPA is always more persistent than glyphosate when formed from glyphosate, its degradation rate can be faster than that of glyphosate. The kinetics also suggest that apart from glyphosate being transformed to AMPA, the sarcosine pathway can be just as significant. The long persistence of glyphosate was also confirmed in the lysimeter study, where glyphosate+AMPA residues constituted 59 % of the initial amount of glyphosate added to the clay soil 748 d after application. Despite large amounts of precipitation in the autumn and winter after application, however, these residues were mainly located in the topsoil, and only 0.009 and 0.019 % of the initial amount of glyphosate added leached during the whole study period in the sand and clay, respectively. No leaching of AMPA occurred in the sand, whereas 0.03 g/ha leached in the clay soil.

Materials and methods

Lysimeter Experiment

Soil Characteristics, Lysimeter Collection, and Management

Three undisturbed soil columns of a sandy soil and four of a clay soil were used. The smaller number of sand columns was based on the fact that sandy soils are usually more homogeneous and therefore show less variability in flow processes (Bergström and Shirmohammadi, 1999). Some physical and chemical properties of the two soils used are listed in Table 1. The soil columns were collected using coring equipment in which a polyvinyl chloride pipe (1.18–m long and 0.295–m inner diam.) is gently pushed into the soil by a steel cylinder with cutting teeth, which rotates around the pipe as it penetrates the soil (Persson and Bergström, 1991). After collection at the two field sites, Lanna in southwest Sweden (58°21′ N, 13°08′ E) and Nåntuna close to Uppsala (59°49′ N, 17°39′ E), the columns were prepared for gravity drainage by removing about 0.07 m of soil at the base, which was replaced by gravel, two stainless steel meshes, and a fiberglass lid, giving a final length of the soil columns of ~1.05 m. The lysimeters were then placed in vertical pipes permanently installed below ground at a lysimeter station located at the Swedish University of Agricultural Sciences in Uppsala, Sweden (Bergström, 1992).

Table 1. Selected soil characteristics of the Lanna clay and the Nåntuna sand. Standard laboratory methods were used throughout (Bergström et al., 1994).

Layer	Soil texture (Gr/Sa/Si/Cl)†	Organic matter	Cation exchange capacity	Bulk density	pH‡	Water content at tensions (cm)		
						0§	100	15,000
cm	%		$cmol_c\ kg^{-1}$	$g\ cm^{-3}$		$m^3\ m^{-3}$		
Clay								
0–30	1.3/6.0/46.2/46.5	4.4	28.4	1.24	7.2	0.524	0.359	0.193
30–60	0.6/2.7/40.6/56.1	0	33.6	1.43	7.4	0.477	–	0.249
60–90	0.2/1.8/37.4/60.6	0	–	1.46	7.4	0.468	–	0.297
Sand								
0–30	0/87.8/4.5/7.7	2.0	4.7	1.43	7.4	0.448	0.180	0.034
30–60	0/95.4/4.6/0	1.0	1.8	1.47	6.4	0.427	0.165	0.019
60–90	–	1.0	1.4	1.46	7.0	0.450	0.065	0.016

† Gr = gravel, >2 mm; Sa = sand, 0.06–2 mm; Si = silt, 0.002–0.06 mm; Cl = clay, <0.002 mm.

‡ Determined in water.

§ Equivalent to porosity.

All management practices performed on the lysimeters were intended to reproduce field conditions as closely as possible. Just before sowing in each year, the soil in each lysimeter was hand–tilled to simulate light harrowing. Spring barley (Hordeum distichum L.) was sown at a rate of 2 g per lysimeter on 21 May 2006, 26 May 2007, and 30 May 2008. On each occasion, mineral fertilizers were applied at rates of 100 kg N/ha, 22 kg P/ha, and 56 kg K/ha. The barley was harvested on 1 Sept. 2006, 28 Sept. 2007, and 16 Sept. 2008 by cutting the aboveground plant parts at ground level.

In addition to natural precipitation, all lysimeters received supplemental irrigation on two occasions during the 2–yr experimental period (in total, 22 mm). On each occasion, water was added with spray bottles over a few hours at rates typical of heavy rain storms, but not exceeding the infiltration capacity of the soil.

Chemical Application

Glyphosate was applied to two lysimeters of the sand soil and to three lysimeters of the clay soil on 18 Sept. 2006 at a rate corresponding to 1.54 kg a.s./ha, which represents a normal dose in Swedish cereal production systems. Radiolabeled [^{14}C] glyphosate (ARC 1313 glyphosate-[phosphonomethyl–^{14}C], 50 mCi/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO) was used to obtain fast screening of the leachate samples using scintillation counting analysis. The radiolabeled portion (5.32 MBq) was mixed with formulated (Roundup BIO, contains 486 g glyphosate/L as isopropylamin salt, Monsanto Crop Sciences), unlabeled glyphosate (in total 10.5 mg/lysimeter), which was dissolved in 11 mL (0.16 mm) of water. This solution was applied to the lysimeters by dripping it on the soil surface using a syringe. After the solution had been applied, 5 mL (0.07 mm) of water was drawn up into the syringe and also applied to each lysimeter. In addition to glyphosate, KBr at a rate of 0.268 g Br^- per lysimeter (~40 kg Br^-/ha) was applied to provide information on the movement of water through the soil columns. The KBr was dissolved in water (0.4 g KBr in 5 mL), which was applied separately to the lysimeters, also using a syringe.

Soil and Water Sampling

On 17 Oct. 2007, samples of the topsoil (0–30 cm) and subsoil (30–80 cm) of each soil were collected for determination of adsorption and degradation characteristics. These samples were taken from the lysimeter of each soil used as control (i.e., no glyphosate added). Three soil cores from each lysimeter were collected with a tube drill. The individual samples were then mixed by layers into a topsoil and a subsoil sample for each lysimeter. On 5 Oct. 2008, after leaching measurements were terminated, soil samples were collected from the lysimeters to which glyphosate had been applied to determine the residual amounts of glyphosate and AMPA about 2 yr after application. Three cores from each lysimeter were taken with a tube drill and divided into three layers (0–30, 30–60, and 60–90 cm), which were pooled to one sample for each lysimeter and layer. After collection, all soil samples were stored in a freezer (–20°C) until analyzed.

Leachate from the lysimeters was collected and weighed each week during the 2–yr period when

drainage water was available. After collection, all leachate samples were stored in a freezer (–20°C) until analyzed. The amount of ^{14}C was measured in 10 mL of the leachate using a Beckman LS 6000TA liquid scintillation counter (Beckman Coulter Inc, Fullerton, CA) after addition of 10 mL of Insta–Gel Plus (PerkinElmer, Waltham, MA).

Adsorption Study

The adsorption study was performed according to the OECD 106 guideline (OECD, 2001). Adsorption data were obtained at five different concentrations in two replicate samples. Four grams dry weight (DW) of field–moist soil were shaken at 200 rpm on a shaker for pre–equilibration with 39 mL of 0.01 M $CaCl_2$ for 24 h at 20°C in 50–mL plastic tubes. Thereafter, the soil slurry was spiked with 1 mL of a mixture of labeled (1.98 kBq) and unlabeled glyphosate in 0.01 M $CaCl_2$ to give five initial concentrations in the range 0.1 to 10 µg/g dw of soil. After shaking for 24 h, the tubes were centrifuged for 20 min at 4000 rpm and then the radioactivity was measured in 10 mL of the supernatant. Tubes without soil and ^{14}C–labeled glyphosate were included for subtraction of background radiation, and tubes without soil were used to give the initial amount of ^{14}C activity added. No significant adsorption of glyphosate occurred on the plastic tubes. A pre–study showed that adsorption equilibrium was obtained after 24 h of contact time between soil and solution, which also indicates that negligible amounts of AMPA had been formed.

Adsorption data were fitted by nonlinear regression to the Freundlich adsorption isotherm:

[1]

$$c_{soil} = K_f c_{aq}^{1/n}$$

where c_{soil} (µg/g) is the adsorbed amount, c_{aq} (µg/mL) is the concentration in the aqueous phase, K_f [$\mu g^{1-1/n}$ $(mL)^{1/n}$/g] is the Freundlich adsorption coefficient, and $1/n$ (–) the measure of nonlinearity.

Degradation Study

Glyphosate dissolved in water (1.4 mg/mL) was applied dropwise (1.0 mL) to 15 g of fresh soil. The soil was dried and mixed, after which an additional amount of fresh soil (to give 140 g DW in total) was thoroughly mixed into the spiked soil to give an initial concentration of 10 µg glyphosate per g DW of soil. Portions corresponding to 10 g of dry soil were transferred to 50–mL plastic tubes. The water content was adjusted to 60 % of the water–holding capacity. The tubes were sealed with plastic caps that allow gas exchange and incubated at 20°C in the dark. After 2, 4, 8, 16, 32, and 64 d, two tubes were put in the freezer (−20°C) until analysis for residual concentrations of glyphosate and the metabolite AMPA. The weight of the tubes was measured once a week during the incubation, and when necessary, the moisture content was adjusted to 60 % of the water–holding capacity.

Residual values of glyphosate were used for a least squares fitting procedure to determine values of the parameters of the function for first order exponential decay:

[2]

$$c_G(t) = c_{G0} e^{-k t}$$

where c_G (mg/kg) is the residual concentration of glyphosate at time t days after application, c_{G0} (mg/kg) is the initial concentration of glyphosate, and k (d^{-1}) is the first–order rate coefficient for degradation.

A branched reaction scheme was applied to describe the degradation of glyphosate to AMPA and sarcosine (Karpouzas and Singh, 2006; Borggaard and Gimsing, 2008) and the degradation of AMPA (Figure 1). According to this scheme and assuming first–order kinetics, the rate of AMPA formation and degradation is then

[3]

$$\frac{dc_A}{dt} = 0.66 k_1 c_G - k_2 c_A$$

where c_A (mg/kg) is the concentration of AMPA at the time t. Because the concentrations of glyphosate and AMPA were expressed in units mg/kg, the value of c_{G0} obtained from Eq. [2] was multiplied by the stoichiometric factor 0.66 (i.e., the ratio of the molecular weights of the dominant species of AMPA and

glyphosate at pH 7) in these calculations. The equation describing the concentration of AMPA was obtained by combining Eq. [2] and [3], and integrating:

[4]

$$c_A = \frac{0.66 k_1 c_{G0}}{k_2 - k}(e^{-kt} - e^{-k_2 t})$$

In this branched pathway, k for glyphosate degradation in Eq. [2] equals the sum of k_1 for AMPA formation and k_3 for sarcosine formation. Then $k_3 = k - k_1$ and the fractions of glyphosate transformed into AMPA and sarcosine are k_1/k and k_3/k, respectively. Since no more than 100 % of the glyphosate can be transformed into AMPA, the upper limit for k_1 is k, in which case $k_3 = 0$. The maximum concentration of AMPA, c_{Amax}, occurs at time t_{Amax} when $dC_A/dt = 0$. Inserting this value into Eq. [3], replacing c_G and c_A in Eq. [3] by their expressions in Eq. [2] and [4], respectively, and rearranging gives the following:

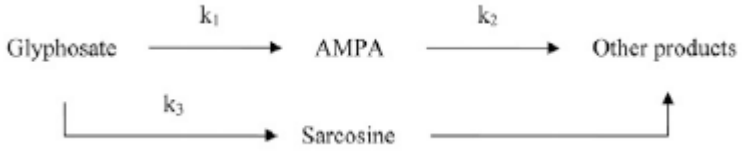
[5]

$$t_{A\,max} = \frac{\ln(k) - \ln(k_2)}{k - k_2}$$

Nonlinear Regression

Least squares fits of data on adsorption and on residual values of glyphosate and AMPA were fitted to their respective equations by nonlinear regression. Residual values of AMPA were fitted using the values of c_{GO} and k for glyphosate degradation as obtained from Eq. [2]. The calculations were performed on a PC with the application SigmaPlot for Windows version 10.0 (Systat Software, Inc., San Jose, CA); the nonlinear regression method is based on the Levenberg and Marquardt method.

Figure 1. Branched reaction scheme with the first-order rate coefficients k_1 and k_3 for the degradation of glyphosate to aminomethylphosphonic acid (AMPA) and sarcosine, respectively, and k_2 for the degradation of AMPA



Glyphosate and AMPA Analyses

Reagents

Analytical standards used for calibration were (trivial names in italics): N−(phosphonomethyl) glycine, *glyphosate*, (Riedel−de−Haën, Sigma−Aldrich, Sweden AB) and (aminomethyl) phosphonic acid, *AMPA*, (Dr. Ehrenstorfer GmbH, Augsburg, Germany). Internal standards were ^{13}C; ^{15}N; ^{2}D−labeled AMPA and ^{13}C; and ^{15}N−labeled glyphosate (LCG Standards AB, Borås, Sweden). Concentrated HCl, ethyl acetate, and NaOH (analytical reagent grade from VWR, Stockholm, Sweden), were used for extraction and solvation. The AG1−X8, 100−200, formate form (Bio Rad Laboratories, Sundbyberg, Sweden) and Isolute C18 EC 200 mg (Sorbent AB, Gothenburg, Sweden) were used for ion exchange and clean−up. Trifl uoroacetic anhydride (TFAA) and trifluoroethanol (TFE), both analytical reagent grade from Sigma Aldrich Sweden AB (Stockholm, Sweden), were used for the derivatization. The 0.22−µm glass fiber filters # GSWP04700 were from Millipore VWR (Stockholm, Sweden).

Calibration

Stock solutions of glyphosate and AMPA were diluted in water to concentrations of 100 µg/mL and stored at +4°C. A solution containing 1 µg/mL of glyphosate and AMPA was prepared daily as a working standard. The labeled glyphosate and AMPA were diluted in deionized water to a concentration of 1 µg/mL and stored at −20°C in 2−mL portions.

Clean−Up and Derivatization: Water Samples

A 50−mL volume of a water sample and 0.1 µg each of glyphosate and AMPA internal standard were adjusted to pH 2 with 6 M HCl in a plastic tube. The sample was left to precipitate for 1 h and centrifuged at 5000 rpm for 10 min. The upper, clear phase was adjusted to pH 7 to 8. Ag1−X8 (2.3 g) was weighed

into an empty 6 mL−plastic column equipped with a piece of cotton at the bottom, and the column was wetted with deionized water. A 3−mL (200 mg) C18 SPE column was activated with 3 mL of methanol and 3 mL of water and connected on top of the AG1−X8 column. An empty 75−mL plastic column was connected on top of the C18 and Ag1−X8 columns, and the sample was applied at a rate of 2 mL/min. The two upper columns were removed and the analytes were eluted with 3×4 mL of 0.6 M HCl at a rate of 1 mL/min and collected in a 100−mL pear−shaped fl ask. The sample was evaporated to approximately 2 mL under vacuum, quantitatively transferred to an 8−mL glass tube and evaporated to dryness under an air stream at 50°C. The derivatization was performed by adding 1 mL of trifluoroethanol and 2 mL of trifluoroacetic anhydride, and the sample was held at 100°C for 1 h. After being cooled to room temperature, the sample was evaporated under nitrogen and redissolved in 1.00 mL of ethyl acetate before analysis.

Clean−Up and Derivatization: Particle−Bound Glyphosate and AMPA in Leachate

Leachate samples from three lysimeters of each soil were analyzed for particle−bound glyphosate and AMPA. These samples comprised two samples from the untreated lysimeters and four samples from the glyphosate−treated lysimeters on sampling occasions when the highest concentrations of glyphosate and AMPA were detected in the leachate. A 300−mL portion of each sample was filtered through a 0.22−µm glass fiber filter. The filter was weighed before and after filtration, dried at 105°C, and the dry weight of the particles was calculated. The dry filter and the particles were analyzed for glyphosate and AMPA by extraction with 7 mL of 0.1 M NaOH following the same procedure as for soil samples (see below).

Clean−Up and Derivatization: Soil Sample

Ten grams of soil were extracted with 40 mL (for the degradation study) or 75 mL (for the lysimeter soil residue analysis) of 0.1 M NaOH by shaking for 30 min at 200 rpm, sonicated for 10 min and centrifuged for 10 min at 5000 rpm. The internal standards (0.1 µg each of glyphosate and AMPA) were added to a portion (40 µL and 4 mL for the degradation and the lysimeter studies, respectively) of the clear upper part of the sample, which was then analyzed according to the procedures described for the water samples. The portion from the degradation study was evaporated and derivatized directly after precipitation of the extract, since no column clean−up was needed due to the high residual concentrations in these samples.

Instrumentation

The gas chomatography−mass spectrometry (GC−MS) analyses were performed with a Hewlett−Packard 6890 GC (Agilent Technologies Sweden AB), equipped with a 30 m by 0.25 mm i.d. (0.25−µm fi lm thickness) fused silica capillary column (HP−5 for GC–MS), a mass spectrometer 5973, a split/splitless injector, and the software Chemstation, all from Agilent Technologies (Kista, Sweden). One microliter of the samples was injected (in the splitless mode at 270°C, oven temperature 70°C). After 2 min, the oven temperature was raised to 170°C at 30°C/min and then from 170 to 250°C at 120°C/min. Helium (N47 grade, 99.997 %) was used as the carrier gas and the flow rate was 1.2 mL/min. The mass spectrometer was operated in the electron impact (EI) mode; the transfer line and manifold temperatures were 270 and 230°C, respectively. Fragment ions were detected by selected ion monitoring (SIM) and used for identification of the AMPA and glyphosate derivates as shown in Table 2.

Table 2. Molecular weights, retention times (RT) and specific selected ions for compound derivatives.

Molecule	Molecular weight	RT (min)	Quantification ion (m/z, % relative abundance)	Qualification ion (m/z, % relative abundance)
AMPA†	371	4.49	126 (100)	302 (23)
AMPA‡	375	4.49	130 (100)	306 (22)
Glyphosate	511	5.35	411 (100)	384 (50)
Glyphosate‡	513	5.35	413 (100)	386 (48)

† AMPA = aminomethylphosphonic acid.

‡ Internal standard.

Verification of compound identification was based on comparison of the areas of the selected ions in the samples with those of the standards. For quantification, the response areas for AMPA and glyphosate target ions were calculated in relation to those of the internal standards. The response was found to be

linear in the practical concentration range (2.5−100 pg) of individual components injected.
The quantification levels for glyphosate and AMPA were 0.1 µg/L in water and 0.01 µg/g in soil. In some samples, however, the quantification level was higher due to the specific background.

Results

Adsorption of Glyphosate

The high correlation coefficients (R^2 ≥0.997; Table 3) obtained when sorption data for both soils and soil layers were fitted to the Freundlich adsorption isotherm show that they could be accurately described by this model. The values of the K_f parameter obtained were considerably higher in the clay soil than in the sand and are similar to values previously reported for glyphosate sorption to soils of similar textures (Vereecken, 2005). In the sand, K_f was higher in the topsoil than in the sub−soil, whereas the opposite was true for the clay. The correlation between K_f and the amount of clay in the different soils was 0.987. Although based on only four soils (topsoil and subsoil in the respective soils), this result supports the generally held view that glyphosate is primarily sorbed to clay particles and their associated iron oxides (Vereecken, 2005). Normalization of the distribution coefficients for glyphosate should therefore also account for the amount of clay and oxides present in soil and not organic carbon only, which is used to calculate K_{oc}. The 1/n parameter, which expresses the degree of linear relationship between c_{soil} and c_{aq}, was close to 1 for both layers of the clay soil and the sand topsoil, showing an almost constant distribution coefficient between sorbed and dissolved glyphosate in these soil layers in the range of concentrations studied. In the subsoil of the sand, the parameter 1/n was 0.82, indicating that the availability of sites for sorption in this layer becomes limiting at high glyphosate concentrations.

Table 3. Freundlich coefficients (K_f) (± SE, n = 10) for adsorption of glyphosate obtained by nonlinear regression according to Eq. [1].

Soil	K_f	1/n	R^2
	$\mu g^{1-n}(mL)^n g^{-1}$	–	–
Sand topsoil	40.0 ± 2.9	0.92 ± 0.04	0.997
Sand subsoil	28.7 ± 1.2	0.82 ± 0.02	0.998
Clay topsoil	118 ± 4.4	0.95 ± 0.01	1.000
Clay subsoil	165 ± 10.7	1.03 ± 0.02	0.999

Degradation of Glyphosate and AMPA

Best fits of glyphosate and AMPA residue data to Eq. [2] and [4], respectively, are shown in Figure 2 and the parameter values obtained in Table 4 and Table 5, respectively. Initial extraction efficiencies of glyphosate were 112 to 123 % as shown by comparing the initial concentrations obtained (Table 4) with the nominal value of 10 µg/g DW. All parameter values were significantly different from zero (p <0.05, n = 12). The models gave good fits of the data for all soils (R^2 ≥0.90), except for glyphosate in the clay subsoil (R^2 = 0.56). This poor fit could be due to difficulties in getting glyphosate homogeneously distributed in this clay−rich (56.1 %) subsoil with no organic matter (0 %). Another explanation could be that the R^2 values obtained by nonlinear and linear regression are not comparable. In nonlinear regression, R^2 refers to the fraction of the variance explained and is the model efficiency (EF). A disadvantage of EF is its dependency on the slope of the curve, as it is always relatively small for relatively flat decline patterns, or can even be negative for curves describing for instance formation and degradation of metabolites, irrespective of the scatter of measured data around the calculated curve (FOCUS, 2005). Therefore, from visual inspection of the fits to the data (Figure 2) and from the generally small standard errors in the parameters determined (Table 4 and 5), we concluded that the equations provide relevant quantitative information.

Figure 2. Best fits (A) to Eq. [2] of data on glyphosate and (B) to Eq. [4] of data on aminomethylphosphonic acid (AMPA) concentrations for sand topsoil (•), sand subsoil (○), clay topsoil (▼), and clay subsoil (^) (mean ± SE, n = 2). dw = dry weight.

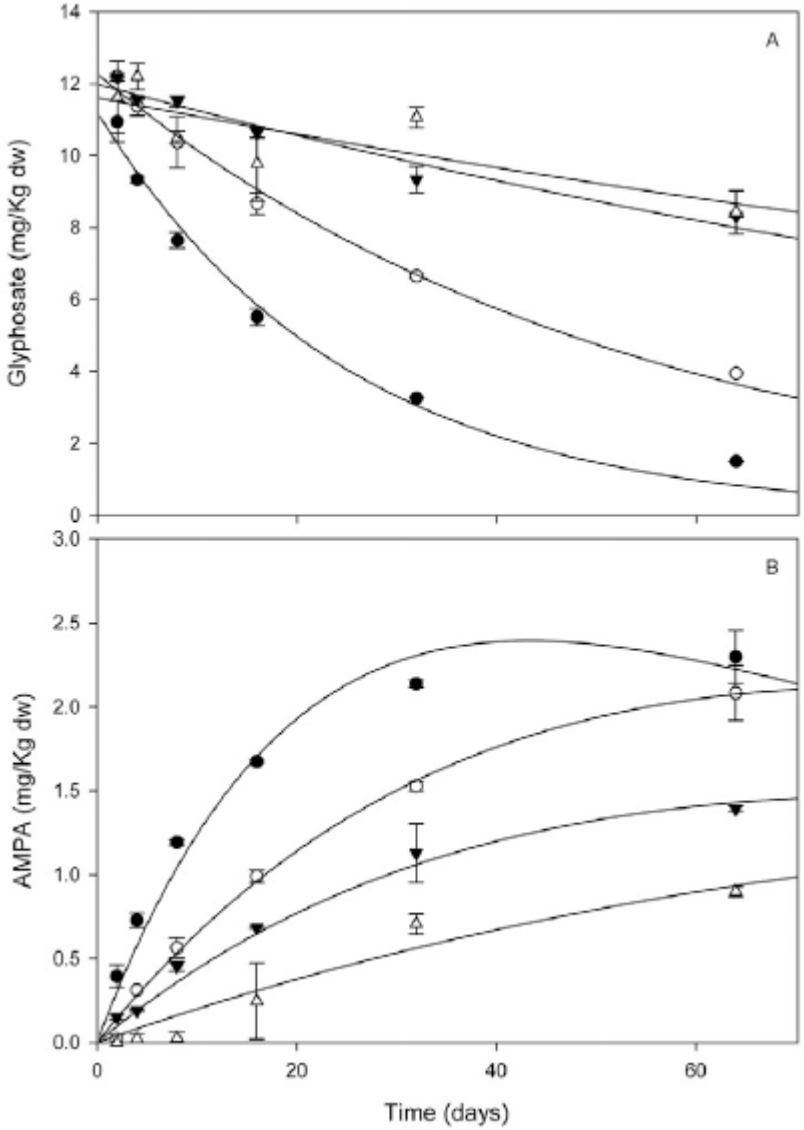


Table 4. Coefficients (± SE, n = 12) obtained by nonlinear regression for degradation of glyphosate according to first−order kinetics (Eq. [2]).

Soil	c_{00}†	k	R^2	$t_{1/2}$‡	DT_{90}§
	mg kg^{-1}	d^{-1}	–	d	d
Sand topsoil	11.21 ± 0.33	0.041 ± 0.003	0.978	16.9	56.2
Sand subsoil	12.27 ± 0.19	0.019 ± 0.001	0.985	36.5	121
Clay topsoil	11.99 ± 0.15	0.0063 ± 0.0005	0.948	110	365
Clay subsoil	11.61 ± 0.41	0.0046 ± 0.0014	0.562	151	501

† c_{00} = initial concentration of glyphosate; k = first-order rate coefficient for degradation; t1/2, = half-life; DT_{90} = time for 90% degradation.

‡ Calculated as ln(2)/k.

§ Calculated as ln(10)/k.

Table 5. Coefficients (± SE, n = 12) obtained by nonlinear regression for formation and degradation of aminomethylphosphonic acid (AMPA) according to Eq. [4].

Soil	k_1†	k_2	$t_{1/2}$‡	R^2
	d^{-1}		d	–
Sand topsoil	0.0216 ± 0.0011	0.0115 ± 0.0019	60.4	0.965
Sand subsoil	0.0092 ± 0.0005	0.0076 ± 0.0018	91.3	0.988
Clay topsoil	0.0063§ ± 0.0005	0.0199 ± 0.0013	34.9	0.973
Clay subsoil	0.0028 ± 0.0006	0.0071 ± 0.0087	97.6	0.901

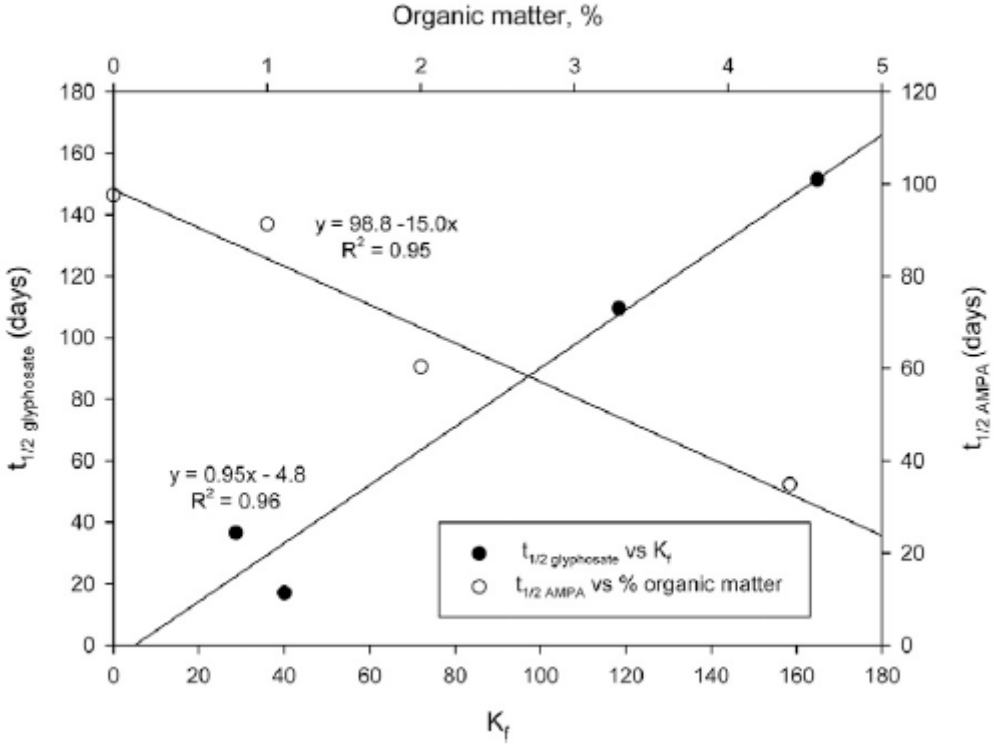
† k_1 = first-order rate coefficient for degradation of glyphosate to AMPA; k_2 = first-order rate coefficient for degradation of AMPA ; $t_{1/2}$ = half-life.

‡ Calculated as ln(2)/k_2.

§ In the clay topsoil, k_1 became 0.0073, i.e., larger than k (first-order rate coefficient for degradation; Table 4), leading to a formation fraction > 1, and was therefore set equal to k.

Glyphosate degraded relatively rapidly in the sand, with a half−life of 16.9 and 36.5 d in the topsoil and subsoil, respectively (Table 4). In the clay, very long half−life values of 110 and 151 d were obtained, and remarkable values of 365 and 500 d for 90 % degradation (DT_{90}). These half−life values are within the range previously reported for glyphosate degradation in agricultural soils (Giesy et al., 2000). There was a high correlation between half−life and K_f (Figure 3), suggesting that adsorption is important for the amount of glyphosate available in the soil water for degradation.

Figure 3. Relationship between half−life ($t_{1/2}$) for glyphosate and Freundlich adsorption coefficient (K_f), and between $t_{1/2}$ for aminomethylphosphonic acid (AMPA) and % organic matter.



The concentration of AMPA steadily increased during the incubation period of 64 d in all soils except the sand topsoil (Figure 2B), where it peaked after 43.4 d at 2.4 mg of AMPA/kg, representing 32.4 % of the initial amount of glyphosate added (Table 6). The degradation rate of AMPA, as quantified by k_2, gave a half−life of 35 to 98 d, with slower rates in the subsoil (Table 5). The correlation between these half−life values and the amount of organic matter was −0.973 (Figure 3), suggesting that increasing amounts of organic matter, or perhaps AMPA−degrading microorganisms dwelling there, increase degradation rates.

Table 6. Derived parameter values on fraction of glyphosate degraded to aminomethylphosphonic acid (AMPA) ($k1/k$), rate constant for formation of sarcosine (k_3), incubation time (t_{Amax}) at which the AMPA−concentration peaks (c_{Amax}), and c_{Amax} as fraction of initially added glyphosate.

Soil	k_1/k†	k_3‡	t_{Amax}§	c_{Amax}¶	c_{Amax} fraction#
	–	d^{-1}	d	mg kg^{-1}	%
Sand topsoil	0.53	0.0190	43.4	2.40	32.4
Sand subsoil	0.48	0.0098	80.4	2.12	26.2
Clay topsoil	1	0	84.7	1.47	18.6
Clay subsoil	0.61	0.0018	174	1.34	17.5

† Values of k (first-order rate coefficient for glyphosate degradation) from Eq. [2] (Table 4) and of k_1 (first-order rate coefficient for degradation of glyphosate to AMPA) from Eq. [4] (Table 5).

‡ $k_3 = k - k_1$.

§ Calculated according to Eq. [5].

¶ Obtained by inserting derived values of k, k_1, k_2, c_{GO}, and t_{Amax} into Eq. [4].

Calculated as $(c_{Amax} \times 1.52 \times 100)/c_{GO}$, where 1.52 is the stoichiometric factor for conversion of AMPA to glyphosate concentration.

The degradation of AMPA is reported to be slower than that of glyphosate (Giesy et al., 2000). In the Footprint database, AMPA is classified as persistent, with a typical half−life of 151 d, compared with 12 d for glyphosate (Footprint, 2009). The fact that AMPA is formed when glyphosate is degraded clearly means that the persistence of AMPA has to be equal to or longer than that of glyphosate. However, we did not find any previous study in which the degradation of AMPA was studied and compared with that of glyphosate in the same soil. In a study where glyphosate degraded with a half−life of 9 d, Simonsen et al. (2008) estimated a half−life of 32 d for AMPA from the descending part of data on AMPA residues. However, this is a worst−case scenario as these data represent the sum of AMPA formation from degradation of the glyphosate still present and AMPA degradation. This does not reveal how fast the AMPA molecule per se is degraded. Our data suggest that the AMPA degradation rate can be faster than that for glyphosate, for instance in soils with high clay content, which slows down glyphosate degradation, and high organic matter content, which stimulates AMPA degradation (Figure 3).

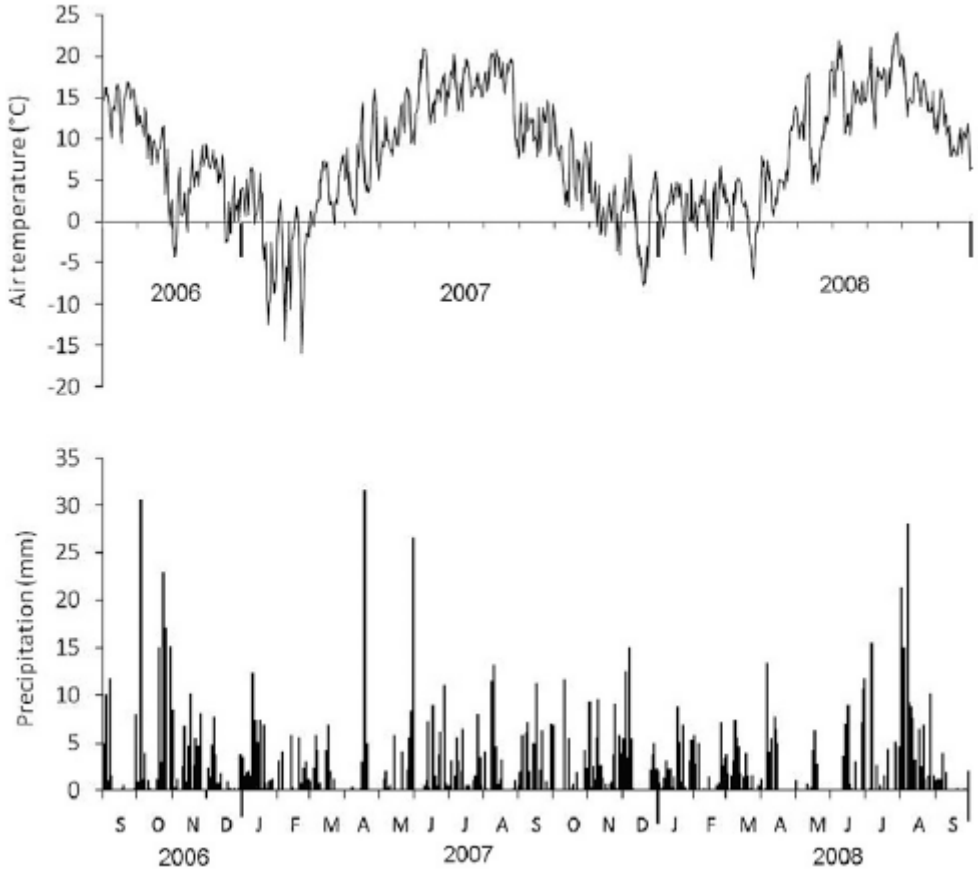
Microbial degradation is the main process controlling the disappearance of glyphosate in soil, and there are two well−described biological pathways for such degradation that give AMPA and sarcosine as the respective metabolites (Karpouzas and Singh, 2006; Borggaard and Gimsing, 2008). It has recently been shown that ligninolytic enzymes can also transform glyphosate into AMPA (Pizzul et al., 2009). Because AMPA is the only significant soil metabolite found in soil degradation studies, it is frequently suggested that metabolism of glyphosate in soil usually proceeds via the AMPA pathway (Giesy et al., 2000; Karpouzas and Singh, 2006). However, the fractions of AMPA formed in our study (48−100 %, Table 6) suggest that both pathways can be active in soil, with up to 52 % not following the AMPA pathway. Reasons for the sarcosine pathway not being considered significant in soil could be that soil residues of sarcosine are not determined in most studies and that sarcosine rapidly degrades to glycine (Karpouzas and Singh, 2006) in biologically active soil.

Precipitation and Drainage Conditions

Daily precipitation and average air temperatures at the lysimeter station are shown in Figure 4. Over the 2−yr study period (15 Sept. 2006−15 Sept. 2008), cumulative precipitation was 1192 mm, which, in combination with supplemental irrigation, resulted in a total water input to the lysimeters of 1214 mm. This total water input is slightly higher (10 %) than the long−term average precipitation for the Uppsala region (554 mm/yr). Average air temperature during the experimental period (7.8°C) was also higher than the long−term average at Uppsala (5.3°C).

A few weeks after glyphosate was applied, from 30 September onward, rain events were quite frequent (Figure 4), and precipitation totaled 232 mm by the end of 2006. This clearly created worst−case conditions for leaching of the herbicide, and the average amounts of leachate were 169 and 156 mm from the sand and clay soil, respectively, during this period. Peak weekly amounts of leachate, reaching 42 (sand) and 33 (clay) mm, occurred 8 wk after herbicide application. During 2007, precipitation was close to the normal for the area, although quite unevenly distributed. During periods with low evaporation (November, December, and January), monthly precipitation was about 60 mm, which was clearly above the average and increased the risk of leaching. In 2008, precipitation was again above normal, causing large amounts of leachate.

Figure 4. Average daily temperature (upper graph) and daily precipitation (lower graph) at the lysimeter site during the experimental period.



The cumulative amounts of leachate from the lysimeters each year are shown in Table 7. In total over the 2−yr period, the amount of leachate was 572 (± 17) mm from the sand and 461 (± 15) from the clay soil. In relation to water input, these amounts constituted 47 and 38 % of precipitation plus irrigation, which is considerably higher than in other similar leaching studies performed in Sweden (Bergström and Jokela, 2001).

Table 7. Water inputs to the lysimeters and mean annual amounts of leachate from the lysimeters to which glyphosate was applied (± SD; n = 2 for sand, n = 3 for clay)

Year	No. of days	Precipitation + irrigation	Leachate	
			Sand lysimeters	Clay lysimeters
		mm		
2006†	104	232	169 (± 1.4)	156 (± 4.7)
2007	365	550	229 (± 3.2)	158 (± 9.1)
2008‡	261	432§	174 (± 13)	148 (± 4.0)
Total	730	1214	572 (± 17)	461 (± 15)

† Measurements made during the period 18 September to 31 December.

‡ Measurements made during the period 1 January to 18 September.

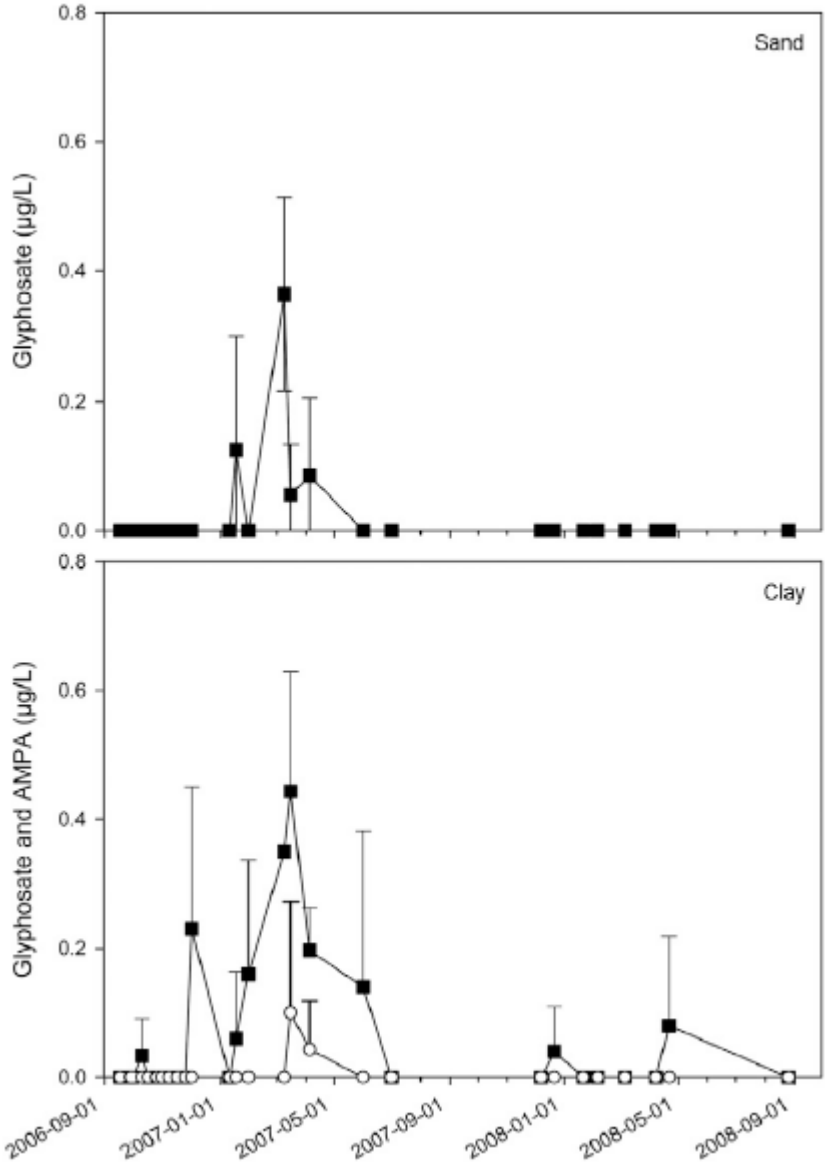
§ Includes 22 mm irrigation.

Leaching of Glyphosate and AMPA

Average concentrations of glyphosate and AMPA in leachate are shown in Figure 5. In the sand, the average peak concentration of glyphosate reached 0.36 µg/L in the beginning of March 2007, when temperatures were consistently above freezing, about 25 wk after pesticide application. During this period, the amount of leachate was about 250 mm (i.e., equivalent to 1.5 effective pore volumes).

Thereafter the glyphosate concentration decreased and the average concentration was below 0.1 µg/L from 16 March 2007 onward. This leaching pattern indicates limited preferential transport of the herbicide through the sand profile, although some preferential transport must have occurred considering the strong sorption of glyphosate (Table 3) and thereby expected large retardation. This is a flow behavior reported in several other leaching studies in sandy soils (e.g., Bergström and Shirmohammadi, 1999). The fact that the glyphosate peak occurred about 15 wk later than the corresponding bromide peak is a reflection of bromide being a nonreactive tracer. In the clay soil, the initial glyphosate peak occurred in the beginning of December and reached 0.23 µg/L after about 150 mm of water (i.e., equivalent to 0.8 effective pore volumes) had leached out of the soil columns. This considerably smaller amount of leachate suggests that glyphosate was partly transported through preferential flow paths in the clay profile, as was the case for bromide. This flow pattern has been documented earlier in this clay soil for reactive solutes (Djodjic et al., 1999; Bergström, 1995) and for nonreactive tracers (Bergström and Jarvis, 1993; Bergström and Shirmohammadi, 1999). However, the highest glyphosate peak (0.44 µg/L) in leachate from the clay soil coincided with that in the sand, i.e., in the beginning of March 2007. This glyphosate peak was washed out of the columns slightly earlier than the corresponding bromide peak, which was rather unexpected. Apart from preferential flow, another explanation could be that the highly water−soluble bromide diffused into micropores in the clay soil relatively soon after application and once in these pores it was largely protected from percolating water (Bergström and Stenström, 1998). From July 2007 onward, the average glyphosate concentration in clay soil leachate was <0.1 µg/L, although single samples had concentrations slightly exceeding the detection limit (0.1 µg/L). Average concentrations of AMPA in leachate were at or below 0.1 µg/L in both soils (Figure 5), with the highest concentration (0.30 µg/L) in a sample from one of the clay lysimeters. The average total amount of glyphosate that leached from the sand was 0.13 (± 0.03) g/ha and from the clay soil 0.28 (± 0.08) g/ha. These amounts correspond to 0.009 and 0.019 % of the amount of glyphosate applied to the soils. No leaching of AMPA occurred in the sand, whereas 0.03 g/ha leached in the clay soil. Total leaching of the ^{14}C applied in September 2006 was on average 0.31 % from the sand soil and 0.25 % from the clay. This shows that constituents other than glyphosate and AMPA that were not positively identified formed the major proportion of the total radioactivity in leachate. The leaching rates determined in this study are quite small compared with those in many other studies. For example, in a study performed by Al−Rajab et al. (2008), which included microlysimeters of three soils (clay loam, silty clay loam, and sandy loam), the amounts of glyphosate leached during 11 mo ranged between 0.11 and 0.28 % of the amount applied. However, there are also studies showing similar results to those obtained in the present experiment. In a study in France performed using lysimeters filled with calcareous soil (Landry et al., 2005), leaching of glyphosate was between 0.02 and 0.06 % of that applied after 680 mm of rainfall. Similarly, Cheah et al. (1997) recovered 0.04 to 0.07 % of applied glyphosate in lysimeter leachate after 200 mm of simulated rainfall. However, the conditions in all the above−mentioned studies were quite different from those in this study; the lysimeters were only 9.8 to 25 cm long, the experimental periods were considerably shorter (a few days to 1 yr), and the amounts of rainfall were much smaller (200 to 869 mm). These differences certainly have to be taken into account in a comparison of results.

Figure 5. Average concentrations of glyphosate (■) and aminomethylphosphonic acid (AMPA) (○) in the leachate (mean + SD, n = 2 for sand and n = 3 for clay). No AMPA was found in the leachate from sand.



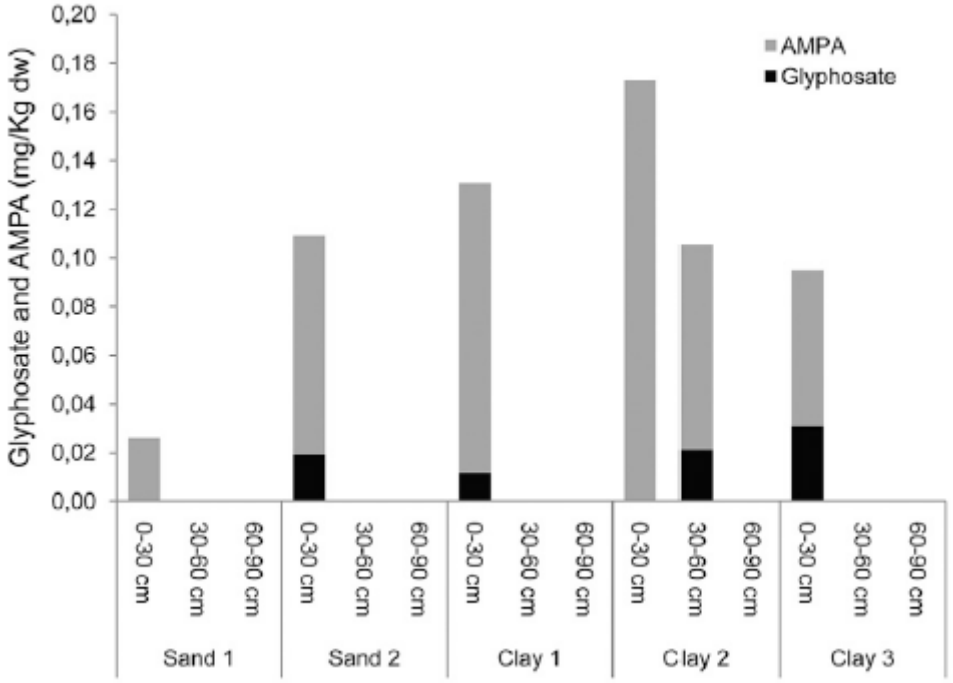
No glyphosate or AMPA was determined to be particle−bound, even though large quantities of particles were present in leachate from the clay soil. It is noteworthy that the particles were operationally defined as those being retained on a 0.22−µm glass−fiber filter. Some studies have shown that colloid−facilitated transport of glyphosate can occur. For example, de Jonge et al. (2000) showed in a study on lysimeters filled with undisturbed topsoil of a sandy loam that 1 to 27 % of leached glyphosate was particle−bound. Considering the overall low total concentrations of glyphosate in the present study (Figure 5), the particle−bound proportion would be below the detection limit (0.1 µg/L) if it constituted less than 25 % of what was leached. It is also important to bear in mind that topsoil lysimeters only include about 30 % of the profiles used in this study and may in fact, as indicated above, generate results that are quite atypical of results obtained in full−length lysimeters, such as those used here. The underlying subsoil can act as a sink or source for particles leaching through the soil profile.

The trend for glyphosate to leach in larger amounts from clay soils than from sandy soils is relatively well documented. In a Danish study, this was attributed to periods of high intensity rainfall shortly after application, when glyphosate was located on the soil surface and thereby exposed to rapid water transport in clay macropores extending up to the surface (Kjaer et al., 2003).

Residues of Glyphosate and AMPA in Soil

Residues of glyphosate and AMPA in the 0− to 30−, 30− to 60−, and 60− to 90−cm soil layers 748 d after application are shown in Figure 6. Residues were found in the 0− to 30−cm layer in all lysimeters and also in the 30− to 60−cm layer in one of the lysimeters with clay soil, possibly due to preferential flow in clay macropores and translocation in plant roots (Laitinen et al., 2007). No residues were found in the 60− to 90−cm layer in any of the lysimeters. Considering the worst−case conditions prevailing for leaching after application of glyphosate in the autumn of 2006, these results confirm the generally low mobility found for these compounds (Giesy et al., 2000; Vereecken, 2005).

Figure 6. Residues of glyphosate and aminomethylphosphonic acid (AMPA) in the 0− to 30−, 30− to 60−, and 60− to 90−cm soil layers 748 d after application. dw = dry weight.



No glyphosate was detected in one of the sand lysimeters, and 0.019 mg/kg remained at 0 to 30 cm in the other one. Low concentrations could be expected from the fast degradation in the sand topsoil (laboratory half−life 16.9 d). The concentrations of AMPA (0.026 and 0.090 mg/kg) remaining can be due to a combination of slow degradation (laboratory half-life 60.4 d) and continuous supply from degradation of remaining glyphosate. Related to the initial amount of glyphosate added, the remaining glyphosate residues represented 2.7 % and total residues of glyphosate + AMPA, calculated as glyphosate equivalents, represented 27 %.

In the clay soil, glyphosate and AMPA were found in all three lysimeters, probably due to very slow degradation of glyphosate in the topsoil and subsoil (Table 4), and thereby a long−term supply of AMPA, slow degradation of AMPA in the clay subsoil, and 100 % formation of AMPA from glyphosate degradation in the topsoil. Glyphosate residues represented 5.1 % and total residues 59 % of the initial amount of glyphosate added. Similar field persistence of glyphosate and AMPA residues was found in a sandy soil in Finland, where total residues in the 0− to 60−cm layer accounted for 72 % of the amount applied 20 mo after application (Laitinen et al., 2009).

Conclusion

The influence of adsorption on glyphosate degradation was confirmed, giving very slow degradation in the clay soil. The kinetics of AMPA residues suggest that although AMPA is always more persistent than glyphosate when formed from glyphosate, its degradation can be faster, for instance in soils with a high clay content, which slows down glyphosate degradation, and a high organic matter content, which stimulates AMPA degradation. The kinetics also suggest that apart from glyphosate being transformed to AMPA, the sarcosine pathway can be just as significant. The long persistence of glyphosate was also

confirmed in the lysimeter study, where glyphosate+AMPA residues constituted 59 % of the initial amount of glyphosate added to the clay soil 748 d after application. However, despite quite frequent rain events and large amounts of precipitation in the autumn and winter after application, these residues were mainly located in the topsoil, confirming the generally low mobility reported for these compounds. This conclusion is also supported by the small amounts of glyphosate and AMPA leached during the whole study period. Possible residues of glyphosate and AMPA due to transport on particles >0.22 µm were below the limit of detection (0.1 µg/L), and this does not appear to be an important transport mechanism in the soils included in this study.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a lysimeters experiment including derivation of sorption parameters and degradation data for glyphosate and AMPA in two Swedish agricultural soils. Chemical purity of the test substances is not reported, no mass balances or tabulated results per sample point are provided.

Lysimeter experiment: Not all required information is reported to check the validity of the study.

The study is therefore classified as reliable with restrictions (Category 2).

Sorption experiment: The experiment was conducted according the OECD 106 guideline for glyphosate. Not all required information is provided to check the validity of the study (see above, additionally LoD not sensitive enough).

The study is therefore classified as reliable with restrictions (Category 2).

Degradation experiment: The soil degradation of glyphosate and AMPA was considered. Not all required information is provided to check the validity of the study (see above, additionally mass of soil <50 g).

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.4.3
Report author	Candela, L. Et al.
Report year	2010
Report title	Glyphosate transport through weathered granite soils under irrigated and non–irrigated conditions–Barcelona, Spain
Document No	The Science of the total environment, (2010), Vol. 408, No. 12, pp. 2509–16.
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Experimental conditions not sufficiently described to evaluate validity of the results)

2.Full summary of the study according to OECD format

The transport of Glyphosate ([N–phosphonomethyl] glycine), AMPA (aminomethylphosphonic acid, CH_6NO_3P), and Bromide (Br^-) has been studied, in the Mediterranean Maresme area of Spain, north of Barcelona, where groundwater is located at a depth of 5.5 m. The unsaturated zone of weathered granite soils was characterized in adjacent irrigated and non–irrigated experimental plots where 11 and 10 boreholes were drilled, respectively. At the non–irrigated plot, the first half of the period was affected by a persistent and intense rainfall. After 69 days of application, residues of Glyphosate up to 73.6 μg/g were detected till a depth of 0.5 m under irrigated conditions, AMPA, analyzed only in the irrigated plot was detected till a depth of 0.5 m. According to the retardation coefficient of Glyphosate as compared to that of Br^- for the topsoil and subsoil (80 and 83, respectively) and the maximum observed migration depth of Br^- (2.9 m) Glyphosate and AMPA should have been detected till a depth of 0.05 m only. Such migration could be related to the low content of organic matter and clays in the soils; recharge generated by irrigation and heavy rain, and possible preferential solute transport and/or colloidal mediated transport.

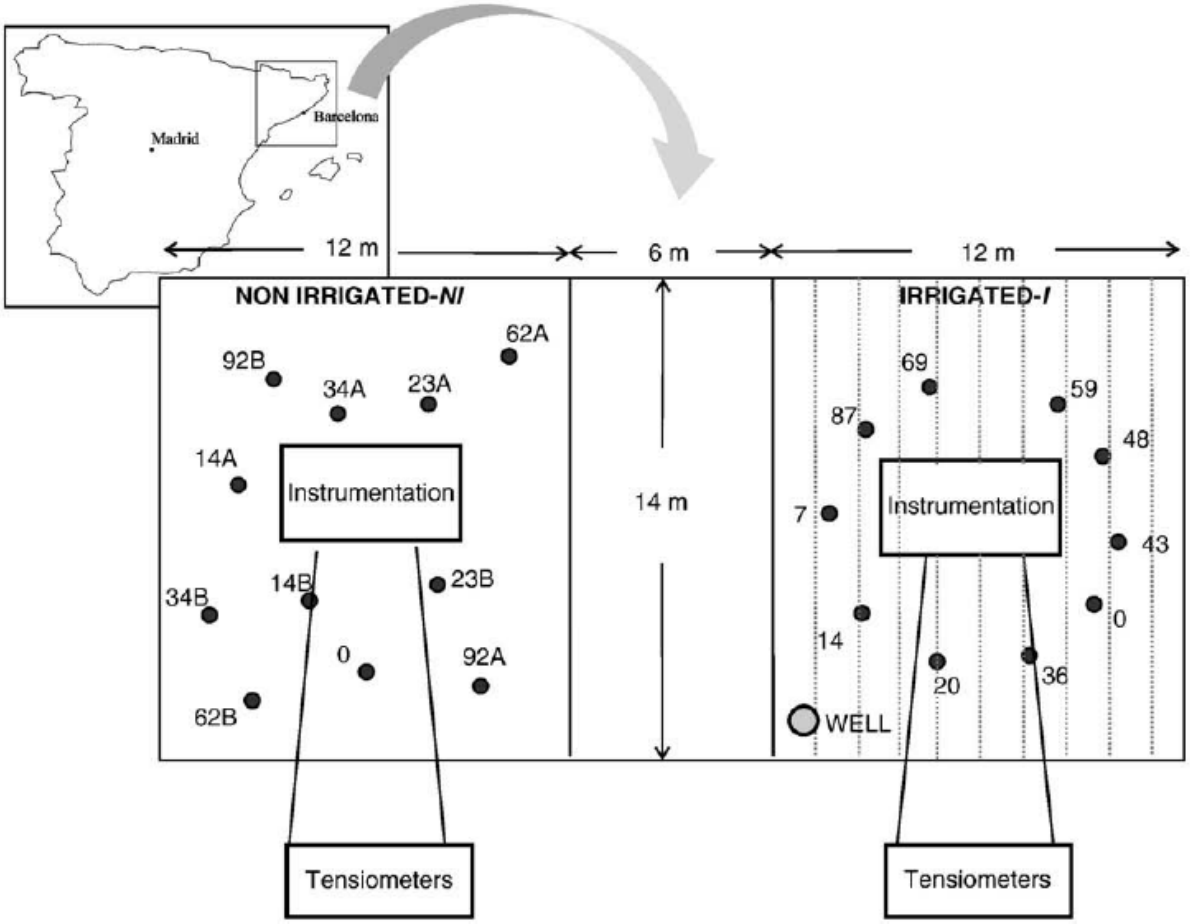
Materials and methods

Experimental site

The experimental site was located in a narrow coastal strip composed of weathered granite in the IRTA agricultural station of the Maresme region, located 30 km North of Barcelona– Spain (Figure 1). The area, under no–tillage farming, was only covered by a wheat crop to protect the soil from erosion for more than ten years. Groundwater was at a depth of 5.5 m; the hydrology of the study site has been described in detail by Guimerà et al. (1995).

Two individual plots of approximately 168 m^2 each, separated by a control area of 84 m^2, were selected. Initially, the weeds covering both plots were manually removed to allow installation of the irrigation and vadose zone monitoring equipment. Subsequently, the wheat cover was allowed to redevelop, prior to herbicide application. The upward–downward flux of water in the unsaturated zone was monitored by 7 tensiometers (Soilmoisture®). At the beginning of the experiment duplicate tensiometer sets were installed by manual drilling, in the middle of the plots, at a depth of 0.30, 0.60 and 0.90 m 106 and one tensiometer was installed at a depth of 1.20 m (Figure 1). Instrumentation remained in place until the end of the experimental activities.

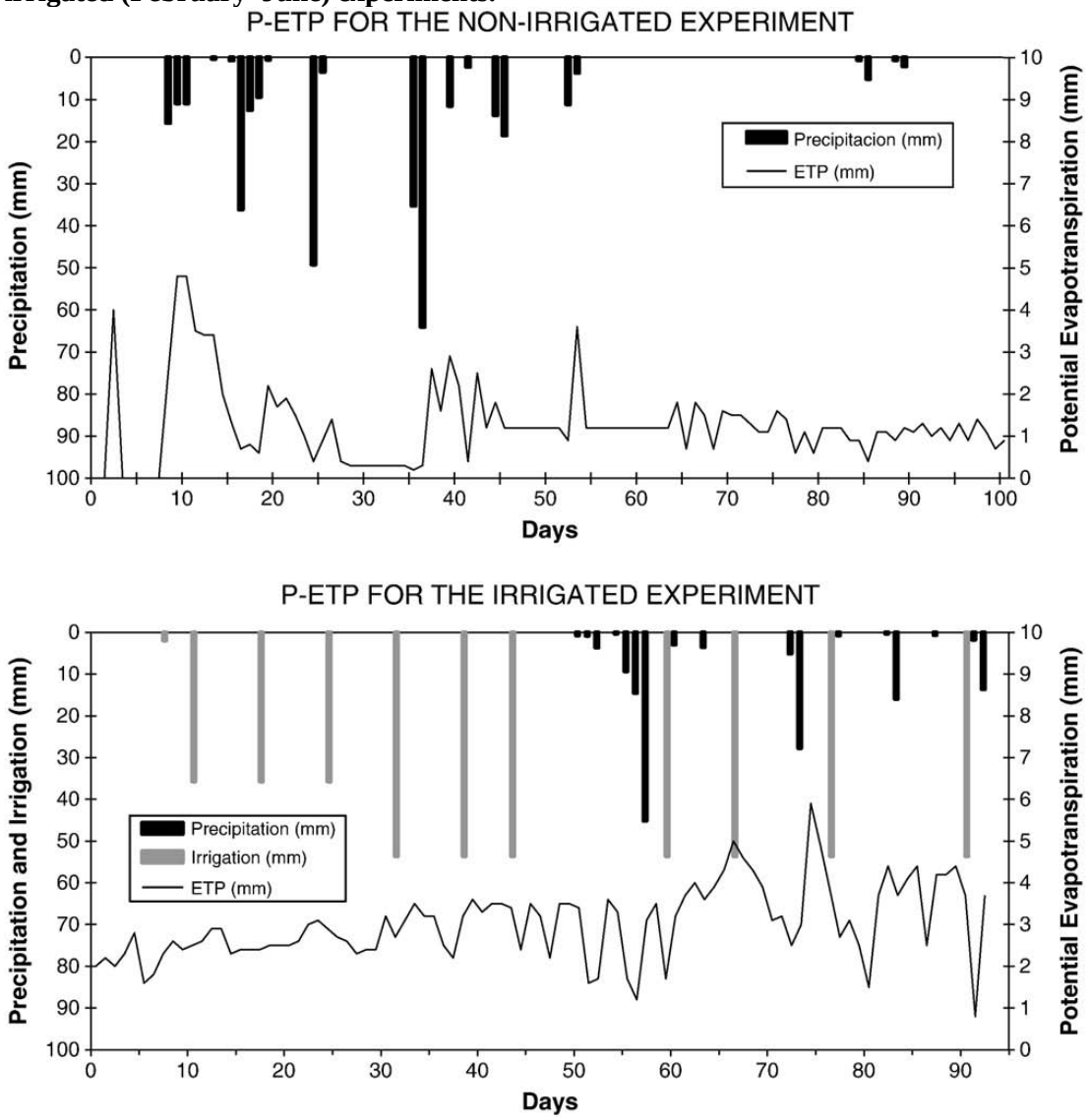
Figure 1. Study area location and non–irrigated (NI) and irrigated (I) experimental plots. The location of in situ field instrumentation and drillings is shown for the two sites; 0 denotes location of background drillings; duplicate drillings for *NI* are denoted as A and B (e.g., 1A and 1B). The vertical dashed lines (in *I*) denote the location of soak bands. The location of a groundwater well is also shown.



For initial characterization of the unsaturated zone profile, before starting the experiments, two boreholes were drilled (location denoted as "0" in the non–irrigated–*NI* and irrigated–*I* plots) and undisturbed soil samples were taken down to 4.50 m. In both plots soil matrix characterization and the monitoring of pore water content was performed by destructive sampling. The amount of pesticide in the vadose zone at given times was determined in undisturbed soil sampling. Groundwater quality was monitored in an existing pumping well in plot *I* (Figure 1).

Precipitation amounts for both experimental periods were provided by the IRTA meteorological station. Irrigation was based on soak bands that were installed in the *I* plot (Figure 1) with a separation of 0.30 m in order to obtain a uniform spatial distribution of water. Two irrigation doses of 36 mm per week, were applied during two hours for the first three weeks of the study period (March I-14 to I-27). Subsequently, the amount of irrigation increased to 53 mm per week (see Figure 2).

Figure 2. Precipitation and evapotranspiration during the non-irrigated (September–December) and irrigated (February–June) experiments.



Application of Glyphosate and bromide

Both Glyphosate (*Roundup*®, 36 % p/v, Montsanto Europe S.A.), and bromide (NaBr; conservative tracer) were applied under non–irrigated (*NI*) and irrigated (*I*) conditions (Figure 1). The first field experiment, non–irrigated, was conducted during the rainy season (September to December, 1994) and sampling and monitoring activities extended over 92 days. During the second field experiment, irrigated, that lasted 87 days, the area was irrigated from February to May (1995).

Glyphosate, along with a solution of NaBr, was applied on the soil surface on September 13 on the *NI* plot and on March 7 on the *I* plot using an automated spray system to ensure uniformity. The pesticide and bromide solutions were prepared at the study site before application. The concentrations of BrNa solutions for the *NI* and *I* plots were of 20 g/L and 17 g/L of BrNa respectively. Glyphosate solution was prepared by mixing 400 cm^3 and 420 cm^3 of a commercial 36 % (p/v) Glyphosate EC formulation with 20 and 21 L of groundwater for the *NI* and *I* plots, respectively. This procedure of pesticide application follows standard agricultural practice in the Maresme area.

Vadose zone soil and water sampling methodology

Soil samples were obtained with a hollow–stem auger after pesticide and bromide application in both field plots. A random sampling scheme with duplicate soil cores was applied in the non–irrigated area (Figure 1; *NI*–0(A,B) to 92 (A,B)) where undisturbed soil cores were taken at 0.20 m intervals till a

depth of 1 m, and at 0.50 m intervals below it (Figure 1). Due to field and experimental constraints a circular sampling pattern and single cores, where undisturbed soil samples were obtained at 0.20 m intervals, was applied in the irrigated experimental plot (Figure 1; *I*–0 to 69). To prevent possible contamination from overlying layers, two samples of the soil to be analyzed were taken from the inner part of each core, after discarding the top and the bottom portions of it. One sample was carefully wrapped in aluminum foil, and frozen until pesticide and Br laboratory analyses. The other one was used for the determination of volumetric water content, saturated and unsaturated hydraulic conductivity, and bulk density (following ASTM 1993 standards) clay content and clay type (RX diffraction) and organic matter content. Also pH, CEC and Al and Fe oxides were determined in samples following standard techniques described in Melo (1996) and Candela et al. (2007).

During the field experiments (Tables 1A and 1B), groundwater samples, soil cores and soil–water potential measurements from the tensiometers were obtained after each rain or irrigation episode. Groundwater samples were obtained with a bailer from the existing open well where also the depth of the water table was monitored. Due to analytical constrains, the concentration of AMPA was monitored in the irrigated plot only.

Table 1A. Sampling dates and precipitation amounts for the non–irrigated (NI) experiment conducted in 1994 (September–December).

Sampling survey	NI-0 Background	NI-14	NI-23	NI-34	NI-62	NI-92
Drilling date	Sep 6[a]	Sep 27	Oct 6	Oct 17	Nov 14	Dec14
Precipitation (mm)[b]	–	52.6	110.5	49.1	7.2	–
Days after glyphosate and NaBr application[c]	−7	14	23	34	62	92

[a] September 6 (*NI*-0), soil profile characterization.
[b] Cumulative values for the time interval between sampling. Total precipitation: 219.4 mm.
[c] Glyphosate and NaBr application on September 13.

Table 1B. Sampling dates and precipitation amounts for the irrigated (I) experiment conducted in 1995 (February–June).

Sampling survey	*I*-0 background	I-7	I-14	I-20	I-36	I-43	I-48	I-59	I-69	I-87
Drilling date	Feb 28[a]	Mar 14	Mar 21	Mar 27	Apr 12	Apr 19	Apr 24	May 5	May 15	Jun 2
Irrigation mm/week[b]	36	36	36	53	53	53	53	53	53	53
Precipitation (mm)[b]	–	–	–	–	–	0.7	4.7	75.3	32.8	32.9
Days after glyphosate and NaBr application[c]	−7	7	14	20	36	43	48	59	69	87

[a] February 28 (*I*-0), soil profile characterization.
[b] Total irrigation: 479 mm. Total precipitation: 146.4 mm. Precipitation (January–March 15) 8.6 mm.
[c] Glyphosate and NaBr application on March 7.

The total length of the sampled soil cores in each survey was determined according to: (a) the depth of penetration of water through the unsaturated zone as predicted from in situ tensiometer readings, (b) the hydraulic conductivity of soil samples as determined in the laboratory, (c) the predicted theoretical depth reached by the center of mass of Br^-, and (d) the retardation factor, R (Ghodrati and Jury, 1992) of glyphosate as determined in batch experiments for soils and sediments of the area. However, as a safety measure, soil drillings and sampling depths were always greater than the calculated theoretical depth of penetration of Glyphosate.

Chemical analyses

Chemical analysis of glyphosate and AMPA residues in soil and water samples was performed using an HPLC method (Hewtlett Packard, HPLC ChemStation G1034A) based on reversed–phase chromatography, with flourescent detection using pre-column derivatization with FMOC (9–fluorenylmethylchloroformate) to give the flourescent derivative. The liquid chromatography coupled column (LC–LC) methodology described by Sancho et al. (1996) was used to confirm the presence of glyphosate and AMPA residues in positive samples. The LC–LC technique presents several advantages, such as improved sensitivity, selectivity, and sample throughput. The detection limit of glyphosate and

AMPA was 6 ng/g and 4 ng/g for soil, and 0.15 µg/L and 0.1 µg/L for water samples respectively, with extraction efficiency greater than 95 % for both analytes. Bromide content was determined by ionic chromatography (VYDAC column) and the detection limit was 0.1 ng/g.

Results

Soil properties

The soil profile, a Typic Xerorthent (Soil Survey Staff, 1999), is very homogeneous and consists of medium to coarse sand size with low clay content (clay, 5 %; silt, 20 %; sand, 75 %). The clay fraction is mainly composed of smectite, illite and kaolinite. The soil had no visual structure except for the presence of a coarse sand layer at 1.50–1.90 m and granite debris at a depth of about 4.50 m. However, according to physico–chemical properties a top soil layer and a subsoil horizon may be distinguished. The soil chemical properties determined from samples at a depth of 0–0.20 m (top soil) and 0.70–1 m (subsoil horizon) respectively, are: cation exchange capacity (CEC), 5.2 and 4.6 meq.100/g; pH (1:1 in H_2O), 7.9 and 7.3; organic matter 1.1 and 0.09 (%); P 0.2 mg.100/g (top soil), total Fe_2O_3, 1.92 and 5.43 g.100/g; and total Al_2O_3 1.75 and 7.22 g.100/g. Average values of soil bulk density from field samples were 1.65 and 1.7 g/cm^3 for the top soil and subsoil, respectively. Residues of Glyphosate and Br^- were not detected along vadose zone profile before the experiments (*I*–0 and *NI*–0; Figure 3 A and B).

Figure 3A. volumetric content of water, bromide and glyphosate in the different soil profiles for the non–irrigated plot (September–December 1994). *NI*–0: soil profile prior to pesticide and bromide application. The high water content level (0.20–0.14 cm^3/cm^3) at a depth of 1.5 m reflects the presence of a coarse sand layer (LoD: 6 ng/g Glyphosate; 4 ng/g AMPA; 0.1 ng/g Br).

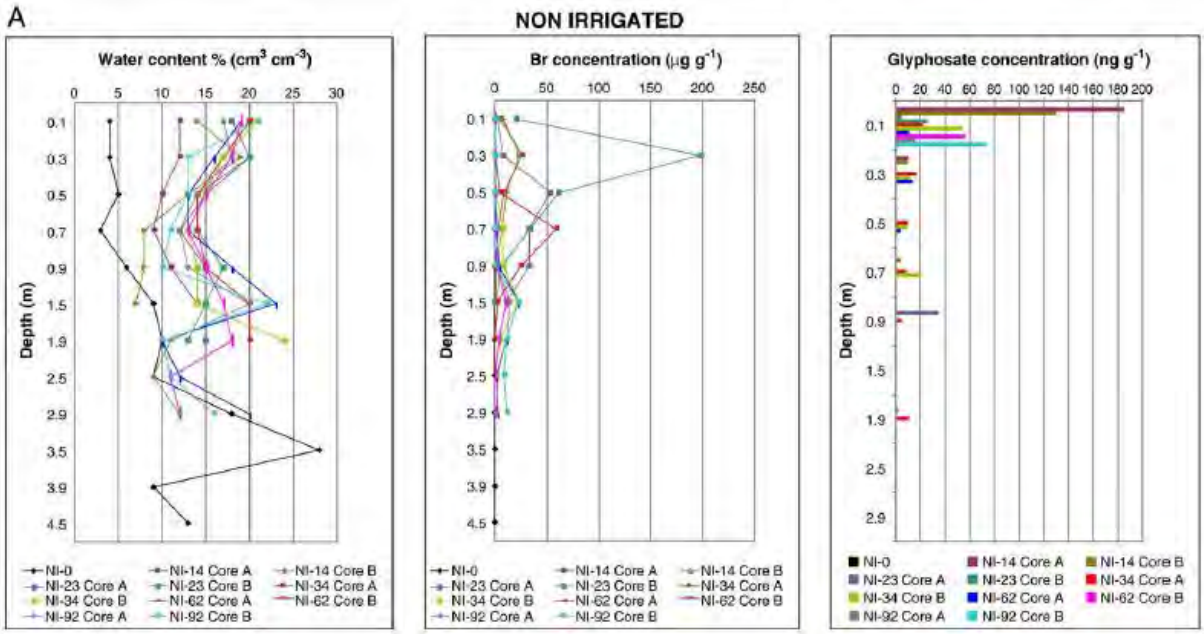
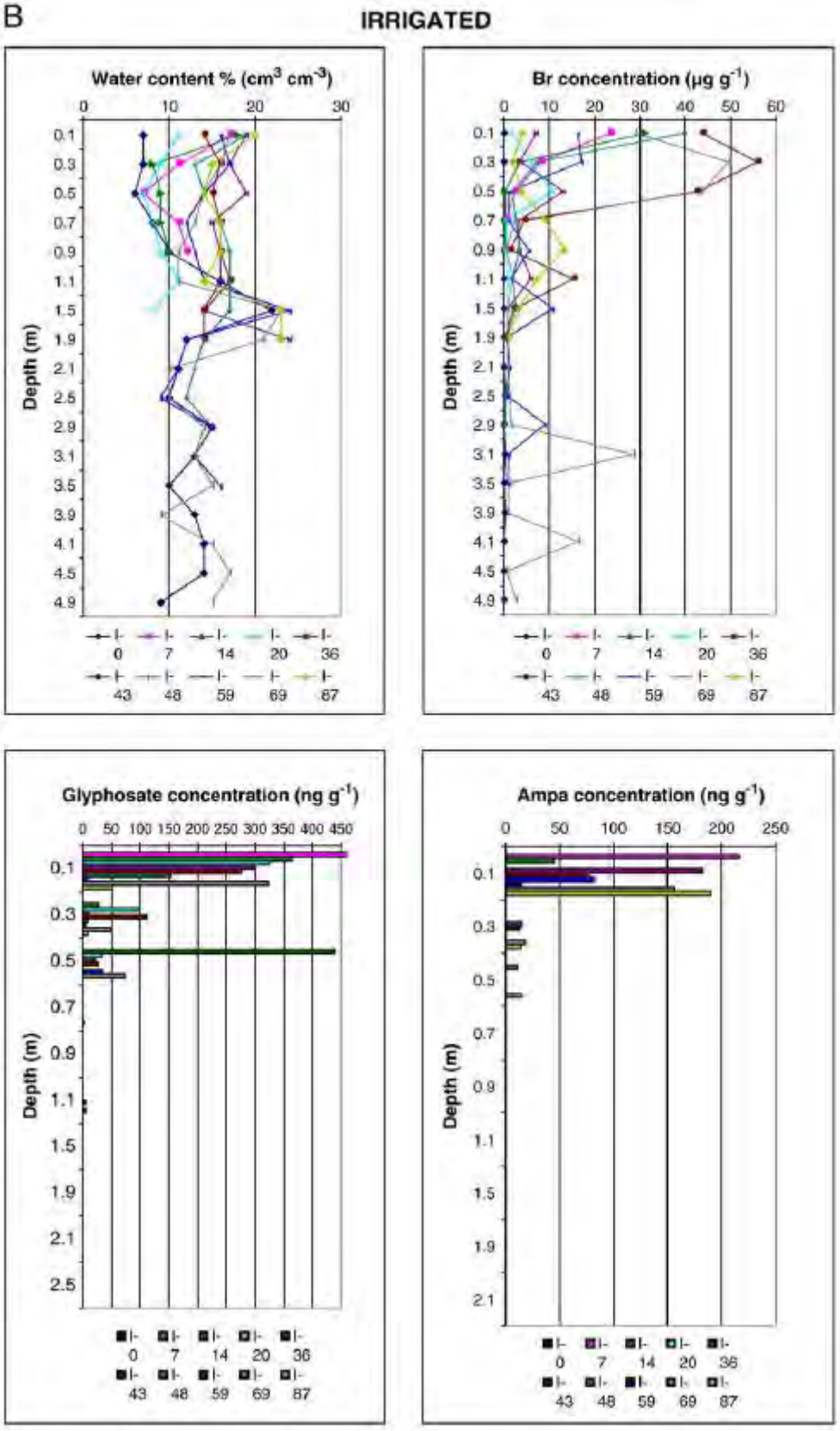


Figure 3B. volumetric content of water and bromide in the different soil profiles for the irrigated experiment (March–June 1995). *I*–0: soil profile prior to pesticide and bromide application. The high water content level (0.22–0.15 cm^3/cm^3) at a depth of 1.5 m reflects the presence of a coarse sand layer. (LoD: 6 ng/g Glyphosate; 4 ng/g AMPA; 0.1 ng/g Br).



Non-irrigated plot

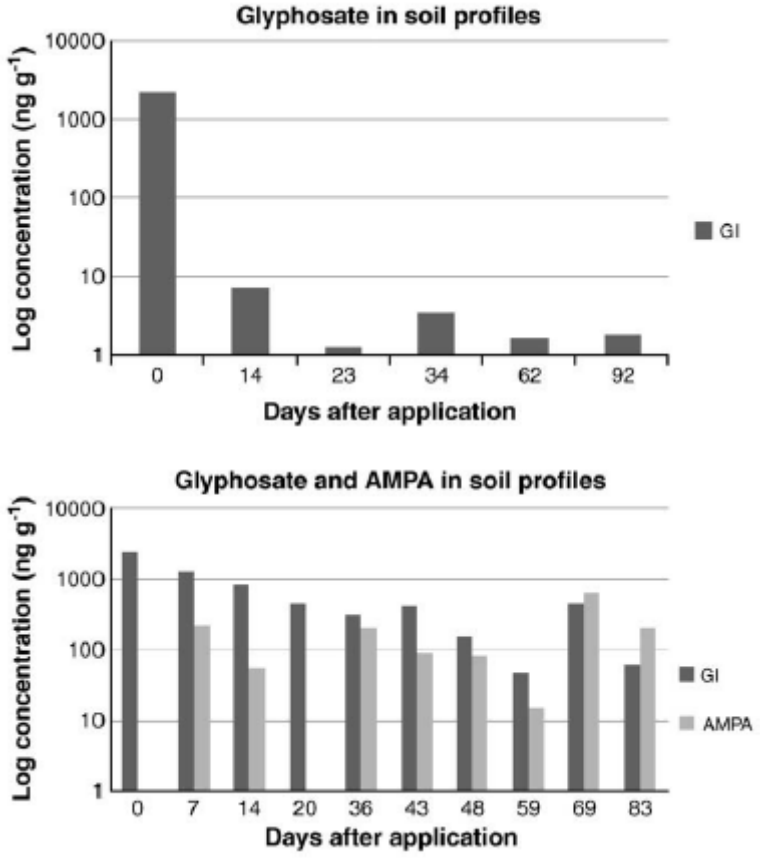
During the non–irrigated experiment the average temperature was 14.7°C, rainfall accounted for

219.4 mm, and more than 50 % of the total precipitation (163 mm) was due to three storm events in September and October. For this same time period, evapotranspiration was 118.9 mm (see Figure 2). In this plot the water content till a depth of 1.5 m was extremely low (6 % background average) due to lack of precipitation and high temperature during the summer period (*NI*, Figure 3A). After the first rain event, a week after Br and pesticide application, the movement of the wetting front is clearly observed (*NI*-14). In the upper 1.50 m the water content increases up to 20 % at the end of the experimental period and soil–water content seems to stabilize after 34 days (*NI*–34). The greatest water content along the profile was observed in the coarse sand layer at 1.5 m depth.

Maximum concentration of Glyphosate in the unsaturated zone were detected at a depth 0–0.30 m, except for *NI–23A* and *NI–34B* where residues were also detected at a depth of 0.9 and 0.7 m, respectively. The depth of penetration in individual cores varied widely. Glyphosate residues were also detected along the unsaturated zone, at concentration below the detection limit (LoD), up to a depth of 0.90 and 1.90 m after 23 and 34 days of application. After 14 days, the residual amount was 7 % of the total applied mass. After 23 days and till the end of the experiment, residual amounts account for 1 %. Glyphosate half–life (or half concentration time) calculated from in situ experimental values was 7 days, although it may be even lower considering that the first sampling campaign was undertaken after 14 days of pesticide application.

Figure 4 presents the amount of pesticide remaining in the soil profile till the end of the experiment for each core and sampled borehole. Mass estimation refers to the initial applied dose. A rapid initial dissipation phase, followed by a slower one is observed after 23 days. Degradation rate, estimated from logarithmic pesticide concentration vs. time (best fit equation) was 1.52 days. However, the small value of the correlation coefficient obtained ($R^2 = 0.4$) indicates the low accuracy of the calculations and the associated uncertainty.

Figure 4. Residual mass of glyphosate and AMPA remaining in soil profile as a function of time. Non–irrigated and irrigated experiment.



Irrigated plot

In the irrigated plot experiment carried out during springtime, the total amount of water applied was three times higher than that of the NI plot as precipitation accounted for 146.4 mm and irrigation for 483 mm. The average temperature was of 12.3°C, and evapotranspiration (266.6 mm) was greater than in the non–irrigated experiment. The background average water content in the soil profile up to 1.5 m was 10.9 %. From *I–36* (when the irrigation dose is increased), until the end of the experiment the soil profile water content is quite constant (Figure 3B, water content). The increase in water content at 1.50–1.90 m is due to the presence of a coarse sand layer.

As shown in Figure 3B, maximum concentration of Glyphosate was always detected between the first 0–0.5 m of the soil profile and concentration values were greater than those found in the non–irrigated plot. Residues of Glyphosate (below LoD) were still found at 1.50 m after 69 days of application and continued to be detected after 87 days. Residual amount of Glyphosate in soil profile after 14 days was 34 % of the applied dose, being reduced to 2 % after 59 day, and up to the end of the experiment (Figure 4). Field half–life (or half concentration time) was around 7 days and estimated degradation rate was 0.04 days ($R^2 = 0.6$).

Glyphosate and bromide were not detected in groundwater samples obtained with a bailer along all the monitoring periods.

Discussion

For the non–irrigated experiment (NI) Br concentration along the soil profile was clearly affected by the rain episodes, and was detected up to a depth of 150 cm after 14 days of application, implying a flow velocity of 10 cm/day calculated according to Burns (1975). The observed deficit at *NI–14* profile (55 % recovery of applied dose) could be attributed to the uptake of bromide by plants (Kung, 1990a). After decomposition of plant residues, bromide may return to the soil and can be accounted for as an external input in the bulk mass balance. In the irrigated plot (Figure 3B) tracer distribution over depth is fully controlled by irrigation dose, and Br^- concentration presents lower variability.

As shown in the results of both field experiments, concentrations of Glyphosate were detected, much deeper than expected according to the distribution coefficients calculated for the surface soil ($K_f = 93$), and subsoil ($K_f = 154$) in batch experiments (Melo, 1996; Candela et al., 2007). The retardation factor (R, Ghodrati and Jury, 1992) of Glyphosate, as compared to that of Br for the topsoil and subsoil, is 80 and 83, respectively. Considering a worst–case scenario (R = 80) and the maximum migration depth of Br (2.90 m; and 4.90 m Figure 3A, B), then, the maximum transport depth of Glyphosate should have been 0.05 m only. We hypothesize that the deep transfer of both glyphosate and AMPA can be the result of: (a) preferential transport along the unsaturated zone (Kung, 1990b; Van den Bosch et al., 1999; Scorza et al., 2004; Coppola et al., 2009), and/or (b) colloidal mediated transport of both components (Vereecken, 2005; Borggaard and Gimsing, 2008), a process that can be inferred from their relatively large K_f values.

The mobility of strongly adsorbing compounds as Glyphosate (Veiga et al., 2001; Kjaer et al., 2005; Vereecken, 2005, among others) has already been shown for pesticides such as propiconazole and fempropimorph (Krongvang et al., 2004), regardless of how strongly they were found to be adsorbed under equilibrium conditions in the laboratory. For the two experiments reported here, the observed differences in soil profile distribution, and rate of degradation are probably conditioned by climatic factors prevailing during the experiments (autumn and springtime), agricultural practices (dryland–irrigated), inherent variability of soil spatial parameters, land cover and roughness of soil surface.

At the *NI* experiment the presence of glyphosate at greater depth than expected may be the consequence of rainfall events. In the *NI–34* profile, Glyphosate was detected along all the sampled profile showing a high concentration (20.3 ng/g) at 0.70 m although according to batch experiments (Candela et al., 2007), after 34 days the pesticide should have been retained in the upper part of the soil. The high precipitation registered immediately after pesticide application could induce a rapid flux of water through the unsaturated zone, inhibiting adsorption onto soil particles. This process could be favored by

the amount of Glyphosate available and the initial low water content in soil before rain which could promote the existence of preferential solute transport. In sandy soils with no visible structure in the top 1 m, preferential flow appears to be dependent on soil moisture and water flow tends to be channeled through low moisture zones. This effect has been observed by Kladivko et al. (1999) and Nolan et al. (2008). Previous laboratory soil column experiments carried out with the same soils and pesticide demonstrated the importance of non–equilibrium sorption under flow conditions. Mass loss is larger for longer residence times associated either to low pore–water velocity or long soil column lengths.

Mobility of AMPA is lower than Glyphosate and residues were only detected in the 0–0.30 m interval. Considering the molecular weight of both compounds, a 0.6 ratio glyphosate/AMPA concentration in soil and water samples could be expected. However, AMPA concentrations detected in soil samples only accounted for 15 % of glyphosate degradation (Figure 4). A slower glyphosate/AMPA transformation over time, or even AMPA degradation could explain the missing amount of herbicide. The analysis on dissipation of Glyphosate and AMPA formation was not the objective of this research and the available data are not sufficient to assess the importance of biological and chemical transformation of Glyphosate. Analysis of AMPA formation (0.08 days according to best fit equation) are highly uncertain due to the low correlation coefficient obtained ($R^2 = 0.295$).

Very little is known about the nature and kinetics of this process (Grunewald et al., 2001), therefore, to gain insight into it, soil microbiological activity and the fast mineralization of both Glyphosate and AMPA should be the subject of future research.

Based on the non–reacting behavior of Br and the reduced mobility of pesticide induced by adsorption, estimation of glyphosate percentage found 3 times deeper than predicted, calculated following the Ghodrati and Jury (1992) approach, would account for 18 % and 28 %for the non–irrigated and irrigated areas, respectively (Table 2). Note that in the non–irrigated area the transport of the pesticide is clearly influenced by the two rain events (NI–34 and NI–62; Figure 2A), a phenomenon not observed in the irrigated plot where water infiltration is mainly conditioned by continuous irrigation.

Table 2. Percentage of glyphosate found three times deeper than predicted (ZG) for the different soil profiles considering achievement of equilibrium adsorption.

Non irrigated (NI)			Irrigated (I)		
Profile	$3Z_C$ (cm)	PF (%)	Profile	$3Z_C$ (cm)	PF (%)
NI-14A	0.6	5	I-7	0.2	0
N1-14B	0.6	9	I-14	0.1	56
NI-23A	1.0	0	I-20	0.6	28
NI-23B	1.0	0	I-36	0.2	10
NI-34A	1.4	64	I-43	0.6	33
NI-34B	2.4	43	I-48	0.2	5
NI-62A	3.0	62	I-59	0.6	82
NI-62B	3.0	0	I-69	0.6	28
NI-92A	3.4	0	I-87	1.3	15
NI-92B	3.8	0			
Average (%)		18	Average (%)		28

Z_C : theoretical depth to the center of mass of the pesticide (Ghodrati and Jury, 1992). PF: preferential flow; $Z_C = Z_{Br}/R$, where R is the retardation factor and Z_{Br} the depth reached by the center of mass of a pulse of the conservative tracer (Br). Center of mass depth of tracer (Z_{Br}) was estimated at each sampling profile (see Fig 3).

Although in both plots detectable amounts of tracer and pesticide have been found in the soil profile, direct comparison of results is not possible as they are conditioned by climatic parameters and water application regime. The experiment under non–irrigated conditions was undertaken in autumn; the most important aspect of the precipitation pattern is its concentration in a few unevenly distributed events of heavy storms, characteristics of the Mediterranean environment. Evapotranspiration presents a decreasing trend and water content in soil profile was low at the beginning of the exercise. For the irrigated experiment, spring climatic conditions prevail and evapotranspiration is much higher than in autumn. The weekly applied irrigation dose controls water content in soil presenting a more uniform distribution along the soil profile and experimental period.

As far as the authors are aware, such deep penetration of Glyphosate has not been reported from field studies for granite soils, such as the studied ones in the Maresme area of Spain.

Conclusion

Glyhosate is commonly considered a pesticide strongly sorbed on soils, presenting a low risk for groundwater pollution due to the phosphonate functional group strong adsorption to clay minerals, Fe and Al–oxides and OM according to laboratory experiments. A problem is whether pesticide parameters measured in the laboratory are representative for predicting pesticide behaviour under field conditions. Field investigation and monitoring of pesticide leaching present the complexity of profiling pesticide concentration in soil and the difficulty of sampling pesticide migration through preferential flow paths.

As shown in the field experiments described above, Glyphosate deep leaching in a weathered granite soil profile was observed under natural field conditions regardless of the irrigated or non–irrigated conditions and climatic season. Laboratory miscible displacement experiments performed with the same soils showed that Glyhosate adsorption in soils is essentially a kinetic process and depends on the pore water velocity and residence time of soil solutions. If flow velocities are slow and enough time is given to react with the soil matrix, surface complexation and precipitation takes place. Complexation with iron and aluminum oxides, transition metals or alkaline–earth metals has been reported in literature (Sprankle et al., 1975; Vereecken, 2005). Since Glyphosate adsorption is not an instantaneous process, needing time to attain equilibrium conditions, under heavy rain or irrigation just after its application on soil surface, it could leach more than predicted.

Given the typical conditions of the Maresme region vadose zone, highly permeable medium–coarse sand with low organic matter and clay content and containing Al, Fe, oxides and hydroxides, the principal mechanism affecting Glyphosate transport through the vadose zone may not be chemical equilibrium with the solid matrix alone. At field scale two possible explanations accounting for physical non–equilibrium will be of decisive importance on the transport of pesticide through the vadose zone. Since the phosphate compound of the molecule can be strongly adsorbed by Al, Fe, oxides and hydroxides, organic matter and humic acids of colloidal size, transport of colloid–bound Glyphosate and AMPA and also, preferential flow pathways driven by rainfall events or water application dose is likely. Presence of Glyphosate residues below detection level at depth up to 1.10 and 1.9 m in the irrigated and non–irrigated plot suggests that the pesticide may migrate into deep soil layers. This observation emphasizes the potential risk of Glyphosate transport to groundwater.

At field scale, the half–life was found to be shorter than 7 days in both experiments, much shorter than values reported in the literature (47 days average). This can be attributed to the fact that under field conditions a multitude of factors and processes contribute to herbicide disappearance, while laboratory studies are generally designed to study one of these processes. It is important to note here that these results are conditioned by the low correlation coefficient obtained (best fit equation). From the limited information obtained during the experimental study, AMPA accumulation in soil from pesticide degradation accounts for 15 % of the initial herbicide application. The parent compound transformation rate is always superior to the above–mentioned rate, leading to the conclusion that Glyphosate/AMPA transformation is a slow process or rapid AMPA degradation has occurred. Whether the transformation of the herbicide during the course of the experiment is basically controlled by chemical or biochemical processes is unknown and more research is needed regarding microbiological transformation.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a leaching experiment with glyphosate in an agricultural area in Spain. Leaching over a period of several months in spring and in autumn was observed under irrigated and un–irrigated conditions. Glyphosate and AMPA were found in in deeper soil layers than expected from the calculations based on a tracer experiment. However, only assumptions for reasons of deeper leaching

were provided, and based on the provided information (i.e. soil structure etc.) no profound explanation can be established. Duration of the study is in addition not long enough to evaluate the leaching behavior for a long–time perspective. Details regarding field sampling and sample handling practices and analysis are not sufficient to classify the study as fully reliable.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Cassigneul, A. et al.
Report year	2016
Report title	Fate of glyphosate and degradates in cover crop residues and underlying soil: A laboratory study
Document No	Science of the Total Environment 545–546 (2016) 582–590
Guidelines followed in study	OECD 106 (2000)
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Reliable with restrictions (Not all information reported to check validity of experiment against current guidelines, not all parameters reported to evaluate kinetic behavior)

2.Full summary of the study according to OECD format

The increasing use of cover crops (CC) may lead to an increase in glyphosate application for their destruction. Sorption and degradation of ^{14}C–glyphosate on and within 4 decaying CC–amended soils were compared to its fate in a bare soil. ^{14}C–Glyphosate and its metabolites distribution between mineralized, water–soluble, NH_4OH–soluble and non–extractable fractions was determined at 5 dates during a 20°C/84–d period. The presence of CC extends ^{14}C–glyphosate degradation half–life from 7 to 28 days depending on the CC. ^{14}C–Glyphosate dissipation occurred mainly through mineralization in soils and through mineralization and bound residue formation in decaying CC. Differences in sorption and degradation levels were attributed to differences in composition and availability to microorganisms. CC– and soil–specific dissipation patterns were established with the help of explicit relationships between extractability and microbial activity.

Materials and Methods

Soil and mulch sampling
Common vetch (*Vicia sativa*), white mustard (*Sinapis alba*), hybrid ryegrass (*Lolium hybridum*) and a mixture of common vetch + oat (*Avena sativa*) were grown as cover crops (CC) on the Lamothe INP–EI Purpan experimental station (near Toulouse, SW France) on a clay loam soil from June to September 2012. Prior to this cover crop, the whole field had grown a durum wheat–sunflower rotation without glyphosate application for more than 10 years. Aerial parts of the 4 cover crops were collected, dried at 40°C and cut into 1 cm square pieces. The underlying 0–5 cm topsoil was collected, sieved (5 mm) and stored at 4°C. CC–associated soils were sampled on each CC plot to record any possible plant–specific soil–borne microbial populations.

Herbicides
Both experiments were conducted with a mixture of technical–grade and [phosphonomethyl–^{14}C] glyphosate (Sigma–Aldrich), prepared in 0.01 M $CaCl_2$. Specific radioactivity and radiochemical purities of GLY were 81.4 MBq/mmol and 98.8 %, respectively.

Experiment 1: glyphosate adsorption on decaying cover crop residues
Incubations and CC characterization/description
CC– were subjected to accelerated decomposition in the dark for 6, 28 or 56 days at 28°C and in non–limiting moisture conditions. Each CC–was moistened and placed on top of its associated soil in a plastic tray (24 ∗ 37 ∗ 7 cm). Soils had been previously brought to field capacity (pF 2.5). At days 0, 6, 28 and 56 of the incubation, CC were dried, ground and analyzed (i) in duplicate for their carbon and nitrogen

content and (ii) on a single aliquot for their biochemical composition as assessed by Van Soest fractionation (Van Soest and Robertson, 1979). CC and soils characteristics are described in Table 1.

Table 1 Cover crops (a) and associated soils (b) characteristics at different incubation times. OM: organic matter, SOL: water–soluble, NDF: neutral detergent fiber soluble, HEM: hemicellulose–like, CEL: cellulose–like, LIC: lignin–like, C: carbon, N: nitrogen.

(a)

CC	Incubation Time (days)	OM	SOL	NDF	HEM	CEL	LIC	C ($mg \cdot g^{-1}$)	N ($mg \cdot g^{-1}$)	C/N
Vetch + oat	0	89.1	31.3	7.1	21.1	33.7	6.8	427.6	34.7	12.3
	6	81.0	29.4	12.0	12.7	38.7	7.1	427.5	34.8	12.3
	28	73.8	27.8	18.7	16.2	23.7	13.6	370.6	37.7	9.8
	56	75.8	18.8	24.3	18.2	23.5	15.2	374.0	36.5	10.3
Vetch	0	88.6	27.4	18.5	13.9	31.1	9.1	432.0	44.3	9.8
	6	66.8	29.6	13.0	11.6	33.9	11.9	352.7	27.9	12.6
	28	62.8	19.0	20.1	14.7	23.8	22.3	321.2	33.5	9.6
	56	54.2	7.4	30.3	11.5	27.1	23.6	290.3	29.9	9.7
White mustard	0	64.0	14.4	23.0	24.3	31.4	6.9	391.9	40.4	9.7
	6	62.8	35.9	24.7	7.8	22.1	9.6	323.4	29.5	11.0
	28	63.9	16.1	19.8	14.8	26.8	22.5	317.6	32.8	9.7
	56	69.9	23.1	20.8	15.2	25.5	15.4	336.1	30.5	11.0
Ryegrass	0	87.8	37.7	3.1	28.8	26.8	3.6	423.9	34.9	12.1
	6	72.1	24.4	26.1	15.1	18.2	16.1	377.6	44.6	8.5
	28	74.9	39.5	9.6	14.2	28.1	8.6	382.3	33.9	11.3
	56	72.6	23.5	27.3	15.7	19.2	14.3	380.9	43.2	8.8

(b)

CC	pH	OM (%)	C/N	N (%)	Organic C (%)	CEC (méq/100 g)	$CaCO_3$ (%)	CaO exangeable ($mg \cdot kg^{-1}$)	K_2O exangeable ($mg \cdot kg^{-1}$)	MgO exangeable ($mg \cdot kg^{-1}$)	P_2O_5 Olsen ($mg \cdot kg^{-1}$)
Vetch + oat	7.4	2.1	8.2	0.148	1.21	19.5	<0.1	4671	158	734	29.4
Vetch	7.5	1.8	7.4	0.138	1.02	16.2	<0.1	4255	153	582	20.3
White mustard	7.4	1.8	7.5	0.138	1.04	17.5	<0.1	4721	147	631	33.3
Ryegrass	7.4	2.2	9.2	0.140	1.28	17.0	<0.1	4387	184	645	24.0

Sorption characterization

Sorption of glyphosate onto CC residues and soil was determined using a batch equilibration technique, as detailed in Cassigneul et al. (2015). The sorbent:glyphosate solution ratio was 1:9 (g/mL) for soil and 1:5.8 for CC residues. Amounts of sorbed glyphosate were described using the partition coefficient K_d (L/kg) and the normalized organic carbon content K_d i.e. K_{oc} (L/kg OC)

Experiment 2: glyphosate degradation in microcosms of soil and cover crop residues

Microcosm setup/construction/description – Microcosms, i.e. cylinders containing soil (118 g dw) covered by CC mulch (2.5 g dw), were set up as detailed in Aslam et al. (2014). The amount of mulch corresponds to 8 t/ha of biomass in the field, soil and mulch densities being 1.2 g/cm^3 and 0.05 g/cm^3 respectively. This amount of biomass was chosen to ensure a sufficient soil coverage given our objectives. After determination of their retention curve using pressure plates, water content of both soil and mulch was brought to field capacity (pF 2.5) in order to ensure water availability to microorganisms. Microcosms were placed in a 2 L hermetically sealed jar and incubated in the dark (20 ± 1°C). To maintain constant soil moisture, a 10 mL vial filled with deionized water was placed in each jar and water content was adjusted weekly by weighing and adding water as necessary. Two 84–days incubations were performed, both with treatments including a bare soil (control) and 4 studied CC amended soils, but with and without ^{14}C–glyphosate application. The aim was to characterize separately (i) glyphosate fate in 'soil + mulch' and (ii) carbon mineralization from mulch. Each treatment was repeated thrice.

Organic C mineralization – CO_2–C produced by soil respiration and mulch decomposition was trapped in a vial containing 20 mL of 0.1 M NaOH, which was replaced weekly throughout the incubation. From a 1 mL aliquot, CO_2–C was analyzed by colorimetry on a continuous flow–analyzer. Net mineralization of CC carbon was calculated by subtracting the mineralization measured in the control soil treatment from that of the CC–amended treatment, and expressing the difference as a percentage of the initially–introduced organic carbon content.

Degradation study: pesticide monitoring in soil and mulch samples – At day 0, the recommended rate of glyphosate (2 L/ha) was applied at the microcosm surface (soil or mulch) in 2 mL of aqueous solution

with a micropipette. The water volume thus added had been subtracted from the total amount of water that had to be added to reach the targeted water content.

Mineralized fraction – $^{14}CO_2$–C originating from glyphosate mineralization in the mulch and/or underlying soil was trapped by the same procedure as total CO_2–C. The vials containing 20 mL of 0.1 M NaOH were replaced weekly throughout the incubation.

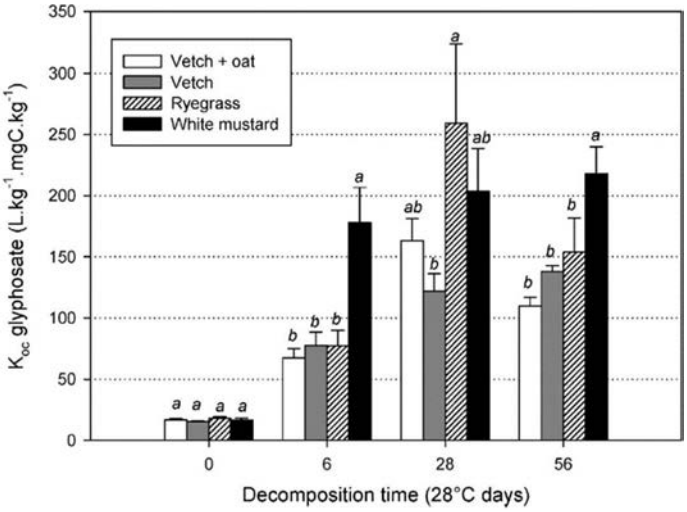


Figure 1. Sorption of glyphosate on cover crop residues. Letters correspond to LSD grouping within a single incubation time.

Extractable fractions – At 0, 7, 22, 49 and 84 DAT (days after treatment), microcosms were destructively sampled. Soils (top 1 cm) and mulches were separately submitted to 4 sequential extractions. Substrates were placed in polypropylene tubes containing solvent and shaken in a rotary shaker for 24 h in the dark at room temperature. The substrate:solvent ratio was 1:20 (g/g) and 1:3 (g/g) for mulches and soils respectively. Extractions were performed first with $CaCl_2$ (0.01 M) and then 3 times with NH_4OH (0.1 M), providing access to the weakly–sorbed and to the strongly–sorbed ^{14}C–glyphosate. Between each extraction, tubes were centrifuged for 10 min at 10,000 g and 6000 g for the mulch and the soil respectively. Supernatants were sampled for radioactivity counting, and the remaining volumes were stored at 4°C until HPLC analysis.

Non–extractable fraction – CC or soil material pellets remaining after the last extraction were oven–dried for 72 h (40°C) and ground for 10 min (Retsch GmbH, Germany). Duplicate aliquots of 500 mg were burnt in a Sample Oxidizer 307 where evolved $^{14}CO_2$ was trapped in a scintillation vial containing Oxysolve T. The vial was immediately subjected to scintillation counting.

Analytical determinations – Radioactivity content in the liquid samples was measured by scintillation counting from a 1 mL aliquot mixed with 10 mL of scintillation liquid (Ultima Gold™ XR, Perkin Elmer, USA), using a Packard Tri–Card counter (GMI, Inc., USA). To prevent a chemiluminescence reaction, NaOH and NH_4OH scintillation vials were submitted to a 24 h period in the dark prior to counting. A blank sample containing solvent or NaOH solution was inserted in each counting series. To determine the amount of glyphosate and metabolites, soil and mulch extracts containing sufficient radioactivity (83.3 Bq/mL) were previously filtered, concentrated by evaporation under vacuum at 50°C (Rotavapor ®, Büchi, Switzerland), and centrifuged to ensure maximum particle removal. Samples of NH_4OH extracts included the extracts of the 3 successive extractions. HPLC analysis was performed coupled with a Flexar (PerkinElmer, USA) coupled with a radioactive flow detector (Radiomatic Flow Scintillation Analyzer 150TR, PerkinElmer, USA). Samples (200–500 µL) were injected into an Allsep ™ A–2 anion exchanger column (100 mm × 4.6 mm, 7 µm, Grace Davison Discovery Science, USA) preceded by a GA–1 Anion guard column (7.5 × 4.6 mm, Grace Davison Discovery Science, USA) to ensure an efficient separation, eluted with a KH_2PO_4 solution (0.34 g/L) adjusted to pH 2 with a 85 %

H_3PO_4 solution. The mobile phase flow was 10^{-3} L/min. Under these conditions, the retention time was 3–5 min for GLY and 1–3 min for its main metabolite.

Glyphosate in the extracts was identified by comparison with the standard solution on the basis of retention time. Other detected peaks were considered as "main metabolite" (MM) or "unidentified" (UI) peaks. The main metabolite was suspected to be AMPA from previous experience with the same analytical method but, in the absence of a radiolabeled standard, this could not be verified in this particular experiment. The area of each peak was integrated (Chromera ® chromatography Data System, PerkinElmer) and expressed as a percentage of initial radioactivity applied in the microcosm.

Degradation half–life modeling.
The percentage of glyphosate in the extractable fraction in the microcosm (corresponding to the extractable fraction in the mulch + the extractable fraction in the underlying soil) was fitted to a single first–order kinetic model with untransformed data, $C(t) = C_0e–kt$ where C(t) is the measured concentration in glyphosate at time t, C_0 is the initial concentration measured immediately after application, and k is the first–order rate constant (day^{-1}). Following this model, the degradation half–life (DT_{50}), time (days) for 50 % disappearance of the initial amount of glyphosate, was calculated for $C = C_0/2$ and corresponds to the ln 2/k ratio.

Data analysis
Data handling and modeling – The radioactivity measured in the different glyphosate fractions was expressed as a percentage of the initially applied radioactivity. Cumulative net CO_2–C and $^{14}CO_2$–C mineralization were fitted to an exponential model that describes mineralization at incubation time t as $a_{MIN} * (1–\exp.(–k_{MIN} * t))$. The parameters α_{MIN} and k_{MIN} describe the maximum % C mineralized, and the rate at which it is reached, respectively. The kinetics data for extractable and non–extractable fractions proportions were fitted to an exponential model with 2 ($y = a_{EXT} * \exp.(–k_{EXT} * t)$) and 3 ($y = y_0 + a_{NER} * (1–\exp.(–k_{NER} * t))$) parameters respectively. In the former case, α_{EXT} is the initial extractable proportion and k_{EXT} the rate of decrease, and in the latter case y_0 is the initial NER proportion, a_{NER} the direction of variation, increase or decrease in NER and k_{NER} the rate of NER variation.

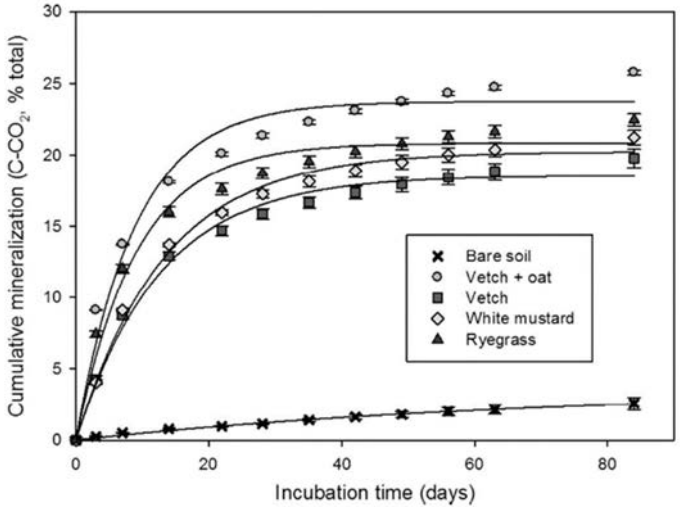


Figure 2. Organic carbon mineralization. Error bars represent the standard error of the mean of 3 replicates.

Statistical analysis
Analyses of variance were performed to ascertain whether each glyphosate fraction proportion was influenced by the incubation time, the treatment or the compartment (soil or mulch) at/on which it was measured. Then, for each fraction, a Fisher's LSD test was used to rank the treatments or compartments. Additionally, an analysis of the correlations between the different glyphosate fractions was carried out at the column and compartment level, with treatments considered together and alone. Parameters of the

different kinetic models were also subjected to analysis of variance and post–hoc LSD Fisher test to rank the different treatments, with a level of significance set at 0.05.

Results

Adsorption

Sorption was significantly higher on soil than on cover crop residues (Figure 1). K_d was 53 and 8 times higher on soil than on fresh and decomposed (56–d) CC residues, respectively. Furthermore, the statistical analysis performed within the CC revealed a significant effect of both decomposition degree and CC type. Sorption increased with the decomposition degree of cover crops ($p < 0.0001$), K_d and K_{oc} for decomposed CC (56 d) being on average 8 or 9 times higher than those measured on fresh CC (0 d). K_d was significantly higher on white mustard than on other CC for 6– and 56–d old CC, being 57 L/kg and 75 L/kg while other CC averaged 28 and 50 L/kg, respectively. For 28–d old CC, K_d was significantly higher on ryegrass (99 L/kg) than on vetch (39 L/kg), other CC being intermediate (66 L/kg). In CC, the analysis of correlations between sorption coefficients and organic matter descriptors did not show any significant relations for K_d. K_{oc} was inversely correlated with the hemicellulose–like fraction ($r = -0.55$, $p < 0.05$).

Degradation study

Mulch characteristics during incubation – During the whole incubation period, 2.6 % of the microcosms' carbon content was mineralized from the bare soil. In CC–amended soils, carbon mineralization ranged from 19 % (vetch) to 25 % (vetch + oat) (Figure 2), corresponding to a weight loss of approximately 35 %. Water content remained constant, being 17 % (w:w) in the soil and 60–72 % according to the mulch (data not shown).

Variability across intercepting material, plant type and time – Glyphosate recovery in the microcosms averaged 90 % of the initially applied dose (Figure 3). ^{14}C glyphosate fractions were significantly influenced by time, intercepting material (i.e. decaying residue or soil) and plant type (i.e. cover crop species).

Mineralized fraction – Glyphosate mineralization started immediately after application, without any lag phase. It fitted the chosen exponential model well ($R^2 > 0.95$), with parameter k_{MIN}, the speed at which the maximum is reached, and a, the maximum value reached. Treatments differed significantly from each other for the parameter a_{MIN} (Table 2), with a higher cumulative glyphosate mineralization in the bare soil microcosms, compared to the mineralisation in the CC–amended microcosms, with values ranging from 13.0 to 15.8 % (Table 2). Analysis of the plant type effect showed that the maximum mineralized glyphosate was reached significantly faster in ryegrass (Table 2).

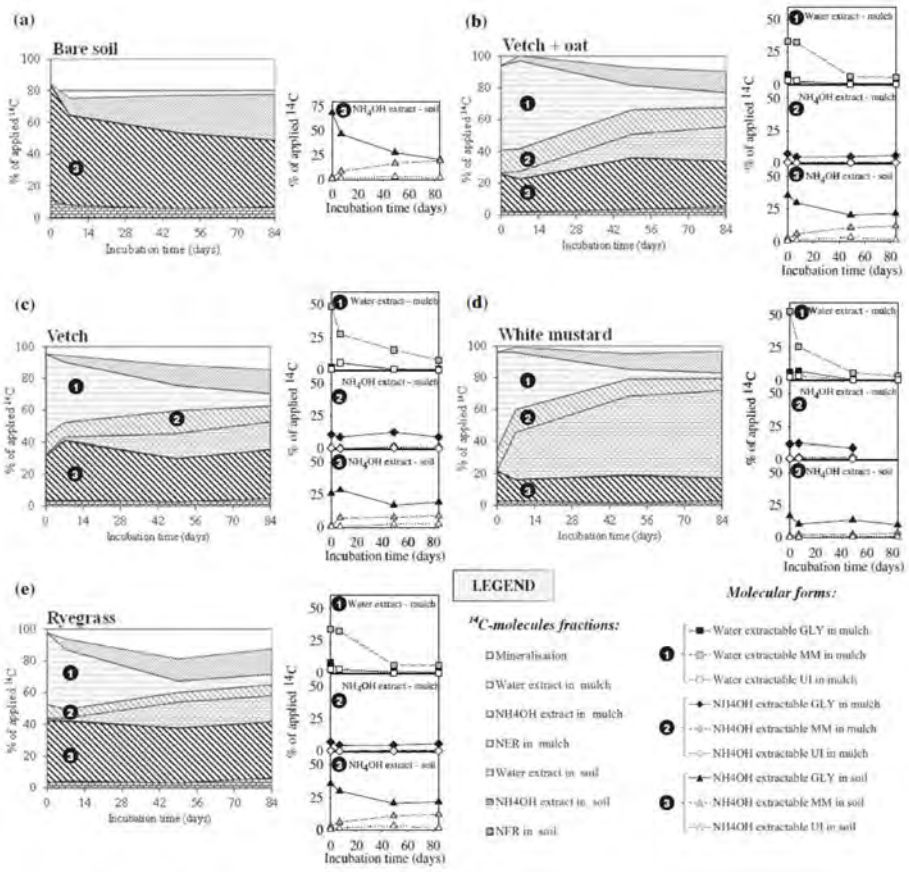


Figure 3. Fate of glyphosate in the microcosms. Letters indicate the treatment (a: bare soil, b: vetch + oat, c: vetch, d: white mustard, e: ryegrass) and numbers 1, 2 or 3 indicate the fraction within which molecular forms were analyzed (water- or NH_4OH–extractable in mulch and/or soil). Results are expressed as % of applied ^{14}C.

Extractable fraction – Total extractable fraction (corresponding to the water and NH_4OH extracts) was well fitted by the chosen exponential model, with R^2 >0.8 and R^2 = 0.65 for CC–amended and bare soil treatments respectively. The extractable fraction decreased over time for all treatments (Figure 3). Differences were observed between the treatments, extractability falling faster in white mustard than in other treatments (param b_{EXT}) (Table 2). At the end of the experiment, significantly less glyphosate was extractable in the white mustard treatment. The extractable fraction is separated into a water–extractable and an ammonia–extractable fraction, for which more details of molecular forms are given below. The water–extractable fraction decreased rapidly from 52.7 ± 3.4 to 7.0 ± 1.3 % of the applied ^{14}C between 0 DAT and 84 DAT in the mulch compartment (Figure 3, fraction ❶) while it remained low in the soil compartment (<1 % of applied ^{14}C). A larger proportion of ^{14}C was extracted with water in the vetch + oat microcosms, until 49 days of incubation (Figure 3b). In mulches, more metabolites than glyphosate (GLY) were found in the water extracts. Both GLY and its main metabolite decreased during incubation, averaging 7.9 ± 2.7 % to 0.9 ± 0.3 % and 45.0 ± 4.7 % to 6.7 ± 1.2 % of the applied ^{14}C between 0 DAT and 84 DAT, respectively. The ammonia–extractable fraction remained stable with time in the mulch compartment and varied with no clear trend in the soil compartment while it decreased during incubation in the bare soil treatment (Figure 3, fractions ❷ and ❸). In the bare soil treatment, GLY proportions decreased from 70 to 20 % of the applied dose between 0 DAT and 84 DAT, whereas MM proportions increased from 2 to 19 % in the same period (Figure 3a). In all CC–amended treatments, the GLY proportion decreased from an average of 26.5 ± 4.7 % to 14.4 ± 2.1 % and from 11.9 ± 0.1 % to 8.8 ± 1.3 % in soil and in mulch compartments between 0 DAT and 84 DAT, respectively. Meanwhile, MM proportion (i) increased from 1.5 ± 0.3 % to 8.5 ± 1.9 % in soils and (ii) averaged 1.01 ± 0.15 % in mulches.

Table 2 Fraction–dynamics model parameters. Letters correspond to LSD groups

Fraction	Treatment	y_0			$a_{i\ (i\ =\ MIN,\ EXT,\ NER)}$			$k_{i\ (i\ =\ MIN,\ EXT,\ NER)}$		
		Value	Effect of mulch vs. bare soil	Effect of plant type	Value	Effect of mulch vs. bare soil	Effect of plant type	Value	Effect of mulch vs. bare soil	Effect of plant type
Mineralized fraction $y = a * (1\text{-}\exp.(-kt))$	Bare soil	–	–	–	27.1 ± 0.9	a	–	0.06 ± 0.01	ab	–
	Vetch + oat	–	–	–	15.0 ± 2.5	b	ns	0.04 ± 0.01	b	b
	Vetch	–	–	–	15.8 ± 1.4	b	ns	0.04 ± 0.01	b	b
	White mustard	–	–	–	13.0 ± 0.4	b	ns	0.04 ± 0.01	b	b
	Ryegrass	–	–	–	15.1 ± 0.2	b	ns	0.08 ± 0.01	a	a
Extractable fraction $y = a * \exp.(-kt)$	Bare soil	–	–	–	66.5 ± 0.6	b	–	0.01 ± 0.00	b	–
	Vetch + oat	–	–	–	92.2 ± 0.5	a	ns	0.00 ± 0.00	b	b
	Vetch	–	–	–	89.6 ± 0.8	ab	ns	0.01 ± 0.00	b	b
	White mustard	–	–	–	84.9 ± 7.8	ab	ns	0.02 ± 0.00	a	a
	Ryegrass	–	–	–	89.0 ± 0.8	ab	ns	0.01 ± 0.00	b	b
Non-extractable fraction $y = y_0 + a * (1\text{-}\exp.(-kt))$	Bare soil	8.9 ± 1.0	a	–	−2.7 ± 0.9	c	–	0.12 ± 0.04	a	–
	Vetch + oat	4.1 ± 1.0	b	ns	59.8 ± 27.7	a	ns	0.01 ± 0.01	b	b
	Vetch	3.1 ± 0.7	b	ns	28.2 ± 6.3	b	ns	0.02 ± 0.01	b	b
	White mustard	3.4 ± 1.0	b	ns	53.2 ± 2.3	ab	ns	0.05 ± 0.01	b	a
	Ryegrass	3.6 ± 0.9	b	ns	31.1 ± 5.9	ab	ns	0.02 ± 0.01	b	b

Non–extractable fraction – The NER fraction increased with time for CC–amended treatments, especially in the mulch compartment. On the contrary, NER decreased in the bare soil from 11 to 7 % of the applied dose between 0 and 84 DAT (a <0, Table 2). By comparison, in the soil compartment below the mulch NER increased from 3 to 5 % or remained constant (white mustard) (Figure 3, bricks symbols). At the end of the experiment, three statistical groups differing in their NER proportions were distinguished: (i) white mustard with 59.7 ± 2.8 %, (ii) the 3 other mulches with 27.2 ± 0.8 % and (iii) bare soil with 9.0 ± 1.1 % of the initially applied ^{14}C. The modeling of NER formation showed that NER formation rate was significantly greater in white mustard (k_{NER} parameter) than in other mulches (Table 2).

Glyphosate degradation half–life – In presence of a cover crop mulch, glyphosate degradation half–life was longer than in bare soil, being respectively 28–47 days and 20 days (Table 3). DT_{50} values showed that glyphosate persistence was increased in the presence of a mulch layer at the soil surface, whatever the type of mulch.

Table 3 Glyphosate half–life (DT_{50}) calculated from fitting of experimental data to $C = C_0.e^{-kt}$ model. Data are mean ± standard–error.

Treatment	DT_{50} (days)	LSD group	R^2
Bare soil	21 ± 1	*b*	0.91
Vetch + oat	28 ± 10	*ab*	0.80
Vetch	47 ± 4	*a*	0.74
White mustard	43	*ab*	0.77
Ryegrass	38 ± 5	*ab*	0.73

Correlation between processes – Considering all treatments, glyphosate mineralization was (i) positively correlated with carbon mineralization (r = 0.80 for CC– amended and r = 0.99 for bare soil) and non–extractable fraction (r = 0.54 for CC–amended and r = 0.99 for bare soil, p <0.05); (ii) negatively correlated with water–extractable fraction (r = −0.69 for CC–amended and r = −0.97 for bare soil, p <0.01). Furthermore, NER fraction was (i) positively correlated with mineralized glyphosate fraction (r >0.96, p <0.01) and carbon mineralization (r = 0.55 for CC–amended and r = 0.99 for bare soil, p <0.05) and (ii) negatively correlated with water–extractable fraction (r <−0.96, p <0.05) in bare soil and all CC–treatments except vetch. Vetch specific correlations were found between the ammonia–extractable fraction and carbon (r = −0.98) and glyphosate (r = −0.99) mineralization. At the compartment level, the analysis revealed a correlation of NER formation either with water–extractable fraction in mulch (r = −0.98) or with ammonia extractable fraction in soil (r = −0.94).

Discussion

Glyphosate fate depends on the intercepting material

After application, glyphosate fate presented specificities according to the intercepting material, i.e. soil or CC mulch. It was much strongly retained by soil than by mulch, being mainly extractable with ammonia and with water, respectively. These results are in agreement with the sorption measurements (Figure 1) and are mainly explained by the sorption affinity of glyphosate to soil mineral constituents (clays, oxides). Furthermore, despite a high microbial activity in the mulches, reflected by the carbon mineralization (Figure 2), glyphosate mineralization in the presence of mulch was lowered as compared to the bare soil treatment. These observations partly suggest a difference in glyphosate accessibility to microorganisms in the two compartments. In soil, although glyphosate is strongly retained, as stated above, the herbicide remains accessible for a complete biological degradation. These results are in agreement with those of Schnurer et al. (2006) who observed biodegradation of soil–sorbed glyphosate. In mulches, the absence of change in the molecular forms with time in the ammonia extracts (Figure 3, fraction ❷) suggests that mulch–sorbed molecules were not available for microorganisms. In contrast, soluble glyphosate and its degradates are available in the mulch–water extracts, as shown by the decrease in their respective proportion (Figure 3, fraction ❶). However, microbial populations that colonize the decaying mulch are not as efficient as soil microbial population in mineralizing glyphosate. NER formation is generally considered to be the result of either microbial incorporation of pesticide, physical entrapment in the nanoporosity, chemical stabilization by bounding, or diffusion to less accessible sites during a long period of contact. In this study, NER formation was positively correlated to glyphosate mineralization by microorganisms in both soil and mulch compartments. As the mulch compartment is prone to a higher microbial activity (Figure 2), NER formation is clearly one of the main dissipation pathways of glyphosate in mulches, while it is a minor pathway for soils. In our study, NER proportion was negatively correlated either to sorbed (ammonia extracted) glyphosate fraction in soil or to soluble (water extracted) glyphosate fraction in mulches. In CC mulches, the decrease with time in soluble glyphosate is combined with a weak mineralization and its nearly constant sorbed proportion (recovery of glyphosate in the ammonia fraction). This supports the hypothesis of a direct transfer from the ‘soluble’ to the ‘NER’ fraction.

Glyphosate fate as influenced by the nature of the intercepting plant material

Glyphosate fate in the mulch compartment is similar whatever the mulch, i.e. the time evolutions of different fractions are generally similar. However, two of the four cover crop species stand out from the others. Glyphosate was less mineralized in ryegrass than in other cover crops, which we cannot explain, and NER formation is maximal in white mustard. This latter result was not expected but can be explained in view of the results of the sorption study where white mustard was the mulch which maximized sorption at day 6 and 56.

Glyphosate fate in cover crop residues and environmental risk assessment

In this study, glyphosate fate was studied at a fine scale by considering several fractions. The results can be interpreted at a broader scale by considering only two fractions: (i) the dissipated glyphosate i.e. the glyphosate mineralized as CO_2 and immobilized as NER; and (ii) the available glyphosate and metabolites i.e. the molecules which remain available and could be leached in field conditions. At this scale, except for the white mustard treatment, both dissipated and available glyphosate were statistically the same in all treatments. This does not lead to the conclusion that glyphosate fate is not influenced by the presence of a cover crop since (i) dissipation pathways are treatment–specific, i.e. mineralization and metabolites (AMPA) formation are greater in bare soil and more non–extractable residues are formed in CC–amended treatments; and (ii) the NER formation pathway in mulch is time–dependent, leading to a potential decrease in availability of glyphosate in CC–amended treatment. According to the mechanisms potentially involved in NER formation routes we have proposed for mulch, such release cannot be excluded. The extent to which these results can be extrapolated to field conditions will be determined by (i) weather conditions, especially during the time between application and the first rain and the temperature; (ii) agricultural practices, especially cover crop incorporation and fertilization; and (iii) mulch biomass, coverage, and contact with soil as well as soil type.

Conclusions

This study aimed at evaluating the effects of a mulch of cover crop residues located at the soil surface on the environmental behavior of glyphosate. In the presence of a cover crop mulch, glyphosate and its metabolite remained mainly water–soluble, but with time, a higher proportion of the herbicide became non–extractable. Unlike in soil conditions, bound residue formation was the main process involved in glyphosate dissipation in cover crop mulches. Variations in the intensity of each process were observed among the four cover crop residues studied, but remained unexplained by the biochemical composition of the residues. Finally, degradation half–life of glyphosate was increased with all type of mulches. Such results suggest a greater risk of glyphosate transfer by leaching in conservation agriculture systems.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes the degradation and sorption of Glyphosate to soil considering cover crops. The article is well described and provides potential endpoints for degradation and sorption. However, the available information does not allow to check the validity against current guidelines, and not enough parameters are provided to evaluate the kinetic behavior.

The article is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Dollinger, J., et al.
Report year	2018
Report title	Contrasting soil property patterns between ditch bed and neighbouring field profiles evidence the need of specific approaches when assessing water and pesticide fate in farmed landscapes
Document No	Geoderma 309 (2018) 50–59
Guidelines followed in study	OECD 106
Deviations from current test guideline	study not sufficiently described to check validity of the results
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (study not sufficiently described to check validity of the results)

2. Full summary of the study according to OECD format

The authors' aim was to evaluate the specificity of ditch material properties to determine whether ditches require an approach that differs from that of field soils when studying water and pesticide fate in farmed landscapes. The authors thus analysed the variations in the pedological, herbicide sorption and flow properties of soil materials along a 2D cross-section of an intermittently flooded ditch in the Roujan catchment of southern France. They found that the upper part of the ditch bed soil profile is composed of 3 horizons that formed after the original creation of the ditch, most likely via the deposition of field-eroded particles and the accumulation of organic matter. These specific horizons have greater porosity, mostly due to their dense root systems, and contain up to 2 times more organic carbon than the neighbouring banks or field soils. Consequently, the hydraulic conductivity is greater, and the sorption of hydrophobic herbicides is up to 2 times greater in ditch bed materials than it is in soils located farther away from the ditch surface. Moreover, significant macroporal flow was evidenced in both profiles but with different contribution to the global flow. The contrasts in the hydrodynamic and sorption properties between both the ditch bed and banks materials likely results in significantly different water and pesticide infiltration patterns in ditches compared to crop fields. Given these differences, they recommend investigating the specific properties of ditch beds when studying and modelling water and pesticide fate in croplands.

Materials and methods

Study site

The studied ditch is located near the outlet of the Roujan catchment (Herault, France). This 91 ha catchment (Figure 1 and Table 1) is cultivated mainly by vineyards and a dense network of ditches, 11 km total length, was implemented between the vine fields. Except on the plateau, the soils are directly developed over the Miocene loose sandstone and are organized along a toposequence. The soils depth increase and soil texture evolve consistently with the colluvial accumulations of clay and gravels in the glacis. Nearby the study site the soil is classified as a gleyic cambisol (IUSS Working Group WRB, 2014). A perennial groundwater has developed on the bottom part of the catchment (Figure 1) and >5 km of ditches (47 % of the total length of ditches) drain this area.

The catchment is subjected to semi-arid Mediterranean climate characterized by scarce high-intensity rainfall events. This specific precipitation pattern results in the periodic flooding of ditches and the rapid fluctuation of the shallow water table in the bottom part of the catchment. The high reactivity of the water table leads to the alternation of downward and upward fluxes in ditch beds during storm events. The studied ditch is chosen near the catchment outlet in order to: i) represent the typical functions of ditch in a perennial groundwater environment (Figure 1) and ii) be representative of the soil type and ditch characteristics combination that prevail in the 33 ha of the bottom part of the catchment.

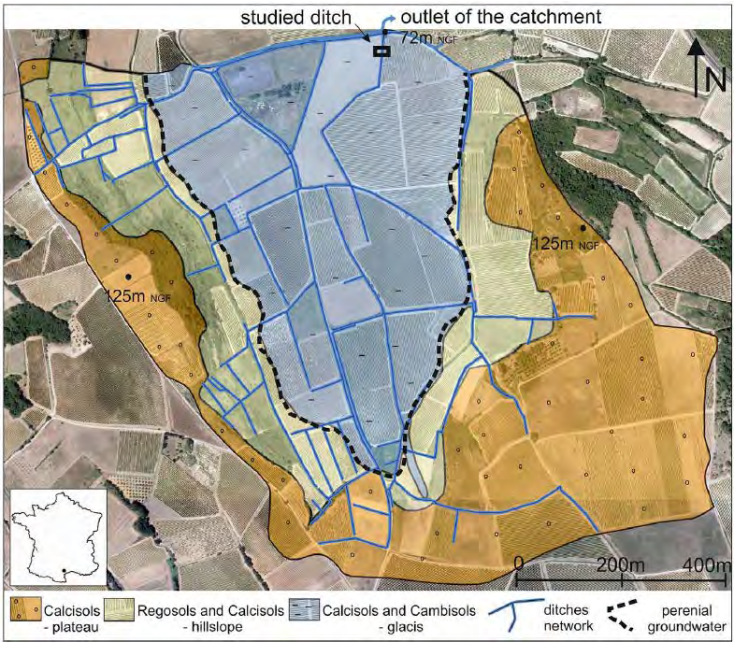


Figure 1. **The ditch network over the Roujan catchment in relation with the soils and the perennial groundwater.**

Table 1 **The spatial variability of ditches network over the catchment.**

Topographic position	Dominant soil types (WRB 2014)	Soil depth	Surface area	Total length of ditch in the network
		m	ha	km
Plateau	Calcisols	0.4–2	36	1.49
Hillslope	Regosols and calcisols	0.2–1.5	22	4.78
Glacis	Calcisols and cambisols	1–4	33	5.48
Catchment			*91*	*11.75*

Experimental design

Characterization of soil properties and core sampling along the cross section

For characterizing and sampling soil heterogeneity of the ditch soil and its vicinity, a 1.50-m-wide, 1.50-m-deep trench was excavated across the ditch in February 2014. The studied ditch is densely vegetated, and roots are present along the entire soil profile to a depth of 1.5 m (Figure 2A).

A series of morphological parameters, including texture, structure, colour, stone and root abundances, were observed in the field. Soil horizons were determined based on these observations. Bulk densities (ρ_b) were measured by core sampling with 100-cm^3 cylinders, using 6 replicates per horizon. The ρ_b was determined as the ratio between the dry soil mass and the total core sampling volume. Samples of over 500 g were collected from each horizon for further laboratory characterization. Particle size distribution, pH, cation exchange capacity (CEC), organic carbon content (OC), and calcium carbonate ($CaCO_3$) content were measured at the INRA-ARRAS Laboratory (France) (see Table 2).

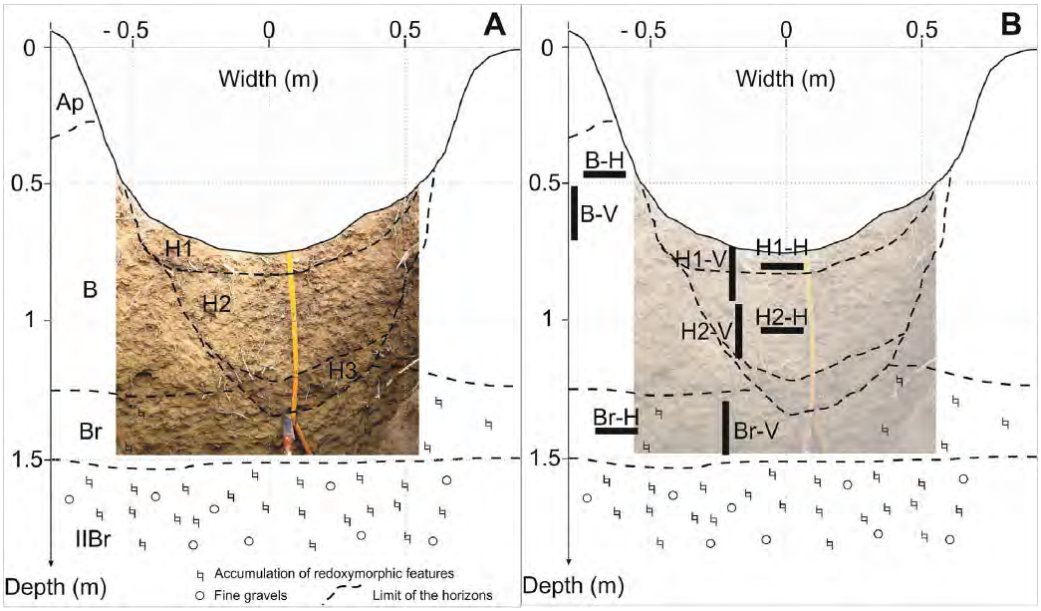


Figure 2. **Morphology of the ditch cross-section soil profile. A) Description of the soil profile, B) core sampling scheme. The black lines represent core sampling locations within the soil profile. V and H represent the cores sampling axis direction being, respectively, vertical and horizontal.**

Table 2 **Physico-chemical properties of ditch-bed and banks soils.**

Horizon	Depth from field topsoil	Structure	Sand	Silt	Clay	OC	CEC	pH	$CaCO_3$	ρ_b
	m		%	%	%	%	cmol kg^{-1}		g kg^{-1}	g cm^{-3}
B	0.4–1.30	Polyhedral subangular blocky – hydromorphic features	7.7	57.2	35.1	0.96	14.2	8.71	247.0	1.36 ± 0.01
H1	0.75–0.82	Stratified	35.9	39.1	25.0	1.58	12.5	8.44	150.0	1.25 ± 0.06
H2	0.82–1.15	Granular	40.3	33.1	26.6	1.56	12.1	8.54	160.0	1.26 ± 0.04
H3	1.15–1.35	Stratified and subangular blocky – hydromorphic features	10.4	56.7	32.9	1.17	14.0	8.59	248.0	1.21 ± 0.05
Br	1.30–1.50	Polyhedral blocky – hydromorphic and redoximorphic features	4.7	58.5	36.8	0.73	14.4	8.63	285.0	1.48 ± 0.03

Four undisturbed soil cores were sampled from each horizon except in the Ap horizon of the bank profile that has no counterpart in the ditch soil profile. These cores were collected by gently pushing stainless-steel cylinders with internal diameters of 15 cm and heights of 20 cm in the soil until the soil surface was approximately 5 cm from the top of the cylinder. The soil around the cylinders was then excavated to facilitate the undisturbed extraction of the monoliths. To characterize the anisotropy of downward vs. lateral water and solute flow, a series of monoliths was sampled vertically and a second series was sampled horizontally (Figure 2B). Due to the length of the sampled cores, the core sampled in the first horizon below the ditch also included the top of the second horizon; because the third horizon below the ditch was too narrow, it could not be sampled. After extraction, cores were stored at 4°C until undergoing tracer experiments.

Tracer displacement experiments

Tracer displacement experiments were performed on soil cores sampled vertically and horizontally in the ditch bed and bank profiles (Figure 2B) in order to characterize the water flow patterns of these materials. Because bromide is only present at trace concentrations in the environment and rarely sorbs to soil particles, it was selected as a conservative tracer of water flow for these displacement experiments.

Stainless-steel grids with 6-mm-diameter holes were sealed at the bottom of the columns to prevent soil loss occurring during the infiltration experiments without disturbing the water flux in the columns. Prior to tracer injections, the columns were gradually saturated via capillarity for 48 h to prevent the trapping of gas bubbles in soil pores.

The tracer solutions used for the displacement experiments contained 800 mg/L of bromide (Br^-). At the beginning of the displacement experiments, the saturated columns were manually ponded with a

30-mm water height of the tracer solution. This water height was chosen to mimic the infiltration conditions in the Roujan catchment during intermittent flooding and corresponds to the water level commonly monitored in ditches during flood events with a 1-month return period. The water height was kept constant during the infiltration by adjusting the supply of the tracer solution. A total of 85 mm of solution was supplied to the columns. Among the 16 columns, the pore volumes ranged from 63 to 82 mm. Therefore, the volume of tracer solution supplied during the displacement experiment was always higher than the pore volume of the columns. When the solution supply stopped, the decrease in water head was monitored during the remaining period of ponded water infiltration. Just after all the ponded solution had infiltrated, the columns were ponded again with a constant head of 30 mm, and the columns were flushed with 85 mm of tap water, following the same procedure. During the infiltration and flushing periods, 50 ml fractions of the percolates were collected in glass containers at the outlets of the columns. The sampling frequency varied from 15 s to about 6 min, depending on the columns drainage fluxes. The outlet flowrates were monitored using the timing of sample collection and their precise weights. The inlet flowrates were monitored by weighing the injection tank at 1-s intervals.

The concentrations of bromide in the percolate samples were measured using an ion-specific electrode (Hanna Instruments, HI4002, Lingolsheim). These concentration values were cross-validated with ion chromatography measurements of randomly selected samples. A good fit was found between the ion-specific electrode and the ion chromatograph results (data not shown). The electrical conductivity and pH were also measured in the percolate samples.

Dye tracing of the active macroporosity

Following the displacement experiments, dye tracing was performed on the columns to visualize and quantify the active macroporosity. The dye tracing experiments were also used to visualize the presence or absence of sidewall flow. The displacement experiments were validated when sidewall flow was absent or weak and discontinuous along the sides of the columns. In contrast, when continuous sidewall flow was detected along the sides of the columns, the corresponding displacement experiments were dismissed. A total of 8 columns, containing one sample per horizon and one sample per direction (vertical/lateral) were validated based on dye staining experiments.

The infiltration conditions of dye tracing were identical to those of the displacement experiments, as the fraction of active porosity mobilized for percolation in structured soils likely varies with initial moisture and water head conditions. The columns were thus saturated again via capillarity for 48 h; then, 57 mm of the fluorescent dye sulforhodamine B at a concentration 1 g/L was percolated through the columns with a constant water head of 30 mm. At a concentration of 1 g/L, the sorption sites of sulforhodamine B on soils in contact from all horizons were saturated, which guaranteed homogeneous staining among the columns. After percolation, the columns were sliced into cross-sections approximately 2 cm in height. A marker was placed on the sides of the slices to determine the orientation and superposition of the 7 slices within a given column. The slices were then imaged in a dark chamber with homogeneous LED lighting (3800 K) using a digital camera that was equipped with a 28-mm lens and was positioned 65 cm above the slice. The image resolution was 300 dpi, which corresponds to a pixel size of 71 µm. The illumination and hue saturation of the raw images were corrected using Nikon Capture NX2 software based on the grey and colour scales positioned next to the column slices during imaging. The RGB channels were split, and the colour thresholds were adjusted in each of the channels. The minimum/maximum thresholds applied to all images were 9/255, 118/253 and 4/255 for the R, G and B channels, respectively. The RGB channels were then merged, and the image was binarized. Both bright and dark isolated pixels were removed using the 'Noise' function of the ImageJ software, with a radius of 10 pixels, for white and black pixels, successively. The respective areas of both bright and dark pixels relative to the total area of the column cross-section were then calculated with ImageJ. The dark areas correspond to the stained areas on the cross-sections of the columns.

The volume of macroporosity mobilized during percolation relative to the total porosity (ω) was estimated from the dye coverage area. As dye diffusion in the matrix is limited, due to its short infiltration time, ω_i was calculated for each column cross-section (i) by multiplying the average dye coverage area per column slice (i.e., top and bottom coverage) by the slice height and dividing it by the

total porosity (i.e., the total volume of soil in the slice multiplied by the soil porosity). The average ω per column was also calculated as a geometric mean of the respective ω_i values of the 7 column slices (i).

Inverse modelling of transport properties

Water flow and transport equations

Inverse modelling was performed with the HYDRUS-1D model that solves the Richards and convection-dispersion equations. Four modelling approaches were compared in the first place: single porosity, dual porosity, dual porosity + mobile-immobile (DP + MIM) and dual permeability (see Šimůnek et al., 2003 for a detailed description of these approaches). Only the dual-permeability model provided satisfactory fits of the tracer displacement experiments for most of the columns and is thereby considered in this paper. The column H2-H was the only exception for which the model DP + MIM was better adapted than the dual permeability model. DP + MIM was used to simulate the bromide breakthrough curve of H2-H but is not described in this paper (for the description of the model please refer to Šimůnek et al., 2003). Equations of the dual permeability model are briefly reviewed below.

The dual permeability model assumes that flow and solute transport occur within and between two distinct compartments, namely the macropore compartment, consisting in inter-aggregate or fracture porosities, and the micropore or matrix compartment, consisting in intra-aggregate porosity. The water flow equations in the macroporal and matrix compartments are assumed similar by HYDRUS 1D and given by:

$$\frac{\partial \theta_f(h_f)}{\partial t} = \frac{\partial}{\partial z}\left[K_f(h_f)\left(\frac{\partial h_f}{\partial z} + 1\right)\right] - S_f(h_f) - \frac{\Gamma_w}{\omega} \qquad (1a)$$

$$\frac{\partial \theta_s(h_s)}{\partial t} = \frac{\partial}{\partial z}\left[K_s(h_s)\left(\frac{\partial h_s}{\partial z} + 1\right)\right] - S_s(h_s) - \frac{\Gamma_w}{1-\omega} \qquad (1b)$$

where subscript *f* and *s* respectively refers to the fast macroporal compartment and the slow matrix compartment, θ is the water content [L^3/L^3], *h* is the pressure head [L], *K(h)* is the unsaturated hydraulic conductivity function, *S* is a sink or source term [T^{-1}], ω is the ratio of the macroporal volume of fast to the total poral volume of the soil (dimensionless) and Γ_w is the transfer rate between the two compartments [T^{-1}]. The water retention curve $\theta(h)$ and the unsaturated hydraulic function *K(h)* are defined for both compartments using the van Genuchten model. *K(h)* is described as the product of the relative hydraulic conductivity function *Kr* (dimensionless) and the saturated hydraulic conductivity *Ks* [L/T].

The transport equations associated with the dual-permeability formulation for water flow are based on the classical convection-dispersion equation for both the fast macroporal compartment and the slow matrix compartment with an exchange term between the two compartments:

$$\frac{\partial \theta_f c_f}{\partial t} + \rho\frac{\partial s_f}{\partial t} = \frac{\partial}{\partial z}\left(\theta_f D_f \frac{\partial c_f}{\partial z}\right) - \frac{\partial q_f c_f}{\partial z} - \phi_f - \frac{\Gamma_s}{\omega} \qquad (2a)$$

$$\frac{\partial \theta_s c_s}{\partial t} + \rho\frac{\partial s_s}{\partial t} = \frac{\partial}{\partial z}\left(\theta_s D_s \frac{\partial c_s}{\partial z}\right) - \frac{\partial q_s c_s}{\partial z} - \phi_s + \frac{\Gamma_s}{1-\omega} \qquad (2b)$$

$$\Gamma_s = \omega_{dp}(1-\omega)\theta_s(c_f - c_s) + \Gamma_w c^* \qquad (2c)$$

where *c* is the solute concentration [M/L^3], *s* is the sorbed solute concentration [M/M], ρ is the bulk density [M/L^3], *D* is the dispersion coefficient accounting for both molecular diffusion and hydrodynamic dispersion [L^2/T], *q* is the Darcian flux [L/T] ϕ is a sink-source term [$M/(L^3\ T)$], Γs is the mass transfer term for solute between the macroporal and the matrix compartments [$M/(L^3\ T)$] and c^* is equal to c_f. for $\Gamma_w > 0$ c_m for $\Gamma_w < 0$.

Inverse modelling design

The 0.15 m-soil profiles were densely discretized with 101 nodes to facilitate numerical convergence. An initial hydrostatic equilibrium with a zero-pressure at the top of the soil columns was considered. A variable head was imposed at the upper boundary condition. It was fixed at the ponding head value (3 cm) during the injection and rinsing phases and varied between both phases to correspond to the ponding height decreases monitored during the experiments (see Section *Tracer displacement experiments*). The eight following parameters were fitted against the cumulative water outflows heights and bromide concentrations at the outlet of the soil column: θs_s, θs_f, Ks_s, Ks_f, ω, $Disp_s$, $Disp_f$, ω_{dp} with θs and θr respectively the saturated and residual soil water content and *Disp*, the dispersion coefficient [L]. To avoid local minimum, the stability of the fitted parameter set estimated was evaluated using different sets of initial parameters, including the estimated sets themselves. The other hydrodynamic parameters were set according to the textural composition and bulk density of the soils using Rosetta, except θr_f that was set to zero. Note however that since the soil column remained saturated during the whole experiment, the van Genuchten parameters alpha, n and l were not sensitive. The Bromide diffusion coefficient was fixed to $1.67\ 10^{-9}\ m^2\ s^{-1}$. K_{sat} was not adjusted but calculated from the experimental outflow data using Darcy's law. Θs was also not adjusted but calculated from the bulk density data using a pedotransfer function.

Sorption properties of selected herbicides

Two herbicides, diuron and glyphosate, were selected to assess the heterogeneity of sorption properties along the profile of the ditch cross-section. Diuron was extensively used on the Roujan catchment for weed control in vineyards. After it was banned from the list of allowed active molecules in France in 2008, it was replaced by the broad-spectrum herbicide glyphosate. Both herbicides were still measured in the water column of the ditch at the outlet of the catchment in 2016. Glyphosate and diuron exhibit very different physicochemical properties (Table 3), which may lead to contrasting sorptive patterns along the soil profiles.

The adsorption parameters were assessed according to the procedure described in Dollinger et al. (2016), which was adapted from the OECD Guideline 106. Briefly, the soils were air-dried to a target humidity of 10 % then sieved to a size of 2 mm. 10 mL of the ^{14}C-labelled pesticide solution, with concentrations ranging from 5 to 1000 µg/L, were equilibrated with 1 and 2 g of dry soil in glass centrifuge tubes for glyphosate and diuron adsorption experiments, respectively. The tubes were shaken for 24 h, and the radioactivity in the supernatant was measured after centrifugation. Pesticide concentrations in soils were assessed by mass balance between initial and equilibrium concentrations. Both linear (Eq. 3) and Freundlich models (Eq. 4) were fitted to the experimental data.

$$C_s = Kd\ C_w \quad (3)$$

$$C_s = Kf\ C_w^{\,n} \quad (4)$$

$$H = \frac{n_{des}}{n_{ads}} \quad (5)$$

where C_s is the amount of sorbed pesticides in the soil at equilibrium ($\mu g\ kg^{-1}$), C_w is the equilibrium concentration in the supernatant (µg/L), *Kd* is the linear sorption coefficient (L/kg), *Kf* ($\mu g^{(1-n)}\ L^n/kg$) and n are the Freundlich coefficients and H is the apparent hysteresis index with n the non-linearity parameter of the Freundlich model and subscripts *ads* and *des* standing for adsorption and desorption isotherms, respectively.

The detailed procedure for the determination of herbicide desorption parameters can be found in Dollinger et al. (2016). Briefly, after 24 h of equilibration with a 100 µg/L pesticide solution, the activity in the supernatant was measured and the residual supernatant was removed. An equivalent volume of fresh electrolyte was added, and the tubes were shaken again for 24 h. Five successive desorption steps of 24 h each were then performed. The amount of pesticides sorbed to soils at each step was calculated

by mass balance based on radioactivity counting, and experimental data were fitted to Freundlich isotherms (Eq. 5). The hysteresis between adsorption and the corresponding desorption isotherms was represented by the H parameter (Eq. 3), which was calculated as proposed by Barriuso et al. (1994). Sorption is considered to be hysteretic when H <0.7; the lower the value of H is, the more irreversible the sorption is.

Table 3 **Physico-chemical properties of the studied pesticides.**

Properties		Glyphosate	Diuron
Formula		$C_3H_8NO_5P$	$C_9H_{10}Cl_2N_2O$
Molecular mass	g mol^{-1}	169.1	233.1
Aqueous solubility at 20 °C	g l^{-1}	10.5 to 12.0	0.42
Log Kow at pH 7		− 4.1 to − 3.2	2.7
pKa_1 – pKa_2 – pKa_3		2.2–5.5–10.2	13.2

From ANSES, 2017, FOOTPRINT, 2015, ChemID, 2017 and chemicalize.org 2017.

Results

Morphology of the ditch bed and bank profiles

Based on field morphological descriptions, two different soil profiles were distinguished along the cross-section: (i) the bank profile, which is composed of 4 horizons, and (ii) the ditch bed profile, which is composed of 5 horizons (Table 2 and Figure 2).

The bank soil profile corresponds to the soil pit observed in the vicinity of the ditch by Andrieux et al. (1993). According to the World Reference Base, this soil is a tilled gleyic Cambisol (colluvic, clayic). The structure of the first horizon (Ap, which extends from the surface to a depth of 0.4 m) is affected by tillage and deep ploughing operations. The upper cambic horizons B and Br (described in Table 1) are developed above another deep cambic horizon IIBr (Figure 2A) that feature both a high clay content and high bulk density values. However, more hydromorphic features and denser root systems are observed closer to the ditch bank surface than they are in the bank soil profile, which is located further away. The ditch bed soil profile corresponds to a succession of 3 ditch-specific horizons (H1, H2, and H3) and the Br and IIBr horizons, which are shared with the bank profile. The H1, H2, and H3 horizons are significantly different from the other horizons. H1 and H2, which are enriched in sand and have platy structures, are different from the bank horizon B, which is siltier and is dominated by a subangular blocky structure. The third horizon, H3, is similar to the bank horizon B in terms of texture but features a stratified structure that differs from that of the bank horizons. These differences indicate that the H1, H2, and H3 horizons were formed by the deposition of field-eroded particles during successive flood events subsequent to the creation of the ditch. The contours of the 3 horizons specific to the ditch bed (H1, H2 and H3; Figure 2A) thus likely delimit the section of the original ditch. The shape of the horizons is probably due to the regular management of the ditch, including dredging operations. At the location where the profiles were observed, the original ditch only slightly incises the Br horizon that prevailed prior to the creation of the ditch.

The B and Br horizons have very similar physicochemical properties (Table 2), although horizon B has a slightly greater organic carbon content and a lower bulk density. However, the porosities of horizons B and Br are larger in the vicinity of the ditch surface due to the higher density of the ditch vegetation root system. The upper two ditch bed soil horizons (H1 and H2) contain 1.5 to 2 times more organic carbon than horizon B, which is consistent with the presence of vegetation and higher water contents during the year. Moreover, the bulk densities of the specific ditch bed horizons are significantly lower than those of the other horizons, which is in accordance with their textures, organic matter contents, and dense root channels network. Therefore, the overall porosity of the ditch bed soil profile is higher than that of the bank profile.

In summary, the ditch bed profile and the bank profile have contrasting textural, chemical and structural

properties. Moreover, the vertical gradient of the analysed soil properties across the ditch bed horizons is sharper than that across the bank horizons. Both lateral and vertical gradients of soil properties, such as organic matter content or bulk density, are present within the limited spatial area of one square metre between the ditch and the bank. It is therefore expected that flow and sorption properties differ between the ditch and bank soils.

Heterogeneity and anisotropy of water pathways and associated soil pore structure

The results of the displacement experiments and dye staining of the active macroporosity (ω_{dye}) allowed us to compare the hydraulic conductivity and preferential flow patterns of the different horizons and sampling axes in the two soil profiles (Figure 3, Table 4) and to relate these flow patterns to the macroporosity patterns (Figures 4 and 5). The inverse modelling procedure provides a complementary estimation of the flow mechanisms and soil hydrodynamic properties (Table 4). The main interest of the modelling results is the opportunity to estimate the contribution of the fast flow to the global outflow.

The horizontal and vertical saturated hydraulic conductivity values (K_{sat}) at the column scale calculated from the percolation flux data range from very large ($1.7 \cdot 10^{-4}$ m s^{-1} for H2-V) to rather small ($6.9 \cdot 10^{-6}$ m s^{-1} for Br-H) values (Table 4). Regardless of the horizon, no systematic differences were observed in the measured K_{sat} values between the two sampling axes. With the exception of the second horizon in the ditch bed (H2), the anisotropy of the hydraulic conductivity was small in all samples. Therefore, a mean saturated hydraulic conductivity value was calculated for each horizon, and these values are reported in Table 4. The mean saturated hydraulic conductivity of the B horizon is slightly smaller than those of the H1 and the H2 horizons, despite important differences in their textures, organic matter contents and structures, as observed in Section *Morphology of the ditch bed and bank profiles*. Horizon H2 is the most conductive horizon due to the large value of its observed vertical K_{sat}, which may be caused by specific local macropore features, such as the snail shells observed in this horizon. The Br horizon is 4 to 15 times less conductive than the other horizons. Accordingly, as generally observed in structured soils, both ditch bed and bank soil profiles exhibit decreasing soil hydraulic conductivity with depth (e.g., Sammartino et al., 2015; Udawatta and Anderson, 2008), but the upper horizons of the ditch bed profile have higher permeability values than those of the bank profile.

The dye tracing experiments reveal information about the active macroporosity patterns (Figure 4) and, thus, about the heterogeneity of the soil structures between the horizons. In all columns, the dye percolated across the column demonstrating the presence of connected macroporosity along the height of the column. However, the magnitude of this connected macroporosity varied greatly between the horizons. Roots were found to be the main source of flow paths in most horizons, as most stained areas surrounded living or decayed root channels. However, not all living or decayed root channels were stained. Roots were present throughout the entirety of both profiles, but denser networks were located near the ditch surface. Consistently, on average, the active porosity was largest in the cores sampled in the upper horizons of the ditch bed profile (Figures 2 & 4, Table 4). The B horizon exhibits a large anisotropy in ω_{dye}, yielding a very large value of approximately 20 % in the horizontal direction. This anisotropy may be partly explained by the fact that the sampling location of the horizontal column is almost in the ditch sidewalls and is slightly further away for the vertical column (Figure 2). The H1 and H2 horizons both exhibit a large active porosity, as H2 has the largest ω_{dye} values of all of the horizons. The numerous snail shells, combined with the granular structure present in H2, are likely responsible for its greater active porosity than H1. The active porosity of the Br horizon is significantly smaller than those of the other horizons. Finally, in accordance with the observed variations in saturated hydraulic conductivity, the ditch bed profile exhibits, on average, a larger active porosity than the bank profile. Indeed, although the linear correlation is not statistically significant, K_{sat} generally increases when ω_{dye} increases (Figure 5).

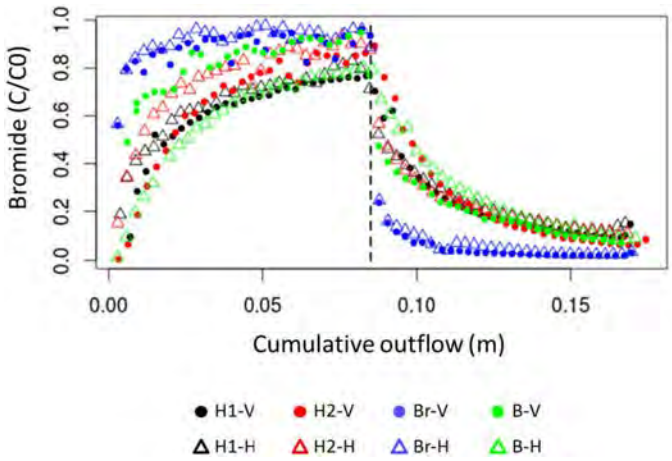


Figure 3. **Bromide breakthrough curves. The black dashed lines represent the shift between contaminated and clear water injection.**

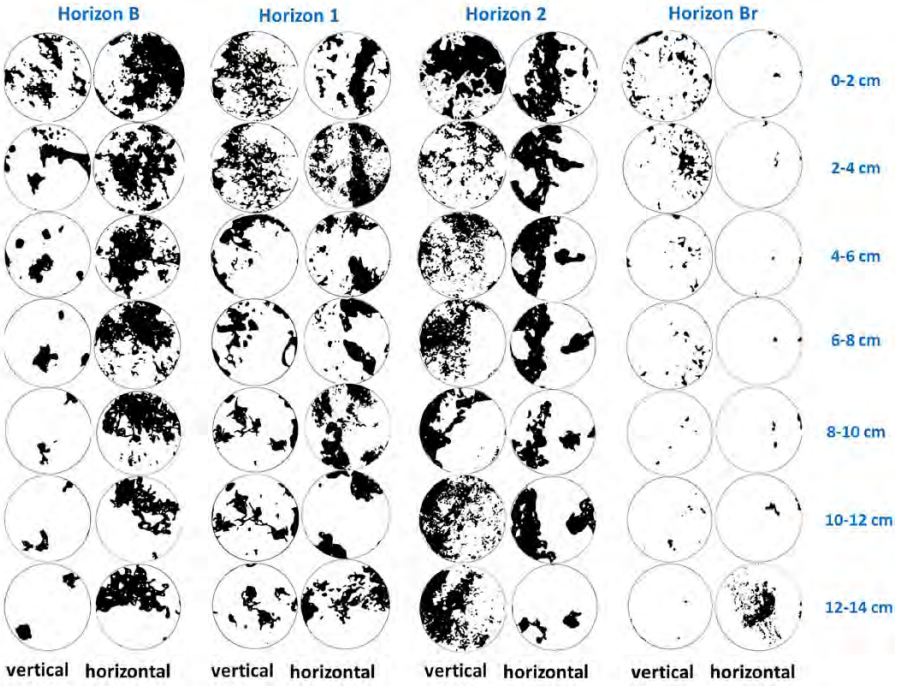


Figure 4. **Imaging of preferential flow patterns within the soil columns. The black areas represent the stained areas at different depths along the soil cores.**

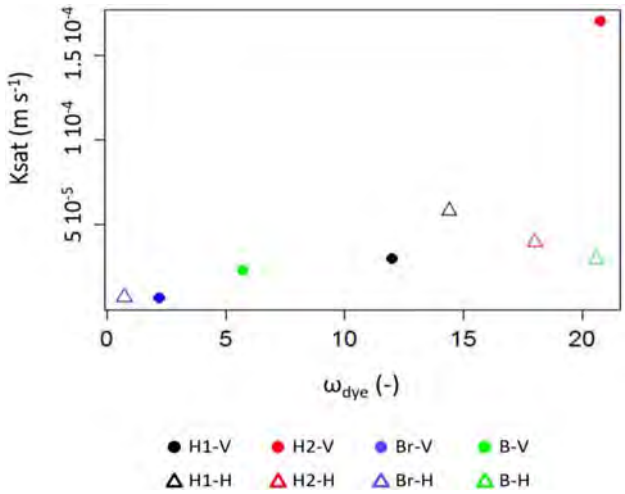


Figure 5. **Evolution of the hydraulic conductivity with the active macroporosity fraction in the set of soil columns.**

Table 4 **Hydrodynamic properties of the ditch-bed and banks soil horizons.**

Columns	[a] K_{sat}	[a] *Mean* K_{sat}*	[a] ω_{dye}	[b] θr_s	[b] θs_s	[b] Ks_s	[b] θs_f	[b] Ks_f	[b] ω	[b] $Disp_s$	[b] $Disp_f$	[b] θs_{Tot}	[a] θs	[b] Relative contribution of preferential flow
	m s^{-1}	m s^{-1}	%	m^3 m^{-3}	m^3 m^{-3}	m s^{-1}	m^3·m^{-3}	m s^{-1}	%	m	m	m^3 m^{-3}	m^3 m^{-3}	%
B-V	$2.34 \cdot 10^{-5}$	$2.68\ 10^{-5}$	5.7 ± 3.5	0.093	0.55	$3.01 \cdot 10^{-5}$	0.150	$3.07 \cdot 10^{-4}$	10.0	0.13	0.5	0.51	0.48	53.1
B-H	$3.02 \cdot 10^{-5}$		20.6 ± 3.5	0.093	0.45	$8.95 \cdot 10^{-6}$	0.492	$7.76 \cdot 10^{-5}$	21.0	0.27	24.25	0.46	0.48	69.7
H1-V	$2.99 \cdot 10^{-5}$	$4.41\ 10^{-5}$	12.0 ± 3.6	0.075	0.41	$1.35 \cdot 10^{-5}$	0.367	$1.13 \cdot 10^{-4}$	17.0	0.1	0.07	0.40	0.52	63.2
H1-H	$5.82 \cdot 10^{-5}$		14.4 ± 2.8	0.075	0.45	$2.26 \cdot 10^{-5}$	0.450	$3.79 \cdot 10^{-4}$	10.0	0.23	30	0.45	0.52	65.1
H2-V	$1.70 \cdot 10^{-4}$	$1.05\ 10^{-4}$	20.8 ± 5.2	0.077	0.55	$4.91 \cdot 10^{-5}$	0.440	$5.98 \cdot 10^{-4}$	22.0	0.1	0.1	0.52	0.52	77.5
H2-H**	$3.99 \cdot 10^{-5}$		18.0 ± 3.5	0.077	0.49	$1.18 \cdot 10^{-5}$	0.430	$3.0 \cdot 10^{-4}$	10.0	0.03	25.84	0.48	0.52	73.3
Br-V	$6.90 \cdot 10^{-6}$	$7.38\ 10^{-6}$	2.2 ± 2.0	0.092	0.40	$1.08 \cdot 10^{-6}$	0.300	$9.80 \cdot 10^{-5}$	6.0	0.1	0.47	0.39	0.44	85.3
Br-H	$7.86 \cdot 10^{-6}$		0.7 ± 1.1	0.092	0.40	$1.08 \cdot 10^{-6}$	0.300	$9.80 \cdot 10^{-5}$	7.0	0.1	0.47	0.39	0.44	87.2

[a] Values obtained from the experimental data.
[b] Values obtained from the inverse modelling of the bromide breakthrough curves.
* Mean K_{sat} is the mean saturated hydraulic conductivity for a given horizon.
** The values of the hydrodynamic parameters obtained from inverse modelling result from adjustments of a dual permeability model except for the column H2-H for which a dual porosity + mobile-immobile model was used. θr_F always equals 0. For H2-H the saturated water content of immobile compartment: $\theta s_{IM} = 0.35$, the Ks_s and Ks_f are the K_{sat} values in the slow and fast porous compartments and the K_{sat} of the immobile compartment is null.

Figure 3 depicts the succession of bromide concentrations measured at the column outlets during displacement experiments. The results are expressed as the ratio of outlet to inlet concentrations. These concentration evolutions could all be satisfactorily simulated using a dual-permeability model except the H2-H for which a dual-porosity model with a mobile-immobile conceptualization of the matrix compartment was needed. For each set of parameters allowing a good reconstitution of the water flow and the bromide leaching pattern (Table 4), the estimated ω were statistically equivalent to the one obtained with dye tracing (linear correlation: slope = 0.97, intercept = 0, $R^2 = 0.92$, p-value = $5 \cdot 10^{-5}$). This highlights the reliability of the simulated parameters, despite the equifinality issue inherent to the large number of fitted parameters.

For all displacement experiments, quantifiable bromide concentrations were measured in the first 50 ml of leachates, which were collected between 15 s and 7 min after injection began. This suggests that preferential flow occurred in all of the columns (e.g., Paradelo et al., 2016), which is consistent with the observation of connected macroporosity in the columns. Additionally, two major shapes of breakthrough curves can be distinguished.

In the columns of horizons B and H, the curve features a gentle increase and decrease in concentration during the injection and rinsing phases, respectively, as well as a maximum concentration that is less than the injected concentration. If it is assumed that macropore flow occurred almost instantaneously at a concentration close to the injected concentration, it follows that, throughout the displacement experiment in these columns, matrix flow was a significant contributor to outflow, as bromide concentration remained below the injected concentration. Although the volume of the injected bromide

solution was chosen to be larger than the overall pore volume of the columns, this volume was likely not sufficient to ensure a renewal of matrix pore water. This hypothesis is confirmed by the modelling results indicating that even if preferential flow contributed up to 77 % to the global outflow for this group of columns, the hydraulic conductivity of the fracture never exceeded 25 time that of the matrix (Table 4).

The other breakthrough curve shape is observed in the columns of the Br horizon and exhibits a sharp increase and early plateau in bromide concentrations during the injection phase, in which the plateau concentration is close to the injected concentration value. Additionally, a sharp decrease in concentration is observed during the rinsing phase. This pattern is not consistent with the small observed macroporosity of the Br horizon but can be explained by the very poor permeability of the soil matrix. In this case, most of the flow bypasses the soil matrix and flows through a few connected macropores. This hypothesis is confirmed by the modelling results indicating that preferential flow contributed to >85 % to the global outflow for this group of columns and that the hydraulic conductivity of the fracture was >90 time higher than that of the matrix (Table 4).

In accordance with recent studies relating soil macroporosity and hydraulic conductivity in structured soils, K_{sat} generally rises along with an ω increase (Figure 5). As ω is related to the root channels density, which decreases with the distance from the ditch surface, the saturated hydraulic conductivity is overall greater in the upper horizons of the ditch bed than in the banks. The contribution of preferential flow to the global outflow, is however greater in the deep bed and bank horizon. This can be explained by the contrasted hydraulic conductivity of the macroporal compartment relative to that of the matrix compartment (Ks_f/Ks_s) and by ω ($R^2 = 0.95$, p-value = $6 \cdot 10^{-4}$).

In sum, it is mainly in their upper horizons that the bank and ditch bed profiles differ in their patterns of water and solute transport. The top horizons of the ditch bed exhibit larger transport properties due to their larger active macroporosities, which are related to their denser rooting patterns. Thus, in contrast with the soil profile from which it originates, the ditch bed profile exhibits larger infiltration and percolation capacities. However, the deeper percolation of water and solutes is limited in both profiles by their common bottom Br horizon, which exhibits low permeability. The differences in the flow patterns may induce significant contrasts in the transfer and retention of herbicides. Indeed, water pathways determine the material surface area that is in contact with the soil solution and its effective contact time during downward seepage. This conditions the herbicide sorption equilibria.

Pesticide sorption heterogeneities among the ditch bed and bank soil profiles

The heterogeneities in herbicide sorption properties among the horizons are presented in Table 5. The H1 and H2 horizons exhibit the greatest diuron adsorption capacities and lowest desorption capacities, whereas adsorption on B and Br is low and very easily reversible. Therefore, the sorption capacities of the ditch bed profile are larger than those of the bank profile. For glyphosate, the adsorption coefficient of the B horizon is higher than that of the H1 horizon but is lower than that of the H2 horizon, and the desorption hysteresis of the B horizon is smaller and larger than those of the H1 and H2 horizons, respectively. The Br horizon exhibits a lower adsorption coefficient than the B horizon, but they both exhibit a similar desorption hysteresis. Based on the properties of these horizons, it remains unclear whether the ditch bed profile or the bank profile has the greater retention capacity.

Table 5 **Sorption coefficients of the herbicides on ditch soils.**

Molecule	Horizon	Kf_{ads}	n_{ads}	Kd_{ads}	Kf_{des}	n_{des}	H
		$\mu g^{(1-n)} l^n kg^{-1}$	–	$l\,kg^{-1}$	$\mu g^{(1-n)} l^n kg^{-1}$	–	–
Diuron	B	4.49	0.84	1.86	110.58	0.12	0.98
	H1	14.79	0.83	5.17	114.66	0.32	0.39
	H2	10.57	0.82	3.52	118.74	0.25	0.31
	Br	4.12	0.81	1.49	114.31	0.09	0.97
Glyphosate	B	157.66	0.94	109.90	666.99	0.28	0.29
	H1	77.60	0.93	51.69	675.64	0.14	0.15
	H2	165.24	0.96	129.86	533.29	0.36	0.37
	Br	124.36	0.91	75.38	505.00	0.29	0.32

In summary, the heterogeneities in the sorption coefficients of the two studied pesticide within a given profile are more substantial under the ditch bed than in the banks. Generally, due to the enrichment in organic matter of the ditch bed horizons, the sorption capacities of hydrophobic molecules in the ditch bed profile should be greater than those in the bank profile. Concerning ionisable compounds with low hydrophobicity, a higher sorption capacity of ditch bed profiles is not straightforward. The desorption hysteresis are generally significant in the ditch bed and are weaker or null in the bank soils. This should lower the release of pesticides previously adsorbed in the ditch bed soils as compared to the bank soils.

Conclusion

This study provides the first description of the range of soil properties influencing the magnitude of the water and pesticide exchanges occurring between surface water and groundwater along a ditch cross section profile. These ditch bed soil properties were also for the first time compared with those of the surrounding field soils. The in-situ and laboratory characterization of the physico-chemical properties evidenced distinct soil profiles between both the ditch bed and banks profiles. The ditch bank profile was equivalent to the surrounding field profile. In particular, the ditch bed upper horizons contain up to 2 times more organic carbon than the bank soils. These upper ditch bed horizons being also located closer to the ditch surface than the bank soils, they contain a denser network of plant roots which increases their active macroporosity and in turn their hydraulic conductivity. The deeper horizons share however, great similarities in both profiles, in particular their poor macroporosity, hydraulic conductivity and organic carbon content.

In conclusion, the physicochemical and sorptive properties of specific ditch bed horizons contrast with those of the ditch banks and neighbouring field soils. These differences may thus have different effects on the risk of groundwater contamination by pesticides. On one hand, ditch beds exhibit higher organic matter contents than field soils, possibly limiting the percolation of hydrophobic pesticides due to increased retention in the soil matrix. On the other hand, the upper horizons of ditch beds present larger active macroporosity and transport property values, which favour percolation. The final balance between the two effects, in terms of overall groundwater contamination risk, depends on the local hydrological conditions.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study reports the properties of a soil from a ditch in an agricultural area in the south of France. Mainly, the hydraulic parameters of the different soil layers of the ditch and the surrounding banks are considered and modelled and tracer experiments with bromide are presented. Sorption experiments with glyphosate were conducted and Freundlich sorption coefficients for the different soil horizons are reported. However, insufficient data to check validity of the experiment are reported (mass balances, chemical properties of test substance, solution agent, analytical information (method,

LOD, LOQ), temperature, considered concentrations, stability of the test item). No actually measured concentrations in the field are reported.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Dollinger, J. et al.
Report year	2015
Report title	Glyphosate sorption to soils and sediments predicted by pedotransfer functions
Document No	Environ Chem Lett (2015) 13:293-307
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Reliable with restrictions (no new experimental data is presented)

2. Full summary of the study according to OECD format

Glyphosate is the most applied herbicide for weed control in agriculture worldwide. Excessive application of glyphosate induces water pollution. The transfer of glyphosate to freshwater and groundwater is largely controlled by glyphosate sorption to soils and sediments. Sorption coefficients are therefore the most sensitive parameters in models used for risk assessment. However, the variations in glyphosate sorption among soils and sediments are poorly understood. Here we review glyphosate sorption parameters and their variation with selected soils and sediment. We use this knowledge to build pedotransfer functions that allow predicting sorption parameters, Kd, Kf and n, for a wide range of soils and sediments. We gathered glyphosate sorption parameters, 101 Kf, n and equivalent Kd, and associated soil properties. These data were then used to perform stepwise multiple regression analyses to build the pedotransfer functions. The linear (Kd) and Freundlich (Kf, n) pedotransfer functions were bench marked against experimental data. We found the following major points: (1). Under current environmental conditions, sorption is best predicted by the Kd pedotransfer function. (2) The pedotransfer function is Kd = 7.20*CEC – 1.31 *Clay + 24.82 (Kd in L/kg, CEC in cmol/kg and clay in %). (3) Cation exchange capacity (CEC) and clay content are the main drivers of Kd variability across soils and sediments. Freundlich parameters are additionally influenced by pH and organic carbon. This suggests that the formation of complexes between glyphosate phosphonate groups and soil–exchanged polyvalent cations dominates sorption across the range of analyzed soils.

Materials and Methods

Physical and chemical properties of glyphosate

Glyphosate [N–(Phosphomethyl)glycine] is a weak acid with strong hydrophilicity and very high water solubility (Table 1). Speciation of this zwitterionic molecule varies with the pH of the surrounding environment (Figure 1). The main species within the soil pH range are GH_2^- and GH^{2-}, corresponding to net negative charges of one and two, respectively (Figure 1).

Table 1 Physicochemical properties of glyphosate

Properties		References
Formula	$C_3H_8NO_5P$	ANSES (2015), FOOTPRINT (2015)
Molecular mass (g mol^{-1})	169.1	ANSES (2015), FOOTPRINT (2015)
Aqueous solubility at 20 °C (g L^{-1})	10.5 to 12.0	ANSES (2015), FOOTPRINT (2015)
Log Kow at pH 7	−4.1 to −3.2	ANSES (2015), FOOTPRINT (2015)
pKa_1–pKa_2–pKa_3	2.2–5.5–10.2	ANSES (2015), FOOTPRINT (2015)
Vapor pressure at 25 °C (mPa)	1.31×10^{-2}	ANSES (2015), FOOTPRINT (2015)
Henry's law constant at 25 °C (Pa m^3 mol^{-1})	2.10×10^{-7}	ANSES (2015), FOOTPRINT (2015)

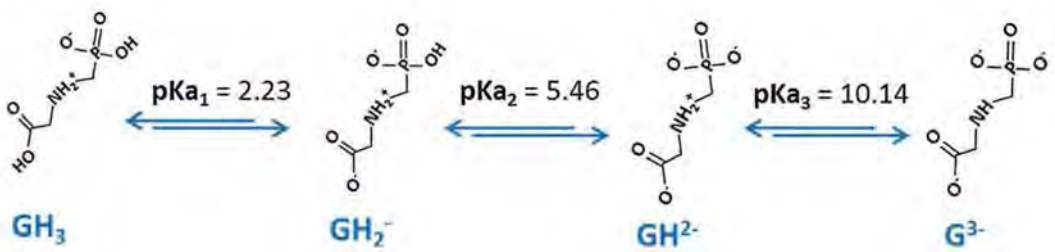


Figure 1 Speciation of glyphosate through the entire soil pH range from Albers et al. (2009), Borggaard (2011) and Maqueda et al. (1998)

Data mining

We extensively reviewed the literature to assemble a database of observed glyphosate sorption coefficients to both soils and sediments and the associated substrate properties (Table 2). We found 23 studies reporting sorption parameters for one or more soils or sediments. The soils or sediments for which glyphosate sorption measurements were carried out originated from four continents (Europe, Asia and North and South America) and exhibited highly varied texture and properties. The experimental conditions varied greatly. For example, the initial concentrations in the liquid phase ranged from 0.01 to more than 1000 mg/L. Only coefficients of sorption to unmodified soils or sediments were included in the database. Measured coefficients of sorption to organic soils were included in the database, but only those measured for sorption to mineral soils, i.e., with an organic matter content lower than 20 % (IUSS 2014) were used for the statistical analyses. Several studies have reported that sorption coefficients depend strongly on the background electrolyte. Therefore, only sorption coefficients obtained with classical background electrolyte, either Milli–Q water or $CaCl_2$, were included in the database. Among the 101 sorption parameters registered in the database (Table 2), 69 were measured with $CaCl_2$, as the background electrolyte. Statistical analyses were only performed for sorption parameters measured with $CaCl_2$ (designated as "sample A").

Sorption isotherms

For sample A, approximately two–thirds of the sorption models were nonlinear Freundlich (Table 3). To establish a pedotransfer function for Kd, we approximated equivalent Kd values by linearizing the Freundlich models over the actual range of the initial aqueous concentrations of the batch experiment used for model fitting (Table 3). The relative difference between Kf and its equivalent, Kd (Kd_{eq}), was approximately 30 % on average.

Statistical analyses

Pedotransfer functions aim to predict the sorption parameters Kd, Kf and n from selected substrate properties. Some of the properties, especially CEC, iron– and aluminum oxides or phosphorus content, were not available for all soils or sediments (Table 2). This lack of data induced a subsampling of sample A for the establishment of pedotransfer functions for the Kd and Kf parameters. This sample is designated as "sample B". The sample used for the establishment of the pedotransfer function for the n parameter excluded sorption studies that investigated only one concentration and, thereby, did not consider the possibility that n differs from 1. This sample is designated as “sample C”.

The statistical analyses were performed using the R statistical computing software. Correlation analyses were performed using the default "1 m" function of the R software. The three pedotransfer functions for the estimation of linear and nonlinear sorption models were established by forward and backward stepwise multiple regression analyses of the substrate properties and the Kd_{eq}, Kf and n parameters. The stepwise multiple regression analyses were performed using the default "step" function of the R software.

The validity of the Kd pedotransfer function is strongly supported by the fact that sorption processes do not depend on the pesticide concentration. However, in sample A, the n values ranged from 0.48 to 1.05, with a mean value of 0.83, indicating saturation of the sorption sites at high glyphosate concentrations. A complementary multiple regression between n, the substrate properties and the experimental conditions (C_{max} and *R)* was performed. The resulting equation (see Eq. 4) indicates the linearity range under various conditions.

Finally, we evaluated the accuracy of the predicted equilibrium partitioning of glyphosate between the soil and water by using the sorption parameters provided by the Kd or Kf/n pedotransfer functions. The evaluation was performed for 11 initial concentrations (0.01 , 0.04, 0. 10, 0.40, 1,4, 10, 40, 100, 400 and 1000 mg/L) in the liquid phase by comparing the predicted soil–to–water glyphosate concentration ratios, as obtained by the pedotransfer–estimated sorption parameters, to those obtained by the batch–fitted sorption parameters. The aqueous and soil concentrations were calculated for all sample B soils and sediments using the numerical solver as described previously.

Table 2 Glyphosate sorption parameters and associated soil or sediment properties' database

Substrate type and origin	Texture	pH	CEC	Organic carbon	Clay	Phosphorus	Fe_{ox} and Al_{ox}	Kf	n	Aqueous concentration min–max	Solid/ liquid ratio	Aqueous phase	References
	Sand-silt-clay (%)		cmol kg^{-1}	%	%	mg kg^{-1}	g kg^{-1}	L kg^{-1} n^{-1}		mg L^{-1}	g mL^{-1}		
Soil (Italy)	63.4:22.6:14.0	8.11[a]	na	0.70	14.00	na	na	43.01	0.79	0.20–120.00	1/5	$CaCl_2$	Accinelli et al. (2005)
Soil (USA)	93.5:2.7:3.8	7.20 [a]	na	0.94	3.80	na	na	62.16	0.88	0.20–120.00	1/5	$CaCl_2$	Accinelli et al. (2005)
Soil (France)	Sandy loam	5.10 [a]	na	0.82	10.50	1240.00	298.73	34.50	0.97	0.73–60.13	1/5	$CaCl_2$	Al-Rajab et al. (2008)
Soil (France)	Silt clay loam	6.30 [a]	na	1.45	30.60	3240.00	48.57	33.60	1.00	0.73–60.13	1/5	$CaCl_2$	Al-Rajab et al. (2008)
Soil (France)	Clay loam	7.90 [a]	na	1.91	34.90	2740.00	35.67	16.60	1.00	0.73–60.13	1/5	$CaCl_2$	Al-Rajab et al. (2008)
Soil (Finland)	Sandy loam	6.10[b]	na	0.47	17.00	1.60	na	166.00	0.97	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Clay	5.80[b]	na	2.88	46.00	3.00	na	55.00	0.92	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Clay	5.60[b]	na	0.54	58.00	1.10	na	249.00	0.91	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Sandy loam	5.80[b]	na	2.57	13.00	27.40	na	44.00	0.90	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Sandy loam	5.70[b]	na	0.72	4.00	17.10	na	55.00	1.00	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Clay	6.00[b]	na	7.06	41.00	4.10	9.66	97.00	1.03	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Clay	6.00[b]	na	2.96	47.00	1.50	11.22	41.00	1.02	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Sandy loam	6.40[b]	na	5.93	4.00	3.30	9.26	97.00	0.85	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Sandy loam	5.90[b]	na	1.77	4.00	0.90	6.70	51.00	0.86	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Clay	8.10[b]	na	2.67	41.00	38.70	na	58.00	0.93	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Clay	7.90[b]	na	2.50	30.00	22.40	na	113.00	0.87	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Sandy loam	7.10[b]	na	2.35	21.00	8.80	na	93.00	0.90	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Sandy loam	6.80[b]	na	0.75	8.00	2.80	na	90.00	0.86	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Clay	6.00[b]	na	7.05	41.00	5.80	na	179.00	1.26	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Sandy loam	6.30[b]	na	5.93	4.00	6.40	na	121.00	0.98	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Silty loam	5.40[b]	na	7.90	5.00	10.00	4.48	159.00	0.93	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Silty loam	5.60[b]	na	4.50	4.00	3.60	4.29	102.00	1.05	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Silty loam	5.40[b]	na	1.30	8.00	3.50	4.57	37.00	0.76	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Muddy clay	6.90[b]	na	12.60	57.00	52.00	na	84.00	0.91	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Soil (Finland)	Organic soil	5.20[b]	na	26.00	79.00	5.10	17.09	303.00	1.11	2.00–10.00	1/5	H_2O	Autio et al. (2004)
Sediment (France)	35.9:39.1:25.0	8.44[a]	12.50	1.58	25.00	19.00	2.78	51.69	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Sediment (France)	40.3:33.1:26.6	8.54[a]	12.10	1.56	26.60	6.00	2.62	129.86	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Sediment (France)	4.7:58.5:36.8	8.63[a]	14.40	0.73	36.80	5.00	3.27	75.38	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Sediment (France)	7.7:57.2:35.1	8.71[a]	14.20	0.96	35.10	5.00	3.48	109.90	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Sediment (France)	9.7:54.6:35.7	7.30[a]	23.20	0.54	35.70	11.00		302.63	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Sediment (France)	25.5:42.0:32.5	7.93[a]	26.10	3.88	32.50	73.00	na	262.41	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Sediment (France)	77.9:13.9:8.2	6.03[a]	6.34	0.47	8.20	142.00	na	89.07	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Sediment (France)	7.8:62.6:29.6	6.19[a]	17.60	3.67	29.60	144.00	na	318.82	1.00	0.005–1.00	1/10	$CaCl_2$	Bailly et al. (2015)
Soil (Sweden)	7.3:46.2:46.5	7.20[b]	28.40	4.00	46.50	na	na	118.00	0.95	0.01–0.10	1/10	$CaCl_2$	Bergström et al. (2011)
Soil (Sweden)	3.3:40.6:56.1	7.40[a]	33.60	0.00	56.10	na	na	165.00	1.03	0.01–0.10	1/10	$CaCl_2$	Bergström et al. (2011)

Table 2 continued

Substrate type and origin	Texture	pH	CEC	Organic carbon	Clay	Phosphorus	Fe_{ox} and Al_{ox}	Kf	n	Aqueous concentration min–max	Solid/liquid ratio	Aqueous phase	References
	Sand-silt-clay (%)		cmol kg^{-1}	%	%	mg kg^{-1}	g kg^{-1}	L kg^{-1} n^{-1}		mg L^{-1}	g mL^{-1}		
Soil (Sweden)	87.8:4.5:7.7	7.40[a]	4.70	2.00	7.70	na	na	40.00	0.92	0.01–0.10	1/10	$CaCl_2$	Bergström et al. (2011)
Soil (Sweden)	95.4:4.6:0.0	6.40[a]	1.80	1.00	0.00	na	na	28.70	0.82	0.01–0.10	1/10	$CaCl_2$	Bergström et al. (2011)
Soil (Spain)	82.0:11.0:7.0	7.90[b]	5.20[c]	1.00	7.00	na	36.70	93.00	0.78	0.10–5.00	2/5	H_2O	Candela et al. (2007)
Soil (Spain)	59.0:13.0:10.0	7.30[a]	4.60[c]	0.00	10.00	na	126.50	154.00	0.74	0.10–5.00	2/5	H_2O	Candela et al. (2007)
Soil (Malaysia)	Sandy loam	6.70	7.10[c]	1.30	10.00	na	na	83.80	0.85	0.10–5.00	1/5	$CaCl_2$	Cheah et al. (1997)
Soil (Malaysia)	Muck	4.70	54.10[c]	30.50	32.50	na	na	417.00	0.78	0.10–5.00	1/5	$CaCl_2$	Cheah et al. (1997)
Soil (France)	10.0:44.0:46.0	7.60[b]		3.90	46.00	na	4.40	33.90	1.00	0.10–5.00	1/5	H_2O	Dousset et al. (2007)
Soil (France)	53.0:25.0:22.0	6.60[a]		1.50	22.00	na	5.10	51.80	1.00	0.10–5.00	1/5	H_2O	Dousset et al. (2007)
Soil (Denmark)	Sandy	7.67	12.60	1.70	15.00	na	na	30.90	0.78	0.03–67.00	1/20	H_2O	de Jonge and Wollesen de Jonge (1999)
Soil (Denmark)	Sandy	6.13	12.60	1.70	15.50	na	na	78.50	0.75	0.03–67.00	1/20	$CaCl_2$	de Jonge and Wollesen de Jonge (1999)
Soil (Denmark)	Sandy	6.30	12.60	1.70	15.50	na	na	78.40	0.75	0.03–67.00	1/20	$CaCl_2$	de Jonge and Wollesen de Jonge (1999)
Soil (Denmark)	Sandy	6.53	12.60	1.70	15.50	na	na	48.40	0.77	0.03–67.00	1/20	$CaCl_2$	de Jonge and Wollesen de Jonge (1999)
Soil (Chili)	Clay loam	4.79[a]		6.60	18.00	na	na	12.13	1.00	1000.00–2500.00	1/10	H_2O	Kogan et al. (2003)
Soil (Finland)	Clay	6.02[a]		7.10	41.00	4.20	9.66	98.69	1.02	2.00–10.00	1/5	H_2O	Laitinen et al. (2008)
Sediment (Germany)	Sandy	7.7[c]		0.34	0.00	0.00	0.61	1.89	0.48	0.10–100.00	1/2	$CaCl_2$	Litz et al. (2011)
Soil (France)	Rendzina over limestone	8.4[a]	6.40	1.86	8.80	3590.00	2.51	17.60	0.76	0.20–10.00	1/5	H_2O	Mamy and Barriuso (2005, 2007)
Soil (France)	Rendzina over limestone	8.2[a]	7.10	1.86	9.30	2720.00	2.58	34.80	0.80	0.20–10.00	1/5	$CaCl_2$	Mamy and Barriuso (2005, 2007)
Soil (France)	Clay loam	8.36[a]	17.80	2.00	37.60	2490.00	15.20	32.90	0.86	0.20–10.00	1/5	H_2O	Mamy and Barriuso (2005, 2007)
Soil (France)	Clay loam	8.2[a]	20.60	1.69	37.70	2760.00	14.96	41.90	0.80	0.20–10.00	1/5	$CaCl_2$	Mamy and Barriuso (2005, 2007)
Soil (France)	Clay loam	6.3[a]	16.40	1.01	27.40	1460.00	8.58	60.50	0.88	0.20–10.00	1/5	H_2O	Mamy and Barriuso (2005, 2007)
Soil (France)	Clay loam	7.6[a]	15.90	0.96	23.50	1310.00	7.00	276.00	0.77	0.20–10.00	1/5	$CaCl_2$	Mamy and Barriuso (2005, 2007)
Soil (Greece)	13.4:64.1:22.6	8.0[a]	28.30[c]	0.00	22.60	na	3.40	13.79	0.77	50.00–300.00	1/25	$CaCl_2$	Piccolo et al. (1994)
Soil (UK)	46.3:36.8:17.0	5.8[a]	18.30[c]	3.73	17.00	na	63.10	40.64	0.77	50.00–300.00	1/25	$CaCl_2$	Piccolo et al. (1994)
Soil (Germany)	81.5:12.6:6.0	4.6[a]	30.70[c]	9.23	6.00	na	14.80	51.14	0.58	50.00–300.00	1/25	$CaCl_2$	Piccolo et al. (1994)
Soil (France)	1.7:82.4:16.0	8.3[a]	11.40[c]	0.45	16.00	na	22.80	152.85	0.44	50.00–300.00	1/25	$CaCl_2$	Piccolo et al. (1994)
Soil (Brazil)	46.0:7.0:47.0	5.2[a]	14.18	3.20	47.00	89.00	222.80	162.90	0.98	0.42–6.72	1/5	$CaCl_2$	Prata et al. (2003)
Soil (Brazil)	38.0:60.0:56.0	5.0[a]	12.32	2.50	56.00	59.00	277.10	215.70	0.99	0.42–6.72	1/5	$CaCl_2$	Prata et al. (2003)

Substrate type and origin	Texture	pH	CEC	Organic carbon	Clay	Phosphorus	Fe_{ox} and Al_{ox}	Kf	n	Aqueous concentration min–max	Solid/liquid ratio	Aqueous phase	References
	Sand-silt-clay (%)		cmol kg^{-1}	%	%	mg kg^{-1}	g kg^{-1}	L kg^{-1} n^{-1}		mg L^{-1}	g mL^{-1}		
Soil (Denmark)	Sandy	5.3[a]	na	na	1.64	na	2.21	403.50	1.00	0.30	1/5	$CaCl_2$	Strange-Hansen et al. (2004)
Soil (China)	–	7.20[a]	23.60	4.57	34.30	na	209.50	31.19	0.82	21.00–169.00	1/25	H_2O	Wang et al. (2006)
wetland Sediment (Canada)	Sandy	6.70[a]	13.30	3.20	9.10	na	na	172.90	1.00	1.00	1/10	$CaCl_2$	Xu et al. (2009)
Wetland sediment (Canada)	Sandy	6.40[a]	16.80	3.80	11.30	na	na	152.60	1.00	1.00	1/10	$CaCl_2$	Xu et al. (2009)
Wetland sediment (Canada)	Sandy	6.40[a]	24.80	6.80	4.90	na	na	251.90	1.00	1.00	1/10	$CaCl_2$	Xu et al. (2009)
Wetland sediment (Canada)	Sandy	7.20[a]	33.10	8.70	5.60	na	na	193.10	1.00	1.00	1/10	$CaCl_2$	Xu et al. (2009)
Wetland sediment (Canada)	Sandy	7.10[a]	31.90	9.60	6.70	na	na	124.90	1.00	1.00	1/10	$CaCl_2$	Xu et al. (2009)
Soil (Denmark)	Sandy	3.70[b]	na	1.32	4.20	50.55	3.21	107.40	0.62	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	3.60[b]	na	1.06	4.20	63.49	2.47	80.60	0.62	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	3.60[b]	na	1.28	4.20	87.39	2.98	83.50	0.66	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	3.80[b]	na	1.06	4.20	72.15	2.53	79.40	0.68	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.20[b]	na	1.20	4.20	18.32	2.73	121.20	0.60	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.30[b]	na	1.33	4.20	27.70	3.36	141.20	0.63	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.20[b]	na	1.30	4.20	44.58	3.43	118.10	0.67	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.30[b]	na	1.40	4.20	44.50	3.24	111.50	0.62	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.50[b]	na	1.26	4.20	12.07	3.23	126.80	0.56	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.70[b]	na	1.26	4.20	18.56	2.95	120.00	0.58	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.60[b]	na	1.21	4.20	36.31	2.76	92.00	0.59	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	4.90[b]	na	1.36	4.20	32.08	3.27	116.20	0.61	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	5.20[b]	na	1.33	4.20	9.08	3.46	154.00	0.62	0.04—65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	5.50[b]	na	1.14	4.20	13.07	3.44	138.80	0.65	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	5.40[b]	na	1.29	4.20	24.28	3.65	136.60	0.63	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy	5.50[b]	na	1.20	4.20	25.57	3.32	119.80	0.63	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy loam	6.20[b]	na	1.28	10.80	6.19	6.45	214.70	0.56	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy loam	6.20[b]	na	1.26	10.80	13.26	5.26	165.10	0.60	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy loam	6.30[b]	na	1.25	10.80	24.52	5.35	137.60	0.67	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy loam	6.40[b]	na	1.23	10.80	58.74	5.71	106.40	0.71	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy loam	6.30[b]	na	1.21	10.80	8.75	5.28	171.70	0.58	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy loam	6.30[b]	na	1.40	10.80	15.56	5.04	144.00	0.59	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)
Soil (Denmark)	Sandy loam	6.30[b]	na	1.44	10.80	24.79	4.94	151.30	0.65	0.04–65.00	1/18	$CaCl_2$	de Jonge et al. (2001)

Table 2 continued

Substrate type and origin	Texture Sand-silt-clay (%)	pH	CEC cmol kg^{-1}	Organic carbon %	Clay %	Phosphorus mg kg^{-1}	Fe_{ox} and Al_{ox} g kg^{-1}	Kf L kg^{-1} n^{-1}	n	Aqueous concentration min–max mg L^{-1}	Solid/liquid ratio g mL^{-1}	Aqueous phase	References
Soil (Brazil)	81.7:2.0:16.3	4.00[a]	2.00	0.83	16.30	28.77	212.00	31.32	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	81.7:2.0:16.3	5.00[a]	2.00	0.83	16.30	28.77	212.00	17.89	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	81.7:2.0:16.3	6.00[a]	2.00	0.83	16.30	28.77	212.00	19.59	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	81.7:2.0:16.3	7.00[a]	2.00	0.83	16.30	28.77	212.00	15.52	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	27.7:42.0:30.3	4.00[a]	4.00	0.38	30.30	3.12	289.40	0.87	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	27.7:42.0:30.3	5.00[a]	4.00	0.38	30.30	3.12	289.40	0.83	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	27.7:42.0:30.3	6.00[a]	4.00	0.38	30.30	3.12	289.40	1.37	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	27.7:42.0:30.3	7.00[a]	4.00	0.38	30.30	3.12	289.40	0.90	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	19.7:26.0:54.3	4.00[a]	11.00	2.56	54.30	16.93	236.70	4.45	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	19.7:26.0:54.3	5.00[a]	11.00	2.56	54.30	16.93	236.70	3.44	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	19.7:26.0:54.3	6.00[a]	11.00	2.56	54.30	16.93	236.70	2.51	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)
Soil (Brazil)	19.7:26.0:54.3	7.00[a]	11.00	2.56	54.30	16.93	236.70	2.01	1.00	3405.00	2/5	$CaCl_2$	da Cruz et al. (2007)

Note that sorption parameters included in the database were measured on unmodified soil or sediment and with background electrolyte being either water (H_2O) or calcium chloride ($CaCl_2$). Some parameters were not included in the database because of unit incoherencies (e.g., Jacobsen et al. 2008)

na not available

[a] pH_{H2O}

[b] pH_{CaCl2}

[c] CEC meq/100 g

Table 3 Statistical characteristics of the database subsamples

	Sample A					Sample B					Sample C				
	Nobs.	Mean	Median	Min	Max	Nobs.	Mean	Median	Min	Max	Nobs.	Mean	Median	Min	Max
Kf (L kg^{-1} n^{-1})	69	108.16	111.50	0.83	403.50	36	96.32	63.00	0.83	297.02	51	118.89	118.10	1.89	297.02
n	69	0.83	0.88	0.48	1.05	36	0.94	1.00	0.75	1.05	51	0.77	0.75	0.48	1.05
Kd_{eq} (L kg^{-1})	69	73.96	38.89	0.06	403.50	36	87.02	39.05	0.83	318.82	51	72.61	44.78	0.06	318.82
pH	69	6.10	6.30	3.60	8.71	36	6.68	6.85	4.00	8.71	51	6.20	6.30	3.60	8.71
OC (%)	69	1.79	1.29	0.00	9.60	36	2.62	1.69	0.00	9.60	51	1.50	1.29	0.00	6.44
CEC (cmol kg^{-1})	36	13.25	12.55	1.80	33.60	36	13.25	12.55	1.80	33.60	19	15.21	12.60	1.80	33.60
Clay (%)	69	18.32	10.80	0.00	56.10	36	25.36	24.25	0.00	56.10	51	16.11	10.80	0.00	56.10
P_{OLSEN} (mg kg^{-1})	52	297.88	25.18	0.00	3240.00	26	321.32	16.93	3.12	2760.00	40	382.36	29.89	0.00	3240.00
Fe_{ox}–Al_{ox} (g kg−1)	49	80.87	5.04	0.61	298.73	19	157.32	212.00	2.58	289.40	36	28.00	3.44	0.61	298.73

Sample A all data with $CaCl_2$ as the background electrolyte. *Sample B* data used for the calibration of Kd and Kf pedotransfer functions. *Sample C* data used for the calibration of the **n** pedotransfer function

OC organic carbon, *CEC* cation exchange capacity, Fe_{ox}–Al_{ox} iron- and aluminum oxides, *Nobs.* number of observations

Results and Discussion

Database and sample characteristics

The soils and sediments used in the glyphosate sorption measurements displayed great variability in their origins and properties. This variability was preserved in the subsampling of the database for pedotransfer function calibration, as seen in Table 3. Indeed, the three subsamples of the database displayed similar distributions of properties and parameters values. The 0.01–1000 mg/L concentration range (Table 4) was also preserved by the subsampling of the database. This range covers all possible environmental glyphosate concentrations from concentrations found during spraying to those found in runoff and groundwater. It is interesting to note that the data presented in Table 5 exhibited highly significant correlations between some basic soil properties: The CEC was correlated with organic carbon or iron– and aluminum oxides, and the clay content was correlated with iron– and aluminum oxides. In contrast, there was no correlation between clay and CEC, suggesting a large influence of the within–sample variation in clay mineralogy (Table 5).

Table 4 Initial aqueous concentration used for the linear approximation if Freundlich isotherms

	Initial aqueous concentrations (mg L^{-1})	Frequency of use in experimental design (%)
Class 1	0.01–0.02–0.04–0.06–0.08	56
Class 2	0.10–0.20–0.40–0.60–0.80	59
Class 3	1.00–2.00–4.00–6.00–8.00	55
Class 4	10.00–20.00–40.00–60.00–80.00	32
Class 5	100.00–200.00–400.00–600.00–800.00–1000.00	12

Table 5 The Pearson correlation coefficients matrix among soil properties

Parameters	CEC (cmol kg^{-1})	OC (%)	Clay (%)	Phosphorus (mg kg^{-1})	Fe_{ox} and Al_{ox} (g kg^{-1})
pH	0.407*	NS	0.250*	0.339*	NS
	(36)	(69)	(69)	(52)	(49)
CEC	–	0.666***	NS	NS	−0.691**
		(36)	(36)	(26)	(19)
OC	–	–	NS	NS	NS
			(69)	(52)	(49)
Clay	–	–	–	NS	0.631***
				(52)	(49)
Phosphorus	–	–	–	–	NS
					(49)

The number in brackets corresponds to the number of observations for the given correlation. "***", "**" and "*" represent correlation significance levels of 0.001, 0.01 and 0.05, respectively

NS correlation is not significant, *OC* organic carbon, *CEC* cation exchange capacity, Fe_{ox}–Al_{ox} iron- and aluminum oxides

Table 6 The Pearson correlation coefficients matrix between sorption parameters and soil properties or experimental conditions

Parameters	pH	CEC (cmol kg^{-1})	OC (%)	Clay (%)	Phosphorus (mg kg^{-1})	Fe_{ox} and Al_{ox} (g kg^{-1})	C_{max} (mg L^{-1})	log(R)
Kd_{eq}	NS	0.659***	0.380**	NS	NS	NS	−0.364**	NS
	(69)	(36)	(69)	(69)	(52)	(49)	(69)	(69)
Kf	NS	0.659***	0.255*	NS	NS	−0.527***	−0.551***	−0.432***
	(69)	(36)	(69)	(69)	(52)	(49)	(69)	(69)
n	0.531***	NS	0.351*	0.760***	0.361*	0.560***	−0.666***	0.489***
	(51)	(19)	(51)	(51)	(40)	(36)	(51)	(51)

The number in brackets corresponds to the number of observations for the given correlation. "***", "**", and "*", represent correlation significance levels of 0.001, 0.01 and 0.05, respectively

NS correlation is not significant, *OC* organic carbon, *CEC* cation exchange capacity, Fe_{ox}–Al_{ox} iron- and aluminum oxides, C_{max} maximal initial concentration (mg L^{-1}), *log(R)* log-transformed solid–liquid ratio (g mL^{-1})

Table 7 Pedotransfer function for the estimation of linear (Kd) and Freundlich (Kf–n) sorption isotherms

Pedotransfer function	Sample	Soil parameters	Equation	R^2	RMSEP
Kd	B	CEC, Clay	Kd = 24.821 + 7.199 * CEC − 1.307 * Clay	0.48***	7.59 (8.7 %)[a]
Kf	B	CEC, Clay, OC	Kf = 50.904 + 9.246 * CEC − 1.985*Clay − 11.811 * OC	0.52***	16.33 (16.9 %)[b]
n	C	Clay, pH	*n* = 0.505 + 0.007 * Clay + 0.024 * pH	0.62***	0.006 (0.8 %)[c]

OC organic carbon (%), *CEC* cation exchange capacity (cmol kg^{-1}), clay (%)

*** Correlations are significant at the level 0.001

[a] RMSEP expressed as a percentage of the mean Kd value (87.02 L kg^{-1})

[b] RMSEP expressed as a percentage of the mean Kf value (96.32 L kg^{-1} n^{-1})

[c] RMSEP expressed as a percentage of the mean n value (0.77)

Glyphosate sorption: mechanisms and prediction

The Pearson correlation coefficients (Table 6) showed that the Kd, q and Kf values are primarily correlated with CEC and, secondarily, with organic carbon content and Fe_{ox}–Al_{ox} content. They also show that n exhibits significant correlation with all of the selected soil properties, with the exception of CEC. The multiple regression analysis (Table 7) provided pedotransfer functions that accurately fit the observed Kd_{eq}, Kf and n values. The functions account for 48–62 % of the variation in the sorption parameters. Visual inspection of the disparity between the measured and predicted Kd_{eq}, Kf and n sorption parameters did not reveal systematic departures from the regression, except for one outlier corresponding to high Kd_{eq} and Kf values measured on a sediment containing a particularly high organic carbon content (Figure 2). The multiple regression analyses high–lighted the points that CEC is the main predictor of Kd_{eq} and Kf variation and that clay is a useful predictor. Furthermore, we found that organic carbon was a predictor for Kf only. The analyses also revealed that clay and pH are significant predictors of *n*. These results suggest that the formation of complexes between the glyphosate phosphonate groups and the soil exchanged polyvalent cations is the dominating sorption mechanism across the entire range of analyzed soils. This is indicated by the primary role of CEC in controlling Kd_{eq} and Kf variability. Given the high correlation between CEC and Fe_{ox}–Al_{ox} in our sample, it is likely that the influence of the latter property was masked by that of the former.

Additionally, we found that clay content explained only approximately 5 % of the Kd_{eq} and Kf variability (Tables 6, 7). Significant correlations were found between organic carbon and Kd_{eq} or Kf (Table 6), although organic carbon only slightly increased the R^2 value obtained in the multiple regression analyses of Kf. Organic carbon appeared to be strongly correlated with CEC, indicating the significant contribution of organic matter to CEC; this correlation may explain the correlation of organic carbon with the sorption parameters. There is a general consensus that a rise in pH negatively affects the sorption of glyphosate. However, the multiple regression analyses did not detect any influence of pH on Kd_{eq}, and Kf variability.

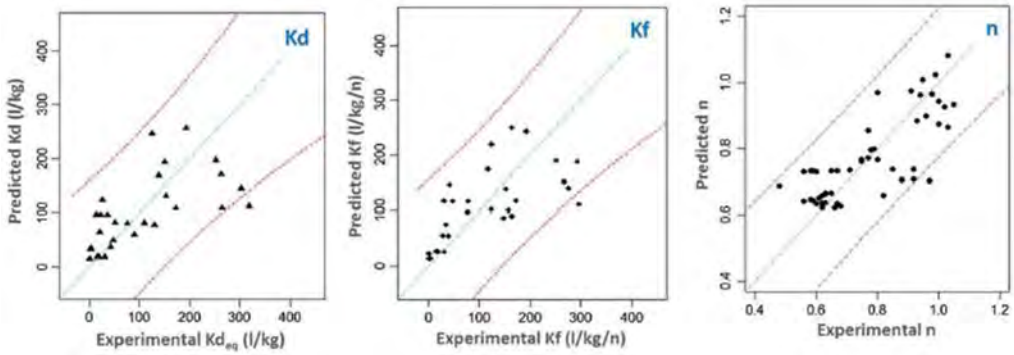


Figure 2 Multiple regression analysis of the sorption coefficients (Kd, Kf, *n)* and soil properties. The sorption coefficients predicted from the pedotransfer functions presented in Table 7 were plotted against the sorption coefficienls (Kf, *n*) fitted from the experimental data and for Kd_{eq}, against the linearized sorption coefficients.

Here, pH and clay explained most of the n parameter variability (Tables 6 and 7). The positive correlation of n with pH may be related to the increased negative charges for both glyphosate (Figure 1) and the soil, favoring the formation of complexes with soil–exchanged polyvalent cations. Despite the increasing electrostatic repulsion, a rise in pH appears to reduce the potential saturation of sorption sites for high initial concentrations by favoring cation bridging between glyphosate and the soil. The variability of the sorption parameters that is not predicted by the multiple regressions may be largely attributed to the varying experimental conditions among the studies measuring glyphosate sorption to soils and sediments (Table 2). If different parameters are considered to be possible predictors in the multiple regressions, they enable a fit to a regression function (Eq. 4) with a better performance (R^2 = 0.69) than that of the regression using only basic soil properties as predictors for *n*.

$$n = 0.920 - 0.028 \times \log(C_{max}\ (mg/L)) + 0.064 \times \log (R(g\ m/L)) + 0.005 \times clay\ (\%) \qquad (4)$$

A small R implies a limited amount of sorption sites.

Correlations between Kf, Kd_{eq} and the solid–to–liquid ratio or the maximal initial concentration (Table 6) are further evidence of the influence of the experimental conditions on the sorption. However, unlike the case of the *n* parameter, inclusion of the experimental conditions (C_{max}, *R*) in the multiple regression analyses did not increase the predictive performance of the regression for Kd_{eq} and Kf. It must be noted that the pedotransfer functions could be improved with additional experimental sorption studies designed to closely mimic the environmental conditions and with pH and CEC analyzed with the standardized methods [pH_{H2O}, Metson CEC (cmol/kg)].

Use of pedotransfer functions for risk assessment

The linear sorption coefficient Kd can be predicted by a pedotransfer function requiring the knowledge of only two properties, the clay content and CEC. The prediction performance is good with an RMSEP of less than 10 % of the mean glyphosate Kd_{eq} (Table 7). However, Figure 3a shows that the errors in the predicted soil–to–water concentration ratios vary largely according to the initial concentration of water. The errors are moderate for initial liquid–phase concentrations below 10 mg/L, indicating that the Kd pedotransfer function predicts sorption relatively accurately for concentrations below this

threshold. The 10 mg/L may correspond to the threshold above which the concentration independence of the sorption process can no longer be assumed. This assumption can be checked by examining the variation in n given by Eq. 4. Figure 4 presents the departure from linearity assumed to occur when *n* is below 0.9 across a range of clay content values and initial glyphosate concentrations.

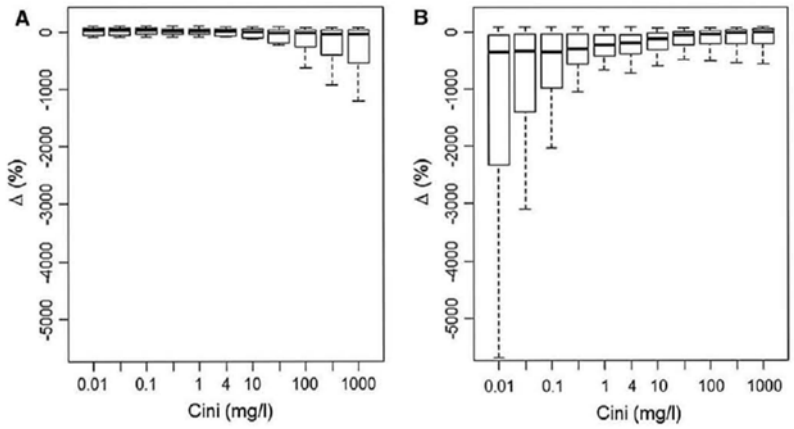


Figure 3 Distributions of the prediction errors for linear sorption isotherms and nonlinear sorption isotherms. Δ represents the difference (%) at a given initial concentration between the predicted and the measured ratio of concentrations between soil and water. a Ratios predicted by the Kd pedotransfer function (linear isotherm estimation) and b ratios predicted by the Kf and *n* pedotransfer function (nonlinear isotherm estimation)

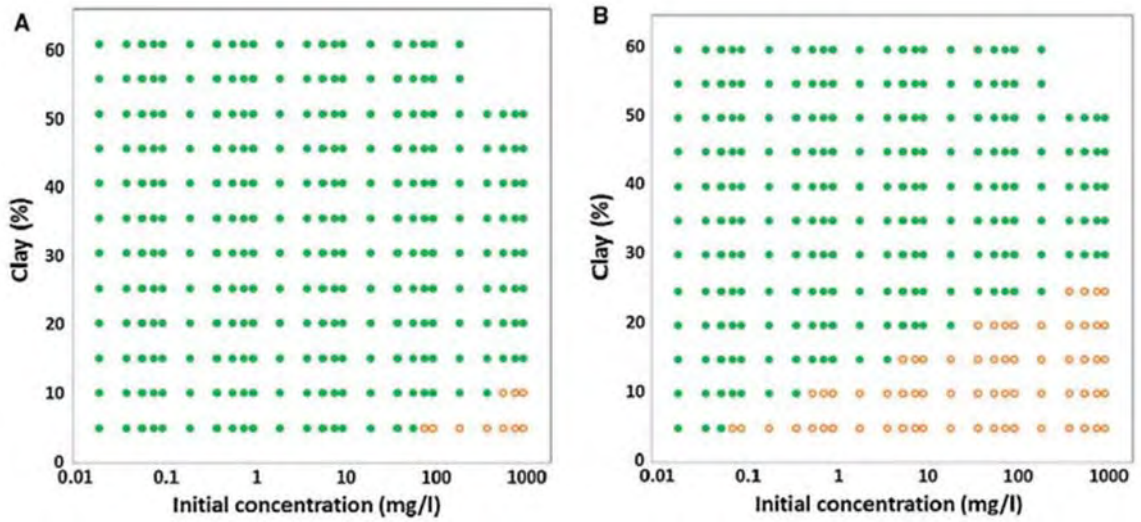


Figure 4 Linearity range of sorption isotherms in relation to the clay content and initial glyphosate concentrations in the liquid phase. Plain green dots represent 0.9 <n <1.05, and empty orange dots represent n values lower than 0.9 (bottom right), A n values were calculated from Eq. 4 with a solid–to–liquid ratio of 1:1 (g/ml). B n values were calculated from Eq. 4 with a solid–to–liquid ratio of 1:20 (g/ml). Note that for a solid–to–liquid ratio of 1:1, the saturation of sorption sites occurs at initial concentration higher than 100 mg/L for clay content varying between 0 and 10 %, whereas for the 1:20 ratio, the saturation for the same clay content starts at initial concentrations of approximately 0.1 mg/L.

The Freundlich isotherms can be satisfactorily predicted by two pedotransfer functions requiring the knowledge of four properties, namely the organic carbon and clay contents, CEC and pH (Table 7). As seen in Figure 4, the prediction errors of the soil–to–water concentration ratio exceed 1000 % for concentrations between 0.01 and 0.40 mg/L and 500 % for concentrations up to 10 mg/L (Figure 3b). Thus, the sorption estimated by the combination of *n* and Kf pedotransfer functions is significantly underestimated for initial concentrations below 10 mg/L. This may be due to the multiplication of

properties used to estimate the sorption parameters and the accumulation of inherent bias of the two pedotransfer functions. However, it must also be noted that for concentrations higher than 10 mg/L, the predictions using the estimated Freundlich model parameters show slightly smaller errors than those using the estimated linear isotherms. The application of the Kf pedotransfer function is therefore only advisable for estimating sorption for very high liquid–phase concentrations, a condition that is relatively rarely found in the current environmental conditions.

Conclusion

Sorption to soils and sediments controls the fate of glyphosate in the environment and thus the potential risk of freshwater and groundwater contamination. Glyphosate sorption appeared to be controlled mainly by cation exchange capacity, clay and organic carbon content and pH. This suggests that the mechanism driving glyphosate sorption over the range of soil and sediment investigated is the complex formation between the phosphonate group of glyphosate and the soil–exchanged polyvalent cations. Robust pedotransfer function for the estimation of glyphosate Kd was built from multiple regression analysis of the literature data. This Kd pedotransfer function enables prediction of glyphosate sorption for a wide range of soils and sediments with a limited number of properties and with reasonable accuracy for most environmental conditions.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The article estimates pedotransfer functions for the adsorption of glyphosate to soil based on based on review of existing literature data. No new experimental data is presented.
The article is therefore classified as reliable with restrictions (Category 2).

Te

1. Information on the study

Data point:	KCA 7.1.2.1.1
Report author	Ghafoor, A. et al.
Report year	2011
Report title	Measurements and modeling of pesticide persistence in soil at the catchment scale
Document No	The Science of the total environment (2011) Vol. 409, No. 10, pp. 1900–8.
Guidelines followed in study	OECD 106 Guidance
Deviations from current test guideline	Not sufficient information provided to check validity
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Not all information provided to check validity of the results, no results reported for adsorption experiment)

2. Full summary of the study according to OECD format

Degradation of pesticides in soils is both spatially variable and also one of the most sensitive factors determining losses to surface water and groundwater. To date, no general guidance is available on suitable approaches for dealing with spatial variation in pesticide degradation in catchment or regional scale modeling applications. The purpose of the study was therefore to study the influence of various soil physical, chemical and microbiological characteristics on pesticide persistence in the contrasting cultivated soils found in a small (13 km^2) agricultural catchment in Sweden and to develop and test a simple model approach that could support catchment scale modeling. Persistence of bentazone, glyphosate and isoproturon was investigated in laboratory incubation experiments. Degradation rate constants were highly variable with coefficients of variation ranging between 42 and 64 % for the three herbicides. Multiple linear regression analysis and Mallows Cp statistic were employed to select the best set of independent parameters accounting for the variation in degradation. Soil pH and the proportion of active microorganisms (r) together explained 69 % of the variation in the bentazone degradation rate constant; the Freundlich sorption co–efficient (K_f) and soil laccase activity together explained 88 % of the variation in degradation rate of glyphosate, while soil pH was a significant predictor ($p < 0.05$) for isoproturon persistence. However, correlations between many potential predictor variables made clear interpretations of the statistical analysis difficult. Multiplicative models based on two predictors chosen 'a priori', one accounting for microbial activity (e.g. microbial respiration, laccase activity or the surrogate variable soil organic carbon, SOC) and one accounting for the effects of sorption on bioavailability, showed promise to support predictions of degradation for large–scale modeling applications, explaining up to 50 % of the variation in herbicide persistence.

Materials and methods

Study site and soils

The study was carried out in the E21 monitoring catchment in Östergötland, southern Sweden. The total catchment area of 13 km^2 consists of 95 % agricultural land, with main crops of winter and spring sown cereals, rape, potatoes and peas. The soils, which are derived from glacial and post–glacial fluvial sediments and glacial till (moraine), have a wide range of texture, from loamy sand to clay. Soil samples were collected from 60 locations in the catchment (1 location every 20 ha) on a grid pattern. Five soil samples from each location were taken in the surface 20 cm, bulked, homogenized by passing through a 2 mm sieve, put into plastic bags and stored at 4°C until use (within 48 days). Sixteen of these sampled locations were selected for further study to cover the range of measured textures, organic matter contents and pH values.

Soil pH was measured on fresh samples after shaking the samples in de–ionised water (1:2.5) at room temperature (Swedish Standard Institute, 1994). Particle size distributions were evaluated using the standard pipette method (Day, 1965). The contents of clay, sand, and silt are usually correlated with one another (Iqbal et al., 2005). Thus the geometric mean particle diameter, d_g, was derived from the fundamental particle size classes as (Shirazi and Boersma, 1984):

[1]

$$d_g = exp(\sum_i m_i ln(X_i))$$

where *m* is the mass fraction of particle size class i and X is the mean diameter of that class. For the Swedish system, x–values are 0.001, 0.03 and 1.03 mm for clay, silt, and sand, respectively. Total organic C and N were measured using a Leco CN 2000 (LECO Corp., St. Joseph, MI, USA). Water contents at a pressure potential of –100 cm (pF 2) were measured on a sand table (Jamison, 1958). Ammonium–lactate extractable phosphorus and potassium were measured according to the method described by Egner et al. (1960).

The physical and chemical characteristics of the 16 soils are given in Table 1. There was a relatively wide range of SOC contents, ranging from 0.9 to 10.2 %. Soil pH ranged from 6.0 to 7.6. Soil texture is very variable for such a small catchment: clay, sand and silt contents ranged from 4–45 %, 12–87 %, and 8–54 % respectively, and 8 of the 11 USDA texture classes are represented. Ammonium–lactate extractable phosphorus and potassium ranged from 56–148 mg/kg and 54–209 mg/kg, respectively.

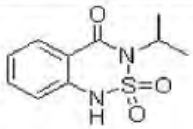
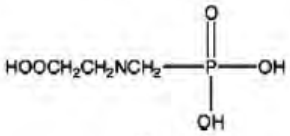
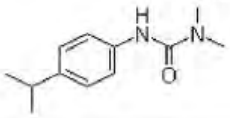
Table 1. Physico–chemical properties of soils.

Soils	pH	Texture			Textural class	SOC	$CaCO_3$	Total N	Water content at pF_2 (−100 cm)	Geometric mean particle diameter (d_g)	Available P	Available K
		Sand	Silt	Clay								
		%	%	%	International	%	%	%	g/g	mm	mg kg^{-1}	mg kg^{-1}
1	7.6	49	32	18	Loam	1.6	9.2	0.16	0.253	0.095	58	68
2	6.2	87	8	4	Sand	1.2	0.1	0.08	0.115	0.588	63	69
3	7.0	43	27	30	Clay Loam	2.3	0.1	0.22	0.363	0.049	99	116
4	7.1	58	25	17	Sandy Loam	2.1	0.4	0.21	0.3	0.131	89	84
5	6.9	68	17	15	Sandy Loam	2.1	0.2	0.21	0.285	0.199	111	114
6	6.5	70	21	9	Sandy Loam	1.1	0.1	0.09	0.175	0.263	90	120
7	6.5	85	9	6	Loamy Sand	1.6	0.1	0.15	0.169	0.494	159	70
8	7.6	55	28	17	Sandy Loam	2.6	0.2	0.25	0.265	0.118	56	68
9	6.4	12	45	44	Silty Clay	6.7	0.3	0.54	0.643	0.010	57	205
10	6.9	17	54	29	Silty Clay Loam	10.2	0.5	0.87	0.540	0.020	73	170
11	6.9	22	33	45	Clay	2.5	0.9	0.25	0.383	0.014	134	162
12	7.3	56	24	20	Sandy Loam	5.4	0.4	0.53	0.477	0.110	142	209
13	6.0	63	27	10	Sandy Loam	0.9	0.1	0.08	0.240	0.198	132	126
14	6.1	83	11	6	Loamy Sand	1.3	0.1	0.13	0.227	0.460	148	54
15	7.5	35	39	25	Clay Loam	3	0.1	0.28	0.410	0.046	101	97
16	7.1	31	40	29	Clay Loam	1.9	0.2	0.19	0.347	0.033	89	164

Chemicals

Unlabelled isoproturon (N,N–dimethyl–N′–[4–(1–methylethyl) phenyl]urea; 99 % purity), bentazone (3–(1–methylethyl)–1 H–2,1,3–benzothiadiazin–4(3 H)–one 2,2–dioxide; 97 % purity) and glyphosate (N–(phosphonomethyl)glycine, 98 % purity) were purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Ring (^{14}C) isoproturon (4.044 MBq/mg; purity >95 %) and [P–methylene–^{14}C]glyphosate (5.155 MBq/mg, purity >99 %) were purchased from Izotop, Institute of Isotopes, Budapest, Hungary. ^{14}C–labelled bentazone (3–(1–methylethyl–1 H–2,1,3–benzothiadiazin–4(3 H)–one 2,2–dioxide–[phenyl–U–^{14}C]; 5.211 MBq/mg; 100 % purity) was a gift from BASF, Limburgerhof, Germany. Table 2 gives the structural formulae and some physical and chemical properties of the three compounds. The 3–methyl–2–benzothiazolinone hydrazone (MBTH), 3–(dimethylamino) benzoic acid (DMAB) and 2,2′–azinobis(3–ethyl–benzthiazoline–6–sulfonic acid) (ABTS) were supplied by Sigma–Aldrich Sweden AB.

Table 2. Selected pesticides and their properties (data from the e–Pesticide Manual (3.0), British Crop Protection Council, 2003).

Herbicides	Structural formulae	pKa	mol wt	Solubility in water (mg L^{-1})
Bentazone		3.3	240.3	570
Glyphosate		2.3, 5.7,10.2	169.1	10,500
Isoproturon		n.a	206.3	65

Degradation

Incubation experiments for each soil/pesticide combination were carried out on two replicate samples. A sub–sample of each soil (7 g) was spiked with glyphosate dissolved in water (0.7 mg herbicide/mL water) or isoproturon or bentazone dissolved in methanol (0.7 mg herbicide/mL methanol). The soil was dried, after which an additional amount of fresh soil (63 g) was thoroughly mixed into the spiked soil to give an initial concentration of 10 µg/g dry weight (d.w.) of soil (procedure adopted from Brinch et al. (2002)). Water contents were adjusted to and maintained at pF 2 throughout the experiment by the addition of de–ionized water as necessary. The samples were incubated in aerated glass tubes in the dark at 20°C for 64 days. Duplicate samples (5 g) were taken after 0, 2, 4, 8, 16, 32 and 64 days of incubation for measurement of the residual concentrations of glyphosate, isoproturon, and bentazone.

Analyses of bentazone and isoproturon in soil samples were carried out by HPLC as described by Larsbo et al. (2009), while for glyphosate, the GC–MS method developed by Börjesson and Torstensson (2000) was employed. The data from the incubation study were fitted to first–order degradation kinetics using non–linear regression:

[2]

$$c = c_0 e^{-kt}$$

where c is the mass of compound in the soil (µg/g) at a given time t (days), c_0 is the original mass of compound added to the soil (µg/g), and k (day^{-1}) is the first–order degradation rate coefficient. Degradation half–lives (DT_{50}, days) were calculated as ln(2)/k.

Adsorption

The adsorption experiments were carried out according to the OECD 106 guideline (Organization for Economic Cooperation and Development, 2000) on two replicates. Soil (four grams d.w. for glyphosate, two grams d.w. for isoproturon and bentazone) was shaken to pre–equilibrate with 0.01 M $CaCl_2$ (39 mL for glyphosate, 1.5 mL for bentazone and isoproturon) for 24 h at 20°C in test tubes (50 mL plastic tubes (Sarstedt) for glyphosate and 10 mL glass tubes for bentazone and isoproturon). Thereafter, the soil slurry was spiked with a 1 mL mixture of labeled (ca 7000–11000 dpm) and unlabeled pesticides in 0.01 M $CaCl_2$ to give 5 initial concentrations in the range of 0.1–10 µg/g soil. The tubes were shaken for 24 h and then centrifuged for 20 min at 4000 rpm. After mixing with 10 mL of Insta–Gel Plus (glyphosate) or 6 mL of Ultima Gold emulsifying cocktail (bentazone and isoproturon) (PerkinElmer, Waltham, MA, USA), the radioactivity was measured in the supernatant (10 mL for glyphosate, 1 mL for bentazone and isoproturon) using a LS 6000TA liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). Tubes without soil and ^{14}C–labelled substances were included for subtraction of background radiation and tubes without soil were used to give the initial amount of ^{14}C activity. No significant adsorption of the tested substances occurred on the tubes.

The sorption measurements for each pesticide in the 16 soils were fitted to the Freundlich equation using

non–linear regression:
[3]

$$S = K_f c_e^n$$

where S is the adsorbed amount (μg/g), c_e is the equilibrium concentration (μg/mL), K_f is the Freundlich constant ($\mu g^{1-n}\, mL^n/g$), and n (–) is an exponent that expresses the degree of isotherm nonlinearity.

Manganese peroxidase and laccase enzyme activities
Manganese peroxidase (MnP, EC 1.11.1.13) activity
Ten g of soil was mixed with 20 mL of a 1:1 mixture of 500 mM lactic acid/sodium succinate buffer (pH 4.5) in a Waring blender and homogenized for 3×30 seconds at high speed. The aliquots were centrifuged in 50 mL centrifuge tubes at 4000 rpm for 15 min at 4°C. The supernatant was filtered through 0.45 μm filter paper. Manganese peroxidase (MnP) activity was measured according to the method described by Castillo et al. (1994). Briefly, the assay is based on the oxidative coupling of MBTH and DMAB in the presence of H_2O_2, Mn^{+2} and MnP. This reaction gives a deep purple–blue color with a broad absorption band with a peak at 590 nm. The reaction mixture contained 300 μL 6.6 mM DMAB, 100 μL 1.4 mM MBTH, 30 μL 30 mM$MnSO_4$, 10 μL 10 mM H_2O_2 and 1.56 mL of sample extract in a total volume of 2 mL. A reagent blank without any sample extract was also run. Time zero was registered at the moment of addition of H_2O_2 and the increase in absorbance was then followed at 590 nm for 5 min by using a Shimadzu UV 1800–A spectrophotometer fitted with a time scan function. The initial rates were calculated by using linear regression. MnP activity (mU/min/g soil) in soil was calculated as:
[4]

$$\text{Unit of enzyme } g^{-1}\text{soil} = \frac{\text{Abs}/\textit{min} \times 0.002}{E_m \times \text{mL of sample} \times \text{Dry weight of soil} \times \text{mL of buffer added}}$$

where E_m is the molar extinction coefficient (0.053 μM^{-1}/cm). One unit is defined as the amount of enzyme needed to form 1 μmol of product in 1 min.

Laccase (EC 1.10.3.2) activity
Laccase activity was measured by monitoring the oxidation of ABTS (Wolfenden and Willson, 1982) in a citrate/phosphate (100 mM citrate, 200 mM phosphate) buffer (pH 4.5) at 420 nm. Briefly, five g of soil was extracted with 20 mL 100 mM citrate/phosphate buffer (pH 4.5) for 1 h and then centrifuged for 15 min at 4000 rpm. The supernatant was filtered through a 0.45 μm filter. The reaction mixture contained 900 μL soil extract and 100 μL 30 m MABTS solution. The absorbance was measured at 420 nm at 25°C for 1 min with Shimadzu UV 1800–A spectrophotometer. Absorbance per minute (Abs/min) was calculated from the linear range of the curve and laccase activity was calculated as:
Unit of enzyme g^{-1}soil
[5]

$$= \frac{\text{Abs}/\ \textit{min} \times \text{mL of buffer added} \times 1000 \times \text{Volume}_{\text{reaction mixture}(\mu L)}}{E_m \times \text{Volume}_{\text{enzyme solution}(\mu L)} \times \text{Dry weight of soil}}$$

where Em is 0.036 μM^{-1}/cm.

Respiration
Respiration was measured as described previously (Stenström et al., 2001) with some modifications. Two replicates of soil (10 g d.w.) were weighed into 250 mL respirometric jars. The jars were installed inside a respirometer, and the accumulation of CO_2 trapped in KOH solution (0.2 M; 10 mL) was determined automatically twice every hour for each jar by measuring the electrical conductivity. The soil samples were incubated until a constant basal respiration rate (BR) was established (after about 3 days) at a constant temperature of 22°C and with a moisture content adjusted to pF 2. A substrate was prepared, consisting of glucose (7.5 g), $(NH_4)_2SO_4$ (1.13 g), KH_2PO_4 (0.35 g) and talcum powder (10 g), and 0.19 g of this mixture was thoroughly mixed into each jar. Empty jars were incubated as controls. The BR was calculated by linear regression of accumulated CO_2 produced versus time. The

instantaneous rate of CO_2 formation after addition of the substrate (substrate–induced respiration, SIR) was calculated using non–linear regression. The SIR was divided into the CO_2 production rate of active, exponentiallly growing (r) and dormant, non–growing (*K*) microorganisms as described by Stenström et al. (2001).

^{14}C–DHP mineralization

Synthetic ^{14}C–ring–labeled dehydrogenated polymerizate (^{14}C–DHP) of coniferyl alcohol (gift from Paul Ander, Department of Forest Products, SLU) with a molecular weight of 4–10 kDa and a specific activity of 0.16 MBq/mg was used to quantify lignin degrading activity in situ. The ^{14}C–DHP was added as a DMF–water suspension to 10 g dw of soil in 20 mL plastic jars. The final radioactivity was approximately 13,000 dpm per sample. The water contents were adjusted to pF 2. The plastic jars were each installed into air–tight glass jars together with scintillation vials containing NaOH (0.2 M; 4 mL) to trap carbon dioxide. The glass jars were incubated in the dark at 20°C and the base traps were changed regularly. The amount of ^{14}C in the base traps was measured on an LS 6000TA liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA) after mixing with 4 mL of Insta–gel Plus and incubated in the dark overnight. The ^{14}C liberated was corrected for the background radiation in controls without soil. Kinetic parameters describing ^{14}C–DHP mineralization were determined by non–linear regression according to first–order kinetics:

[6]

$$P = P_{max}\left(1-e^{-kt}\right)$$

where P is the accumulated ^{14}C–CO_2 released (% of the added ^{14}C) at time t, P_{max} is the maximum ^{14}C mineralized (% of applied) and k is the mineralization rate constant (day^{-1}).

Statistical analysis

General regression models (GRM) for best–subset–regression were fitted to the data, where replicate 1 was cross–validated with replicate 2 under the assumption of homogeneous variance. Hence, the two replicates were pooled for variance estimation, and all possible combinations of regressors examined with respect to explanatory power of the response variable (k). When the best–subset–regression models were built, our objective was to identify the subset of explanatory variables that combine optimal orthogonality with maximum explanatory power, in order to explain the variance of the response variable across soil samples. Orthogonality is synonymous with independence across regressor variables, and many methods have been suggested for estimating the ideal subset. Mallow's Cp statistic (Mallows, 1973) is an effective way to punish a linear combination of potential regressors with respect to multi–collinearity and accumulated error (Ryan, 1997). Mallow's Cp is identical to Akaike's information criteria when the generic variance σ^2 is known 'a priori'. Since we estimate σ^2 from our data, Mallow's Cp is a better choice. Statistical analyses were performed with STATISTICA™ (StatSoft, 1995).

Results and discussion

Degradation

The results from the degradation experiments are presented in Table 3. Bentazone, glyphosate and isoproturon degradation in soils generally followed first–order kinetics (all R^2>0.91 and statistically significant at $p<0.001$). The degradation rate constants listed in Table 3 show considerable differences between soils with coefficients of variation ranging from 42 to 64 % for the three compounds. Degradation rate constants for bentazone were in the range 0.005–0.034/day which corresponds to half–lives of 20 to 139 days. Our data are consistent with those (8–133 days) reported by others (Rodriguez–Cruz et al., 2006; Thorstensen and Lode, 2001). Degradation rate constants for isoproturon were in the range 0.011–0.104/day which corresponds to half–lives of 7–63 days. Again, this degree of variation is similar to that reported in the literature for isoproturon, with values ranging from 1.4 to 40 days (Larsbo et al., 2009; Rodriguez–Cruz et al., 2006; Walker et al., 2001). The degradation rates of glyphosate (0.006–0.05/day, which correspond to half–lives of 14–116 days) are also consistent with other studies where the DT_{50} values in a variety of different soil types have been reported in the range of 1.7 to 197.3 days (Giesy et al., 2000; Sorensen et al., 2006).

Table 3. Degradation rate constant of bentazone, isoproturon, and glyphosate in different soils.

Soils	Bentazone		Isoproturon		Glyphosate	
	k (day^{-1})	R^2	k (day^{-1})	R^2	k (day^{-1})	R^2
1	0.024 ± 0.002[a]	0.963	0.045 ± 0.002	0.981	0.044 ± 0.006	0.93
2	0.013 ± 0.001	0.984	0.031 ± 0.000	0.977	0.018 ± 0.001	0.95
3	0.019 ± 0.001	0.980	0.024 ± 0.001	0.986	0.032 ± 0.002	0.86
4	0.021 ± 0.001	0.986	0.044 ± 0.003	0.940	0.046 ± 0.002	0.88
5	0.015 ± 0.001	0.959	0.016 ± 0.001	0.980	0.031 ± 0.001	0.87
6	0.015 ± 0.002	0.972	0.041 ± 0.001	0.968	0.033 ± 0.003	0.97
7	0.010 ± 0.000	0.983	0.032 ± 0.005	0.990	0.024 ± 0.000	0.95
8	0.018 ± 0.002	0.976	0.062 ± 0.006	0.968	0.050 ± 0.001	0.89
9	0.032 ± 0.001	0.984	0.027 ± 0.000	0.941	0.017 ± 0.004	0.98
10	0.034 ± 0.001	0.986	0.015 ± 0.001	0.960	0.006 ± 0.001	0.95
11	0.014 ± 0.000	0.936	0.027 ± 0.003	0.983	0.013 ± 0.001	0.96
12	0.015 ± 0.002	0.961	0.034 ± 0.001	0.967	0.029 ± 0.001	0.96
13	0.006 ± 0.000	0.987	0.023 ± 0.003	0.964	0.022 ± 0.000	0.95
14	0.005 ± 0.000	0.910	0.011 ± 0.001	0.933	0.027 ± 0.000	0.97
15	0.017 ± 0.002	0.985	0.104 ± 0.002	0.937	0.028 ± 0.001	0.96
16	0.023 ± 0.002	0.968	0.077 ± 0.007	0.939	0.032 ± 0.004	0.98
Mean	0.018		0.039		0.028	
CV %	46		64		42	

[a] indicates the ± standard deviation of two replicates.

Correlations between variables

Soil physical, chemical and microbial parameters

Correlations between basic soil properties, microbiological parameters, sorption strength and the degradation rate of pesticides are reported in Table 4. As is quite typical, the sandy soils in our catchment (large dg values) generally had lower pH and SOC contents than the finer–textured loamy and clayey soils (Table 4). Activities of ligninolytic enzymes (MnP and laccase) were highly variable in our soils. Sinsabaugh et al. (2008) found a coefficient of variation for phenol oxidase (e.g., laccase) and peroxidase (e.g., MnP) activities among ecosystems of nearly 300 %. This variability can be attributed to differences in both the enzymology of various enzyme–producing white–rot species and differences in growth and enzyme production responses of the fungi to different soil and environmental factors (Sinsabaugh, 2010). Correlation analysis suggested that significantly higher enzyme activities in our soils were associated with higher soil organic carbon and soil pH (Table 4). MnP was positively correlated with SOC ($r = 0.78$; $p < 0.0001$). For peat soils, Sinsabaugh et al. (2008) also found that peroxidase activity increased with SOC. The activity of laccase was positively correlated with soil pH ($r = 0.55$; $p < 0.001$). This has also been found in other studies (Sinsabaugh, 2010; Sinsabaugh et al., 2008). Laccases deprotonate at high soil pH, which reduces their redox potential and increases their solubility, both of which may enhance their reaction potential (Sinsabaugh, 2010). Because laccases are widely produced for varied purposes, it is arguable that the diversity of the soil enzyme pool and potentially its range of action may also increase with soil pH (Sinsabaugh et al., 2008). Soil pH is also known to be an important predictor of microbial diversity (Sinsabaugh, 2010). Thus, SIR was positively correlated with soil pH, as well as SOC and available potassium, whereas it was negatively correlated with d_g and available P. The proportion of active microorganisms (r) was positively correlated with SOC, available K, and MnP whereas it was negatively correlated with d_g.

Table 4.Linear correlation coefficients.

Laccase	SIR	r	DHP k	Bentazone k	Isoproturon k	Glyphosate k
0.324						
−0.084	0.646***					
−0.504**	−0.019	0.13				
0.204	0.761***	0.787***	0.077			
0.546**	0.429*	−0.054	−0.274	0.130		
0.588**	−0.026	−0.536**	−0.012	−0.10	0.413	
−0.423	0.525**	0.555**	0.347	0.274	−0.14	−0.538**
−0.225	0.629***	0.761***	0.284	0.694	−0.23	0.413
−0.404	−0.078	0.498**	−0.162	0.112	0.11	−0.924***

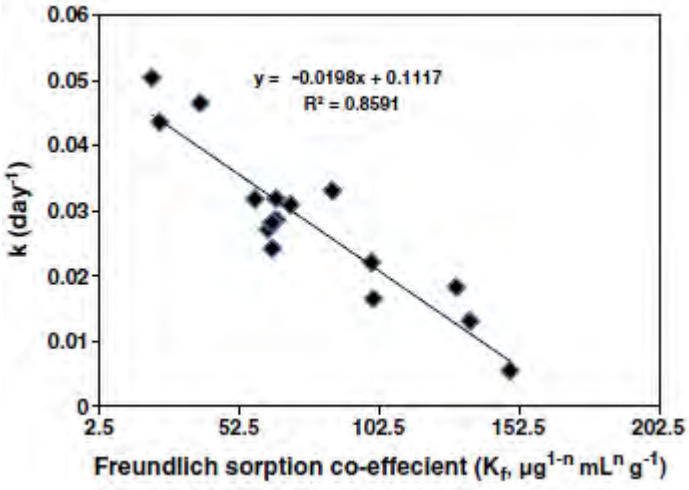
	pH	c_g	SOC	P	K (Potassium)	MnP
d_g	0.621**					
SOC	0.186	−0.479*				
P	−0.32	0.299	−0.254			
K (potassium)	0.057	−0.605**	0.645***	0.018		
MnP	−0.132	−0.172	0.777***	0.043	0.700**	
Laccase	0.546**	−0.305	−0.274	−0.432*	−0.26	−0.588**
SIR	0.458*	−0.699***	0.494*	−0.489*	0.516**	0.323
r (active)	−0.015	−0.455*	0.834***	−0.448*	0.579**	0.548**
DHP k	0.099	−0.032	0.509**	0.229	0.253	0.649***
Bentazone k	0.449*	−0.655**	0.736***	−0.706***	0.477*	0.361
Isoproturon k	0.592**	−0.295	−0.169	−0.268	−0.115	−0.308
Glyphosate k	0.516**	−0.053	−0.503**	−0.223	−0.492*	−0.503**
Bentazone K_f	−0.286	−0.178	0.551**	0.035	0.610**	0.803***
Isoproturon K_f	0.025	−0.496*	0.814***	−0.221	0.697***	0.719***
Glyphosate K_f	−0.523**	0.059	0.428*	0.054	0.444*	0.363

*, **, and *** indicate significance at $p<0.1$, 0.05 and 0.01, respectively.

Pesticide degradation

Glyphosate degradation was significantly positively correlated to soil pH and laccase activity and negatively correlated with SOC and K_f. The strong relationship between glyphosate degradation and the Freundlich sorption coefficient is illustrated in Figure 1. A negative correlation between glyphosate adsorption and degradation in soil has also been reported by others (Zablotowicz et al., 2009).

Figure 1. Relationship between the degradation rate constant k (day^{-1}) for glyphosate and the Freundlich sorption coefficient (K_f, $\mu g^{1-n} mL^n g^{-1}$).

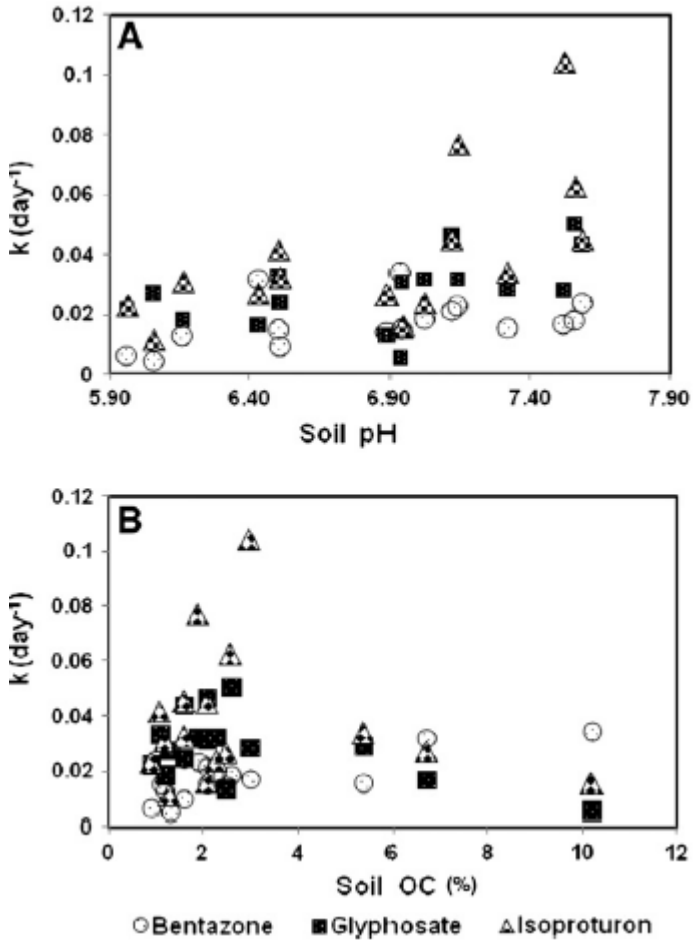


In this catchment, the finer-textured loamy and clay soils of higher pH showed faster degradation than sandy soils of low pH (Figure 2A and Table 3). Soil bacterial diversity and richness decline as pH decreases (Sinsabaugh et al., 2008) and other studies have also found pesticide persistence to increase as soil pH decreases (Walker et al., 2001). However, caution should be exercised in interpreting our data, since pH and SOC are strongly (positively) correlated if the three locations with highly organic (peaty) topsoils are excluded.

Figure 2B shows that the influence of SOC on pesticide degradation was rather complicated. An increase in soil organic matter increases biological activity and pesticide degradation rates in soil by providing conditions favorable to microbial growth (Thorstensen and Lode, 2001). On the other hand, pesticide sorption in soil, which is often positively related to SOC (Kah et al., 2007), may reduce the bioavailability of pesticides (Boivin et al., 2005; Kah et al., 2007; Sorensen et al., 2006; Thorstensen and Lode, 2001). Many studies have therefore demonstrated strong negative relationships between pesticide sorption and degradation in soils (Bolan and Baskaran, 1996; Dyson et al., 2002; Lehmann et al., 1992). In our study we observed a strong negative relationship between glyphosate degradation and its Freundlich sorption coefficient (Figure 1). The competing effects of organic matter content on microbial activity and sorption (bio–availability) mean that both positive and negative relationships

between sorption and degradation have been reported, as well as non–monotonic relation–ships which display an optimum (Bolan and Baskaran, 1996), as in Figure 2B for isoproturon.

Figure 2. Relationships between soil pH (A) and organic carbon content (B) and the degradation rate constants of all three pesticides.



Regression analysis

Table 5 shows the results of best-subset regression analysis. The use of Mallows Cp led to the selection of five out of 12 potential regressor variables: soil pH, SOC, r, laccase, and K_f. Soil pH and r together explained 69 % of the total variation in the bentazone degradation rate constant. There was, however, a problem with this model, arising from the skewed distribution of r, which resulted in heteroscedasticity. After applying Box–Cox transformation to the original r variable (rBC), we obtained a regression equation for which the residual distributions were approximately normal homoscedastic:

[7]

$$k = 0.006(\text{pH}) + 0.004(\text{rBC}) - 0.012$$

$$R^2 = 0.57, \text{Adj } R^2 = 0.51, F(2,14) = 8.75, p < 0.01$$

As an alternative model, pH and SOC explained 56 % of the variation in bentazone degradation, with an acceptable behaviour of the residuals (Table 5). This is because SOC and *r* are strongly correlated (Table 4). For glyphosate, 88 % of the variation in degradation rate coefficient could be explained by the Freundlich co–efficient K_f and soil laccase activity. Soil pH was the most significant predictor (pb 0.05) for isoproturon degradation and the inclusion of two more terms (SOC and r) significantly increased R^2 from 0.29 to 0.42 (Table 5).

Table 5. Best subset regression models relating first–order degradation rate constants for bentazone, glyphosate and isoproturon to soil parameters (β is the unscaled regression coefficient).

Pesticide	Intercept	Soil pH	SOC	r	Laccase	K_f	Overall performance
Bentazone	−0.030	β = 0.006 p < 0.05 F (1, 15) = 8.25	–	β = 0.007 p < 0.001 F (1, 15) = 25.2	–	–	Adj R^2 = 0.69 F (2, 14) = 17.8 p < 0.001
	−0.024	β = 0.005 p = 0.07 F (1, 15) = 3.85	β = 0.002 p < 0.01 F (1, 15) = 13.9	–	–	–	Adj R^2 = 0.56 F (2, 14) = 10.6 p < 0.01
Glyphosate	0.04	–	–	–	β = 0.000011 p < 0.05 F (1, 15) = 7.5	β = −0.0002 p < 0.0001 F (1, 15) = 60.7	Adj R^2 = 0.88 F (2, 14) = 56.5 p < 0.000
Isoproturon	−0.14	β = 0.030 p < 0.05 F (1, 15) = 7.08	–	–	–	–	Adj R^2 = 0.29 F (1, 15) = 7.08 p < 0.05
	−0.17	β = 0.032 p < 0.01 F (1, 15) = 11.6	β = −0.008 p < 0.05 F (1, 15) = 4.9	β = 0.02 p = 0.13 F (1, 15) = 2.7	–	–	Adj R^2 = 0.42 F (3, 13) = 4.57 p < 0.05

As discussed above, these predictor variables are more or less strongly correlated, both with each other and with other potential predictors (Table 4). Furthermore, multiple linear regression models comprising linear additive terms (e.g. for SOC and K_f) cannot reproduce observed non–monotonic relationships between degradation rate coefficients and either sorption constants or soil organic carbon content (see Figure 2B). It may therefore be more fruitful to develop models based on a mechanistic understanding of the processes controlling degradation. For microbial degradation, Allen and Walker (1987) suggested that degradation rates should be controlled by some measure of microbial activity multiplied by a factor related to the bio–availability of the compound. We can write:

[8]

$$k = k_{ref} \cdot (B)^m \cdot (A)^n$$

where *k* is the degradation rate constant, k_{ref} is a pesticide–specific reference rate coefficient which, in addition to the influence of variables not included in the model, should be related to the inherent degradability of the compound as determined by its molecular structure, *m* and *n* are constants, *A* is some measure of microbial activity and B is some measure of bioavailability. We tested six different forms of Eq. 8, combining three potential descriptors of microbial activity (laccase activity, SIR, and SOC) with two for bioavailability, K_f or the calculated fraction of pesticide in soil solution, Fs, given by:

[9]

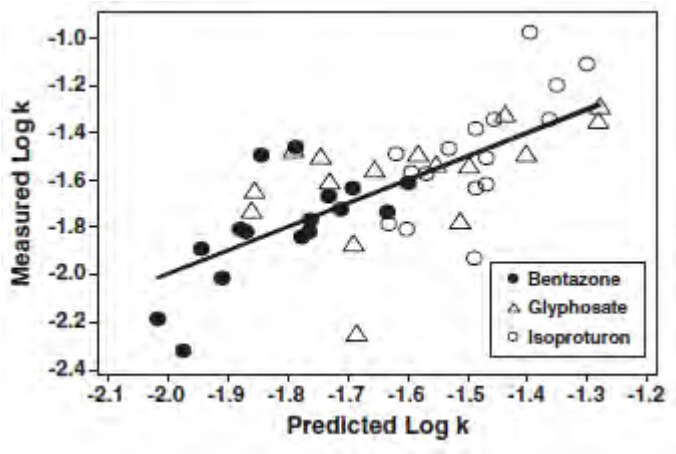
$$F_s = \frac{m_g}{m_g + K_f c_e^{n-1}}$$

where m_g is the gravimetric water content at pF 2 and c_e is the equilibrium concentration of the pesticide in solution at the start of the incubation experiment, which was iteratively calculated from the applied amount, the gravimetric moisture content and the parameters of the Freundlich equation. Although pH could also have been considered, we chose SOC as a surrogate variable for microbial activity, since it was strongly correlated to the microbial parameters MnP, SIR and r.

The data for all three pesticides were fitted to the logarithmic form of Eq. (8). The parameter values were estimated by introducing three 'class' variables into the data set (B, G, and I for bentazone, glyphosate and isoproturon, respectively, which take values of either 1 or zero) and k_{ref} values were obtained as regression coefficients for G, I and B. Table 6 shows that several of the models fitted the data well, especially models 1, 3 and 5, which had R^2 values ranging from 39 to 50 % and regression co–efficients that were all significant ($p<0.1$). As an example, Figure 3 shows a comparison of measured k with predictions using model 1 (i.e. Eq. (8) based on K_f and SIR). In contrast, model 6 (using Fs with SOC as a surrogate measure of microbial activity) gave the poorest results, with no significant effect of SOC and clear bias in the residuals (not shown). However, after excluding glyphosate from the model, the overall regression became highly significant ($p<0.0001$) and the distribution of residuals was unbiased. It is interesting that bioavailability, as reflected in the parameter F_s, emerges here as a

significant factor controlling degradation rates of bentazone and isoproturon, something which was not readily apparent from the classical correlation and regression analysis. Furthermore, although K_f is a very good predictor of glyphosate degradation (Figure 1), F_s is not. The reason for this is not clear.

Figure 3. Comparison of measured degradation rate constants with those predicted using model 1 (see Table 6).



No single model of pesticide degradation can be generally valid (Kah et al., 2007) as the mode of degradation varies considerably between compounds (e.g. chemical hydrolysis, co–metabolic or metabolic microbial degradation). However, although further testing is required, these results suggest that at least for some particular classes of pesticides, a multiplicative model based on soil organic carbon content and the sorption co–efficient (e.g. models 3 and 6, Table 6) may be an effective and practical way to account for the effects of microbial activity and bio-availability on pesticide degradation in the context of modeling applications at catchment or regional scales.

Table 6. Parameter values and their significance for different models developed to predict degradation rate for all three pesticides together.

With K_f in the models					With F_s in models				
Model	Parameters	Values	P value	Centered R^2	Model	Parameters	Values	P value	Centered R^2
1	Log ($k_{ref\,(B)}$)	−2.274	<.0001	0.50	4	Log ($k_{ref\,(B)}$)	−1.797	<.0001	0.42
	Log ($k_{ref\,(G)}$)	−0.893	0.0006			Log ($k_{ref\,(G)}$)	−0.659	0.1515	
	Log ($k_{ref\,(I)}$)	−1.472	<.0001			Log ($k_{ref\,(I)}$)	−1.182	<.0001	
	Log K_f	−0.500	0.0005			Log F_s	0.433	0.0179	
	Log SIR	0.366	0.0006			Log SIR	0.176	0.1134	
2	Log ($k_{ref\,(B)}$)	−2.825	<.0001	0.45	5	Log ($k_{ref\,(B)}$)	−2.586	<.0001	0.49
	Log ($k_{ref\,(G)}$)	−2.106	<.0001			Log ($k_{ref\,(G)}$)	−1.510	0.0076	
	Log ($k_{ref\,(I)}$)	−2.296	<.0001			Log ($k_{ref\,(I)}$)	−1.991	<.0001	
	Log K_f	−0.218	0.1408			Log F_s	0.404	0.0149	
	Log Laccase	0.353	0.0057			Log Laccase	0.344	0.0038	
3	Log ($k_{ref\,(B)}$)	−2.166	<.0001	0.39	6	Log ($k_{ref\,(B)}$)	−1.659	<.0001	0.38
	Log ($k_{ref\,(G)}$)	−0.715	0.0179			Log ($k_{ref\,(G)}$)	−0.295	0.4889	
	Log ($k_{ref\,(I)}$)	−1.336	<.0001			Log ($k_{ref\,(I)}$)	−0.972	<.0001	
	Log K_f	−0.530	0.0029			Log F_s	0.538	0.0030	
	Log SOC	0.242	0.0773			Log SOC	−0.029	0.8036	

3. Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

The study describes degradation and sorption experiments with glyphosate among other substances on several Swedish agricultural soils. The analytical methods used in both experiments are not explained in detail, no LoD or LoQ are provided. For the sorption experiment, no results are provided. No mass balances and measurement per sample date are provided for both experiments.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.4.1.1
Report author	Gjettermann, B. et al.
Report year	2011
Report title	Kinetics of Glyphosate Desorption from Mobilized Soil Particles
Document No	Soil Science Society of America journal (2011), Vol 75, No 2, pp. 434–443
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Study not sufficiently described to check validity of results)

2. Full summary of the study according to OECD format

Desorption kinetics of chemical compounds can be important both for their mobility in soil and for the significance of particle–facilitated transport. We studied desorption of glyphosate [N–(phosphonomethyl) glycine] on mobilized particles from two soil columns (50–cm height, 30–cm diameter), i.e., particles leached by free drainage from the bottom and particles mobilized by splash erosion and collected next to the top of the column. Leaching and splash erosion were driven by three, 30–mm irrigation events following surface application of ^{14}C–labeled glyphosate. Fresh leachate samples were investigated within 30 min of sampling, and desorption from splash–eroded particles in suspension (100 mg solid/L) was followed for 48 h (starting 2.0 min after immersion). Glyphosate concentrations were determined by measuring the ^{14}C activity using liquid scintillation counting. Similar fractional amounts of glyphosate (on average, 10–20 % in 20 min) desorbed from leached and from splash–eroded particles (>20 nm) shortly after leaching or immersion, respectively, indicating that the processes of desorption from the different sources of particles were similar. In leachate, about 45 to 79 % remained particle bound after 20 min, while calculated values at equilibrium were 20 % or less. Equilibrium was established after about 5 to 10 h in suspensions with splash–eroded particles, except for one sample. These direct observations, supported by estimated values of the Damköhler number, lead to the conclusion that desorption kinetics are important for evaluating the significance of dissolved and particle–facilitated transport of glyphosate. To quantify particle–facilitated glyphosate transport, the water and solid phases in the leachate should consequently be separated within a few minutes after leaching.

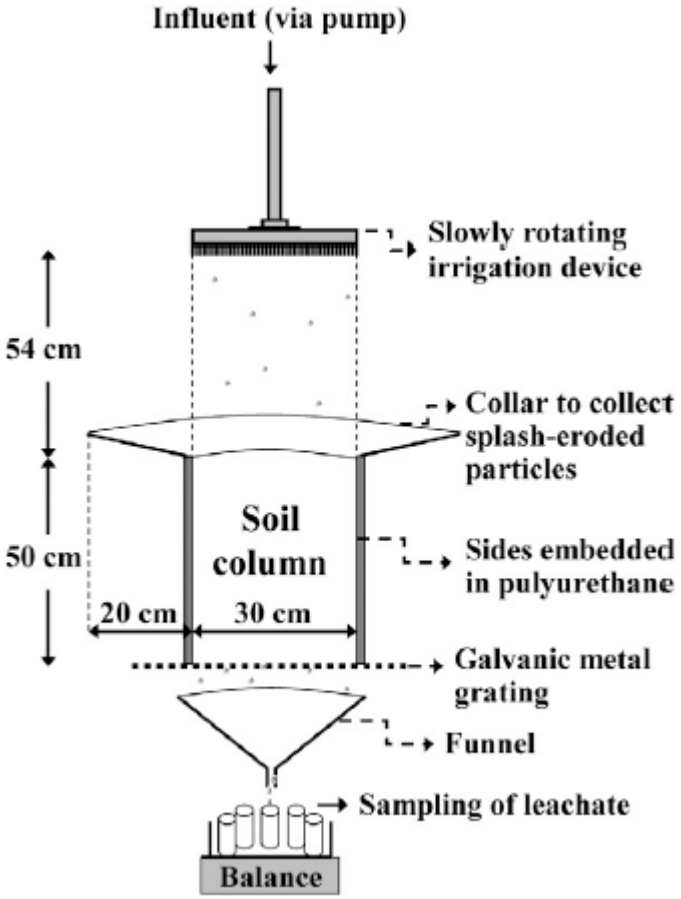
Materials and methods

Experimental Setup

The investigated sandy loam soil and parts of the experimental setup have previously been described in detail by Gjettermann et al. (2009), who focused on the influence of soil structure on particle–facilitated pesticide leaching. Two undisturbed cylindrical soil columns (B1 and B2) were carefully excavated from a recently tilled (plowed and drilled) experimental plot, encapsulated with polyurethane foam, and trimmed at the bottom end (50–cm cylinder length, 30–cm diameter). As reference, two columns (A1 and A2) were excavated from an untilled plot. The coding of the columns used in the glyphosate experiments by Gjettermann et al. (2009) has been kept to facilitate comparison. Particular care was observed not to disturb the surfaces of the columns or to block large macropores while trimming the bottom ends. The investigated sandy loam soil (an Agrudalf) is located at the University of Copenhagen research farm Roerrendegaard at Taastrup, Denmark, and has previously been described by Petersen et al. (2001). The contents of coarse sand (200–2000 μm), fine sand (20–200 μm), silt (2–20 μm), clay (<2 μm), and organic C in the upper 30 cm were 29, 40, 18.5, 12.5, and 1.2 %, respectively. A schematic presentation of the experimental setup is given in Figure 1. All irrigation water applied to the columns (influent) had a composition similar to rainwater (Miljøstyrelsen, 1996) containing 0.017 mmol/L

$CaCO_3$, 0.018 mmol/L KNO_3, 0.021 mmol/L $MgSO_4$, 0.126 mmol/L NaCl, and 0.94 mmol/L NH_4Cl. The pH was 6.32 and the electrical conductivity was 0.047 mS cm^{-1}. Water was applied to the column with a pump (FMI Pump QG 150, Fluid Metering Inc., Syosset, NY) through a motor–driven, slowly rotating sprinkling device with 90 syringe needles (Trumo, 25G), to ensure a uniform application rate of 15.0 mm/h (1.06 L/h). The sprinkling device was placed 54 cm above the surface of the column. The drop size was determined at the used intensity by sampling and weighing about 20 drops (10 repetitions). The mass of a drop was 6.4 ± 0.5 mg, corresponding to a (spherical) drop diameter of 2.3 ± 0.9 mm.

Figure 1. Schematic illustration of the experimental setup for the leaching experiments and for the collection of splash–eroded soil particles.



The columns were rewetted at the start of the experiment by irrigation, which was stopped 35 min after the first appearance of leachate. One day later, ^{14}C–labeled glyphosate mixed with the commercial glyphosate product Roundup Bio (Monsanto Europe, Antwerp, Belgium) was applied uniformly to the surface. The glyphosate stock solution had a specific concentration activity of 0.80 MBq/mg and contained 4.4 % ^{14}C–labeled glyphosate, 93.5 % unlabeled glyphosate, and 2.1 % aminomethylphosphonic acid (AMPA). The applied dose of glyphosate (12.5 mg/column) was comparable to current agricultural practice. A total of 13 mL of solution (stock solution and rinse water) was applied during the glyphosate application.

Three irrigations were applied to the soil columns 5, 8, and 12 d after rewetting, respectively. Each event lasted 2.0 h and had a constant intensity of 15.0 mm/h. Water drained freely from the column during and after each irrigation event. The mass of drainage water (leachate) was measured continuously. The leachate was sampled continuously, yielding a total of 21 samples, each containing 30 to 50 mL of leachate. The top ends of the columns were covered with plastic whenever possible to minimize evaporation.

A new plastic collar was mounted around the tops of the columns before each irrigation event to collect water splashes and soil particles (splash–eroded particles) that were eroded by drops and thrown over the sides (height up to 1 cm above the soil surface) with the droplets. All of these water droplets were collected on the collar. The water droplets generally evaporated within a few hours. The particles left were scraped off the collar 24 h after the irrigation event, allowed to air dry, and sieved through a 100-µm sieve. The mass of air–dry particles <100 µm was determined. This material was used for the desorption experiments.

Measurements

The ^{14}C activity of unfiltered and filtered leachate samples was measured with a Wallac 1414 (Perkin Elmer Corp., Waltham, MA) liquid scintillation counter (LSC) using 10 mL of scintillation cocktail (InstaGel, PerkinElmer) to a 9–mL sample. Using ^{14}C–labeled pesticides has the consequence that also metabolites, for example the major metabolite of ^{14}C–glyphosate (^{14}C–AMPA) were measured by LSC. The detection limit of the ^{14}C LSC analysis was 16.4 disintegrations min^{-1} (0.038 µg ^{14}C–glyphosate/L) and the method had trueness for quantification on ^{14}C standard buttons (PerkinElmer) of 100.2 ± 0.8 %. The effect of quenching was automatically adjusted by the LSC, and increasing quench induced by increasing particle concentration was accurately measured. Gjettermann et al. (2009) found good agreement between these determinations and direct chemical measurements of glyphosate plus AMPA, and they showed that AMPA constituted only a minor part (up to 17.5 %) in leachate samples from the investigated columns.

Particle concentration in the leachate was determined indirectly from the measured turbidity. Turbidity was measured with a turbidity meter (Tintometer GmbH, Dortmund, Germany). Samples were shaken and immediately transferred to glass vials. Turbidity was then measured after exactly 60 s. With the chosen procedure, isolated soil particles were <30 to 50 µm (equivalent spherical diameter), assuming a particle density of 1600 to 2650 kg/m^3. The mass of particles was estimated in 70 randomly selected leachate samples of known volume to establish a relationship between turbidity and concentration of particles. The samples were centrifuged (30 min at 4100 × *g*) and washed twice with deionized water. Finally, the particles were dried at 105°C before determining the mass. The correlation between turbidity *T* (in nephelometric turbidity units [NTU]) and the concentration of soil particles in the leachate was used to calculate the concentration of particles: concentration of particles (mg/L) = 110 ln(*T*) − 241 (R^2 = 0.74, 70 samples). For turbidity <20 NTU, equivalent to particle concentrations of less than approximately 88 mg/L, the relationship was poor and this limit was therefore used as the detection limit.

Desorption was investigated in five leachate samples from each of the tilled soil columns (Samples 2, 8, and 14 from the first irrigation event and Sample 21 from the other two events; see Table 1). The samples were selected to illustrate the development in glyphosate levels during the three irrigation events. Only one leachate sample (Sample 2, first irrigation) from each of the untilled columns was investigated, the amount of sediment being too small (considerably below the detection limit) and the uncertainty of the determination on individual samples too high during the later phases of the drainage events (Gjettermann et al., 2009). Approximately 40 mL of leachate sample was collected and a stopwatch was activated. A 10–mL sample was immediately filtered (0.02–µm inorganic, anopore filter, Frisenette, Knebel, Denmark) into a clean glass and the time (about 1.5 min) was recorded. Nine milliliters of the filtrate was later extracted for ^{14}C–activity measurement. After 5 min, another 10 mL of leachate was extracted and filtered for activity measurement. This was repeated after another 5 to 10 min and, if the amount of the original sample allowed it, after approximately 30 min. The so–called reaction time associated with each filtration, t_r, was assigned as the time span from the midpoint [$(t_{beg} + t_{end})/2$] of the sampling interval to when filtration had just been performed.

The concentrations of soil particles in the leachate used for the desorption experiments were not measured but estimated as the average of measured concentrations in the directly preceding and succeeding samples (first irrigation) or as the concentration measured in the directly preceding sample (second and third irrigations). The sample size did not allow combined determination of both particle concentration and desorption, and larger samples would have compromised the need for fast separation of colloids from the water phase. The uncertainty associated with this procedure was estimated from concentration difference between consecutive samples measured by Gjettermann et al. (2009), the

absolute average concentration difference being assigned as *D*.

Detectable splash erosion occurred from both of the tilled columns (B) in all events, but not from the untilled columns (A). Sieved and air–dried, splash–eroded particles generated during each irrigation event from the tilled columns were immersed (at time zero) in stirred artificial rainwater (irrigation water) yielding a suspended particle concentration of $C_{particle}$ = 100 mg/L. Samples of 10 mL were extracted and filtered, and the ^{14}C activity of the filtered samples was determined five or six times, typically 2.0, 10.0, 60, 120, 1440, and 2880 min after immersion (equivalent to t_r) as described above for the leachate.

Table 1 summarizes all analytical results available on the leachates.

Table 1. Overview of analyses conducted on leachate samples from each of the three irrigation events.

Sample no.	First irrigation	Second and third irrigation
1	pesticide (colloidal and soluble fractions), ph, conductivity and turbidity	
2	sorption and desorption kinetics of pesticide	total concentration of pesticide, colloidal and soluble organic c, ph, conductivity and turbidity.
3–7	pesticide (colloidal and soluble fractions), ph, conductivity and turbidity	
8	sorption and desorption kinetics of pesticide	total concentration of pesticide, colloidal and soluble organic c, ph, conductivity and turbidity.
9–13	pesticide (colloidal and soluble fractions), ph, conductivity and turbidity	
14	sorption and desorption kinetics of pesticide	total concentration of pesticide, colloidal and soluble organic c, ph, conductivity and turbidity.
15–20	pesticide (colloidal and soluble fractions), ph, conductivity and turbidity	
21		sorption and desorption kinetics of pesticide

Data Analyses

The specific activity of the applied glyphosate stock solution was checked by LSC analysis at each application time. The measured specific activity was used to convert the measured ^{14}C activity into glyphosate concentration units. The concentration of particle–bound glyphosate (C_p) in the leachate was defined as the difference between the measured concentration in the suspension (total concentration, Cs) and the measured concentration in the filtrate (dissolved glyphosate, Cd). The particle–bound fraction of leached glyphosate was calculated as C_p/C_s. Rates of change of the particle–bound fraction were estimated by least squares linear regression, i.e., from fitting experimental data to the simple approach presented by

[1]

$$\frac{100C_p}{C_s} = -\alpha t_r + \beta$$

where the rate α (% min^{-1}) and β (%) are constants (positive α values indicate desorption) and t_r is the time of reaction (min).

Equation [1] was also fitted to the first data points from experiments with splash–eroded particles in an attempt to obtain similar time scales of desorption from the different sampling types of particles (leached and splash eroded). In this analysis, C_s was obtained as the sum of particle–bound and dissolved concentrations at equilibrium ($C_{p,eq}$ and $C_{d,eq}$, respectively), and C_p was calculated as the difference between C_s and C_d.

At long experimental periods ($0 < t_r \leq 48$ h), however, the desorption from splash–eroded particles was not linear and the points were therefore also described according to

[2]

$$\frac{dC_d}{dt} = -k\left(C_d - C_{d,eq}\right)$$

where C_d (µg/L) is the dissolved concentration (<20 nm), t is the time (h), $C_{d,eq}$ (µg/L) is the "equilibrium" dissolved concentration, and k is a rate constant (h^{-1}). This approach has been termed a linear driving force approximation (LeVan et al., 1997) or a first–order mass transfer model (Lick et al., 1997). Assuming that C_d at time t = 0 h when the particles were suspended [$C_d(0)$] = 0, Eq. [2] can be integrated into

[3]
$$C_d = C_{d,eq}\left[1 - \exp\left(-kt_r\right)\right]$$

where t_r is the time of reaction. The Solver function in Excel (Wraith and Or, 1998) was used to adjust the model parameters $C_{d,eq}$ and k by minimizing the difference between predicted and measured C_d values (maximizing R^2). Because the chemical or physical processes involved in the desorption are expected to be similar in the two data sets, the choice of a linear vs. an exponential model is based solely on the number of data points available and the time scale used to observe the different particles.

For the splash–eroded particles, the total content of glyphosate in the sample was not measured (the results had to be discarded due to an error in the laboratory). It therefore had to be estimated. Gjettermann et al. (2009) reported a K_d value of 503 L/kg for the bulk topsoil (and 496 L/kg for AMPA). It has previously been shown that particles larger than about 0.1 mm are not present in drainage from the investigated field site (Holm et al., 2003), indicating that coarse sand and parts of the fi ne sand fraction either are not mobile or are immobilized on the way through the soil column. For this soil, it may generally be expected that the Fe and Al oxides that sorb glyphosate is mainly present in the fraction <20 µm. Hence, K_d for the investigated leached particles will be larger than that for the bulk soil. Based on the texture of the topsoil, 40 % of the constituents were >0.100 mm. Hence, an estimate of K_d was obtained as 503 L/kg / 0.60 = 8.4 × 102 L/kg. This is a conservative estimate because it assumes no sorting of particles below the 0.1–mm limit within the soil columns. Estimates of the concentration of particle–bound glyphosate at equilibrium, $C_{p,eq}$ (µg/L) were obtained from the fitted $C_{d,eq}$, the soil/water ratio (particle concentration $C_{particle}$, kg/L), and K_d as $C_{p,eq} = C_{particle}K_dC_{d,eq}$. Hence, in the absence of direct measurements, the total glyphosate concentration was calculated as

[4]
$$\begin{aligned} C_s &= C_{d,eq} + C_{p,eq} \\ &= C_{d,eq}\left(1 + C_{particle}K_d\right) \end{aligned}$$

The Damköhler number, D_a, is a measure of the relative importance of kinetics to equilibrium processes in transport (Bold et al., 2003). The D_a is defined as the ratio between the transport and the reaction time scales, and can be calculated as

[5]
$$D_a = \frac{k}{\left(U/L\right)}$$

where L (cm) is the transport distance (e.g., length of column, cm) and U (cm/h) is the water velocity in the soil.

Results and Discussion

Desorption in Leachate from Tilled Soil

Measured dissolved glyphosate concentrations in the leachate from the tilled soil generally increased with time (Figure 2), and the particle–bound fraction decreased (Figure 3). Thus, one immediate finding of the experiment is that considerable amounts of glyphosate desorbed from leached soil particles

(>20 nm) during the investigated period (about 20 min). Desorption was particularly large for the first irrigation on Column B1 (Figure 2), probably reflecting leaching of highly pesticide–enriched particles. Thus, the initially (about 1.5 min after sampling) measured concentration of glyphosate on particles was 19 to 24 mg/kg for this irrigation event, while it was between 7.7 and 3.5 mg/kg for the other irrigations on Column B1 and all irrigations on Column B2. The data do not indicate that concentrations of dissolved glyphosate reached stable levels.

The concentration of leached particles, $C_{particle}$ (Table 2), could be a critical factor for the α values describing glyphosate desorption. Higher concentrations of particles should result in lower desorption rates due to a higher final equilibrium value (c.f. Eq. [4]). The particle concentrations showed little variation from sample to sample within events (Table 2, D ≤24 mg/L), although it often varied significantly from the beginning to the end of an irrigation event (Gjettermann et al., 2009). Thus, the uncertainty associated with the estimated $C_{particle}$ values in Table 2 is probably on the order of 24 mg/L or less. The concentrations ranged between 123 and 292 mg/L and were higher for Column B1 than for Column B2. The expected dissolved mass fraction at equilibrium, $C_{d,eq}/C_s$ can be estimated by rearranging Eq. [4] and inserting the measured particle concentrations from Table 2. According to this calculation, the mass of sorbed glyphosate at equilibrium in the leached samples will account for 20 % or less of the mass in solution. Hence, with the investigated range of particle concentrations and the high initial fractions of particle–bound glyphosate (Figure 3), the samples are far from equilibrium and particle concentrations should not be important for the relative amount of desorbed pesticide or the desorption rates.

Figure 2. Concentration of dissolved glyphosate (C_d) in leachates from two soil columns, B1 (left) and B2 (right), at different reaction times (t_r, 0–30 min): (a) and (b) data for the first irrigation event (Samples 2, 8, and 14); (c) and (d) data for the second and third irrigation events (Sample 21).

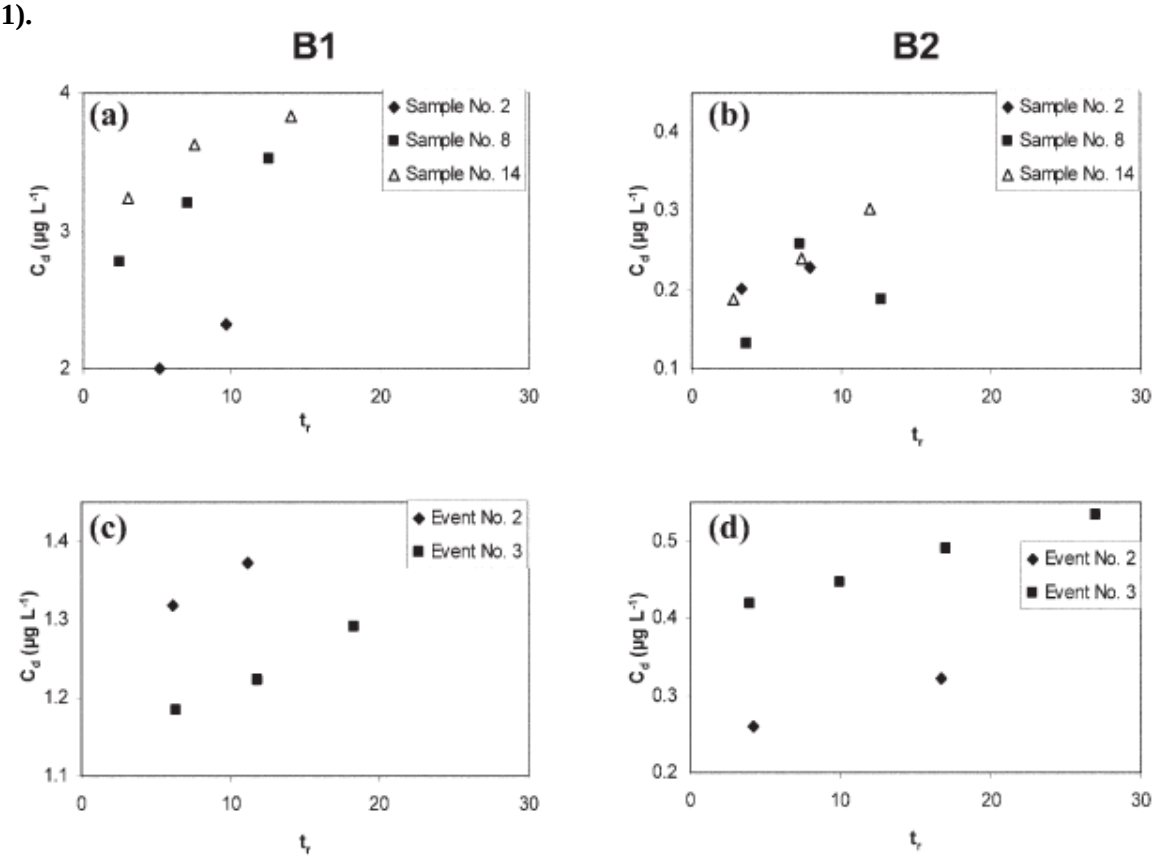


Table 2. Relative desorption rate (α), intercept parameter (β), concentration of soil particles ($C_{particle}$) in the investigated leachate, and average absolute particle concentration difference between consecutive samples (D) derived from experiments on leachates; α, β, and coefficient of determination (R^2) obtained by fitting data from different soil columns (B1, B2, A1, and A2), irrigation events (1–3), and samples (2, 8, 14, and 21) to Eq. [1].

Column	Irrigation no.	Sample no.	α	β	R^2†	$C_{particle}$	D
			% min^{-1}	%		— mg L^{-1} —	
B1	1	2	0.94	78		292	
	1	8	0.80	72	0.98	276	9 (6)‡
	1	14	0.56	67	0.92	268	
	2	21	0.41	53		212	13 (19)
	3	21	0.33	58	0.99	263	6 (5)
B2	1	2	0.48	85		199	
	1	8	0.39	87	0.11	134	24 (20)
	1	14	0.98	89	1.00	151	
	2	21	0.69	67		123	14 (13)
	3	21	0.51	60	0.99	166	11 (13)
A1	1	2	0.37	21		264	23(21)
A2	1	2	−1.03	8	1.00	190	21(26)

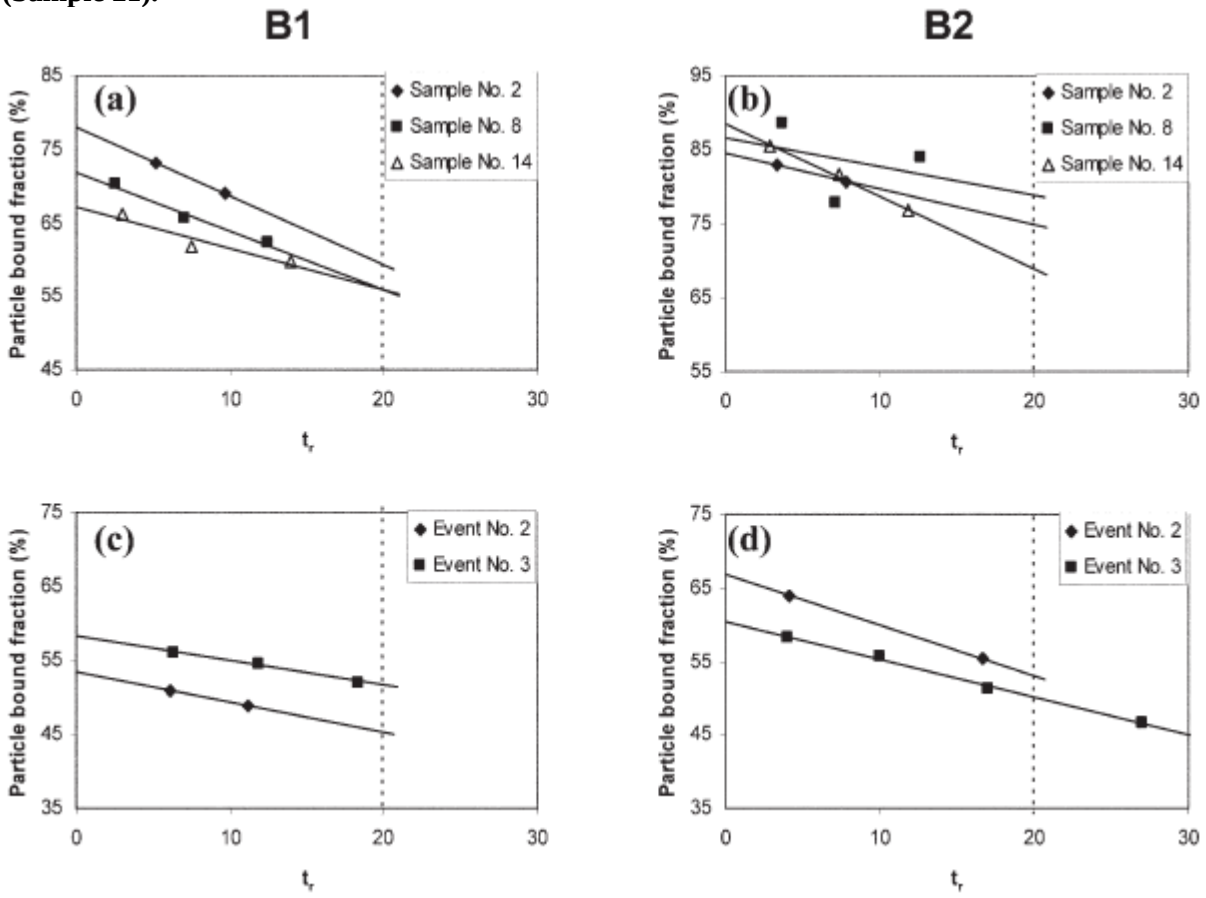
† Not applicable when the number of samples was <3.
‡ Standard deviation in brackets ($14 < n < 21$).

In general, Eq. [1] fitted well to the measured fractions of particle–bound glyphosate (Figure 3). The coefficients of determination were high ($R^2 \geq 0.92$), except for Sample 8 from the first irrigation on Column B2 (Table 2). By using this equation with the parameter values from Table 2, it was estimated that 7 to 20 % (on average, 12 %) of the leached glyphosate was desorbed from soil particles (>20 nm) within the first 20 min after sampling, corresponding approximately to the time scale of the observations. The reaction time (t_r) associated with the first filtration varied somewhat between events due to differing lengths of the sampling intervals. The 20–min relative desorption may be overestimated if the last measureements were close to the equilibrium concentrations (which was probably not the case according to the above calculations), and it may be underestimated if desorption took place much faster before the first filtrations (1.5 min after sampling).

The reaction time, defined as the time from the midpoint of the sampling interval, can be considered as an estimate of the time span after leaching. Hence, an estimate of the particle–bound fraction of glyphosate at a given time after leaching can be obtained from Eq. [1] by using parameters from Table 2. The data indicate that 45 to 79 % of the leached glyphosate was still particle bound 20 min after leaching (Figure 3). Thus, the rates of desorption measured shortly after sampling could not fully account for the amounts of glyphosate being desorbed 20 min after leaching.

The particle–bound fraction measure in leachate from the tilled soil 1.5 min after sampling varied between 51 and 89 % (Figure 3). This is in accordance with results reported by Gjettermann et al. (2009). It is probable that such figures depend considerably on the conditions that eventually lead to bypass flow and leaching. The applied methods were chosen to minimize desorption in the leachate before sampling and phase separation.

Figure 3. Particle (>20–nm) bound fraction of glyphosate in leachates from two soil columns, B1 (left) and B2 (right), at different reaction times (t_r, 0–30 min): (a) and (b) data for the first irrigation event (Samples 2, 8, and 14); (c) and (d) data for the second and third irrigation events (Sample 21).



The particle–bound fractions of glyphosate measured in the leachate from the two untilled soil columns (A1 and A2) 1.5 min after sampling were 19 and 14 %, respectively. This conforms to previously reported results that the fraction of particle–bound glyphosate in recently produced leachate can be much smalller with a minimally disturbed soil structure than with a tilled structure (Gjettermann et al., 2009). The particle–bound fraction decreased with time after sampling in the A1 sample, indicating desorption, whereas it increased in the A2 sample, indicating sorption (Table 2). By inserting the estimated K_d (= 8.4×10^2 L/kg) in Eq. [4], the fractions of particle–bound glyphosate at equilibrium were estimated to be 18 and 14 % for the A1 and A2 samples, respectively. Hence, leached glyphosate from the untilled soil columns appears to have been close to equilibrium, which is probably why both sorption and desorption may have occurred, as indicated by the measurements.

The individual desorption rates are relatively uncertain, being based on only two to four measured particle–bound fractions (Figure 3). More observations could have been obtained, but only if the sample sizes had been increased correspondingly. This would have increased the time of reaction and hence desorption taking place before the measurements. The trends observed are similar for all samples, however, corroborating the conclusion that the particle–bound glyphosate in the solution leaching from the tilled columns was not in equilibrium with the surrounding water phase.

Desorption from Splash–Eroded Particles

Noticeable splash erosion occurred during all irrigation events involving the tilled columns. The amounts of (air–dry) splash–eroded particles varied between 31 and 70 mg per event independent of irrigation number and column. All fine–earth particle sizes were present, in accordance with earlier findings that eroded material is typically unsorted (Heilig et al., 2001; Hairsine and Rose, 1991; Al–Durrah and Bradford, 1982). Larger particles were removed by using the 100–µm sieve in consequence

of the earlier reported finding that particles smaller than about 0.1 mm are not present in drainage water from the investigated field site (Holm et al., 2003). The fine particles released considerable amounts of glyphosate after being suspended. Hence, dissolved glyphosate concentrations increased with time, with gradually decreasing rates (Figure 4a and 4b). The rates were still relatively high after 1 h. After a few hours, concentrations were high compared with concentrations measured in most leachates (Figure 2), except from the first irrigation on Column B1. An equilibrium concentration of dissolved glyphosate appeared to be reached after about 5 to 10 h, except for the first irrigation event on Column B2; equilibrium was not attained within 48 h in this case. Glyphosate desorption decreased successively with irrigation event number.

Equation [3] fitted well to the measured dissolved concentration as a function of time (0–48 h) (Figure 4a and 4b; Table 3). Coefficients of determination, R^2, varied between 0.87 and 0.98. The rate constant of desorption, k, was found to be in the range 0.57 to 1.19 h^{-1}, largest for the first irrigation event on Column B1. The equilibrium concentration, $C_{d,eq}$, was in the range 1.56 to 4.1 µg/L, decreasing successively with each additional irrigation event.

Table 3. Parameters and key data derived from experiments on splash-eroded particles. Rate constants (k), dissolved concentrations at equilibrium ($C_{d,eq}$), and coefficient of determination (R^2) obtained by fitting Eq. [3] to all data (reaction time t_r 0–48 h) from the two columns (B1 and B2) and three irrigation events. Relative desorption rate (α), intercept parameter (β), and R^2 obtained by fitting Eq. [1] to data for relatively short time scales: results based on the first three data points (t_r = 2, 10, and 60 min) and desorption rate based on the first two data points (t_r = 2 and 10 min).

Column	Irrigation no.	From Eq. [3], 0–48 h			From Eq. [1]			
					2–60 min			2–10 min
		k	$C_{d,eq}$	R^2	α	β	R^2	α
		h^{-1}	µg L^{-1}		% min^{-1}	%		% min^{-1}
B1	1	1.19	4.0	0.98	0.56	71	0.94	1.62
	2	0.75	2.19	0.91	0.54	79	0.98	1.09
	3	0.59	1.83	0.94	0.48	82	0.98	1.06
B2	1	0.57	4.1	0.92	0.41	81	0.99	0.79
	2	0.58	2.59	0.95	0.55	87	1.00	0.40
	3	0.99	1.56	0.87	0.50	73	0.98	1.10

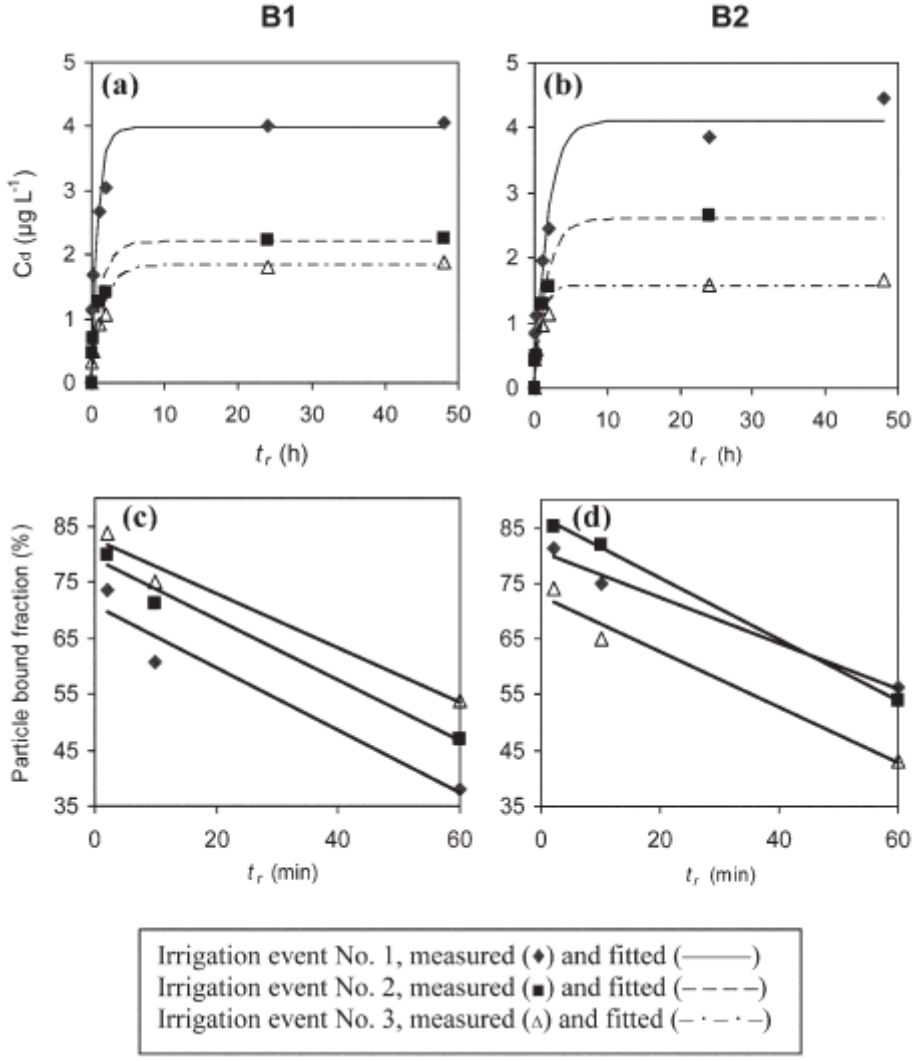
The equilibrium concentrations, $C_{d,eq}$, the previously estimated K_d = 8.4 × 102 L/kg, and the constant particle concentration $C_{particle}$ = 100 × 10^{-6} kg/L were used when calculating total concentrations (Eq. [4]), particle–bound fractions, and relative desorption rates (Eq. [1]). Accordingly, the fitted dissolved concentrations at equilibrium (Table 3) represent about 92 % of the total concentrations. The linear relationship Eq. [1] fitted well to the three data points representing the particle-bound fraction vs. time (2 to 60 min) after dissolving the splash–eroded particles, R^2 being in the range 0.94 to 1.00 (Figure 4c and 4d; Table 3). The relative desorption rates (α) were estimated to be in the range 0.41 to 0.56 % min^{-1} (average 0.51 % min^{-1}), with no systematic dependence on irrigation event or column (Table 3). Hence, at these rates, 8 to 11 % (average 10 %) would be desorbed during a 20–min time period. The rates tended to be slightly smaller than desorption rates measured in the leachate at a similar or somewhat shorter time scale (α for Columns B1 and B2 in Table 2, average value 0.61 % min^{-1}). For the period 2 to 10 min, the measured relative desorption rates were in the range 0.40 to 1.62 % min^{-1} (Table 3; average value 1.01 % min^{-1}), i.e., generally somewhat larger than for the period 2 to 60 min. This was expected also from the good fit of all the data to Eq. [3]. The rates obtained for the 2– to 10–min period were of the same order of magnitude, although generally larger than desorption rates measured in the leachate at a similar or somewhat longer time scale (α for Columns B1 and B2 in Table 2). Calculated from rates obtained for the 2– to 10–min period, 8 to 32 % (average 20 %) would desorb in 20 min right after the first fractionation. Overall, similar desorption rates were found for leached and splash–eroded particles when determined at similar time scales. This indicates that similar desorption processes were involved for the two types of particles.

The initial glyphosate concentrations (mg/kg) were somewhat higher on splash–eroded particles than on leached particles. For the first measurements on leached particles made 1.5 min after sampling, the range of concentrations was 4 to 24 mg/kg; for measurements on splash–eroded particles made 2.0 min after immersion, the range of estimated concentrations was 13 to 36 mg/kg. The concentrations decreased systematically with succeeding irrigation event for both types of particles. The splash–eroded particles may have been enriched with glyphosate when the water droplets evaporated on the collar after irrigation; however, concentrations on splash–eroded particles from the first irrigations (about 44 mg/kg according to Table 3 and Eq. [4]) were within a realistic range for the uppermost soil layer shortly after spraying. Thus, by assuming that the applied glyphosate was distributed in the uppermost 2– to 5–mm soil layer having a bulk density of 1.6 g/cm^3, an expected average glyphosate concentration of 55 to 22 mg/kg can be calculated for the layer.

The splash–eroded particles were air dry, and the particle–bound fraction of the glyphosate must therefore have been close to 100 % right before the particles were immersed in water (at $t_r = 0$). At t_r = 2.00 min, however, the particle–bound fraction had already decreased to between 74 and 85 % (Figure 4c and 4d). It is difficult to model this very rapid decrease as a function of time when seen at the shorter time scales. It indicates that a fraction (up to 26 %) of the glyphosate could have been very weakly bound. Physical effects of the immersion may also have affected the rapid glyphosate release.

From the linear models shown in Figure 4c and 4d, it can be calculated that about 60 to 76 % of the glyphosate was still particle bound 20 min after immersion of the particles in water. The values are of a similar magnitude as the estimated particle–bound fractions in the leachate 20 min after leaching (45–79 %, cf. above). In the study on leachate, it is probable that desorption had taken place in wet fractions of the soil columns before leaching and from leached particles in the leachate before the first fractionation. This may to some extent have reduced the initially measured fraction of particle–bound glyphosate and the measured desorption rates.

Figure 4. Desorption of glyphosate in suspensions containing splash–eroded soil particles from two soil columns, B1 (left) and B2 (right): (a) and (b) concentration of dissolved glyphosate (C_d) monitored at long time scales of reaction ($0 < t_r \leq 48$ h), the curves represent least squares fits of Eq. [3] to data points; (c) and (d) particle–bound fraction monitored at short time scales (2–60 min), the lines represent least squares fits of Eq. [1] to data points.



Can Desorption Kinetics be Ignored in Glyphosate Transport?

Bold et al. (2003) investigated the significance of kinetics in contaminant transport using an intraparticle diffusion model to account for the kinetic contaminant–particle interaction. They showed by sensitivity analysis that kinetic limitations of contaminant–particle interactions have to be taken into account for $0.01 < D_a < 100$. They also concluded that for $D_a < 0.01$, desorption of contaminants from particles is so slow that it can be neglected.

A range of possible outcomes of D_a for the present column experiments was estimated based on desorption rate coefficients obtained in the 0– to 48–h experiments on splash–eroded particles (k values in Table 3). The fluid velocity inside the column depends on whether it is moving through macropores or the matrix. Two extreme boundaries could be: (i) transport exclusively through a water–filled continuous macropore from the surface to the bottom of the column, and (ii) transport exclusively thought the soil matrix. For extreme (i), a continuous macropore with a diameter of 0.6 cm (area = 0.28 cm^2), and steady–state condition, the irrigating rate (1060 cm^3/h) would give rise to an average fluid velocity of approximately 3700 cm/h. For extreme (ii), a homogeneous soil matrix (column area = 707 cm^2), steady-state condition, and a water content equal to field capacity (about 30 %), the irrigation would give rise to an average fluid velocity of approximately 5 cm/h. For extreme (i), the D_a would be

in the range of 0.01 to 0.02 (cf. Eq. [4]), depending on the value of *k*. For extreme (ii), the D_a would be in the range of 6 to 12. These intervals, even the one for homogeneous matrix flow, are within the critical range estimated by Bold et al. (2003), indicating that kinetic limitations of glyphosate–particle interactions have to be taken into account in describing the transport. In reality, bypass flow and glyphosate transport below the 25–cm depth took place almost exclusively in earthworm channels in the size range 2 to 8 mm (Gjettermann et al., 2009), indicating conditions much closer to extreme (i) than (ii). Although we realize that the measured rates are not necessarily representative of the conditions throughout the soil columns, the results of this analysis indicate that particle mobilization and particle–facilitated transport could play a critical role in pesticide leaching under such conditions.

The interaction between contaminants and mobile particles has, in many studies, been described as an instantaneous equilibrium process (e.g., Prechtel et al., 2002; Villholth et al., 2000). To our knowledge, no study has described the importance of desorption kinetic behavior of contaminants in structured soil with special attention to facilitated transport. Turner et al. (2006), however, revealed that Cs desorption from illite particles was slower than Sr desorption and demonstrated that this difference in desorption kinetics resulted in greater colloid–facilitated transport of Cs in columns packed with a quartz porous medium. They estimated D_a to be in the range of 0.00035 to 0.086 for Cs and 0.97 to 2.0 for Sr. Van de Weerd and Leijnse (1997) also found that desorption of Am from humic particles was a slow process that could only be described by taking into account a kinetic interaction between Am and humic particles. These findings combined with the current investigations show that it is important to consider desorption kinetics as an integral part of the transport process when considering particle–facilitated transport of glyphosate and other non-instantaneously desorbing contaminants.

Conclusion

Glyphosate desorbed with similar fractional rates from leached and from splash–eroded particles (>20 nm) when investigated at similar relatively short time scales. Thus, 7 to 20 % of the total amount of leached glyphosate (average 12 %) desorbed in 20 min shortly after leaching, while on average between 10 and 20 % desorbed from splash-eroded soil particles in suspension in 20 min shortly after immersion. The similarities support the view that the particles investigated and the processes of desorption were similar for the two types of material. Concentrations of glyphosate on leached particles were always somewhat lower than concentrations on splash–eroded particles.

Equilibrium concentrations were generally obtained within 5 to 10 h in suspensions containing splash–eroded particles. Hence, depending on the time of fractionation of the collected samples (in the interval 0–10 h), very different relative amounts of particle–bound glyphosate may be found; to quantify particle–facilitated glyphosate transport, the water and solid phases should be separated immediately after leaching. Furthermore, an analysis of the Damköhler number indicates that desorption kinetics is important for glyphosate transport and for the significance of particle–facilitated transport.

2. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a leaching experiment with glyphosate in soil columns. The desorption of glyphosate from soil particles and its effect on interpretation of leaching experiments was in the focus of the study. Not all necessary information was reported to check the validity of the results (no mass balances, study set-up not clearly described, insufficient information on soil properties and soil origin, test item not sufficiently described, Temperature not provided, molecular identity of desorbed radioactivity not determined).

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.4.1.1
Report author	Gjettermann, B. et al.
Report year	2011
Report title	Evaluation of Sampling Strategies for Pesticides in a Macroporous Sandy Loam Soil
Document No	Soil & sediment contamination (2011), Vol 20, No 5–8, pp. 986–994
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Not sufficient information available to check validity of the results, scope of the study is not on leaching of glyphosate itself but on evaluating the usage of a dye to improve sampling strategies)

2 Full summary of the study according to OECD format

It is not straightforward to sample and demonstrate the presence and transport of pesticides in heterogeneous soil. Following leaching experiments with four differently structured 50–cm–long soil columns (tilled and untilled soil), the objective of this study was to investigate the extent that visual tracing of the dye Brilliant Blue could support in soil sampling for two strongly sorbing pesticides (^{14}C–labeled glyphosate and pendimethalin). About 830 samples were collected. No pesticide was found below 10–25 cm depth by random sampling, even though 0.21–0.31 % of the applied amounts were leached, and 0.18 % of the soil volume was sampled. With similar sampling efforts, the pesticides could generally be traced throughout the columns by sampling from stained soil volumes, only. None of the two particular sampling strategies for pesticides produced accurate mass balances or balances that were obviously better than the other. No pesticide was detected outside stained soil volumes, except for glyphosate in one sample. Below 30 cm, stained soil comprized on average 5 % of the total soil volume, leaving 95 % as expectedly pesticide–free. The results suggest that much more efficient sampling for sorbing pesticides can be obtained by using the dye and focusing on stained soil volumes.

Materials and methods

Soil Columns

The macroporous Rorrendegaard sandy loam soil investigated in this experiment is developed on till from the Weichselian glaciation. The contents of coarse sand (200–2000 µm), fine sand (20–200 µm), silt (2–20 µm), and clay (<2 µm) in the upper 30 cm is 29 %, 40 %, 18.5 %, and 12.5 %, respectively, and the organic C content is 1.2 %. At 50 cm depth, the number of vertically oriented earthworm channels (diameter: 3–8 mm) is typically in the range 200–600 m^{-2}. The soil has previously been described in detail by Petersen et al. (2001) and Gjettermann et al. (2009).

Undisturbed soil columns (diameter: 30 cm; 0–60 cm soil depth) were sampled in late autumn from two experimental plots with different tillage treatments (A and B). For each of the previous nine years the same cereal crop (wheat or barley) had been grown in the plots. Plot A had not been tilled for one year, and it had not been subjected to deep (>4–6 cm), loosening tillage for eight years. Plot B had been under traditional tillage (including annual ploughing) for at least nine years. It had been ploughed and drilled for wheat one month before sampling, and a new wheat crop had just been established. Treatment A (untilled) gave rise to a relatively stable soil structure with vertically oriented earthworm channels from the partially covered surface (old wheat stubble, weeds, and moss) to the bottom of the columns. Treatment B (tilled) did result in a more variable structure with stubbles being heterogeneously incorporated in the plough layer (0–25 cm). Fewer vertically oriented earthworm channels penetrated all the way to the surface. Columns (two per treatment) were manually excavated and encapsulated with

polyurethane to stabilize and seal the walls. Care was observed not to disturb the surface structure. The columns were trimmed to 50 cm length from the bottom end, avoiding sealing macropores, and placed on a galvanized metal grating. The columns were sealed with plastic foil at the upper end and stored at 2–3°C whenever not used in the experiments.

Leaching Experiments

The leaching experiments have been described in detail by Gjettermann et al. (2009) and were in brief as follows: Columns were rewetted by irrigation one day before pesticide application. Each pesticide was applied uniformly to the surface of one column per treatment. The pesticides were applied in doses similar to the ones used in agriculture, i.e. 12.51 mg glyphosate/column and 14.24 mg pendimethalin/column. Glyphosate was taken from a stock solution made from the commercial product Roundup Bio, ^{14}C–glyphosate, and blank formulation (all from Monsanto). The stock solution had a specific concentration activity of 0.80 MBq/mg. It contained both glyphosate and its major metabolite, AMPA (aminomethylphosphonic acid, CH_6NO_3P) (2.034 g/L in total) distributed on ^{14}C–glyphosate (4.4 %), unlabeled glyphosate (93.5 %), and AMPA (2.1 %). Pendimethalin was taken from another stock solution made from the commercial product Stomp mixed with ^{14}C–pendimethalin (both from BASF). This stock solution had a specific concentration activity of 4.55 MBq/mg. It contained ^{14}C–pendimethalin (4.2 %) and unlabeled pendimethalin (95.8 %).

Leaching was driven by irrigation water having a composition similar to rain water (Gjettermann et al., 2009). The water was applied uniformly at a fixed intensity (15 mm/h) to the top of the columns through a rotating irrigation device. Each column received three 2.0 hours irrigation events 5, 8, and 12 days after rewetting, respectively. Thus 30 mm of irrigation water was applied in each event, corresponding approximately to 7.6 % of the total soil pore volume. A 15 mm/h rain event in 2 h may be considered as an extreme for Danish conditions expected to occur about once every 10 years, even though short–time rain intensities are frequently much higher (Madsen et al., 2009). Water was allowed to drain freely from the bottom of the columns. Pesticide contents in the leachate were determined by measuring the ^{14}C–activity with liquid scintillation counting. Brilliant Blue was applied to the four columns (one per combination of soil treatment and pesticide) after the pesticide–leaching experiments. The dye was applied in aqueous solution (4.0 g/L) as a standard irrigation (i.e. 15 mm/h in two hours) after rewetting.

Sampling

Samples were obtained from 9 or 10 separate column sections prepared 1–2 days after dye application. Initially, the columns were sectioned into 7 or 8 depth intervals (cylindrical slices). All columns were cut at 15, 20, 25, 30, and 40 cm depth using a steel thread or a narrow–bladed saw to minimize smearing. Two more cross-sections were made at depths below 15 cm in three columns, whereas only one cross–section was obtained in one column representing treatment B. All cross–sections were carefully cleaned for traces of soil materials and dye being smeared during the cutting procedure. They were then subjected to intensive diffuse light and photographed using a 3.0 Mpx camera.

Soil sampling within the slices was conducted according to three different strategies: (1) 5–10 soil samples were taken from different, intensively blue–colored soil volumes in the vicinity of dyed (flow active) macropores; (2) 10 core samples were taken randomly within non–colored areas (as determined at the top–end); and (3) 10 completely randomized core samples were collected. Thus, typically 25–30 soil samples were taken per slice. However, due to extensive staining making it difficult to avoid blue soil, sampling according to strategy 2 was not performed above 5–10 cm depth. Furthermore, special procedures were followed in the uppermost slice. Following strategy 3, 10 samples were taken randomly in the 0–0.5 cm and the 0.5–1.5 cm depth intervals (the uppermost 1.5 cm was not included when sampling below). With strategy 1, sampling started at 0.5 cm depth (treatment A) or 1.5 cm depth (treatment B) because it was not possible to identify flow active macropores in the uppermost layer. Sampling according to strategy 1 was accompliced by scraping 1–2 mm of stained soil from the inside of biopores or cracks using a spatula. Sampling following strategies 2 and 3 was supported by coordinates generated by a random number generating program. It was done using a drill (diameter: 4.0 mm) throughout the entire soil layer. Hence with 10 samples per layer, roughly 0.18 % of the total soil volume was sampled. A total of 185–240 soil samples were obtained per column. Similar samples, according to the sampling strategy, were pooled within each soil layer.

Analyses
The pooled soil samples were air–dried, grounded using a ball–mill (350 rpm for 1 min), and mixed carefully. ^{14}C–activity was measured by LSC after heating of 250 mg soil to 800°C in a constant flow of oxygen (Packard Sample Oxidizer Model 507) followed by ^{14}C–CO_2 absorption by Carbosorb E (Packard) and Permafluor E+ (Packard). Two replicates were analyzed from each pooled soil sample. The concentrations of pesticides in soil were calculated from the specific concentration activity of the ^{14}C–labeled pesticides and the relationship between labeled and unlabeled pesticide in the applied pesticide solutions. The detection limits for glyphosate and pendimethalin in soil were 0.01 and 0.005 mg/kg, respectively.

All the blue-stained representations of flow patterns appearing on photos of the cross–sections were manually transferred to transparent plastic sheets, and the new binominal representations (images showing either color or no color) were digitized using the procedures described by Petersen et al. (1997). The only distinction made in this process was whether or not blue dye was visible on the photos as evaluated by one person. The photos were handled in systematic order governed by a random serial number assigned to each. The digitized images were then scaled in two mutually perpendicular directions, and the fractional dye–stained area (DC, %) was determined using the image processing program ImageJ (Collins, 2007). The thickness of the uppermost completely dyed in soil layer (maximum depth with DC = 100 %) was measured. Fractional volume of dyed soil in a given soil layer was calculated as the average of DC observed at the top and bottom ends.

Mass balances for the pesticides were established based on sampling strategy 1 and 3, respectively. For strategy 1, measured pesticide concentration in soil was multiplied with fractional volume of dyed soil and by the mass of soil to get the pesticide content of a given soil layer. Concentrations obtained with strategy 3 were applied in the uppermost 0.5 cm (treatment A) or 1.5 cm (treatment B) layers in the lack of strategy 1 observations. For strategy 3, the pesticide content of a given soil layer was obtained by multiplying the measured concentration by the mass of soil. A dry bulk density of 1.60 g/cm^3 (average value for all columns) was applied throughout in these calculations.

Results and Discussion

Distribution of Pesticides
Significant pesticide concentrations (above the detection limits) could be traced all the way through the columns by sampling strategy 1, except for glyphosate in treatment B at 25–50 cm depth. The concentrations generally decreased with depth (Figure 1). Below 30 cm depth, the pendimethalin concentrations approached the detection limits.

No significant amounts of the pesticides were found using sampling strategy 2, except in one sample (glyphosate in treatment A at 20–25 cm depth, cf. Figure 1). Thus, as a rule, pesticides were not detected by sampling outside blue-stained areas, not even at 5–10 cm depth, where considerable amounts of pesticide were found with the other sampling strategies. This is particularly noteworthy because three irrigations were carried out after pesticide application prior to application of the dye solution. Stronger sorption of the pesticides than of the dye may be part of the explanation. For the investigated (top–) soil, Gjettermann et al. (2009) reported soil–water partition coefficients (K_d–values) of 503 L/kg for glyphosate and 242 L/kg for pendimethalin. Hence, both pesticides sorb strongly to the soil material. For Brilliant Blue, Flury and Flühler (1995) have reported much smaller K_d–values in the range 0.19–5.78 L/kg. Somewhat stronger sorption than measured by Flury and Flühler has been found for soils rich in clay minerals (German–Heins and Flury, 2000; Ketelsen and Meyer–Windel, 1999). It should be noticed that the sampling strategy being based on coring from upper surfaces of the slices does not completely exclude the inclusion of blue-stained soil material.

It was not possible in any case to trace the pesticides all the way through the columns by using strategy 3. With treatment A, glyphosate was not found below 10 cm depth and pendimethalin not below 20 cm. The pesticides tended to be found at slightly greater depths with treatment B. However, no significant concentrations were found below 25 cm depth. Hence with completely randomized sampling, pesticides

were not found in significant amounts in the soil below 10–25 cm depth even though significant amounts (0.21–0.31 % of applied) were leached (Table 1) and 0.18 % of the soil volume was sampled. It was noticed that the strategy generally led to the inclusion of some blue–colored soil in the pooled samples when applied above 15–20 cm. Pesticide concentrations decreased strongly with depth in the 0–5 cm depth interval (Figure 1). By far the highest concentrations were found in the uppermost 5 mm of soil being completely dyed–in for both tillage treatments. Glyphosate concentrations measured in this layer were 8.59 and 10.5 mg/kg for the A and B treatment, respectively, while the corresponding numbers for pendimethalin were 24.6 and 14.1 mg/kg. Significant pesticide concentrations measured according to strategy 1 were always higher than concentrations obtained at the corresponding depths by strategy 3 (Figure 1). The differences between the two repeated measurements of pesticide concentrations of the soil samples were negligible (not shown).

Figure 1. Glyphosate and pendimethalin concentration (C_G and C_P, respectively) as a function of soil column depth obtained by the sampling strategies 1 (dyed), 2 (non–dyed), and 3 (random). Data for the two tillage treatments (A and B, average of 2 repeated measurements). Notice the broken 2nd axes.

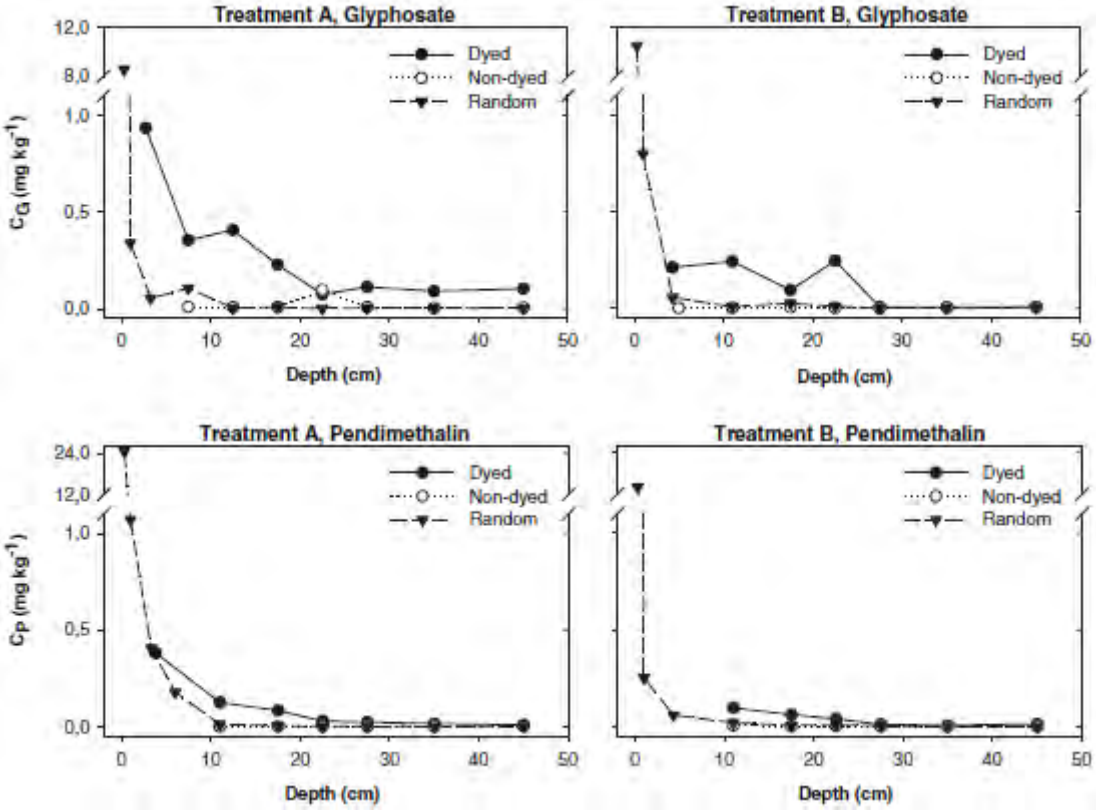


Table 1. Amounts of pesticides retrieved in columns estimated from two different column sampling strategies (1 and 3), and amounts lost with leachate (% of applied)

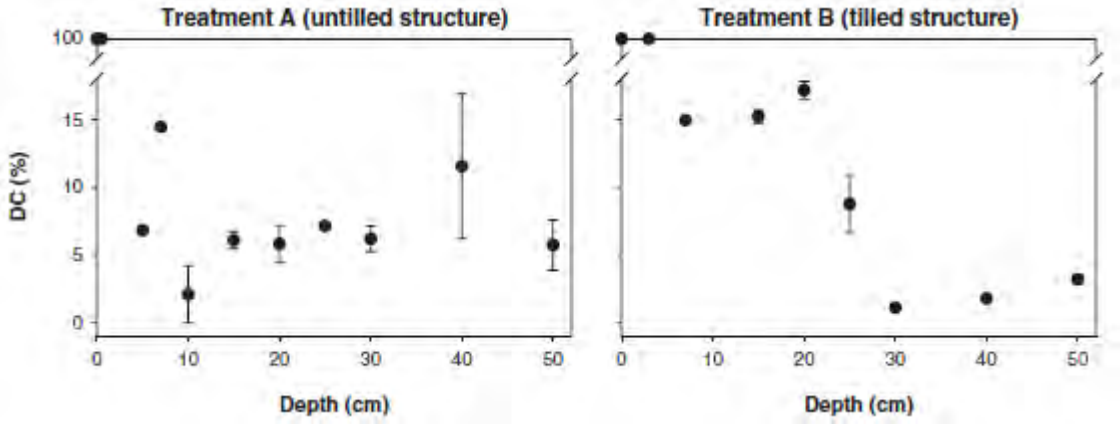
	Treatment A		Treatment B	
	Strategy 1	Strategy 3	Strategy 1	Strategy 3
Glyphosate found in column	63	50	67	61
Glyphosate leached	0.31	0.31	0.21	0.21
Pendimethalin found in column	110	123	65	63
Pendimethalin leached	0.23	0.23	0.21	0.21

Applied amounts per column, Glyphosate: 12.5 mg; Pendimethalin: 14.2 mg.

Dye Patterns

The thickness of the uppermost completely dyed–in soil layer was about 0.5 cm for treatment A and about 3 cm for treatment B. Thus, the fractional volume of dyed soil was 100 % above 0.5–cm depth in columns subjected to treatment A and above 3 cm in columns subjected to treatment B. The fractional area covered with dye (DC) rapidly decreased with depth right below these depths. In the topsoil, DC tended to be larger for treatment B than for treatment A, whereas the opposite trend was observed in the subsoil (Figure 2).

Figure 2. Fractional area covered with dye (DC) at different soil depths. Average values for each of the two tillage treatments (A and B, n = 2) with the range shown by the bars. Notice the broken 2nd axis.



Below 30–cm depth, the stained flow pathways were mainly concentrated around vertically oriented earthworm channels comprising a relatively small fraction of the total soil volume. The dye had typically penetrated less than 1–2 cm into the soil matrix from these flow active macropores (4–10 per column). This is more than previously reported from studies conducted under field conditions (Petersen et al., 1997), probably due to the wet conditions prevailing in the columns with drainage at atmospheric pressure from 50–cm depth. For treatment A, a considerable fraction of the stained soil volume was found at the column walls in connection with large flow–active macropores that were cut during the excavation process. On average for all columns, the fractional volume of dyed soil below 30–cm depth comprized 5 %, leaving about 95 % as unstained and expectedly pesticide free.

Mass Balances

Under typical field conditions, half–lives (DT_{50} values) for glyphosate and pendimethalin are about 12 and 90 days, respectively (PPDB, 2010). However, under the low temperatures prevailing in the columns, both pesticides are expected to be slowly degradable. Furthermore, any non–volatile metabolites containing the ^{14}C would be included in the measurements. The columns were sealed with plastic foil, except when used in the experiments. Hence, losses due to degradation and evaporation are expected to be very small. Also, the fraction of applied pesticide (^{14}C) being leached was small (0.21–0.31 %) and unimportant for the mass balance (Table 1). Consequently, we expected a recovery close to 100 % based on the soil sampling alone. We found between 50 and 123 % with sampling strategy 3, and between 63 and 110 % with strategy 1, respectively (Table 1). Hence, none of the sampling strategies resulted in the expected (slightly less than) 100 % recovery in the columns although the balances tended to be better for strategy 1 than 3.

Both methods of constructing a mass balance obviously had large uncertainties. The largest concentrations (and amounts) of pesticide were found in the uppermost 0.5 cm of the profile, and the major uncertainty appears to be the sampling of this top layer. If, for instance, the actual depth of sampling was 6 mm rather than 5, the error to the mass balance would be 8–19 % for the investigated columns. However, any difference in mass recovery obtained with the two sampling strategies is not related to this uncertainty, since the same sampling of this uppermost thin soil layer was used in both cases. The method based on sampling strategy 1 does correctly include some pesticide from lower parts

of the columns. However, it may be biased if sampling for the pesticides did not fully represent the stained soil volumes. The mass recovery was of the same order of magnitude or considerably better than that obtained by Flury et al. (1995) working with a systematic very dense two–dimensional sampling scheme for herbicides in structured field soil.

Tracing Pesticides in Macroporous Soil

The magnitude of preferential flow contributing to the leaching of pesticide is difficult to quantify from studies on soil samples. Prichard et al. (2005) investigated the predominant source of pesticide residues detected in domestic wells located in an area with cracking clay soil. Although preferential flow through macropores within the field was a potential pathway, pesticide residues were retained in the top 15 cm of the soil. They deduced that the contribution of preferential transport to leaching was insignificant, despite the fact that lack of correlation of pesticide data between soil and water samples has previously been documented for soils with preferential flow (e.g. Sanchez et al., 2006; Laabs et al., 2000; Malone et al., 2000). Sanchez et al. (2006) found high concentrations of methidathion in the upper 25 cm of a soil profile but very low concentrations below this depth. They attributed high concentrations found sometimes in leachates from deeper layers to preferential flow processes. Laabs et al. (2000) similarly suggested that absence of pesticide residues in soil at depths below 25 cm combined with observed leaching indicated non–chromatographic transport of these substances in the soil profile. Malone et al. (2000) concluded that a sampling strategy including the mixing of horizontal slices with dimension 3.75 × 30 × 30 cm was not well suited to trace the movement of pesticides in the subsoil of structured soils. The present study support the interpretations made by Laabs et al. (2000), Malone et al. (2000), and Sanchez et al. (2006).

As expected, visible traces of Brilliant Blue were indicators for the occurrence of the pesticides in the soil. The measurements strongly suggest that both pesticides were transported exclusively within some fraction of the stained soil volume. With transport concentrated on a few macropores as in the subsoil of the present study, it should be possible to sample virtually all dye (and pesticide) at a given depth. Further developed, strategy 1 could therefore be used as the basis for better quantification of strongly sorbing pesticides in macroporous subsoil profiles. It is likely that the dye tracer should be applied under similar conditions (e.g. soil structure, soil moisture content, irrigation/precipitation amount and intensity) as the pesticides themselves.

Conclusions

Visible traces of Brilliant Blue transported under similar conditions as the two pesticides indicated the occurrence of both pesticides in the soil. The results suggest that efficient sampling for sorbing pesticides can be obtained by using the dye and focusing on stained soil volumes. None of the investigated sampling strategies led to mass balances that were accurate enough to detect small but significant amounts of pesticide leaching.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a leaching experiment on soil columns with a dye and glyphosate and pendimethalin. Some important information about study conditions are missing: agricultural use of the soil, temperature, soil parameters, details on analytics and on substance identification, sample storage conditions before analysis.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Gómez Ortiz, A., et al.
Report year	2017
Report title	Sorption and desorption of glyphosate in mollisols and ultisols soils of Argentina
Document No	Environmental Toxicology and Chemistry, Vol. 36, No. 10, pp. 2587–2592, 2017
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Reliable with restrictions (Soils were from outside EU (Argentina))

2. Full summary of the study according to OECD format

In Argentina, glyphosate use has increased exponentially in recent years as a result of the widespread adoption of no-till management combined with genetically modified glyphosate-resistant crops. This massive use of glyphosate has created concern about its potential environmental impact. Sorption–desorption of glyphosate was studied in 3 Argentinean soils with contrasting characteristics. Glyphosate sorption isotherms were modeled using the Freundlich equation to estimate the sorption coefficient (K_f). Glyphosate sorption was high, and the K_f varied from 115.6 to 1612 $mg^{1-1/n}L^{1/n}/kg$. Cerro Azul soil had the highest glyphosate sorption capacity as a result of a combination of factors such as higher clay content, cation exchange capacity, total iron, and aluminum oxides, and lower available phosphorus and pH. Desorption isotherms were also modeled using the Freundlich equation. In general, desorption was very low (<12%). The low values of hysteresis coefficient confirm that glyphosate strongly sorbs to the soils and that it is almost an irreversible process. Anguil soil had a significantly higher desorption coefficient (K_{fd}) than the other soils, associated with its lower clay content and higher pH and phosphorus. Glyphosate high sorption and low desorption to the studied soils may prevent groundwater contamination. However, it may also affect its bioavailability, increasing its persistence and favoring its accumulation in the environment. The results of the present study contribute to the knowledge and characterization of glyphosate retention in different soils.

Materials and Methods

Soils

Soil samples were taken from agricultural fields of Cerro, Tandil, and Anguil, The studied soils are located in areas of high agronomic land use and have different edaphoclimatic conditions. Four composite soil samples from the top 15 cm of topsoil were collected from each field. Samples were homogenized, air-dried, and sieved to a particle size of 2 mm. A subsample of each replicate was used for physicochemical analysis of the soils (see Table 1). Particle size distribution was measured using the pipette method; organic carbon content was measured according to the Walkley-Black method; CEC was determined by displacement with 1M ammonium acetate at pH 7; soil pH was measured by electrode in a soil:water ratio of 1:2.5; available phosphorus (P-Bray) was determined according to Bray and Kurtz; total iron (Fe) was determined by atomic absorption spectrophotometry; and exchangeable aluminum (Al) was measured according to the Al method.

Table 1. Main characteristics of the sampled locations and soil physicochemical properties

	Soil		
	Anguil	Cerro Azul	Tandil
Altitude (masl)	157	280	256
Annual average temperature (°C)	15.3	20.5	13.7
Mean annual precipitation (mm)	760	1844	993
Latitude	36°35′54″S	27°39′42″S	37°36′0.1″S
Longitude	63°58′31″W	55°26′25″W	59°04′29″W
Soil type	Mollisol	Ultisol	Mollisol
Main textural class	Loam	Clay	Loam
pH	6.3 A	4.9 C	5.4 B
Clay (%)	14.7 C	78.5 A	23.0 B
Silt (%)	45.6 A	15.4 C	40.9 B
Sand (%)	39.6 A	6.1 C	36.0 B
Organic carbon (%)	1.3 C	2.4 B	3.4 A
P-Bray (mg/kg)	29.6 A	7.6 C	17.1 B
CEC (meq/100 g)	17.4 C	20.6 B	25.2 A
Ca^{2+} (meq/100 g)	8.1 B	5.6 B	14.7 A
Mg^{2+} (meq/100 g)	2.9 B	3.2 B	5.1 A
K^{+} (meq/100 g)	3.2 A	1.2 A	2.8 A
Na^{+} (meq/100 g)	0.3 A	0.2 A	0.5 A
Al^{3+} (meq/100 g)[b]	0.15 B	0.69 A	0.11 B
Total Fe (%)[b]	1.08 B	8.40 A	0.81 B

[a]Different letters indicate differences among soils ($p < 0.05$).
[b]From Gianelli et al. [8].
CEC = cation exchange capacity; P-Bray = available phosphorus.

Chemicals

Stock solutions for the standard curves and the isotherm study solutions were prepared using analytical pure glyphosate (99.9%). For analytical procedures HPLC - grade methanol and HPLC - grade acetonitrile were purchased commercially. Nanopure water was obtained by purifying demineralized water.

Sorption isotherms

The sorption isotherms were performed according to the batch equilibrium method. First, 2 g of soil was shaken with 40 mL of a 0.01M $CaCl_2$ solution. After 24 h, glyphosate was spiked at different initial concentrations (C_0): 0, 0.5, 1, 5, 10, and 20 mg/L. The suspensions were shaken for another 24 h at constant temperature (20°C). Afterward, tubes were centrifuged, and an aliquot (3 mL) of the aqueous solution was analyzed for glyphosate concentration. Each initial concentration was tested by duplicate for each soil sample. These laboratory duplicates were averaged, finally obtaining data of 4 replicate isotherms per soil.

Desorption isotherms

The desorption isotherms were performed using the spiked soil with the C_0: 5 mg/L solution from the sorption isotherm studies. This concentration is equivalent to the commonly used dose in the field per year (6 L/ha/yr) considering 5 cm depth of soil. After the sorption study, the aqueous phase was carefully discarded to avoid any soil loss during manipulation. The volume of the solution that was removed was replaced with 0.01M $CaCl_2$, and the soil was re-suspended and shaken at a constant temperature for another 24 h. Then, samples were centrifuged and glyphosate was measured in the aqueous solution to quantify the glyphosate that desorbed from the soil matrix. This procedure was repeated at 48 and 72 h by removing the aqueous solution and adding again $CaCl_2$. The amount of adsorbed glyphosate at each desorption step was calculated as the difference between the initially adsorbed concentration and the desorbed amount.

Glyphosate analysis

To quantify the remaining glyphosate in the aqueous solution, an aliquot of 3 mL was transferred to a 15-mL polyethylene flask, and 0.5 mL of borate buffer solution (0.04 mM $Na_2B_4O_7 \cdot 10\ H_2O$, pH 9) and

0.5 mL of acetonitrile were added. Samples were shaken vigorously, then derivatized with 0.5 mL of 9-fluorenylmethylchloroformate dissolved in acetonitrile (6 g/L) and incubated overnight at room temperature. As a cleanup step, CH_2Cl_2 was added to the samples to remove any organic impurities and minimize matrix effects. The aqueous fraction was separated from the organic solvent by centrifuging. The supernatant was collected and filtered and then analyzed by liquid chromatography coupled to a tandem mass spectrometer (MS/MS).
Chromatographic analysis was carried out using a Waters ACQUITY1 ultra-performance liquid chromatography system. Target molecules were detected by a triple quadrupole MS/MS Quattro Premier XE (Waters). The equipment was operated with an electrospray ionization source in positive mode. To take into account the matrix effect of each soil, standard curves were prepared using a background solution of each soil obtained after shaking with $CaCl_2$ 0.01 M. After separating the solid phase from the aqueous phase, the solution was used to prepare each point of the standard curves by adding the corresponding glyphosate concentration. A sample without any glyphosate was also analyzed to check the concentration of presorbed glyphosate. In all cases, the background solution had non-detectable levels of glyphosate. The limit of detection was 0.1 µg/L, and the limit of quantification was 0.5 µg/L.

Sorption modeling
Following the experimental design proposed by the Organisation for Economic Co-operation and Development Guidelines for the Testing of Chemicals, Test No. 106, the measured glyphosate in the aqueous solution was used to estimate the remaining glyphosate sorbed to the soil (Cs).

$$C_s = M_s/M_{soil} = (C_0 - C_w)V_0/M_{soil} \quad (1)$$

where C_s is the concentration of glyphosate adsorbed to the soil at equilibrium (mg/kg), M_s is the mass of glyphosate sorbed to the soil at sorption equilibrium (mg), M_{soil} is the dry mass of the soil sample (kg), C_0 is the initial tested concentration of glyphosate in contact with the soil sample (mg/L), C_w is the analytically measured mass concentration of glyphosate in the aqueous phase at sorption equilibrium (mg/L), and V_0 is the initial volume of the aqueous phase in contact with the soil sample (mL).

The Freundlich equation was used to describe sorption and desorption isotherms

$$C_s = K_f C_w^{1/n} \quad (2)$$

where K_f ($mg^{1-1/n} L^{1/n}/kg$) is the Freundlich sorption coefficient and 1/n is the Freundlich exponent (K_f and 1/n will hereafter refer to sorption and K_{fd} and $1/n_d$ to desorption). The K_f coefficient indicates the affinity of the substance to the soil matrix, and 1/n indicates the degree of linearity between the amounts adsorbed and the concentration in the solution.

The hysteresis coefficient (H) for the sorption/desorption isotherms was calculated according to the equation

$$H = (1/n_d)/(1/n) \quad (3)$$

where 1/n and $1/n_d$ are the Freundlich slopes obtained for the sorption and desorption isotherms, respectively.

Statistical analysis
For the isotherm sorption and desorption studies, each soil sample was analyzed in duplicate. Laboratory duplicate samples were averaged, and the isotherm curves were then modeled using the NLIN procedure of SAS software. Statistical analyses of the soil properties and of the estimated sorption and desorption parameters were performed using a completely randomized design with 4 replicates per soil. Analysis of variance was performed using the PROC GLM procedure to evaluate differences in the Freundlich parameters at a significance level of 5%.

Results and Discussion

Soil characteristics

Tandil and Anguil soils correspond to a loam texture, while Cerro Azul is classified as clay. Cerro Azul soil had a significantly higher clay content, followed by Tandil and then Anguil ($p<0.05$). On the other hand, the organic carbon content and CEC were significantly higher in Tandil, followed by Cerro Azul and Anguil soil ($p<0.05$). Anguil soil had significantly higher pH and P-Bray values than Tandil and Cerro Azul ($p<0.05$). Regarding the exchangeable cations, significant differences were observed only for Ca^{2+} and Mg^{2+}, following the order Tandil>Cerro Azul>Anguil ($p<0.05$). The highest Al^{3+} and Fe contents were found in Cerro Azul soil, denoting its Ultisol origin.

Sorption isotherms

Glyphosate sorption and desorption isotherms are shown in Figure 1. The K_f values for glyphosate were very high and ranged from 115.6 to 1612 (Table 2), being generally higher than those usually reported in the literature. Glyphosate K_f was significantly higher in Cerro Azul compared with Tandil and Anguil soil ($p<0.05$) (Table 2). The values of 1/n ranged from 0.4 to 0.8 (Table 2). Isotherms exhibited an L-type (1/n<1) curve according to the classification of Giles et al. This indicates that sorption is not constant as the concentration of the herbicide increases and that the sorption sites become saturated with increasing glyphosate concentration. In the case of Tandil and Anguil soils, glyphosate was almost completely sorbed to the soil at low initial concentrations; and as the concentration increased, sorption became less efficient (Figure 1). Isotherms of this type occur when the adsorbent has a high initial affinity for the herbicide until the sorption sites become saturated. In contrast, the Cerro Azul isotherm exhibits an almost linear relationship between the amount of sorbed glyphosate and its concentration at equilibrium in the solution (Figure 1), with 1/n values closer to 1 (Table 2). Therefore, it can be assumed that the number of sorption sites remains almost constant even at high concentrations. The reason glyphosate sorption was significantly higher in Cerro Azul soil can be explained by the soil's textural composition. At the soil's pH, the negatively charged glyphosate molecule can be complexed with cations released from the clays via a cation exchange reaction with solution protons. On the other hand, Fe and Al oxides also play an important role in glyphosate sorption because the phosphonate group of glyphosate establishes coordination links with the interchangeable surfaces of Fe^{3+} and Al^{3+} cations. In this sense, the lower soil pH of Cerro Azul could also be favoring sorption via Fe and Al oxides because as the pH decreases, these oxides become more protonated, increasing the affinity toward the negatively charged glyphosate molecule. Therefore, aside from cation exchange reactions, glyphosate may strongly bond through ligand exchange with the metal ions (Fe or Al) at the surface of the clay minerals. This mechanism has been proposed for other organic weak acids, and hence it can be applied to glyphosate.

Table 2. Glyphosate Freundlich sorption and desorption parameters for Anguil, Cerro Azul, and Tandil soils[a]

	Sorption			Desorption[b]							
							Percentage[c]				
Soil	K_f ($mg^{1-1/n}L^{1/n}/kg$)	$1/n$	r^2	K_{fd} ($mg^{1-1/n}L^{1/n}/kg$)	$1/n_d$	r^2	1°	2°	3°	Total[d]	H[e]
Cerro Azul	1612.0 (859.8) A	0.8 (0.5) A	0.97–0.99	101.2 (2.9) C	0.01 (0.0) C	0.99–0.99	0.7 (0.1)	0.6 (0.0)	0.5 (0.1)	1.6 (0.8)	0.01 (0.0) B
Tandil	412.6 (50.9) B	0.5 (0.07) AB	0.98–0.99	105.4 (1.7) B	0.02 (0.0) B	0.99–0.99	0.8 (0.1)	0.6 (0.0)	0.3 (0.0)	1.9 (0.5)	0.04 (0.0) B
Anguil	115.6 (12.9) B	0.4 (0.2) B	0.90–0.99	117.5 (0.6) A	0.20 (0.0) A	0.99–0.99	4.5 (0.3)	3.6 (0.1)	3.3 (0.3)	12.3 (4.1)	0.4 (0.2) A

[a]Mean values of 4 replicates; standard deviation in parentheses. Different letters indicate significant differences among soils ($p < 0.05$).
[b]Desorption from initial glyphosate aqueous concentration $C_0 = 5$ mg/L.
[c]Percentage of desorbed glyphosate in the 1°, 2°, and 3° desorption cycle.
[d]Total desorbed glyphosate after 3 successive desorption cycles.
[e]Hysteresis coefficient ($H = 1/n_d/1/n$).

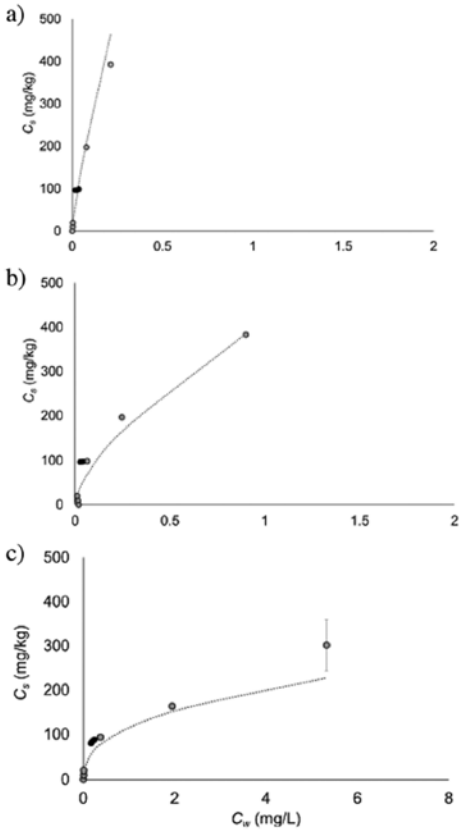


Figure 1. Adsorption (gray dots) and desorption (black dots) isotherms for (a) Cerro Azul, (b) Tandil, and (c) Anguil soils. Error bars represent standard deviation. Black dotted line represents the Freundlich model fit. Note different x axis scale for Anguil soil. C_s = concentration of glyphosate adsorbed to the soil at equilibrium; C_w = analytically measured mass concentration of glyphosate in the aqueous phase at sorption equilibrium.

Desorption isotherms

The K_{fd} values of the studied soils ranged from 101.2 to 117.5 $mg^{1-1/n}kg^{-1}L^{1/n}$ (Table 2). Anguil soil had the highest K_{fd}, while Cerro Azul had a significantly lower desorption coefficient than the rest ($p<0.05$). The total desorbed glyphosate at the end of the desorption study was 1.6 and 1.9% for Cerro Azul and Tandil, respectively, whereas in Anguil soil desorption reached 12% (Table 2). The values of $1/n_d$ ranged from 0.01 to 0.2 (Table 2). The irreversibility of glyphosate sorption was confirmed by the lower values of $1/n_d$ with respect to 1/n. The more pronounced curvature of the desorption isotherms suggests that more energy is required to desorb the molecules than that needed for the sorption process. In consequence, hysteresis coefficients were low, ranging from 0.01 to 0.4 (Table 2). When comparing the 3 soils, desorption and hysteresis coefficients were significantly higher in Anguil. This can be explained by the lower clay content and lower CEC, as well as the significantly higher pH and available phosphorus, which affect glyphosate sorption mechanisms in an inverse way, as explained before. Nevertheless, desorption hysteresis can be considered significant in all the studied soils because the hysteresis coefficient was <0.7, indicating that glyphosate sorption is nearly an irreversible process.

The fact that glyphosate binds strongly to the studied soils and that desorption was very low has a major implication for glyphosate bioavailability. Glyphosate's biological degradation is strongly limited in soils that have high glyphosate affinity and low desorption.

The results obtained in the present study indicate that sorption of glyphosate increases in soils with high contents of Al^{3+}, Fe, and clays as well as low pH and phosphorus content. This situation favors greater glyphosate retention and, therefore, lower desorption, which would reduce the likelihood of leaching and therefore the potential risk of groundwater contamination. However, glyphosate bioavailability can also be reduced, increasing its persistence and therefore contributing to its accumulation in the environment. These results contribute to the knowledge about glyphosate retention in soils and allow the identification of behavior patterns of this extensively applied herbicide in different edaphic scenarios.

This is of major importance for the development of decision-making tools and criteria to reduce the potential negative impacts on soil and groundwater resources.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes a adsorption / desorption experiment with glyphosate on three different agricultural soils from Argentina. The soils do not reflect current EU conditions. Furthermore, no labelled test item was used and no mass balances were provided.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Jodeh S., et al. .
Report year	2014
Report title	Fate and Mobility of Glyphosate Leachate in Palestinian Soil Using Soil Column
Document No	Journal of Materials and Environmental Sciences (6) (2014) 2008–2016
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions for the adsorption experiment Not reliable for the column leaching experiment (Used soil does not reflect EU conditions (soil, climate))

2. Full summary of the study according to OECD format

In recent years, pesticides were used heavily in Palestine, which led to the contamination of soil and water and causing many diseases. Many studies focused on the impact of pollutants such as pesticides and oil on soil, humans, animals, plants and the environment in general. Using column study the amount of glyphosate in soil decreases with increasing depth of soil, where it is for 0–30 cm (11 ppm) >30–60 cm (6 ppm) >60–100 cm (2 ppm) due to organic content and metal oxides founded in soil that can form stable complexes with glyphosate. When we increased the concentration of glyphosate, the amount of glyphosate (contaminant) in leachate where found to be 25 x (15.96 ppm) >15 x (3.91) >5 x (3 ppm) column. The behavior of glyphosate leachate fits the first order reaction and the isotherm is in according with the Freundlich adsorption equation with R^2 value 0.98, *k* value 6.4 and n value 1.07 which indicates good adsorption to soil.

Materials and Methods

Chemicals

Glyphosate (purity 98.5 %) was purchased commercially. Other chemicals like carbon disulfide, copper nitrate and chloroform were available at the university department of chemistry. All chemicals and solvents used in the experiment were of high performance liquid chromatography and high purity.

Acid digestion of soil

To find the metals in soil and $HClO_4$ (70 %) and HF (40 %) were added then heated to incipient (near dryness). HF were added again and heated to dryness then $HClO_4$ and distilled water were added and heated to incipient. The remaining residue was dissolved in HCl and water. Volume was made up to the 100 mL volume and stored in polyethylene bottle. Fe and Cu in the supernatant were determined by AAS. The physicochemical soil properties (Table 1) were determined using standard methods.

Sampling site and Collection

The soil was sampled in three layers; 0–30 cm, 30–60 cm and 60–100 cm from agricultural locations in Nablus, Mount Gerizim before herbicide treatment of the fields. The soil samples were mixed well separately. The soil used for chemical analysis was air dried, sieved to 2 mm stored in the dark at room temperature and protected from humidity. Basic physicochemical properties of soil were conducted on soil before any treatment with glyphosate**.**

Table. 1: Physico–chemical characteristics of the soil column.

Soil texture • Sand [%] • Silt [%] • Clay [%] •	35% 57.5% 7.5%
Moisture **%**	3.3%
Moisture correction factor (mcf)	1.033
pH	7.62
Organic Carbon %	2.11%
Organic Matter %	3.63%
Conductivity(µs)	530
N%	0.1934%
Ca CO_3 (mg)	0.795
Cu (mg/kg)	44
Fe (mg/kg)	1982.27
Available Phosphorous (P) (mg/kg)	62.41

Leachate extraction columns

Leachate extraction columns consist of four columns of polyvinyl chloride (PVC) pipe. A metal mesh screen was placed at the bottom end of each column and a plastic bottle was placed under each column to collect water. Soil column was washed with distilled water to remove air bubbles from soil and to ensure that the pH of leachate water from each column is neutral.

Glyphosate application to soil–column experiment

Glyphosate contains the monoisopropylamine salt of glyphosate (N–(phosphonomethyl)–glycine) (360 g/L) was applied to each column with concentrations; 5 X, 15 X and 25 X, where X equals amount of glyphosate applied to soil yearly (nearly 2 L/dunom), numbers (5, 15, 25) are the years of applying glyphosate to soil. Blank soil samples were used as controls without glyphosate addition. The concentrations of glyphosate added to soil columns are listed in Table 2.

Leachate

Leachate was collected from each column in plastic bottle at the end of every period. Leachate volumes were determined gravimetrically. Leachate water was centrifuged to remove solid particles and then the supernatant was filtered before analysis. Glyphosate extracted by the method described below and derivatized using the method shown below then measured by Spectrometer at 435 nm.

Procedure for Solid–Phase Extraction (SPE) of glyphosate from water samples

A cation exchange resin was used for the pre concentration and cleanup of glyphosate. A slurry of the Amberlite IR–120, Na–ion exchange resin (cationic) was made in 10 mL distilled water and packed into a narrow glass column, plugged with glass wool at the bottom. The resin was rinsed with distilled water and then with 1 M HCl at a flow rate of 2 mL/min several times before sample application. The pH of water sample spiked with glyphosate was adjusted to 2 and amine group of glyphosate was converted into its protonated form. The protonated sample (25 mL) was passed through the column at a flow rate of 0.5 mL/min in order to have maximum exchange of protonated sample. After the loading step, the sorbent was washed with 25 mL of 2 M NaCl solution (used as eluent) at the same flow rate. The eluted solution was evaporated to about 10 mL at 70ºC then evaluated by the proposed method.

Table. 2: Main characteristics of soil after application of glyphosate at different depths.

Column	Depth (cm)	PH	C %	O.M %	N %	Available P mg/kg	$CaCO_3$ mg/kg	Fe mg/kg	Cu mg/kg
Blank	0-30	7.45	1.56	2.69	0.145	7.91	0.211	1941	43
	3-60	7.78	1.53	2.64	0.082	5.3	0.245	1997	38
	60-100	7.7	1.36	2.33	0.024	5.27	0.292	2008	52
5x	0-30	7.55	2.08	3.58	0.321	66.62	0.147	1853	30
	30-60	7.86	2.05	3.53	0.270	48.49	0.161	1953	35
	60-100	7.72	2.03	3.49	0.250	45.71	0.199	2000	64
15x	0-30	7.68	2.08	3.58	0.373	72.57	0.194	1909	35
	30-60	7.75	2.02	3.48	0.356	66.1	0.197	2053	44
	60-100	7.88	1.99	3.42	0.305	53.26	0.208	2103	52
25x	0-30	7.49	2.21	3.80	0.425	95.04	0.178	1909	24
	30-60	7.56	2.05	3.53	0.375	88.31	0.206	1985	29
	60-100	7.66	2.01	3.46	0.319	74.13	0.200	2032	34

Derivatization procedure of glyphosate

Glyphosate was derivatized using carbon disulfide to convert the amine group into dithiocarbamic acid. The dithiocarbamate group was used as chelating group for reaction with transition metal ion Cu (II). The resultant yellow colored complex was measured at 435 nm using UV–Spectrophotometer. Carbon disulfide (1 % CS_2) solution was prepared and an aliquot of glyphosate were added to a series of 100 mL separating funnels followed by the addition of CS_2 solution. Then the mixture was shaken for 3 minutes for the formation of dithiocarbamic acid. An ammonical solution of Cu(II) (1000 mg/L) was added to the mixture, shaken again vigorously to form complex with dithiocarbamic acid and then kept for separation of two phases. The yellow colored chloroform layer containing the complex was separated in a 10 mL flask and diluted with ethanol. The absorbance of the complex was measured at 435 nm.

Soil columns after glyphosate application

At the end of the experiment, soil columns were cut into three parts. Three samples were taken from each part, air dried and stored in an air tight polythene bottle to analyze their parameters in soil lab at An Najah National University. Glyphosate were extracted from the three parts of soil columns, derivatized and measured spectrophotometrically.

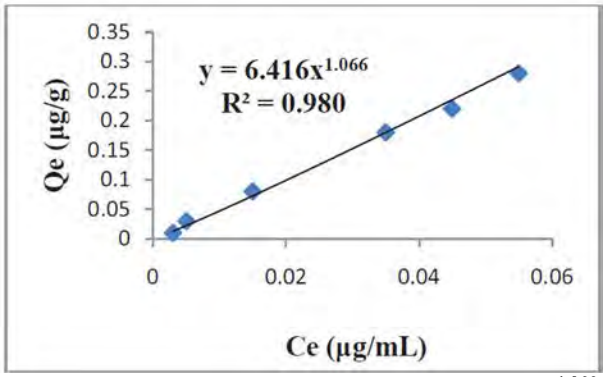


Figure 1: Adsorption isotherm of glyphosate for Palestenian soil ($Qe = 6.416C_e^{1.066}$, $r^2 = 0.9803$).

Batch sorption experiment

Sorption kinetics was analyzed by altering the contact time at a constant concentration of 20 and 30 ppm per vessel for determination of an appropriate equilibrium time at room temperature for the sorption isotherm experiments. They were shaken for 1, 2, 4, 6, 8, 24, 48 and 72 hours, respectively. Samples were equilibrated and processed.

Table. 3: Freundlich isotherm constants for glyphosate.

Coefficient	K	1/n	n	R^2
Glyphosate	6.41	0.93	1.07	0.98

Adsorption isotherm experiment

Soil samples were air–dried, sieved, stored in the dark at room temperature (23°C), and protected from humidity. Sorption experiments were carried out using the standard batch equilibration method. A series of five selected glyphosate concentrations were carried out to determine the adsorption isotherms of glyphosate on soil. The adsorption measuring steps were as follows:

- 200 mL of a PTFE vessels containing 25 g air dried weight soil.
- 100 mL aqueous solutions containing 0–50 mg/L glyphosate were equilibrated for 24 h at room temperature on a reciprocating shaker at low speed 120 excursions per minute.
- The supernatant equilibrium concentration is obtained after centrifuging at 3000 rpm (round per minute) for 20 minutes.
- Blank without glyphosate was also equilibrated. The equilibrium concentrations of each soil were measured spectrophotometrically after derivatization.

Consequently, the differences between the initial and equilibrium concentrations were assumed to be due to sorption onto soil. Sorption isotherms were obtained by plotting the amount of glyphosate sorbed per weight of soil at equilibrium (Qe, µg/g) versus the amount of glyphosate per volume of solution at equilibrium (Ce, µg/mL). The sorption data were described using the Freundlich equation:

$$Qe = K_f . C_e^{nf} \qquad \text{eq. 1}$$

where Qe is the concentration of glyphosate sorbed onto the solid phase (µg/g), Ce is the concentration of glyphosate in solution at equilibrium (µg m/L), and Kf (in $\mu g^{1-nf}\, mL^{nf}\, g^{-1}$) and nf are empirical constants which are related to the adsorption phenomenon and calculated by regression analysis. Kf can be considered as a characterisation of the intensity of sorption, modulated by the deviation from the unity of the nf exponent.

Glyphosate extraction from soil samples

Homogenized soil sample (10 g) was extracted for 60 min with 25 mL of 2 M NH_4OH solution. The extraction was repeated three times. The pH of eluted sample was re–adjusted to pH 5.4 and was evaluated by the proposed method. Each recovery was performed in triplicates.

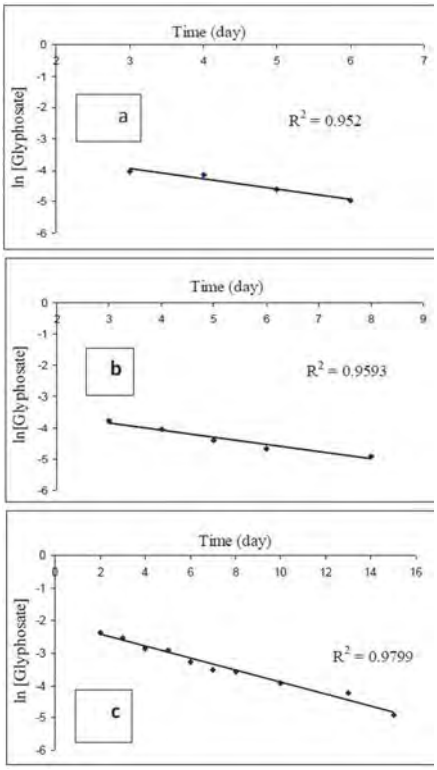


Figure. 2: Plot of time vs. Ln concentration for 5 X (a), for 10 X and (c) for 25 X times glyphosate

Results and Discussion

Batch sorption experiments

The sorption kinetics of the soil were studied to determine an appropriate shaking time for the sorption isotherm experiments. Readings were recorded until 72 hours, no changes in concentrations were observed after 24 hours for all samples, and therefore 24 hours were chosen as equilibrium time for the sorption isotherm experiment due to the quick degradation of glyphosate. The equilibrium adsorption data over the range of concentrations studied here were used to fit Freundlich adsorption equation (eq. 1). The values of n within the range of 2–10 represent good adsorption. Higher values of *k* indicate high adsorption capacity. The isotherm equilibrium results for the examined soil are shown in Figure 1. Freundlich isotherm constants (*k* & n) for glyphosate, the correlation coefficient "R" were obtained from Figure 1. and listed in Table 2. Glyphosate sorption at 25°C in the studied soils was evidenced to be a kinetics process, with a reasonable equilibration time of 24 hours. Literature usually reports Freundlich adsorption constants for glyphosate adsorption by soils which are consistent with that founded in our study. It is indicated from Table 3 and Figure 1. that "n" of glyphosate adsorption is higher than 1. The adsorption isotherms for the soil is of S–type, which indicates the easiness of the adsorption, mainly at higher concentrations.

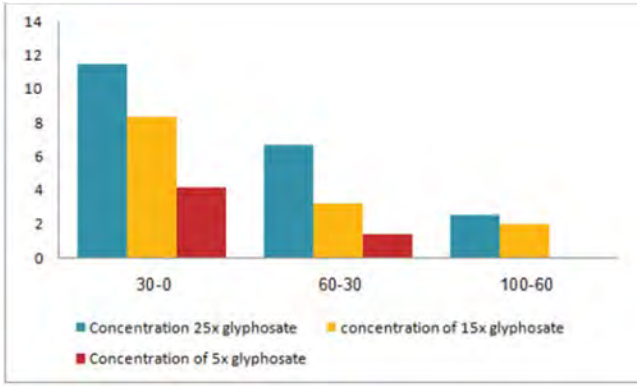


Figure 3: Concentration (mg/L) of glyphosate in soil column at different depths**.**

Glyphosate in leachate

It is indicated that the amount of glyphosate detected in leachate decreases with increasing time. It takes time for 25 x >15 x >5 x until the inability to detect glyphosate in leachate for concentrations less than 1 ppm. Doubling the concentration of glyphosate increases the amount glyphosate (contaminant) in leachate. The above resulting curves shows that the best fit of the glyphosate degradation data was obtained using a first–order reaction as shown in Figure 2. DT_{50} values of glyphosate was 2, 3 and 3.75 days for 5 x, 15 x & 25 x column respectively. This indicate relatively rapid degradation.

Glyphosate in column soil

The results indicated that the glyphosate mobility in the soil columns increased with application rate. With more glyphosate applied, more glyphosate in the soil columns was capable of moving out of the columns. Amount of glyphosate detected in soil columns was increased in the order : 25 x >15 x >5 x. The amount of glyphosate was decreased with depth increasing due to decreasing organic content. It means that the adsorption tendency decreases as the depth increases. No glyphosate detected in 60–100 cm depth as shown in Figure 3. This due to low concentration of glyphosate less than 1 ppm that couldn't be measured by the method used here. Lowest concentration was used also most of glyphosate adsorbed on the upper layer of soil (0–30 cm). This study indicates that glyphosate can be extensively mobile in soil environment if it is applied on soils unable to retain the molecule long enough for its microbial degradation. This may also lead to herbicide leaching to lower soil layers where a limited biological activity occurs.

The effect of organic matter

Soil organic matter consists of a variety of components. These include, in varying proportions and many intermediate stages:

- Raw plant residues and microorganisms (1 to 10 %).
- "Active" organic traction (10 to 40 %).
- Resistant or stable organic matter (40 to 60 %) also referred to as humus.

Table 2 shows that organic matter content of the soil at different depths ranges between 2–3.8 % which is considered as a moderate organic matter soil. Organic matter content of the soil at different depths for each column nearly the same as shown in Figure 4. It is indicated that organic matter only may not affect the adsorption of glyphosate at different depths and it could affect sorption in two ways:

- Reducing glyphosate sorption by blocking sorption sites.
- Increasing glyphosate sorption because poorly ordered aluminium and iron oxides with high sorption capacity are favored at higher soil organic matter content.

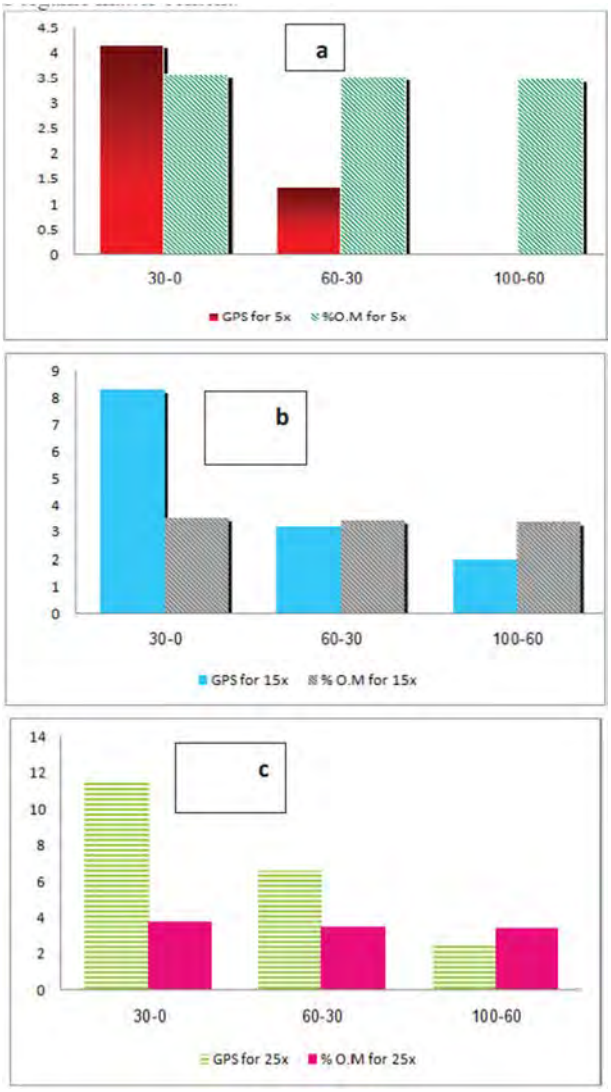


Figure 4: Organic matter content in 5 X (a), 15 X (b) and 25 X (c) column and concentrations of glyphosate at certain depths.

The effect of soil metals

The high sorption values for glyphosate can be in part due to the pH values of soils and to the presence of iron oxides, copper and other metals that can form stable complexes with glyphosate. Glyphosate coordinates strongly to Cu, and Cu–glyphosate complexes formed seem to have higher ability to be adsorbed on the soil than free glyphosate. Copper acts as a bridge between the soil and glyphosate. At these pH values glyphosate is a di–anion and both the carboxylate and the phosphonate functional groups in its molecule are deprotonated, being able to compete for the surface adsorption sites on the metal oxides.

Available phosphorous after glyphosate application

Figure 5 shows that the amount of phosphorous in soil columns after application of glyphosate increased this indicates degradation of glyphosate to its components where phosphorous is one of the degradation products. Glyphosate could be source of phosphorous, nitrogen and carbon in soil as it is shown in Figure 5 and Table 1. The nitrogen content of soil has been increased after glyphosate application to soil columns due to biodegradation of glyphosate.

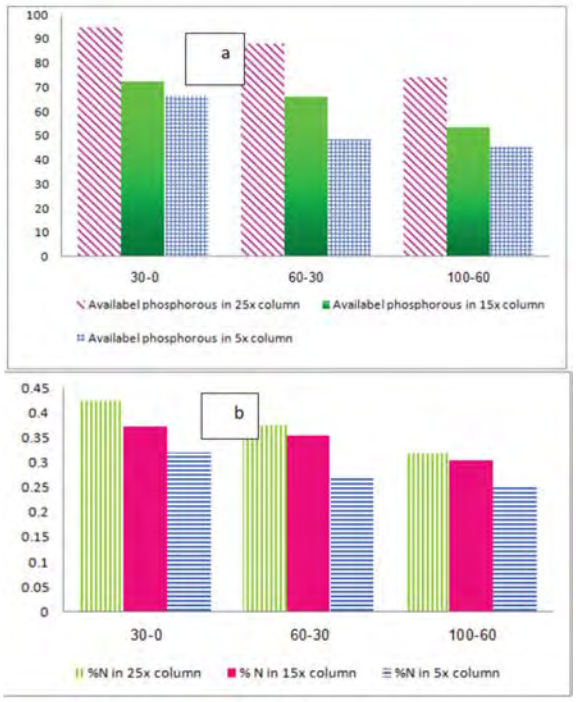


Figure 5: Phosphorous content (a) and nitrogen content (b) in soil columns after application of glyphosate.

Conclusion

Adsorption is an important process in determining the fate of glyphosate in soil. The texture for soil used has been found to be silty clay and the total organic matter (TOM) close to 4 %. Batch equilibrium technique was used to evaluate the extent of glyphosate adsorption on soil as adsorbent. Isotherm is in accord with the Freundlich adsorption equation with R^2 value 0.98; the parameters of this isotherm have been calculated. The adsorption isotherm was fit the S–type isotherm according to Giles. The values of "n" in Freundlich equitation was more than one indicating good adsorption for glyphosate with the soil used. Freundlich constant "*k*" indicates the tendency of glyphosate in this study to be adsorbed on soil particles. *k* increases with increasing the soil minerals and decreases with increasing the depth of soil where the main binding mechanism for glyphosate is the covalent bond between the herbicide and the metals from soil oxides, and so the adsorption decreasing due to decreasing the organic matter content as depth increases. Many factors affect the adsorption of glyphosate as phosphorous content, pH, and temperature. The high sorption values for glyphosate can be in part due to the presence of metal oxides that can form stable complexes with glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes both a column leaching experiment and an adsorption experiment with non-labelled glyphosate with a Palestinian agricultural soil. Due to analytical method insensitivity, the lowest rate examined in the column leaching experiment was 5 times the yearly application rate. In addition, some essential information necessary for assessment of validity of both experiments is not reported (e.g. mass balances).

The article is classified as not reliable (Category 3) for the column leaching experiment and as reliable with restrictions for the adsorption experiment (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.1.1; KCA 7.1.2.1.3; KCA 7.1.3.1.1
Report author	Kanissery, R. G. et al.
Report year	2015
Report title	Effect of Soil Aeration and Phosphate Addition on the Microbial Bioavailability of Carbon-14-Glyphosate
Document No	Journal of Environmental Quality
Guidelines followed in study	USEPA guidelines for adsorption studies (USEPA, 2008)
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (slight differences to EU guidelines, no EU soils; soil deg: insufficient reporting of results for kinetic evaluation accord. current guidelines; ads/des: no single measurements for each concentration reported)

2. Full summary of the study according to OECD format

The adsorption, desorption, degradation, and mineralization of ^{14}C-glyphosate [*N*-(phosphonomethyl)glycine] were examined in Catlin (a fine-silty, mixed, superactive, mesic Oxyaquic Argiudoll), Flanagan (a fine, smectitic, mesic Aquic Argiudoll), and Drummer (a fine-silty, mixed, superactive, mesic Typic Endoaquoll) soils under oxic and anoxic soil conditions. With the exception of the Drummer soil, soil aeration did not significantly alter the adsorption pattern of ^{14}C-glyphosate to soils. Herbicide desorption was generally enhanced with anaerobiosis in all the soil types. Anoxic soils demonstrated slower microbial degradation and mineralization kinetics of ^{14}C-glyphosate than oxic soils in all the soil types studied. Phosphate additions significantly reduced the adsorption of ^{14}C-glyphosate to soils irrespective of soil aeration and confirmed the well-established competitive adsorption theory. The addition of soil phosphate stimulated degradation only in anoxic soils. The results from this research highlight the importance of soil redox conditions as an important factor affecting the bioavailability and mobility of glyphosate in soils.

Materials and Methods

Chemicals

Carbon-14-glyphosate (phosphonomethyl-^{14}C) (specifc activity: 1.85 × 109 Bq/mmol) was obtained from American Radiolabeled Chemicals. Unlabeled glyphosate (chemical purity: 99 %) was procured from Sigma Chemical Company. Organic solvents and water were of Optima grade from Fisher Scientifc and used without further purification.

Soils

The soils used were a Drummer silty clay loam, a Flanagan silt loam, and a Catlin silt loam. The moderately well drained Catlin, the somewhat poorly drained Flanagan, and the poorly drained Drummer soils occur in proximity in landscapes and form a soil catena; they thus have similar parent materials but vary in organic matter content, landscape position, and soil drainage class. The soils were collected (to a depth of 15 cm) from a field with no previous glyphosate application history at the University of Illinois Crop Sciences Research and Education Center in Urbana. All soil samples were sieved through a 2-mm screen and stored at 4°C for 4 weeks. Relevant physical and chemical properties of the three soil types used in this study were analyzed at the A&L Great Lake Laboratories and are listed in Table 1. Each soil type had three replicates for each treatment in the subsequent experiments.

Table 1. Selected properties of the soils used in the experiment (analysis by A&L Great Lakes Laboratories, Inc.).

Soil	pH	Texture†			WHC‡	CEC§	Organic matter¶	P
		Sand	Silt	Clay				
		%				$cmol_c\ kg^{-1}$	%	$mg\ kg^{-1}$
Catlin	7.6	10	58	32	31.4	13.9	3.3	69
Flanagan	6.5	12	56	32	32.0	17.5	3.7	154
Drummer	7.1	14	46	40	33.0	23.8	4.9	81

† Determined by hydrometer method.

‡ Water holding capacity at 33 1/3 kPa (determined by porous plate/pressure apparatus).

§ Cation exchange capacity.

¶ Determined by loss-on-ignition.

Adsorption-Desorption Study

Adsorption isotherms of ^{14}C-glyphosate were determined using the batch equilibrium method for the three soil types. The initial concentrations of ^{14}C-glyphosate (0.1, 1, 5, and 10 mg/L) were prepared in 0.1 mol/L KCl solution and the adsorption experiment followed USEPA guidelines for adsorption studies. Two grams of air-dried soil was equilibrated with 10 mL of ^{14}C-glyphosate in 20-mL Teflon centrifuge tubes in a horizontal shaker (150 rpm) for 24 h (sufficient for apparent equilibrium in a preliminary study) at room temperature (25 ± 1°C). For the anaerobic treatments, 2-g portions of the soil samples contained in the Teflon centrifuge tubes were flooded with sterile, deoxygenated water. Preliminary studies revealed that <5 % of added glyphosate was degraded during 24 h of contact with any of the soils used (data not shown). The tubes were flushed with N_2 gas, sealed, and incubated in an anaerobic chamber from Coy Laboratory Products containing a primary headspace of $N_{2(g)}$ with 5 % $CO_{2(g)}$ and <2 % $H_{2(g)}$ at room temperature (25°C) for 2 wk to allow reduction. To study anaerobic adsorption, ^{14}C-glyphosate was added, using a concentrated O_2-free stock solution, to the reduced (anoxic) soil to attain final concentrations of 0.1, 1, 5, and 10 mg/L. Sealed tubes were equilibrated on a shaker inside the anaerobic chamber for 24 h, where the O_2 was maintained at zero concentration. The effect of phosphate addition on glyphosate adsorption in oxic and anoxic soils was examined by incorporating $CaHPO_4$ into the Catlin, Flanagan, and Drummer soils at an overwhelming concentration (500 mg/kg soil), followed by thorough mixing of the soils before the addition of the herbicide.

At the end of the equilibration period, the soil suspension was centrifuged (15 min, 12,000 × g) and aliquots removed from each tube for a radioactivity assay using a Packard Tri-Carb (1900TR) scintillation counter. Controls (treatment without herbicide) were included for calibration and background correction purposes. The amount of ^{14}C-glyphosate adsorbed to the soil was calculated based on the difference between the initial and final concentrations of herbicide in the solution.

Following equilibration and removal of 5 mL of the initial 10 mL of supernatant, herbicide desorption from the soil was estimated by adding equal amounts of fresh 0.1 mol/L KCl solution to the centrifuge tubes, dispersing the soil aggregates by vibration, and shaking for 24 h. Sampling from the anaerobic soil treatments was handled inside the anaerobic chamber. Soil samples were centrifuged (15 min, 12,000 × g), and an aliquot of the supernatant was removed and analyzed utilizing the radioactivity assay. The desorption process was repeated four times. Desorption was estimated by determining the amount of herbicide (described below) in the soil solution following equilibration and calculated by subtracting the amount of herbicide remaining on the soil surface.

Degradation Study

Microcosm Preparation

Soil incubations were performed for 56 d under reduced (anoxic) or oxidized (oxic) conditions using serum bottle microcosms to determine the degradation kinetics of ^{14}C-glyphosate. No degradation was detected in aqueous or organic stock solutions of glyphosate during the experiment.

Anaerobic incubations: Microcosms consisting of serum bottles (60 mL) were amended with soil (10 g) and were spiked with phosphonomethyl-C-labeled ^{14}C-glyphosate (specifc activity of 3.33 x 103 Bq/mmol, diluted with unlabeled glyphosate) in 50 mL of methanol to produce a final concentration of 2 mg/kg of soil that corresponded to the recommended agricultural application rate. The glyphosate-spiked soils were agitated on a reciprocating shaker for 24 h at room temperature to ensure thorough mixing and to evaporate the solvent. To determine the effect of soil phosphate on glyphosate degradation, $CaHPO_4$ was uniformly mixed into the soils at a concentration of 500 mg/kg soil before the addition of the herbicide. The soil was then flooded with 20 mL of sterile (autoclaved), deoxygenated water to mimic soil saturation by rainfall. The microcosm headspace was flushed with N_2 gas and immediately crimp sealed with a butyl stopper fitted with a vial containing 1 mL of 0.5 mol/L NaOH to trap the mineralized $^{14}CO_2$. These microcosms were incubated in a dark, temperature-controlled chamber at 25°C. Sterilized soil microcosms were included as controls for each soil type. Sterilization was achieved by autoclaving the soils twice at 121°C for 1 h on successive days.

Aerobic incubations: Soil microcosms were built from serum bottles as described above. Sterile, distilled water was added to the glyphosate-spiked soils to adjust the moisture content to about 60 % of the field water-holding capacity. The serum bottles were lightly capped (no crimp seal) with a butyl stopper fitted with a NaOH trap and stored in the dark at 25°C. At 1-wk intervals, the microcosms were aerated by equilibrating the headspace with the atmosphere, and the soil moisture content was adjusted by returning each vessel to its initial weight with sterile, distilled water.

Sample Extraction and Analysis

Anaerobic and aerobic microcosms were destructively sampled at consecutive intervals (0.5, 3, 7, 14, 28, 42, and 56 d) by removing the NaOH trap, followed by agitating the microcosm for 1 min and transferring the contents to a 50-mL Teflon centrifuge tube. Quantification of $^{14}CO_2$ in the NaOH traps was accomplished by direct liquid scintillation spectrometry (LSS) using a Packard Tri-Carb (1900TR) scintillation counter. The solid and liquid phases of the soil slurry were then separated by centrifugation (15 min, 12,000 × g). Aqueous samples were removed and filtered (0.2 µm), and the total aqueous radioactivity was estimated using LSS. The soil was extracted with 20 mL of NaOH (0.1 mol/L) in a Teflon centrifuge tube with horizontal shaking following the method described by Druart et al. (2011). Extracts were centrifuged at 12,000 rpm for 15 min, an aliquot was removed for LSS (to quantify extractable radioactivity), and the supernatant was retained for analysis of the herbicide. The recovery values of glyphosate from oxic soils were 73 to 78 % and 74 to 76 % from anoxic soils. The recovery efficiencies obtained were taken into consideration in the calculations of the results. Soil extract samples containing ^{14}C-glyphosate were analyzed using high-performance liquid chromatography with a Packard Radiomatic Flo-one Beta scintillation detector. Separation was achieved with an isocratic elution of the mobile phase composed of acetonitrile/water (10:90 v/v) through a 4.6 × 150 mm, 5-µm particle size, C_{18} column from Prontosil. Glyphosate had a reproducible retention time of 4.1 min at a flow rate of 1 mL/min.

Data Analysis

The adsorption and desorption parameters of glyphosate under oxic and anoxic conditions for each soil type were calculated using the transformed Freundlich equation; equation: $\log C_s = \log K + 1/n \log C_e$, where C_s is the amount of glyphosate adsorbed to the soil (mg/kg), C_e is the equilibrium concentration in the soil solution (mg/L), and K and $1/n$ are empirical constants that reflect the affinity of the soil for the herbicide and the degree of linearity between the amount adsorbed and the solution concentration, respectively. Regression analysis was performed on adsorption and desorption isotherms to calculate K (intercept) and $1/n$ (slope) values of glyphosate in oxic and anoxic soils. Hereafter, K_{ads} and $1/n_{ads}$ will indicate Freundlich parameters for adsorption, and K_{des} and $1/n_{des}$ will refer to desorption parameters. The data on the degradation of glyphosate in soils were fitted into the first-order kinetics model $C_t = C_0 \exp(-kt)$, where C_0 is the initial concentration (mg/kg soil) of the herbicide in the soil, C_t is the herbicide concentration (mg/kg soil) detected in the soil at time t, and k is the first-order rate constant. Degradation rate constants were calculated by linear regression of the natural logarithm of the percentage of herbicide remaining against the time. The aerobic and anaerobic degradation half-lives ($T_{1/2}$) for each soil type

were calculated using the equation $T_{1/2}$ = ln2/k. The statistical program SAS Version 9.3 from SAS Institute was used to calculate the treatment means and standard errors (n = 3). The experiments were set up as a completely randomized design, and the differences between treatments were evaluated using one-way analysis of variance followed by a least significant difference test at p <0.05.

Results

Adsorption-Desorption

Adsorption data from the experiment were very well fitted by the Freundlich equation (R^2 = 1) for the range of herbicide concentrations (0.1-10 mg/L) and soils tested regardless of the soil redox conditions (Table 2). Among the different soils and treatments, the slope ($1/n_{ads}$) values ranged from 0.76 to 0.93 and the Freundlich adsorption coefficient (K_{ads}) from 62.21 to 103.46. Soil redox conditions did not alter glyphosate adsorption to the Catlin and Flanagan soils, as evident from their nearly equal K_{ads} values. However, the herbicide exhibited a noticeably lower K_{ads} value in the anaerobically treated Drummer soil vs. the aerobic Drummer soil incubations. Further, K_{ads} was observed to be lowest for Catlin and highest for Drummer regardless of the soil redox conditions. A higher K_{ads} indicates a higher adsorption affinity of the herbicide to the soils. Desorption isotherms for glyphosate in all the soils fit well into the Freundlich model (R^2 >0.92). The calculated desorption parameters of glyphosate in the oxic and anoxic soils are presented in Table 3. Freundlich desorption coefficient (K_{des}) values of glyphosate were considerably lower in the anoxic soils than the oxic soils. Among the three soils tested, the highest K_{des} was observed in the Catlin soil irrespective of the soil redox conditions. A higher K_{des} indicates a greater retention of glyphosate on the soil surface.

Table 2. Adsorption (Freundlich model) of ^{14}C-glyphosate in different soil types under oxic and anoxic environmental conditions.

Soil	K_{ads}†		$1/n_{ads}$‡		R^2§	
	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic
Catlin	62.21 (±1.71)¶	72.38 (±5.80)	0.92 (±0.02)	0.76 (±0.05)	0.999	0.999
Flanagan	78.14 (±2.05)	69.64 (±4.02)	0.90 (±0.02)	0.78 (±0.04)	0.999	1.000
Drummer	103.46 (±5.11)	84.82 (±4.36)	0.93 (±0.03)	0.88 (±0.03)	0.998	0.998

† Freundlich adsorption coefficient.

‡ Adsorption isotherm slope.

§ Goodness of fit for Freundlich model.

¶ 95% confidence intervals in parentheses.

Table 3. Desorption (Freundlich model) of ^{14}C-glyphosate in different soil types under oxic and anoxic environmental conditions

Soil	K_{des}†		$1/n_{des}$‡		R^2§	
	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic
Catlin	46.52 (±0.03)¶	42.85 (±0.20)	0.02 (±0.002)	0.28 (±0.003)	0.94	0.94
Flanagan	17.08 (±0.03)	5.81 (±0.02)	0.09 (±0.002)	0.25 (±0.005)	0.92	0.92
Drummer	17.95 (±0.04)	7.75 (±0.05)	0.02 (±0.003)	0.25 (±0.005)	0.92	0.92

† Freundlich desorption coefficient.

‡ Desorption isotherm slope.

§ Goodness of fit for Freundlich model.

¶ 95% confidence intervals in parentheses.

Degradation and Mineralization

Figures 1a to 1c depict the degradation pattern of ^{14}C-glyphosate in the Catlin, Flanagan, and Drummer soils incubated under oxic and anoxic conditions. The first-order parameters including the rate constant (k) and degradation half-life ($T_{1/2}$) of the ^{14}C-glyphosate in the different soil types and redox conditions are presented in Table 4. The ^{14}C-glyphosate degradation followed first-order kinetics in all the nonsterile oxic and anoxic soils, as obvious from their R^2 values (0.83 -1.00). The loss of herbicide from the sterile soil control microcosms was not substantial in either aerobic or anaerobic incubations (Figure 1a -1c). In all three soil types studied, the aerobic $T_{1/2}$ values (15 – 18 d) calculated for glyphosate were

significantly lower than the corresponding anaerobic values (42 -51 d). The $T_{1/2}$ of the herbicide in the Catlin, Flanagan, and Drummer soils were comparable in the aerobic incubations. On the other hand, compared with the other soils, glyphosate degradation was relatively slow in the Flanagan soil in the anaerobic incubations. Figures 1d to 1f illustrate the comparative microbial mineralization trends of glyphosate amendments observed as the amount of $^{14}CO_2$ measured from the alkali trap from aerobic and anaerobic soil microcosms. More than half (53 – 63 %) of the radioactivity in the applied ^{14}C-glyphosate was mineralized as $^{14}CO_2$ from the oxic soils, and only 38 to 41 % of the applied ^{14}C-glyphosate was mineralized in the anaerobic microcosms by the end of incubation. Conversely, aerobically or anaerobically incubated sterilized microcosms had little or no mineralization of the herbicide in all the soil types considered. Another interesting observation from the study is the absence of a lag phase before the evolution of $^{14}CO_2$ from the soils. The evolution of $^{14}CO_2$ from soils was evident immediately after Day Zero of the incubation in both oxic and anoxic soils. Glyphosate mineralization in oxic soils was initially rapid, followed by a gradually decreasing rate. However, in anoxic soils, mineralization of the glyphosate started out slowly and steadily increased toward the end of incubation.

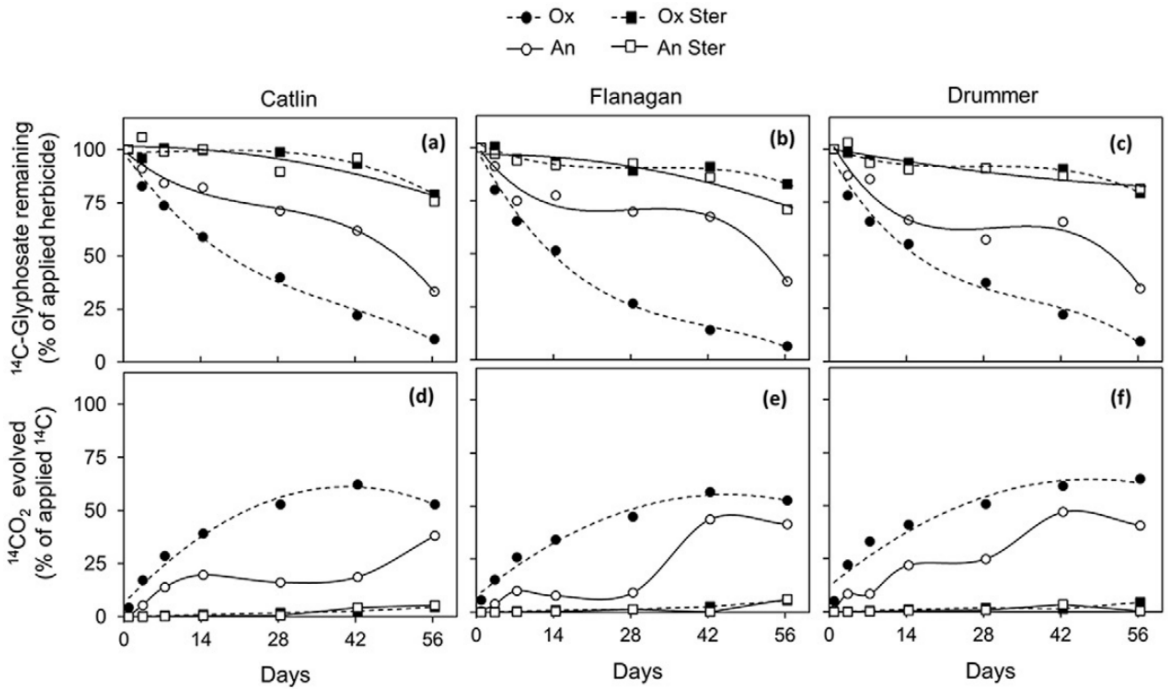


Figure 1. **(a,b,c) Degradation kinetics and (d,e,f) mineralization patterns of ^{14}C-glyphosate under oxic (Ox) and anoxic (An) soil conditions in Catlin, Flanagan, and Drummer soils. Data from oxic (Ox Ster) and anoxic (An Ster) sterilized control soils are also shown.**

Table 4. Degradation (first-order kinetics) parameters of ^{14}C-glyphosate in different soil types under oxic and anoxic environmental conditions.

Soil	k†		$T_{1/2}$‡		R^2§	
	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic
	d^{-1}		d			
Catlin	0.038 (0.003)¶	0.016 (0.005)	18 c# (209)	42 b (154)	0.99 (0.73)	0.88 (0.68)
Flanagan	0.048 (0.003)	0.014 (0.005)	15 c (228)	51 a (140)	1.00 (0.84)	0.81 (0.85)
Drummer	0.038 (0.003)	0.015 (0.004)	18 c (210)	45 b (200)	1.00 (0.85)	0.83 (0.86)

† Rate constant.

‡ Degradation half-life.

§ Goodness of fit for first-order degradation model.

¶ Corresponding values for the sterilized soil control in parentheses.

Means followed by the same letter are not significantly different ($p < 0.05$).

Effect of Phosphate Addition on Adsorption and Degradation

The addition of phosphate to the Catlin, Drummer, and Flanagan soils significantly reduced the ^{14}C-glyphosate adsorption to oxic and anoxic soils (Figure 2a - 2c). Moreover, the extent of the reduction in herbicide adsorption was more pronounced in the oxic soils. Phosphate additions did not improve or had no effect on the degradation of ^{14}C-glyphosate in the oxic soils, as observed from the degradation half-life values ($T_{1/2}$) of the herbicide in the respective soils (Figure 2b). Conversely, the presence of soil phosphate significantly enhanced the anaerobic degradation of ^{14}C-glyphosate in all three soil types studied (Figure 2d).

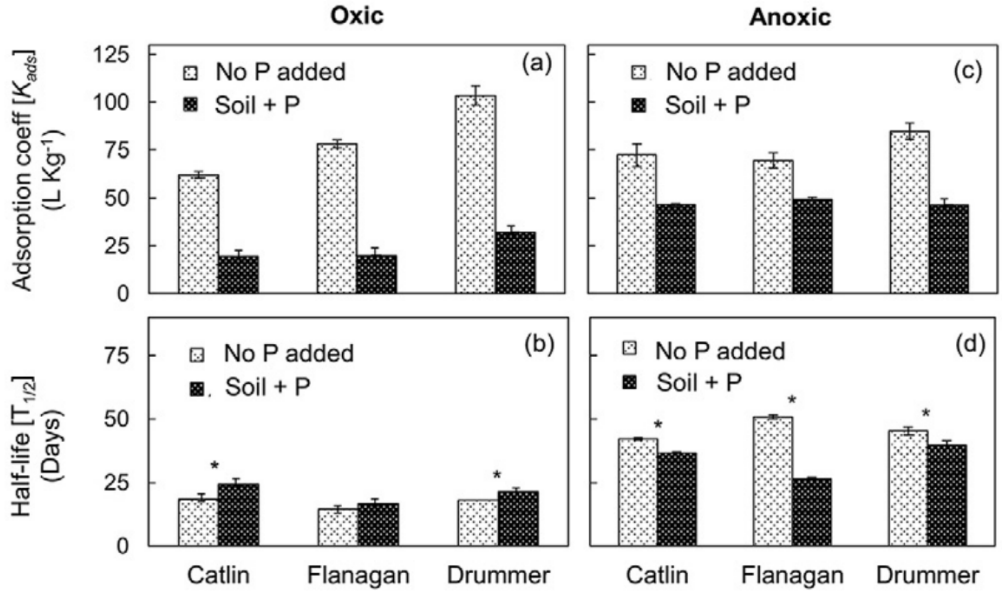


Figure 2. **Effect of phosphate addition (500 mg/kg soil) on the adsorption and degradation of ^{14}C-glyphosate in oxic and anoxic soils: comparison of (a,c) the adsorption coefficient and (b,d) the degradation half-life of glyphosate in oxic and anoxic soils without (No P added) and with (Soil + P) phosphate amendment.**
***Signifcantly different at p <0.05. Error bars represent standard errors (n = 3).**

Discussion

Adsorption-Desorption

High K_{ads} values of ^{14}C-glyphosate from the present study (62.21-103.46) clearly indicate a strong adsorption affinity of the herbicide to the soils (Table 2). The results obtained from this study were comparable to reported K_{ads} values (33-152.9) for glyphosate. The greatest extent of ^{14}C-glyphosate desorption was observed in the soils having the least adsorption (Table 3). Relatively lower K_{des} values in the anaerobic treatments than the corresponding aerobic treatments in all the tested soils indicate that desorption of the herbicide was enhanced in the anoxic, reduced soils. Increased desorption of the herbicide under anoxic conditions may result in an enhanced bioavailability of glyphosate, increasing the risk of movement or crop damage and possibly enhancing degradation of the herbicide under anoxic soil conditions.

Degradation and Mineralization

Degradation of ^{14}C-glyphosate occurred more rapidly in the aerobically incubated Catlin, Flanagan, and Drummer soils than in the corresponding anaerobic incubations, as evident from the significantly lower aerobic $T_{1/2}$ values (Table 4). This concurs with previous studies. Glyphosate degradation could be inferred to be a purely microbially mediated process because practically no degradation or mineralization occurred in the sterile control soils in any soil type or redox condition. The slow start in the anaerobic mineralization may be ascribed to the acclimation of specialized herbicide degrading microbial populations in the anoxic soil.

Impact of Soil Phosphate

Suppression of glyphosate adsorption in both oxic and anoxic soils with phosphate addition explicitly demonstrated the competition for adsorption sites between glyphosate and phosphate despite differences in redox conditions (Figure 2). Several studies have confirmed similar competitive adsorption of glyphosate and phosphate on Al^{3+} and Fe^{3+} surface sites in soil. The effect of phosphate addition on the enhanced microbial bioavailability of glyphosate was found only in the anoxic soils, where the $T_{1/2}$ of glyphosate was noticeably reduced in all the soil types treated anaerobically with phosphate (Figure 2). Phosphate addition did not stimulate glyphosate degradation in oxic soils.

Implications

This study examined the significance of oxic and anoxic soil conditions on the microbial bioavailability of glyphosate in soils. Although ^{14}C-glyphosate was highly adsorbed to the soils regardless of the soil type and redox conditions, desorption or release of the adsorbed herbicide was enhanced in anoxic soils. The degradation and mineralization of ^{14}C-glyphosate exhibited slower kinetics in anoxic soils than oxic soils in all the soil types investigated. The addition of phosphate to the soil suppressed the adsorption of glyphosate in both oxic and anoxic soils and improved the degradation rate in anoxic soils. The effects of anaerobiosis on the observed K_{ads} and K_{des} suggest greater glyphosate bioavailability in saturated soils. Significant decreases in degradation kinetics observed under anaerobiosis across soils could confer a greater potential for transport in water and subsequent environmental impacts. These findings are based on soils in corn (*Zea mays* L.) - soybean [*Glycine max* (L.) Merr.] rotations from the Upper Midwest and may not reflect outcomes in soils in warmer climates or situations involving frequent flooding cycles, such as in wetland rice (*Oryza sativa* L.) production or crop areas in river floodplains. The conflicting observations between oxic and anoxic soil conditions on the environmental fate of glyphosate in the presence of soil phosphate requires additional research attention.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes the sorption and degradation behavior of glyphosate in three different US soils under consideration of aerobic and anaerobic conditions and the addition of phosphates. The sorption experiment is well described according to the USEPA guidelines, however not sufficient results are reported (ads/des results at each concentration not available numerically) to check the validity of the article.

The degradation experiment was conducted in a microcosm with insufficient description of results for calculating degradation or dissipation endpoints according current guidelines.

The article is therefore classified as reliable with restrictions (Category 2).

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
General criteria for reliability considered for all data requirements indicated by the corresponding EU data points as specified in EC Regulation (EU) No 283/2013	1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines met.	No
	2. Previous exposure to other chemicals is documented (where relevant).	No
	3. The test substance is dissolved in water or non-toxic solvent	Yes
	4. Glyphosate, when the test substance, is sufficiently documented - identity of the test material reported (i.e. purity, source, content, storage conditions)	Yes

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
	5. Only glyphosate is the tested substance (excluding mixture), and information on application of glyphosate is described	Yes
	6. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite)	Yes
	7. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes
	8. Analytical verifications performed in test media (concentration)/ collected samples, stability of glyphosate in test media documented	No
	9. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information	Yes
	10. Assessment of the statistical power of the assay is possible with reported data.	Yes
	11. If statistical methodology was applied for findings reported, then the data analysis applied is clearly reported (e.g., checking the plots and confidence intervals)	Yes
	12. Field locations relevant/comparable to European conditions. Soils not completely matching the OECD criteria but from Europe or to some extent representative for the European Agriculture.	Yes
	13. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5 %), bulk density, water retention, microbial biomass (~1 % of organic carbon)	Yes
	14. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass	No
	15. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year	Yes
	16. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	Yes
	17. Data on precipitation is recorded	No
	18. The temperature was in the range between 20-25°C and the moisture was reported	Yes
	19. The presence of glyphosate identified in samples collected from groundwater, soil, surface waters, sediments or air from European areas	No
	20. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate	Yes
	21. Analytical methods clearly described and adequate Statement of specificity and sensitivity of the analytical methods is included	Yes
	22. Radiolabel characterization: purity, specific activity, location of label	Yes
	23. If degradation kinetics are included: expect to see data tables provided, model description. Statistical parameters for kinetic fit.	No

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
	24. Glyphosate monitoring data: description of matrix analysed, and analytical methods fully described as above.	No
	25. For environmental fate studies: clear description of application rate and relevance to approved uses.	No

1. Information on the study

Data point:	KCA 7.1.4.3
Report author	Kjaer, J. et al.
Report year	2011
Report title	Transport modes and pathways of the strongly sorbing pesticides glyphosate and pendimethalin through structured drained soils
Document No	Chemosphere, (2011) Vol. 84, No. 4, pp. 471–9
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Yes, conducted at officially recognised testing facilities (Geological Survey of Denmark and Greenland)
Acceptability/Reliability:	Reliable with restrictions (Substance properties not sufficiently described, no evaluation of the residues in soil layers after finalization of the study was conducted, duration of the study too short)

2.Full summary of the study according to OECD format

Leaching of the strongly sorbing pesticides glyphosate and pendimethalin was evaluated in an 8–month field study focussing on preferential flow and particle–facilitated transport, both of which may enhance the leaching of such pesticides in structured soils. Glyphosate mainly sorbs to mineral sorption sites, while pendimethalin mainly sorbs to organic sorption sites. The two pesticides were applied in equal dosage to a structured, tile–drained soil, and the concentration of the pesticides was then measured in drainage water sampled flow–proportionally. The leaching pattern of glyphosate resembled that of pendimethalin, suggesting that the leaching potential of pesticides sorbed to either the inorganic or organic soil fractions is high in structured soils. Both glyphosate and pendimethalin leached from the root zone, with the average concentration in the drainage water being 3.5 and 2.7 µg/L, respectively. Particle–facilitated transport (particles >0.24 µm) accounted for only a small proportion of the observed leaching (13–16 % for glyphosate and 16–31 % for pendimethalin). Drain–connected macropores located above or in the vicinity of the drains facilitated very rapid transport of pesticide to the drains. That the concentration of glyphosate and pendimethalin in the drainage water remained high (>0.1 µg/L) for up to 7 d after a precipitation event indicates that macropores between the drains connected to underlying fractures were able to transport strongly sorbing pesticides in the dissolved phase. Lateral transport of dissolved pesticide via such discontinuities implies that strongly sorbing pesticides such as glyphosate and pendimethalin could potentially be present in high concentrations (>0.1 µg/L) in both water originating from the drainage system and the shallow groundwater located at the depth of the drainage system.

Materials and methods

Chemicals

Glyphosate [N–(phosphonomethyl)glycine] – the active ingredient in Roundup – is a broad–spectrum, post–emergence, non–selective herbicide that is one of the most used herbicides worldwide. In Denmark, glyphosate is the herbicide sold in the largest quantities; in 2003, glyphosate sales for agricultural purposes accounted for 44 % of all herbicide sales. By 2008, this had increased to 52 % (Danish Environmental Protection Agency, 2004, 2009). Pendimethalin [N–(1–ethylpropyl)–2,6–dinitro–3,4–xylidine] is a selective herbicide used to control most annual grasses and certain broadleaf weeds both pre–emergence (i.e. before weed seeds have sprouted) and early post–emergence. Pendimethalin ranks fourth among the herbicides used in Demark, accounting for 5 % of all herbicide sales for agricultural use in 2003 and 6 % in 2008 (Danish Environmental Protection Agency, 2004, 2009). Solubility of glyphosate is 10500 mg/L while that of pendimethalin is 0.33 mg/L pesticide properties database available at http://sitem.herts.ac.uk/aeru/footprint/index2.htm.

Site description

The study was conducted at the Estrup field research site in Denmark, a virtually flat systematically tile–drained loamy field located on glacial till with a cultivated area of 1.26 ha. The tile drains are located at an average depth of 1 m b.g.s., and the water table is relatively shallow, located 1–3 m b.g.s. The uppermost meter of the soil is heavily fractured and bioturbated, with plough layer containing 100–1000 biopores/m^2 (Lindhardt et al., 2001). The geological structure is complex, comprising a clay till core with deposits of different age and composition. Of three pedological profiles available for the site, one is classified as Aquic Argiudoll, one as Abruptic Argiudoll and one as Fragiaquic Glossudalf. Details on soil properties are reported in Table 1 and geological properties are further described in Lindhardt et al. (2001).

Agricultural management

After maize (Zea mays L.) had been harvested on 13 October 2005, glyphosate (1.44 kg/ha active ingredient; 4.0 L/ha Round–up Bio) and pendimethalin (1.44 kg/ha active ingredient; 3.6 L/ha Stomp) were applied simultaneously together with 30.0 kg/ha of potassium bromide as tracer on 9 November 2005. On 12 April 2006 the field was ploughed to a depth of 18 cm. Spring barley was sown on 27 April 2006. Whereas glyphosate had been applied previously (13 October 2000 and 2 September 2002) the field had been treated with pendimethalin 7 October 1997. The minor residues of glyphosate (0.01–0.03 µg/L) found in the drainage water before the current application of pesticides (Figure 2) is thus likely to derive from these previous treatment.

Monitoring and sample preparation

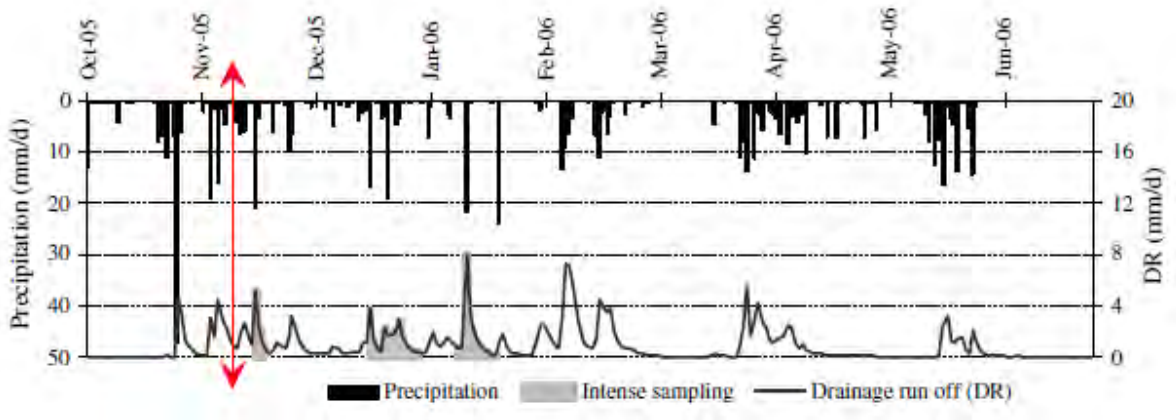
For a period of 8 months following application of the glyphosate and pendimethalin the concentration of the pesticides and bromide was measured on a weekly basis in drainage water sampled flow–proportionally. In addition, more intense sampling of drainage water was performed in connection with three flow events triggered by precipitation on 14 November 2005, 16 December 2005 and 11 January 2006 (Figure 1) in order to enable detailed description of the transport of water and pesticides. Sampling lasted for 2, 13 and 9 d, respectively. Flow events are characterised by an initial rapid rise in the hydrograph followed by a less rapid drop (tailing). During these events, drainage water subsamples were collected for every 2 mm of drainage runoff using a refrigerated Isco sampler (Teledyne Isco, Inc., US) containing eight 2–L borosilicate bottles. Within 24 h of the onset of the flow events, each bottle from the Isco sampler was shaken thoroughly to resuspend the sediment. The particles in the individual samples were then separated by centrifugation at 3500 rpm using Teflon vials. The time required for separation of particles ≥0.24 µm was calculated according to Gimbert et al. (2005). The supernatant was removed using a pipette, cleaned with 20 % HCl. The supernatant of samples to be analysed for pendimethalin was placed in glass bottles and preserved by adjusting to pH 2.0 with sulphuric acid. The pellets were flushed into a glass bottle using demineralised water and preserved using sulphuric acid. The samples to be analysed for pendimethalin were stored at 2°C until analysis. The supernatant of samples to be analysed for glyphosate and AMPA was pipetted into polypropylene (PP) bottles and adjusted to pH 2.0 with sulphuric acid. The pellets were flushed into PP bottles and adjusted to pH 2.0 with sulphuric acid. The latter two types of sample were stored at –18°C until analysis. As the flow event on 16 December 2005 occurred at a weekend, it was not possible to conduct particle separation on the samples. With all the samples collected on a weekly basis and the intensive samples collected following the flow event on 16 December 2005, pesticide concentrations were measured on the entire water sample. Thus the reported concentrations refer to the total concentration of both dissolved and particle–bound pesticide. With samples collected intensively following the flow events on 14 November 2005 and 11 January 2006, pesticide concentrations are reported for both particle–bound pesticide (concentration in the pellets) and dissolved pesticide (concentration in the supernatant). Furthermore, measurements of turbidity, chloride concentration and conductivity were conducted on all water samples obtained from the Isco sampler.

Table 1 Physical and chemical properties of the soil.

Profile[a]	Horizon	Depth	Clay[b] (%)	Silt[b] (%)	Sand[b] (%)	OM (%)	C/N	CEC (meq 100 g^{-1})	pH_{CaCl2}	Fe (mg kg^{-1})	Al (mg kg^{-1})
Estrup 2											
	Ap	0–26	13.8	12.7	70.8	2.7	13	12.1	6.5	2044	808
	Bt(g)	26–45	36.3	15.3	47.8	0.5	6	13.9	6.3	4144	1748
	Bt(g)2	45–121	33.0	15.9	50.9	0.2	4	16.8	6.6	2294	1034
	Cc[c]	121–150	31.1	24.9	7.5	0.5	6	19.7	7.5	2290	568
Estrup 3											
	Ap	0–28	9.9	7.1	77.5	5.5	17	15	7.0	1648	1024
	Bs/Bhs	28–58	8.8	4.7	85.7	0.8	12	10.2	6.6	1730	1340
	Bt(g)	58–115	12.2	4.3	83.1	0.4	8	9.2	4.2	1702	916
	2C	115–185	38.9	24.1	26.9	10.1	39	40.5	4.5	1576	2934

nd.: not determined; OM: organic matter determined as 1.72 total organic carbon; Fe and Al: oxalate extractable Fe and Al determined by the methods of McKeague and Day (1966).
a Profiles are classified as Abruptic Argiudoll (Estrup 2) and Fragiaquic lossudalf (Estrup 3).
b Clay: <2 µm; Silt: 2–20 µm; Sand: 20–2000 µm.
c Contains 36.1% $CaCO_3$. Contains 20.0% $CaCO_3$.

Figure 1. Precipitation (hanging bars on primary axis) and drainage runoff (solid line on secondary axis). The red vertical arrow indicates the date of application. The shaded grey area beneath the solid line indicates the flow events that were intensively monitored (see Section 2.4).



Methods of analysis

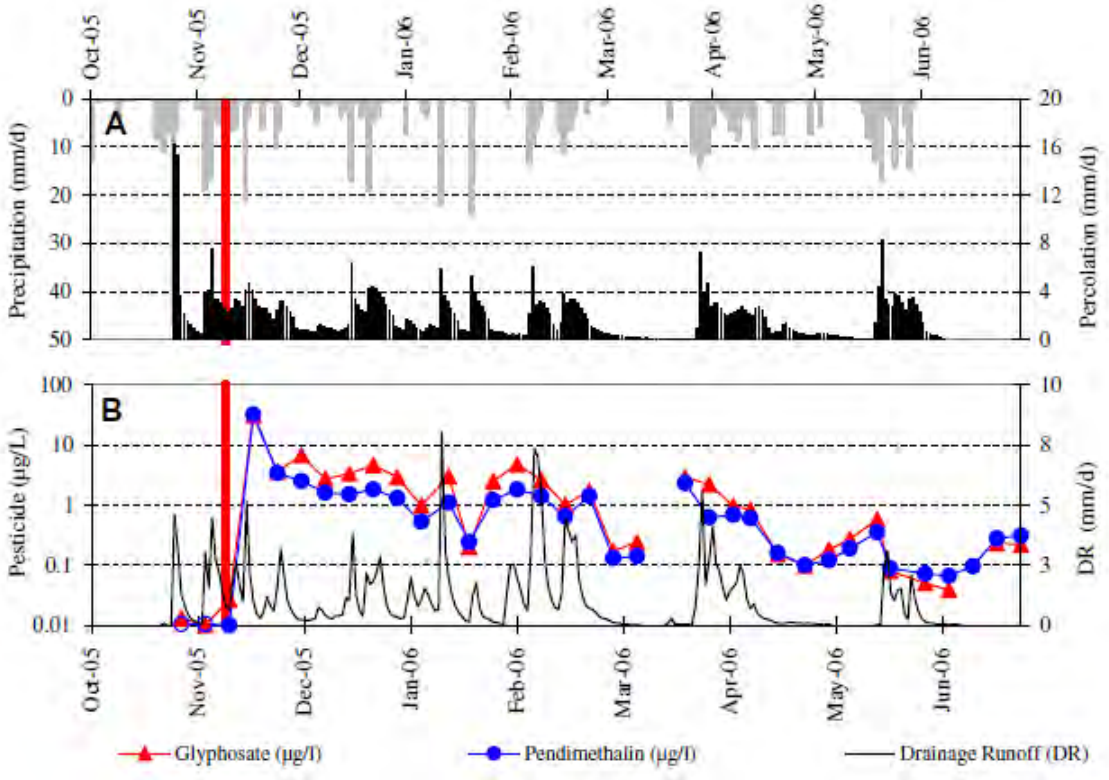
Glyphosate

The preserved water samples were first concentrated on a column of Chelex 100 resin, iron form 100–200 mesh from Bio–Rad. After washing with 0.1 M HCl, the analytes were eluted with 6 M HCl. The eluate was further cleaned on a column of AG 1–X8 resin, chloride form 200–400 mesh. The eluate was evaporated to dryness under nitrogen and redissolved in 200 µL of water–methanol–HCl (160:40:2.7). Derivatisation was carried out with 1 mL of trifluoroacetic anhydride–2,2,3,3,4,4,4–heptafluoro–1–butanol (2:1). The derivatives of glyphosate were measured by GC–MS using a 5 % phenyl methylsiloxane GC–column (HP–5) with the MS in electron impact (EI) mode. 2 µL sample was injected by splitless injection at 280°C with oven temperature at 65°C. After 2 min the oven temperature was raised to 310°C at 20°C min^{-1} and held at 310°C for 4 min. The glyphosate derivatives were identified by MS using m/z 612, 611 and 584. The calculations were made using the internal standard procedure with glyphosate–$^{13}C^{15}N$ as the internal standard. The LOD (limit of detection) was below 0.01 µg/L. The preserved pellet samples were treated with 1 M ammonia prior to analysis in order to extract the glyphosate from the solids. The extract was then diluted with water, adjusted to pH 2.0 with HCl, and analysed as described above for the water samples.

Inorganic analysis

The water samples were analysed for turbidity, conductivity and chloride concentration. Turbidity was measured with an infrared LED light source using a pHotoFlex Turb photometer (WTW Gmbh, Weilheim, Germany). Conductivity was measured using a Cond 340i conductivity pocket meter (WTW Gmbh, Weilheim, Germany). Chloride concentration was measured using a FIAstar™ 5000 flow injection analyser (Foss Analytical AB, Höganäs, Sweden).

Figure 2. Precipitation and simulated percolation (A) together with concentration of pendimethalin and glyphosate (B) in the drainage runoff (DR on secondary axis). The red vertical lines indicate the date of application. The open circles indicate concentrations below the LOD (0.01 µg/L).



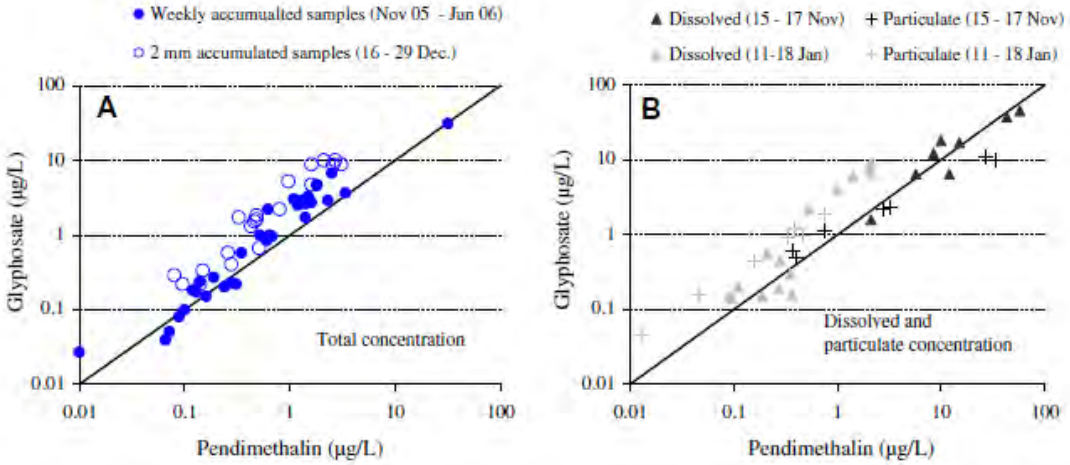
Results and Discussion

Leaching of glyphosate and pendimethalin

The leaching pattern of glyphosate resembled that of pendimethalin (Figure 2), thus suggesting (i) that the leaching potential of strongly bound pesticides from structured soil is high both with pesticides that bind to soil organic matter (e.g. pendimethalin) or to the inorganic fraction (e.g. glyphosate) and (ii) that the pathways governing the transport of these two pesticides are similar. Both glyphosate and pendimethalin leached from the root zone in average concentrations considerably exceeding the EU limit value for groundwater (0.1 µg/L) during the 8–month drainage flow period (Figure 2). The average concentration of glyphosate and pendimethalin in the drainage water was 3.5 and 2.7 µg/L, respectively. Both pesticides were found in all of the weekly drainage water samples. Among the 32 samples collected after pesticide application, the concentration exceeded 0.1 µg/L in 29 (Figure 2). The similarity of the leaching patterns of the two pesticides was reflected in the close correlation between the measured concentration of pendimethalin and glyphosate (Figure 3). R^2 for measured total concentration (both dissolved and particle–bound) in samples collected (i) on a weekly basis during the entire monitoring period (32 samples) and (ii) for every 2 mm of drainage runoff occurring during a 13–d period in December (20 samples) was 0.962 and 0.963, respectively (Figure 3A). A similar tendency was found when comparing the particulate and dissolved concentrations of pendimethalin and glyphosate measured during two individual flow events, R^2 being 0.943 for dissolved pesticide (19 samples) and 0.928 for particle–bound pesticide (10 samples) (Figure 3B). Pesticide leaching was governed by preferential transport, as evidenced by the soil hydraulic properties (Kjær et al., 2005) and fast solute transport. Piston flow through the low–permeable soil matrix would entail a transport time to the drainage system of about 98 d (Kjær et al., 2007). However, glyphosate and pendimethalin were detected in drainage water samples as early as 8 d after application. This finding is thus consistent with previous transport studies conducted at the Estrup site (Kjær et al., 2005, 2007), as well as other field studies demonstrating rapid macropore–mediated transport of pesticides (for a review see Jarvis (2007)). As both glyphosate

and pendimethalin leached in high concentrations following the same transport pathways, the difference in sorption characteristics of glyphosate, which sorbs strongly to the inorganic soil fraction, and pendimethalin, which sorbs strongly to the soil organic fraction, had little impact on leaching in this structured soil. Our finding is in line with previous studies showing that differences in the leaching of pesticides that differ widely in sorption properties are significantly reduced in the presence of macropore flow (Larsson and Jarvis, 1999). Likewise, Flury (1996) concluded from the transport studies of Kladivko et al. (1991), Traub–Eberhard et al. (1995) and Flury et al. (1995) that part of the various pesticides applied simultaneously to the soil surface moved through structured soil in an identical manner irrespective of their chemical properties.

Figure 3. Measured concentration of glyphosate and pendimethalin in drainage water samples collected after pesticide application. A (left): Total concentration (both dissolved and particle–bound) in samples collected on either a weekly basis during the entire monitoring period (closed circles) or for every 2 mm of drainage runoff during a 13–d period in December 2005. B (right): Concentration of dissolved (triangles) and particle–bound (crosses) pesticide in samples collected for every 2 mm of drainage runoff during two selected flow events in November 2005 (black) and January 2006 (grey). Sampling periods are indicated in parentheses.

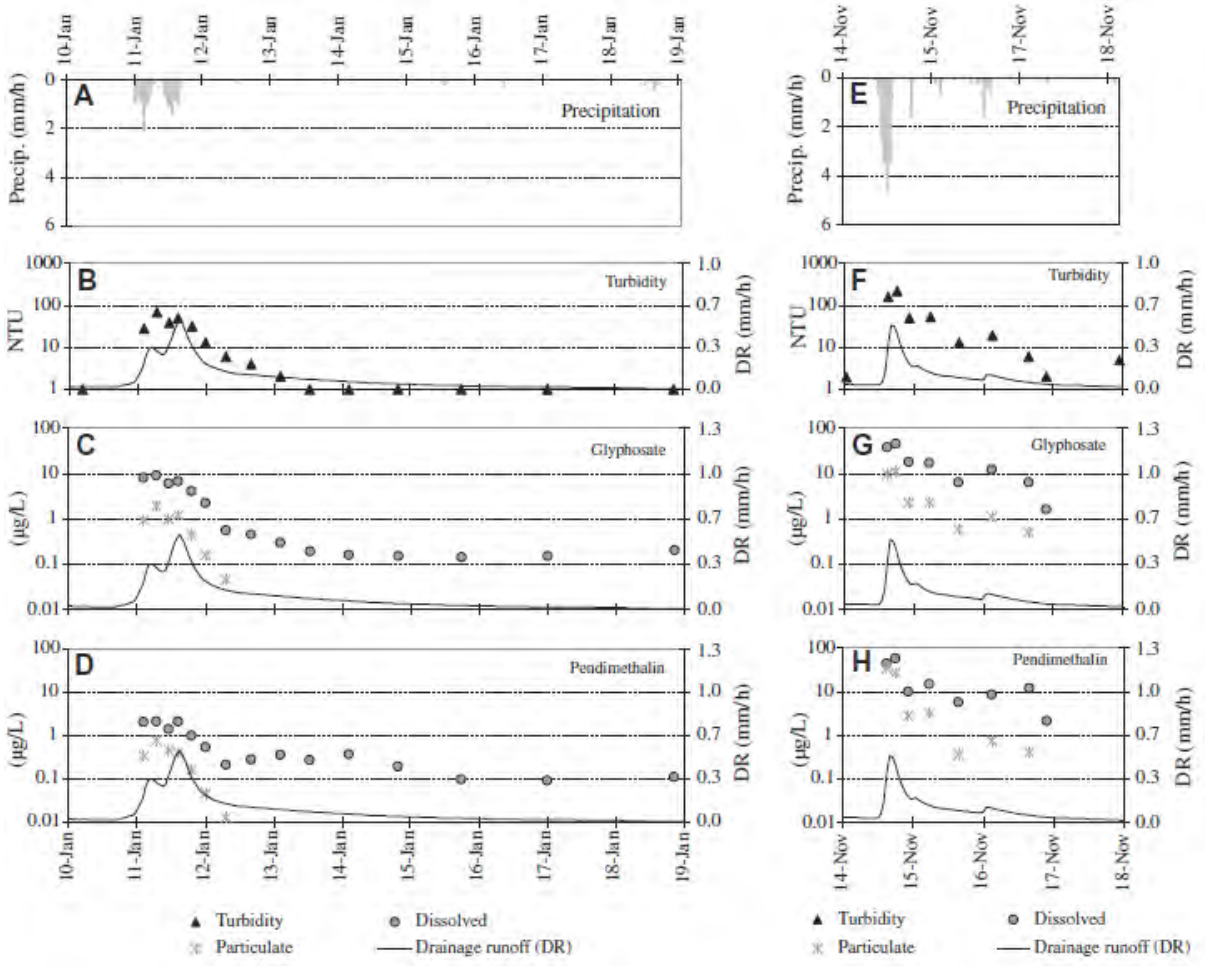


Quantitative impact of particle–facilitated transport on total leaching

Measured concentration of particle–bound pesticides were marked lower than that of dissolved pesticides, ratio between measured concentration of dissolved pesticides and particle–bound ranging between 4–14 and 1.2–30 for glyphosate and pendimethalin respectively. Intensive monitoring of two individual flow events (Figure 4) suggested that particle–facilitated transport (particles >0.24 µm) accounted for only a small proportion of the observed leaching (13–16 % of the leached mass of glyphosate and 16–31 % of the leached mass of pendimethalin). These values are in line with the few available field studies quantifying particle–facilitated transport of strongly sorbing pesticides. In Danish drainage water studies using a cut–off size of 0.7 µm, Petersen et al. (2003) found that 9 % of the leached pesticide was particle bound. Correspondingly, Vilholdt et al. (2000), using a cut–off size of 0.24 µm, found that 6 % of the leached pesticide was particle bound. In laboratory experiments with undisturbed 20–cm soil columns, de Jonge et al. (2000) found that particle–facilitated transport (particles >0.24 µm) accounted for <1–27 % of total glyphosate leaching. In a study by Gjettermann et al. (2009) using intact soil columns from ploughed and minimal tillage cultivation systems, colloid–facilitated glyphosate leaching (cut–off size >0.02 µm) accounted for 68 ± 10 % of total glyphosate leaching from the ploughed system as compared to only 17 ± 12 % from the minimal tillage system. That leaching of particle–bound glyphosate from the ploughed soil was markedly greater than that seen in our study and previous studies may be attributable to differences in experimental conditions, e.g. ploughing before or after pesticide application and precipitation intensity. In our field study the total amount of precipitation and maximum precipitation intensity were 12 mm within 11 h and 2.1 mm h^{-1} (11 January 2006) and 18 mm within 10 h and 4.6 mm h^{-1} (14 November 2005). In the study of Gjettermann et al. (2009) glyphosate was applied to the soil 1 d after the last of two rewettings and the soil then irrigated twice

for 2 h using 15 mm h^{-1} on days 5, 8 and 12 following the last rewetting. In Denmark such high precipitation intensity is rare during the period relevant for autumn application of glyphosate (September–November). Analysis of precipitation data collected in a national grid of approximately 60 automatic climate stations run by the Danish Meteorological Institute revealed that there had only been 8 precipitation events exceeding 15 mm h^{-1} during the preceding 10 years (Birgit Sørensen, personal communication). The combination of wet, loose soil and very intensive precipitation shortly after the application of pesticide is likely to result in greater contact between pesticide and soil particles and enable greater mobilisation of soil particles. In soils having had time to consolidate, such as the minimal tillage soil studied by Gjettermann et al. (2009) and in the present study (ploughed 7 months before pesticide application) fewer particles will be available for contact with the pesticide.

Figure 4. Hourly precipitation (grey hanging bars in A and E) together with turbidity (B and F), particulate (crosses) and dissolved (circles) glyphosate (C and G), particulate (crosses) and dissolved (circles) pendimethalin (D and H) in the drainage runoff (DR on the secondary axis) following flow events on 14 November 2005 (right) and 11 January 2006 (left).

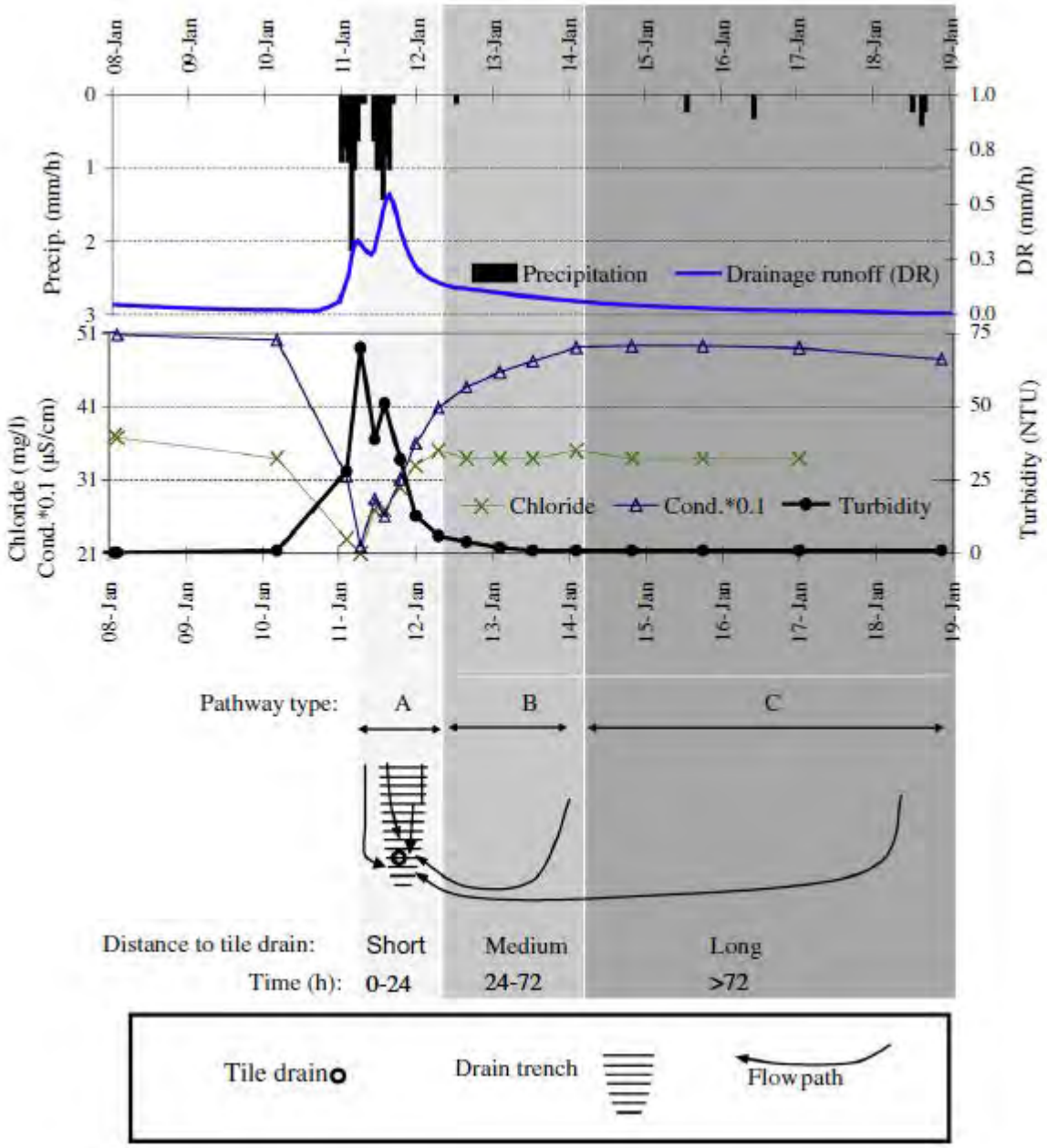


Water flow pathways

Drainage water consists of a mixture of water of different origins, with the dominant flow pathways varying over the course of time (Jacobsen and Kjær, 2007). Knowledge of the dominant transport pathways is thus important for the interpretation of measured pesticide concentrations. The transport pathways during the flow event of 11 January 2006 are indicated by the measured turbidity, chloride concentration and conductivity (Figure 5). Thus the chloride concentration and conductivity decreased markedly during the first precipitation event, while the turbidity increased. This indicates rapid transport of precipitation with low chloride concentration and conductivity, probably through drain–connected macropores. During the 24–h period from the end of the first precipitation event the turbidity decreased, while the chloride concentration and conductivity increased. This indicates that water entered the

drainage system not only from macropores connected directly to the drains but also from the vicinity of the drain pipe, i.e. the trench dug when installing the tile drain system. Transport pathways is likely also to involve a lateral component in the shallow saturated zone through natural macro pores aided by gradients generated by inter–drain mounding of the water table during the high–flow condition following the rain event (Figure 5). Water from here would have a relative short travelling distance before entering the drainage system. These transport pathways characterised by having a short flow path to the drain and being active during the first 24–h period are designated type A (Figure 5). From 24 to 72 h the turbidity remained low and below the detection level of 1 NTU (nephelometric turbidity units), the chloride concentration plateaued out and the conductivity continued to increase. That the chloride concentration returned to the ‘‘background level’’ indicates cessation of the rapid entrance of precipitation low in chloride. Instead the length of the pathway increased with drainage water entering from in between the drain trench, designated pathway B in Figure 5. During these longer transport pathways, the particles are filtered by the soil causing the turbidity to decrease below the detection limit, while the longer retention time allows the infiltrating water to interact with the soil matrix causing the conductivity to increase. By 72 h after the end of the first precipitation event the conductivity, chloride concentration and turbidity had returned to their background levels, and the drainage water is dominated by the longer transport pathways (designated type C). These transport pathways are likely to comprise precipitation that has infiltrated vertically some distance from the drain trench and subsequently been transported laterally to the drain via the saturated layer.

Figure 5. Hourly precipitation and drainage runoff together with measured chloride concentration, conductivity and turbidity (lower graph). The shaded areas indicate the dominant transport pathways (types A–C) feeding into the sampled drainage water during the flow event. While ‘‘time’’ and ‘‘pathway types’’ are classified directly from measured data, ‘‘distance to tile drain’’ and shown water flow pathways are indicative providing our interpretation of measured data. (see Section *“Through which pathways do strongly sorbing compounds enter the drainage system?”*).



Pesticide transportation pathway

During the 24–h period following cessation of the first precipitation event on 11 January 2006 the leaching pattern was similar for both particles, particle–bound pesticide and dissolved pesticide (Figure 4), thus indicating that all three follow the same transport pathways, presumably involving drain–connected macropores located above or in the vicinity of the drains or rapid lateral transport near the drain line (type A in Figure 5). Thereafter the leaching of particles and particle–bound pesticide ceased, whereas dissolved pesticide continued to leach in high concentrations (>0.1 µg/L) for up to 7 d after the precipitation had stopped (Figure 4). This ‘‘tailing’’ of dissolved pesticides indicates that the transport pathways involve transport through macropores between drains followed by lateral transport to the

drains (types B and C; Figure 5). Moreover, it indicates that while particles (indicated by elevated turbidity) and particle–bound pesticide seems to be retained in the soil during the lateral transport in between the drain, dissolved pesticide can be transported laterally through the saturated zone to the drainage system. The leaching pattern following the flow event on 14 November 2005 (Figure 4) was very similar to that observed after the flow event on 11 January 2006, although sampling conditions precluded the recording of transport occurring through pathways B and C. The flow event on 11 January 2006 was characterised by high precipitation (12 mm) followed by 7 d virtually free of precipitation (1 mm in total). Such conditions are ideal for describing variation in flow pathways over time and capturing the transport involving all three pathways (A–C; Figure 5). In contrast, the flow event on 14 November 2005 was characterised by one major (18 mm on 14 November) and several minor precipitation events (6 mm in total), and sampling was performed for just 2 d (Figure 4. E–H). The conditions were ideal for descryibing transport pathway A, but inadequate for describing pathways B and C (Figure 5). The fact that turbidity remained high for a much longer period (approx. 24 h) during the November 2005 event than during the January 2006 event (Figure 4E–H) is attributable to the minor precipitation events on 16 and 17 November 2005 and resultant rapid preferential transport of leachate via pathway A (Figure 5). The direct transport from surface layers to drains via macropores (pathway A in Figure 5) reported here is in line with previous observations. Thus several studies report that the soil surface can be in direct contact with drains through macropores comprised of old root channels or earthworm burrows (Nielsen et al., 2010; Nuutinen and Butt, 2003; Shipitalo and Gibbs, 2000). The same pathways were also responsible for the leaching of colloid–size particles (Nielsen et al., 2011) and the strongly sorbing pesticides (both dissolved and particle–bound) pendimethalin (Petersen et al., 2003) and prochloraz (Vilholdt et al., 2000) on drained, loamy soils. The observed transport pathway involving transport through macropores located between drains followed by lateral transport to the drains (pathways B and C; Figure 5) is presumably attributable to connectivity between the vertical biopores and the three–dimensional fracture system in the soil, which enables rapid, lateral transport in the soil (Rosenbom et al., 2008; Nilsson et al., 2000, 2001; McKay et al., 1999). Studies of the transport of two fluorescent tracers in clayey till (Rosenbom et al., 2008) indicate that during periods of continuous drainage runoff the extent of rapid macropore transport in the soil between the drain lines is determined by the degree of connectivity between root zone biopores and high–permeability fractures. Evidence that such connectivity enables leaching of solutes from the surface of fractured till is also provided by forced gradient tracer experiments conducted at three different locations (Ringe, Avedøre and Lillebæk) in Denmark (Nilsson et al., 2000, 2001; McKay et al., 1999). These transport studies were all performed with conservative or slightly sorbing tracers (chloride, bromide, bacteriophage tracer PRD–1, colloidal tracer, sulforhodamine B, and acid yellow). Similar studies addressing the potential of pathways B and C (Figure 5) to transport strongly sorbing pesticides are very limited, however. Transport of the strongly sorbing pesticides pendimethalin and prochloraz in drained structured soil has been studied by Vilholdt et al. (2000) and Petersen et al. (2003). However, the study design, while suitable for describing vertical transport from the top soil to the vicinity of the drain line (pathways A and B), was unsuitable for describing transport involving vertical infiltration between the tile drains followed by a subsequent lateral transport to the drain (pathway C). In Vilholdt et al. (2000), pesticide sampling was performed 2.5 m either side of the drain trajectory up to 7.5 h following a precipitation event. The study of Petersen et al. (2003) was conducted on a very well drained soil with limited lateral water flow (drainage runoff accounting for only 2–10 % of total precipitation input during the sampling period) with most pesticide samples being collected within 36 h of the precipitation event. Under such conditions, lateral transport of pesticides (pathway C) is unlikely. The leaching pattern found in our study indicate that while particles (indicated by elevated turbidity) and particle–bound pesticides were retained in the soil during lateral transport, dissolved glyphosate and pendimethalin were transported through the saturated zone to the drainage system. Similar findings suggesting that dissolved, strongly sorbing pesticide can be transported over long distances via discontinuities are provided by Gooddy et al. (2007), who studied the concentration of dissolved and particle–bound diuron and its metabolites in chalk groundwater sampled 30 m b.g.s. Most of the pesticide–colloid complexes (particles >0.1 μm) formed in the soil were removed during migration of the water through the 30 m deep, unsaturated zone and/or the saturated zone, whereas pesticides in soluble form was detectable in the groundwater 30 m b.g.s. Moreover, in a study of the transport of brilliant blue, bromide and micropheres along macropores in sandy loam, Nielsen et al. (2011) found that while colloid–size particles were trapped in the bottom of the biopores, dissolved tracer (brilliant blue and bromide) migrated further into the soil.

Conclusion

Pesticides leaching from the unsaturated zone may eventually pose a risk to the aquatic environment. The present 8–month study of a loamy field demonstrates that:
Strongly bound pesticides, whether bound to the organic or inorganic soil fraction, may leach from the root zone and enter the aquatic environment in average concentrations exceeding 0.1 µg/L. Particle–facilitated transport (particles >0.24 µm) accounted for only a small proportion of observed leaching (13–16 % for glyphosate and 16–31 % for pendimethalin). The pathway by which these strongly sorbing compounds entered the drainage system involved transport through drain–connected macropores (above or in the vicinity of the drains) as well as the macropores situated between the drains and connected to underlying fractures. Particle–bound pesticide (particles >0.24 µm) was transported solely by vertical transport in macropores and rapid lateral transport occurring nearby the drain line, whereas dissolved pesticide was also transported laterally over larger distances through the saturated zone via discontinuities in the soil. This newly identified transport pathway whereby dissolved pesticides are transported laterally via discontinuities in the soil needs to be taken into account when assessing the risk posed by pesticides to the aquatic environment. Our findings imply that strongly sorbed pesticides such as glyphosate and pendimethalin may be present in high concentrations (>0.1 µg/L) in both the water flowing from the drainage system and in the shallow groundwater located at the depth of the drainage system.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes a leaching experiment with glyphosate and pendimethalin in a Danish agricultural soil over eight months. The substance properties are sufficiently reported. With regard to the data requirement, the study is too short for a comprehensive evaluation of the leaching behavior. In addition, no residues were determined in different soil layers after finalization of the study, and sample storage stability prior to analysis was not established.

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Munira, S., et al.
Report year	2016
Report title	Phosphate fertilizer impacts on glyphosate sorption by soil
Document No	Chemosphere 153 (2016) 471-477
Guidelines followed in study	OECD 106 (2000)
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (no European climate and soil conditions, validity check for study could not finalized)

2. Full summary of the study according to OECD format

This research examined the impact of field-aged phosphate and cadmium (Cd) concentrations, and fresh phosphate co-applications, on glyphosate sorption by soil. Soil samples were collected in 2013 from research plots that had received, from 2002 to 2009, annual applications of mono ammonium phosphate (MAP) at 20, 40 and 80 kg P/ha and from products containing 0.4, 70 or 210 mg Cd/kg as an impurity. A series of batch equilibrium experiments were carried out to quantify the glyphosate sorption distribution constant, Kd. Extractable Cd concentrations in soil had no significant effect on glyphosate sorption. Glyphosate Kd values significantly decreased with increasing Olsen-P concentrations in soil, regardless of the pH conditions studied. Experiments repeated with a commercially available glyphosate formulation showed statistically similar results as the experiments performed with analytical-grade glyphosate. Co-applications of MAP with glyphosate also reduced the available sorption sites to retain glyphosate, but less so when soils already contain large amounts of phosphate. Glyphosate Kd values in soils ranged from 173 to 939 L/kg under very strong to strongly acidic condition but the Kd was always <100 L/kg under moderately acidic to slightly alkaline conditions. The highest Olsen-P concentrations in soil reduced Kd values by 25 - 44% relative to control soils suggesting that, under moderately acidic to slightly alkaline conditions, glyphosate may become mobile by water in soils with high phosphate levels. Otherwise, glyphosate residues in agricultural soils are more likely to be transported off-site by wind and water-eroded sediments than by leaching or runoff.

Materials and Methods

Experimental design and soil characteristics

Soil samples (0 -15 cm) with a sandy clay loam texture were collected in the spring 2013 from research plots situated under a durum wheat and flax rotation near Carman, Manitoba, Canada. The soil is classified as an Orthic Black Chernozem. The experimental plot was a randomized complete block design with 10 treatments and 4 replicates per treatment. In each of the forty plots, the composite soil sample consisted of ten samples collected in the plot using a Dutch augur. Treatments were a control (neither phosphate nor Cd applications), and plots receiving from 2002 to 2009 annual applications of mono ammonium phosphate (MAP) fertilizers that originated from three different phosphate rock sources containing 0.4, 70 or 210 mg Cd/kg, or low, medium and high Cd, respectively (Grant et al., 2013). MAP from these three sources was applied to plots at 20, 40 and 80 kg P/ha, or 20P, 40P and 80P, respectively. For all plots that received MAP, 20 kg P/ha was placed near the seed to enhance fertilizer use efficiency, a common practice in Canadian Prairie agriculture. For the 40 and 80 kg P/ha treatments, to avoid seedling toxicity, the additional MAP was broadcasted and then incorporated in soil. From 2010 to 2013, the rotation was continued but no phosphate or Cd was applied. Nitrogen fertilizer varied by year to optimize yields. The typical rate of N applied was 90 kg N/ha in durum wheat and 50 kg N/ha in flax. Soil samples were air-dried and sieved (<2 mm) prior to soil properties analysis and sorption studies. Soil was digested with nitric acid and total Cd was determined by inductively coupled plasma (ICP). Extractable Cd was extracted with diethylene triamine pentaacetic acid (DTPA) ICP. Various factors have been shown to influence the efficiency of micronutrient extraction by DTPA,

including extraction temperature and shaking time. Available phosphate was extracted using Olsen ($NaHCO_3$) phosphorus test. Soil physical and chemical properties that are known to influence glyphosate and phosphate sorption by soil, but did not significant vary across the plots by treatment, were also determined. Soil organic carbon content was determined using combustion technique with a high temperature induction furnace. Extractable Fe_2O_3 and Al_2O_3 were extracted with DTPA and 0.01 M $CaCl_2$, respectively, and extracts were analyzed by ICP. Extractable Ca was also measured by ICP using ammonium acetate as an extractant. Results were soil organic carbon content: 2.80% (mean) ±0.04 (standard error) (n = 16, number of plots analyzed); extractable Fe_2O_3: 246 ± 5 mg/kg (n = 40), extractable Al_2O_3: 6.4 ± 0.65 mg/kg (n = 16); and extractable Ca: 2252 ± 40.57 mg/kg (n = 16). Given that the study focused on Cd and P applications as treatments, the concentrations of extractable and total Cd, as well as Olsen-P in all plots were determined. We did not expect to see treatment differences for the other parameters that were measured (i.e., extractable Fe_2O_3, Al_2O_3, and Ca). Fe_2O_3 was also measured in all plots as previous studies have demonstrated that there is a strong positive association between Fe_2O_3 concentrations and phosphate or glyphosate sorption in soils. Since our results indicated no treatment differences induced by Cd and P applications on Fe_2O_3 concentrations extractable Al_2O_3, and Ca were quantified for 16 plots only (i.e., Control, 20P, 40P and 80P plots).

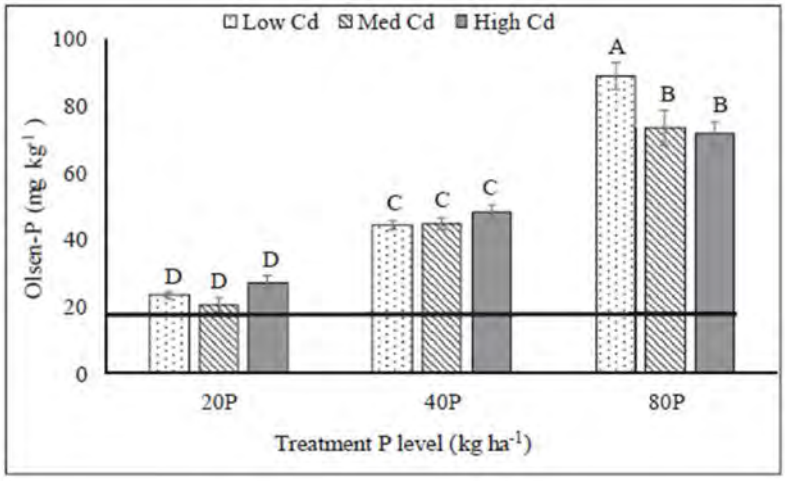


Fig. 1. Effect of phosphate fertilizers with different Cd levels on Olsen P concentrations in soil. The solid line indicates the concentration of Olsen P in control plots.

Sorption studies

Chemicals used in the sorption studies were: analytical grade ammonium phosphate monobasic (98% chemical purity) and glyphosate (99.9% purity), ^{14}C-labelled glyphosate [phosphonomethyl-^{14}C] (99% radiochemical purity; specific activity 50 µCi), and Roundup Ultra 2 (49% active). Active ingredient was potassium salt of N-(phosphonomethyl) glycine. Glyphosate sorption was determined by batch equilibrium with the initial solution containing 1 mg/L glyphosate and 6.67 x 10^4 Bq/L ^{14}C-labelled glyphosate. Batch equilibrium procedures followed the OECD guideline 106 using a soil/solution ratio of 1:5 and an equilibrium time of 24 h (OECD, 2000). Initial solution was added to soil in centrifuge Teflon tubes (duplicates) and slurries were rotated in the dark for 24 h. A constant 5 ^{0}C temperature was utilized to minimize risks for biodegradation. Equilibrium solution was centrifuged and subsamples of supernatant were added in duplicated scintillation vials containing Scintisafe scintillation cocktail. Vials were lightly shaken and stored in the dark for 24 h to disperse the chemiluminescence before the radioactivity was measured. Radioactivity was quantified by Liquid Scintillation Counting (LSC) with automated quench correction (#H method). The glyphosate sorption distribution constant, Kd (L/kg) was calculated by Cs/Ce, whereby Cs = glyphosate sorption by soil at equilibrium (mg/kg), and Ce = glyphosate concentration of equilibrium solution (mg/L). The effects of field-aged phosphate and Cd concentrations on glyphosate sorption were examined at pH conditions ranging from 3.6 to 7.3. This first experiment utilized soils from all forty plots and the range in pH was induced using different types of ions in the initial solution (0.01M HCl, 0.01M $CaCl_2$, 0.01M KCl, 0.01M KOH or dH_2O). For the control and high Cd 80P plots, the experiments were repeated but then using the Tier 2 parallel method

with tubes being sampled at 1, 2, 4, 6, 8 and 24 h. The two subsequent experiments utilized soils from the plots labelled as low Cd and with 20P, 40P or 80P levels. In one experiment, for slurry pH conditions ranging from 3.6 to 7.3, batch equilibriums procedures were repeated but using Roundup Ultra 2 in 0.01 M HCl, 0.01 M $CaCl_2$, 0.01 M KCl, 0.01 M KOH or dH_2O to verify experimental results for a formulated product. In the other experiment, for slurry pH conditions range from 4.7 to 5.4, the effect of fresh phosphate additions on glyphosate sorption by soil was examined by adding analytical grade MAP to analytical glyphosate in 0.01 M $CaCl_2$, 0.01 M KCl and dH_2O solutions. The amounts of MAP added was equivalent to 11, 22 and 44 mg P/kg, or an estimated 20, 40 and 80 P kg/ha, respectively, assuming the fertilizer being present in the top 15 cm layer of a soil with a bulk density of 1200 kg/m^3.

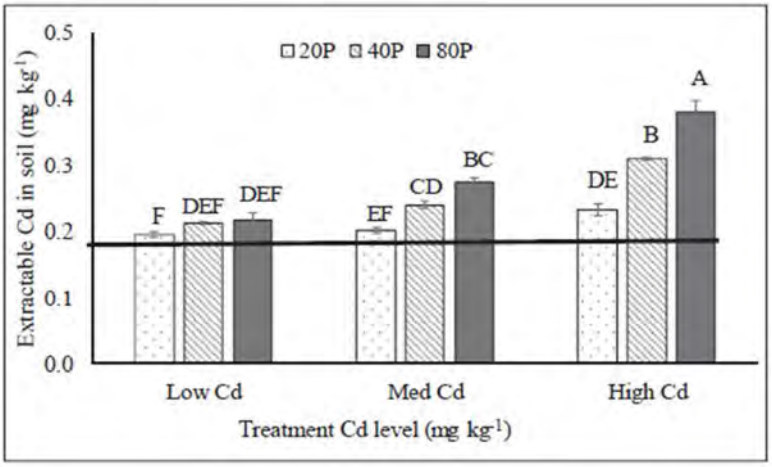


Fig. 2. Effect of phosphate fertilizers with different Cd levels on DTPA-extractable Cd in soil. The solid line indicates the concentration of extractable Cd in control plots.

Statistical analyses were completed using SAS software version 9.3 for Windows. Two-way analysis of variance (ANOVA) and multiple means comparison (Tukey's) tests were conducted to determine the effect of phosphate fertilizer (20P, 40P, 80P) and Cd (low, medium, high) treatment on Olsen-P concentrations, extractable Cd concentrations and total Cd concentrations in soil. For each pH (ionic solution), multiple linear regression analyses were carried out to predict glyphosate Kd values by using Olsen-P and extractable Cd concentrations as independent variables. Repeated measure analysis was used to determine the effect of shaking time (0.5, 1, 2, 4, 6, 8 and 24 h) by using phosphate levels and time as independent variables. Two way ANOVA and multiple means comparison (Tukey's) tests were utilized to quantify the effects of field-aged (20P, 40P, 80P) and fresh phosphate additions (11, 22 and 44 mg P/kg) on glyphosate Kd values. One-way ANOVA and multiple means comparison (Tukey's) tests were applied to quantify the impact of using Roundup Ultra 2 versus analytical-grade glyphosate on Kd values in soils.

Results and Discussion

Glyphosate Kd values ranged from 43 to 1173 L/kg which is in agreement with glyphosate Kd values reported in agricultural soils. There were no significant differences in glyphosate sorption by soil when using either Roundup Ultra 2 or analytical-grade glyphosate, suggesting that other ingredients in the commercial formulation had no impact on the sorption behaviour of the active ingredient glyphosate in soil. The additions of MAP fertilizers from 2002 to 2009 had a significant effect on phosphate concentrations in 2013 (Fig. 1). Olsen-P concentrations ranged from 13 to 99 mg/kg across plots and significantly decreased from 80P > 40 P > 20P plots. Total Cd concentrations in soil ranged from 0.42 to 0.98 mg/kg across plots but there were no significant treatment effects. Thus, the amount of Cd in the MAP fertilizers applied had no significant effect on the total Cd concentrations in 2013. DTPA-extractable Cd concentration ranged from 0.19 to 0.41 mg/kg, within the typical range of 0.1 - 0.5 mg/kg reported for soils (International Cadmium Association, 2015). There was a significant interaction, between the rate of phosphate fertilizer applied and the amount of Cd that the phosphate fertilizer

contained, on extractable Cd concentrations in soil (Fig. 2). For the 80P plots, extractable Cd concentrations significantly decreased in the order of high Cd > med Cd > low Cd. For the 40P plots, extractable Cd concentrations significantly decreased in the order of high Cd > (med Cd = low Cd). In 20P plots, only the high and low Cd treatments had significantly different extractable Cd concentrations. Despite these significant differences, extractable Cd concentrations in soil had no significant influence on glyphosate Kd values. The Cd concentrations in our field plots are those typically encountered in agricultural soils, but we recognize that in a batch equilibrium experiment, Zhou et al. (2004) demonstrated that the co-application of exceptionally large quantities of Cd to glyphosate solutions (i.e., 562 mg Cd/kg soil) can increase glyphosate sorption by approximately 1.6 times fold, relative to control soil. Increased Olsen-P concentrations in soil was a significant factor ($P < 0.0001$) in the regression analysis to explain reduced glyphosate Kd values in soil.

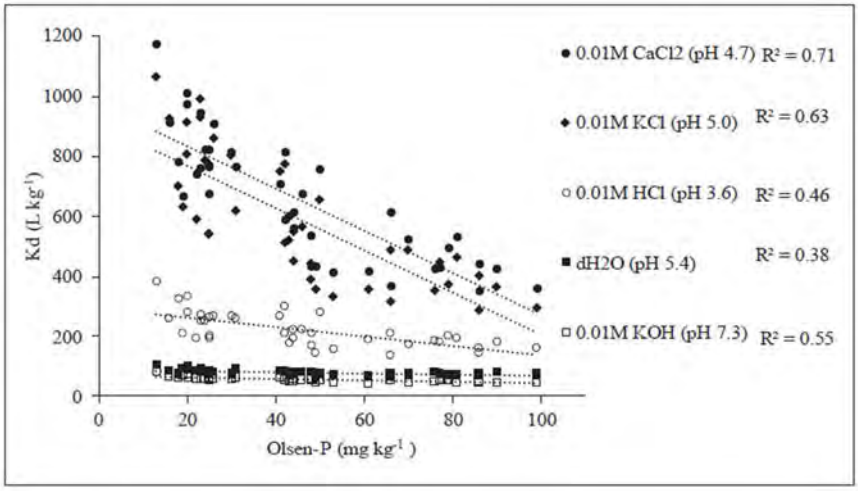


Fig. 3. Relation between Olsen-P concentrations in soil and the glyphosate sorption distribution constant, Kd, with soil slurries being under different pH conditions. All regression equations are significant at $P < 0.0001$

Regardless of the ionic solution used in the batch equilibrium experiments, increased Olsen P concentrations significantly decreased glyphosate sorption by soil (Fig. 3). A maximum reduction in glyphosate sorption occurred at a pH of 5 (0.01 M KCl solution) when the Olsen-P concentrations was on average 89 mg/kg Olsen P and the glyphosate Kd value was reduced by 57%, relative to the control plots that contained on average 18.75 mg/kg Olsen-P (Fig. 3). Our results are in agreement with the findings of de Jonge et al. (2001) who also reported that field-aged phosphate in soil reduces glyphosate sorption by soil. The iron oxides content of the Orthic Black Chernozem used is within the range of that observed in other Prairie soils in Canada suggesting the competitive effect of phosphate on glyphosate sorption could be applicable to a wider range of soils in the Prairie region of Canada particularly with low pH and high Fe content. At pH 5.4, in both 80P and control, time had no significant effect on glyphosate Kd values and sorption was always significantly smaller in 80P than control plots. For all other pH conditions, glyphosate sorption approached equilibrium at approximately 8 h because there were no significant differences in glyphosate Kd values between 8 and 24 h (Fig. 4). For these pH conditions, glyphosate sorption was almost always significantly smaller in 80P than control plots, regardless of the time, except for 0.5, 1 and 2 h under pH 3.6 and 0.5 h under pH 5.0 (Fig. 4). In general, longer shaking hours resulted in greater numerically differences in glyphosate Kd values between control and 80P plots. Regardless of the ionic solution used (Fig. 5), there was a significant interaction ($P < 0.01$) between field-aged and fresh phosphate on glyphosate sorption. In general, regardless of the amount of aged phosphate in soil, the addition of fresh MAP to the ionic solutions numerically reduced glyphosate Kd values, suggesting that phosphate and glyphosate compete for the same sorption sites in soil and that phosphate is preferentially sorbed when added with glyphosate to soil. Additions of 11 mg P/kg to the 0.01 M $CaCl_2$ solutions had no significant effect on glyphosate Kd values, except in the 20 P plots containing relatively small Olsen-P concentrations (Fig. 5). The addition of 22 or 44 mg P/kg to the 0.01 M $CaCl_2$ solutions always significantly reduced glyphosate Kd values, except the addition of 22 mg P/kg to 80 P plots (Fig. 5). For the largest co-application (44 mg P/kg), glyphosate Kd values were reduced on average by 52% in 20P plots, but by only 37% in the 80P plots. Additions of 11, 22 or

44 mg P/kg to 0.01 M KCl solutions always significantly reduced glyphosate Kd values except for 80 P plots for which only the addition of 44 mg P/kg resulted in a significant reduction in glyphosate Kd values (Fig. 5).

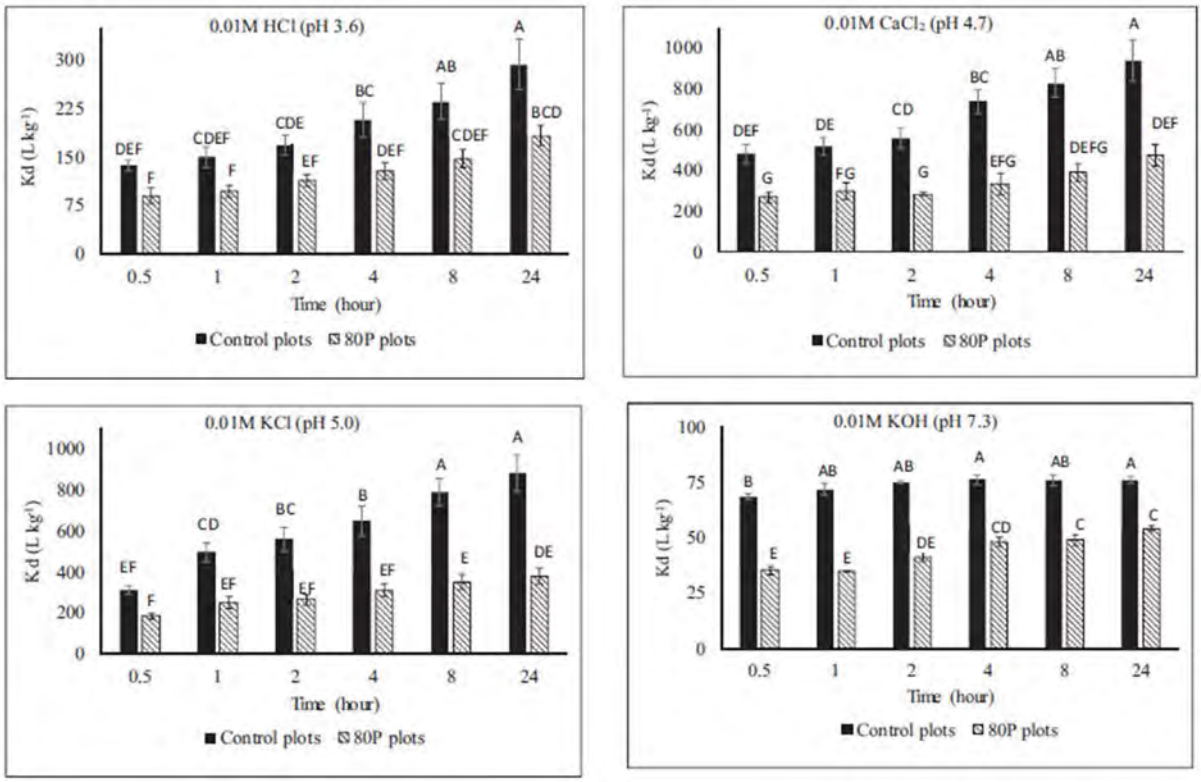


Fig. 4. Time dependent sorption study of glyphosate Kd values in control and 80P plots.

For the 44 mg P/kg co-application, glyphosate Kd values were reduced on average by 54% in 20P plots, but by 42% in the 80P plots. Thus, the largest impact of fresh MAP applications on reducing sorption sites for glyphosate occurred in soils with smaller field-aged phosphate concentrations because more sorption sites were available for competition in the plots that had low field-aged phosphate concentrations. In general, glyphosate Kd values were largest at pH 4.7 (0.01 M $CaCl_2$) when glyphosate molecules mainly exist as H_2G^- (~85%) and HG^{2-} (~15%), and at pH 5.0 (0.01M KCl) when glyphosate molecules mainly exist as H_2G^- (~75%) and HG^{2-} (~25%). The soil used in this study had already a relatively large Ca^{2+} content (2252 ± 40.57 mg kg^{-1}), and using 0.01 M KCl, would allow K^+ to replace Ca^{2+} on the exchange site of organic-clay complexes which may interact with glyphosate forming stable complexes. Glyphosate Kd values were greater at pH 3.6 (0.01 M HCl), than pH 5.4 (dH_2O) (Fig. 3). At pH 3.6, a greater amount of soil colloids is net positively-charged, promoting the sorption of glyphosate molecules that mainly exist as H_2G^- (~95%) and H_3G (~5%). Sorption was less at pH 5.4 than at pH 3.6 because the amount of negatively- charged soil colloids increases with soil pH, and glyphosate molecules mainly exist as $H2G^-$ (~60%) and $HG2^-$ (~40%) at pH 5.4. The lowest sorption was observed at pH 7.3 (0.01 M KOH), as the negatively charged soil colloids increased and glyphosate molecules existed as $HG2^-$ (~100%).

Conclusion

Analytical-grade glyphosate showed similar results as a commercially-available glyphosate formulation. Long-term additions of phosphate fertilizers to soils will reduce the capacity of the soil to bind glyphosate under a wide range of pH conditions, but the impurities of Cd in these fertilizers have no impact on glyphosate sorption.

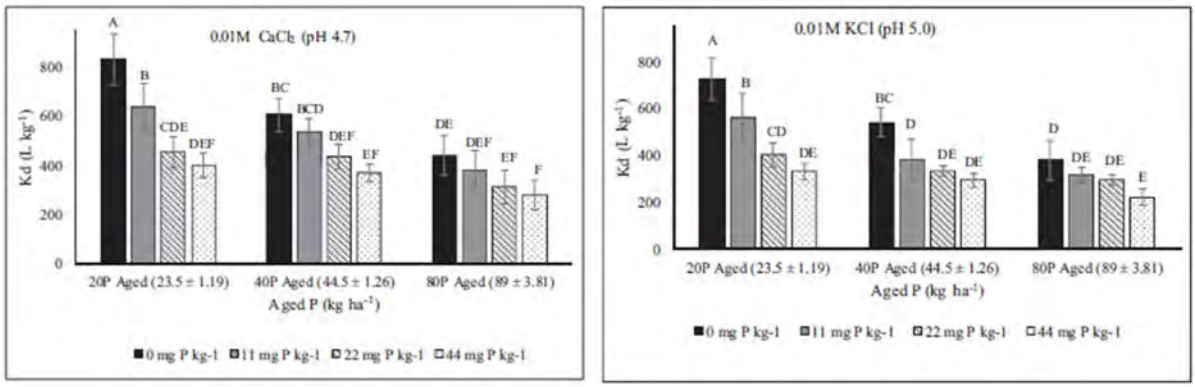


Fig. 5. Effect of co-applying mono ammonium phosphate with glyphosate in solution, for batch equilibrium experiments using 0.01 M $CaCl_2$ (pH 4.7) and 0.01 M KCl (pH 5.0).

Fresh applications of phosphate fertilizers to most soils will significantly reduce the availability of sorption sites for glyphosate. However, this reduction in sorption site availability will be small in soils that have exceptionally high phosphate levels and do not have many sorption sites available for phosphate or glyphosate. Cd concentrations typically found in agricultural fields are not high enough to influence the binding capacity of glyphosate in soil.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes a sorption experiment with glyphosate on a Canadian agricultural soil considering different treatments with phosphate fertilizer. Some information on soil and study design are not reported (soil characteristics, mass balances, amount of soil), so no final validity check is possible. The temperature of the experiment was set to 5°C.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Sirajum Munira & Annemieke Farenhorst
Report year	2017
Report title	Sorption and desorption of glyphosate, MCPA and tetracycline and their mixtures in soil as influenced by phosphate
Document No	Journal of Environmental Science and Health, Part B 2017, VOL. 0, NO. 0, 1–9
Guidelines followed in study	OECD 106 (2000)
Deviations from current test guideline	Temperature: 5°C, 0.01 M KCl
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (not valid against current guidelines)

2. Full summary of the study according to OECD format

Phosphate fertilizers and herbicides such as glyphosate and MCPA are commonly applied to agricultural land, and antibiotics such as tetracycline have been detected in soils following the application of livestock manures and biosolids to agricultural land. Utilizing a range of batch equilibrium experiments, this research examined the competitive sorption interactions of these chemicals in soil. Soil samples (0–15 cm) collected from long–term experimental plots contained Olsen P concentrations in the typical (13 to 20 mg/kg) and elevated (81 to 99 mg/kg) range of build–up phosphate in agricultural soils. The elevated Olsen P concentrations in field soils significantly reduced glyphosate sorption up to 50 %, but had no significant impact on MCPA and tetracycline sorption. Fresh phosphate additions in the laboratory, introduced to soil prior to, or at the same time with the other chemical applications, had a greater impact on reducing glyphosate sorption (up to 45 %) than on reducing tetracycline (up to 13 %) and MCPA (up to 8 %) sorption. The impact of fresh phosphate additions on the desorption of these three chemicals was also statistically significant, but numerically very small namely <1 % for glyphosate and tetracycline and 3 % for MCPA. The presence of MCPA significantly reduced sorption and increased desorption of glyphosate, but only when MCPA was present at concentrations much greater than environmentally relevant and there was no phosphate added to the MCPA solution. Tetracycline addition had no significant effect on glyphosate sorption and desorption in soil. For the four chemicals studied, we conclude that when mixtures of phosphate, herbicides and antibiotics are present in soil, the greatest influence of their competitive interactions is phosphate decreasing glyphosate sorption and the presence of phosphate in solution lessens the potential impact of MCPA on glyphosate sorption. The presence of chemical mixtures in soil solution has an overall greater impact on the sorption than desorption of individual organic chemicals in soil.

Materials and Methods

Chemicals

Analytical grade glyphosate (99.9 %), MCPA (99 %), tetracycline (98 %), potassium dihydrogen phosphate (KH_2PO_4), (99 %) and potassium chloride (100 %) were obtained commercially. Radioactive [phosphonomethyl–^{14}C] glyphosate (99 %; specific activity 50 mCi/mmol), [2–methyl–4–chlorophenoxyacetic acid ^{14}C] MCPA (98 %; specific activity 55 mCi/mmol) and [7–^{3}H (N)] tetracycline (98 % radiochemical purity; specific activity 20 Ci/mmol) were obtained commercially.

Table 1. Selected soil physical and chemical properties as mean with standard error.

Organic Carbon [a] (%)	pH[b]	Fe_2O_3[c] (mg kg^{-1})	Al_2O_3[c] (mg kg^{-1})	Ca[d] (mg kg^{-1})	Clay[e] %	Silt[e] %	Sand[e] %
2.81 ± 0.04	4.7± 0.02	237±7.93	6.41±0.64	2,252±35	20	20	60

[a]Soil organic carbon content was determined using combustion technique with a high temperature induction furnace.[39]
[b]Soil pH was determined using a 10 ml 0.01M $CaCl_2$ solution and 2 g soil solution ratio.[40]
[c]Extractable Fe and Al were extracted with diethylenetriaminepentaacetic acid (*DTPA*) [41] and 0.01M $CaCl_2$, [42] respectively, and extracts were analyzed by ICP.
[d]Extractable Ca was also measured by ICP using ammonium acetate as an extractant [43]
[e]data adapted from Grant et al.[36]

Soil characteristics and experimental design

Soil samples (0–15 cm) were collected in spring 2013 from experimental plots that were arranged in a randomized complete block design with four replications and were located at the University of Manitoba Carman Field Research Station, Manitoba, Canada. All plots were under a flax and durum wheat rotation and received urea fertilizers at an annual rate of 50 and 90 kg N/ha, respectively. For this study, samples were collected from the replicated plots that had also received eight years (2002–2009) of annual mono ammonium phosphate (MAP) applications at rates of 80 kg P/ha, as well as from control plots that did not receive MAP application during these years. The rotation was continued from 2010 to 2013 but after 2010 no phosphate was applied. In each plot, composite soil samples were collected using a Dutch auger with ten samples per plot and the auger was cleaned in between plots. The soil is classified as an Orthic Black Chernozem based on the Canadian System of Soil Classification, which is approximately equivalent to the Udic Boroll subgroup in the U.S. Soil Taxonomy. Key soil properties are listed Table 1.

Impact of phosphate in solution on herbicides and antibiotic sorption and desorption

Batch equilibrium procedures using 50–mL centrifuge Teflon tubes (duplicates) followed the OECD guideline 106 with air–dried soil (2 g) and a soil/solution ratio of 1:5 with 0.01 M KCl as the background electrolyte Soil slurries were rotated in the dark at 5°C from 0 to 24 h (pre–incubation), from 24 to 48 h (sorption) and from 48 to 72 h (desorption) with phosphate added at 0 h, 24 h and/or 48 h, or never added, depending on the treatment (Table 2). Radiolabelled chemical solutions contained 1 mg/L analytical–grade glyphosate, MCPA or tetracycline, with 6.67 x 10^5 Bq/L ^{14}C–labelled glyphosate, 3.83 x 10^5 Bq/L ^{14}C–labelled MCPA or 4.17 x 10^5 Bq/L ^{3}H–labelled tetracycline, respectively. The concentration 1 mg/L represented environmentally–relevant concentrations of herbicides and antibiotics detected in agricultural soils or animal manure. At 48 h, tubes were centrifuged at 10,000 rev/min for 10 min and subsamples (1 mL) of the supernatant (duplicates) were added to scintillation vials (7 mL) containing 5 mL 30 % Scintisafe scintillation cocktail (Fisher Scientific, Fair Lawn, NJ). Radioactivity was quantified by Liquid Scintillation Counting (LSC) with automated quench correction (#H method). The sorption distribution constant, Kd (L/kg), of glyphosate, MCPA or tetracycline was quantified by Cs/Ce, where Cs is the concentration of the organic chemical in soil at equilibrium (mg/kg) and Ce is the concentration of the organic chemical in the equilibrium solution (mg/L). The concentration of the organic chemical in soil was calculated by the difference between the radioactivity in the initial solution and the equilibrium solution. The soil organic carbon coefficient, Koc (L/kg) of glyphosate, MCPA or tetracycline was calculated by dividing the Kd value by 0.0281 which was the fraction of soil organic carbon in soil.

Table 2. Addition of phosphate during pre–incubation, sorption and desorption steps.

Code	Pre-incubation from 0 h to 24 h	Sorption from 24 h to 48 h	Desorption from 48 to 72 h
n,n,n	No P added	No P added	No P added
n,n,P	No P added	No P added	P added at 48 h
P,n,n	P added at 0 h	No P added	No P added
P,n,P	P added at 0 h	No P added	P added at 48 h
n,P,P	No P added	P added at 24 h	P added at 48 h

n = no phosphate added during pre-incubation, sorption and/or desorption step; P = phosphate added at time 0 h at the start of the pre-incubation step or at time 24 h at the start of the sorption step; or at time 48 h at the start of the desorption step.

Impacts of MCPA and tetracycline in solution on glyphosate sorption and desorption in the presence and absence of fresh phosphate

Experiments followed similar protocols as described for *n,n,n; n,n,P*; and *P,n,P* in Table 2 above and also added to soil (at 0 h) were MCPA, tetracycline (Tetra), or their mixtures (M/T). MCPA, Tetra, and M/T were added at concentrations of 1 or 11 mg/L. The glyphosate solution was always added at 24 h and contained 1 mg/L analytical–grade glyphosate with 6.67 x 10^5 Bq/L ^{14}C–labelled glyphosate.

Table 3. Effect of phosphate fertilizer on MCPA and tetracycline sorption and desorption in soil. See Table 2 for an explanation of the treatment labels.

	Kd (L kg^{-1})		Desorption (%)	
Treatment	MCPA	Tetracycline	MCPA	Tetracycline
n,n,n	5.37 A	134.49 A	27.45 B	0.51 B
n,n,P	5.28 A	129.02 A	29.63 A	0.73 A
P,n,n	5.00 B	117.50 B	29.04 A	0.69 A
P,n,P	5.00 B	122.55 B	30.18 A	0.71 A
n,P,P	4.99 B	117.55 B	29.91 A	0.74 A

Effect of the pre–sorbed phosphate on the sorption of glyphosate, MCPA and tetracycline

This batch equilibrium experiment only used the soil samples obtained from the plots that had not received phosphate fertilizer applications.

Effect of the pre–sorbed MCPA on glyphosate sorption

Experiments followed similar protocols as described for the pre–sorbed phosphate above. The glyphosate solution contained 1 mg/L analytical–grade glyphosate with 6.67 x 10^5 Bq/L ^{14}C–labelled glyphosate.

Statistical analysis

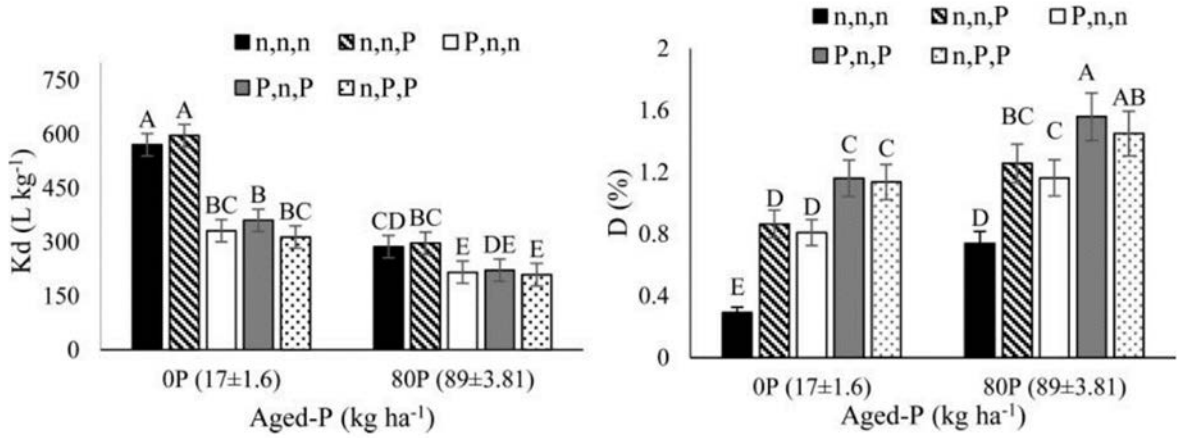
Statistical analyses were carried out using SAS software version 9.4 for Windows. Prior to each analysis, data sets were checked for outliers, normality of residuals and homogeneity of variances. Residuals were normally distributed and variances were homogeneous. For the Kd values, data were analyzed by using normal distribution and for the % desorption by beta distribution. Two–way ANOVA in PROC GLIMMIX was used to quantify the effect of field aged–P (0P, 80P) and fresh–P addition (0, 11 mg/L) on Kd values and % desorption of MCPA, tetracycline, and glyphosate in soil. One–way ANOVA in PROC GLIMMIX was utilized to determine the effect of retained phosphate in soil on glyphosate, MCPA and tetracycline sorption, and of retained MCPA in soil on glyphosate sorption. Both in the presence and absence of fresh phosphate, two–way ANOVAs in PROC GLIMMIX were carried out to quantify the effect of field aged–P (0P, 80P) and of the concentrations (0, 1, 11 mg/L) of MCPA, tetracycline, or MCPA–tetracycline mixtures on glyphosate Kd values. For fresh phosphate added at

48 h only, or at both 0 h and 48 h, and in the absence of fresh phosphate, two way ANOVAs in PROC GLIMMIX were carried out to quantify the effect of field aged–P (0P, 80P) and of the concentration (0, 1, 11 mg/L) of MCPA, tetracycline, or MCPA–tetracycline mixtures on the percent of glyphosate desorbed. For all ANOVAs, the separation of treatment means was performed using the Tukey's test ($p < 0.05$).

Results

Kd values on average ranged from 209 to 596 L/kg for glyphosate (Figure 1), from 118 to 135 L/kg for tetracycline, and from 4.99 to 5.37 L/kg for MCPA (Table 3). Koc values ranged from 6105 to 25,496 L/kg for glyphosate, from 3,928 to 4,901 L/kg for tetracycline, and from 156 to 209 L/kg for MCPA. These results are within the ranges observed in previous studies of the sorption of glyphosate, tetracycline and MCPA in soils. Glyphosate (<2 %) (Figure 1) and tetracycline (<1 %) desorption was always small but MCPA desorption ranged from 26 to 31 % (Table 3). Phosphate significantly reduced glyphosate sorption in soil (Figure 1). Without laboratory-added phosphate, glyphosate Kd values were 50 % smaller in soil containing 81 to 99 mg/kg Olsen P than in soil containing 13 to 20 mg/kg Olsen P. Regardless of whether MCPA, tetracycline or MCPA/tetracycline mixture were added to soils in the laboratory, field aged–P always significantly reduced glyphosate Kd values (Table 4). When phosphate was added to soil solution at either 0 h or 24 h, it had the same significant effect on reducing glyphosate sorption with glyphosate Kd values being reduced by 37–45 % in field soils containing 13 to 20 mg P/kg, and by 23–27 % in field soils containing 81 to 99 mg P/kg (Figure 1).

Figure 1. Effect of phosphate fertilizer on glyphosate sorption and desorption in soil. Potassium dihydrogen phosphate was added prior or during glyphosate addition for the sorption study and prior, during and/or post stage of glyphosate addition for the desorption study (see Table 2 for labels and details).



In the presorbed phosphate experiment, the soil retained 9.8, 18.5 and 32.4 mg P/kg for the additions of 11, 22, 44 mg P/L respectively, and glyphosate sorption was significantly reduced by 41 % (11 mg P/L), 52 % (22 mg P/L) and 65 % (44 mg P/L) (Figure 2).The amount of field aged–P in soil had no significant impact on MCPA and tetracycline sorption in soil. However, fresh phosphate added to soil solution significantly reduced tetracycline Kd values by 8–13 % and MCPA Kd values by 7–8 % (Table 3). The competitive effect of phosphate on MCPA and tetracycline sorption was not dependent on when the phosphate was added in the laboratory (either 0 h or 24 h) (Table 3). In the pre–sorbed phosphate experiment, phosphate significantly reduced MCPA sorption by 10 % and tetracycline sorption by 8 % for the addition of 44 mg P/L (Table 5, or Figure 2). However, there was no impact on MCPA or tetracycline sorption when phosphate additions were 11 or 22 mg P/L. Glyphosate desorption was significantly greater in field soils containing 81 to 99 mg/kg Olsen P (0.74 %) than in soils containing 13 to 20 mg/kg Olsen P (0.29 %) (Figure 1). Regardless of whether MCPA, tetracycline or MCPA/tetracycline mixture were added to soils in the laboratory, field aged–P always significantly increased glyphosate desorption. Fresh phosphate additions at 0 h, 24 h or/and 48 h to soil solutions in the laboratory also significantly increased glyphosate desorption by 0.52–0.84 % in soils containing 13 to 20 mg/kg Olsen P and by 0.52–0.82 % in field soils containing 81 to 99 mg/kg Olsen P (Figure 1). The amount of field aged–P in soil had no significant impact on MCPA and tetracycline desorption in

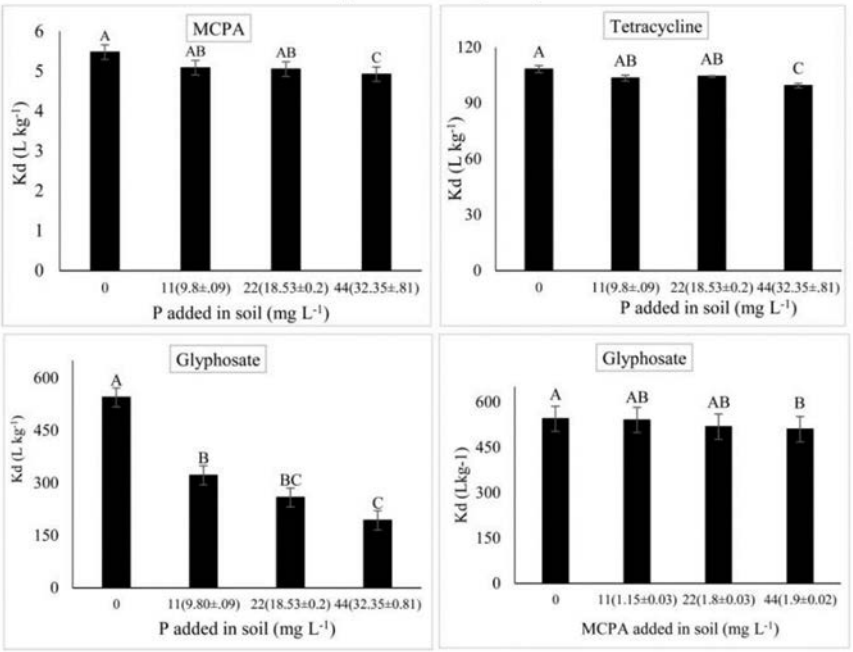
soil, but the addition of fresh phosphate to soil solutions in the laboratory significantly increased desorption of MCPA by 2–3 % and tetracycline by 0.18–0.23 % (Table 3).

Table 4. Effect of MCPA (0, 1, 11 mg/L), tetracycline (0, 1, 11 mg/L) and MCPA/tetracycline mixtures (0, 1, 11 mg/L) on sorption and desorption of glyphosate in soil in the presence and absence of phosphate.

		No P		P at 48 h		P at 0 h and 48 h	
Chemicals	Concentration (mg L^{-1})	Kd (Lkg^{-1})	D (%)	Kd (Lkg^{-1})	D (%)	Kd (Lkg^{-1})	D (%)
MCPA	0	428.48 A	0.52 A	445.99 A	1.10 A	290.80 A	1.38 A
	1	409.73 A	0.53 A	424.99 A	1.11 A	271.09 A	1.42 A
	11	370.88 B	0.60 B	382.32 B	1.16 A	278.44 A	1.43 A
Tetracycline	0	428.48 A	0.52 A	445.99 A	1.10 A	290.80 A	1.38 A
	1	415.64 A	0.54 A	426.02 A	1.04 A	283.50 A	1.36 A
	11	415.94 A	0.55 A	426.02 A	1.08 A	271.72 A	1.45 A
MCPA-tetracycline mixtures	0	428.48 A	0.52 A	445.99 A	1.10 A	290.80 A	1.38 A
	1	426.02 A	0.48 A	444.58 A	1.12 A	283.50 A	1.39 A
	11	318.05 B	0.66 B	386.72 B	1.15 A	290.51 A	1.44 A

The competitive effect of phosphate on MCPA, tetracycline and glyphosate desorption was not dependent when phosphate was added to soil solution (either at 0 h, 24 h or 48 h). The number of times that phosphate was added had no significant effect on MCPA and tetracycline desorption (Table 3). However, glyphosate desorption was greater when phosphate was added twice (*P,n,P*, or *n,P,P*) rather than once (*P,n,n* or *n,n,P*) but glyphosate desorption remained <2 % in all cases (Figure 1). MCPA and MCPA/tetracycline mixtures added at 11 mg/L significantly reduced glyphosate Kd values and increased glyphosate desorption, but only when no phosphate was added to the soil solution (Figure 3, Table 4). MCPA and MCPA/tetracycline mixtures added at 1 mg/L had no significant effect on glyphosate sorption and desorption (Table 4). Tetracycline had no significant effect on glyphosate Kd values and desorption, regardless of whether it was added to soil at 1 or 11 mg/L, and whether or not phosphate was added to soil solution (Table 4). Thus, the effect of MCPA/tetracycline mixtures on glyphosate sorption and desorption was due to MCPA.MCPA addition significantly reduced glyphosate Kd values by 14 % (Figure 3) and glyphosate desorption by 0.1 % (Figure 3). In the pre–sorbed MCPA experiment, the addition of 11, 22, 44 mg MCPA/L the soil retained 1.2, 1.8 and 1.9 mg MCPA/kg, respectively. The pre–sorbed MCPA significantly reduced glyphosate sorption by 6 % for the addition of MCPA at 44 mg/L, but there was no impact on glyphosate sorption when additions were at 11 or 22 mg/L (Table 5, Table 3 S, or Figure 2).

Figure 2. Effect of pre–sorbed phosphate concentrations on MCPA, tetracycline and glyphosate sorption, and of pre–sorbed MCPA concentrations on glyphosate sorption in soil. Numbers on x–axis in parenthesis refer to mean (± standard error) of measured pre–sorbed phosphate and MCPA.



Discussion

The addition of phosphate at either 0 h or 24 h yielded the same impact on glyphosate sorption (Figure 1), in agreement with the findings of Gimsing et al. (2004) who also reported that the timing of phosphate additions had no significant effect. Glyphosate and phosphate have shown to compete for the same sorption sites in soil. Application of phosphate with glyphosate in solution reduced glyphosate sorption because phosphate is preferentially sorbed over glyphosate by available sorption sites. Glyphosate Kd values were significantly smaller in soils containing elevated Olsen P concentrations than in soils containing typical Olsen P concentrations. This elevated Olsen P concentrations resulted from eight years of annual phosphate application from 2002 to 2009, with soils being sampled for this study in 2013. These results indicate that phosphate persists in agricultural soils and occupies sorption sites that otherwise would be available sorption sites for glyphosate. In–addition, in the pre–sorbed phosphate experiment, glyphosate sorption was also reduced with increasing phosphate application to soil thus indicating that phosphate from recently fertilizer applications will also occupy sorption sites otherwise available for glyphosate sorption. Given the moderately acidic conditions (soil pH 5), the sorption sites that phosphate occupies are positively charged Fe/Al–oxides. When phosphate ($H_2PO_4^{-1}$) is retained by Fe/Al–oxides, the Fe/Al–oxides will yield a net negative charge, leading to an electrostatic repulsion between the Fe/Al–oxides and glyphosate (H_2G^-) in soil. However, a portion of glyphosate molecules that were sorbed by available positively charged Fe/Al–oxides. The addition of phosphate after this sorption increased glyphosate desorption (Figure 1) possibly because phosphate is able to displace glyphosate bound to Fe/Al–oxides as the bonding forces between phosphate and Fe/Al–oxides are stronger than the bonding forces between glyphosate and Fe/Al–oxides. Under the experimental conditions with the soil slurries being at a pH 5, the molecules of MCPA (pKa D 3.73) are predominantly negatively–charged.

Table 5. Effect of pre–sorbed phosphate (0, 11, 22, 44 mg/L) on glyphosate, MCPA and tetracycline sorption and pre–sorbed MCPA on glyphosate sorption (L/kg) in soil.

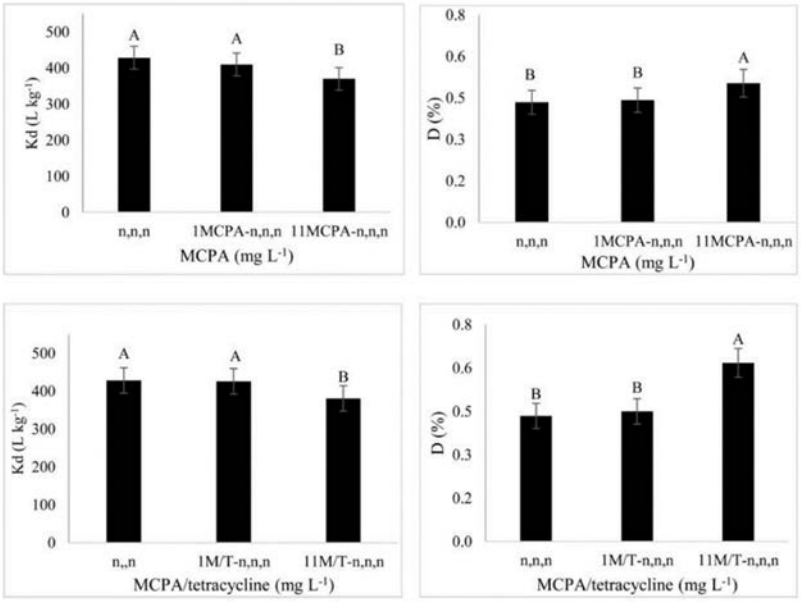
Concentration (mg L^{-1})	Glyphosate	MCPA	Tetracycline	Glyphosate
0	544.60 A	5.48 A	108.22 A	544.6 A
11	321.78 B	5.09 AB	103.39 AB	540.8 AB
22	258.49 BC	5.05 AB	104.35 AB	518.25 AB
44	192.96 C	4.93 C	99.32 C	510.25 B

MCPA and tetracycline sorption was only significantly reduced at the highest rate because more Fe/Al–oxides were net negatively charged and repelling MCPA and tetracycline molecules. The effect of phosphate on reducing sorption was less for MCPA and tetracycline than for glyphosate. Under moderately acidic conditions, Fe/Al–oxides are the dominant sorption sites for glyphosate and phosphate because both have a phosphonic acid group. However, MCPA (i.e., carboxyl and phenyl groups) and tetracycline (i.e., tricarbonylamide carbonyl, amine and hydroxyl groups) have other functional groups and sorption sites for MCPA and tetracycline can include under moderately acidic conditions humic substances and clay minerals in addition to Fe/Al–oxides in soils. MCPA had no longer a significant effect on glyphosate sorption when phosphate was added to the soil solution. The molecular size of phosphate (0.25 nm) is smaller than glyphosate (0.43 nm) and MCPA (0.77 nm). Therefore, it is possible that phosphate is preferentially sorbed over glyphosate and MCPA. Thus, when both phosphate and MCPA were added to the soil solution, phosphate occupied the sorption sites that may otherwise be available to MCPA and suppressed the effect of MCPA on glyphosate sorption. In the pre–sorbed experiment, in the absence of phosphate additions, MCPA reduced glyphosate sorption because pre–sorbed MCPA occupied some sorption sites which may otherwise be accessible to glyphosate.

MCPA was weakly retained with Koc values ranging from 156 to 209 L/kg while glyphosate and tetracycline were strongly retained with Koc values ranging from 6,105 to 25,496 and 3,928 to 4,901 L/kg, respectively. It has been reported that organic molecules are considered relatively mobile when Koc value ranges from 150 to 500 L/kg. Thus, given these Koc values, MCPA is relatively mobile in soil because it is only weakly retained, unlike glyphosate and tetracycline. Glyphosate is very strongly retained in soil and is less likely to be mobile in matrix flow than MCPA, regardless of the amounts of

phosphate or MCPA that can compete with glyphosate for sorption sites in soil. In contrast, the presence of recent phosphate applications to agricultural soils may increase the mobility of MCPA to deeper depths but only when applied at relatively large phosphate fertilizer rates.

Figure 3. Effect of MCPA and MCPA/tetracycline mixtures on glyphosate sorption and desorption in soil. Potassium dihydrogen phosphate with MCPA or MCPA/tetracycline were added prior glyphosate for the sorption study and prior, or post stage of glyphosate addition for the desorption study: (see Table 2 for labels and details).



Conclusion

Field–aged phosphate had no significant effect on MCPA and tetracycline sorption and desorption but significantly reduced glyphosate sorption up to 50 % and increased glyphosate desorption by 0.45 %. Pre–sorbed phosphate had a greater impact on reducing glyphosate sorption than on reducing MCPA and tetracycline sorption. The addition of fresh phosphate in the laboratory also significantly decreased glyphosate sorption (up to 45 %) and increased glyphosate desorption (up to 0.87 %) and the impact on reducing MCPA and tetracycline sorption (<13 %) and increasing MCPA and tetracycline desorption (<3 %) was significant but smaller than the impact on glyphosate. Glyphosate and tetracycline were strongly retained in soil with Kd values >100 L/kg and desorption less than 2 %. In contrast, MCPA was weakly retained in soil with Kd values <6 L/kg and desorption was above 25 %. Hence, even in soils with a large phosphate build–up, glyphosate will be less mobile in matrix flow than MCPA. MCPA but not tetracycline additions significantly decreased glyphosate sorption, but only when MCPA was present at concentrations ten times greater than typically detected in agricultural soils and there was no phosphate added to the herbicide solutions.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The article describes an OECD 106 experiment with glyphosate on a Canadian soil considering the influence of phosphate additions. The article shows some deviations from the validity criteria for EU guidelines (Temperature, usage of 0.01 M KCl instead of 0.01 M $CaCl_2$).

The article is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Munira, S., et al.
Report year	2018
Report title	Phosphate and glyphosate sorption in soils following long-term phosphate applications
Document No	Geoderma 313 (2018) 146–153
Guidelines followed in study	OECD 106 (2000)
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (No all validity criteria met for OECD 106, e.g. Temperature)

2. Full summary of the study according to OECD format

Phosphate and glyphosate molecules compete for sorption sites in soil. The objective of this study was to quantify the impact of Olsen P concentrations in two contrasting soils on phosphate and glyphosate sorption. Soils were a sandy clay loam soil rich in iron oxides (SCL-Fe_2O_3) and a clay loam soil rich in calcium carbonates (CL-$CaCO_3$). The phosphate Freundlich sorption coefficient (K_f) ranged from 3 to 68 $L^{1/n}$ $mg^{1-1/n}$ kg^{-1} in the SCL-Fe_2O_3 and from 21 to 76 $L^{1/n}$ $mg^{1-1/n}$ kg^{-1} in the CL-$CaCO_3$. Glyphosate sorption coefficient (Kd) ranged from 293 to 1173 L/kg in the SCL-Fe_2O_3 but only 99 to 141 L/kg in the CL-$CaCO_3$. Glyphosate K_d and phosphate K_f values decreased significantly with increasing Olsen P concentrations in both soils. Glyphosate K_d values were further significantly reduced when phosphate was added to the slurry solutions, but phosphate K_f values were not impacted by the presence of glyphosate in solutions. We conclude that annual phosphate fertilizer applications leave phosphate concentrations in Prairie soils to the extent that soils have a lesser capacity to retain glyphosate and phosphate that are subsequently applied, but glyphosate residues will not influence phosphate sorption.

Methods

Chemicals

Chemicals used were analytical grade glyphosate (99.9% purity) from Sigma-Aldrich Co., St. Louis, MO; [phosphonomethyl-^{14}C]glyphosate (99% radiochemical purity; specific activity 50 mCi/mmol) from American Radiolabeled Chemicals Inc., St. Louis, MO; Roundup Ultra2® (49% active ingredient and 51% other ingredients, CAS No. 70901-12-1) from Monsanto Chemical Company; and analytical grade potassium dihydrogen phosphate (KH_2PO_4) (99% chemical purity), potassium chloride (100% chemical purity) and calcium chloride, dehydrate (> 95% chemical purity) from Fisher Scientific, Fair Lawn, NJ.

Soil characteristics and experimental design

This study utilized soil samples (0–15 cm) obtained from long-term experimental plots under a durum wheat and flax rotation near Carman (49° 29.7′ N, 98° 2.4′ W) and near Forrest (50° 1.2′ N, 99° 53.3′ W) Manitoba, Canada. Soil profiles at both sites were classified based on the Canadian System of Soil Classification as Orthic Black Chernozems, which is equivalent to the Udic Boroll subgroup in the U.S. Soil Taxonomy. The experimental design at each site was a randomized complete block design with four mono ammonium phosphate fertilizer treatments and four replicates plots. Treatments were a control (no phosphate applications), and plots receiving annual applications of mono ammonium phosphate fertilizers at 20, 40, and 80 kg P/ha or 20P, 40P, and 80P, respectively, from 2002 to 2009. For all plots that received mono ammonium phosphate, 20 kg P/ha was placed near the seed to enhance fertilizer use efficiency, a common practice in Canadian Prairie agriculture. For the 40 and 80 kg P/ha treatments, to avoid seedling toxicity, the additional mono ammonium

phosphate was broadcast and then incorporated. From 2010 to 2013, the rotation was continued but no phosphate was applied. Application of urea fertilizer differed by year. Generally, durum wheat received 90 kg N/ha and flax 50 kg N/ha· From each plot, composite samples were collected in spring, 2013 using a Dutch auger with ten (Carman) to eight (Forrest) samples per plot and cleaning the auger between plots. Soil samples were air-dried and sieved (< 2 mm) prior to soil property analyses and sorption experiments. The Carman soil has a sandy clay loam texture and is relatively high in iron oxides (SCL-Fe_2O_3), whereas the Forrest soil has a clay loam texture and is relatively high in calcium carbonates (CL- $CaCO_3$) (Table 1). Available phosphate was extracted using the Olsen (0.5 M $NaHCO_3$, pH 8.5) phosphorus test. 2 g of air-dried soil and 40 mL of 0.5 N $NaHCO_3$ solution was mixed in a 50 mL Erlenmeyer flask. Flasks (duplicates) were shaken horizontally (200 excursions/min). Equilibrium solutions were filtered through Whatman No. 2 filter paper and phosphate concentrations were determined colorimetrically.

Table 1. Selected soil physical and chemical properties as mean with standard error.

Soil	Organic Carbon[a] (%)	pH[b]	Fe_2O_3[c] (mg kg^{-1})	Al_2O_3[c] (mg kg^{-1})	Ca[d] (mg kg^{-1})	Clay[e] %	Silt[e] %	Sand[e] %
SCL-Fe_2O_3	2.81 ± 0.04	4.7 ± 0.02	237 ± 7.93	6.41 ± 0.64	2252 ± 35	20	20	60
CL-$CaCO_3$	3.2 ± 0.07	7.3 ± 0.02	12.52 ± 0.22	1.07 ± 0.47	4791 ± 158	30	39	31

[a] Soil organic carbon content was determined using combustion technique with a high temperature induction furnace (Nelson and Somemers, 1996).
[b] Soil pH was determined using a 10 mL 0.01 M $CaCl_2$ solution and 2 g soil solution ratio (Jones, 2001).
[c] Extractable Fe and Al were extracted with diethylenetriaminepentaacetic acid (*DTPA*) (Whitney, 2011) and 0.01 M $CaCl_2$, (Barnhisel and Bertsch, 1982) respectively, and extracts were analyzed by *ICP*.
[d] Extractable Ca was also measured by *ICP* using ammonium acetate as an extractant (Warncke and Brown, 2011)
[e] Data adapted Grant et al. (2013)

Phosphate sorption

Phosphate sorption was determined by batch equilibrium using either 0.01 M $CaCl_2$ or 0.01 M KCl as the background electrolyte. Batch equilibrium procedures followed standard protocols using a soil/solution ratio of 1:10 and an equilibrium time of 24 h. Two experiments were conducted utilizing soil samples: (1) from all plots at each site to quantify the effect of Olsen P concentrations on phosphate sorption in soil and (2) from control and 80P plots at each site to quantify the effect of Roundup Ultra2 additions to soil slurries on phosphate sorption in soil.

Effect of field-aged phosphate concentrations on sorption of phosphate

In the first experiment, potassium dihydrogen phosphate solutions (20 mL) at concentrations of 5, 10, 25, 50, 100, 150, 250 or 500 mg P/L were added to air-dried soil (2 g) in 50-mL centrifuge tubes (duplicates) and shaken horizontally (120 excursions/min) at room temperature (23 ± 2 °C) for 24 h. Equilibrium solution was centrifuged (6100 G for 10 min) and filtered (0.45 μm). Phosphate concentration was determined colorimetrically by the molybdate blue method. Linearized Freundlich isotherm has been specified as: The phosphate sorption coefficient, K_f ($L^{1/n}$ $mg^{1-1/n}$ kg^{-1}), was calculated using the linearized form of Freundlich equation: log q= log K_f + 1/n log C. Where q represents phosphate sorption in soil at equilibrium (mg/kg), C represents phosphate concentration of equilibrium solution (mg/L), and 1/n represents the Freundlich slope. In addition, the Freundlich P sorption isotherm was used to determine the equilibrium P concentration (EPCo) at log q = 0, which is the concentration at which neither sorption nor desorption occurs and hence can be used to define whether a soil is likely to act as a sink (sorption) or source (desorption) of P. EPCo levels above 0.025 mg/L suggest an increased risk of eutrophication because of P transport in soluble form.

Effect of glyphosate formulation on sorption of phosphate

In the second experiment, stock solutions of 150 mg P/L were prepared with and without 100 mg/L Roundup Ultra2 in the solution. The 100 mg/L Roundup Ultra 2 was equivalent to 378 mg glyphosate/kg soil. The 150 mg P/L solution was used because previous studies have proposed that this parameter (P150) is the most optimum single point in the isotherm reflective of the phosphate sorption capacity in soils. Batch equilibrium procedures were carried out as described above. The phosphate sorption

coefficient, Kd (L/kg), was calculated by q/C, where q represents phosphate sorption by soil at equilibrium (mg/kg) and C represents phosphate concentration of equilibrium solution (mg/L).

Glyphosate sorption

Glyphosate sorption was determined by batch equilibrium with the initial glyphosate solution containing 1 mg/L analytical-grade glyphosate and 6.67 × 104 Bq/L ^{14}C–labelled glyphosate. Two experiments were conducted utilizing soil samples: (1) from all plots to quantify at each site the effect of Olsen P concentrations on glyphosate sorption, and (2) from control and 80P plots to quantify at each site the effect of fresh phosphate additions to soil slurries on glyphosate sorption in soil.

Impact of field-aged phosphate concentrations on sorption of glyphosate

Batch equilibrium procedures followed the OECD guideline 106 using a soil/solution ratio of 1:5, an equilibrium time of 24 h and 0.01 M $CaCl_2$ or 0.01 M KCl as background electrolyte. Glyphosate solutions (10 mL) were added to air-dried soil (2 g) in 50-mL centrifuge Teflon tubes (duplicates) and slurries were rotated in the dark at 5 °C for 24 h. Equilibrium solution was centrifuged (6100 G for 10 min) and subsamples (1 mL) of supernatant were added in duplicated 7-mL scintillation vials containing 5 mL of 30% Scintisafe scintillation cocktail (Fisher Scientific, Fair Lawn, NJ). Radioactivity was quantified by Liquid Scintillation Counting (LSC) with automated quench correction (#H method) (LS 6500 Beckman Instruments, Fullerton, CA). The glyphosate sorption distribution constant, Kd (L/kg), was calculated by Cs/Ce, where Cs represents glyphosate sorption by soil at equilibrium (mg/kg) and Ce represents glyphosate con- centration of equilibrium solution (mg/L). The difference between the added radioactivity and radioactivity in the supernatant was assumed to be the proportion of glyphosate having been sorbed.

Impact of fresh phosphate addition on sorption of glyphosate

Experiments followed similar batch equilibrium sorption protocols as described above. In this experiment, potassium dihydrogen phosphate was added to the initial glyphosate solution at rates equivalent to 11, 22 and 44 mg P/kg soil, or an estimated 20, 40 and 80 P kg/ha, respectively, when assuming the fertilizer being present in the top 15-cm layer of a soil with a bulk density of 1200 kg/m^3.

Statistical analysis

Statistical analyses were carried out using SAS software version 9.3 for Windows (SAS Institute Inc. 2002–2010). Prior to each analysis, data sets were checked for outliers, normality of residuals and homogeneity of variances. Residuals were normally distributed and variances were homogeneous. The paired *t*-test ($P < 0.05$) was used to test for the effect of background electrolyte solution (0.01 M $CaCl_2$ versus 0.01 M KCl) on glyphosate K_d or phosphate K_f and EPCo. For both background electrolyte solutions and at each site, simple linear regression analyses ($P < 0.05$) were carried out to estimate glyphosate K_d and phosphate K_f values using Olsen P concentration as the independent variable. In each of the glyphosate K_d and phosphate K_f figures, the slopes of regression lines developed for SCL-Fe_2O_3 and CL- $CaCO_3$ were compared by including dummy variables in PROC REG to test whether the responses of sorption to increasing Olsen P concentrations was influenced by soil type. Simple linear regression analysis was also carried out to estimate glyphosate K_d values by using the added fresh phosphate concentration as an independent variable. The slopes of the regression lines developed for the 0P (control) and 80P plots in both soils were compared by including dummy variables in PROC REG to test whether the responses of sorption to increasing potassium dihydrogen phosphate concentration was influenced by Olsen P concentrations (0P, 80P). Simple linear regression analyses were carried out to determine the relationship between glyphosate K_d and phosphate K_f values by using K_f as an independent variable. Simple linear regression analyses ($P < 0.05$) were also carried out to estimate EPCo values by using Olsen P as an independent variable for CL-$CaCO_3$ soil. Graphical plot fitting of

EPCo as a function of Olsen P showed that data did not fit well with simple linear regression for the SCL-Fe_2O_3 soil.

Results

Effect of background electrolyte solutions on sorption of phosphate and glyphosate

The types of ions in solution had a significant effect on phosphate and glyphosate sorption, except for glyphosate sorption in the CL-$CaCO_3$ soil (Table 2). Phosphate K_f values in both soils were significantly greater in experiments with 0.01 M $CaCl_2$ than experiments with 0.01 M KCl (Table 2).

Table 2. Statistical parameters (Paired t-tests) on the effect of background electrolyte solution (0.01 M $CaCl_2$ versus 0.01 M KCl) on glyphosate (L/kg) and phosphate sorption coefficient ($L^{1/n}$ mg $^{1-1/n}$ kg^{-1}) in soils.

Sorption Parameter	Soil	Mean		DF	t Value	Pr > |t|
		0.01 M $CaCl_2$	0.01 M KCl			
Phosphate sorption coefficient, K_f	SCL-Fe_2O_3	38.47	23.11	15	11.14	< 0.0001
	CL-$CaCO_3$	54.08	36.04	15	10.94	< 0.0001
Glyphosate sorption coefficient, K_d	SCL-Fe_2O_3	703	632	15	5.89	< 0.0001
	CL-$CaCO_3$	116	117	15	− 1.55	0.1430
Phosphate equilibrium concentration, EPCo	SCL-Fe_2O_3	0.007	0.04	15	− 2.29	< 0.0366
	CL-$CaCO_3$	0.006	0.015	15	− 4.72	< 0.0003

Phosphate K_f values were on average 54 $L^{1/n}$ mg $^{1-1/n}$ kg^{-1} in CL-$CaCO_3$ and 38 $L^{1/n}$ mg $^{1-1/n}$ kg^{-1} SCL-Fe_2O_3 with $CaCl_2$ but on average 36 L1/n mg $^{1-1/n}$ kg^{-1} in CL-$CaCO_3$ and 23 $L^{1/n}$ mg $^{1-1/n}$ kg^{-1} SCL-Fe_2O_3 with KCl. Thus, when 0.01 M $CaCl_2$ was used with the SCL- Fe_2O_3 and CL-$CaCO_3$ soils but also when KCl was used with the CL-$CaCO_3$ soil, phosphate likely formed stable complexes with a portion of Ca^{2+} in soil solution and precipitated. In batch equilibrium experiments with 0.01 M $CaCl_2$, precipitation with Ca^{2+} occurs more readily for phosphate than glyphosate. For glyphosate sorption, K_d values were on average 116 L/kg in CL-$CaCO_3$ and 703 L/kg SCL- Fe_2O_3 with $CaCl_2$, and on average 117 L/kg in CL-$CaCO_3$ and 632 L/kg SCL-Fe_2O_3 with KCl. In calcareous soils, Ca^{2+} in forms a bridge between negatively charged soil colloids and glyphosate molecules in soil and, because of the already high free calcium content in the CL-$CaCO_3$ soil, the addition of Ca with 0.01 M $CaCl_2$ solution had no impact on glyphosate sorption. For the SCL-Fe_2O_3 soil, glyphosate sorption was greater with 0.01 M $CaCl_2$ than 0.01 M KCl, suggesting that glyphosate was able to form complexes with Ca^{2+} in solution for enhanced sorption.

Effect of field-aged phosphate concentrations on sorption of phosphate

Despite being exposed to similar long-term phosphate fertilizer treatments, Olsen P ranged from 13 to 99 mg/kg in the acidic SCL-Fe_2O_3 soil but only from 8 to 48 mg/kg in the calcareous CL-$CaCO_3$ soil. Olsen P concentrations by treatment were on average 17 (control), 24 (20P), 44 (40P) and 89 (80P) mg/kg in the SCL- Fe_2O_3 soil and 13 (control), 18 (20P), 24 (40P) and 41 (80P) mg/kg in the CL-$CaCO_3$ soil. The Olsen P test was originally developed for calcareous soils and can overestimate plant available P in acidic soils, such as the SCL-Fe_2O_3. Olsen P measures the $NaHCO_3$ extractable phosphate in soil, but calcareous soil may also contain slow release inorganic phosphate (apatite minerals) extracted by 1 M HCl. Olsen P concentrations ranged from 8 to 99 mg/kg in this research which is within the typical range of 8 to 114 mg/kg that has been reported for soils in North America. Hence, the findings from this research on the sorption pattern of phosphate and glyphosate in soil would be applicable to a wider range of soils in North America. Phosphate K_f values significantly decreased with the increasing concentrations of Olsen P in soil (Fig. 1).

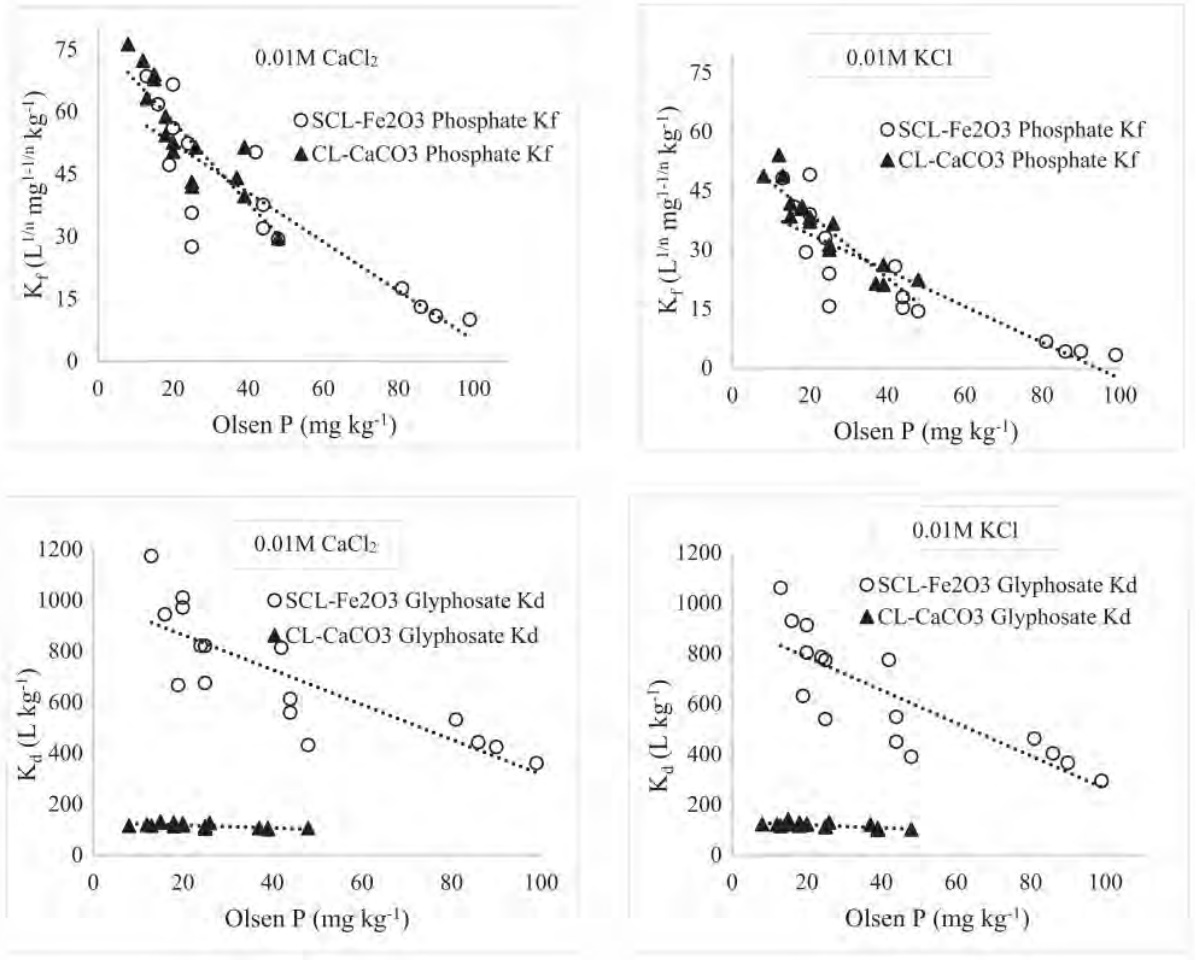


Fig. 1. Effect of Olsen P concentrations in soil on glyphosate and phosphate sorption in SCL-Fe_2O_3 and CL-$CaCO_3$ soils, as determined by batch equilibrium experiments using 0.01 M $CaCl_2$ or 0.01 M KCl as background electrolyte solutions. All regression equations are significant at $P < 0.05$.

The SCL- Fe_2O_3 and CL-$CaCO_3$ soils showed relatively similar phosphate sorption (Fig. 1). Phosphate K_f values ranged from 3.2 to 68 $L^{1/n}$ mg $^{1-1/n}$ kg^{-1} in the SCL- Fe_2O_3 soil with 1/n values between 0.37 and 0.92, and from 21 to 76 $L^{1/n}$ mg $^{1-1/n}$ kg^{-1} in the CL-$CaCO_3$ soil with 1/n values between 0.68 and 0.92. These values are within the range of other studies (Bertrand et al., 2003; Jalali, 2007; Shafqat and Pierzynski, 2014). A maximum reduction of phosphate K_f value was observed in SCL- Fe_2O_3 soil. The phosphate K_f value in SCL- Fe_2O_3 was reduced by 95% in soil containing 99 mg/kg Olsen P relative to

soil containing 13 mg/kg Olsen P. Thus, P accumulation in soil reduced the capacity of soil to hold Wang et al. (2015) also reported that sorption of P decreased with the in- creasing concentrations of Olsen P because long-term application of P fertilizer leads to the accumulation of P in soil. In their study, they showed that long-term (5 to 15 years) application of phosphate significantly reduced phosphate sorption by 56% in soil containing 53 mg/kg Olsen P relative to soil containing 15 mg/kg Olsen P. Olsen P concentrations significantly predicted phosphate K_f (Fig. 1) in both SCL- Fe_2O_3 and CL-$CaCO_3$. The effect of Olsen P concentrations on reducing phosphate sorption was more pronounced for SCL- Fe_2O_3 than CL-$CaCO_3$. For the phosphate K_f, the regression slopes were significantly different between the soils in case of 0.01 M KCl but not with 0.01 M $CaCl_2$ because the presence of Ca in solution led to the possibility of precipitation of phosphate-Ca^{2+} complexes in both soils. Generally, in calcareous soil, Ca forms precipitation with the added phosphate in soil solution. For 0.01 M KCl, the CL- $CaCO_3$ showed a significantly steeper slope than SCL- Fe_2O_3 (Fig. 1) because, with increasing Olsen P concentrations, more sorption sites remained available in SCL- Fe_2O_3. CL-$CaCO_3$ soil has less sorption sites available for the added phosphate than SCL- Fe_2O_3 soil because calcareous soils contain slow-release phosphate (e.g, octacalcium phosphate and apatite) which occupy sorption sites that otherwise would be available for the added phosphate.

EPCo significantly increased with increasing concentrations of Olsen P in both SCL- Fe_2O_3 and CL-$CaCO_3$ (Fig. 2). EPCo values ranged from 0 to 0.281 mg/L, depending on the background electrolyte solution and soil (Fig. 2). EPCo values in both soils were significantly greater in the experiments with 0.01 M KCl than experiments with 0.01 M $CaCl_2$ (Table 2) because of the formation of Ca^{2+}-phosphate complexes in both soils with 0.01 M $CaCl_2$. All EPCo levels were below the threshold value of 0.025 mg/L except in the 80P plots. The average calculated EPCo values for the four replicated 80P plots was 0.031 mg/L for CL-$CaCO_3$ and 0.190 mg/L for SCL- Fe_2O_3 with 0.01 M KCl, and 0.025 mg/L for SCL- Fe_2O_3 with 0.01 M $CaCl_2$. Although this suggest that prairie soils have a low risk for soluble P transport, a recent review reported that a significant portion of phosphate in Prairie soils can be transported as dissolved P during snow melt runoff. Phosphate can be transported from the agricultural soil when phosphate fertilizer is applied in excess of crop requirements and also from plant residues during snow melt.

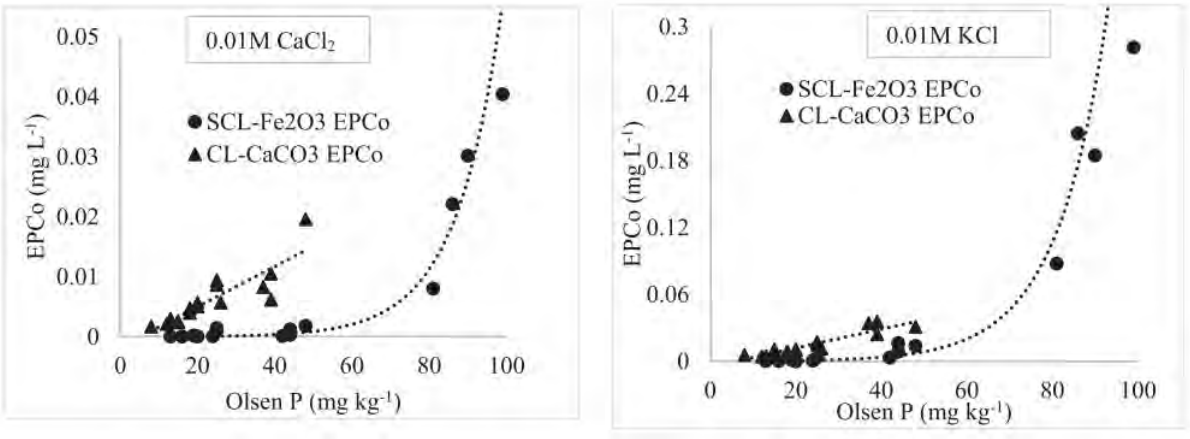


Fig. 2. Effect of Olsen P concentrations in soil on the phosphate equilibrium concentration, (EPCo) in SCL-Fe_2O_3 and CL-$CaCO_3$ soils determined by batch equilibrium experiments using 0.01 M $CaCl_2$ or 0.01 M KCl as background electrolyte solutions. Olsen P All regression equations are significant at $P < 0.05$.

Effect of glyphosate formulation on sorption of phosphate

Commercially available glyphosate formulation had no impact on phosphate sorption in soil because there were no significant differences in phosphate sorption between treatments with and without

Roundup Ultra2 additions to soil slurries. Gimsing and Borggaard (2001) also found that, when glyphosate was added following phosphate additions to goethite, glyphosate did not displace the sorbed phosphate. In a recent article that was published in the magazine "No-Till Farmer", a statement was made that "20-25 percent of the dissolved reactive phosphorus in runoff is caused by glyphosate [use]" because of the assumption that glyphosate residues in soil decreases phosphate retention in soil. However, in our batch-equilibrium study that utilized very high rates of Roundup Ultra2, there was no significant difference in phosphate sorption between treatments with and without Roundup Ultra2 additions to soil slurries. Thus, given our findings, the recent concerns stated in Barrera (2016) are unlikely to be applicable to the Prairie soils that were included in our studies.

Effect of field-aged phosphate on sorption of glyphosate

Glyphosate K_d values significantly decreased with the increasing concentrations of Olsen P in both SCL-Fe_2O_3 and CL-$CaCO_3$ (Fig. 1). Glyphosate K_d values ranged from 293 to 1173 L/kg in the acidic SCL-Fe_2O_3 soil and from only 99 to 141 L/kg in the calcareous CL- $CaCO_3$ soil (Fig. 1), and these values are within the range of other studies (Farenhorst et al., 2008; Kumari et al., 2016; Sørensen et al., 2006). Long-term application of phosphate fertilizer in soil reduced glyphosate sorption because pre-sorbed phosphate occupied the sorption sites that would otherwise be available to glyphosate. A maximum reduction in glyphosate K_d value was observed in SCL- Fe_2O_3 soil. The K_d value was reduced by 75% in soil containing 99 mg/kg Olsen P relative to soil containing 13 mg/kg Olsen P in SCL- Fe_2O_3. Thus, results indicate that glyphosate and phosphate compete for the same sorption sites in soil. Similar observations have been made by de Jonge et al. (2001) who reported that long-term (60 to 100 years) application of phosphate significantly reduced glyphosate sorption by 50% in soil containing 59 mg/kg Olsen P relative to soil containing 6 mg/kg Olsen P.

Olsen P concentrations significantly predicted glyphosate K_d (Fig. 1) in both SCL- Fe_2O_3 and CL-$CaCO_3$. With both 0.01 M $CaCl_2$ and 0.01 M KCl, the slopes of the regressions predicting glyphosate K_d were significantly different between soils with the SCL- Fe_2O_3 showing steeper slopes than CL-$CaCO_3$ (Fig. 1). Regardless of the solution used, the sorption of glyphosate was greater in SCL- Fe_2O_3 than CL-$CaCO_3$ be- cause of the importance of Fe_2O_3 in providing sorption sites for the negatively charged glyphosate in acidic soils. Research findings indicate that the presence of iron-oxide and soil pH had a stronger influence on glyphosate than phosphate sorption. The SCL- Fe_2O_3 soil contained 94% more Fe-oxides and 83% more Al-oxides than the CL-$CaCO_3$ soil (Table 1), and glyphosate sorption was greater in SCL-Fe_2O_3 soil because glyphosate sorption has been shown to be positively correlated with Fe/Al-oxides. In addition, glyphosate sorption was greater in SCL- Fe_2O_3 (pH 4.7 to 5) than CL-$CaCO_3$ (pH 7.3 to 7.5) soil because glyphosate sorption is negatively correlated with soil pH. This is because with increasing soil pH, an increasing portion of the glyphosate molecules become negatively charged with glyphosate molecules existing as HG^{2-} (~ 100%) (net negative charge of glyphosate is 2^-) at pH 7.3–7.5, and soil colloid deprotonation increases with soil colloids having a net negative charge in Prairie soils when soil pH > 6. Hence, regardless of the background electrolyte solutions, the sorption of glyphosate was always relatively low in the CL-$CaCO_3$ soil (Fig. 1). Thus, the effect of Olsen P concentrations on reducing glyphosate sorption was more pronounced for SCL-Fe_2O_3 than CL-$CaCO_3$. For example, with 0.01 M KCl, glyphosate K_d was reduced by 39% when the phosphate concentration increased from 17 mg/kg (control) to 44 mg/kg (40P plots) in SCL-Fe_2O_3 but by only 11% when the phosphate concentration increased from 13 mg/kg (control) to 41 mg/kg (80P plots) in CL-$CaCO_3$.

Association between glyphosate K_d and phosphate K_f in relation to field-aged phosphate

Phosphate K_f and glyphosate K_d values were positively correlated (Fig. 3). Thus, agreeing with previous studies suggesting phosphate and glyphosate have similar sorption pattern in soil. However, regardless of the background electrolyte solution, phosphate K_f and glyphosate K_d were more strongly correlated in SCL-Fe_2O_3 than CL-$CaCO_3$. Hence, glyphosate and phosphate may compete more strongly for

sorption sites in acidic soils with high Fe/Al-oxides content than in calcareous soils. In both soils and under both electrolyte background solutions, phosphate sorption was more strongly reduced by Olsen P concentrations than glyphosate sorption was reduced by Olsen P concentrations. Thus, long-term application of phosphate fertilizer has an overall greater impact on reducing phosphate sorption than glyphosate sorption.

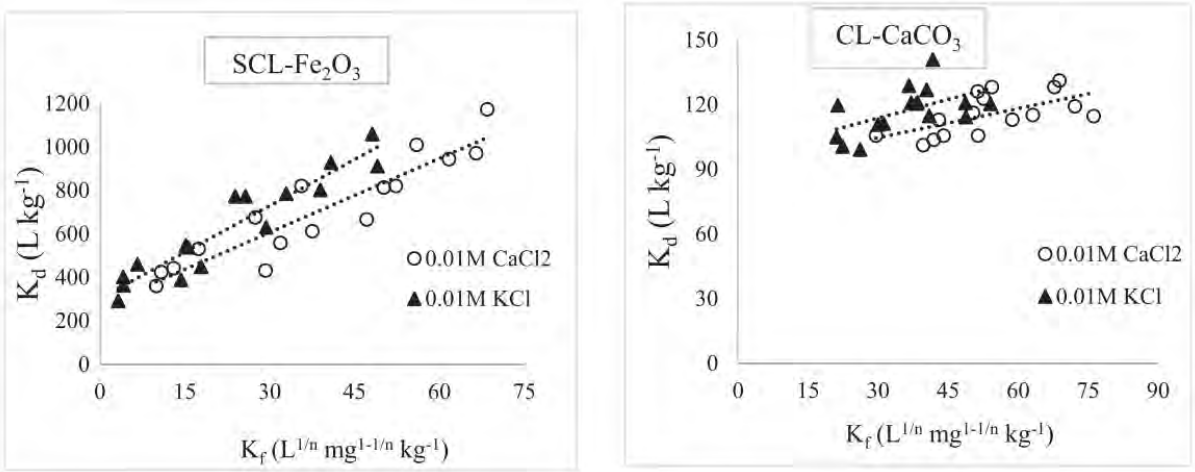


Fig. 3. Association between glyphosate K_d and Phosphate K_f in SCL-Fe_2O_3 and CL-$CaCO_3$ soils with sorption being determined by batch equilibrium experiments using 0.01 M $CaCl_2$ or 0.01 M KCl as background electrolyte solutions. All regression equations are significant at $P < 0.05$.

Effect of fresh phosphate addition on the sorption of glyphosate

Regardless of the background electrolyte solution and soil, the potassium dihydrogen phosphate additions to soil slurries significantly decreased glyphosate K_d values (Fig. 4). Addition of fresh phosphate significantly reduced glyphosate sorption because the chemicals competed for the same sorption sites as they have similar phosphonate functional groups. Gimsing and Borggaard (2002) studied the competitive sorption effect of fresh phosphate on glyphosate in soil and concluded that phosphate is preferentially sorbed over glyphosate. In addition to this, sorption of phosphate lowers the zero point charge of sorption sites such as Fe/Al-oxides, potentially increases the net negative charge on the oxide surfaces and thereby increasing the electrostatic repulsion between glyphosate and soil oxides.

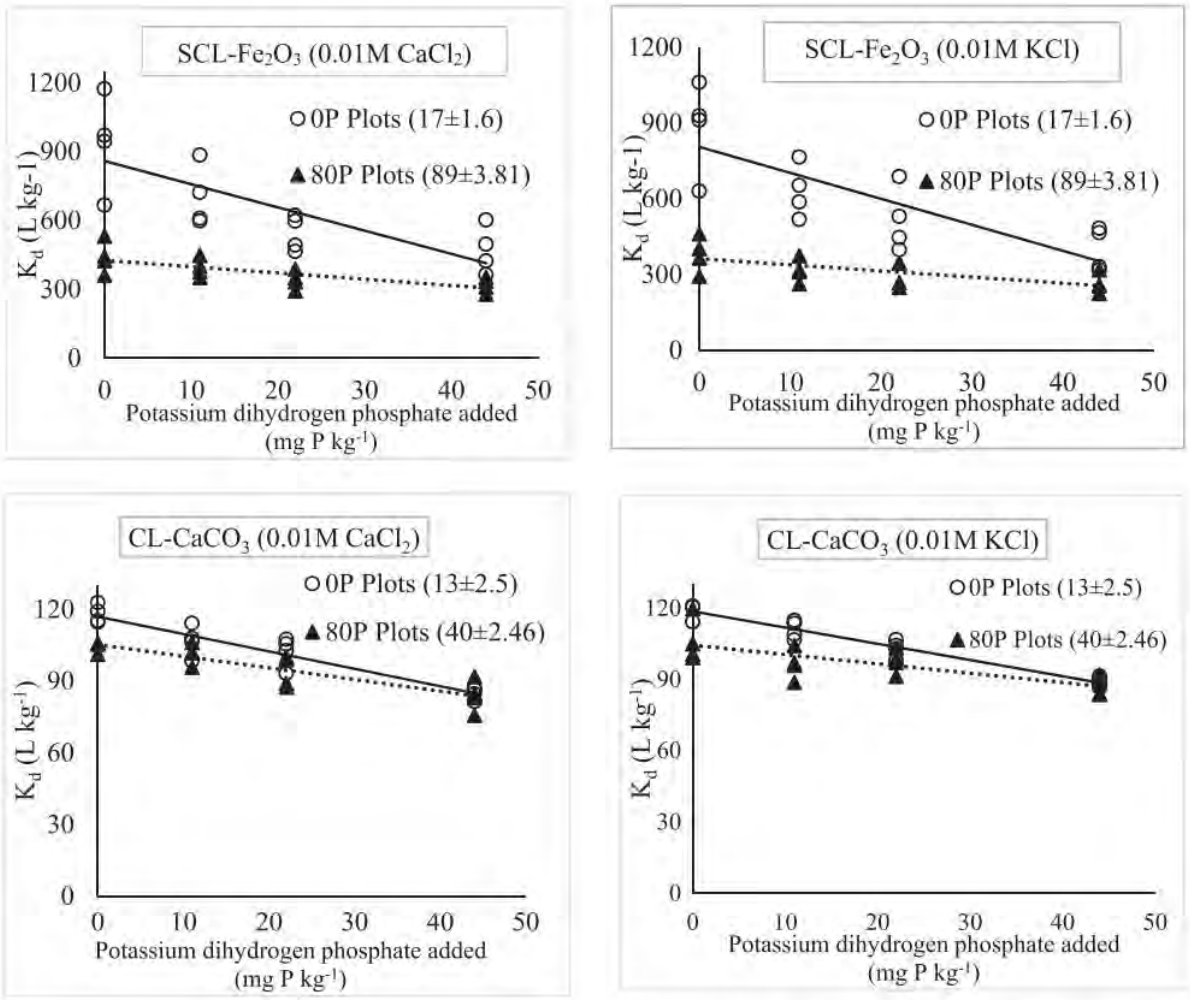


Fig. 4. Effect of potassium dihydrogen phosphate concentrations on glyphosate sorption in SCL-Fe_2O_3 and CL-$CaCO_3$ soils with low (0P) or high (80P) Olsen P concentrations. Potassium dihydrogen phosphate was added to glyphosate in soil sluries during batch equilibrium experiments using 0.01 M $CaCl_2$ and 0.01 M KCl. All regression equations are significant at $P < 0.05$. The values in parentheses in each legend represent mean values of Olsen P and standard error.

Fresh phosphate significantly predicted glyphosate K_d (Fig. 4) in both SCL- Fe_2O_3 and CL-$CaCO_3$. The regression slope was significantly steeper for 0P plots (control) than 80P plots in both soils and regardless of the background electrolyte solution (Fig. 4). Thus, the effect of potassium dihydrogen phosphate addition in reducing glyphosate K_d values was less in soils that had greater Olsen P concentrations because less sorption sites were available for the added phosphate to compete with glyphosate molecules. This impact of phosphate already in soil was larger in SCL- Fe_2O_3 than CL-$CaCO_3$ because in CL-$CaCO_3$ soil at pH 7.3–7.5, glyphosate molecule existed as HG^{2-} (~ 100%) leading to less sorption, both in the presence and absence of fresh phosphate. Thus, the competitive effect of phosphate on glyphosate is stronger in soils that are acidic and contain substantial amount of Fe-oxides than in calcareous soils.

Conclusion

The sorption of phosphate and glyphosate was reduced due to the long-term addition of phosphate fertilizer in two Prairie soils. The impact of Olsen P on reducing glyphosate sorption was more

pronounced in the acidic (iron-oxide rich) sandy clay loam than the calcareous (calcium carbonate rich) clay loam soil, both with or without the addition of potassium dihydrogen phosphate. Regardless of the background electrolyte and soil type, phosphate sorption was more strongly reduced by the Olsen P concentrations than glyphosate sorption. The reduction of glyphosate sorption due to the application of potassium dihydrogen phosphate was greater in soils containing low Olsen P concentrations. The equilibrium phosphate concentration was above the threshold level for eutrophication only in soils that had exceptionally high phosphate concentrations i.e., the soils had received annual applications of mono ammonium phosphate at rates of 80 kg/ha for eight years. Commercially formulated glyphosate had no influence on phosphate sorption suggesting that glyphosate residues in soils have no impact on phosphate sorption or mobility.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes a sorption experiment with phosphate and glyphosate to Canadian agricultural soils. Some validation criteria of the underlying OECD 106 study protocol were not met, or insufficient information is reported.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.4.2
Report author	Marco Napoli; Stefano Cecchi; Camillo A. Zanchi; Simone Orlandini
Report year	2015
Report title	Leaching of Glyphosate and Aminomethylphosphonic Acid through Silty Clay Soil Columns under Outdoor Conditions
Document No	Journal of Environmental Quality 44:1667–1673 (2015)
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Not sufficiently described to check validity against current guideline)

2. Full summary of the study according to OECD format

Glyphosate [*N*-(phosphono-methyl)-glycine] is the main herbicide used in the Chianti vineyards. Considering the pollution risk of the water table and that the vineyard tile drain may deliver this pollutant into nearby streams, the objective of the present study was to estimate the leaching losses of glyphosate under natural rainfall conditions in a silty clay soil in the Chianti area. The leaching of glyphosate and its metabolite (aminomethylphosphonic acid [AMPA]) through soils was studied in 1-m-deep soil columns under outdoor conditions over a 3-yr period. Glyphosate was detected in the leachates for up to 26 d after treatments at concentrations ranging between 0.5 and 13.5 µg/L. The final peak (0.28 µg/L) appeared in the leachates approximately 319 d after the first annual treatment. Aminomethylphosphonic acid first appeared (21.3 µg/L) in the soil leachate 6.8 d after the first annual treatment. Aminomethylphosphonic acid detection frequency and measured concentration in the leachates were more than that observed for the glyphosate. Aminomethylphosphonic acid was detected in 20% of the soil leachates at concentrations ranging from 1 to 24.9 µg/L. No extractable glyphosate was detected in the soil profile. However, the AMPA content in the lowest layer ranged from 13.4 to 21.1 mg/kg, and on the surface layer, it ranged from 86.7 to 94 mg/kg. Overall, these results indicate that both glyphosate and AMPA leaching through a 1-m soil column may be potential groundwater contaminants.

Materials and methods

Borate buffer (0.05 M) was prepared by dissolving 1.9 g disodium-tetraborate-decahydrate in 100 mL ultra-pure water. The FMOC-Cl solutions (1 g/L) were prepared by dissolving 10 mg FMOC-C1 in 10 mL acetonitrile. Glyphosate and AMPA working standard (30 µg/L) were prepared by dissolving glyphosate and AMPA in ultra-pure water. Working standards were stored at 4°C for no more than 1 wk.

In summer 2006, three lysimeters were installed at a lysimeter station in Montepaldi, San Casciano Val di Pesa, Tuscany, Italy. Each lysimeter consisted of a cube-shape casing (1-m edge) made of 4-mm-thick stainless steel sheet. At the bottom end of each lysimeter, a polyethylene corrugated drainage pipe was installed to collect the leachate. During the summer of 2006, the containers were filled with a silty clay soil collected from a nearby Chianti vineyard that had been mechanically weeded over the previous 3 yr. The soil was taken from the 0- to 100-cm layer of three randomly selected vine interrows. The soil was then taken and placed in the lysimeter, taking care to maintain the profile's natural order of layers.
During the monitoring period, hourly temperature and rainfall data were measured by a meteorological station located 300 m from the experimental site. The annual mean temperature and precipitation at the study site were 14.6°C and 914 mm/yr, respectively.

The commercial formulations of glyphosate (360 g/ L a.i.) were applied in the study area at a dose

of 2 L/ha per application. There were one to two spring applications along each vine row, covering a strip of ~1 m. This implies that along the treated strip, the concentration of the active ingredient ranged from 70 to 150 mg/m^2 depending on the number of spring applications. For the lysimeter study, the concentration data associated with the two spring applications was modeled. Therefore, in the middle of March and in the middle of May, glyphosate was applied to each lysimeters .An aqueous solution of herbicide was sprayed onto the surface of the soils to simulate an application rate of 0.72 kg/ha a.i.

Drainage water was collected after each rainfall event from 1 Mar. 2007 to 28 Feb. 2010. To ensure limited degradation, leachate volumes were determined gravimetrically and then preserved in the dark at −20°C for a maximum of 25 d until analysis. On 26 Feb. 2007 and then at the end of each year (i.e., the last week of February), the soil was sampled in triplicate for each of the lysimeters, which were separated into six layers (0–5, 5–20, 20–40, 40–60, 60–80, and 80–100 cm), air-dried, weighed, and sieved. The chemical and physical analyses were performed on air-dried, 2-mm fractions taken from each layers. The soil characteristics are listed in Table 1.

Table 1. Principal chemical and physical properties of study soil; organic matter and carbonates in percentage of the weight of the 2-mm sieved soil; soil electric conductivity (EC) and cation-exchange capacity (CEC) are reported.

Layer	Particle-size distribution (USDA)						Bulk density	Organic matter	Total carbonates	EC	CEC
	Gravel	Fine earth									
		Coarse sand	Fine sand	Coarse silt	Fine silt	Clay					
	%						kg m^{-3}	%		dS m^{-1}	cmol kg^{-1}
0–5 cm	18.2	7.7	8.6	14.7	28.5	40.5	1332	0.81 ± 0.21	14.8 ± 0.2	0.21	23.6 ± 0.5
5–20 cm	19.6	13.1	14.4	13.3	16.2	43.0	1347	0.64 ± 0.22	14.7 ± 0.2	0.2	22.6 ± 0.5
20–40 cm	20.7	15.0	15.2	13.7	14.8	41.3	1410	0.43 ± 0.17	14.7 ± 0.2	0.2	22.1 ± 0.5
40–60 cm	19.9	20.0	18.3	14.5	14.0	33.2	1421	0.37 ± 0.19	14.8 ± 0.2	0.21	20.5 ± 0.5
60–80 cm	22.3	25.0	19.7	14.2	13.4	27.7	1469	0.35 ± 0.07	14.9 ± 0.2	0.2	20.6 ± 0.5
80–100 cm	21.6	26.1	19.5	14.4	13.1	26.9	1488	0.33 ± 0.08	15.1 ± 0.2	0.2	20.2 ± 0.5

Water samples were filtered through 1-mm glass-fiber filters. The liquid was immediately derivatized. The herbicide residues in the sediment, along with the residues in the soil samples, were extracted first by ultrasonic extraction in methanol after which the derivatization procedure was used. To reduce the sorption of glyphosate and AMPA from the methanol-extracted solutions onto glassware surfaces, water and soil samples were dispensed in parallel into plastic vials. Methanol (50 mL) was added to soil samples (50 mg) that had been dried and sieved. The soil suspension was mixed for 60 min and then left at 20°C for 24 h to allow complete solvent evaporation. Then, 15 g of soil was added to 40 mL of solvent and sonicated at 30 to 40 kHz for 30 min. Extracts were filtered through Whatman 40 filter paper, and the filtrate was evaporated on a rotary vacuum evaporator at 40°C to dryness. The residue of herbicide extract was dissolved in 5.0 mL of water and then collected in plastic vials for the derivatization procedure. Sediment extraction was performed as depicted for soil samples.
Following Le Bot et al. (2002), 3-mL samples were derivatized by adding 0.5 mL borate buffer and, after mixing, 500 µL FMOC-Cl solution. Then, samples were shaken for 1 h and incubated, allowing the reaction to take place for 15 h at room temperature (20°C). Derivatization was performed in the dark. The reaction was stopped by adding formic acid at about pH 3.0. The samples were washed with 2 mL diethyl ether to eliminate excess derivatization reagent.

The solid-phase extraction (SPE) was performed by means of a Dionex AutoTrace 280 SPE autosampler (Thermo Scientific). Glyphosate and AMPA were analyzed by liquid chromatography–electrospray ionization–tandem mass spectrometry (TSQ Vantage triple quadrupole mass spectrometer, Thermo Scientific), which comprise an analytical column (Syncronis C8, 2.1 by 150 mm, 5 mm, Thermo Scientific) and a column guard (Syncronis C8, 2.1 by 10 mm, 5 mm, Thermo Scientific). Each standard and sample (3 mL) were injected onto the analytical column and then eluted in gradient mode using a binary solvent mix comprising 99% 5 mM ammonium acetate and 1% acetonitrile (mobile phase A) and 99% acetonitrile and 1% 5 mM ammonium acetate (mobile phase B). The mobile phase flow rate was 0.3 mL/min. Analyses were performed in negative ionization mode with a spray voltage of 3.5 kV. The source temperature and the ion transfer tube temperature were 325°C and 250°C, respectively. The minimum

detectable level (MDL) was 0.1 µg/L for glyphosate and AMPA in leachates and 10 µg/kg in soil.

Results

The daily rainfall and the glyphosate and AMPA concentrations in the leachates are presented in Fig. 1.

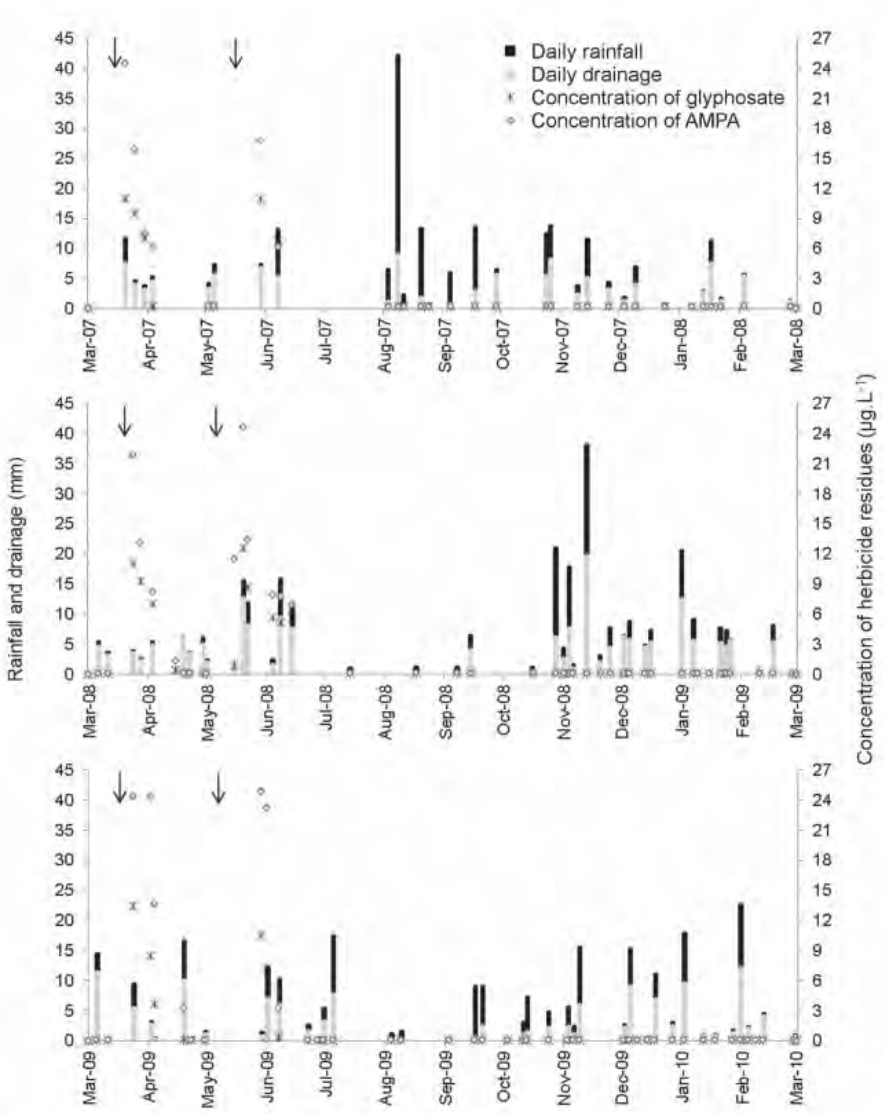


Fig. 1. Daily rainfall and drainage, and concentrations of glyphosate and AMPA measured in the leachates of the vineyard soil from March 2007 to March 2010. Herbicide dates of application are indicated with arrows.

The cumulative rainfall amounts for the period from 1 March to 28 February of the subsequent year were 524, 751, and 1429 mm during the first, second, and third year of the experiment, respectively. During the monitoring period, glyphosate was detected in 3% of the soil leachates at concentrations ranging from 0.2 to 1 µg/L and in 16% of the leachates at concentrations ranging from 1 to 13.47 µg/L. Glyphosate appeared at high concentrations (12.1 ± 1.3 µg/L) in the soil leachates 9.3 ± 4 d after each treatment.

Glyphosate was detected in the leachates for 25.8 ± 8.3 d after treatments at concentrations exceeding 0.5 µg/L. During the latter, average drainage of 15.5 ± 2.9 mm was measured, corresponding to 22.9 ± 6.7 mm of measured rainfall. Thereafter, the glyphosate concentration in leachates decreased to 0.1 µg/L. At the end of each trial year, the final glyphosate peaks appeared in the leachates between late January and early February (about 318.9 ± 8 d after the first annual treatment) at an average concentration of 0.3 µg/L.

Similar to the results for glyphosate, AMPA first appeared at an average concentration of 21.3 ± 6.2 µg/L in the soil leachate approximately 6.8 ± 1.2 d after each treatment. Aminomethylphosphonic acid was detected more frequently than glyphosate; it was detected in 13% of the leachates from soil at concentrations ranging from 0.2 to 1 µg/L and in 20% of the leachates from soil at concentrations ranging from 1 to 24.9 µg/L.

The amounts of water drained from the soil for the period from 1 March to 28 February of the following year, were 113.8, 187.4, and 130.5 mm, respectively, during the first, the second and the third year of the experiment. Approximately 0.19, 0.31, and 0.12% of the amount of glyphosate distributed in the first, second, and third year of the experiment, respectively, were recovered in the leachates as glyphosate, whereas 0.49, 0.78, and 0.48%, respectively, were recovered as AMPA.

On the basis of the analysis, the number of days from the treatment (DN) showed the highest negative correlation with the glyphosate and AMPA concentrations in leachate ($p \leq 0.001$). In contrast, the daily mean temperature (T_{med}) and the daily rainfall (R) showed a positive role in determining the herbicide concentration ($p \leq 0.05$). Since these variables were not autocorrelated, they were selected as independent variables X_1, X_2, and X_3, respectively, for the multiregressive model (Eq. [1]). The multiregression analysis led to the set up of Eq. [2] and [3] for the estimation of glyphosate and AMPA concentration in leachate, respectively:

$$Y_{\text{glyphosate}} = 0.0508T_{\text{med}} - 0.3445\text{DN} - 0.0179R + 13.2308 \quad [2]$$

$$Y_{\text{AMPA}} = 0.1937T_{\text{med}} - 0.6727\text{DN} - 0.1412R + 25.2585 \quad [3]$$

The glyphosate and AMPA concentrations were computed using data measured during the second and the third year of the experiment.

At least for the current study, climatic conditions and for this soil type, Eq. [4] and [5] can be used to determine the number of days free of rain (NR) necessary to ensure a safe threshold for distributing the herbicide.

$$\text{NR}_{\text{glyphosate}} = -2.9028Y_{\text{glyphosate}} + 0.1475T_{\text{med}} - 0.0519R + 38.4058 \quad [4]$$

$$\text{NR}_{\text{AMPA}} = -1.4866Y_{\text{AMPA}} + 0.2879T_{\text{med}} - 0.2099R + 37.5479 \quad [5]$$

No extractable glyphosate was detected in the soil profile. Aminomethylphosphonic acid was found as deep as 100 cm in the soil column. The concentration of AMPA increased with increasing depth, thus indicating a gradual accumulation of AMPA in the lower profile during the 3-yr experimental period. On the contrary, AMPA was distributed throughout the soil columns as shown in Fig. 2.

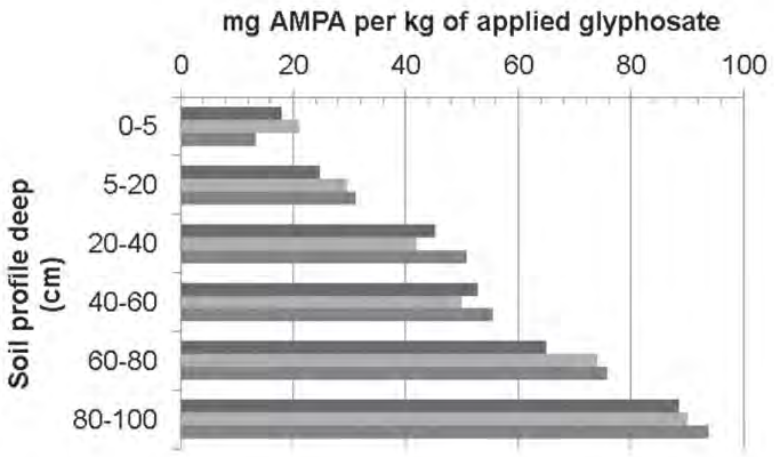


Fig. 2. Distribution profile of aminomethylphosphonic acid (AMPA) in the soil 1 year after the application of glyphosate for the first (dark gray), the second (light gray), and the third (medium gray) year of experiment.

The AMPA content in the surface soil layer ranged between 0.0013 and 0.0021%, based on the amount of glyphosate applied. The AMPA content in the lowest layer ranged between 0.0089 and 0.0094%, based on the amount of glyphosate applied. During the 3 yr, a continuous increase in the concentration of AMPA in the lower layers of the profile was measured; however, there are no statistical data to attribute this to an accumulation effect, but rather to different weather conditions. Finally, at the end of each year of experimentation, the total amount of AMPA recovered in the soil profiles was about 0.03%, based on the amount of glyphosate applied.

The amounts of glyphosate and AMPA, in terms of applied glyphosate, measured in the leachates and in the soil profiles were summed on a yearly basis (Table 2).

Table 2. Mass balance of glyphosate and aminomethylphosphonic acid (AMPA) in leachates and soil profles (in percentage based on the amount of glyphosate applied) for the three experimentation years.

Year	Herbicide leachates		Herbicide in soil		Total residue
	Glyphosate	AMPA	Glyphosate	AMPA	
	%				
First year	0.19	0.49	0	0.03	0.70
Second year	0.31	0.78	0	0.03	1.11
Third year	0.12	0.48	0	0.03	0.63

Conclusion

After a 3-yr experimental period under outdoor conditions, the present work has demonstrated that both glyphosate and AMPA may be transported in leachates through 100 cm of soil profile, thus confirming the high mobility of this herbicide. The mean annual percentage of glyphosate and AMPA, as a percentage of applied glyphosate, recovered in leachates were about 0.2 and 0.58%, respectively. Moreover, results suggested that preferential, flow along with rains that occurred within 2 wk after the treatment, can cause the leaching of glyphosate and AMPA in high concentration. At least in this environment and for this soil, a multiregressive equation was found to determine the number of days free of rain necessary to ensure a safe herbicide distribution. Soil analyses indicated that glyphosate was below detection in 1 yr. On the contrary, the total amount of AMPA, based on the amount of glyphosate

applied, recovered in the soil profiles was around 0.03% at the end of each year of experimentation. Overall, these results suggest that when applied to shallow soils, herbicides can pose a risk of groundwater contamination, and, when applied to pipe-drained crops, contaminated leachate can be transported by the pipe drain to surface waters.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes a lysimeters study with glyphosate using three lysimeters from the Chianti region in Italy. The study is well described, however, there is some information missing to check the validity of the study against current guidelines.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.1.1
Report author	Nguyen, Nghia. K., et al.
Report year	2013
Report title	Soil properties governing biodegradation of the herbicide glyphosate in agricultural soils
Document No	24th Asian Pacific Weed Science Society pg 312-324
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (no sufficient data for evaluation of degradation behaviour reported, only mineralization after 32 days)

2. Full summary of the study according to OECD format

The relationships between soil properties and glyphosate biodegradation in different agricultural soils was investigated in this study. Soils differ hugely in soil texture, soil organic matter content. pH, oxalate extractable $A1^{3+}$ and Fe^{3+}. The biodegradation experiments were conducted under test conditions: water tension of -15 kPa as soil moisture, a soil density of 1.3 g/cm^3 and at 20 °C in the dark. The biodegradation experiments showed that the mineralization of glyphosate in 21 agricultural soils greatly varied. Between 7.6 to 68.7 % of the applied ^{14}C-glyphosate was mineralized to $^{14}C0_2$ in the 21 different soils within 32 days of incubation. The highest and lowest mineralized glyphosates were observed in Feldkirchen (68.7%) and Brejze soil sample (7.6%), respectively. Glyphosate was mineralized rapidly by the microorganisms in the soil solution and the highest mineralization rate was reached shortly after application. The mineralization of glyphosate in soils was individually regulated by exchangeable H^+, soil pH-$CaCl_2$, oxalate extractable AI $^{3+}$ and bacterial cell numbers at the end of the experiments, but it was collectively controlled by exchangeable H^+, Ca^{2+} ions and plant available K. Moreover, soil textures, soil organic content, P_20_5, Cu^{2+}, oxalate extractable Fe^{3+} and CEC were found not to have any correlation with mineralization of glyphosate. The NaOH extractable residues were bioavailable for degradation whereas the bound residues of glyphosate in soils were mostly formed by microbial activity.

Materials and Methods

Soil

The experiment was conducted using 21 agricultural soils typical of Germany and Slovenia. There was a big variation in the different soil characteristics (soil textures, organic matter content, total N, C/N, plant available P, oxalate extractable Al^{3+}, Fe^{3+}, Cu^{2+}, CEC, pH-$CaCl_2$, water content at water potential of -15 kPa and heterotrophic bacteria). All soils were taken from the upper Ap layer of arable fields (0-30 cm), sieved (2 mm) after sampling, homogenized and stored at 4°C in the dark before use. At the beginning of the experiments all soils were conditioned and moistened to a water potential close to -15 kPa at room temperature (20 ± 2 °C) for 2 weeks and compacted to the soil density of 1.3 g/cm^3 to equilibrate the microbial processes and to make sure that all soils have the comparable conditions at the start of the experiments.

Table 1. Some characteristics of soil samples

Name of soil (site of origin)	Sand [%]	Silt [%]	Clay [%]	Water content -15 kPa (%)	pH ($CaCl_2$)	Organic mattter [%]	C [%]	N [%]	P_2O_5 (mg $100g^{-1}$)	K_2O (mg $100g^{-1}$)
1 (Ada-A02)	62.5	27.4	10.1	21.9	5.7	2.9	1.7	0.2	17	4.1
2 (Apace-njiva)	66.4	31.2	2.4	20.7	7.0	2.6	1.5	0.2	4	22.6
3 (Berta-A02)	46.4	39.4	14.2	28.1	5.7	2.5	1.5	0.2	8	12.5
4 (Brezje)	8.3	73.2	18.5	32.7	5.2	2.8	1.6	0.2	5	21.1
5 (Dunja - A06)	62.4	25.9	11.7	17.4	5.4	2.2	1.3	0.2	11	13.2
6 (Feldkirchen)	34.8	47.0	18.2	28.2	7.0	3.4	2.0	0.3	39	9.4
7 (Grace - A13)	50.3	41.3	8.4	21.0	5.4	2.6	1.5	0.2	12	9.6
8 (Hanna - A15)	62.3	24.2	13.5	18.4	5.2	1.7	1.0	0.1	7	8.2
9 (Hohenwart)	67.2	20.5	12.3	22.4	6.2	1.7	1.0	0.1	21	21.1
10 (Joy -A19)	31.6	45.6	22.8	31.9	5.9	2.7	1.6	0.2	34	43.2
11 (Kelheim)	76.2	15.5	8.3	12.5	6.5	1.2	0.7	0.1	23	17.0
12 (Konjise)	33.8	60.2	6.0	34.6	6.9	4.5	2.6	0.2	4	7.9
13 (Lamanose)	10.3	69.6	20.1	35.8	5.8	4.3	2.5	0.3	5	18.7
14 (Lea -A18)	18.9	66.8	14.3	28.9	5.2	1.9	1.1	0.2	6	23.8
15 (Lomanose)	21.9	60.2	17.9	25.8	5.8	1.7	1.0	0.2	11	16.8
16 (Neumark)	85.5	8.8	5.7	12.6	5.2	1.6	0.9	0.1	11	12.2
17 (Pearl - A20)	29.3	51.8	18.9	28.3	5.0	2.3	1.3	0.2	12	31.7
18 (Scheyern Lysi)	17.2	62.6	20.2	30.1	5.5	2.7	1.6	0.2	20	5.3
19 (Skrinjar)	67.5	27.0	5.5	19.2	7.1	1.6	0.9	0.1	21	24.7
20 (Zepovci)	41.3	43.1	15.6	24.0	5.7	2.9	1.7	0.2	11	24.7
21 (Zepovci (Plitv.))	11.8	72.2	16.0	27.4	5.2	1.9	1.1	0.2	8	20.2

Table 2. Some characteristics of soil samples (cont.)

Name of soil (site of origin)	Al_{Ox} (mg $100g^{-1}$)	Fe_{Ox} (mg $100g^{-1}$)	Cu^{2+} (mg kg^{-1})	Ca	Mg	K	Na	II	CEC	Heterotrophic bacteria (x10^7 CFU g^{-1})*
				[$mmol_c$ $100g^{-1}$]						
1	63	198	4	8.5	0.8	1.0	0.04	5.7	16.0	0.3
2	62	248	4	11.1	2.3	0.1	0.04	1.5	15.0	0.5
3	76	265	3	9.0	1.0	0.6	0.04	5.3	15.9	0.4
4	187	518	2	7.2	0.9	0.6	0.07	11.1	19.8	0.1
5	80	211	62	7.0	0.6	0.6	0.04	5.3	13.6	0.3
6	139	310	12	26.4	2.5	0.5	0.05	3.5	32.9	1.1
7	106	259	3	8.7	0.7	0.6	0.04	7.4	17.5	0.6
8	83	215	2	7.2	0.5	0.2	0.04	5.7	13.6	0.5
9	75	206	4	5.5	1.2	0.4	0.05	3.9	11.1	1.6
10	101	320	39	13.1	1.8	0.7	0.06	6.7	22.4	0.9
11	44	132	8	5.5	1.2	0.3	0.05	2.0	9.1	0.8
12	88	381	7	10.8	4.6	0.1	0.06	3.2	18.8	0.1
13	134	456	4	16.4	3.6	0.3	0.06	9.2	29.6	0.4
14	107	345	3	6.4	0.8	0.4	0.07	6.9	14.6	0.3
15	72	252	3	9.5	1.8	0.3	0.09	5.4	17.0	0.7
16	88	110	1	2.6	0.4	0.2	0.05	4.3	7.5	0.7
17	125	319	4	6.9	0.8	0.5	0.05	8.8	17.0	0.8
18	102	349	10	9.1	1.6	0.6	0.06	7.1	18.4	0.9
19	57	257	4	10.8	0.5	0.1	0.06	1.5	13.0	0.5
20	165	476	4	7.8	0.7	0.9	0.05	10.6	20.0	0.3
21	147	430	2	4.4	0.5	0.7	0.10	9.4	15.1	0.1

* CFU = colony-forming unit at the start of degradation experiments

Chemicals

^{14}C-labelled glyphosate [N-(phosphonomethyl)glycine purity >97.0%.] was labelled on the phosphonomethyl group. ^{14}C-glyphosate was mixed with non-labelled glyphosate (purity 98%) resulting in a final specific radioactivity of 1.6 Bq/mg (for degradation experiments). Aminomethylphosphonic acid (AMPA) had the purity of 98 %. Sodium hydroxide (NaOH), monopotassium phosphate (KH_2P0_4), sodium chloride (NaCI), calcium chloride dihydrate ($CaCl_2x2H_20$), NH_4CI, NH_4NO_3, methanol (CH_40), diatomaceous earth, water for chromatography were purchased commercially.

Glyphosate mineralization experiments

Pesticide application procedure - All biodegradation experiments were performed in 4 replicates with 50 g soil (dry mass) for each replicate. ^{14}C -glyphosate was dissolved in autoclaved and distilled water and mixed with non-labeled glyphosate which was also dissolved in sterilized distilled water. This was the application standard solution with a concentration of a.i of 5.42 µg/L and a specific radioactivity of 166.70 Bq/µg. The application standard (0 .089 mL) was applied to an oven dried, pulverized soil and carefully stirred for 1 minute with a spatula. The spiked aliquot was transferred to another glass beaker containing the rest of equilibrated soils and mixed for another 2 min. The total concentration of glyphosate was 10 µg/g in each set corresponding to a total radioactivity of 83,000 Bq.

Test system, experimental conditions and samplings - The spiked soils were compacted to a soil density of 1.3 g/cm^3 and soil water was adjusted to a water potential of -15 kPa. The f1asks were covered with special rubber caps, and incubated at 20 ± 1°C in the dark for a period of 32 days. The soil humidity was controlled weekly. The rubber caps were equipped with an air inlet and outlet system as well as a facility to trap the evolved CO_2. The air exchange system should prevent unaerobiosis in the incubation flasks

and consisted of a canal which was made of a stainless needle with a diameter of 1 mm. To eliminate CO_2 from the ambient air entering the flasks, a 12 mL plastic syringe filled with granular CO_2 absorber (soda lime) was connected to the canal at the top of the cap. Below the cap a small plastic beaker was placed containing 0.1 M NaOH solution to capture $^{14}CO_2$ released from glyphosate mineralization from the soil samples. The NaOH solution was exchanged three times per week and from the collected solution an aliquot of 2 mL was mixed with 3 mL of scintillation cocktail Ultim a Flo AF to determine $^{14}CO_2$ in a liquid scintillation counter. At the end of the experiment, 30 g of each soil sample (dry mass) were used for pore water extraction, 7 g of each soil sample were extracted with NaOH to determine the quantity and quality of the extractable residues as well as to quantify the non-extractable residues, while 1 g of each soil sample was used for cell counts.

NaOH extraction, clean up and HPLC analysis- For NaOH extraction, the method used by Gimsing et al. (2004a) was applied. At the end of the experiment, soil was extracted with 0.1 M NaOH by shaking on overhead shaker for 17 hours. The supernatant was collected after centrifuging for 10 min at 3020 rcf. Radioactivity of the filtered supernatant was measured by scintillation counting using 100 µl of supernatant aliquot and 5mL of scintillation cocktail Ultima Gold XR to quantify the NaOH extractable pesticide residues. Subsequently, extracts were concentrated and cleaned up before injecting to HPLC. Twenty µl of each sample (NaOH extract) were injected via an Auto Sampler AS50 to a HPLC system that was connected with a Radioflow detector LB 509. ^{14}C-glyphosate and its metabolites (AMPA, sarcorsine, glycine, methylamine) were identified by comparison of their retention times with standard substances. After each analysis the column was regenerated with Regenerant-RG019 at a flow velocity (isocratic) of 0.5 mL/min for 30 min.

Quantification of non-extractable ^{14}C-labelled residues - After extraction with 0.1 M NaOH, the rest of radioactivity remaining in the soil was considered as non-extractable residues. Soil material was intensively mixed and homogenized with diatomaceous earth for 2 min in a mortar. Four aliquots of each soil sample were weighed in combustion cups and mixed with 8 drops of saturated aqueous sugar solution to accelerate and ensure a complete oxidation of the ^{14}C. The oxidation step was done with an automatic sample-oxidizer 306. $^{14}CO_2$ from the combustion was trapped in Carbo-Sorb E and mixed with Permaflour E before scintillation counting. The extractable and non-extractable glyphosate residues were calculated after the combustion.

Bacterial cell counts- Bacterial cell counts were performed to count the cultivable and heterotrophic bacteria in the different soils. The method for bacterial cell counts was adapted from Ngigi et al. (2011). Soil bacteria were extracted from the soil by mixing soil with a buffer solution. Before use the buffer solution was autoclaved and shaken vigorously for 1 hour on a shaker at 150 rpm. The soil particles were allowed to sediment for 10 min. Then 0.1 mL of the supernatant was transferred to sterilized buffer solution for further dilution steps. A total of 4 dilutions (10^{-1} to 10^{-4}) were established. Finally, 0.1 mL of each dilution was spread in triplicates on Lysogeny broth (LB) agar media. This medium was also autoclaved before use. The number of CFU was determined after three days of incubation at 25 ^{0}C by counting.

Table 3. Behavior of ^{14}C-glyphosate in different soils

Soil	Cum. Min (%)[a] (1)	NaOH extract. residues (%)[a]* (2)	Non-extract. residues (%)[a] (3)	Total recovery** (%)[a] (4)	Quality of NaOH extract. residues		
					Glyphosate (%) (5)	AMPA[d] (%) (6)	Unknown (%) (7)
1	44.7	48.3	4.8	97.8	37.7	2.3	8.3
2	67.3	24.5	9.6	101.4	18.9	2.2	3.4
3	48.9	42.7	6.3	97.9	34.4	0.0	8.3
4	7.6	91.0	2.5	101.1	88.0	0.0	3.0
5	39.5	53.9	3.9	97.3	45.2	0.0	8.7
6	68.7	23.3	9.0	101.0	12.2	7.6	3.6
7	35.5	57.7	3.7	96.9	44.1	2.8	10.8
8	32.2	59.8	4.1	96.1	54.0	0.0	5.8
9	55.8	37.7	6.3	99.8	24.8	5.7	7.3
10	47.9	46.9	8.2	103.0	31.3	7.1	8.5
11	51.8	37.3	6.2	95.3	25.1	4.1	8.1
12	49.1	35.7	9.5	94.3	23.3	4.5	7.9
13	25.5	64.4	6.7	96.6	45.0	8.3	11.1
14	37.3	55.7	5.4	98.4	30.0	0.0	25.7
15	43.7	46.8	6.4	96.9	30.4	8.0	8.4
16	31.2	63.0	3.1	97.3	48.8	2.5	11.7
17	31.5	63.6	3.7	98.8	29.5	0.0	34.1
18	32.5	59.8	5.0	97.3	40.9	5.5	13.4
19	61.6	28.8	11.4	101.8	16.9	4.8	7.1
20	19.5	73.3	4.1	96.9	65.8	0.0	7.5
21	18.4	78.5	2.7	99.6	55.9	11.3	11.3

a) % of applied ^{14}C-glyphosate after 32 days; the mean value is presented
b) Aminomethylphosphonic acid
* Total NaOH extractable residues (2) = (5) + (6) + (7)
** Total recovery (4) = (1) + (2) + (3)

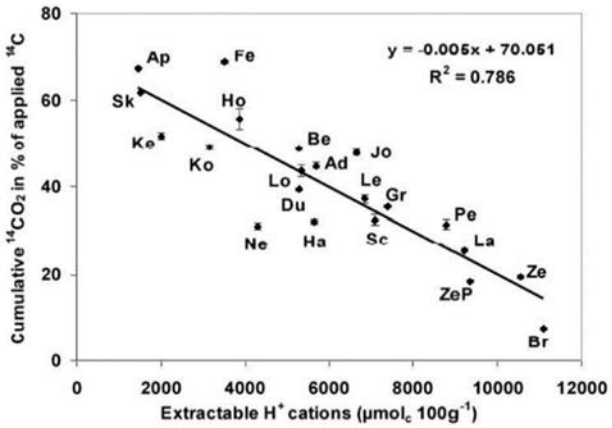


Fig. 1. Correlation between cumulative mineralization of glyphosate and extractable H^+ cations in soils (bars indicate standard deviation of 4 samples).

Statistical analysis

The data were statistically analysed using analysis of variance (ANOVA) and multiple regression analysis.

Results and Discussion

The main aim in this part of the study is to check the correlation between soil parameters and glyphosate mineralization. The selected soil parameters for correlation were exchangeable [H^+], silt, clay, soil organic matter, C, N, C/N, P_2O_5, Cu^{2+}, oxalate extractable Al^{3+}, oxalate extractable Fe^{3+}, K_2O, $CFU_{beginning}$ and CFU_{end}, Ca^{2+}, Mg^{2+}, K^+, Na^+, CEC, and Ph.

Mineralization of glyphosate

After 33 days of incubation a big variance of cumulative mineralization can be observed. Between 7.6 to 68.7 % of the applied ^{14}C -glyphosate was mineralized to $^{14}CO_2$ in the 21 different soil types (Table 3). Shortly after application, a high amount of glyphosate was mineralized. The lowest mineralization of ^{14}C-glyphosate was identified in Brezje soil while the highest mineralization of ^{14}C-glyphosate was obtained in Feldkirchen and Apace-njiva soils. Low mineralization of glyphosate was also observed in

Zepovci, Zepovci(Plitv.) and Lamanose soils. In these 3 soils less than 30% of the initial glyphosate was mineralized after 32 days. In contrast, other soils had a higher mineralization activity and $^{14}CO_2$ production after 32 days reached 31.2-68.7% of the initial glyphosate. A big difference in biomineralization of glyphosate among 21 soils indicates that agricultural soils have difference in ability to degrade glyphosate. The firstly rapid mineralization of glyphosate was observed for most soils during the first 4 days without a lag phase, but mineralization rates subsequently decreased over time, as found in other earlier studies (von Wiren-Lehr et al., 1997; Gimsing et al., 2004a). At the end of the biodegradation experiments, mass balances were established. Mass balances of ^{14}C-glyphosate are presented in Table 3. In all soils, the ^{14}C mass balances were quite good: over 94 % of the totally applied ^{14}C-glyphosate was recovered at the end of the biodegradation experiments.

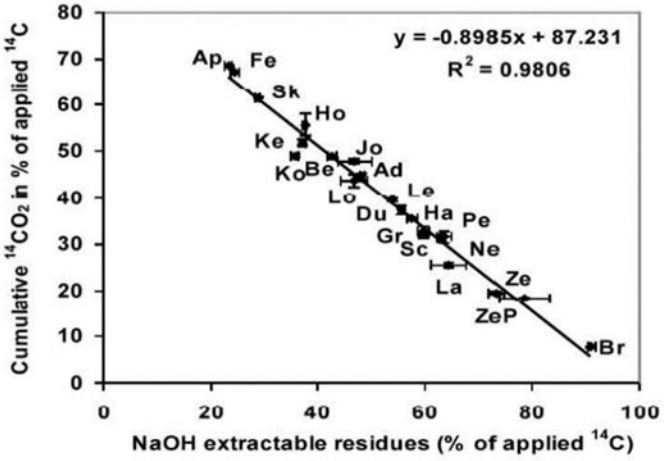


Fig. 2. Correlation between cumulative mineralization of glyphosate and NaOH extractable residues (bars indicate standard deviation of 4 samples).

Identification of the parameters governing mineralization of glyphosate

In order to identify the factors which govern glyphosate mineralization in the 21 soils, soil parameters, NaOH extractable residues, ^{14}C-glyphosate residues, non-extractable residues and the mineralized glyphosate were compared at the end of the biodegradation experiments and several significant correlations could be discovered.

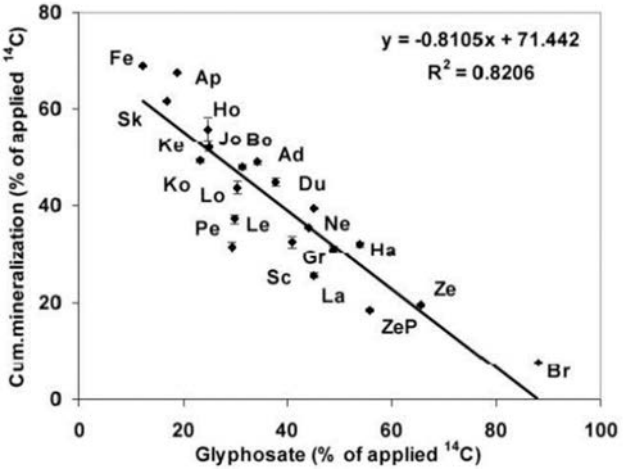


Fig. 3. Correlation between cumulative mineralization of glyphosate and glyphosate residues (from extractable residues) (bars indicate standard deviation of 4 samples).

Relationship between mineralized glyphosate and extractable acidity (extractable H^+ cations)

As can be seen in Fig. 1, according to univariate correlation analysis there was highly significant and negative correlation between the cumulative mineralization glyphosate and extractable H+ cations (p =

0.000). This illustrates that the extractable H+ cations interfered the mineralization process in soils. Therefore, the assessment of extractable H+ cations in soils appears suitable for ranking of soil according to the mineralization of the compound.

Relationship between mineralized glyphosate and NaOH extractable residues
NaOH extractable residues of the 21 investigated soils were performed after 32 days. The results show that the NaOH extractable fraction in all soils was relatively high and very various. Approximately between 23 and 91 % of initial glyphosate after 32 days incubation was extracted with NaOH 0.1 M (Table 3). Soils with higher mineralization had lower NaOH extractable fraction. A correlation was performed to check the relationship between mineralized glyphosate and NaOH extractable residues. Fig. 2 shows that there was a negative correlation between mineralized glyphosate within 32 days and NaOH extractable residues ($p = 0.0000$). This shows that NaOH extractable residues were non-available for microorganisms to be degraded.

Relationship between mineralized glyphosate and ^{14}C-glyphosate residues from extractable pool ^{14}C-glyphosate is the major component in the NaOH extract as compared to AMPA and unknown metabolites (Table 3). To test whether there is any relationship between the mineralized glyphosate and NaOH extractable residues, we calculated correlation between both values. There is exist significantly negative correlation between ^{14}C-glyphosate residues from extractable pool and mineralized glyphosate ($p = 0.0000$; Fig. 3). This indicates that in soils with low mineralization glyphosate is present in a high amount and that this glyphosate could not be degraded/mineralized because it was adsorbed to Al- or Fe-oxides.

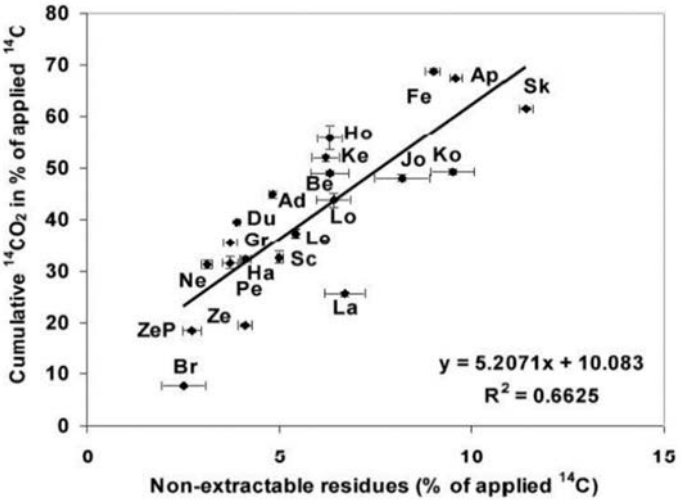


Fig. 4. Correlation between cumulative mineralization and non-extractable residues (bars indicate standard deviation of 4 samples).

Relationship between mineralized glyphosate and non-extractable residues The amount of non-extractable residues was relatively low. It varied between 2.5 % and 11.4 % of the initial glyphosate (Table 3). The non-extractable residues and mineralized glyphosate were compared together to see whether there is any relationship between both parameters. As can be seen from Fig. 4, a significant and positive correlation between mineralized glyphosate and non-extractable residues ($p = 0.0000$) was found. The high mineralization of glyphosate in soils coincided with non-extractable residues at the end of the experiment.

Relationship between mineralized glyphosate and bacterial cell counts
There was a significantly positive correlation between mineralization of glyphosate and bacterial cell counts ($p = 0.003$; Fig. 5). This shows that the mineralization of glyphosate in soils is limited not only by availability of glyphosate and its degradation products, but also by the bacterial activity. Therefore, it can be assumed that the bacterial cell numbers at the end of the experiment seemed to be the degrading

microorganisms for glyphosate in soils and it was likely that microbes capable of degrading glyphosate aerobically exist in soils.

The interacting junctions of the different soil parameters on mineralized glyphosate

In order to investigate the interacting functions of the different soil parameters on cumulative glyphosate mineralization, a multiple regression analysis was used. The input parameters were extractable H^+ cations, silt, clay, soil organic matter, C, N, C/N, plant available P, Cu^{2+}, oxalate extractable AI^{3+}, oxalate extractable Fe^{3+}, plant available K, Ca^{2+}, Mg^{2+}, K^+, Na^+, CEC, pH , CFUs at beginning and CFUs at the end of the experiments. The result of multiple regression analysis reveals extractable H^+ cations, Ca^{2+} and plant available K as key parameters governing glyphosate mineralization in the 21 tested soils and Ca^{2+} and plant available K contributes additionally to extractable H^+ cations to the mineralization of glyphosate. In this multiple regress ion, extractable H+ cations has a negative correlation with mineralization of glyphosate, whereas exchangeable Ca^{2+} and plant available K have a positive correlation with cumulative mineralization of glyphosate. Once again, this result indicates that extractable H^+ cations is an important factor which reduces the bioavailability of glyphosate in soils, and as a consequence the mineralization of glyphosate is reduced. Regarding Ca^{2+} and plant available K, cumulative mineralization was found to be positively correlated with exchangeable Ca^{2+} and plant available K, respectively. Therefore, it is proposed in this study that a complexation between glyphosate with exchangeable Ca^{2+}/plant available K will not reduce the bioavailability and mineralization of glyphosate. In the contrary, Ca^{2+}-glyphosate complexes may be transported more efficiently across microbial cell walls than sole glyphosate compound as it has already been argued for Cu^{2+} complexes in literature (Kools et aI. , 2005). However, these mechanisms have not been documented and should be clarified.

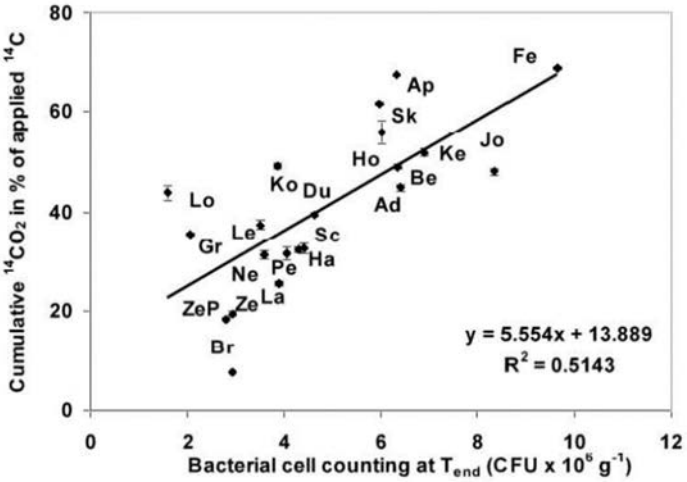


Fig. 5. Correlation between cumulative mineralization of glyphosate and bacterial cell counting (bars indicate standard deviation of 4 sample) .

Conclusions

Degradation of glyphosate in soils greatly varies. depending on soil properties. Mineralized glyphosate is affected by extractable acidity (H^+ cations) and bacterial cell counts. Sorption behavior and bioavailability of glyphosate in soil are important to regulate mineralization. extractable H^+ cations. Ca^{2+} ions and plant available K have been identified as important soil parameters that collectively control the mineralization of glyphosate in soil. Glyphosate that is absorbed by AI/Fe-oxides and extractable H^+ cations can be extractable with NaOH 0.1 M, but it is not available for degradation by soil microorganisms. Non-extractable residues of glyphosate which have been identified as a result of microbial activity.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes the dissipation of glyphosate in agricultural soils in Europe. Experimental details were well described. However, insufficient data for evaluation of degradation behaviour are reported, only mineralization after 32 days.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.1.1
Report author	Norgaard, T., et al.
Report year	2015
Report title	Can Simple Soil Parameters Explain Field-Scale Variations in Glyphosate-, Bromoxyniloctanoate-, Diflufenican-, and Bentazone Mineralization?
Document No	Water, Air, & Soil Pollution (2015) 226: 262
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (not sufficient data provided to check validity against current guidelines)

2. Full summary of the study according to OECD format

The large spatial heterogeneity in soil physico-chemical and microbial parameters challenges our ability to predict and model pesticide leaching from agricultural land. Microbial mineralization of pesticides is an important process with respect to pesticide leaching since mineralization is the major process for the complete degradation of pesticides without generation of metabolites. The aim of our study was to determine field-scale variation in the potential for mineralization of the herbicides glyphosate, bromoxyniloctanoate, diflufenican, and bentazone and to investigate whether this variation can be predicted by variations in basic soil parameters. Sixty-five soil samples were sampled from an agricultural, loamy field in Silstrup, Denmark, from a 60×165 m rectangular grid. The mineralization potential of the four pesticides was determined using a 96-well microplate ^{14}C-radiorespirometric method. Initial mineralization rates were determined using first-order kinetics for glyphosate and bromoxyniloctanoate and zero-order kinetics for diflufenican and bentazone. The mineralization rates of the four pesticides varied between the different pesticides and the different soil samples, but we could not establish correlations between the pesticide mineralization rates and the measured soil parameters. Only the glyphosate mineralization rates showed slightly increasing mineralization potentials towards the northern area of the field, with increasing clay and decreasing OC contents. The mineralization potentials for glyphosate and bentazone were compared with 9-years leaching data from two horizontal wells 3.5 m below the field. The field-scale leaching patterns, however, could not be explained by the pesticide mineralization data. Instead, field-scale pesticide leaching may have been governed by soil structure and preferential flow events.

Materials and Methods

Field Site

The agricultural test field (Silstrup, northwestern Jutland, Denmark) was a conventionally cultivated, loamy field with a cultivated area of 1.69 ha. The climate is coastal, cold temperate. The field has been cultivated as part of a routine agricultural practice with management and pesticide records dating back to 1983. Glyphosate was sprayed on the field five times since 1983, bentazone was sprayed four times, bromoxyniloctanoate only once, and diflufenican not at all. Application dates for the pesticides and their commercial formulations are shown in Table 1. Two horizontal wells, H1 and H2 (Fig. 1), are located 3.5 m below the surface, and each consists of three screen sections of 18 m. Water samples from the middle screen section of each well (H1.2 and H2.2) have been analyzed for pesticides every month, and the samples from the outer screen sections (H1.1, H1.3, H2.1, and H2.3) have been analyzed twice a year (Rosenbom et al. 2010). During 9 years screening (2000–2009), pesticides were detected in 44 % of the water samples from the middle section of the northern horizontal well (H1) whereas only 5 % of the water samples from the middle screen section of the southern horizontal well (H2) contained detectable pesticide concentrations. In the outer screen sections of the northern well (H1.1 and H1.3),

pesticides were detected in 30 and 27 % of the water samples whereas there were no pesticide detections in the outer screen sections of H2 (Norgaard et al. 2012). Consequently, pesticides seem to leach only from the northern part of the field.

Table 1. Pesticide application history. There is no record of which Roundup formulation was applied in 1988 and 1999

	Application date	Formulation
Glyphosate	25 October 1988	Roundup (unknown)
	10 October 1994	Touchdown
	5 August 1999	Roundup (unknown)
	25 October 2001	Roundup Bio
	15 September 2003	Roundup Bio
Bromoxyniloctanoate	20 April 1999	Oxitril
Bentazone	24 May 1994	Basagran 480
	17 June 1994	Basagran 480
	17 May 2003	Basagran 480
	19 May 2009	Fighter 480

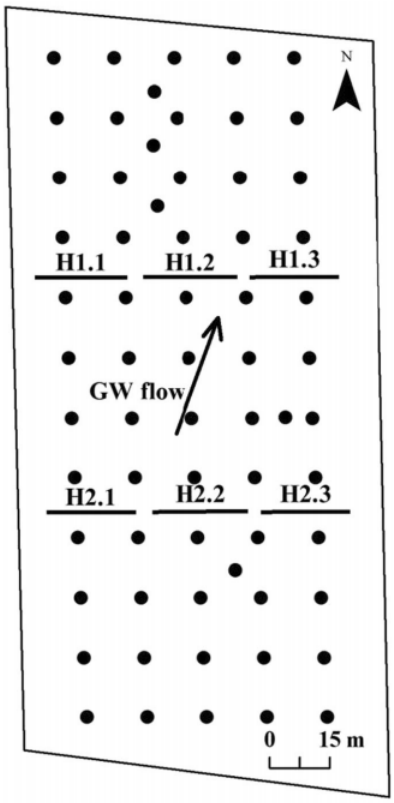


Fig. 1 Schematic presentation of the Silstrup field. Sample positions are indicated by the black dots. The horizontal wells (H1 and H2) and the screen sections in each well are indicated by the lines. The arrow indicates the groundwater flow direction

Soil Sampling

Sixty-five samples were sampled from a 60 × 165 m rectangular field with a distance of 15 m between sampling points (Fig. 1) on 6 December 2011. Soil was sampled from the plough layer at a depth of approximately 8–16 cm. First, the upper 8-cm top soil was removed and then a sample was taken by pounding a sterile 50-ml centrifuge tube (upside down) into the ground until the tube was almost full

and then the tube was sealed. In the lab, each sample was homogenized by sieving twice through a sterile 4-mm mesh. The soil was further mixed thoroughly and stored at 2 °C for 1 week.

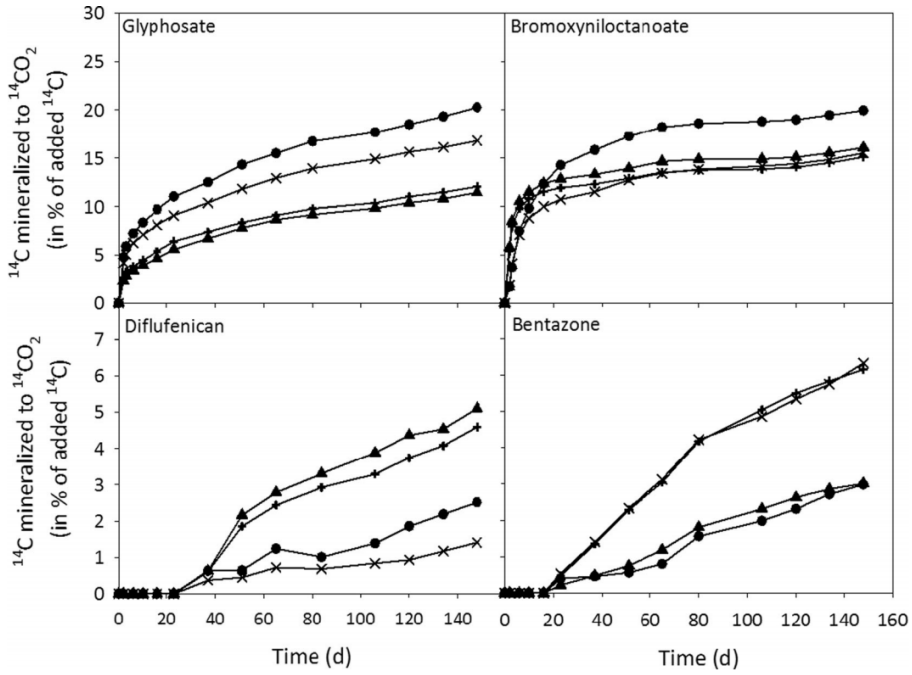


Fig. 2. Mineralization curves depicting the variation in mineralization within the field. The curves represent for each herbicide the two soil samples with the lowest initial mineralization rate and the two with the highest

Physical and Chemical Soil Analyses

Soil texture was determined according to Gee and Or (2002) using a combined sieve/hydrometer method. Organic carbon was determined on a LECO analyzer coupled with an infrared CO_2 detector. Bulk density was determined from weights of 20×20 cm intact soil columns after drying at 105 °C for 2 weeks. The soil pH was measured in a soil/water suspension of 1:4 (*v/v*), and the soil electrical conductivity (EC) was measured in a soil/water suspension of 1:9 (*v/v*). Oxalate-extractable iron, aluminum, and phosphorus were determined at AGROLAB GmbH, Germany, using the procedure described by Schoumans (2000). The Dexter index (Dexter *n*) for each soil sample was calculated as the ratio (*w/w*) between clay and organic carbon.

Table 2. Basic soil parameters. Minimum, maximum, mean, and coefficient of variation (CV) of soil texture, organic carbon (OC), Dexter n, bulk density, oxalate-extractable aluminum (Al), oxalate-extractable iron (Fe), and oxalate-extractable phosphorus (P), pH, and electrical conductivity (EC)

	Clay (<2 μm)	Silt (2–50 μm)	Sand (0.05–2 mm)	OC	Dexter *n*	Bulk density	Al	Fe	P	pH	EC
	kg kg^{-1}	kg kg^{-1}	kg kg^{-1}	kg kg^{-1}	–	g cm^{-3}	mmol kg^{-1}	mmol kg^{-1}	mmol kg^{-1}	–	mS cm^{-1}
Min. value	0.14	0.23	0.45	0.017	6.75	1.39	24	33	7.60	6.39	0.40
Max. value	0.19	0.33	0.59	0.022	10.43	1.60	36	53	15	7.45	0.71
Mean	0.16	0.30	0.51	0.02	8.11	1.50	28.3	43.3	10.6	6.75	0.47
CV	8.4	5.5	4.2	6.9	11.7	3.4	7.7	10.4	14.2	2.2	11.0

Mineralization Potentials

The mineralization potential of the four pesticides was tested using a modified version of a radiorespirometric microplate method. [P-methylene-^{14}C]glyphosate (>99 % radiochemical purity) was purchased from IZOTOP, Institute of Isotopes (Budapest, Hungary). Radioactive pesticide solutions (10

mg/mL, approximately 870 Bq/mL) were prepared by dissolving appropriate amounts of radioactive pesticide and the corresponding non-labeled pesticide in sterile water. For each of the 65 homogenized soil samples, subsamples of 0.5 g were transferred to microplate wells, one microplate for each of the four pesticides and one subsample per pesticide. The microplates were 96-well polypropylene microplates (Nunc 278752) with a well volume of 2.0 mL to minimize oxygen depletion. Fifty microliters of ^{14}C-labeled pesticide solution was added to all wells, corresponding to an initial pesticide concentration of 1 mg/kg soil. The microplates were sealed with PCR sealing tapes on which 96 $^{14}CO_2$ traps (Ca(OH)-amended filter paper discs) were placed in a pattern corresponding to the microplate wells. Polyurethane foam sheets (the size of a microplate lid) were placed on top of the sealing tapes, microplate lids were added, and the plates and lids were held tightly together with strong rubber bands. The sealing tapes were changed after approximately 2, 3, 6, 10 16, 23, 37, 51, 65, 80, 106, 120, 134, and 148 days of incubation at 10 °C. The trapped $^{14}CO_2$ from each well, captured on the $Ca(OH)_2$-impregnated filters, was quantified from a standard series of $NaH^{14}CO_3$ using digital autoradiography and subsequent digital image analysis as described by Hybholt et al. (2011).

Mineralization Kinetics

A two-parameter exponential model (first-order kinetics, Eq. 1) was used to fit the mineralization curves for glyphosate and bromoxyniloctanoate.

$$y = a \cdot (1 - e^{-bt}) \qquad (1)$$

where y is the accumulated $^{14}CO_2$ (% of added ^{14}C) released at time t (day), a is the maximum ^{14}C mineralized (% of added ^{14}C), and b is the mineralization rate constant (day^{-1}).

Since we were interested in estimating the in-situ mineralization potentials, we fitted only the first 23 days of mineralization, where the mineralization followed first-order kinetics. The initial rate at time zero was then calculated from the first derivative function (Eq. 2).

$$dy/dt_0 = ba \qquad (2)$$

A linear regression model (Eq. 3) was used to fit the mineralization curves for diflufenican and bentazone.

$$y = a + bt \qquad (3)$$

For diflufenican and bentazone, the models are based on the mineralization data from days 23–84 and 16–65, respectively. This was done in order to capture the initial, linear part of the mineralization curves from the first detection of mineralization in each of the two cases. The slope of the linear models was used as an estimate of the initial mineralization rate.

Two-Dimensional Interpolation and Statistical Analysis

The spatial, field-scale variation in soil texture, organic carbon content and the mineralization rates were mapped using minimum curvature interpolation with regularized spline interpolation in ArcMap 10.1. The number of points used in the calculation of each interpolated cell was set to 12 and the weight parameter to 0.1. The mineralization rates were correlated to soil physical and chemical parameters using the linear correlation coefficient (R^2), as it shows the fraction of the variation in the mineralization potentials that can be explained by the variation in the physical or chemical soil parameters. Coefficients of variation (CVs) for the pesticide mineralization rates and the soil parameters were calculated as the standard deviation divided by the mean and are given as percentage.

Most Probable Number of Pesticide Degraders

The most probable numbers (MPNs) of cultivable glyphosate-, bromoxyniloctanoate-, diflufenican-, and bentazone degraders were estimated by a modification of the above microplate radiotracer method. To represent the gradients in clay and organic carbon across the field, selected samples were pooled into groups with high clay and low organic carbon content, low clay and high organic carbon content, and intermediate clay and organic carbon content (five to seven subsamples for each group: group A with

17.6–18.9 % clay and 1.8– 1.9 % organic carbon, group B with 14.2–14.3 % clay and 2.0–2.1 % organic carbon, and group C with 16.1– 6.2 % clay and 1.9–2.0 % organic carbon).
A well was considered mineralization-positive if the accumulated amount of $^{14}CO_2$ at the end of the experiment (148 days at 10 °C) exceeded 5 % of the initially added ^{14}C-labeled pesticide. The MPNs were calculated according to Hurley and Roscoe (1983) from the distributions of positive and negative microplate wells. The lower detection limit was calculated by assuming only one mineralization-positive well at the lowest dilution (10 - fold), and the upper limit was calculated from only one mineralization-negative well at the highest dilution (21,870-fold).

Results

Pesticide Mineralization

The MPNs of the microbial degrader populations were for glyphosate and bromoxyniloctanoate above the detection limit of 6.6×10^4 cells/g soil, which indicates a large potential for microbial degradation of these pesticides. This was reflected in the rapid mineralization without any-lag phase of both glyphosate and bromoxyniloctanoate (Fig. 2). After a fast, immediate phase, the mineralization of glyphosate leveled off at 10–20 % and bromoxyniloctanoate at 13–26 %. Diflufenican and bentazone both showed slow linear mineralization with a lag-phase, and both pesticides reached very low mineralization levels (diflufenican 1–5 %, bentazone 3–7 %) within the 148 days of the experiment (Fig. 2). The first mineralization was detected on day 23 for bentazone and on day 37 for diflufenican. We did not detect any microorganisms that could utilize diflufenican or bentazone as a source of carbon and energy (MPN <4 cells/g soil), which probably explains the long lag-phases in the mineralization of these two herbicides. The mineralization of bromoxyniloctanoate showed the best model fits (R^2 = 0.980–0.996, average 0.992, Fig. 3), whereas the glyphosate mineralization was slightly underestimated within the first 3 days and slightly overestimated the following 13 days (R^2 = 0.933–0.987, average 0.968). The model fits for diflufenican mineralization (R^2 = 0.734–0.995, average 0.963) and bentazone mineralization (R^2 = 0.850–1.00, average 0.992) were more variable.

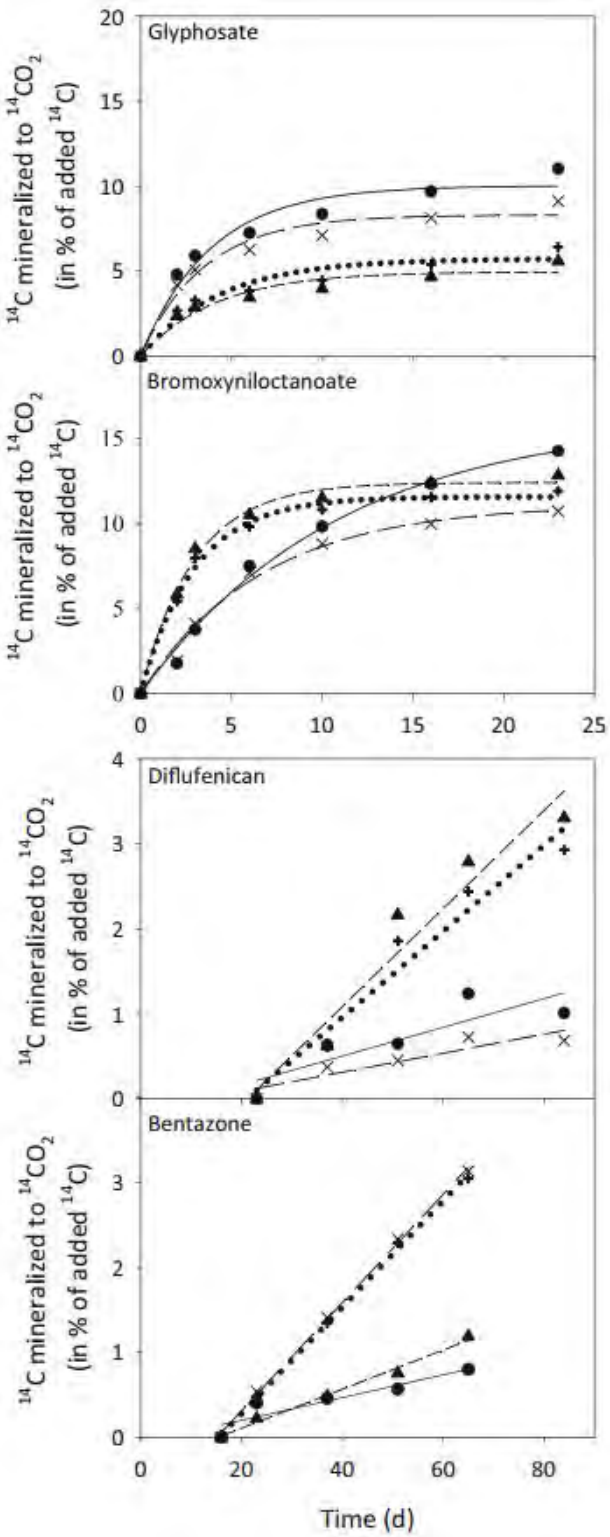


Fig. 3 Examples of herbicide mineralizations and the corresponding model fits. The data represent for each herbicide the two soil samples with the lowest initial mineralization rate and the two with the highest initial mineralization rate within the fitted time period.

Field-Scale Variation in Pesticide Mineralization Rates

The spatial variability of the initial mineralization rates, derived either from the initial rate at time zero for glyphosate and bromoxyniloctanoate or as the slope of the linear regression models for diflufenican and bentazone, is depicted in Fig. 4. Throughout the field, the initial glyphosate mineralization rates varied from 12.1 to 26.0 µg/(kg day) (average 17.1 µg/(kg day), CV=16.7 %), with a slight indication

of lower mineralization in the southern part of the field. Bromoxyniloctanoate had the largest initial mineralization rates varying from 14.9 to 42.0 µg/(kg day) (average 29.6 µg/(kg day), CV=16.5 %). Diflufenican and bentazone showed very limited mineralization of only 0.11– 0.58 µg/(kg day) (average 0.32 µg/(kg day), CV= 24.7 %) and 0.13–0.64 µg/(kg day) (average 0.47 µg/(kg day), CV= 22.4 %).

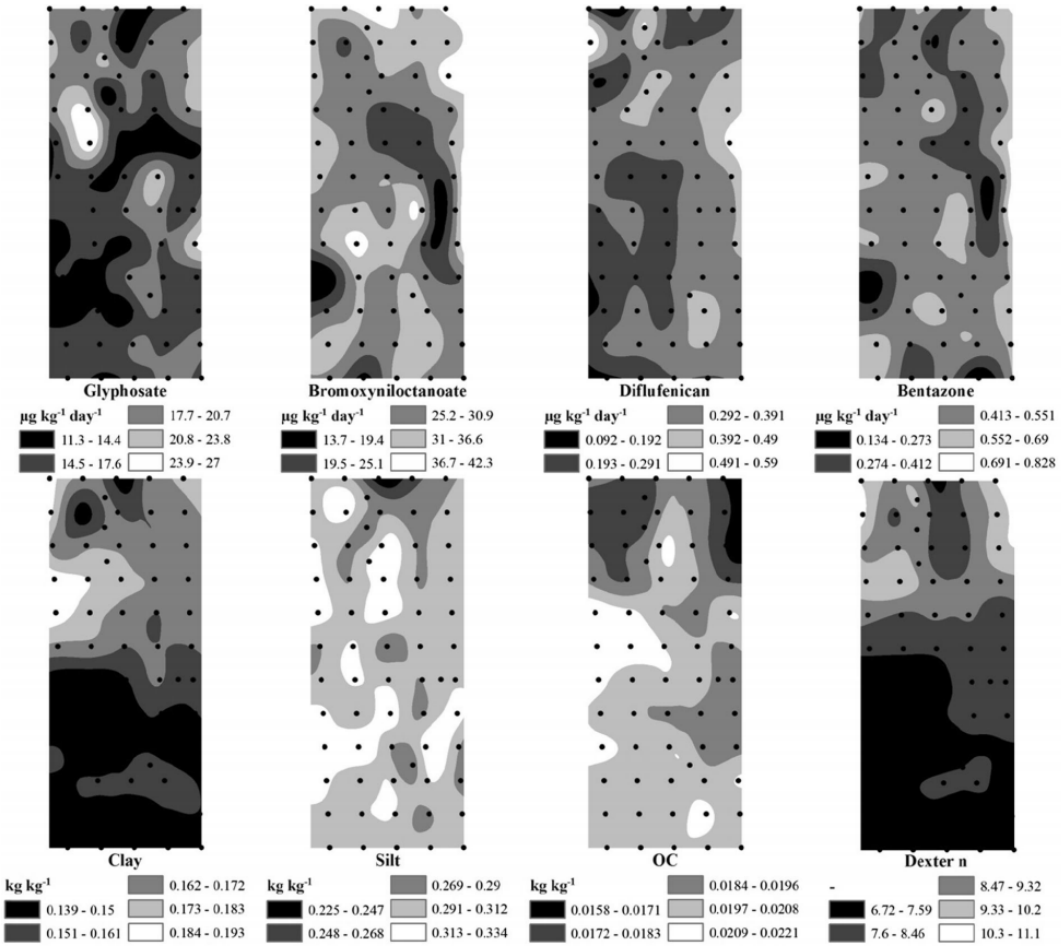


Fig. 4. Spatial distributions of herbicide mineralization rate, clay-, silt-, sand-, and organic carbon (OC) content, and Dexter n (clay/OC ratio). The dots denote the sampling points (n=65).

Table 3. The linear correlation coefficients (R^2) between the pesticide mineralization rates and the basic soil parameters

	Clay (<2 µm)	Silt (2–50 µm)	Sand (0.05–2 mm)	OC	Dexter *n*	Bulk density	Al	Fe	P	pH	EC
Glyphosate	0.09	0.01	0.00	0.11	0.17	0.06	0.06	0.03	0.10	0.00	0.00
Bromoxyniloctanoate	0.01	0.10	0.04	0.04	0.04	0.01	0.08	0.04	0.05	0.05	0.01
Diflufenican	0.06	0.02	0.00	0.06	0.10	0.03	0.01	0.01	0.07	0.00	0.00
Bentazone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01

Mineralization Rates and Soil Characteristics

The range, mean, and CV for the measured soil parameters are reported in Table 2. Gradients in clay and organic carbon content run in opposite directions within the field. Thus, highest clay contents and lowest organic carbon contents were found in the northern part of the field and lowest clay and highest organic carbon contents were found in the southern part of the field (Fig. 4). The ratio between clay and organic carbon, Dexter *n*, therefore increased from south to north (Fig. 4). The mineralization rates for each of the four pesticides generally showed no correlation or very little correlation to the soil parameters

(Table 3). The highest correlation was between the glyphosate mineralization and the Dexter *n*, but this correlation was also weak (R^2=0.17). Linear correlations between the mineralization rates of the four pesticides are reported in Table 4. As in Table 3, the correlation coefficients are weak and the strongest correlation was between the mineralization rates of bromoxyniloctanoate and bentazone (R^2=0.16).

Table 4. The linear correlation coefficients (R^2) between the pesticide mineralization rates

	Glyphosate	Bromoxyniloctanoate	Diflufenican	Bentazone
Glyphosate	1.00	0.07	0.08	0.01
Bromoxyniloctanoate		1.00	0.02	0.16
Diflufenican			1.00	0.03
Bentazone				1.00

Table 5. Number of groundwater samples from H1 and H2 analyzed for glyphosate, aminomethylphosphonic acid (AMPA), bentazone, and 2-amino-N-isopropylbenzamide

	Number of samples, *n*					
	H1.1	H1.2	H1.3	H2.1	H2.2	H2.3
Glyphosate	8	48	8	10	46	8
AMPA	8	48	8	10	46	8
Bentazone	10	63	10	7	46	6
2-Amino-*N*-isopropylbenzamide	4	29	4	4	29	4

Field-Scale Leaching

Water from the two horizontal wells (H1 and H2, Fig. 1) was analyzed for glyphosate and bentazone and their main metabolites aminomethylphosphonic acid (AMPA) and 2-amino-N-isopropylbenzamide (Table 5). Glyphosate was applied five times on the field during the period from 1988 to soil sampling in 2011 with two applications within the monitoring period (2001 and 2003, Table 1). The glyphosate and AMPA contents in water from different subsections of H1 and H2 were analyzed from 2001 to 2005. Glyphosate was not detected in any of the samples. The glyphosate degradation product AMPA, however, was detected in 6.3 % of the analyzed samples from H1.2 and 25 % of the analyzed samples from H1.3 (Fig. 5). None of the AMPA concentrations exceeded the drinking water quality criterion of 0.1 μg/L. Bentazone was applied four times on the field from 1994 to 2011, and two of these applications were within the monitoring program (2003 and 2009, Table 1). Bentazone was analyzed for in the periods from 2003 to 2006 and from 2009 to 2011. The bentazone metabolite was analyzed for only in the period from 2003 to 2006. In total, bentazone was detected in 20 % of the samples from H1, 19.5 % of the H1.2 samples, and 10 % of the H1.3 samples (Fig. 5). One of the detections in H1.2 was above the criterion of 0.1 μg/L. The bentazone degradation product, 2-amino-N-isopropylbenzamide, was not detected in any of the analyzed samples. The metabolite, however, was analyzed for only in the period from 2003 to 2006, whereas bentazone was analyzed for in the periods from 2003 to 2006 and from 2009 to 2011. The horizontal monitoring well, H2, was suspended from 2009.

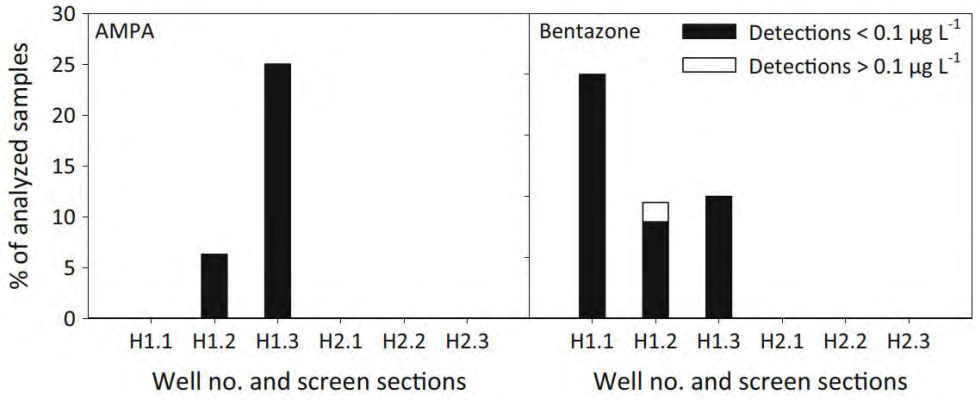


Fig. 5. Percentage of samples AMPA Bentazone from the two horizontal wells, H1 and H2 (Fig. 1), containing detectable levels of the glyphosate degradation product AMPA or bentazone. Water was collected monthly (H1.2 and H2.2, n=29–63) or half yearly (remaining filter sections, n=4–10)

Discussion

In this study, we have investigated the potential mineralization of four herbicides commonly used in agriculture (Miljøstyrelsen 2014). These herbicides represent different physico-chemical properties with very different literature reports on hydrophobicity and sorption. Glyphosate was an easily mineralized, hydrophilic compound with strong sorption to clay loam. Bromoxyniloctanoate was also easily mineralized and strongly sorbing, but hydrophobic. Diflufenican was difficult to mineralize, hydrophobic, and strongly sorbing, and bentazone was also difficult to mineralize, in spite of being hydrophilic with low sorption.

It is clear from the above that bioavailability, expressed as the soil/water distribution coefficient K_d, did not determine the different mineralization patterns between the four herbicides. One reason could be that we added the Tween-80 detergent to the solutions of bromoxyniloctanoate and diflufenican to be able to handle these compounds in aqueous solution. Also, bentazone was not mineralized to any great extent in spite of high bioavailability, which suggest a microbiological limitation rather than a physico-chemical limitation. Glyphosate, in contrast, was easily mineralized in spite of a high distribution coefficient and thus low bioavailability, indicating that sorption may be less important when degraders are very numerous in the soil.

We used first-order kinetics to quantify the mineralization of glyphosate and bromoxyniloctanoate for the first 23 days. Linear regression was used to quantify the mineralization of diflufenican and bentazone covering the time periods 23–84 and 16–65 days, respectively. The linear mineralization patterns indicate that these pesticides were probably mineralized by slow co-metabolic metabolism without growth of the degrader organisms, which is consistent with the absence of bacteria that could utilize them for growth. The 2–3-week delay in mineralization may imply that the degrader organisms were fungi.

The mineralization potentials of bromoxyniloctanoate, diflufenican, and bentazone did not correlate with the gradients in clay and organic carbon across the field or any other of the measured soil parameters. Only the glyphosate mineralization rates tended to increase towards the northern part of the field, correlating slightly with increasing clay and decreasing organic carbon contents (Table 3). The highest correlation was, however, between the glyphosate mineralization and Dexter *n*, so that it was the ratio between clay and organic carbon more than the total contents that influenced the glyphosate mineralization.

Our results indicate that the development of generally valid models for predicting pesticide mineralization across field sites, based on simple soil characteristics and in-vitro mineralization rates, may be unrealistic. Furthermore, if the mineralization of two or more of the herbicides were determined by the same soil parameters, we would have seen correlations between these herbicides, which were not the case (Table 4). It seems difficult to connect pesticide mineralization (or degradation) and specific topsoil parameters, but what about pesticide mineralization and leaching? Though included in the analyses, we did not detect glyphosate in the samples from the horizontal monitoring wells, but the glyphosate degradation product, AMPA, was detected. In contrast, only bentazone was detected, and

not the degradation product, 2-amino-Nisopropylbenzamide. All detections of AMPA and bentazone were from the H1 well that collected water from the northern part of the field. Neither AMPA nor bentazone was detected in the samples from H2 which collected water from the southern part of the field. This pattern does not correspond well with the rather random distribution of mineralization potentials of the two herbicides (Fig. 4).

Conclusion

Glyphosate was an easily mineralized, hydrophilic compound with strong sorption to clay loam. Bromoxyniloctanoate was also easily mineralized and strongly sorbing, but hydrophobic. Diflufenican was difficult to mineralize, hydrophobic, and strongly sorbing, and bentazone was also difficult to mineralize, in spite of being hydrophilic with low sorption. It is clear from the above that bioavailability, expressed as the soil/water distribution coefficient K_d, did not determine the different mineralization patterns between the four herbicides. The linear mineralization patterns indicate that these pesticides were probably mineralized by slow co-metabolic metabolism without growth of the degrader organisms, which is consistent with the absence of bacteria that could utilize them for growth.
The mineralization potentials of bromoxyniloctanoate, diflufenican, and bentazone did not correlate with the gradients in clay and organic carbon across the field or any other of the measured soil parameters. Only the glyphosate mineralization rates tended to increase towards the northern part of the field, correlating slightly with increasing clay and decreasing organic carbon contents. Our results indicate that the development of generally valid models for predicting pesticide mineralization across field sites, based on simple soil characteristics and in-vitro mineralization rates, may be unrealistic. Furthermore, if the mineralization of two or more of the herbicides were determined by the same soil parameters, we would have seen correlations between these herbicides, which were not the case (Table 4). It seems difficult to connect pesticide mineralization (or degradation) and specific topsoil parameters.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study reports the results from an aerobic soil mineralization experiment with glyphosate (among other pesticides) in the lab. In parallel the leaching behavior of the substances in the field where the soils used in the mineralization experiment come from, was investigated. The study does not follow guideline procedures. The provided data does not allow to check the validity of the study against current guidelines. Furthermore, no data on glyphosate content per sampling data and no half-lives were provided, i.e. no endpoint is available.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.2.1
Report author	Passeport, E., et al.
Report year	2014
Report title	Dynamics and mitigation of six pesticides in a "Wet" forest buffer zone
Document No	Environmental Science and Pollution Research (2014) 21:4883–4894
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Not all parameters are reported to check validity of the study)

2. Full summary of the study according to OECD format

Pesticide pollution is one of the main current threats on water quality. This paper presents the potential and functioning principles of a "Wet" forest buffer zone for reducing concentrations and loads of glyphosate, isoproturon, metazachlor, azoxystrobin, epoxiconazole, and cyproconazole. A tracer injection experiment was conducted in the field in a forest buffer zone at Bray (France). A fine time-scale sampling enabled to illustrate that interactions between pesticides and forest buffer substrates (soil and organic rich litter layer), had a retarding effect on molecule transfer. Low concentrations were observed for all pesticides at the forest buffer outlet thus demonstrating the efficiency of "Wet" forest buffer zone for pesticide dissipation. Pesticide masses injected in the forest buffer inlet directly determined concentration peaks observed at the outlet. Rapid and partially reversible adsorption was likely the major process affecting pesticide transfer for short retention times (a few hours to a few days). Remobilization of metazachlor, isoproturon, desmethylisoproturon, and AMPA was observed when non-contaminated water flows passed through the forest buffer. Our data suggest that pesticide sorption properties alone could not explain the complex reaction mechanisms that affected pesticide transfer in the forest buffer. Nevertheless, the thick layer of organic matter litter on the top of the forest soil was a key parameter, which enhanced partially reversible sorption of pesticide, thus retarded their transfer, decreased concentration peaks, and likely increased degradation of the pesticides. Consequently, to limit pesticide pollution transported by surface water, the use of already existing forest areas as buffer zones should be equally considered as the most commonly implemented grass buffer strips.

Materials and Methods

The forest buffer zone is located at the outlet of a tile drained agricultural watershed at Bray (France).

Chemicals

An injection solution was prepared with six pesticides and potassium bromide as a conservative tracer. Pesticides were provided by farmers and diluted in deionized water before injection. Commercial solutions that were used are indicated into parentheses: three herbicides, glyphosate (Glyphogan), isoproturon (Isoproturon), and metazachlor (Novall), and three fungicides, azoxystrobin (Priori Xtra), cyproconazole (Amistar Xtra), and epoxiconazole (Opus) were selected for their contrasting properties and wide use in agriculture.

Tracer Experiment

The forest buffer tracer experiment took place for a period of 14 days, from 19 February 2009, 10:50 to 5 March 2009, 13:20 in a reduced portion of the forest buffer, using watershed outlet flows as incoming flows into the forest buffer. The experimental plot was delimited with soil border levees leading to a 54 m^2 surface area (36 m×1.5 m). Only one significant rainfall event occurred on 308.5 h after the start

of the experiment, with a cumulative rainfall depth of 9.94 mm, measured with the on-site tipping bucket rain gauge (R01 3030A Danae, Précis Mécanique, Bezons, France). Water temperature was 5.9±3.7 °C during the course of the experiment, and was close to or greater than monthly averages. The inlet flow rate was 0.32±0.08 L/s. At the outlet, a flow restriction helped manually measuring flow rates by frequently timing the filling of a container with a known volume. Water from the watershed was allowed to flow through the forest buffer experimental plot on 18 February 2009 at 15:50, in order to saturate the soil and ensure a permanent flow rate for the next day injection. Two peristaltic pumps (Eijkelkamp 12 V SDEC Reignac-sur-Indre, France) were used to ensure a 0.30 L/s injection flow rate during 78 s. Grab water samples or samples collected by means of a time-dependent automated sampler (ISCO 3700 Neotek, Trappes, France) were taken at the outlet of the experimental plot. The sampling frequency was modified along the course of the experiment: every 15 min for the first 7 h, every 30 min until 28.5 h after the start of the experiment, then every 3 h until 94 h since injection, and every 10 h from days 4 to 10 following the start of the experiment. Finally, five grab water samples were taken at forest buffer inlet to control pesticides' background concentrations coming from the artificially drained watershed.

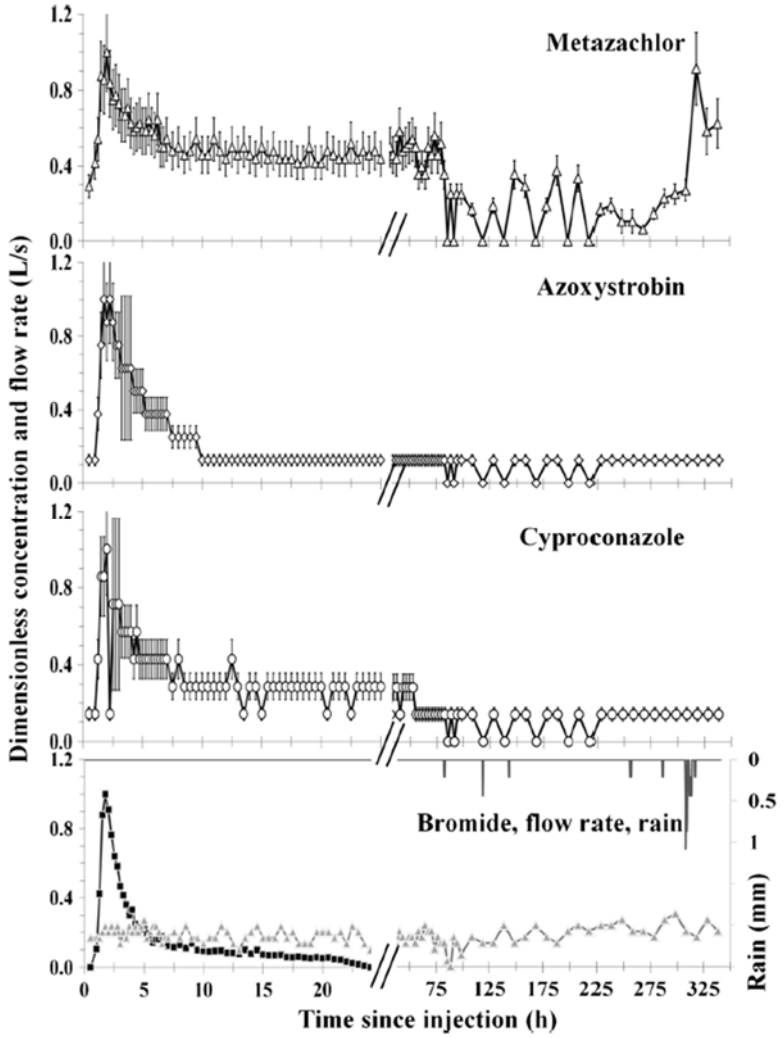


Figure 1. Flowrate at the forest outlet (gray triangle, bottom panel, in liter per second), and dimensionless (C/C_{max}) concentration pattern during the first 24 h (left panels) and the next 350 h (right panels) after injection, for molecules that exhibited the clearest transfer pattern: metazachlor (white triangles), azoxystrobin (white diamonds), cyproconazole (white circles), and bromide (black squares). The double slash bars (//) indicate a change in time step. C concentration at time t; C_{max} peak concentration measured 2 h (metazachlor, azoxystrobin, and cyproconazole) and 1.8 h (bromide) after injection. No rainfall event occurred during the first 24 h; rain beyond 24 h (bottom-most right-hand side panel) is plotted on the right hand vertical axis, in reverse order. Error bars correspond to dimensionless expanded uncertainties, i.e., expanded uncertainties on concentrations (U, coverage factor = 2), divided by C_{max}

Analytical method

Water sample analysis
Subsamples were taken from water samples, filtered and analyzed for bromide with ion chromatography and an IonPac AS9-HC column. The limit of quantification (LQ) was 1 mg/L. Metazachlor, cyproconazole, epoxiconazole, azoxystrobin, isoproturon and two of its metabolites, desmethylisoproturon and 1-(4-isopropylphenyl)urea, were extracted by solid-phase extraction on pre-filtered samples, and then analyzed by high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (LCMS-MS). Limits of quantification were 0.02 µg/L for these seven pesticides and metabolites. Glyphosate and its main metabolite, AMPA, were first derivatized with 9-fluorenylmethyl chloroformate (FMOC) before LC-MS-MS analysis (LQ=0.1 µg/L for both glyphosate and AMPA).

Litter and soil sampling and analysis
Litter, and soil grab samples were taken in the forest experimental plot at the end of the tracer experiment. Another litter and soil samples were collected outside the experimental plot to compare with those collected inside the experimental plot. All samples were frozen before pesticide analysis. Glyphosate and AMPA were extracted by ultrasonic waves in water, then derivatized with FMOC and analyzed by LCMS-MS, whereas extraction for the other molecules from soil samples was carried out with ultrasonic waves in acetone. Extracts were analyzed by LC-MS-MS. Litter samples were treated with an internal procedure developed by the laboratory (Institut Pasteur de Lille). Limits of quantification were 0.01 mg/kg dry weight for each compound.

Data analysis
The hydraulic retention time was calculated based on the bromide conservative tracer using the moment theory on residence time distribution (see Passeport et al. (2010), Kadlec and Wallace (2008)).

Statistical analyses
Pearson correlation coefficients were determined with the R software to detect possible correlations among pesticide concentrations, injected masses, and pesticide physico-chemical properties.

Table 1. Forest buffer inlet concentrations

Molecule	Pesticide inlet concentrations (µg/L)				
	Time from injection (h)				
	LQ[a]	−0.58	6.75	24.17	339
Glyphosate	0.1	n.d.[b]	<LQ	<LQ	n.d.
AMPA	0.1	0.30	<LQ	<LQ	0.30
Isoproturon	0.02	1.60	1.20	1.30	1.40
Desmethylisoproturon	0.02	0.12	0.11	0.10	0.11
1-(4-isopropylphenyl)urea	0.02	<LQ	<LQ	<LQ	n.d.
Metazachlor	0.02	0.29	0.30	0.25	0.19
Epoxiconazole	0.02	<LQ	0.02	n.d.	n.d.
Azoxystrobin	0.02	<LQ	<LQ	n.d.	n.d.
Cyproconazole	0.02	<LQ	<LQ	n.d.	n.d.

[a] Limit of quantification
[b] *n.d.* is "not detected"

Results

Hydrology
Water ran off through the forest buffer experimental plot as a shallow sheet flow with an average outlet flow rate of 0.18± 0.11 L/s (average ± expanded uncertainty for 95 % confidence interval). Bromide

started to be detected 1 h after injection and reached a concentration peak 1.8 h after injection (Fig. 1). Bromide recovery rate and hydraulic residence time were 74 % and 6.3 h, respectively.

Inlet water quality

During the experiment, watershed tile-drain flows continuously entered the experimental plot at a controlled flow rate of 0.3 L/s. We determined that some of the studied pesticides also entered the experimental plot via watershed flows during the course of the experiment. Non-negligible concentrations of isoproturon, desmethylisoproturon, glyphosate, AMPA and metazachlor were measured (Table 1). Epoxiconazole was detected once (6.8 h after injection) but with a concentration at the limit of quantification. The most recent applications of glyphosate and metazachlor on the Bray watershed were approximately 16 months before the start of the experiment.

Table 2. Tracer experiment dynamics characteristics and mass recovery rates

Molecule	Peak conc ± U(C) (µg/L)	Peak conc time (h after injection)	Percent recovery (%)	Time for conc reaching < LQ (h after injection)
Bromide	1750	1.75	74	24.0
Glyphosate	0.05±0.03	NA[a]	NA	NA
AMPA	0.30±0.08	1.75	NA	NA
Isoproturon	1.70±0.41	2.50	NA	NA
Desmethylisoproturon	0.14±0.03	NA	NA	NA
1-(4-isopropylphenyl)urea	0.02±0.01	NA	NA	NA
Metazachlor	0.48±0.10	2.00	NA	NA
Epoxiconazole	0.04±0.01	2.75	NA	NA
Azoxystrobin	0.08±0.02	2.00	22	10.0
Cyproconazole	0.07±0.02	2.00	45	13.5

[a] NA means Not Available, when peak concentration ("Peak Conc") time could not be clearly identified and mass balances could not be reasonably calculated due to a large portion of the concentration dataset below limits of quantifications

Pesticide dynamics description

Table 2 presents the main characteristics for pesticide concentration peaks, dynamics and mass balances. Apart from isoproturon, concentrations were lower than 0.50 µg/L for AMPA and metazachlor, and did not exceed 0.15 µg/L for the other pesticides (glyphosate, azoxystrobin, epoxiconazole, cyproconazole, desmethylisoproturon, and 1-(4-isopropylphenyl)urea). Only injections of metazachlor, azoxystrobin and cyproconazole resulted in a clear transfer pattern at the forest plot outlet (Fig. 1). Two hours after injection, these pesticides exhibited concentration peaks of 0.48 ± 0.10, 0.08 ± 0.02, and 0.07 ± 0.02 µg/L for metazachlor, azoxystrobin, and cyproconazole, respectively. These concentration peaks were observed closely after that of the conservative tracer, which was recorded 1.8 h after injection (Table 2). For glyphosate, AMPA, epoxiconazole, and 1-(4-isopropylphenyl)urea, concentrations at the forest plot outlet were so low that only a qualitative assessment of the data can reasonably be performed. In addition, high background concentration levels of isoproturon and desmethylisoproturon hindered an accurate quantitative analysis of the data for these two molecules. In all water samples, glyphosate concentrations were below the LQ and those for AMPA never exceeded 0.30 ± 0.08 µg/L. No temporal variation was observed for these molecules, besides two small AMPA concentration rises, one after injection (between 1.8 and 3.8 h) and a second one after the rainfall event (between 318.5 and 328.5 h). Concentration peaks for the injected molecules were significantly correlated (p value=1.75×10^{-5}) with background concentrations, highlighting the strong influence that this artifact exerted on the results. The second strongest correlation (despite not significant at a α=5 % significance level) was between pesticide concentration peaks and injected masses. With this small dataset, no statistically significant correlations were found between the ratios and the pesticide sorption properties.

Discussion

Hydrology

The ratio between outlet and inlet flow rates (0.61), and the bromide recovery rate (74 %) are suggestive of some water losses outside the experimental plot, via infiltration, possibly due to poor soil levee compaction, earthworm burrows, and tree roots.

Forest buffer efficiency for pesticide removal
A key conclusion of our study relies on the fact that, for most pesticides, very low concentrations were measured at the forest outlet, thus demonstrating the efficiency of such buffer zones for pesticide removal.

Sorption as part of a complex set of removal processes
The high sorption coefficients of glyphosate, AMPA and epoxiconazole may partly explain their low concentrations measured at the forest outlet. Contrary to glyphosate and AMPA, epoxiconazole was detected on dead leaves at the forest plot inlet and middle zones 14 days after injection even after large rainfall events. This supports a possible strong adsorption of epoxiconazole onto the forest litter. Because epoxiconazole was not detected in the soil below the litter layer, it is likely that the litter layer acted as a key sorption material that prevents strongly sorbing pesticides from leaching to deep soil horizons.

Degradation and remobilization of pesticides
Due to the moderately long half-lives of their parent molecules, glyphosate and isoproturon, the detection of AMPA and desmethylisoproturon at the beginning of the experiment can hardly be attributed to the injected parent molecules. It should be noted that AMPA, isoproturon, and desmethylisoproturon were detected at the forest plot inlet indicating that these molecules were also transferred to the experimental plot from the tile-drain watershed. Glyphosate and isoproturon were applied previously on the agricultural watershed and may have been partially degraded in the catchment and forest buffer soils thus generating these metabolites.

"Dry" vs. "Wet" buffer zone
In this study, the "Wet" forest buffer soil had a high clay content thus limiting downward infiltration. Even if water losses via infiltration might occur, it could not explain alone the observed pesticide removal. It is a fundamental difference with "Dry" buffer zones like grass areas, where infiltration plays a crucial role. The second major difference between grass and forest buffer zones lies in the presence of thick litter layer rich in organic matter in the latter. The litter provides many sorption sites for pesticides and is biologically active, thereby biodegrading retained pesticides. Consequently, when buffer zone soil is saturated, pesticide sorption and degradation should more easily occur in forested areas than in grass areas, provided that the contaminated water runs off through the litter layer as a shallow and slow water flow.

Conclusions

The objective of this experiment was to demonstrate at the field scale the potential of forest buffer zones to reduce the concentrations and loads of pesticides presenting a wide range of physico-chemical properties. Very low concentrations were measured at the forest outlet thus suggesting a potential of the forest buffer to effectively reduce the pollution with pesticides. Understanding processes, which govern the removal of pesticides through the forest buffer was beyond the scope of this study. However, the fine sampling frequency used in this study helped to provide some explanations about the observed dynamics of pesticide transfer through the forest buffer zone. At short time-scales (lower than a month), retention processes are suspected to dominate. Our results highlighted the dual role of organic matter. On the one hand, organic substrates enabled rapid adsorption of pesticides transported in highly contaminated flows. On the other hand, when fresher (i.e., less contaminated) flows crossed the forest buffer, previously adsorbed pesticides were shown to desorb thus being released back to the water column. Organic matter also plays an indirect role in this process as it supports growth of microbial populations. Any forested area adequately located in the landscape could be used as an efficient buffer zone for reducing pesticide pollution. Indeed, even old wood that were not necessarily well maintained could be good candidates for buffering pesticide contaminated flows provided a thick litter layer has

had time to accumulate over time. At a short time scale (here approx. 350 h), highly organic material would therefore mainly act as a retarding factor that temporarily affect pesticide dynamics. For extended periods of water retention, degradation reactions leading to metabolites are likely to occur, however, more research is needed to confirm the extent of pesticide degradation that could be achieved. The results of this study are suggestive of a high potential of "Wet" forest buffer zone for the reduction of downstream pesticide concentrations and loads. Further research should investigate the efficiency of forest buffers for pesticide removal (1) under various climatic conditions, and for a wide range of forest buffer (2) sizes and shapes, and (3) locations in the watershed (headstream vs. downstream). Such results are needed to better understand pesticide fate and the role of the litter layer, and to establish guidelines to design forest buffer zones and incorporate them in land management strategies.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes the mitigation of glyphosate among other pesticides by a wet forest buffer zone in France. Not all required parameters are reported to check validity of the study (e.g. information on test substance, analytical method, characterization of soil).
The study is classified reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Rampoldi, E., et al.
Report year	2014
Report title	Carbon-14-Glyphosate Behavior in Relationship to Pedoclimatic Conditions and Crop Sequence
Document No	Journal of Environmental Quality 43:558–567 (2014)
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (No EU conditions, missing information for validity check)

2. Full summary of the study according to OECD format

The recognition of glyphosate [(*N*-phosphonomethyl) glycine] behavioral patterns can be readily examined using a pedoclimatic gradient. In the present study, glyphosate adsorption–desorption and degradation were examined under different scenarios in relationship to soil properties and soil use applications. Three sites with varied pedoclimatic conditions and two crop sequences were selected. Adsorption–desorption and glyphosate distribution in mineralized, extractable, and non-extractable fractions were assessed under laboratory conditions. Glyphosate sorption was characterized by isotherms and glyphosate degradation using the distribution of ^{14}C-glyphosate radioactivity among mineralized fractions, two extractable fractions (in water, ER1; in NH_4OH, ER2), and non-extractable fractions. Results showed sorption indices (distribution coefficient K_d and Freundlich sorption coefficient K_f: 13.4 ± 0.3–64.1 ± 0.9 L/kg and 16.2– 60.6, respectively), and hysteresis increased among soil sites associated with decreasing soil particle size <2 μm, soil organic matter, and other soil properties associated with soil granulometry. A multiple stepwise regression analysis was applied to estimate the relationship between K_d values and soil properties. Cation exchange capacity, water field capacity, and Bray-1 P were the soil properties retained in the equation. Soils under continuous soybean [*Glycine max* (L.) Merr.] (monoculture) treatment exhibited reduced glyphosate adsorption and decreased hysteresis desorption relative to soils under rotation. To our knowledge, these results are the first to demonstrate that soils with identical properties exhibited different glyphosate retention capacities based on crop sequence. We propose possible explanations for this observation. Our results suggested that characterization of the variability in soil property gradients can serve to determine glyphosate behavioral patterns, which can establish a criterion for use in reducing potential environmental risks.

Materials and Methods

Soils

The province of Cordoba, Argentina, is characterized by broken relief to the west and plains in the central and eastern parts. The dominant parent materials are sediments transported by wind, called *loess*, from the mountain range of Los Andes. Three sites were selected: Pampa de Pocho (PP), Manfredi (M), and Marcos Juarez (MJ). At each site, two crop sequences were investigated, a monoculture of soybean with four glyphosate applications of 6 L/ha (2880 g a.i./ha) during the year and a soybean–maize rotation with only one glyphosate application of 2 L/ha (960 g a.i./ha). The soil was sampled at 0-5 cm. All samples were characterized by particle size determined by sedimentation, water-holding capacity (WHC) by membrane pressure plate, and the permanent wilting point (PWP) by ceramic pressure plate. Soil pH in water (soil/water, 1:1), and total organic C content (TOC) by wet combustion, extractable P by Bray 1, cation-exchange capacity (CEC) by NH_4OAc saturation, exchangeable Ca^{2+} and Mg^{2+} by complexometric titration with ethylenediaminetetraacetic acid, and exchangeable Na^+ and K^+ by flame photometer.

Table 1

Main characteristics of the three soils under two cropping sequences, a soybean monoculture and a soybean–maize rotation.

Property	Marcos Juárez		Manfredi		Pampa de Pocho	
Altitude, m asl	110		292		1026	
Annual avg. temperature, °C	17.9		16.8		16.6	
Mean annual precipitation, mm	931		787		523	
Soil type	Typic Argiudoll		Typic Haplustoll		Entic Haplustoll	
Main textural class†	clay loam		loam		sandy loam	
	Monoculture	Rotation	Monoculture	Rotation	Monoculture	Rotation
pH	5.2 ± 0.1	5.5 ± 0.2	6.1 ± 0.1	6.3 ± 0.1	6.7 ± 0.1	6.2 ± 0.1
Water holding capacity, g kg^{-1}	300	280	220	250	80	150
Permanent wilting point, g kg^{-1}	130	130	100	100	40	70
Clay, g kg^{-1}	278 ± 2	242 ± 18	200 ± 4	216 ± 18	146 ± 15	170 ± 8
Silt, g kg^{-1}	580 ± 9	602 ± 15	558 ± 20	520 ± 20	88 ± 11	268 ± 21
Sand, g kg^{-1}	142 ± 7	156 ± 3	242 ± 5	264 ± 5	766 ± 22	562 ± 15
Total organic C, g kg^{-1}	17.2 ± 0.9	17.0 ± 0.1	14.3 ± 0.27	15.8 ± 0.5	7.3 ± 0.9	9.9 ± 0.3
Bray-1 P, mg kg^{-1}	58.0 ± 1.0	57.0 ± 1.0	53.0 ± 1.0	55.0 ± 1.0	12.0 ± 0.3	45.0 ± 1.0
Cation exchange capacity, cmol kg^{-1}	19.6 ± 0.1	18.8 ± 0.2	18.9 ± 0.1	19.0 ± 0.1	15.0 ± 0.1	9.0 ± 0.1
Ca, cmol kg^{-1}	11.8 ± 0.1	10.9 ± 0.1	12.1 ± 0.1	12.1 ± 0.1	9.4 ± 0.1	6.3 ± 0.1
Mg, cmol kg^{-1}	3.2 ± 0.1	3.4 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.0 ± 0.1	1.5 ± 0.1
K, cmol kg^{-1}	2.3 ± 0.1	2.5 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.2 ± 0.1	0.7 ± 0.1
Na, cmol kg^{-1}	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

Soil size fractionation was done by dispersion of soils in water. The fractions 2000 to 200, 200 to 50, and <50 µm were recovered from the dispersed suspension by sieving and dried at 50°C. The soil weight and organic C concentration in each fraction were quantified.

Carbon-14-Glyphosate Retention

A solution of [methyl-^{14}C]glyphosate was purchased from Sigma Chemical Co. (81 MBq/mmol, 99.2% radiopurity) and was prepared in Milli-Q water by isotopic dilution with unlabeled glyphosate (>99% purity) at six different concentrations (0.2, 0.5, 1, 2, 5, and 10 mg/L). Each solution contained 0.166 MBq/L. Two-gram subsamples of air-dried soil were placed in 25-mL Corex glass centrifuge tubes and 10 mL of ^{14}C-glyphosate solution at one of the six different concentrations was added. Blanks without soil were included and each soil and glyphosate concentration combination was prepared in triplicate. The tubes were shaken by rotation for 24 h at 20 ± 2°C in the darkness. After shaking, the tubes were centrifuged for 15 min at 1800 x g and supernatant removed. The ^{14}C-glyphosate concentrations in the supernatant solution were calculated with a Packard Tri-Carb 2100 TR liquid scintillation counter (Packard Instruments) from the supernatant radioactivity measurements. The amount of sorbed glyphosate per mass of soil was calculated from the difference in herbicide concentration before and after sorption. Desorption of ^{14}C-glyphosate was studied in all samples initially treated with 10 mg glyphosate/L during the adsorption study. After sorption equilibration, most of the supernatant was removed and replaced by an equivalent volume of Milli-Q water. The tubes were vortexed to disperse the soil pellets, and the suspensions were mechanically shaken for 24 h at 20 ± 2°C.The suspensions were then centrifuged for 15 min at 1899 x g, and the supernatant was again replaced with Milli-Q water. Five successive desorption treatments were done for each sample. The supernatant radioactivity was determined after each desorption to quantify the amount of desorbed herbicide.

Table 2

Freundlich sorption–desorption isotherm parameters (adsorption $K_{f,ads}$ and n_{ads}, desorption $K_{f,des}$ and n_{des}, and hysteretic index H) and distribution coefficients (K_d) of glyphosate in three soils under two cropping sequences.

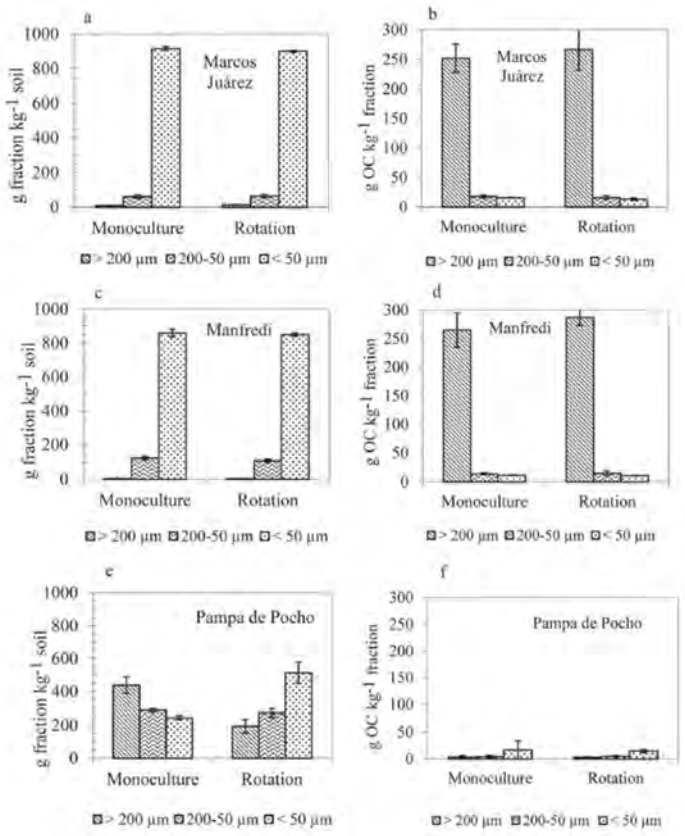
Location	Cropping sequence	Sorption					Desorption			H
		$K_{f,ads}$	n_{ads}	R^2	K_d	R^2	K_{fdes}	n_{des}	R^2	
					L kg^{-1}					
Marcos Juárez	monoculture	48.8 ± 0.3	0.91 ± 0.01	0.99	50.1 ± 0.5	0.98	44.9 ± 0.6	0.17 ± 0.01	0.90	0.19 ± 0.02
	rotation	60.6 ± 0.8	0.90 ± 0.02	0.99	64.1 ± 0.9	0.95	45.2 ± 0.5	0.11 ± 0.01	0.93	0.12 ± 0.03
Manfredi	monoculture	29.3 ± 0.1	0.84 ± 0.01	0.99	28.4 ± 0.5	0.97	36.3 ± 0.3	0.23 ± 0.01	0.95	0.27 ± 0.02
	rotation	42.6 ± 0.4	0.86 ± 0.02	0.99	43.7 ± 0.7	0.88	43.1 ± 0.5	0.16 ± 0.01	0.85	0.19 ± 0.03
Pampa de Pocho	monoculture	16.2 ± 0.2	0.77 ± 0.01	0.99	13.4 ± 0.3	0.96	24.7 ± 0.3	0.30 ± 0.02	0.90	0.39 ± 0.03
	rotation	20.3 ± 0.1	0.85 ± 0.01	0.99	18.7 ± 0.3	0.98	26.5 ± 0.3	0.16 ± 0.01	0.87	0.19 ± 0.02

Carbon-14-Glyphosate Behavior

The mineralization of ^{14}C-glyphosate was followed during laboratory incubations (in triplicate) of 49 d at 28 ± 1°C in the dark. One milliliter of the ^{14}C-glyphosate solution was added to 10 g of each soil. The soil water content was adjusted to 85% of WHC of each soil with Milli-Q water, taking into account the glyphosate solution. The ^{14}C-CO_2 evolved during the incubation was trapped in NaOH. The vials containing NaOH were sampled and replaced after 3, 7, 14, 21, 28, 35, 42, and 49 d. The total radioactivity content was measured by liquid scintillation counting using a Tri-Carb 2100 TR counter and with external standardization and Ultima Gold XR as a scintillation cocktail.

Extractable and Non-extractable Residues

At the 49th d of the incubation period, four sequential extractions were done for the corresponding soil samples. The extractable fraction of ^{14}C-glyphosate was obtained in two steps. The first extraction was done using 50 mL of Milli-Q water during 24 h, the supernatant was recovered, and the radioactivity was measured by scintillation counting (ER1). After that, three successive extractions were performed, each 24, 24, and 4 h, respectively, with 50 mL of 0.5 mol/L NH_4 OH in glass centrifuge tubes. The three successive extracts were pooled for each soil sample and the radioactivity was measured by scintillation counting (ER2). Radioactivity in the solid soil samples containing non-extractable ^{14}C-glyphosate residues (NER) were recovered and dried at 40°C. The radioactivity was measured on three subsamples (100–200 mg) by scintillation counting after combustion at 800°C under O_2 flow in a sampler oxidizer (Packard) followed by ^{14}C-CO_2 trapping in 8 mL of Carbosorb E (Packard) mixed with 12 mL of Permafluor E+ (Packard).



Distribution of soil mass and organic C (OC) content in three soil size fractions (2000–200, 200–50, and <50 µm) in three soils and two cropping sequences.

Figure 1

Mathematical Adjustment and Statistical Analysis

Sorption Isotherms

The amounts of ^{14}C-glyphosate adsorbed on the soil (x/m, mg glyphosate/kg solid) were calculated as the difference between the initial ^{14}C-glyphosate concentration and the supernatant concentration (C, mg glyphosate/L supernatant solution). Glyphosate sorption isotherms were described by the Freundlich model and the linear model.

Kinetics of Degradation
Cumulative ^{14}C-CO_2 glyphosate and C-CO_2 evolved were adjusted to a first-order model:

Statistical Analysis
An ANOVA procedure was performed using the soil type (location) as the main factor, with six replicates per soil. Fisher's test of comparison of means was used. Multiple regression analysis was also performed between glyphosate K_d values and soil properties: sand, clay, silt, pH, TOC, CEC, Ca^{2+}, Mg^{2+}, Na^+, K^+, WHC, PWP, Bray-1 P, and three organic C fractions (>200, 50–200, and <50 µm). The criteria for the selection of the variables were $p < 0.05$. For each crop sequence (monoculture or rotation), a simple ANOVA by each soil with three replicates was used. The statistics software used was Infostat (Di Rienzo et al., 2009).

Table 3
Stepwise linear regression of glyphosate sorption index K_d.

Variable	Coefficient	SE	*P*	R^2	Adjusted R^2
	−48.04	13.22	0.0027		
Water field capacity	130.68	29.6	0.0006		
Bray-1 P	−0.54	0.14	0.0018		
Cation exchange capacity	3.53	1.4	0.0243		
				0.97	0.96

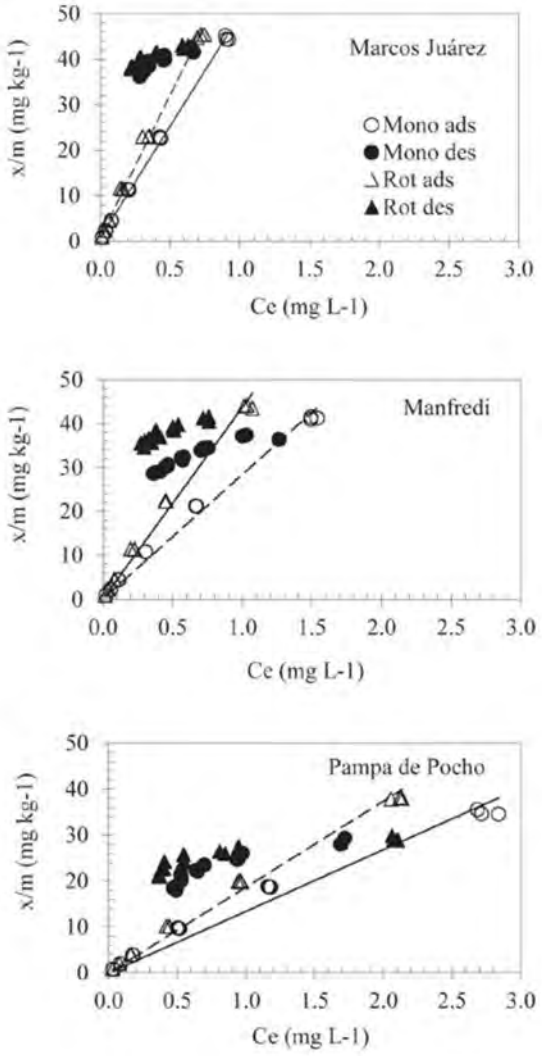
Results and Discussion

Soil Characterization
Edaphoclimatic characteristics from each of the three scenarios studied are shown in Table 1. The PP soils positioned at the northwestern sampling site exhibited a sandy texture, the highest pH and the lowest TOC, CEC, PWP, and WHC. At the extreme southeastern sampling site, the MJ soils showed the lowest pH and the highest TOC, CEC, PWP, WHC, and clay and silt contents. The M soils located in the geographically intermediate sampling site also exhibited intermediate edaphic properties in relationship to the other two sampling sites.

The soil size distribution among fractions ranged from 2000 to 200, 200 to 50, and <50 µm (Fig. 1a, 1c, 1e) and showed granulometric differences among the three sample sites. The M and MJ sites primarily differed in the fraction proportion in micrometers (i.e., 200-50), corresponding to the categories of fine sand and very fine sand. The PP soils differed from the other two sampling sites in the proportion and distribution of the three soil size categories evaluated. Rotation and monoculture treatments revealed identical soil size distributions for M and MJ. However, the two cropping sequence treatments from the PP site were not congruent with M and MJ, and significant differences in soil particle size distribution were observed ($P < 0.05$). Results showed that the coarsest soil size fraction (2000 - 200 µm) containing fresh soil organic matter (SOM) represented the largest organic C concentration in MJ and M soils and both treatments (monoculture and rotation) (Fig. 1b and 1d). Nevertheless, the highest TOC proportion corresponded to humified organic matter associated with a soil size fraction <50 µm, i.e., the highest proportion of this fraction was present in these soils (between 75 and 85%). Carbon enrichment in some of the three soil size fractions, which was associated with soil texture and granulometry, was not found in the PP soils (Fig. 1f).

Carbon-14-Glyphosate Sorption–Desorption
Carbon-14-glyphosate sorption-desorption isotherms obtained from the three scenarios evaluated are shown in Fig. 2. The experimental data were fitted with two mathematical models: the Freundlich adsorption isotherm and the linear model. Empirical evidence indicated that as the value of n_{ads} decreased,

the linear approximation became less satisfactory, especially at high and low concentrations, and discrepancies between K_f and K_d values occurred. Our results showed that the equilibrium concentration range was 0.1 to 1.0 mg/L (MJ soils), 0.1 to 1.5 mg/L (M soils), and 0.1 to 3 mg/L (PP soils), and $K_{f,ads}/K_d$ ranged between 0.9 and 1.1.

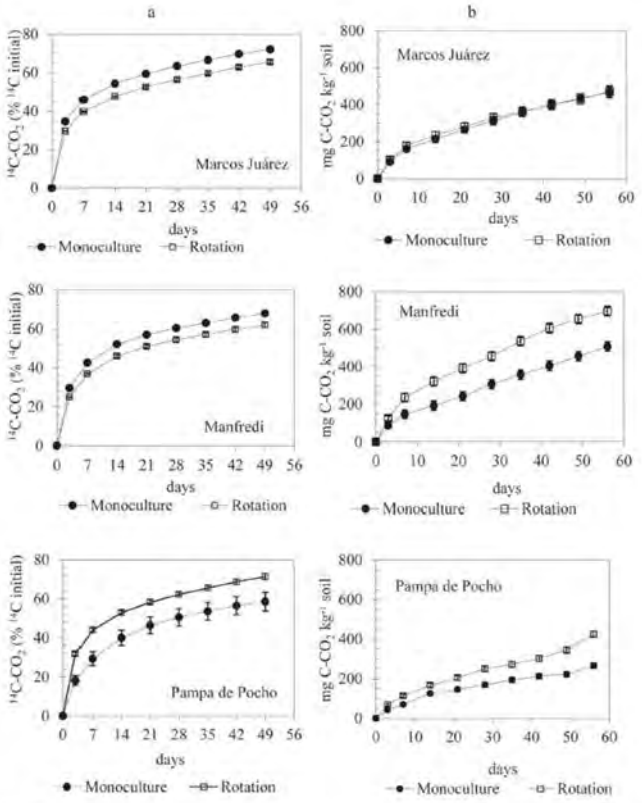


Glyphosate adsorption (open symbols) and desorption (filled symbols) isotherms in three soils and two cropping sequences: monoculture (circles) and rotation (triangles).

Figure 2

The three scenarios examined clearly differed in glyphosate adsorption. The $K_{f,ads}$ and K_d indices showed a threefold increase from PP soils (northwest position) to MJ soils (southeast position). Our results from the three sampling sites showed a geographic gradation in soil characteristics that were associated with glyphosate adsorption, i.e., we detected a relation among geographic position, edaphic characteristics, and glyphosate adsorption capacity. That behavioral pattern was confirmed by adsorption studies. The PP site collective soil characteristics were associated with low glyphosate adsorption capacity, i.e., high pH, low clay content and SOM, while the MJ site exhibited inverse soil attributes and an overall high adsorption capacity. A multiple linear regression analysis was performed to estimate the relationship between glyphosate K_d values and soil properties (Table 3). The PWP, CEC, and Bray-1 P were the regressed variables retained in the analysis, which explained 93% (R^2 = 0.97) of the total variation. Desorption isotherms were fitted for the Freundlich model. An irreversible desorption process was shown by the lack of overlap in the sorption - desorption isotherms (Fig. 2). Glyphosate desorption indices were higher than corresponding adsorption indices. Our study revealed that glyphosate

desorption hysteresis increased from the northwest toward the southeast sampling sites. The *H* indices, which ranged from 0.12 to 0.39, were used to compare the hysteresis degree among soils. Between 15 and 57% of the glyphosate initially applied was desorbed. The PP soils exhibited the highest ^{14}C-glyphosate desorption, with >50% recovered in the first desorption step. On the contrary, for the M and MJ soils in the first desorption step, only about 30% of the total ^{14}C-glyphosate was desorbed. Crop sequence effects were evaluated only for the M and MJ soils. These two sample sites had similar soil characteristics, while the PP site differed and was therefore excluded. The extent of glyphosate adsorption and desorption hysteresis was higher in rotation than monoculture soils (Fig. 3; Table 2). To our knowledge, our results are the first to document that soils with identical properties exhibited different glyphosate retention capacities due to cropping sequence.



Kinetics of (a) ^{14}C-glyphosate mineralization and (b) total organic C mineralization (as an indicator of total microbial activity) during laboratory incubations of the three soils and the two cropping sequences monoculture and rotation. The standard deviations (error bars) are shown when larger than the symbol size.

Figure 3

Carbon-14-Glyphosate Mineralization

Carbon-14-glyphosate mineralization kinetics together with C-CO_2 evolution are shown in Fig. 3. In addition, cumulative glyphosate mineralization and oxidizable C after 49 d of incubation are shown in Table 4. At the end of the incubation period, the ^{14}C-CO_2 released ranged from 61.7 to 72.8% of the ^{14}C applied, and the time needed to reduce the ^{14}C initially applied to 50% was 5.0 ± 0.7 d (calculated from ln2/*k*).

Table 4

Carbon-14-glyphosate mineralized, $C\text{-}CO_2$ expressed as a percentage of the total organic C, and setting parameters for three soils and two cropping sequences using the equation $C_t = C_0[1 - \exp(-kt)]$, where C_t is the percentage of $^{14}C\text{-}CO_2$ or $C\text{-}CO_2$ mineralized at time t, C_0 is the percentage of C potentially mineralizable, k is the daily mineralization rate, and t is time in days.

Soils	Cropping sequence	Mineralization of glyphosate			Mineralization of soil organic C		
		$^{14}C\text{-}CO_2$	C_0	k	$C\text{-}CO_2$	C_0	k
		%		d^{-1}	%		d^{-1}
Marcos Juarez	monoculture	69.1 ± 0.3	62.8 ± 1.3	0.178 ± 0.018	2.8 ± 0.03	3.0 ± 0.11	0.044 ± 0.003
	rotation	63.8 ± 0.2	57.7 ± 1.2	0.170 ± 0.017	2.4 ± 0.02	2.5 ± 0.09	0.053 ± 0.005
Manfredi	monoculture	68.3 ± 0.3	63.1 ± 1.0	0.163 ± 0.012	3.3 ± 0.03	4.1 ± 0.31	0.031 ± 0.004
	rotation	61.6 ± 0.1	57.4 ± 1.0	0.141 ± 0.011	4.3 ± 0.02	4.7 ± 0.21	0.044 ± 0.004
Pampa de Pocho	monoculture	65.3 ± 0.5	60.9 ± 1.2	0.117 ± 0.009	3.2 ± 0.03	3.0 ± 0.11	0.063 ± 0.006
	rotation	74.3 ± 2.8	66.9 ± 1.5	0.159 ± 0.017	3.2 ± 0.03	3.3 ± 0.11	0.054 ± 0.005

Decreased ^{14}C-glyphosate mineralization detected in the MJ and M sites relative to the PP site might be associated with increased glyphosate adsorption in the MJ and M soils. We found that the TMA (total microbial activity) was significantly different ($P < 0.01$) among sampling site soils: M > PP > MJ. The M soils, with the highest TMA, did not have the highest ^{14}C-glyphosate mineralization. Glyphosate mineralization was affected by cropping sequence. At the end of incubation, the $^{14}C\text{-}CO_2$ evolved was monoculture MJ = 69% vs. rotation MJ = 63.6% and monoculture Mm = 68% vs. rotation M = 61.7% ($P < 0.05$). These results provide additional support to our interpretations regarding glyphosate mineralization differences detected among study sites, given that monoculture soils showed reduced glyphosate adsorption and a history of glyphosate use.

Carbon-14-Glyphosate Distribution among Mineralized, Extractable, and Nonextractable Residues

The three study sites differed in the distribution of initial radioactivity applied and in the proportion remaining in ER and NER forms. The lower proportion of ER2 and NER in the PP soils corresponded with soil properties involving low sorbent surfaces. Sequential extraction of ER and NER was conducted following 49 d of incubation; consequently equilibrium between soluble and sorbed forms of glyphosate should have occurred. The PP soil contained 19% ER (ER1 + ER2), which clearly contrasted with 30% ER obtained from the M and MJ soils. The ER1 fraction, extracted with water, represented the weakly adsorbed herbicide and on average was <5% of the total ER for the three sample sites. The ER1 from the PP soils was slightly higher than that from the other two sample sites (MJ and M), indicating weak glyphosate adsorption properties and high sorption reversibility. Nonextractable residues constituted a small fraction (4-6%) of the ^{14}C-glyphosate initially applied. Small differences among soils were observed, such as decreasing order of NER proportions: M > MJ > PP ($P < 0.05$). The M soils showed the highest TMA and NER proportion.

Conclusions

The study of glyphosate retention and degradation processes through a pedoclimatic gradient turned out to be a useful tool to recognize and establish some behavioral patterns. Identification of soil indicators that allow inference of glyphosate behavior is one of the goals in studies of sustainable soil use. We found that along a distance of approximately 280 km, gradual changes in glyphosate behavior were associated with pedoclimatic characteristics. Soil properties associated with soil surface reactivity, such as CEC, WHC, and PWP, increased in from northwest to southeast together with the increase in glyphosate adsorption and the increase in hysteresis of desorption. Changes in the glyphosate distribution between adsorbed and soluble forms establish, in part, a behavior pattern of extractable (ER) and mineralized forms. The extent of glyphosate adsorption and also the hysteresis of desorption were higher in rotation soils than monoculture soils; that is, soils with identical properties exhibited a different glyphosate retention capacity due to the cropping sequence. The results of this study contribute to our understanding of glyphosate behavioral patterns in relation to different edaphoclimatic scenarios and establish criteria for use in reducing potential environmental risks.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes the sorption and degradation behavior of ^{14}C-labelled glyphosate in different agricultural soils from Argentina. Sorption parameters and mineralization of the substance are reported. However, essential parameters are not described, or there are some deviations from current guidelines. In addition, the pedo-climatic conditions do not correspond to EU conditions.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCP 9.2.4
Report author	Rasmussen, S., et al.
Report year	2015
Report title	Effects of Single Rainfall Events on Leaching of Glyphosate and Bentazone on Two Different Soil Types, using the DAISY Model
Document No	Vadose Zone Journal; Advancing Critical Zone Science; Published November 13, 2015
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Reliable with restrictions (Modelling exercise)

2. Full summary of the study according to OECD format

The purpose of the present modeling study was to contribute to an improved understanding of the mechanisms involved in pesticide leaching during a single rainfall event with temporal variability. Rainfall intensity of the first event after pesticide application has great effect on the amount of pesticide transported to groundwater and subsurface drains, especially in soils containing preferential flow pathways. One way to improve the understanding of single event properties on pesticide leaching is to use a transport model. The soil–plant–atmosphere model Daisy was used to simulate pesticide leaching during and after single rainfall events of different durations and intensities. Designed temporally variable single rainfall events based on the Chicago Design Rain were inserted in the original weather file. A combination of different intensities (13, 20, 24, 28, 34, and 39 mm/h) at different event durations (1, 3, 5, and 9 h) where the intensity peak was placed in the middle of the event ware applied, resulting in 24 different design events. The model setup included two different soil types: a coarse sandy soil and a sandy loam containing macropores and subsurface drains. The fates of the herbicides bentazone [3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide] and glyphosate [*N*-(phosphonomethyl) glycine] were simulated. The leaching dynamics of both pesticides showed high variability at the hourly level, illustrating the importance of high model resolution when estimating pesticide leaching. For the coarse sandy soil different intensities did not appear to have an effect, as pesticide leaching was controlled by event volume. In contrast, results for the sandy loam showed an effect of intensity, especially for glyphosate, at initially wet soil conditions. Short intense events (1 h) resulted in high leaching to drains (1.7% of matrix infiltration) compared to events of longer duration (up to 0.4% of matrix infiltration). This indicates that it might be more prudent to view leaching as a risk that occurs under certain conditions, rather than something that can be averaged.

Materials and Methods

Soils

Two different agricultural soils from Denmark were chosen as model soils—a coarse sandy soil and a sandy loam. The DAISY (deterministic and dynamical two-dimensional soil–plant–atmosphere model developed for simulating agrohydrological systems) parameterization of this location (Jutland, Denmark) originates from Jacobsen (1989), and selected soil properties are illustrated in Table 1. Because the coarse sandy soil is considered completely homogeneous in the Ap and C horizons and contains no biopores or subsurface drains, it is modeled in 1D. The sandy loam is a heterogeneous soil developed in a glacial till in the eastern part of Zealand, Denmark. It shows signs of long-term agricultural use with the development of a plow pan that has a lower hydraulic conductivity than the surrounding soil layers (Petersen et al., 2001).

Table 1. Selected soil characteristics of the two locations, coarse sand and sandy loam, used in the DAISY simulations. Data originate from Jacobsen (1989) and Hansen et al. (2012b).

Soil type	Horizon	Soil depth	Clay	Silt	Sand	Humus	K_{sat}
		cm	%				cm h^{-1}
Coarse sand	Ap	0–30	3.8	7.2	86.7	2.3	21.7
	C	30–200	2.8	2.3	94.5	0.4	92.5
Sandy loam	Ap	0–25	10.4	21.6	65.1	2.9	0.174
	Bplow	25–33	14.6	21.1	62.8	1.6	0.046
	Bt	33–120	21.9	19.2	57.4	1.6	0.269
	C	120–200	20.5	23.3	55.2	1.0	1.500

The sandy loam contains biopores and subsurface tile drains. The drains are placed in a depth of 1.1 m, and 16 m apart, and the biopores are divided into classes according to the depth at which they begin and end in the soil profile. Selected soil properties for this location, which originates from Hansen et al. (2012b) are illustrated in Table 1.

Pesticide Management

Bentazone was applied on 17 June with a rate of 960 g/ha, on grass for cutting. Hence, bentazone is located in the crop canopy and will there be mixed with the first rainfall and washed off the plants when the interception capacity is exceeded. Glyphosate was applied on 30 October at a rate of 1440 g/ha on bare soil (stub after harvesting of maize, *Zea mays* L.) (Table 2). Glyphosate will therefore be located at the soil surface, from where it will enter the soil system together with the first rainfall. The fate of both pesticides was simulated for 4 yr, and their leaching at 2 m depth was logged. For the sandy loam, transport of pesticides into drains was included in the leaching assessment.

Table 2. Pesticide management plan used in the DAISY simulations.

Rotation year	Crop	Pesticide	Application date	Dosage
Warm-up	Spring barley			
Warm-up	Spring barley			
Warm-up	Spring barley			
Warm-up	Spring barley w. grass			
1	Grass	Bentazone	17 June	960 g ha^{-1}
2	Grass			
3	Grass			
4	Maize	Glyphosate	30 October	1440 g ha^{-1}
5	Spring barley			
6	Spring barley			
7	Spring barley			
8	Spring barley			

Weather Data

The weather file used in the DAISY simulations was provided by University of Copenhagen and originated from Taastrup, Denmark. It contains hourly values of precipitation, temperature, relative humidity, wind speed, and global radiation and covers the period 1999 to 2008 (8 yr). The weather data covered the 8 yr of pesticide tracking in the model simulations (Table 2). To initiate the soil water content in the model, 4 yr of weather data (1999–2003) and a simple spring barley (*Hordeum vulgare* L.) crop rotation were used as a warm-up period before the first pesticide application. The weather record was then reset as the 8 yr of pesticide simulations began (Table 2). The weather of the warm-up period was kept constant, while the weather of the 8 yr of pesticide simulations were permutated.

Artificial Rainfall Events

A single artificial rainfall event was inserted in the weather file 4 d after application of the pesticide. Intermittent rain that occurred between pesticide application and the artificial rainfall event were removed. The artificial event originates from the CDS rain, developed in the 1950s, for the use in city sewage planning. The durations of the events studied were 1, 3, 5, and 9 h combined with six different levels of maximum intensities of 13, 20, 24, 28, 34, and 39 mm/h (Table 3). When the duration increases, the maximum intensity of the event will be displaced to the middle of the event, and small pre- and post-tails of rain will be added to the event compared to a 1-h event (Fig. 1). Hence, the event volume is connected to the event duration, as increased duration results in increased volume.

Table 3. Characteristics of the inserted Chicago Design Storm rain (CDS rain. All combinations of maximum intensities and durations were investigated at eight different initial conditions, and with different post-event weather.

Max. intensity	Duration	Volume	Repeat interval
mm h^{-1}	h	mm	yr
13	1	13	1
13	3	18	1
13	5	21	1
13	9	25	1
20	1	20	5
20	3	28	5
20	5	33	5
20	9	39	5
24	1	24	10
24	3	33	10
24	5	39	10
24	9	46	10
28	1	28	20
28	3	39	20
28	5	45	20
28	9	53	20
34	1	34	50
34	3	48	50
34	5	55	50
34	9	65	50
39	1	39	100
39	3	55	100
39	5	64	100

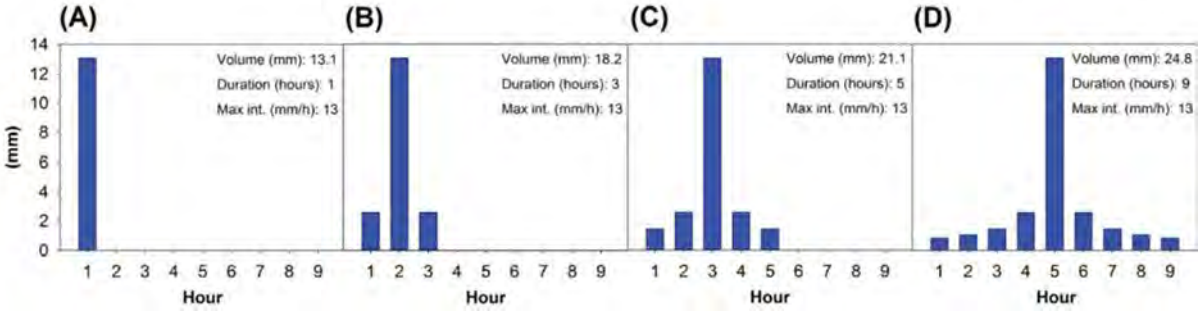


Fig. 1. Examples of Chicago Design Storm rain (CDS-rain) (Kiefer and Chu, 1957), modified by Madsen et al. (2002) and Arnbjerg-Nielsen et al. (2006), inserted in the weather files used in the DAISY simulations. (A) to (D) illustrate the range of event durations investigated and the differences in rain distribution pattern and consequently the differences in total volumes at different durations, with the same maximum intensity.

Results and Discussion

The complete set of total leaching (leaching after 4 yr as percentage of soil input) from the 192 DAISY simulations is illustrated in Fig. 2. A quick overview of the dataset is supplied, as the leaching percentages are shown in a sorted sequence from lowest to highest leaching. It is seen that there is an

effect of soil type on leaching of both pesticides and that bentazone leaching is substantially larger than glyphosate leaching. In the following responsible processes and mechanisms will be described, and where feasible, related to field or laboratory findings. For each of the two soil types, a description of pesticide leaching during the 4-yr simulations (leaching dynamics) will be followed by a clarification of the effects of CDS event structure (event characteristics) and completed with a description of the importance of initial soil water conditions and post-event weather (rotated weather).

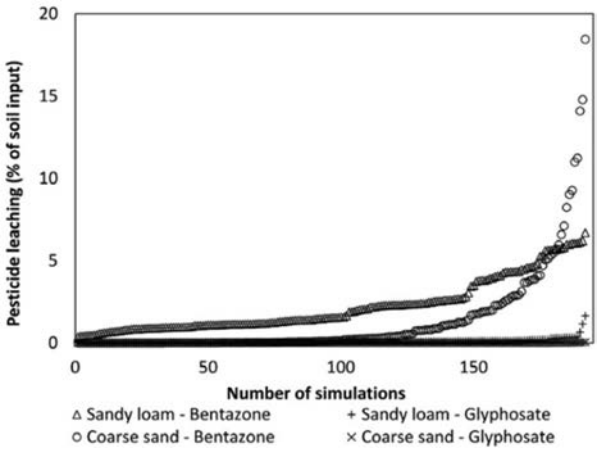


Fig. 2. Overview of all pesticide fate simulations made by the DAISY model. Total bentazone and glyphosate leaching as percentage of soil input (matrix and biopore infiltration), at the coarse sandy and sandy loam soils. The 192 simulations for each pesticide, at each soil type, are sorted from smallest to largest.

Pesticide Leaching in the Coarse Sand—Leaching Dynamics

The pesticides entered the soil with the first rainfall after application and were thereafter transported vertically through the soil profile with the water. Fig. 3 illustrates bentazone and glyphosate leaching (daily values) during 4 yr after pesticide application. The amount of pesticide leaching is calculated at the soil depth of 2 m and is given as the percentage of input to the soil. The average ± SD of all 24 investigated CDS events are shown together with accumulated net precipitation (precipitation minus evapotranspiration) (Fig. 3). The inserted figure illustrates the negative values of accumulated net precipitation during the first 10 d and the importance of glyphosate leaching the first 5 to 10 d. A seasonality in rainfall is seen, as increased accumulated net-precipitation occurs during autumn and winter.

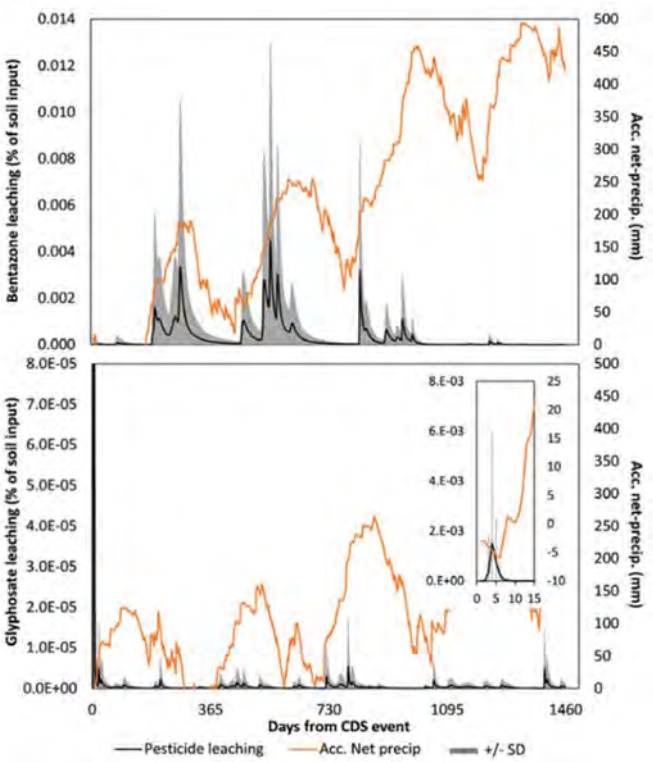


Fig. 3. Four-year pesticide leaching as percentage of soil input (matrix and biopore infiltration) in the coarse sandy soil (daily values). Pesticide leaching is given as an average ± SD of the 24 different events investigated within Weather Rotation 8. The second *y* axis shows the accumulated net precipitation (precipitation minus evapotranspiration), with negative values only shown in the inserted figure. The inserted figure also illustrates the full magnitude of glyphosate leaching.

It can be seen that bentazone leaching took place over a 3-yr period, with the highest leaching occurring during winter. Within the first 10 d after the CDS event only 2.7 x 10^{-5}% of total leached bentazone had reached the 2-m depth, which indicate a limited direct effect of the 24 CDS events. Bentazone was applied 17 June (Table 2), where evapotranspiration most often exceeds the rainfall volume, resulting in dry soil water conditions. The CDS event transports bentazone into the soil profile, but not below the depth of 1 m, where biodegradation is zero in the model. Hence, under these conditions the first rainfall after bentazone application appears of minor importance at the two layered homogeneous coarse sandy soil. In contrast, the direct response of the CDS event on glyphosate leaching (Fig. 3, illustrating variation of the 24 events and effect of WR 8), was found to be substantially larger. Within 10 d after the CDS event 86% of total leached glyphosate had been transported to the 2-m depth. However, total glyphosate leaching (3.5 x 10^{-3}% of soil input) is two magnitudes smaller than total bentazone leaching (4.5 x 10^{-1}% of soil input), which is a result of the higher sorption properties of glyphosate. Glyphosate was applied 30 October, which is a time of year where the net rainfall is substantially larger than at the time of bentazone application (17 June), resulting in wetter soil water conditions, especially at the top 0.5 m. This means that smaller amounts of rainfall is needed to facilitate the transport of glyphosate, and a direct and visible effect of the CDS events is possible (Fig. 3).

Pesticide Leaching in the Coarse Sand—Effect of Single Event Characteristics

Even though the immediate effect of the CDS events were small (Fig. 3), total bentazone leaching at the coarse sandy soil, showed a systematic response to the 24 investigated CDS events. Bentazone leaching increased with increased duration (1, 3, 5, and 9 h) and maximum intensity (13, 20, 24, 28, 34, and 39 mm/h) of the CDS event. This is illustrated in Fig. 4, where the average ± SD of total bentazone leaching of all 8 WRs are shown, but divided into event durations and maximum intensities. This was to be expected, as a higher rainfall volume has a higher potential of transporting pesticides. With increased CDS event volume, the pesticide was transported faster through the degradation zone (upper 1 m), which

left less time for degradation. These results also indicate that higher intensities will result in increased leaching percentages. Glyphosate leaching at the coarse sandy soil showed the same pattern as the one found for bentazone, although only small amounts of glyphosate were leached (<0.025% of soil input).

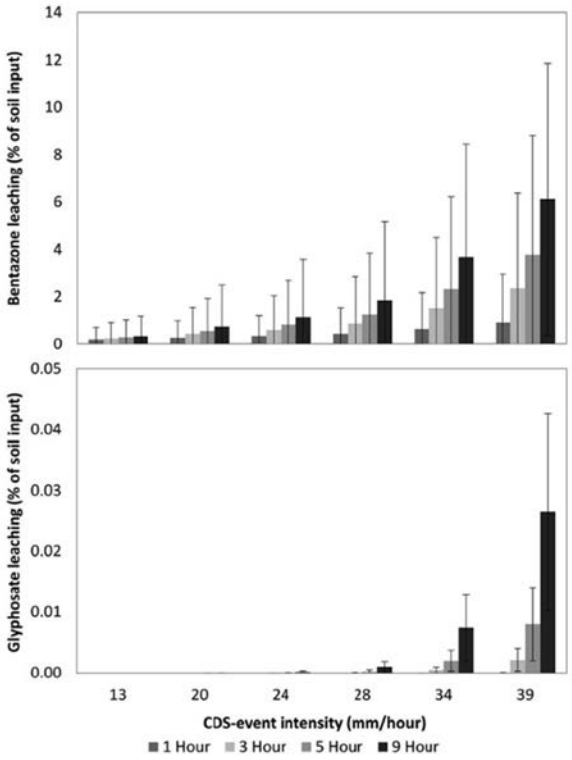


Fig. 4. Average ± SD of 4 yr accumulated pesticide leaching, based on results from all eight weather rotations, shown as percentage of soil input (matrix and biopore infiltration), in the coarse sandy soil, and as a function of Chicago Design Storm (CDS) event intensity and duration.

Pesticide Leaching in the Coarse Sand—Effect of Rotated Weather

The rotated weather produced different soil water conditions at the time of pesticide application, and different post-CDS event weather, which all may influence the pesticide fate. Fig. 5 illustrates these effects on pesticide leaching, where daily values of leaching are given as a function of CDS event volume, for all 8 WRs. These results indicate that the leaching pattern can be explained by the volume of the CDS event, as the accumulated leaching of especially bentazone produced smooth curves at the coarse sandy soil and showed almost no effect of increased intensity. In the case where intensity had an effect, the curves would be irregular, showing a spread of leaching results. This indicates that the effect of intensity shown in Fig. 4 is solely explained by the increase in volume, as intensity increases. The leached amounts of glyphosate were smaller than those of bentazone and showed a steeper curve. This is due to the high number of low leaching percentages even at relatively high CDS event volumes (up to 50 mm). When event volume exceeds 50 mm, a steep increase in leaching percentages was observed (Fig. 5). The difference between the leaching caused by the different WRs in Fig. 5 is either due to the initial soil water conditions (water conditions at the time of pesticide spraying), the post-event rainfall, or a combination thereof. Bentazone leaching at the coarse sandy soil appeared to be mostly affected by CDS event volume. In the case of glyphosate leaching at the coarse sandy soil, no connection to initial soil water conditions or P_{10} was found, except at WR 3 which had the highest P_{10} and the highest leaching.

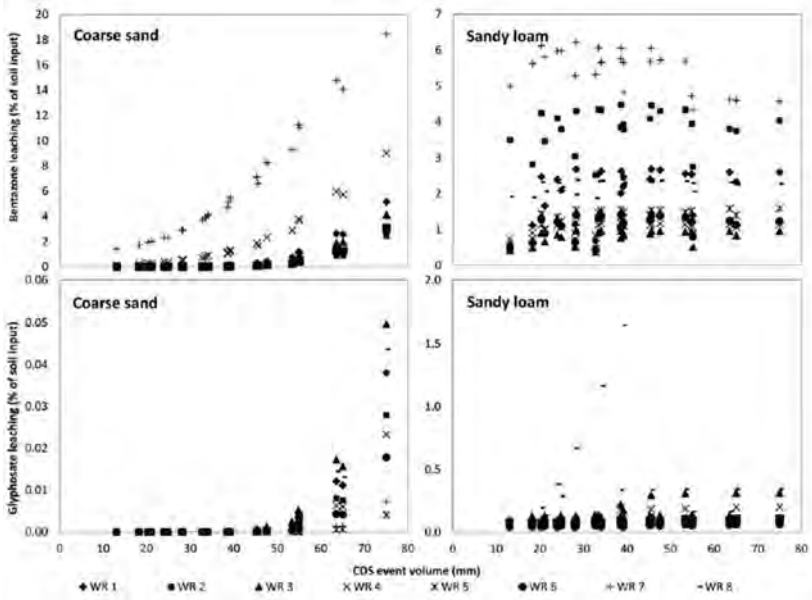


Fig. 5. Four-year cumulative pesticide leaching shown as percentage of soil input (matrix and biopore infiltration), and as a function of Chicago Design Storm (CDS) event volume. Each of the eight weather rotations (WRs) represents a new set of initial soil water conditions and post-CDS event weather conditions. Please note the different scales on the *y* axis.

Pesticide Leaching in the Sandy Loam—Leaching Dynamics

Fig. 6 constitutes an example of how daily values of pesticide leaching (to drain plus leaching at 2 m depth) evolved over the 4 yr period of which the pesticides were tracked. Average ± SD of the 24 CDS events from WR 8 is shown. Compared to similar graphs for the coarse sandy soil (Fig. 2), it can be seen that the largest proportion of both pesticides were leached during or shortly after the CDS event, as was the case for glyphosate at the coarse sandy soil. On average, 87% of total bentazone leaching and 75% of total glyphosate leaching occurred within 24 h from the beginning of the CDS event. Hence, the first single rainfall event after pesticide leaching is of considerable importance on the sandy loam, compared to the coarse sandy soil. Even though both pesticides were affected by the CDS event (Fig. 6), it was found that different processes control the leaching of the two pesticides. It was found that a substantial part of drain leached bentazone was transported via biopores leading directly from the surface to the tile drain. A total of 51% of drain leached bentazone (average of the 24 CDS event in WR 8) completely bypassed the soil matrix where sorption would have occurred. The second way of entering biopores via the soil matrix is how glyphosate predominantly migrated. Only 23% of drain leached glyphosate, was transported directly from the soil surface into the biopores, completely bypassing the soil matrix. Further investigation into the transport mechanisms revealed that the agricultural practice was important, in particular the presence of a plant cover in the model setup. Bentazone was applied on grass for cutting, and thus had to be washed off the crop canopy before getting into contact with the soil surface. The CDS event is the first rainfall after pesticide application and is heavy enough to wash bentazone off the canopy and *at the same time* activate the preferential flow pathways where some led directly to the drain. Glyphosate was applied directly to the soil surface and the main fraction entered the soil matrix together with the very first water that hit the surface. When the pesticide had entered the soil matrix it either sorbed to soil particles or followed the water route in dissolved form through the soil matrix into the biopores and from here to the drain pipe. Despite high sorption of glyphosate a small fraction was present as dissolved glyphosate, which was subject to leaching through transport route B. This explains the dependence of amount of water that comes after the maximum intensity in the CDS event: the hour of maximum intensity does not result in relatively higher transport of glyphosate, since the majority of this water is led directly into macropores and thereby bypassing the soil matrix where glyphosate is located.

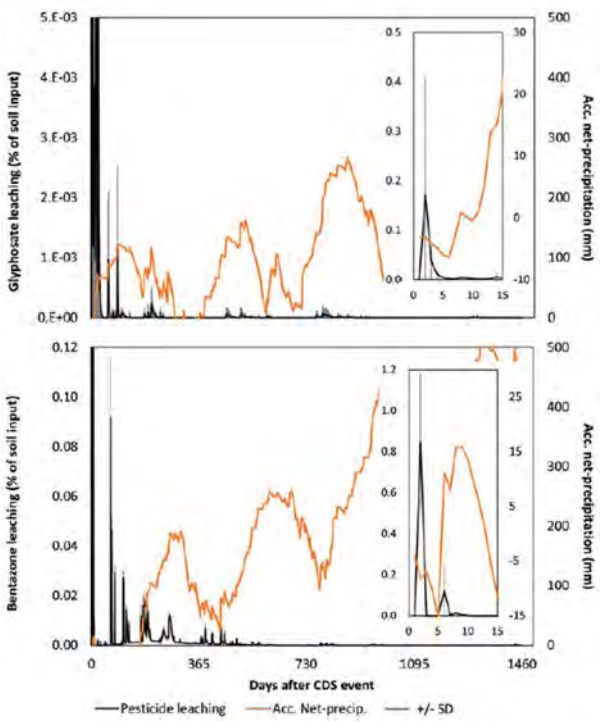


Fig. 6. Four-year pesticide leaching shown as percentage of soil input (matrix and biopore infiltration) in the sandy loam soil (daily values). Pesticide leaching is given as an average ± SD of the 24 different events investigated within Weather Rotation 8. The second *y* axis shows the accumulated net precipitation (precipitation minus evapotranspiration), with negative values only shown in the inserted figures.

Pesticide Leaching in the Sandy Loam—Effect of Single Event Characteristics

Bentazone leaching was found to increase as CDS event volume increased. The CDS events of 3, 5, and 9 h caused leaching that tended to level out as CDS event volumes increased. This trend represents the prevailing leaching patterns, but some WRs showed limited effect of CDS event volume. The differences in leaching dynamics between bentazone and glyphosate in the sandy loam (Fig. 7) were also observed at the 4-yr cumulated leaching (Fig. 8). The applied glyphosate was protected against soil infiltration by small rainfall events when located in a litter layer, and a substantial amount of glyphosate was transported via the biopores to the drains during a heavy event that generated preferential flow. Long CDS event durations resulted in high leaching percentages of glyphosate.

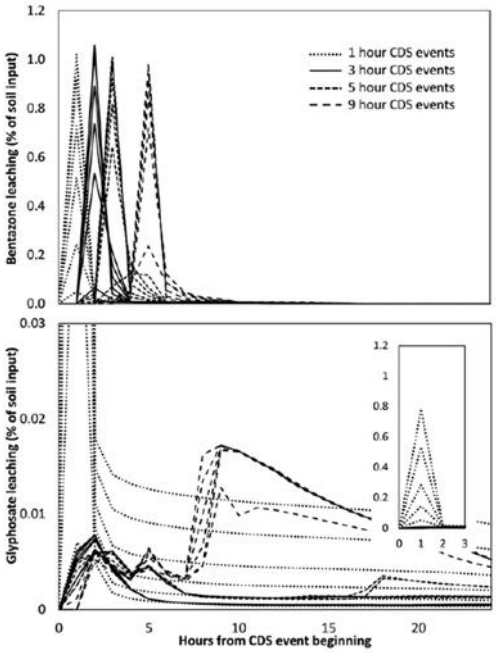


Fig. 7. Four-year pesticide leaching shown as percentage of soil input (matrix and biopore infiltration) in the sandy loam soil (hourly values). Pesticide leaching is shown for each of the 24 different events investigated within Weather Rotation 8, but divided into groups according to the respective Chicago Design Storm (CDS) event duration.

Pesticide Leaching in the Sandy Loam—Effect of Rotated Weather

The smooth curves produced at the coarse sandy soil (Fig. 5) were not reproduced, indicating that CDS event volume alone did not explain the leaching in the sandy loam. The increased bentazone leaching caused by increased CDS event volume (Fig. 8) is not repeated by all WRs, and the effect of CDS event characteristics appear of less importance compared to the effect of initial soil water conditions and post-event weather. Glyphosate leaching appeared to be affected by CDS event intensity to a higher degree than bentazone.

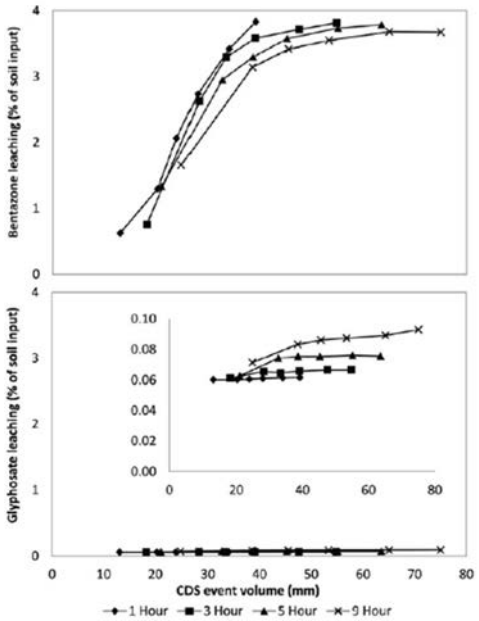


Fig. 8. Four-year accumulated pesticide leaching shown as the percentage of soil input (matrix and biopore infiltration) in the sandy loam and as a function of the Chicago Design Storm (CDS) event volume, Weather Rotation 1.

Simulated Pesticide Concentrations in the Sandy Loam

The simulated concentrations of bentazone and glyphosate in drainage water at WR 8 are shown in Fig. 9. Both pesticide concentrations are seen to peak shortly after the CDS event beginning. Previous studies strongly indicate that glyphosate leaching is highly event driven, where especially rainfall intensity affects the leaching dynamics. High intensity rainfall events occurred as the first rainfall after application at initially wet soil conditions, which supports the findings of this work.

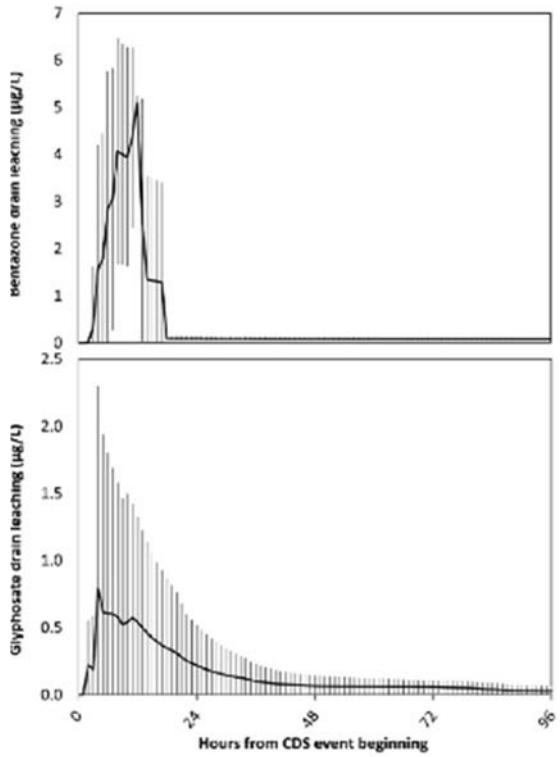


Fig. 9. Pesticide leaching (µg/L), during the first 4 d after the Chicago Design Storm (CDS) event in the sandy loam soil. Pesticide leaching is given as an average ± SD of the 24 different events investigated within Weather Rotation 8. Negative SD values are not shown.

Conclusions

Based on the 192 model simulations, testing the effect on pesticide leaching of duration and intensity of the first rainfall event after pesticide application at an hourly temporal resolution, the following trends were observed: The importance of the first single rainfall event after pesticide application depended highly on soil types. On average, 87% of total bentazone leaching and 75% of total glyphosate leaching occurred within 24 h from the CDS event beginning in the sandy loam. Hence, the first single rainfall event after pesticide leaching is of considerable importance in the sandy loam. Preferential flow transport in the biopores was responsible for this immediate transport of both pesticides. In the coarse sandy soil, the first rainfall event was of minor importance, and the effect was only visible if the soil was relatively wet at the time of application. The influence of rainfall characteristics on pesticide leaching depends on the hydrological conditions of the investigated soil types. In the coarse-textured soil, where non-threshold matrix flow dominates, solute leaching was found to increase with increased rainfall volume, whereas in the sandy loam, varying rainfall intensity also affected pesticide leaching, especially for the strongly sorbing pesticide glyphosate. For strongly sorbing pesticides like glyphosate it might be more prudent to view leaching as a risk that occurs under certain conditions, rather than something that can be averaged. Under most initial conditions glyphosate leaching did not vary much (up to 0.4% of soil input), but at specific initial wet surface conditions glyphosate leaching greatly increased (1.7% of soil input). It may therefore be equally important to have knowledge of the weather preceding the pesticide application, as knowledge of the weather following the pesticide application.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes a modelling experiment for the leaching of glyphosate through two different soil types.
The effect of single rainfall events is analyzed.
The study is classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Sidoli, P., et al.
Report year	2016
Report title	Glyphosate and AMPA adsorption in soils: laboratory experiments and pedotransfer rules
Document No	Environmental Science and Pollution Research (2016) 23:5733–5742
Guidelines followed in study	OECD 106 (2000)
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Not all parameters reported to check the validity criteria for the study)

2. Full summary of the study according to OECD format

Adsorption of the herbicide glyphosate and its main metabolite AMPA (aminomethylphosphonic acid) was investigated on 17 different agricultural soils. Batch equilibration adsorption data are shown by Freundlich adsorption isotherms. Glyphosate adsorption is clearly affected by equilibration concentrations, but the nonlinear AMPA adsorption isotherms indicate saturation of the adsorption sites with increasing equilibrium concentrations. pH_{CaCl2} (i.e. experimental pH) is the major parameter governing glyphosate and AMPA adsorption in soils. However, considering pH_{CaCl2} values, available phosphate amount, and amorphous iron and aluminium oxide contents by using a nonlinear multiple regression equation, obtains the most accurate and powerful pedotransfer rule for predicting the adsorption constants for these two molecules. As amorphous iron and aluminium oxide contents in soil are not systematically determined, we also propose a pedotransfer rule with two variables—pH_{CaCl2} values and available phosphate amount—that remains acceptable for both molecules. Moreover, the use of the commonly measured pH_{water} or pH_{KCl} values gives less accurate results compared to pH_{CaCl2} measurements. To our knowledge, this study is the first AMPA adsorption characterization for a significant number of temperate climate soils.

Materials and Methods

Soil properties

Seventeen surface top soils were sampled in different agricultural plots with variable land uses and fertilization practices under intensive agriculture. The sample site is located in a quaternary fluvio-glacial corridor near Lyon in southeastern France. They are loamy to sandy-loamy soils, characterized by a decarbonation state on surface and large amounts of amorphous iron and aluminium oxides issued from the weathering of primary minerals (Table 1). Fresh soil samples were air-dried, sieved to 2 mm, and stored in the dark at 4 °C, before measuring their physicochemical properties. Crystallized oxy-hydroxides (Fe_{DCB} and Al_{DCB}) were extracted by the Mehra-Jackson method (1960), and amorphous oxy-hydroxides (Fe_{ox} and Al_{ox}) by the Tamm method (1992) (Table 1). The experimental 1:5 soil pH_{CaCl2}, hereafter referred to as 'pH_{CaCl2}', was measured in batch supernatants with a pH microelectrode (Inlab Flex-Micro). These soils showed wide ranges of pH_{CaCl2} (5.1 to 7) and clay content (8.9 to 15.3 %) and, except soil 11, contained less than 2 % organic carbon (Table 1).

Chemical reagents and analysis

Glyphosate adsorption was studied with its ^{14}C-radiolabeled form (phosphonomethyl-^{14}C)-glyphosate (4.36 MBq/mg, radiochemical purity 96.32 %) purchased from Izotop (Hungary). Unlabeled solid glyphosate and AMPA products (purity ≥98 %) were purchased from Dr Ehrenstorfer (CIL Cluzeau, Sainte-Foy la Grande, France). Stock solutions were prepared in MilliQ water (storage at 4 °C for 1 month).

Table 1

Physicochemical properties of studied soils. Crystallized oxy-hydroxides (Fe_{DCB} and Al_{DCB}) and amorphous oxy-hydroxides (Fe_{ox} and Al_{ox}) were extracted by the Mehra-Jackson method (1960) and the Tamm method, respectively

Soil	pH_{CaCl2}	pH_{water}	pH_{KCl}	Organic C (g kg^{-1})	Olsen P (g kg^{-1})	Al_{DCB} (g kg^{-1})	Fe_{DCB} (g kg^{-1})	Al_{ox} (g kg^{-1})	Fe_{ox} (g kg^{-1})	Clay %	Silt %	Sand %	CEC (meq 100 g^{-1})
1	6.8	7.7	6.8	14.1	0.10	2.1	9.9	1.7	3.1	12.3	36.4	49.1	7.6
2	5.1	6.1	4.9	14.0	0.05	11.1	2.7	2.2	2.7	15	37.3	45.5	8.3
3	5.8	6.5	5.7	13.0	0.10	2.4	10.9	2.0	2.9	15.1	39.1	43.6	8.2
4	5.9	6.9	6.0	13.6	0.07	2.6	11.2	1.9	2.8	14.9	38.1	45	8.1
5	5.9	7.5	6.6	16.0	0.09	2.5	12.8	2.0	2.7	15.3	41.5	40.8	8.2
6	5.8	7.0	6.1	14.7	0.07	2.4	11.5	1.9	2.7	15	40.3	42.5	8.3
7	5.9	6.9	6.1	13.4	0.08	2.2	11.5	1.8	2.6	15	39	44.2	8.4
8	6.1	7.2	6.3	8.3	0.11	1.8	8.8	1.5	2.6	11.4	34.3	52.8	6.0
9	6.2	7.3	6.5	14.8	0.06	2.5	13.5	2.1	3.2	15.4	36.3	45.7	8.2
10	5.5	6.7	5.8	17.3	0.06	2.5	11.5	2.0	2.9	14.8	35.6	47.3	7.7
11	7.0	8.0	7.2	23.1	0.20	1.5	8.6	1.2	3.0	11.8	42.3	42.8	9.6
12	6.1	7.0	6.1	16.1	0.08	2.1	11.8	1.9	3.0	13.9	34.1	49.5	8.2
13	5.1	6.3	5.4	12.9	0.04	10.6	2.1	1.8	2.8	14.6	40	43.8	6.6
14	6.2	7.3	6.5	9.2	0.08	1.6	6.8	1.3	2.2	8.9	31.5	58.1	5.0
15	6.3	7.4	6.5	9.1	0.12	1.6	6.9	1.4	2.1	8.9	29.5	60.4	5.6
16	5.4	6.5	5.6	7.2	0.11	1.8	7.7	1.5	2.5	9.7	30.6	58.3	4.4
17	5.9	6.7	5.9	15.0	0.14	2.0	10.4	1.7	2.7	12.7	32.4	52.7	7.6
Mean	5.9	7.0	6.1	13.6	0.09	3.1	9.3	1.8	2.7	13.2	36.4	48.4	7.4
Standard deviation	0.5	0.5	0.5	3.7	0.04	2.8	3.2	0.3	0.3	2.2	3.7	5.9	1.4
Min	5.1	6.1	4.9	7.2	0.04	1.5	2.1	1.2	2.1	8.9	29.5	40.8	4.4
Max	7.0	8.0	7.2	23.1	0.20	11.1	13.5	2.2	3.2	15.4	42.3	60.4	9.6

The glyphosate concentration was obtained by measuring ^{14}C-glyphosate activity, which was counted with a liquidscintillation analyzer (Packard Tricarb® 2300TR). After adding a scintillator (Aquasafe 300 Plus, Zinsser Analytic), the radioactivity was measured in 2 mL of supernatant. The minimal measured ^{14}C-glyphosate radioactivity is 30 dpm/mL which corresponds to 0.09 µg/L.
AMPA analysis was done on an Acquity ultra-performance liquid chromatography system (UPLCTM, Waters) interfaced to a triple quadrupole mass spectrometer (Quattro Premier XE, Waters). Due to its low molecular weight, a derivatization step with FMOC-chloride in the presence of a borate buffer is required prior to analysis. Extraction is done online with an SPE cartouche (Oasis HLB 25 µm 2.1×20 mm) before separation in an Acquity UPLC HSS column (T3 1.8 µm×2.1 mm×100 mm). The quantification limit is 0.05 µg/L.

Isotherm adsorption coefficients (K_f)
Sorption experiments were run according to a normalized method (OECD guideline 106, 2000) with a 1/5 soil-weight/solution-volume ratio in 15-mL centrifuge plastic tube. Equilibrium - tested with a 1 mg/L solution - was obtained after 24 h. After 12 h of pre-equilibration with a $CaCl_2$ solution (0.01 M), the equilibrated soil suspensions were spiked with a pesticide solution and agitated during 24 h (darkness, 20 °C). After centrifugation (3000 rpm, 30 min, 20 °C), the supernatants were filtrated with 0.2 µm cellulose acetate and analyzed for pesticide concentrations. Blanks (each soil without spiking) did not reveal any presence of either molecule in the soils before the experiments. No adsorption was measured on tubes and filters used for batch experiments. The adsorption isotherm was obtained by the relationship between adsorbed concentration per weight (Cs, mg/kg) compared to the equilibrium concentration per volume of solution (Ce, mg/L) according to the Freundlich equation. Six solute concentrations were tested, 0.05, 0.2, 0.5, 1.0, 3.0 and 5.0 mg/L, for both glyphosate and AMPA. For glyphosate, which was studied with its ^{14}C radiolabeled form, the initial radioactivity was 6000 dpm/mL in tubes. The experiments were run as triplicates. The Freundlich parameters Kf and $1/n_f$ were estimated by using a nonlinear fitting programme (XLStat, Excel 5.0).

Table 2

Experimental Freundlich isotherm coefficients $K_{f\text{-}exp}$ (mg kg^{-1}(L mg^{-1})$^{-nf}$) and $1/n_{f\text{-}exp}$ (−) for glyphosate and AMPA, and K_f recalculated for averaged $1/n_{f\text{-}exp}$ glyphosate ($1/n_{f\text{-}avg}$=0.93) and AMPA ($1/n_{f\text{-}avg}$=0.78)

Soils	Glyphosate					AMPA				
	$K_{f\text{-}exp}$	$1/n_{f\text{-}exp}$	R^2	K_f (for $1/n_{f\text{-}avg}$)	R^2	$K_{f\text{-}exp}$	$1/n_{f\text{-}exp}$	R^2	K_f (for $1/n_{f\text{-}avg}$)	R^2
1	34	0.86	1.00	36	1.00	63	0.74	1.00	67	1.00
2	540	0.97	0.99	475	1.00	392	0.80	1.00	365	1.00
3	143	0.93	1.00	143	1.00	140	0.72	1.00	162	1.00
4	92	1.09	1.00	73	0.99	212	0.79	1.00	206	1.00
5	135	0.92	0.99	139	1.00	226	0.81	1.00	208	1.00
6	132	0.90	0.99	140	0.99	191	0.80	1.00	183	1.00
7	101	0.90	1.00	106	0.99	143	0.75	1.00	154	1.00
8	99	0.94	1.00	97	1.00	119	0.81	1.00	111	1.00
9	107	0.91	1.00	110	1.00	158	0.81	0.99	147	0.99
10	233	0.94	1.00	230	1.00	242	0.77	1.00	253	1.00
11	32	0.83	1.00	34	1.00	33	0.78	1.00	33	1.00
12	91	0.94	1.00	89	1.00	134	0.82	1.00	122	1.00
13	285	0.99	0.99	243	0.99	284	0.79	1.00	210	1.00
14	42	0.98	1.00	40	1.00	91	0.80	1.00	87	1.00
15	41	0.88	1.00	43	1.00	56	0.73	1.00	61	1.00
16	233	0.94	1.00	229	1.00	198	0.78	1.00	198	1.00
17	111	0.97	1.00	103	1.00	107	0.82	1.00	99	1.00
Min	32	0.83		34		33	0.72		33	
Max	540	1.09		475		392	0.82		365	
Mean	144	0.93		137		164	0.78		157	
Standard deviation	121	0.06		106		88	0.03		79	

Parametric linear and nonlinear regression for pedotransfer rule determination

The relationship between the K_f parameter and soil properties was studied for each pesticide (XLStat, Excel 5.0) by multiple linear and nonlinear regression analyses.

Results and discussion

Freundlich adsorption isotherms

The Freundlich isotherm equation adjusts accurately the experimental data (R^2>0.99). High experimental glyphosate K_f values, $K_{f\text{-}exp}$, were obtained, ranging between 32 and 540 mg/kg(L/mg)$^{-nf}$ (Table 2) in agreement with previous studies. In the case of AMPA, $K_{f\text{-}exp}$ values between 33 and 392 mg/kg (L/mg)$^{-nf}$ (Table 2) are in the same high adsorption range as glyphosate.

Table 3

Glyphosate and AMPA K_f coefficients calculated by multiple nonlinear regression from Eq. (2): $K_f = C\ e^{\sum_{i=1}^{n} a_i X_i}$

Soil variables X_n	Constant	pH measurements			Olsen P (g kg^{-1})	Al_{ox} (g kg^{-1})	Fe_{ox} (g kg^{-1})	R^2
		1:5 soil pH_{CaCl2}	pH_{KCl}	pH_{water}				
Regression coefficient values	C	a_1	a'_1	a''_1	a_2	a_3	a_4	
4 variables (pH, Olsen P, Al_{ox}, Fe_{ox})								
Glyphosate	**5.1×10^5**	**−1.7**			**−1.1**	**−0.4**	**1.0**	**0.94**
	3.6×10^5		−1.25		−5.6	−0.1	0.1	0.65
	1.3×10^6			−1.30	−7.3	−0.4	0.3	0.61
AMPA	**1.8×10^4**	**−0.9**			**−4.4**	**0.6**	**−0.1**	**0.92**
	3.6×10^3		−0.50		−5.4	1.3	−0.7	0.81
	3.8×10^3			−0.40	−5.4	1.3	−0.8	0.78
2 variables (pH, Olsen P)								
Glyphosate	1.9×10^6	−1.6			−2.6			0.88
	3.5×10^5		−1.24		−5.3			0.65
	6.7×10^5			−1.17	−6.0			0.60
AMPA	6.1×10^4	−0.9			−7.7			0.88
	2.1×10^4		−0.7		−9.6			0.73
	3.2×10^4			−0.7	−9.8			0.70
1 variable (pH)								
Glyphosate	1.33×10^6	−1.59						0.82
	2.23×10^5		−1.24					0.61
	2.74×10^5			−1.12				0.51
AMPA	4.67×10^4	−0.97						0.69
	1.18×10^4		−0.63					0.37
	1.18×10^4			−0.63				0.37

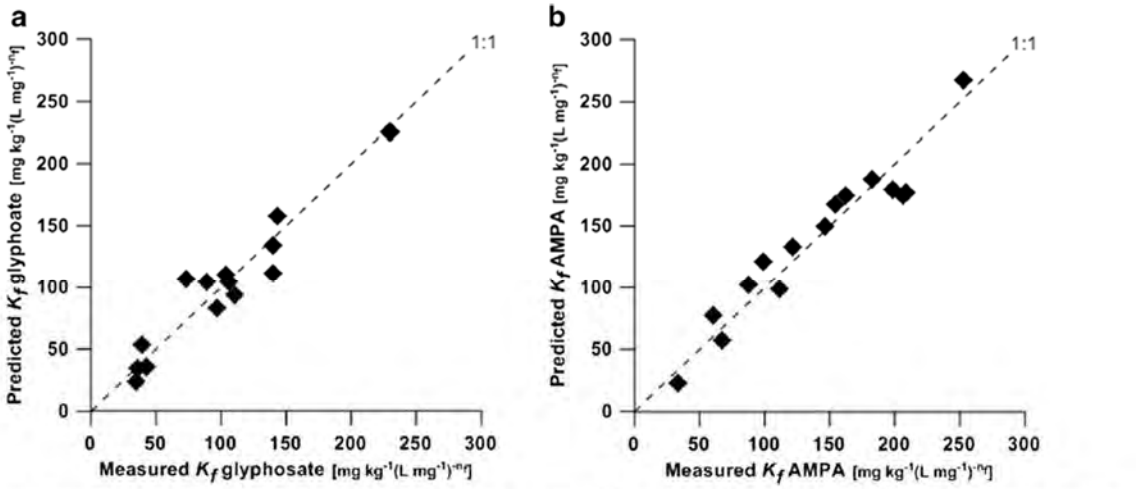
For both molecules, the pedotransfer rule including four variables and the simplified rule including two or one variable(s) are calculated for pH_{CaCl2}, pH_{KCl} or pH_{water}. Value parameters corresponding to the pedotransfer rule including the four pH_{CaCl2}, Olsen P, Al_{ox} and Fe_{ox} variables are indicated in bold

These values are consistent with those obtained by Baez et al. (2015) for molisols and alfisols. Experimental values of the $1/n_{f\text{-}exp}$ coefficients vary between 0.83 and 1.09 for glyphosate, and between 0.72 and 0.82 for AMPA. As the $1/n_{f\text{-}exp}$ values are different, the glyphosate and AMPA $K_{f\text{-}exp}$ datasets cannot be compared directly. Indeed, even if the $K_{f\text{-}exp}$ value is similar, the isotherm can be very different because of the $1/n_{f\text{-}exp}$ value. For each molecule, the $1/n_{f\text{-}exp}$ values had a low standard deviation, allowing to calculate an average $1/n_{f\text{-}exp}$ value, i.e. $1/n_{f\text{-}avg}$, of 0.93 (±0.06) and 0.78 (±0.03) for glyphosate and AMPA, respectively (Table 2). New K_f Freundlich coefficients were recalculated for each soil by using this averaged $1/n_{f\text{-}avg}$ value (Table 2). For both molecules, this second fit to the Freundlich equation is very precise ($R^2 \geq 0.99$) and allows the comparison between soils. For AMPA, the $1/n_{f\text{-}avg}$ value of less than one (i.e. 0.78) indicates that adsorption is strongly limited by the availability of sorption sites. However, the high glyphosate $1/n_{favg}$ value of 0.93 means that adsorption is less governed by the availability of adsorption sites than AMPA. Therefore, despite the similar atomic composition of glyphosate and AMPA, they probably do not sorb in the same way onto the studied soils.

Pedotransfer rule for glyphosate and AMPA adsorption prediction

Regression analysis was restricted to soils with a higher experimental pH_{CaCl2} than both glyphosate pKa_3 and AMPA pKa_2, i.e. $pH_{CaCl2} > 5.4$. This limitation allowed defining adsorption rule when the same ionic form of either glyphosate or AMPA dominates in solution. Thus, soils 2 and 13 (pH_{CaCl2} values=5.1) were excluded from the data analysis. First, a linear multiple regression was tested to relate K_f to every combination of measured soil properties, but the adjustment accuracy was very weak ($R^2 < 0.75$). In our study, nonlinear consideration sharply improves the fit of both glyphosate and AMPA K_f ($R^2 > 0.92$). Of the ten variables studied, nonlinear regression analysis appears optimized when considering the four variables: pH_{CaCl2} value, available phosphate, and amorphous aluminium and iron oxide amount (Table 3 and Fig. 1), for both glyphosate and AMPA K_f adsorption coefficients. As in earlier studies, the highest correlation was found between glyphosate K_f and pH - in our study pH_{CaCl2} - (Table 4). For the pH_{CaCl2} here studied (between 5.4 and 7.0), deprotonation of the phosphonic group results in the dominant glyphosate net-2^- (2^-) and dominant AMPA net-1^- charged (1^-) forms (Fig. 2). For soils with high pH

values, high repulsion forces with negative charges act on the amorphous oxide surfaces and sorption is reduced. In the regression analysis, both glyphosate and AMPA K_f values positively correlate with amorphous iron and aluminium oxides (Table 4), with higher correlations calculated for amorphous aluminium oxides that probably are more reactive in the studied soils. A negative correlation between available phosphate and glyphosate K_f values is observed (Table 4) where both molecules compete for the same adsorption sites on oxide surfaces, thus reducing glyphosate adsorption in the presence of phosphate (Gimsing et al. 2004b).

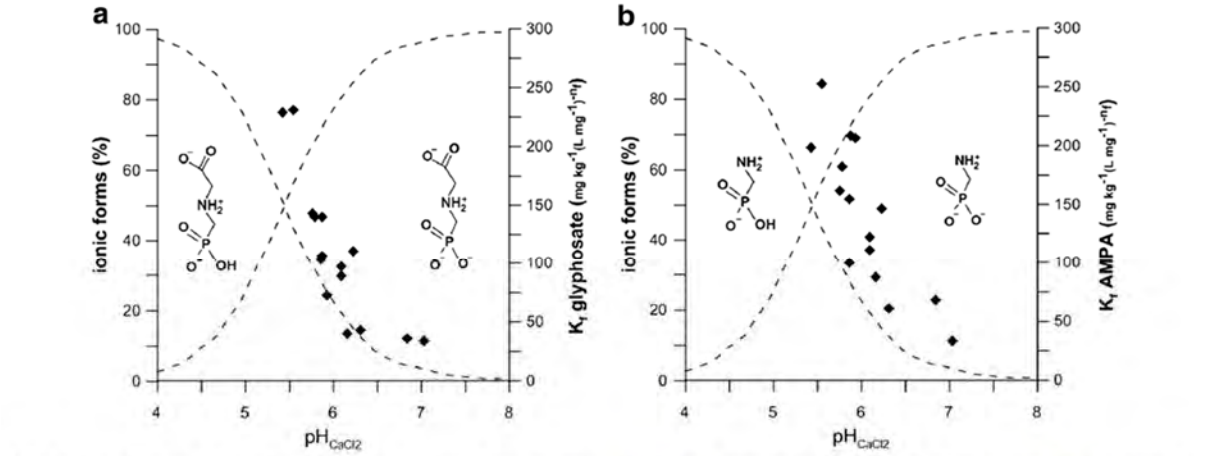


Comparison between measured and predicted K_f coefficients with a nonlinear pedotransfer rule for a glyphosate (with $1/n_{f\text{-avg}}=0.93$) and b AMPA (with $1/n_{f\text{-avg}}=0.78$). The *dotted lines* represent a 1 to 1 straight line. Glyphosate and AMPA pedotransfer rules include pH_{CaCl2}, Olsen P, Al_x and Fe_{ox} variables. Values are presented in Table 3

Figure 1

A negative correlation between AMPA K_f values and available phosphate suggests a similar competition for adsorption on oxide surface sites by reducing AMPA sorption when phosphate is present. The more strongly negative correlation between phosphate and K_f values for AMPA than for glyphosate (Table 4) indicates a higher competitive adsorption between phosphate and AMPA than glyphosate.

To evaluate the sensitivity of the variables affecting the pedotransfer rule, the number of variables was initially reduced to two dominant parameters, i.e. pH_{CaCl2} and available phosphate amount. The resulting equation explains 88 % of the variations in the K_f of glyphosate and AMPA (see R^2, Table 3). Considering only variable pH_{CaCl2} - the most significant of all four variables – decreases the accuracy adjustment for AMPA (R^2 0.69, Table 3), whereas that for glyphosate is only slightly modified (R^2 0.87, Table 3). Thus, it seems possible to arrive at an acceptable estimate of glyphosate adsorption with an equation with just one variable, i.e. pH_{CaCl2}, whereas for AMPA, the two variables pH_{CaCl2} and available phosphate amount are needed.



Distribution of Freundlich coefficients K_f as a function of pH_{CaCl2} and dominance of dissociated glyphosate forms (**a**) and dissociated AMPA forms (**b**) in solution. Bjerrum diagram taken from (Sheals et al. 2002) for glyphosate (**a**) and same diagram suggested as hypothesis for AMPA (**b**). Glyphosate pKa_3=5.46 (Tomlin 1997), AMPA pKa_3=5.4 (Chen et al. 2009)

Figure 2

Constraints in applying pedotransfer adsorption equations

To verify the accuracy of the proposed sorption multiple regression, as Paradelo et al. 2015 showed that this might be dependent upon the study site, we collected published data concerning pedotransfer rules for testing them in our model. To our knowledge, AMPA adsorption instead of glyphosate sorption is rarely described in the literature. Nevertheless, none of the published work describes all four variables - pH_{CaCl2}, available phosphate, and amorphous iron and aluminium contents - for several soils. We thus carried out an in-depth study on the effect of the pH-measuring method on predicting the glyphosate K_f (Table 3). The parameters for the adsorption equations with four and two variables, or even one variable, were recalculated for pH_{water} or pH_{KCl} values, as these are more commonly measured parameters than experimental pH_{CaCl2}. We then did the same work for AMPA equations as a comparison. The choice of pH clearly affected the accuracy of an equation with four variables, as R^2 varied from 0.94 with pH_{CaCl2} to ≤0.65 with pH_{water} and pH_{KCl} for glyphosate, and from 0.92 to ≤0.81 for AMPA. This decrease in the adjustment accuracy was obviously also noted for regressions with two variables - R^2 going from 0.88 to ≤0.65 for glyphosate and from 0.88 to ≤0.73 for AMPA—and one variable (R^2 going from 0.88 to ≤0.61 for glyphosate and from 0.69 to ≤0.37 for AMPA). Glyphosate K_f coefficients are much more affected by the pH measurement method than those of AMPA, but the pH variable in exponential glyphosate equations is systematically associated with higher correlation coefficients than in the AMPA ones (Table 3). These results clearly show that the type of pH measurement plays a crucial role for the prediction of glyphosate and AMPA adsorption coefficients. Since no simple relationship can be established between experimental pH_{CaCl2} and pH_{water} (R^2 0.76) or pH_{KCl} (R^2 0.80), a model validation for glyphosate cannot be based on available published data.

Table 4

Correlation matrix of K_f glyphosate ($1/n_{f\text{-}avg}$=0.93) and K_f AMPA ($1/n_{f\text{-}avg}$ =0.78) with soil parameters by multiple nonlinear regression

Variables	pH $_{CaCl2}$	Olsen P (%)	Al_{ox} (%)	Fe_{ox} (%)
$K_{f\ glyphosate}$	−0.83	−0.36	0.43	0.05
$K_{f\ AMPA}$	−0.82	−0.67	0.68	0.10

Conclusions

High adsorption coefficients calculated for glyphosate and AMPA molecules depend upon the experimental pH_{CaCl2} value (1:5 soil/solution), the available phosphate content, and the amorphous aluminium- and iron oxide contents. These four key soil parameters combined in an exponential regression equation provide a precise description of a pedotransfer rule for K_f prediction. To our knowledge, our AMPA dataset contains the first published data for adsorption on a significant number of natural soils. Because of low $1/n_f$ values, prediction of the AMPA K_f is strongly related to the soil-solution concentration, contrary to glyphosate. Changes in pH strongly affect adsorption by modifying the ionic state of glyphosate and AMPA and the available amorphous oxide surface sorption sites. Phosphate competes with both molecules for adsorption, but more strongly with AMPA. Considering only the two variables, pH_{CaCl2} and available phosphate content, leads to a satisfactory prediction of the adsorption constants. For both molecules, pH_{CaCl2} is the most reliable explanatory variable. However, pH_{CaCl2} is only rarely measured during batch experiments, even though most of such experiments use $CaCl_2$ as the solute. Replacing pH_{CaCl2} by pH_{KCl} or by pH_{water} - that are more frequently measured in soils - as the variable in the pedotransfer rule does not allow adjusting K_f with sufficient precision. Since simple relation does not exist between pH_{KCl} and pH_{CaCl2} - or between pH_{water} and pH_{CaCl2} - a complementary soil characterization with pH_{CaCl2} value therefore appears necessary for the application of the pedotransfer rule. However, such a measurement is easier and faster than the implementation of sorption experiments. The acquisition of supplementary data on various soils will lead to a better validation of the pedotransfer rules for glyphosate and AMPA. Finally, the strong adsorption observed in the studied soils, which are rather depleted in organic carbon, takes place on mineral fractions. Hence, the—little studied—geological materials present in the unsaturated zone might also strongly adsorb glyphosate and AMPA. The adsorption of these molecules on such materials should thus be studied as well.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes and sorption experiment with glyphosate on 17 soils from France. The OECD 106 guideline was considered. However, not all parameters were reported to check the validity of the study. The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1, KCA 7.1.3.1.2
Report author	Skeff, W., et al.
Report year	2018
Report title	Adsorption behaviors of glyphosate, glufosinate, aminomethylphosphonic acid, and 2-aminoethylphosphonic acid on three typical Baltic Sea sediments
Document No	Marine Chemistry 198 (2018) 1–9
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (no agricultural conditions, not sufficient data to check validity of the results)

2. Full summary of the study according to OECD format

A batch experiment was conducted to study the adsorption behaviors of glyphosate, glufosinate, aminomethylphosphonic acid (AMPA), and 2-aminoethylphosphonic acid (2-AEP) in marine sediments (mud, silt, and sand) from the Baltic Sea. The experiment took into account the influence of pH, salinity, and temperature on the adsorption behaviors of the studied compounds. In contrast to glufosinate, glyphosate exhibited an adsorption affinity for the three types of sediments. AMPA and 2-AEP showed similar adsorption behaviors on mud and silt, while their adsorption on sand was negligible. The equilibrium adsorption data for glyphosate, AMPA, and 2-AEP on mud and silt fit well with the linear partitioning and Freundlich isotherms, whereas the data for glyphosate on sand could only be fitted with the Freundlich isotherm. The Freundlich distribution coefficients (k_f) were in the range of 6.1-259.5 L/kg for glyphosate, 9.2-39.5 L/kg for AMPA, and 7.7–38.5 L/kg for 2-AEP under the experimental conditions of pH 8.1, temperature = 21°C, and a salt concentration of 8 g/L. The adsorption kinetic was better described by the pseudo-second-order than the pseudo-first-order model, suggesting chemisorption as the adsorption mechanism. The order of adsorption of the compounds on the sediments was: glyphosate >AMPA ≥2-AEP >glufosinate. The adsorption capacity of sediments followed the sequence: mud >silt >sand. Increasing the pH, salinity, or temperature of the solution significantly reduced the adsorption capacity of the compounds. The data obtained in this study provide valuable information on the fate and distribution of the investigated phosphonates in the Baltic Sea.

Materials and methods

Chemicals and reagents

Standards of glyphosate, a glyphosate internal standard (1-2-$^{13}C_2{}^{15}N$ glyphosate), AMPA, an AMPA internal standard (^{13}C ^{15}N AMPA), and glufosinate were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 2-AEP was supplied by Sigma-Aldrich (Taufkirchen, Germany). Stock solutions of these compounds, except the internal standards, were prepared in polypropylene volumetric flasks at a concentration of 100 mg/L by dissolving 5 mg of each compound in 50 mL of LC-MS grade water (VWR International GmbH, Darmstadt, Germany). The stock solutions were stored at 5°C in the dark. A stock solution (66.6 mM) of 9-fluorenylmethyl chloroformate (FMOC-Cl) (purity 99.0 %, Sigma-Aldrich) was prepared by dissolving 1 g in 58 mL of acetonitrile (Walter-CMP GmbH, Kiel, Germany). Borate buffer at pH 9 was prepared by dissolving 1 g of sodium tetraborate decahydrate (Sigma-Aldrich) in 50 mL of Milli-Q water (Merck KGaA, Darmstadt, Germany). Artificial sea salt, contains all 70 trace elements found in natural seawater in the exact proportions found in nature, was purchased from Tropic Marin®, Germany. Chloroform was supplied by VWR AnalaR Normapure (Germany).

Sediment collection and characterization

Three types of sediment typical of the Baltic Sea were collected from the German Baltic Sea (Figure 1) which are: S1 from Arkona basin (54° 50′ N, 13° 30′ E), S2 from Tromper Wiek (54° 39′ N, 13° 35′ E), and S3 from Oder Bank (54° 04′ N, 14° 03′ E). The sediments were collected using a multiple corer during research cruise EMB76, in June 2014. Samples of the uppermost sediment were sealed in glass jars, stored at -20°C until dry. No sieving was done but large items such as stones, leaves, grass and animals were removed and the samples were manually homogenized. The bulk sediments were freeze-dried using a Chaist ALPHA 1-4 LD freezer dryer and used as sorbents in this study. The grain sizes of the sediments were determined using a CILAS 1180 particle size analyzer. The TOC content of the sediments was analyzed with an elemental analyzer according to (Leipe et al., 2011). The major and trace elements in the sediments were measured using inductively coupled plasma optical emission spectrometry after acid total digested of the samples. The sediment grain sizes were distributed among the different classes: clay (<2 µm), silt (2-63 µm), and sand (>63 µm). The sediment S1 contained 6.6 % clay, 92.3 % silt, and 1.1 % sand, with a median grain size 20.1 µm. The sediment S2 contained 3.6 % clay, 69.9 % silt, and 26.5 % sand, with a median grain size 41.2 µm. The sediment S3 contained 1.7 % clay, 10.7 % silt, and 87.6 % sand, with a median grain size 156.8 µm. The sediment S1, with organic-rich silt-size sediments, was classified as mud, while the sediment S2 as silt and the sediment S3 as fine sand. The sediment TOC, total phosphorus, and major and trace elements followed the order: mud >silt >fine sand.

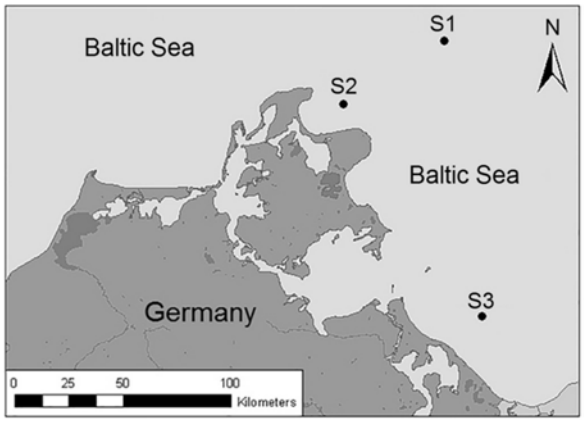


Figure 1. Location of the sampling stations in the German Baltic Sea. The station S1 is in Arkona basin, S2 in Tromper Wiek, and S3 in Oder Bank.

Batch sorption experiment

To investigate the possible adsorption of the analytes onto the walls of the centrifuge tubes, the hydrolysis and degradation of the test compounds during the experiment, a control set of tubes was established in which sediment-free artificial seawater samples were spiked with 250 µg of the analytes/L for 48 h. An additional set of tubes containing sediments with unspiked artificial seawater controlled for possible desorption and the contamination of the sediments and media with the target compounds.

To initiate the experiment, artificial seawater was prepared at a salt concentration of 8 g/L. The pH of the solution was 8.1, measured using a conductivity meter (WTW Inolab cond® 720, Germany). Chloroform (0.1 %) was added to the media to inhibit microbial activity. 1 g dry weight of each sediment type was distributed in 15-mL polypropylene centrifuge tubes and mixed with 10 mL of artificial seawater. The tubes were mechanically shaken and incubated for at least 2 days, after which the samples were centrifuged (Megafuge 1.0, Heraeus Instruments) for 3 min at 2500 rpm. Then, 8 mL of each supernatant was transferred to a sediment-free polypropylene centrifuge tube. The samples were then spiked with the target compounds, well shaken at 300 rpm using a mechanical shaker, and 200 µL were then drawn and analyzed for their initial concentrations of the compounds. Thereafter, the spiked medium was returned to the respective sediment tube, which was then vigorously shaken. This process was conducted in order (i) to avoid any possible adsorption of the compounds onto the sediments at the start of the experiment (T = 0 h) and (ii) to allow the analysis of the phosphonates in same sample matrices during the experimental time, whereas a variety of sample matrices might lead to analytical

errors. The experiment was conducted for 48 h at room temperature (21°C), with samples from the aqueous phase taken for analysis at 0, 1, 3, 5, 7, 24, and 48 h. The phosphonates were tested at the following concentrations: 120, 300, 600, 900, and 1200 µg/L. All experiments were performed in duplicate and each sample was measured in triplicate. The target compounds were measured in the aqueous phase. The amounts adsorbed onto the sediments (q_t, µg/g) at time *t* were calculated according to Eq. (1):

$$q_t = (c_0 - c_t)v/m \quad (1)$$

where c_0 is the initial concentration (µg/L), c_t is the concentration at time *t* (µg/L), v is the volume of the solution (L), and m is the dry mass of the sediment (g).

Analysis of organophosphonates

A volume of 200 µL of each supernatant was transferred to 2-mL reaction tubes (Eppendorf, Germany) and diluted to 700 µL using LC-MS grade water. The samples were then treated with 100 µL of the glyphosate and AMPA internal standard solutions, prepared in the same matrix, to obtain a final concentration of 15 µg/L. To derivatize the samples, the pH was adjusted to 9 using 100 µL of 0.07 M borate buffer, after which 100 µL of 33.3 mM FMOC-Cl in acetonitrile was added. The samples were shake-incubated at room temperature for 4 h to allow complete derivatization, filtered through a 45-µm Phenex-RC 15-mm syringe filter (Phenomenex, Germany), and analyzed by LC-MS/MS according to a previously described method (Skeff et al., 2015, 2016). Glyphosate was quantified using the glyphosate internal standard, and AMPA, glufosinate, and 2-AEP using the AMPA internal standard.

Statistical analysis

All adsorption experiments were conducted in duplicate, and the measurements in triplicate. The adsorption study measured the initial and equilibrium concentrations of the target compounds. A *p*-value <0.05 was considered to indicate statistical significance. A one-way ANOVA followed by a Holm-Sidak post-hoc test was carried out using SigmaPlot software (version 13.0, Systat Software Inc.).

Results

Control experiments

A successful adsorption investigation requires the proper controls to rule out both contamination of the aqueous phase or adsorbents with the sorbates and the loss of the sorbates due either to their degradation during the experiment or their adsorption onto the tubes. Controls for both possibilities were therefore established. Data from the first control experiment, in which the compounds were incubated in artificial seawater without sediments, showed a high degree of measurement stability and thus high biological stability of the sorbates during the 48 h and negligible adsorption onto the tubes as well. Furthermore, the stable measurements indicate that the C-P bonds in the organophosphonates are relatively stable and no hydrolysis occurs. Data from the second control experiment, in which the sediments were incubated without sorbates, failed to show the compounds in the aqueous phase and thus confirmed the lack of contamination or desorption. The results of both control experiments demonstrated the validity of the adsorption study.

Kinetic studies and models

The mechanism of glyphosate, glufosinate, AMPA, and 2-AEP absorption onto marine sediments was examined in kinetic studies. The q_t (µg/g) values of glyphosate, AMPA, and 2-AEP between 0 and 48 h are shown in Figure 2. Whereas glyphosate had an affinity for all three types of sediments, AMPA and 2-AEP adsorbed to mud and silt but not sand. Glufosinate concentrations measured in the aqueous phase remained comparable during the 48 h of the experiment, indicating the lack of significant adsorption (*p* >0.05) onto the sediments. The presence of a methyl group on the phosphonate of glufosinate might obstruct the formation of surface complexes, thus limiting its adsorption compared to glyphosate.

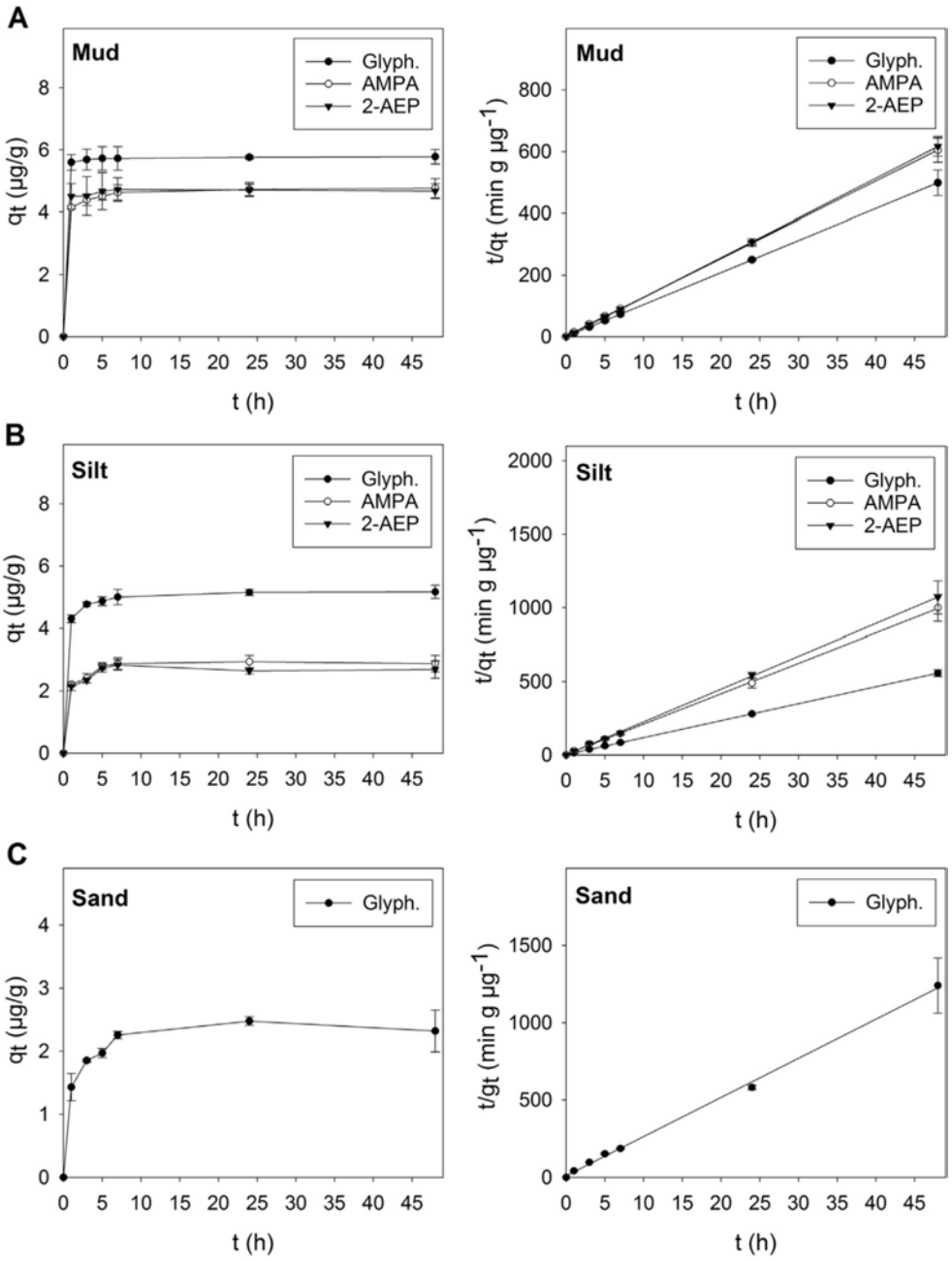


Figure 2. **Adsorption equilibrium time (left) of glyphosate, AMPA, and 2-AEP on A. mud, B. silt, and C. sand, and the respective pseudo-second order kinetics (right). The figures in the left column are based on a concentration of 600 µg of each compound/L.**

As shown in Figure 2, glyphosate, AMPA, and 2-AEP followed similar adsorption kinetics in the mud and silt sediments, and on sand for glyphosate. The adsorption kinetics consisted of two distinct stages: a fast adsorption process in the first hour followed by slow adsorption. The adsorption equilibrium of glyphosate, AMPA, and 2-AEP was reached in 24 h. The amount adsorbed of glyphosate was higher than those of AMPA and 2-AEP for the three types of sediment. The adsorption rate of glyphosate was the highest on mud, followed by silt and sand (96.3 %, 86.2 %, and 38.6 %, respectively). The adsorption rates of AMPA and 2-AEP on mud and silt were similar (~80 % and ~50 %, respectively). The latter observation can be explained that AMPA and 2-AEP own the same functional groups (i.e. each contains phosphonate and amino group), resulting in their similar interactions with sediments.

Lagergren pseudo-first-order and pseudo-second-order models were employed as kinetic models to investigate the rate-controlling steps involved in the adsorption of glyphosate, AMPA, and 2-AEP onto

sediments. The linearized Lagergren pseudo-first-order [Eq. (2)] and pseudo-second-order [Eq. (3)] equations are as follows:

$$\ln(q_e - q_t) = \ln q_e - k_1 t \quad (2)$$

$$t/q_t = 1/k_2 q_e^2 + t/q_e \quad (3)$$

where q_e and q_t are the amount of phosphonates (µg/g) adsorbed onto the marine sediments at equilibrium and time t (min), respectively, and k_1 (min^{-1}) and k_2 (g/µg min) are the equilibrium rate constants of the pseudo-first-order and pseudo-second-order models, respectively. The best-fit model was selected based on the values of the linear regression correlation coefficient (R^2). The pseudo-second-order kinetic model efficiently predicted the kinetic behavior of the three compounds on sediments, based on the high R^2 values (0.9982-0.9999), whereas a poor fit of the data was obtained with the pseudo-first-order kinetic model (R^2 <0.85). The rate constant k_2, the q_e values, and the corresponding linear regression correlation coefficient (R^2) were calculated from the linear plots of t/q_t vs. t (Figure 2) and are shown in Table 1. The good fit obtained with the pseudo-second-order model suggested chemisorption as the rate-limiting step, presumably between the functional groups of the compounds (i.e., the phosphonate, carboxylate, and amino groups) and the sediment surfaces through the sharing or exchange of electrons. As can be seen from Table 1, the calculated adsorption capacity values ($q_{e.cal}$) from the second order model are well comparable to the experimental adsorption capacity values ($q_{e.exp}$). Thus, the adsorption kinetics of the three phosphonates on the sediments is more precisely described by the mechanism of surface site-sorbates reaction of pseudo-second-order model. The adsorption capacity of the three compounds followed the sequence glyphosate >AMPA ≥2-AEP.

Table 1 Pseudo-second order kinetic parameters for the adsorption of glyphosate, aminomethylphosphonic acid (AMPA), and 2-aminoethylphosphonic acid (2-AEP) onto Baltic Sea mud, silt, and fine-sand sediments under the experimental condition of 600 µg/L initial concentrations, 8 g salt/L, pH = 8.1, temperature = 21 °C.

Sediment	Pseudo-second-order				
	Equation	R^2	$q_{e.\ exp}$ (µg/g)	$q_{e.\ cal}$ (µg/g)	K_2 (g/ µg min)
Mud					
Glyphosate	Y = 0.1730X + 0.4567	0.9999	5.7804	5.7789	0.0655
AMPA	Y = 0.2093X + 2.4457	0.9999	4.7778	4.7633	0.0179
2-AEP	Y = 0.2146X + 1.4743	0.9998	4.6772	4.6705	0.0312
Silt					
Glyphosate	Y = 0.1879X + 2.3688	0.9999	4.6998	5.1685	0.0157
AMPA	Y = 0.3371X + 3.6885	0.9997	2.9070	2.9318	0.0308
2-AEP	Y = 0.5931X + 6.7319	0,9997	2.6925	1.6432	0.0522
Sand					
Glyphosate	Y = 0.4218X + 10.449	0.9982	2.3708	2.8043	0.0170
AMPA	NA	NA	NA	NA	NA
2-AEP	NA	NA	NA	NA	NA

NA: not applicable.

Adsorption isotherms

Linear partitioning and Freundlich models are common adsorption isotherms that were applied in this study to describe the adsorption equilibrium of glyphosate, AMPA, and 2-AEP on marine sediments.

Linear partitioning is described by Eq. (4) and the linear formula of the Freundlich isotherm is shown in Eq. (5):

$$q_e = k_d\, c_e \quad (4)$$

$$\log q_e = \log k_f + 1/n\ \log c_e \quad (5)$$

where c_e is the concentration (µg/L) in the aqueous phase at equilibrium, and k_d (L/g) the distribution coefficient for the sediment/solution ratio (1/10). The k_d values (Table 2) were obtained from the slope of the linear plots of q_e (µg/g) vs. c_e (µg/L) (Figure 4). k_f (µg/g) is the Freundlich constant (i.e. sorption capacity), and 1/n an empirical parameter related to the intensity of adsorption. The values for k_f and 1/n (Table 2) were determined from the intercept and slope of the plots log q_e vs. log c_e (Figure 4). As shown in Figure 4, both isotherms described the equilibrium adsorption of the three phosphonates on mud and silt. The Freundlich model had a slightly better fit than the linear partitioning model based on the higher R^2 values (0.96 and 0.99), which suggests that the adsorption takes place on heterogeneous surfaces. It is important to point out that the concentrations of the compounds tested in this study of marine sediments were lower than those typically used in soil adsorption studies, as they were considered representative of conditions in the marine ecosystem. Thus, fitting of the data to both models might be a result of the narrow concentration range (120-1200 µg/L) tested in this study.

The k_d values obtained from linear partitioning were in the range of 55.2 to -259.5 L/kg for glyphosate, 10.0-39.5 L/kg for AMPA, and 7.7-38.5 L/kg for 2-AEP. Data on glyphosate adsorption in the sandy sediment could only be fitted with the Freundlich model (R^2 =0.99), not with linear partitioning (R^2 = 0.607), which suggested adsorption saturation by the sand as the glyphosate concentration increased. The AMPA and 2-AEP concentrations measured in the aqueous phase were relatively stable, indicative of their difficult adsorption onto sand.

In the Freundlich isotherm, higher k_f values represent a larger adsorption capacity. The calculated k_f values for glyphosate, AMPA, and 2-AEP were in the range 129.8-397.7 L/kg, 25.3-73.5 L/kg, and 19.9-70.1 L/kg, respectively. The k_d and k_f values obtained in this study clearly demonstrated the higher adsorption capacity of glyphosate than of the other studied compounds. The parameter 1/n represents the linearity of the relationship between $C_{aq.}$ and $C_{sediment}$, with a lower 1/n value indicating a less homogeneous distribution of the adsorption site energy on the sediments. For all of the tested compounds, the 1/n values were <1: 0.527-0.917 for glyphosate, 0.784-0.899 for AMPA, and 0.779-0.882 for 2-AEP. The higher 1/n value of glyphosate implied that the variability of the sediment adsorption sites had a smaller effect on its adsorption than was the case for AMPA or 2-AEP. The 1/n values for the three compounds decreased according to the sequence mud >silt >sand, reflecting the increasingly difficult (i.e., concentration-dependent) adsorption process.

The influence of sediment organic carbon content on the adsorption of glyphosate, AMPA, and 2-AEP was determined by examining their correlations. The sediment organic carbon normalized distribution coefficient (k_{oc}) was calculated from the Freundlich isotherm using Eq. (6). The results are provided in Table 2:

$$K_{oc} = (k_f \times 100)/TOC\% \quad (6)$$

The k_{oc} values of glyphosate were in the range of 5706-86,540 L/kg, but were higher in the sandy sediment, which had the lowest TOC content (0.15 %). This result demonstrated that sediment organic carbon content is not a determining factor in glyphosate adsorption. For AMPA and 2-AEP, the k_{oc} values decreased with the decreasing TOC content, which suggested that the adsorption of both compounds was more sensitive to the organic carbon content of the sediments than glyphosate. The soil mineral composition, which includes aluminium and iron oxides, is a major factor governing glyphosate and AMPA adsorption. In this study, a positive correlation was also determined between the aluminium and iron contents of the sediments and the adsorption of glyphosate, AMPA, and 2-AEP.

Table 2 Parameters obtained from the linear partitioning and Freundlich adsorption isotherms

of glyphosate, AMPA, and 2-AEP on mud, silt, and sandy sand sediments under the experimental condition of 8 g salt/L, pH =8.1, temperature = 21 °C.

Sediment	Linear partitioning model		Freundlich model			
	K_d (L/kg)	R^2	K_f (L/kg)	K_{oc} (L/kg)	1/n	R^2
Mud						
Glyphosate	259.5	0.994	397.7	7152.9	0.917	0.999
AMPA	39.5	0.992	73.5	1321.9	0.889	0.998
2-AEP	38.5	0.992	70.1	1259.0	0.882	0.994
Silt						
Glyphosate	55.2	0.990	141.5	5706.1	0.849	0.999
AMPA	9.2	0.988	25.3	1019.4	0.784	0.996
2-AEP	7.7	0.987	19.9	802.4	0.779	0.998
Sand						
Glyphosate	6.1	0.607	129.8	86,540.0	0.527	0.992
AMPA	NA	NA	NA	NA	NA	NA
2-AEP	NA	NA	NA	NA	NA	NA

NA: not applicable.

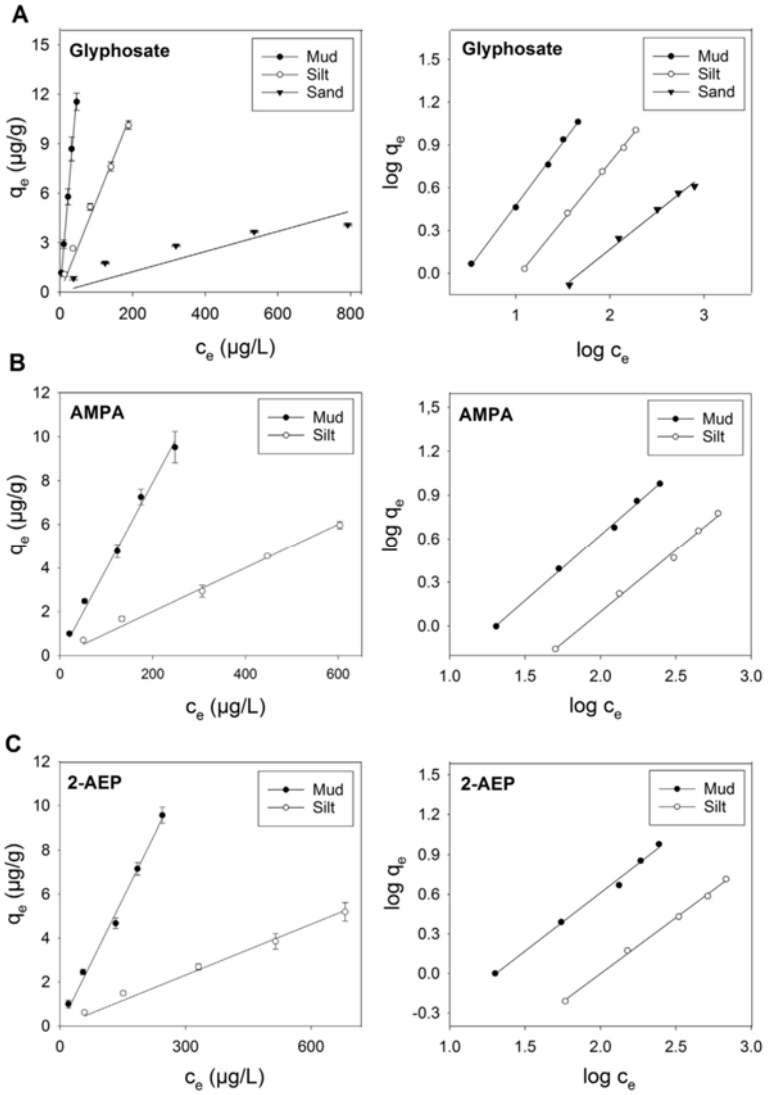


Figure 4.Linear partitioning isotherm (left) and Freundlich isotherm (right) of A. glyphosate, B. AMPA, and C. 2-AEP on marine mud, silt, and sandy sediments.

Effect of environmental factors

The impact of environmental factors, including the pH, salinity, and temperature of the medium, on the adsorption behaviors of glyphosate, AMPA, and 2-AEP was investigated in the mud sediment. The choice of this sediment is due to its greater adsorption capacity for the compounds than silt and sandy sediments. The initial concentrations of the three compounds in the salinity and temperature tests was 300 µg/L, and in the pH test 120 µg/L.

Effect of the medium pH

To elucidate the effect of the pH of the medium on phosphonate adsorption onto the mud sediment, artificial seawater (a salt concentration of 8 g/L, 0.1 % $CHCl_3$) was adjusted to three different pH values (7.3, 8.1, 8.7) reflecting the variability of the pH of Baltic Sea water. The pH was adjusted using concentrated HCl and NaOH. The mud sediment samples were incubated for 48 h in the corresponding medium and then spiked with the test compounds. The experiment was then conducted as described for the standard experiment at 21°C. As seen in Figure 5A, adsorption of the three compounds increased significantly ($p < 0.05$) as the pH decreased from 8.7 to 7.3. The results suggested the similar effects of a change in seawater pH on 2-AEP and AMPA. As the pH of the medium increases, the positive surface charge of the sediment decreases and may become negative. Thus, in this study, the decreased adsorption may have been due to the reduced coordination between the phosphonate group of the compounds and the surface of the sediments. In addition, a higher pH may enhance the release of native organic matter from the sediment into solution, thereby reducing the sediment adsorption capacity of the target compounds.

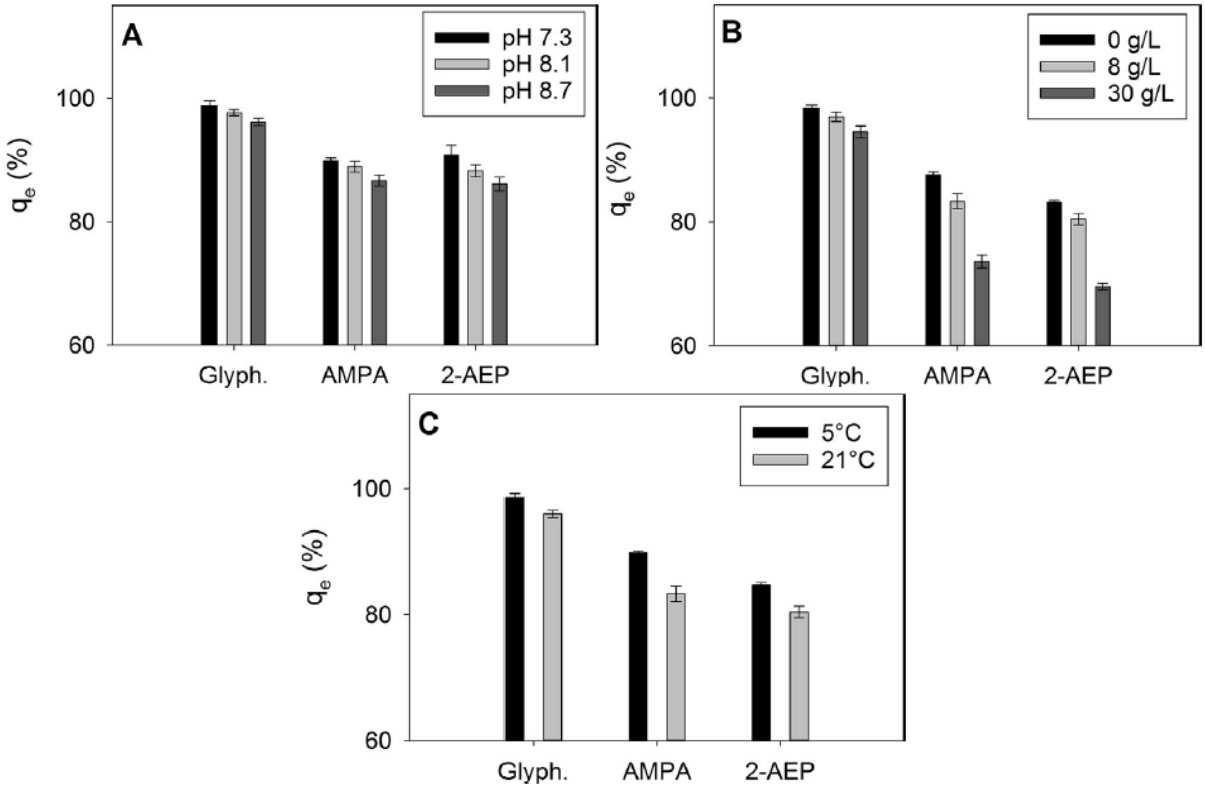


Figure 5. **The influence of A. pH, B. salinity, and C. temperature on the adsorption of glyphosate, AMPA, and 2-AEP onto mud sediment. The data are based on duplicate experiments, each consisting of triplicate measurements. The initial concentrations of the compounds were 120 µg/L in the pH experiment and 300 µg/L in the salinity and temperature experiments.**

Effect of solution salinity

Salinity (ionic strength) may have an important influence on the adsorption behavior of amphoteric compounds, including glyphosate, AMPA, and 2-AEP, in seawater-sediment systems. To investigate its effect, media containing three different salt concentrations (0, 8, 30 g/L) were prepared. The salt-free

medium (0 g/L) consisted of LC-MS grade water, presumably free of salt. The experiment was run at pH 8.1 and 21°C. The results revealed the negative correlation between the adsorption of the compounds and the salinity of the medium (Figure 5B). The adsorption capacity increased significantly ($p < 0.05$) as the salt concentration decreased from 30 g/L to 0 g/L, an effect attributable to ion exchange. At pH 8.1, glyphosate and AMPA carry negative charges related to the phosphonate (both molecules) and carboxylate (glyphosate) groups and positive charges related to the amino group (both compounds). Most sediment surfaces carry a net negative charge but at pH 8.1 positive charges in sediment organic matter might be exposed. Therefore, changing the ionic composition of the medium may influence the adsorption process, by promoting competition for ion-exchangeable sites. Alternatively, complexes between the phosphonates and cations such as Ca^{2+} and Mg^{2+}, present in the medium, may form that have a lower adsorption affinity for the sediments than do free compounds, such that their adsorption decreases with increasing salinity. According to the results, the various salt concentrations had similar effects on the adsorption behaviors of 2-AEP and AMPA, perhaps because they have the same functional groups.

Effect of temperature

To examine the influence of temperature on the adsorption behaviors of glyphosate, AMPA, and 2-AEP, two different temperatures (5°C and 21°C) were tested. As shown in Figure 5C, the amount of adsorbed compounds increased significantly ($p < 0.05$) as the temperature decreased, indicating that adsorption was an exothermic process. The amount of adsorbed glyphosate, AMPA, and 2-AEP increased differentially as the temperature decreased; at rates of 1.5 %, 6.5 %, and 4.3 %, respectively. This may have been due to the different effects of temperature on the water solubility of the compounds. In general, the solubility of chemical substances improves as the temperature rises, such that the amounts entering the solid phase will be lower when equilibrium is reached. Moreover, an increase in the temperature of the medium could increase the solubility of organic matter in sediments, thus increasing the competition with phosphonates for sediment adsorption.

Environmental implications

Mud sediments can act as a sink for glyphosate as well as for AMPA and 2-AEP, based on the high adsorption affinities of these compounds (>96 % and >78 %, respectively). In silt, the three compounds were distributed between the water and adsorption to the sediment, with a higher tendency of the latter. This result clearly supports the need for bioavailability and toxicity studies of benthic as well as pelagic organisms. Sandy sediments had a weak adsorption capacity for glyphosate, and a negligible adsorption capacity for glufosinate, AMPA, and 2-AEP. Therefore, these compounds can be easily moved from Baltic Sea regions characterized by sandy sediments to those with mud or silt sediments. The pH, salinity, and temperature data demonstrated that the variability of these parameters significantly influences the adsorption behaviors of glyphosate, AMPA, and 2-AEP. A decrease in either the seawater pH or the temperature enhanced the adsorption of these compounds onto marine sediments. Thus, their mobility is a more important factor in warmer than in colder marine systems. Furthermore, the warming effect induced by climate change may influence the fate of phosphonates in the marine environment. The negative correlation between salinity and adsorption suggested the greater mobility of these compounds in marine than in freshwater systems. In the Baltic Sea, salinity varies greatly from south to north, and from east to west, increasing from 2 to 4 in the northern area up to 20-30 in the southwestern area of the sea. Thus, the distribution of glyphosate, AMPA, and 2-AEP in Baltic Sea water and sediments is most likely spatially dependent. These results provide basic information about the fate of these phosphonates in the Baltic Sea and highlight the importance of monitoring these compounds in marine water and sediments, especially in semi-closed seas such as the Baltic Sea, where contaminants may cause acute effects.

Conclusion

In this work, the adsorption of glyphosate, glufosinate, AMPA, and 2-AEP onto mud, silt, and sandy sediments of the Baltic Sea was investigated. Glufosinate had no adsorption affinity for any of the sediments tested. Data on the adsorption kinetics of the other compounds could be well fitted with a second-order rate model. The adsorption rate followed the order glyphosate >AMPA ≥2-AEP

>glufosinate. Linear partitioning and Freundlich isotherms described the adsorption of glyphosate, AMPA, and 2-AEP on mud and silt. However, only glyphosate showed important adsorption onto the sandy sediment and its behavior could be well modeled with the Freundlich isotherm. The adsorption capacity of the sediments decreased in the order mud >silt >sand. Inverse correlations between the pH, salinity, and temperature of the medium and the adsorption of glyphosate, AMPA, and 2-AEP were determined. This study showed that a small difference in the chemical structure of amphoteric substances such as glyphosate and glufosinate can lead to large differences in their adsorption behaviors.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes the sorption of glyphosate and AMPA to sediments of the Baltic Sea. The described sediments are not relevant for agricultural land use. Some information is missing to check the validity of the experiment (no mass balances, test items not sufficiently described).

The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.1.1
Report author	Sun et al.
Report year	2019
Report title	Degradation of glyphosate and bioavailability of phosphorus derived from glyphosate in a soil-water system
Document No	Water Research 163 (2019) 114840
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	None
Acceptability/Reliability:	Reliable with restrictions (some validity criteria of the OECD 307 guideline not met, no information about the soil used, no tabulated values available)

2. Full summary of the study according to OECD format

Glyphosate, the most commonly used herbicide in the world, can be degraded into more toxic and persistent products such as aminomethylphosphonic acid (AMPA) or non-toxic products such as sarcosine and glycine. In this study, we used liquid chromatography mass spectrometry (LC-MS) and electrospray ionization (ESI) source Q Extractive Orbitrap mass spectrometry (ESI-Orbitrap MS) to identify glyphosate degradation products and combined with sequential extraction and stable isotopes to investigate the degradation of glyphosate and transformation of phosphorous (P) product in a soil-water system. The LC-MS and ESI-Orbitrap MS results showed that glycine formed during the early stage but was rapidly utilized by soil microorganisms. AMPA started to accumulate at the late stage and was found to be 3-6 times more resistant than glyphosate against degradation; while no sarcosine was formed. The ^{18}O labeling and phosphate oxygen isotope results allowed a clear distinction of the fraction of inorganic P (P_i) derived from glyphosate, about half of which was then rapidly taken up and recycled by soil microorganisms. Our results provide the first evidence of the preferential utilization of glyphosate-derived P_i by microorganisms in the soil-water system. The rapid cycling of P_i derived from this disregarded source has important implications on nutrient management as well as water quality.

Materials and methods

Reagent and chemicals

Glyphosate (≥96 %), (Aminomethyl) phosphonic acid (≥98 %) and 9-Fluorenyl-methoxycarbonyl chloride (FMOC-Cl) (≥97 %) were obtained from Sigma-Aldrich. Isotope labeled compounds including glyphosate-2-^{13}C, ^{15}N, glycine-d_5 and sarcosine-d_3 (methyl-d_3) were purchased from Sigma-Aldrich. Other chemicals including glycine (≥99 %) and sarcosine (≥98 %) were purchased either from Acros Organics or Fisher Scientific. All the reagents were of analytical grade and stock solution were prepared with DI water.

Soil collection and incubation

A typical silt loam soil (0-15 cm depth) from the Agricultural Experiment Station research farm at the University of Delaware was used in this study. The detailed information about the soil characterization has been reported in a previous publication. After removing any plant residues and granular rock particles, the soil samples were air-dried, homogenized, passed through a 2 mm sieve, and stored until analyses.

A flowchart of the experimental and analytical approach used is shown in Figure 1. The first degradation experiment was run to identify glyphosate and its degradation products in soil as well as to determine the degradation kinetics and half-lives of major products. The soil was incubated with 1 μmol/g unlabeled glyphosate at 20ºC in the dark with 60 % water content for 175 d. A separate experiment with

dual isotope (^{13}C and ^{15}N) labeled glyphosate (1 μmol/g) spiked in soil was performed for 35 d to accurately identify degradation products. The control experiment was performed under the same condition but without glyphosate. The natural soil incubation included both biotic and abiotic degradations. Identical experiment run with autoclaved water and soil served as abiotic degradation. At selected time points, 5 g subsamples were collected into 50 mL centrifuge tubes and stored at -20ºC until further analysis. All experiments were run in duplicate under the same condition.

In order to identify P distribution and bioavailability during glyphosate degradation, the second set of experiments was performed in two ^{18}O-labeled waters ($\delta^{18}O_{H2O}$ = -6.51 and + 18.27 ‰). To collect sufficient P for isotope analyses, 5 μmol/g unlabeled glyphosate was spiked into 300 g soil, and incubated with 600 mL ^{18}O-labeled water at 20ºC in the dark for 161 d. The spiked glyphosate concentration is much higher than application dose in agriculture (about 1 kg/ha), but is required to obtain reliable phosphate isotopic analyses. The experimental containers were tightly capped to avoid any water evaporation that compromises the water oxygen isotopes. The containers were shaken every day for ~15 min to homogenize the system and then briefly ventilated to replenish ambient oxygen and to preserve the oxic condition. The control experiments were run under the same condition but in the absence of glyphosate. Subsampling and processing followed a similar procedure as described above.

Extraction and analyses of glyphosate, AMPA, glycine, and sarcosine

The extraction of glyphosate, AMPA, glycine, and sarcosine was based on the published method. Briefly, 1 g lyophilized soil samples degradation experiments were extracted with 5 mL 0.6 M KOH for 1 h by shaking at 140 rpm, then centrifuged at 2755 x g for 30 min. One mL of supernatant was removed and neutralized by HCl and then 0.12 mL of borate buffer (pH=9) and 0.12 mL FMOC-Cl (12 g/L) were added and shaken for 1 min on a vortex mixer. After an overnight reaction at room temperature, the mixture was filtered with a 0.45 μm syringe filter for LC-MS analysis.

Glyphosate, AMPA, glycine, and sarcosine standards were prepared to develop the separation method by using an Acclaim 120, C18 column (2.1 x 250 mm) under a gradient eluent program. After testing and running several programs, the optimized gradient was identified to be effective with a mixture of two mobile phases with a flow rate of 0.35 mL/min with (A) acetonitrile and (B) 5 mmol/L HAc/NH_4Ac: 0-6 min, 20-40 % A, 80-60 % B; 6-9 min, 40-75 % A, 60-25 % B; 9-10.2 min, 75-100 % A, 65-0 % B; 10.2-12 min, 100 % A, 0 % B; 12-12.1 min, 100-20 % A, 0-80 % B; 12.1-14 min, 20 % A, 80 % B.

The chromatographic separation for each sample required 14 min.

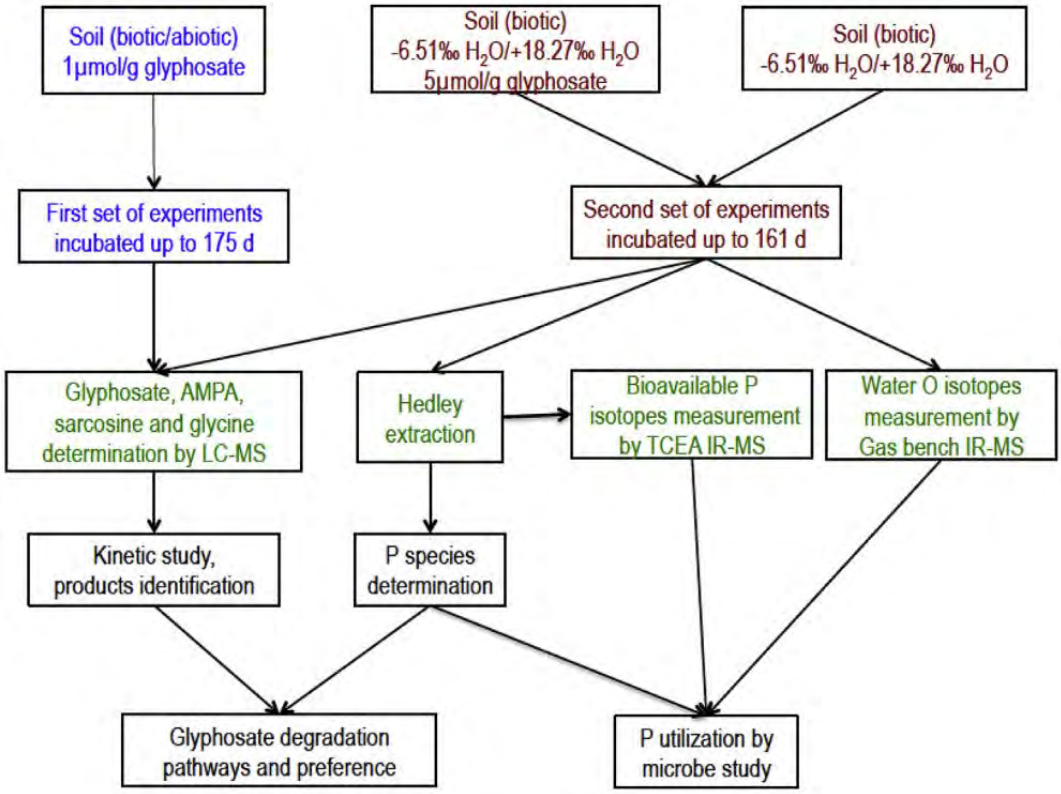


Figure 1. Flowchart outlining glyphosate degradation experiments in the water-soil system.

Glyphosate and its degradation products were identified and quantified by a Waters single quadrupole LC-MS equipped with PDA and SQ detector. The optimized MS parameters are as follows: ESI positive mode, capillary voltage 3 kV, cone voltage 40 V, desolvation temperature 200ºC, desolvation gas flow 650 L/hr, and full mass scan from 100 to 500 m/z. The unlabeled glyphosate and labeled sarcosine were quantified with labeled glyphosate and unlabeled sarcosine as internal standards. Similarly, labeled glycine was quantified by labeled glycine as an external standard to avoid any interference from glycine already present in soil. AMPA was determined by the soil spiked external standards. Labeled glyphosate degradation samples were analyzed with a high resolution mass spectrometry-Q Extractive Orbitrap Mass Spectrometry (Thermo, Germany) at the University of Delaware. Orbitrap MS data were acquired under the positive mode with scan range from 100 to 1000 m/z. Glycine formation during labeled glyphosate degradation were determined by external standard prepared by spiking labeled glycine in soil to avoid the interference of soil original glycine.

The extraction and derivatization methods for glyphosate, AMPA, glycine, and sarcosine were validated by spiking known amounts of these compounds in soil. The recovery ranged from 85 to 107 % for glyphosate, 79-93 % for AMPA, 74-88 % for glycine, and 80-97 % for sarcosine with RSD below 20 %, which is considered satisfactory. The limit of quantification (LOQ) for glyphosate and AMPA is 10 nmol/g soil and for glycine and sarcosine is 50 nmol/g in single quadrupole LC-MS, while it was largely improved by using Orbitrap (0.5 nmol/g).

Distribution of P derived from glyphosate into soil P pools

To differentiate and quantify the distribution of glyphosate-derived P in soil, samples from both control and glyphosate spiked soils (from the second set of experiments) were analyzed. A 0.3 g lyophilized soil was weighed and extracted with 30 mL DI water for 2 h using the modified Hedley et al. (1982) sequential extraction method (Tiessen et al., 1984). The supernatant was collected as H_2O extractable P_i (most labile P_i), and residual soil was extracted with 30 mL of 0.5 M $NaHCO_3$ for 16 h to collect labile and weakly adsorbed P_i. Inorganic P from those two pools represents microbially available P_i. The soil was further extracted for 16 h first with 30 mL of 0.1 M NaOH and then with 1 M HCl to obtain the NaOH extractable P_i (strongly sorbed P, fixed by Fe and Al oxides) and HCl extractable P_i (strongly

fixed Ca-P), respectively. The concentration of P_i in each pool was measured by using the phosphomolybdate blue method. The residual P in the soils after the completion of sequential extraction was quantified using ICP-MS.

Measurement of oxygen isotope ratios
Soil samples from control and glyphosate spiked (5 μmol/g) experiments with two ^{18}O-labeled waters were centrifuged first to extract waters to measure water oxygen isotopes ($\delta\,^{18}O_W$) by CO_2 equilibration method. The measurement was done in a Finnigan GasBench II coupled with an isotope ratio mass spectrometer (IRMS; Thermo, Darmstadt, Germany) in the Environmental Biogeochemistry Laboratory at the University of Delaware.

To understand the P bioavailability, the H_2O- and $NaHCO_3$- extracted P_i pools were combined and processed for the measurement of phosphate oxygen isotope ratios ($\delta\,^{18}O_P$). Five grams of lyophilized soil samples from the second set of degradation experiments were processed following the Joshi et al (2018) method to purify and finally convert P_i into silver phosphate. The O-isotope ratios were measured by a thermochemolysis/elemental analyzer (TC/EA) couples with IRMS. All isotopes from samples and standards were run at least in triplicate.

The measured $\delta\,^{18}O_P$ values of P_i were calibrated against two silver phosphate standards (YR 1aR-2 and YR 3-2, with the $\delta\,^{18}O_P$ values of -5.49 and +33.63 ‰, respectively). Similarly, the $\delta\,^{18}O_W$ values of porewater were calibrated with two USGS water standards ($\delta\,^{18}O_W$ values of -1.97 and -9.25 ‰, respectively). All isotope values are reported in per mil (‰) relative to the Vienna Standard Mean Ocean Water (VSMOW).

Results and Discussion (please refer to the section 3 of the original paper)

Degradation kinetics of glyphosate and its metabolites
The typical chromatography spectra of glyphosate, AMPA, sarcosine, and glycine are shown in Figure 2. Based on the LC-MS results, the concentrations of the compounds were calculated and are shown in Figure 3. Glyphosate gradually degraded over time and the extent of degradation reached >80 % by 35 d of incubation but traces of residual glyphosate were still detected until 175 d.

AMPA, the major metabolite of glyphosate, appeared after several days, accumulated during incubation, and reached its maximum concentration at 35 and 56 d in the experiment with 1 μmol/g and 5 μmol/g glyphosate, respectively. Afterwards, its degradation dominated over accumulation. Neither the degradation of glyphosate nor the formation of AMPA was observed in the sterilized soil incubation (abiotic only experiment), indicating microorganisms play a crucial role in degrading glyphosate in soils.

The degradation of glyphosate with time is often described according to first-order kinetics:

$$\ln(C/C_0) = -kt \qquad (1)$$

$$t_{1/2} = \ln 2/k \qquad (2)$$

where C_0 is the initial concentration, C is the concentration at time t, and k is the degradation rate constant. The maximum accumulated concentration of AMPA is used as its initial concentration since more than 80 % of glyphosate was degraded at the time. The results show that both glyphosate and AMPA degradation follow first order kinetics with a strong correlation coefficient (R^2 >0.85). The calculated half-lives of glyphosate under two sets of experiments are 28.9 and 31.5 d, respectively, consistent with the published results. A calculation based on the maximum amount of AMPA accumulated in the soil shows that the AMPA accounts for 48-68 % of the products from glyphosate degradation. It shows much longer half-lives (138.6 and 173.3 d), which highlights the high risk because of its toxicity and persistence in the environment.

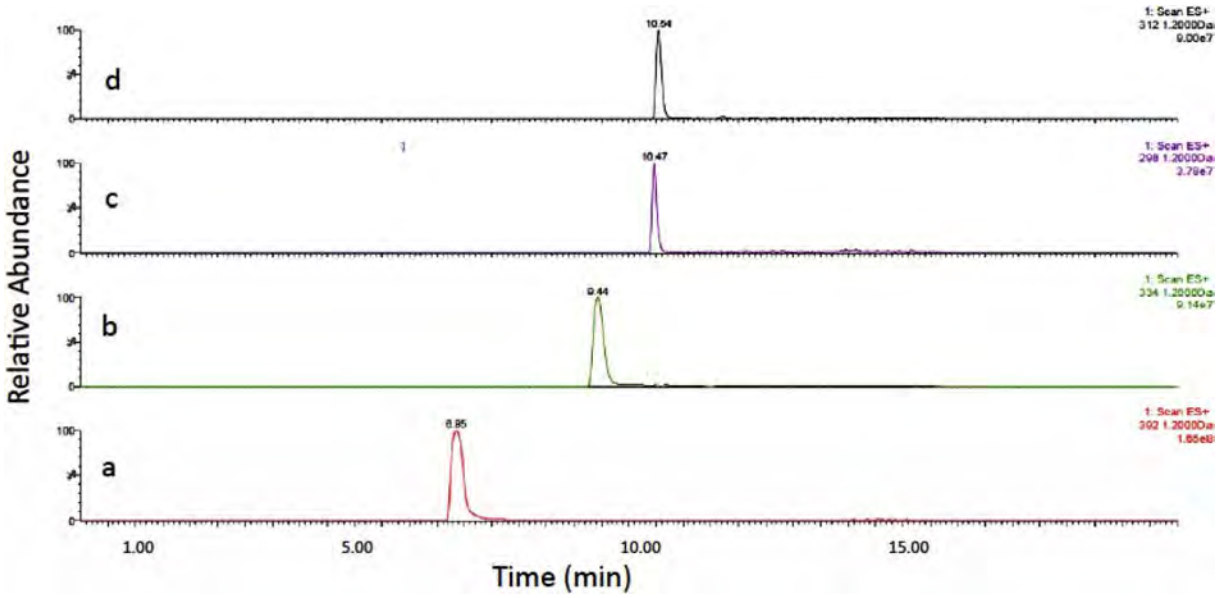


Figure 2. **Typical spectrum of glyphosate, AMPA, glycine and sarcosine analyzed by LC-MS (soil spiked with 1 µmol/g standards). a) glyphosate, b) AMPA, c) glycine, and d) sarcosine.**

Glycine is a common amino acid and commonly present in soil and other environment. The isotope labeled glyphosate provides the reliability of detection because the labeled element is present in glycine as well. Labeled glycine appeared only after few days, accumulated, and reached the highest concentration after 5 d and then decreased but was still detectable after 35 d incubation (Figure 3a).

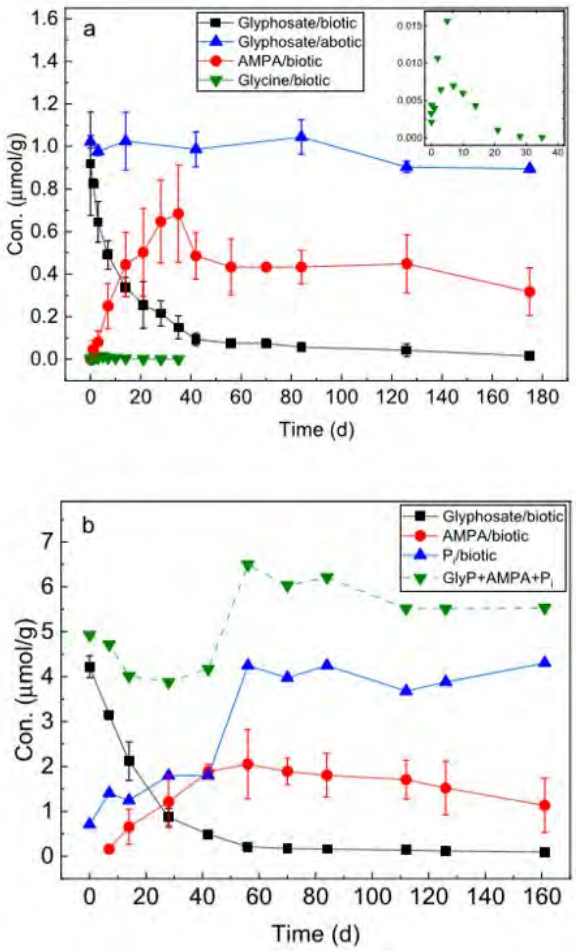


Figure 3. **Kinetics of glyphosate biotic (natural soil) and abiotic (sterilized soil) degradation and its products. a) incubated with 1 µmol/g glyphosate, and b) incubated with 5 µmol/g glyphosate. Please note that the natural soil incubation includes both biotic and abiotic components of degradation.**

The concentration of labeled glycine is low, probably due to glycine derived from glyphosate was readily incorporated into microbial biomass son after it formed. Results from a separate labeled glycine incubation experiment showed a rapid decline of soil-spiked glycine (1 µmol/g) with half-life of 0.89 d (Figure 4). Abiotic experiment showed no significant decline in glycine concentration in sterilized soil, validating methodology as well as indicating that soil microorganisms play a major role in glycine transformation. A recent study of labeled glyphosate reported the distribution of ^{13}C and ^{15}N into several amino acids including glycine, which our results corroborate. These findings, together, confirm that glyphosate derived glycine in the experiments should have rapidly utilized and metabolized by soil microorganisms.

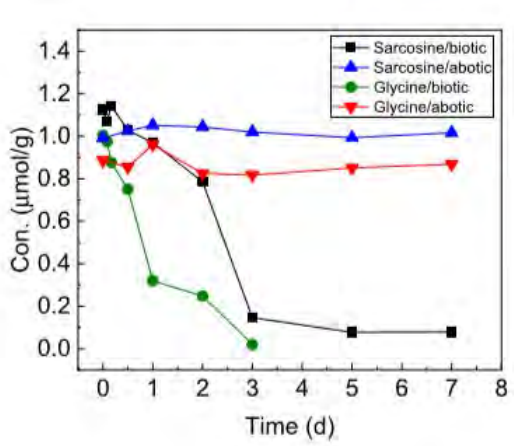


Figure 4. **Biotic (natural soil) and abiotic (sterilized soil) degradation of glycine and sarcosine in soil with spiked concentration of 1 µmol/g of each. Please note that the natural soil incubation includes both biotic and abiotic components of degradation.**

Sarcosine is a commonly recognized precursor to glycine during glyphosate degradation primarily on pure cultures that include bacteria isolated from soils, but rarely from the natural or simulated environments. In this study, sarcosine was not detected in any soil treatments including labeled glyphosate and high glyphosate (5 µmol/g) incubations. There might be three possibilities for the observed results: inefficient extraction from soil, fast oxidation of sarcosine, or presence below the detection limits of the analytical method. However, the recovery test performed by artificially spiking sarcosine in the same soil revealed that the method used could efficiently extract and accurately quantify sarcosine (yield 80-97 %). The individual incubation experiment showed that sarcosine could be degraded fast in the biotic experiment (with half-life of 0.99 d) but no significant decline in sterilized soil, which indicates degradation possible only by soil microorganisms. These lines of evidences suggest analytical method is not the reason, particularly since the high resolution Obitrap MS (detection limit of 0.5 nmol/g of sarcosine and glycine) was used. In the labeled glyphosate degradation experiments, soil samples were collected in several time points (0, 1, 2, 4 h, …until 35 d). The analytical method used successfully monitored the glycine formation and accumulation under extremely low concentration. If sarcosine was actually formed as a precursor to glycine, it should have detected by Obitrap MS since both sarcosine and glycine have similar half-lives. In a recent study, sarcosine was not detected in the abiotic degradation of glyphosate catalyzed by Mn minerals. These authors used advanced analytical methods including NMR, HPLC, and density functional theory (DFT) based electronic structure calculations and concluded that sarcosine was not a necessary intermediate product. Overall, the reliable extraction and analytical methods and intensive time point sampling verified that sarcosine was not formed during glyphosate degradation by soil microorganisms in this study.

Distribution of glyphosate-derived phosphorous in soil

Concentrations of four soil P_i pools in the control and glyphosate-spiked soils during the second set of incubations are shown in Figure 5. The experiments performed in two ^{18}O-labeled waters are considered duplicates because the difference in water oxygen isotopes does not impact the kinetics and extent of glyphosate degradation. Clearly, the control soil without glyphosate already contains high P_i and concentrations of P_i in different pools vary. It is noticeable, however, that the concentrations of P_i in these four pools remained essentially constant during the long-term incubation, with H_2O-P_i (1.01 ± 0.08 µmol/g), $NaHCO_3$-P_i (4.21 ± 0.23 µmol/g), NaOH-P_i (10.08 ± 0.91 µmol/g), and HCl-P_i (0.52 ± 0.06 µmol/g). This means that no significant transfer of P pools and organic-inorganic transformation occurred during the long-term incubation. The NaOH-P_i pool was the largest, indicating that Fe and Al minerals associated P is the major P sink in this soil, which is consistent with several other soils.

The results from the experiment in which glyphosate was spiked show that P_i derived from glyphosate

transferred into different pools, resulting in an increase of corresponding pool size. The maximum concentration of H_2O-P_i was 2.11 µmol/g at 70 d of incubation. The difference between control (soil without glyphosate) and glyphosate spiked soil shows that there was 1.06 µmol/g glyphosate-derived P_i transferred into this pool. Similarly, a significant net increase of P_i was observed in $NaHCO_3$-P_i (1.40 µmol/g), NaOH-P_i (1.93 µmol/g), and HCl-P_i (0.23 µmol/g) pools, with the highest P_i concentrations measured around 56-126 d of incubation. It is interesting that the order of P pool was the same as that in the original (control) soil: NaOH-P_i >$NaHCO_3$-P_i >H_2O-P_i >HCl-P_i. Calculated P mass balance shows that the total increase in P_i among 4 pools was 4.30 µmol/g at the end of incubation, which accounts for ~86 % of spiked glyphosate (5 µmol/g). The residual P in the control and glyphosate spiked soils were similar (7.99 ± 0.69 and 7.67 ± 0.69 µmol/g, respectively), indicating that there was no significant incorporation of glyphosate-derived P in the residual P pool. It also means that the Hedley extraction could efficiently extract almost all P and account P derived from biodegradation of glyphosate.

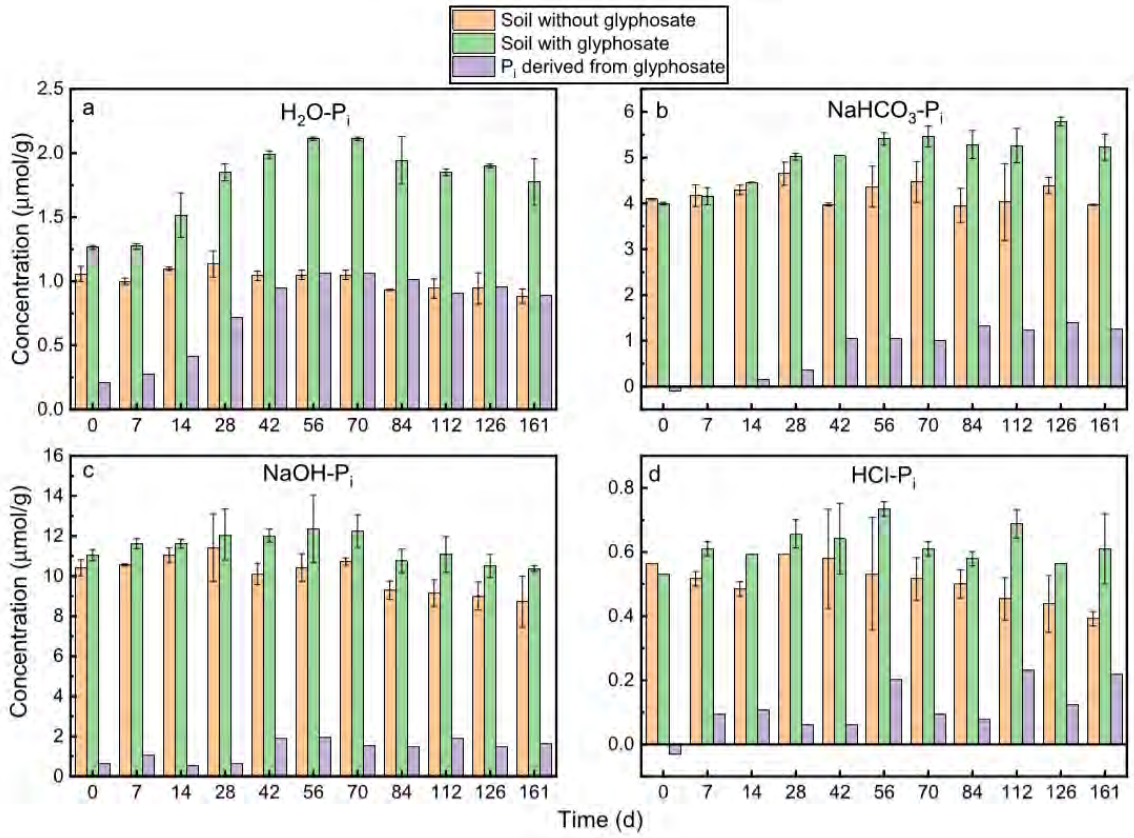


Figure 5. **Concentrations of P in different pools in original soil and glyphosate incubated soil during biotic degradation. H_2O and $NaHCO_3$ extracted P pools are considered bioavailable P in soil. Soil was spiked with 5 µmol/g glyphosate. Glyphosate derived P was calculated as the different between soil with and without glyphosate.**

In terms of distribution P_i derived from glyphosate (Figure 6), the H_2O- and $NaHCO_3$-P_i pools, which are considered readily available P_i for uptake by microorganisms and plant roots, received almost half (44 %) of it. Meanwhile, around 33-38 % of glyphosate P transformed into the NaOH-P_i pool, an unavailable or moderately bioavailable P pool depending on the soil P conditions and plant efficiency and time. This means that this conditionally unavailable P pool might be further transported into open water systems by leaching or soil erosion and could increase the risk of polluting waters. The HCl-P_i, which is not directly utilized by plants and microorganisms and normally remains as an unavailable P pool in agricultural soil, only received 3-5 % of P derived from glyphosate. These results highlight the fact that P load derived from a large amount of glyphosate application (with estimated 130 million kg used in the U.S.) cannot be ignored.

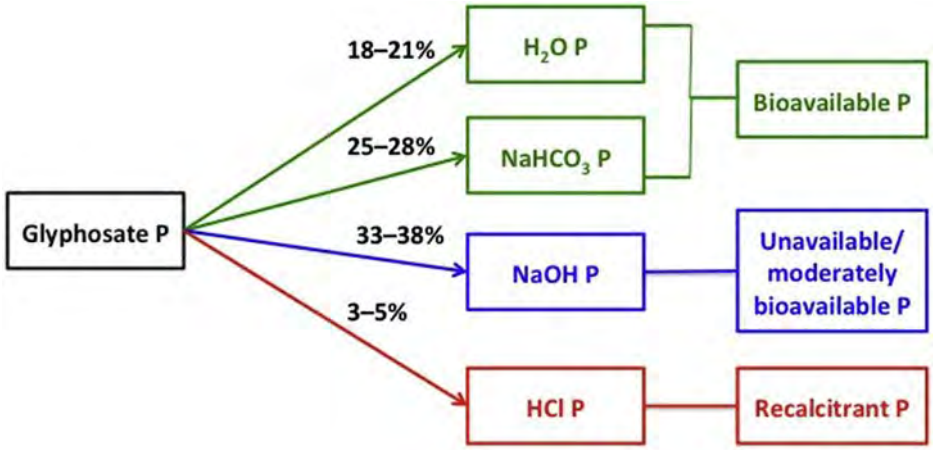


Figure 6. Distribution of glyphosate-derived P to different P pools during its biodegradation in the soil-water system. Soil incubated with 5 µmol/g glyphosate.

Given that the P_i derived from glyphosate is steady means that it was gradually released as the degradation continues and distributed more into the bioavailable pool, and it may be a better P source for plants. Phosphorus fertilizer is the major P supply for plants with estimated 4 billion kg used in the U.S in 2014 with 50-70 % use efficiency. However, its fast P release kinetics do not match the dynamic needs of different crop growth stages well and this offset causes nutrient loss from soil to aquatic systems. Given the slow but steady P release from glyphosate degradation, it might be slightly more synchronous than commercial fertilizers, but still too fast than plant needs. Furthermore, multiple sprays of glyphosate during the crop lifetime (average of 1.6 times per crop year) support the possibility of fractionating more into bioavailable P that plants can readily take up. This demands reconsidering glyphosate not only use as a herbicide but a bonus P source to crops and should be included in estimations of crop P needs to improve the P efficiency of plant uptake as well reducing the P loss from agricultural soils.

Bioavailability of glyphosate-derived phosphorus

Once inside the cell, P_i is involved in several metabolic reactions catalyzed by enzymes including incorporation into cell biomass and ATP-ADP conversion. One of the unique enzymes is pyrophosphatase (PPase), which is highly conserved across all three domains of life, catalyzes the hydrolysis of pyrophosphate into P_i. This is a reversible reaction and leads to exchange of all four O atoms in P_i with O in ambient water and thus achieves O-isotopic equilibrium between phosphate and water. The equilibrium isotope depends on the temperature and water oxygen isotope value.

To further test the bioavailability and rate of microbial utilization of glyphosate-derived Pi, phosphate oxygen isotopes ($\delta^{18}O_P$) of P_i in the soil incubated with and without glyphosate were measured and compared with the equilibrium isotope values calculated from the temperature and oxygen isotopes of water ($\delta^{18}O_w$) in the experiments. The $\delta^{18}O_w$ values remained constant at -6.51 ± 0.30 ‰ and +18.27 ± 0.12 ‰ for two ^{18}O-labeled water experiments in the long-term incubation except at the end of the experiment (161 d), when an inadvertent evaporation resulted in slight enrichment of isotopes (-4.90 ‰ and +20.71 ‰, respectively). The expected isotopic equilibrium value ($\delta^{18}O_{P\text{-}eq}$) was calculated based on the (Chang and Blake, 2015) equation as:

$$\delta^{18}O_{p-eq} = e^{\left(\frac{14.43}{T} - 0.0265\right)} \times (\delta^{18}O_w + 1000) - 1000 \qquad (3)$$

The $\delta^{18}O_{P\text{-}eq}$ values in the experiments incubated with -6.51 ‰ and +18.27 ‰ water are +15.83 ± 0.31 ‰ and +41.16 ± 0.12 ‰ (Figure 7), respectively, and remained constant during the incubation period (except at 161 d, in which water mass was not conserved). The starting isotope values of extracted P_i were consistent in all treatments: 20.77 ± 0.26 ‰, 21.02 ± 0.10 ‰, 21.38 ± 0.42 ‰ and 21.21 ± 0.16 ‰ in two controls (soil without glyphosate) and two glyphosate spiked experiments with -6.51 ‰ and +18.27 ‰ ^{18}O-labeled waters, respectively. It means that there are no different O sources or contaminants that might have impacted isotope values during the incubation period, besides the degradation of glyphosate.

The measured $\delta^{18}O_P$ values in the bioavailable P in ^{18}O spiked (+18.27 ‰) water became gradually heavier (Figure 7a), shifting towards the equilibrium values (+41.16 ‰) and reached 32.04 ‰ at the end of incubation. This result reveals the rapid uptake of the available P by soil microorganisms and the release of cycled P back to the soil. At the early stage, $\delta^{18}O_P$ values of P_i in the soil spiked with glyphosate were consistent with those in original control experiments. However, they became lighter after 14 d and remained 1.2-2.5 ‰ lighter for a long period. This is due to the contribution from a much lighter isotope value of P_i (~4-9 ‰) derived from glyphosate. The newly derived P_i from glyphosate degradation mixed with soil P_i pool and turned them into isotopic lighter and away from the equilibrium value (around +41.2 ‰). This result is consistent with P_i distribution that P_i was heavily released from glyphosate from 14 d to 84 d (Figure 5) and preserving isotope record of the lighter glyphosate derived P_i in the system (see Figure 5). However, the difference in isotope values between those two treatments gradually narrowed and eventually erased at 161 d, indicating that the soil microorganisms were efficient to uptake and cycle almost all of bioavailable P in the soil both from originally present soil and from glyphosate derived P_i.

The isotope trend in the experiments performed in -6.51 ‰ water (Figure 7b) is comparable to heavy water, but with a minor difference. For example, the $\delta^{18}O_P$ values in glyphosate spiked soil became much lighter and reached the equilibrium value sooner than those from control soil (without glyphosate). The reason is that the P_i derived from glyphosate carries much lighter $\delta^{18}O_P$ values (as explained above), which brings the isotope values close to equilibrium (which is lighter: +15.83 ± 0.31 ‰, due to the lighter water oxygen isotopes). The gap between the two treatments was 0.8 ‰, and then increased to 2.3 ‰ due to the large contribution of lighter isotopes of glyphosate derived Pi, but with the enhancement of microbe turnover, it decreased again but still 1.6 ‰ off at the end of the incubation.

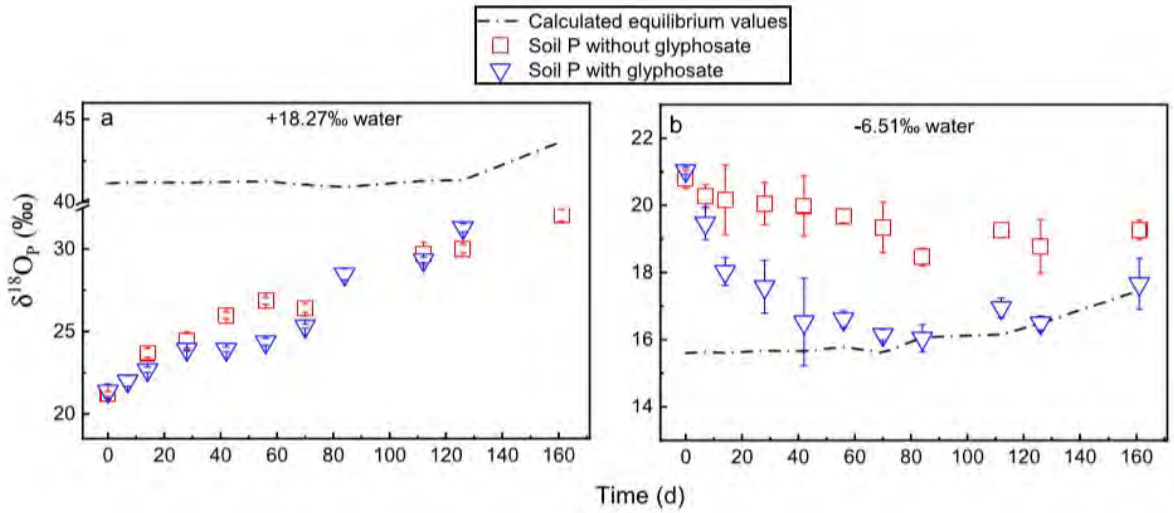


Figure 7. Changes in phosphate oxygen isotopes during glyphosate biodegradation in the water-soil system. The calculated equilibrium values assumes all P is completely recycled by microorganisms. The closer the isotope values toward the equilibrium values, the higher the extent of P cycling.

The observed results explained above provide several new insights on degradation of glyphosate

and its metabolites and recycling of glyphosate derived-P and together have several implications on the fate and impact of glyphosate in soils.

First, it proves that the isotope signature of glyphosate degradation can be detected in the experiments mimicking environmental systems. Second, it indicates that the degradation of glyphosate is faster than the microbial uptake and turn-over of P, so that the unique signature could be measured at the early to middle stage of the reaction.

Third, if the $\delta^{18}O_P$ values of the P derived from organic compounds are farther/closer to the equilibrium range compared to those present in-situ, they could easily shift/overprint bulk isotope value (due to mixing), leading to the inaccurate estimation of the biological activities.

Microbial turnover of P in the soil-water system

To evaluate the extent of P taken up and recycled by soil microorganisms, the P turnover was calculated from the starting $\delta^{18}O_P$ values ($\delta^{18}O_{P\text{-}t0}$) at 0 h, measured values at time t ($\delta^{18}O_{P\text{-}t}$) and the equilibrium values ($\delta^{18}O_{P\text{-}eq}$):

$$\%P\ \ turnover = \frac{(\delta^{18}O_{p-t} - \delta^{18}O_{p-to})}{(\delta^{18}O_{p-eq} - \delta^{18}O_{p-t})} \times 100 \qquad (4)$$

As the equation shows, the closer the values of $\delta^{18}O_{P\text{-}t0}$ to $\delta^{18}O_{P\text{-}eq}$, the higher the microbial turnover efficiency. The results show that P_i in the control experiment was rapidly exchanged by soil microorganisms and driven closer to the equilibrium values, with the turnover efficiency of 22-28 % at 56 d and 45-48 ‰ at 161 d in two ^{18}O-labeled waters (Figure 8). As expected, the efficiency of P turnover was similar irrespective of the starting isotopic values of ^{18}O-labeled water (-6.51 ‰ or +18.27 ‰).

In the glyphosate spiked experiments, the $\delta^{18}O_P$ value at time t ($\delta^{18}O_{P\text{-}t/spike}$) is the sum of glyphosate derived P_i ($\delta^{18}O_{P\text{-}t/gly}$) and the original P_i from control soil ($\delta^{18}O_{P\text{-}t/con}$), which can be calculated from a simple mass balance equation as follows:

$$\delta^{18}O_{p-t/spike} = x\delta^{18}O_{p-t/gly} + (1-X)\delta^{18}O_{p-t/con} \qquad (5)$$

where *x* is the fraction of P_i derived from glyphosate in the spiked samples. We calculated the starting isotope values of glyphosate derived P_i in two ^{18}O-labeled water systems at 0 h using previous results, which are +6.92 ‰ and ±12.14 ‰ in -6.51 ‰ and +18.27 ‰ waters, respectively. Based on the starting values of glyphosate-derived P_i, its microbial turnover was calculated using equation (4). As shown in Figure 8, the trend of P turnover in the soils receiving glyphosate-derived P_i was similar to that of control soil (without glyphosate), but the recycling efficiency was higher (67-75 ‰). Overall, phosphate oxygen isotopes allowed discrimination of sources and variable recycling efficiency of soil P vs glyphosate-derived P_i.

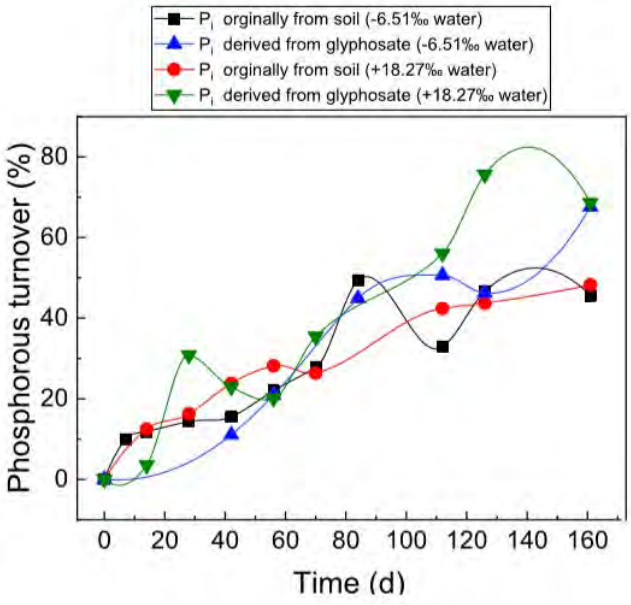


Figure 8. Microbial turnover efficiency of soil P and glyphosate-derived P.

Glyphosate degradation pathways in soil

To understand the degradation pathways and specific preferences in the soil system studied, the released P_i extractable from four pools were combined together. The total P mass from glyphosate source was also calculated by adding glyphosate, AMPA, and released P_i and are shown in Figure 3b. The released P_i steadily increased and reached the peak concentration around 56 d. AMPA remained at the accumulation stage and started to degraded at that time only when more than 80 % of glyphosate was already degraded. There was slight decrease in total P (from original concentration of 4.92 μmol/g) at the early stage of degradation, and then remained almost constant during the incubation period. Consider the efficient extraction of the glyphosate-derived P_i, it implies that there might be some other non-detected P speciation during the early stage of glyphosate degradation besides glyphosate, AMPA, and inorganic P. A potential P compound could be methylphosphonic acid, which can be generated synchronously if glycine forms directly from glyphosate. Based on the data generated in this study and foregoing assumptions and published results, revised pathways and temporal preference of glyphosate degradation in the soil-water system is proposed and shown in Figure 9. Under the action of soil microorganisms, at the early stage of degradation, glyphosate is cleaved at C(3)-N position to form glycine and methyl-phosphonic acid, the latter one is further degraded to form P_i, which accumulates in the system. Another bond cleavage occurs at C(2)-N position and form AMPA and glyoxylic acid. AMPA accumulates at the late stage of degradation. No sarcosine was generated in the soil-water system in this study, so it is not the required intermediate metabolite to form glycine.

Conclusion

In this study, we studied degradation glyphosate and its metabolites and successfully utilized phosphate oxygen isotopes to confirm the biological availability of glyphosate-derived P in the simulated soil-water system. The broader conclusions derived from this study and the implications thereof are as follows:

1) A satisfactory method of extraction and separation of glyphosate and its major metabolites in soil was developed, which could be used to identify the fate of glyphosate in a variety of environments. The absence of degradation in sterilized soil showed the soil microorganisms play the essential role on the degradation of glyphosate. Temporal presence of glycine and AMPA varied as well as their microbial uptake and degradation. AMPA was found to be 3-6 times resistant than glyphosate against degradation, which brings a higher concern to the safety of environment.

2) The distribution of glyphosate-derived P_i in a soil was investigated. About half of the glyphosate-derived P_i transferred into the readily bioavailable P pool. A slow but steady release of P_i from the degradation of glyphosate could mean that its supply could be slightly more synchronous with plant P demand during plant growth especially because it is applied more than one time during a crop cycle. This means that a higher proportion of glyphosate-derived P, than P from commercial fertilizers which release P all at once, could be taken up by plants.

3) Glyphosate-derived P_i has a distinct isotopic signature and can aid in identification of its source. The natural environment, however, is complex and could pose additional challenges, most likely due to the low content of glyphosate and inappropriate sampling time could miss to detect significant offset of isotope values. This is because isotope signature could be erased or overprinted due to biological cycling of glyphosate-derived P.

4) ^{18}O-labeling in water and application of phosphate oxygen isotope method allowed explicit understanding of microbial uptake and extent of biological turnover of glyphosate derived-P. The microbial turnover of original P in soil and glyphosate-derived P was comparable, but it was found that the microorganisms were more efficient to utilize and recycle glyphosate-derived P. The research tool developed could be further used to investigate the extent of microbial activities in soils and other natural environments.

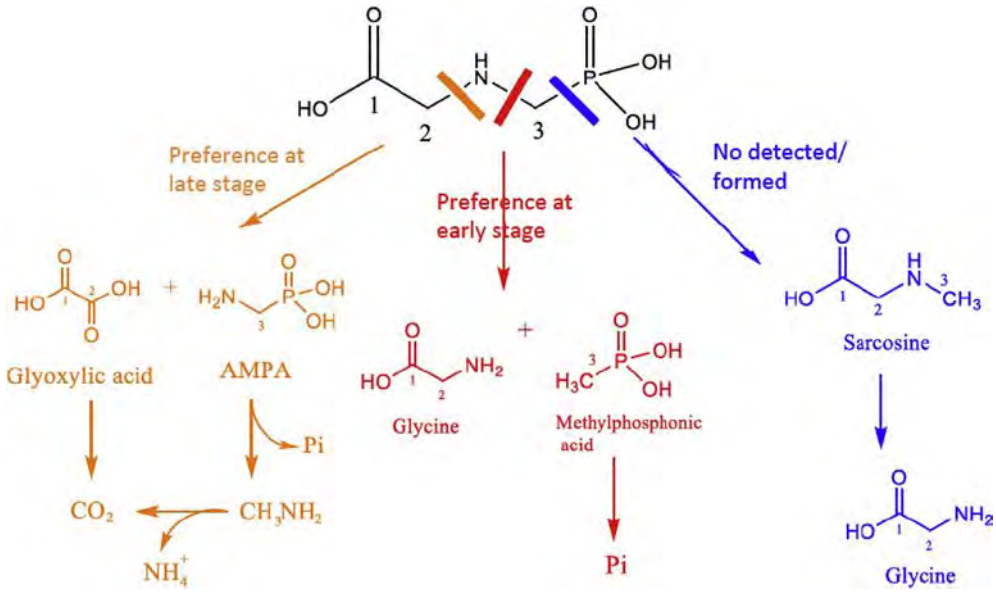


Figure 9. (Bio)degradation pathways of glyphosate and preference of degradation in the water-soil system used in this study.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a soil degradation experiment with glyphosate, where also AMPA was identified as metabolite. The main focus of the article is the transformation of glyphosate-derived phosphorous. In general, the methods and results are well described but the relevant experiment (incubation with 1 µmol/g non-labelled glyphosate for 175 d) shows some deviations from the OECD 307 study guideline: soil properties are not reported, exact soil water content during incubation is unclear, the mass of soil incubated is not clearly stated (1 g soil was used for extraction). Further, concentrations of glyphosate and AMPA are only presented in figures, not as tabulated values. DT_{50} values according to SFO were calculated for glyphosate and AMPA (based on max. concentration) but details on fits and statistics are not provided.

The article is therefore considered as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Tévez, H., et al.
Report year	2015
Report title	pH dependence of Glyphosate adsorption on soil horizons
Document No	Boletín de la Sociedad Geológica Mexicana Vol 67, Num. 3 , 2015, P. 509-516
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (the soil used for the experiment does not reflect European conditions (climate and soil characterization))

2. Full summary of the study according to OECD format

Pesticides bring many problems to the environment and to human health. The first rationale for their use is increased food production. Glyphosate N-(phosphonomethyl)glycine (PMG) is a non-selective, post emergent, and broad spectrum herbicide, very well known for its extensive application in agriculture worldwide. PMG adsorption experiments were carried out in three horizons of a Typic Haplustoll soil from the Province of Santiago del Estero, Argentina. Adsorption isotherms were fitted using Freundlich and Langmuir models. The affinity constants (K_F and K_L), the adsorption intensity (l/n) and the maximum surface coverage (Γ_{max}) were obtained. The results show the dependence of the parameters K_L and Γ_{max} with pH and also with the different horizons and particle size.

Materials and Methods

Chemicals
All chemicals utilized were of analytical reagent grade and were used without further purification. All solutions and soil dispersions were prepared using Milli-Q water. All PMG solution concentrations ranged from 0.05 to 10 mM prepared daily.

Study area
Climate is semiarid mesothermal, with an average annual temperature of 19.6 °C and rainfall of between 600 and 750 mm per year concentrated in the spring-summer period. Samples were taken up to 130 cm of depth from three very well differenced horizons classified as Ap (0 - 18 cm), AB (18 - 50 cm) and BC (105 - 130 cm).

Characterizations
The fresh soil samples were air-dried and ground. pH was measured in 0.0 1 M $CaCl_2$ solution. Organic matter (OM) content and soils chemical analysis were determined by the dichromate oxidation method. The available phosphorus (P) is the inorganic P, that is extractable at pH 8.5 and was determined following the experimental procedure described in Olsen et al., 1954 and Page et al., 1982. The total surface area (Sw) was measured by H_20 adsorption (Torres-Sanchez and Falasca, 1997). The total iron oxides (Fe_{tot}) and amorphous iron oxides (Fe_{amorph}) were established by dithionite (Holmgren, 1967) and oxalate method (McKeague, 1967), respectively. Soils samples were mixed with Lithium Metaborate/Lithium Tetraborate ($LiBO_2$, /$Li_2B_4O_7$) and fused in a furnace. The molten melt was completely dissolved in acidic media of 5 % nitric acid. This solution was analyzed for major and selected trace elements by Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES). The sample composition are reported as oxide percentage. The mineralogical composition and quantitative analysis of the soils were determined by X-ray Diffraction (XRD) and using the Rietveld method (Rietveld, 1969). Point of zero net proton charge (PZNPC) or point of zero salt effect (PZSE) is the pH where the net adsorption of protons and hydroxyl ions on the surfaces is independent of electrolyte concentration. Titration curves, when surface charge is plotted against pH, frequently showed a common intersect ion point that match with PZNPC.

Table 1. Characteristics of agriculture soils profile from Santiago del Estero/Argentina.

Horizon	pH ($CaCl_2$ 1:2.5)	OM ($g\ C.Kg^{-1}$)	P ($\mu g.g^{-1}$)	Sw ($m^2.g^{-1}$)	Fe_{amorph} ($mg.g^{-1}$)	Fe_{tot} ($mg.g^{-1}$)	PZNPC (pH)
Ap	5.90	23.30	43.34	188	0.239	1.66	7.1
AB	5.75	17.10	6.67	259	0.158	1.91	7.4
BC	6.02	12.10	1.19	242	0.095	0.99	8.1

Adsorption experiment

The adsorption of herbicide by the soils was studied using batch experiments. Solutions of different concentration of glyphosate was added to soil samples dispersions. Dispersions were kept in constant agitation overnight at constant pH, ionic strength and room temperature to reach equilibrium. The sample was filtered and adsorbed glyphosate was calculated from the difference between the total added ligand and the supernatant concentration (Ce). PMG was evaluated by ion chromatography. Two plastic anion columns were coupled in series to serve both as pre-column and analytical chromatographic column. The typical experimental error is lower than 5 % for all results.

Table 2. Chemical Analysis of agriculture soils profile from Santiago del Estero, Argentina.

Horizon	Chemical Analysis (%)						
	SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	Na_2O	K_2O
Ap	64.6	12.25	3.51	1.47	1.14	1.70	2.43
AB	63.0	13.65	4.10	1.34	1.40	1.50	2.53
BC	63.0	14.05	4.42	1.44	1.62	1.56	2.66

pH effect

The pH dependence of the glyphosate uptake by soil horizons was investigated using batch isotherm experiments in a pH range from 2 to 8 with a soil concentration of 9.1 g/L and different initial concentrations of PMG at a constant ionic strength of 0. 1 M of KNO_3. The pH was measured using a Metrohm 644 pH-meter with a combined glass microelectrode. Adsorption experiments were conducted in triplicate following the procedure described above. There were no significant differences within each replicate ($p < 0.0\ 1$). The expressed values represent the average of the obtained results.

Isotherms Modeling

The relationship between the ligand uptake and the sorbate equilibrium concentration as constant temperature is known as the adsorption isotherm. The adsorbent capacity of a certain material is related to the material balance adsorption: the sorbate that disappears from solution must be in the adsorbent. Freundlich and Langmuir models were chosen and applied for describing the equilibrium data.

Table 3. Mineralogical Composition of agriculture soils profile from Santiago del Estero, Argentina. Values in parenthesis represent estimated standard deviations.

Horizon	Mineralogical Composition (%)				
	Quartz	Sanidine Feldspar	Andesine Feldspar	Illite	Magnetite
Ap	45.2 (0.4)	9.6 (0.9)	24.7 (0.8)	18.6 (1.4)	1.3 (0.2)
AB	39.8 (0.5)	9.6 (0.8)	23.5 (0.7)	25.9 (1.5)	1.2 (0.3)
BC	46.2 (0.4)	9.3 (0.9)	19.9 (0.9)	24.7 (1.3)	1.2 (0.3)

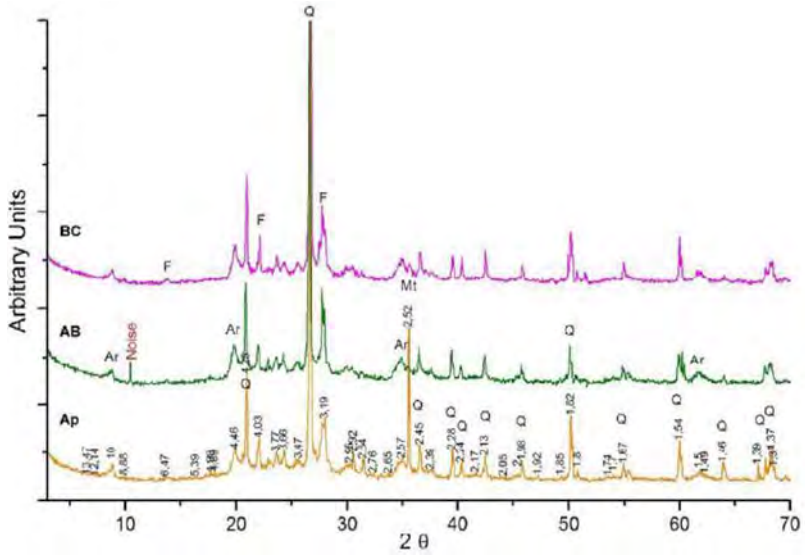


Fig. 1. XRD or the three soil horizons. Q: Quartz, Ar: Clay, F: Feldspar, Mt: Magnetite.

Results and Discussion

Soil characteristics, chemical analysis, mineralogical composition and quantitative analysis are presented in Table 1, 2 and 3 respectively. XRD of the three soil horizons are shown in Figure 1. The experimental curves of PZNPC recorded for the BC horizon are illustrated in Figure 2. Similar behavior was found for all the horizons that showed PZNPC values in the range of 7.1 - 8.1 (Table 1) following the sequence: Ap<AB<BC. PZNPC value can be explained by the absence of clay minerals with a negative permanent charge, while the presence of 2: 1 clays shift the PZNPC to lower pH values (Table 3). The higher PZNPC value for the horizons corresponds to horizon BC that contains similar amount of quartz, lower amount of feldspars (andesine) and high amount of illite. PZNPC increase with andesine feldspar content and OM decrease. The determination coefficients of a linear fit were $R^2_{andesine} = 0.9971$ and $R^2_{OM} = 0.9189$. The analysis of the three parameters variations in a 3D plot presented a determination coefficient of $R^2 = 1.0000$ and a constant variance test of $p < 0.0001$. The PMG adsorption isotherms of soils dispersions equilibrated at different pH values are shown in Figure 3. The Freundlich model parameters values (K_Γ , and l/n) were calculated and are given in Table 4. The 1/n values vary between 0.1 and 1, which indicates that this model could be used for interpreting the data. The correlation between experimental and calculated curves had a p-level between 0.137 and 0.0035; the determination coefficients were between 0.7578 and 0.9953 for different pHs and horizons.

The Langmuir model was also applied to make an interpretation of PMG adsorption isotherms on soil dispersions equilibrated at different pH values. This is shown in Figure 3, where solid lines are calculated using this model and Γ_{max} and K_L are given.

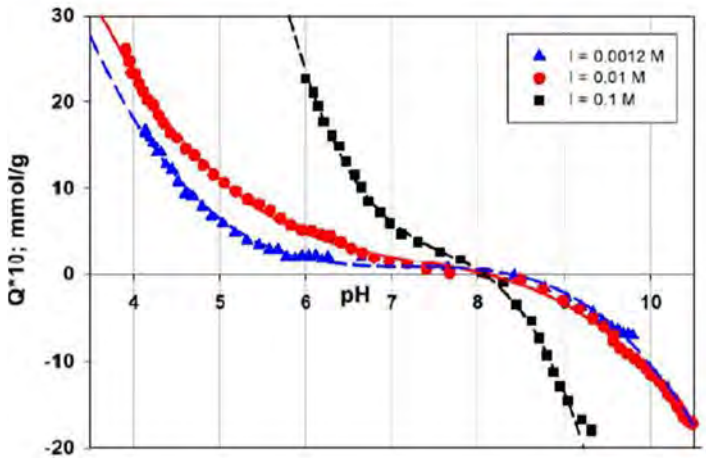


Fig. 2. Potentiometric titration curves or the dispersions or the BC horizon at three ionic strengths ($I = \frac{1}{2}\Sigma_i C_i Z_i$).

The isotherm model parameters were obtained by a non-linear optimization using the Solver-Excel tool. The parameters values were obtained from the plot of the inverse of the surface coverage as a function of the inverse of the equilibrium concentration. Results of the adsorption and surface coverage calculations were normalized with Sw data and the various horizons were contrasted. The correlation between experimental and calculated curves had a p-level between 0.050 and 0.001; the determination coefficients (R^2) obtained were between 0.9300 and 0.9999; and were higher than those obtained using the Freundlich model. Thus, the Langmuir model would better represent the adsorption process of PMG on the Santiago del Estero Province soil.

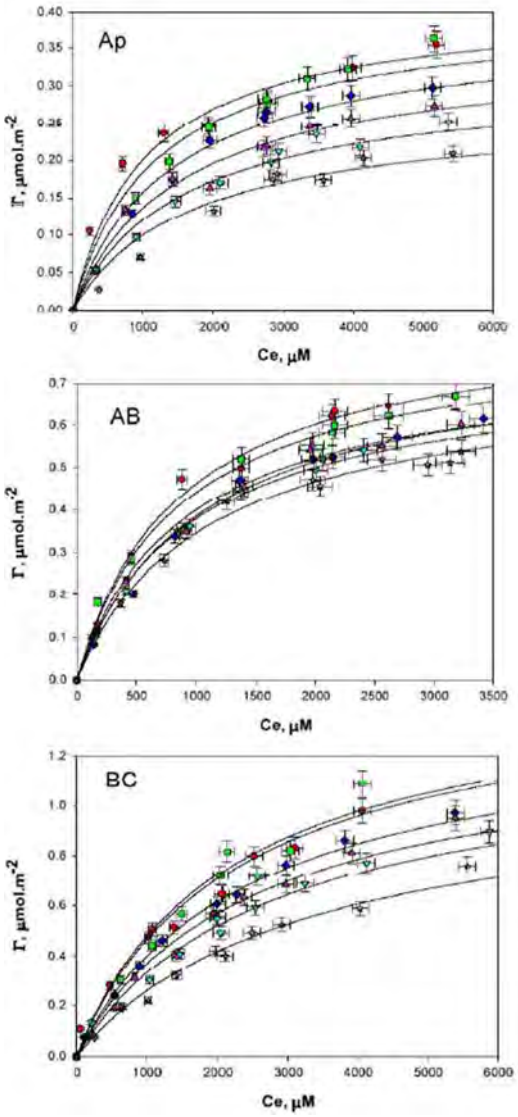


Fig. 3. Adsorption isotherm or PMG on horizon Ap, AB and BC. Solid lines are calculated using Langmuir model with constants and maximum surface coverage detaled in Table 5 . •: pH 2, •:pH 3, +: pH 4, : pH 5, pH 6 and * : pH 8.

The dependence of the surface coverage with PMG concentration in the various horizons at constant pH = 5 is shown in Figure 4. Horizon Γ_{max} sequence is Ap<AB<BC. This behavior is similar to those found for PZNPC. The dependence of the surface coverage with pH in the various horizons is also shown in Figure 3. The adsorption capacity increases from pH 8 to 2. This pH effect was normally observed during the adsorption of anionic species. Consequently, PMG interaction with the surface occurs throughout the anionic chemical groups (carboxylate or phosphonate) and not through the amine gr up (pKa = 10.14) that is positively charged at the studied pH range (Figure 5). The surface coverage decrease, $\Delta\Gamma_{max}$ for horizon Ap is around 41 % for this pHs range (Table 5). This difference is lower for

horizons BC, 27 % , and AB, 12 %. The highest adsorption capacity is obtained by horizon BC followed by horizon AB, and the lowest for horizon Ap. A similar sequence was obtained for PZNPC (Table 1), indicating that the horizon with higher positive surface charge presents higher PMG surface coverage. The ratio of the Γ_{max} of the horizons ($R_{H1/H2}$) was calculated where H1 and H2 denote two different horizons, Γ_{maxH1} and Γ_{maxH2} indicate the maximum coverage of H1 and H2 horizons, respectively. This ratio between the horizons BC and AB was $R_{BC/AB}$ = 46 %, between horizons BC and Ap was $R_{BC/AP}$ = 72 % and between horizon AB and Ap was $R_{AB/Ap}$ = 50 %. These percentages are opposed to the phosphate content that follows the order Ap > AB > BC. The highest adsorption constants correspond to horizon AB (Table 5). The changes in the adsorption affinity between horizon BC and AB reach ΔK_L = 46 % while horizon BC decreases 73 % in respect to horizon Ap.

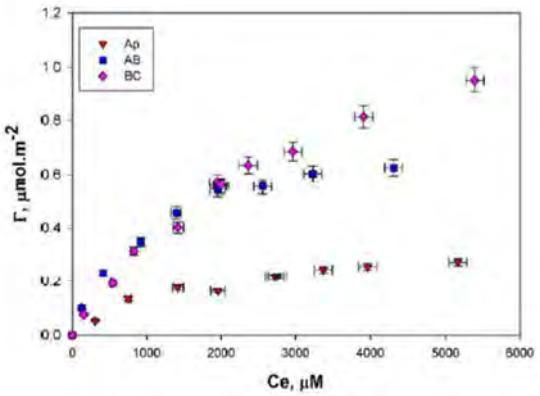


Fig. 4. Adsorption isotherm of PMG on horizon Ap, AB and BC at pH 5.

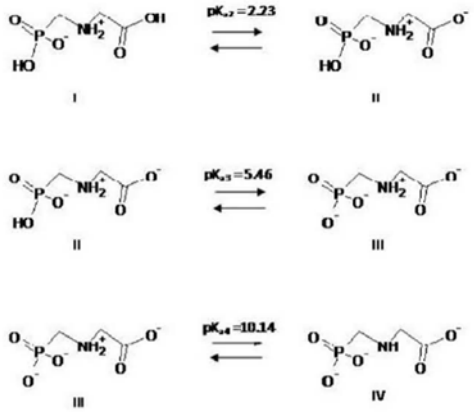


Fig. 5. PMG acid-base equilibrium.

The greater slope of the adsorption curves in the AB horizon indicate that PMG binds more strongly to the active sites of this horizon. Thus, the active site of PMG adsorption on the AB horizon could be the surface iron atoms and the higher adsorption in this horizon is directly related to higher iron content.
The adsorption on horizon BC does not reach maximum coverage in experimental conditions. The adsorption isotherms with a low initial slope describe an adsorption process with characteristic adsorption constants of low energy interaction (Figure 3). The constant and the equilibrium reactions of acid-base dissociation of glyphosate (Barja and dos-Santos-Afonso, 1998) are shown in Figure 5, where I, II and III are the main species presents in the studied pH range.

Table 4 . Freundlich parameters (in $\mu mol^{1-1/n}.m^{-2}$) for glyphosate adsorption on Santiago del Estero Province soils.

Horizon		Ap			AB			BC	
pH	K_F*10^3	1/n	R^2	K_F*10^3	1/n	R^2	K_F*10^3	1/n	R^2
2	7.3	0.46	0.9449	19.7	0.44	0.9743	5.3	0.64	0.9842
3	6.7	0.47	0.9953	18.4	0.45	0.9894	4.9	0.65	0.9759
4	5.9	0.47	0.9646	16.2	0.46	0.9654	4.7	0.63	0.9852
5	5.6	0.46	0.9729	16.2	0.45	0.9652	4.6	0.63	0.9892
6	5.0	0.48	0.7578	15.1	0.46	0.9862	4.3	0.62	0.9812
8	3.8	0.47	0.9453	14.3	0.45	0.9749	3.0	0.64	0.9893

Conclusions

The major factor in PMG adsorption on soil samples is given by the pH, which could be due to the influence of this parameter on the PMG molecule and on the surface charge of the soil particles. PMG adsorption increase with acidity, and this increase correspond to the adsorption of a ligand with a negative net charge. Sorption of glyphosate in soils is similar to the adsorption of the organic molecule on the soil components such as clay minerals, iron oxides and OM. For these soils with a low organic matter contents and/or similar amounts of clay in the various horizons, the adsorption would be determined by the content of phosphorous, iron oxide and the specific surface. Regarding the relative adsorption capacity of the soil, the adsorption process has a different behavior profile, where the deeper horizon (BC) has a higher capacity retention for this herbicide.

Table 5. Langmuir parameters for PMG adsorption on soils or Santiago del Estero Province, Argentina

Horizon		Ap			AB			BC	
pH	Γ_{max}, $\mu mol.m^{-2}$	K_L, $L.mmol^{-1}$	R^2	Γ_{max}, $\mu mol.m^{-2}$	K_L, $L.mmol^{-1}$	R^2	Γ_{max}, $\mu mol.m^{-2}$	K_L, $L.mmol^{-1}$	R^2
2	0.41	0.91	0.9572	0.81	0.93	0.9936	1.50	0.40	0.9331
3	0.40	0.87	0.9723	0.82	0.93	0.9833	1.49	0.42	0.9664
4	0.38	0.73	0.9874	0.78	0.92	0.9925	1.36	0.41	0.9955
5	0.34	0.67	0.9769	0.76	0.92	0.9932	1.27	0.40	0.9854
6	0.31	0.63	0.9633	0.74	0.91	0.9974	1.23	0.39	0.9858
8	0.24	0.61	0.9424	0.72	0.90	0.9926	1.10	0.28	0.9830

The lower adsorption in the AB and Ap horizons could be influenced by the higher content of phosphorus. However, the strength of the interaction, as given by the Langmuir Model Constant K_L is larger on horizon A B and would be linked to the illite and iron oxide content that have a better distribution in AB. It should be noted that the Langmuir adsorption model is the best fit to the adsorption experimental results in these soils, although the Freundlich model has a good fit for some pHs. Given the adsorption extent found in this study, it is expected that pesticides will be retained in these soils. This strong interaction could prevent the pesticides movement into the ground water. On the other hand, this retention rate could result in the release of the herbicide on the environment due to displacement by runoff.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes the adsorption of non-labelled glyphosate on an agricultural soil from Argentina. The pH-dependency was evaluated in addition. The soil and climate conditions do not reflect European conditions. However, the soil characterization and the results for adsorption, Freundlich isotherm and pH dependency are well described.

The study is therefore classified as relevant with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.2.2.1
Report author	Todorovic, G., et al.
Report year	2014
Report title	Influence of soil tillage and erosion on the dispersion of glyphosate and aminomethylphosphonic acid in agricultural soils
Document No	International Agrophysics, 2014, 28, 93-100
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (study design not sufficiently described to relate results to real field conditions, details on analytical method and statistics are missing)

2. Full summary of the study according to OECD format

Erosion processes can strongly influence the dissipation of glyphosate and aminomethylphosphonic acid applied with Roundup Max® in agricultural soils; in addition, the soil structure state shortly before erosive precipitations fall can be a key parameter for the distribution of glyphosate and its metabolite. Field rain simulation experiments showed that severe erosion processes immediately after application of Roundup Max® can lead to serious unexpected glyphosate loss even in soils with a high presumed adsorption like the Cambisols, if their structure is unfavourable. In one of the no-tillage-plot of the Cambisol, up to 47% of the applied glyphosate amount was dissipated with surface run-off. Moreover, at the Chernozem site with high erosion risk and lower adsorption potential, glyphosate could be found in collected percolation water transported far outside the 2x2 m experimental plots. Traces of glyphosate were found also outside the treated agricultural fields.

Materials and Methods

The experiments were carried out where following soil tillage systems were compared in 3 field replications:
– conventional tillage (CT) with plough with and without cover crop during winter period;
– no-tillage (NT) with cover crop during winter period.
The investigated soils were a Chernozem from loess at Pixendorf and a sandy stagnic Cambisol from tertiary carbonate free sediments at Kirchberg, Austria. In order to investigate the influence of erosion and tillage on glyphosate and AMPA, two rain simulation experiments were conducted in 3 field replications (1, 2, 3) within the CT and NT plots. For this, Roundup Max® was applied onto rain simulation soil plots according to the common agricultural practice (2% herbicide solution. In both sites, the vegetation cover degree was typically higher in the NT-plots (80-100% of weed cover) than in the CT-plots (only few yield residues of maize) and the application was carried out in sunny and not windy weather shortly before starting the rain simulation experiment (worst case scenario). The average slope in both sites was 12-15% at the Cambisol and 10% at the Chernozem. Both sites are known as rather erodible. The soil surface of the Chernozem immediately before the rain simulation was crumby; in turn, the cambisol had a crusted, dry, and cracky surface. The rain simulator was designed as a potable equipment, the spray pattern was generated by full jet nozzles, the rain fall intensity was controlled with intermittent spraying.

During 60 min of rain simulation with 30 mm, run-off fractions were collected at different time intervals at the Chernozem and averagely at the Cambisol and cooled in boxes. In the laboratory, the run-off samples were immediately centrifuged to separate the liquid from the solid phase. Immediately after the rain simulation, soil samples were collected within the simulation soil plots of 2x2 m at different depths

(0-2, 2-5, 5-10, 10-15, and 15-20 cm at the Chernozem and at 0-2 and 2-5 cm at the Cambisol). Glyphosate and AMPA were analyzed according to Rampazzo *et al.*, 2013. All physical and chemical analyses on soil samples were carried out according to the standard methods.

Table 1. Fe-oxide distribution in the investigated soils

Site	Soil type (WRB)	Depth (cm)	Fe_o (mg kg^{-1})	Fe_d (mg kg^{-1})	Fe_o/Fe_d
Pixendorf	Chernozem	0-5	983	7 970	0.12
		5-20	1 040	8 378	0.12
Kirchberg	Cambisol	0-5	3 422	14 843	0.23
		5-20	3 726	15 032	0.25

Fe_o – amorphous (weakly crystallized) Fe-oxides, oxalate-soluble; Fe_d – well crystallized Fe-oxides, dithionite-soluble.

Results and Discussion

The Chernozem shows the development from loess with typical silty texture (topsoil 0-20 cm, 12% clay, 65% silt, 23% sand, pH 7.3, 15% $CaCO_3$ and 3% OM), whereas the Cambisol is a loamy sandy soil (topsoil 0-20 cm, 14% clay, 33% silt, 53% sand, pH 5.7, no $CaCO_3$ and 3% OM). The Chernozem exhibited a low content and the Cambisol a high content of Fe oxides and therefore the expected sorption capacity for glyphosate and AMPA was theoretically higher at the Cambisol (Table 1).
Figure 1 shows the amount of total (liquid and solid) run-off after the rain simulation experiments on the Chernozem. Before glyphosate and AMPA were analyzed, a separation of the solid and liquid run-off phase in the laboratory was carried out. The CT-plots produced the highest run-off amounts because of their lower protecting weed cover, causing a splash of the surface by the erosive precipitation with consequent loss of infiltrability. On the other hand, the amount of runoff at the Chernozem was 10 times lower than the Cambisol because of its crumby structure with a better infiltration rate during the rainfall simulation, whereas the soil surface of the Cambisol was compacted and crusty. The different amounts of run-off between the 3 field replications of the Chernozem (Fig. 1) were due to the inhomogeneity of the field conditions. Consequently, the total amount of glyphosate washed out of the plots by liquid run-off at the Chernozem was much higher in the CT-plots than in the NT-plots (Fig. 2).

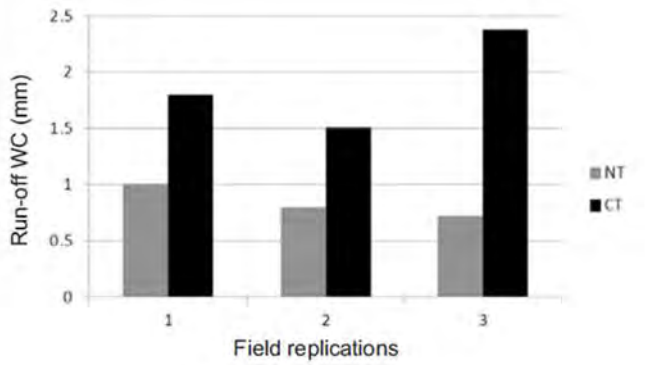


Fig. 1. Chernozem: total run-off of the conventional tillage (CT) and no-tillage (NT) plots in the 3 field replications, WC – water column.

A fractionation of the time-dependent glyphosate contents in run-off-fractions of the Chernozem at time intervals of 15 min is shown in Fig. 3a. As it was expected, the first fraction showed the highest contents in both variables CT and NT and then decreasing with time. The CT-plots showed again higher glyphosate contents than the NT-plots, which instead showed higher glyphosate concentration (less dilution) at the same time (Fig. 3b).

According to Gjettermann *et al.,* 2011, desorption kinetics are important for evaluating the significance of dissolved and particle-facilitated transport of glyphosate. Consequently, the separation from water and solid phases should be done within a short time of minutes. We managed to do this within 30 min from field sampling. The contents of glyphosate and AMPA in the solid phase of run-off in the Chernozem are shown in Figs 3c,d. The glyphosate contents retained by the run-off sediment is an analogue to that in the total and fractionated runoff (Figs 2, 3a), where the first collected fraction of run-off sediment contains the highest amounts of glyphosate which then generally decreases in the following fractions and the CT-plots shows higher amounts than the NT-plots. Analogous is the distribution of AMPA in the sediment (Fig. 3d). Since the loss of glyphosate by run-off was higher in the CT-plots (Fig. 3a), the amount of glyphosate and AMPA adsorbed by the Chernozem immediately after the rain simulation experiments was consequently higher in the NT-plots (Fig. 4). Moreover, there is a clear depth function of the adsorption of glyphosate and AMPA through the soil immediately after Roundup Max® application and rainfall simulation at the Chernozem. The glyphosate and AMPA contents clearly decreased with soil depth.

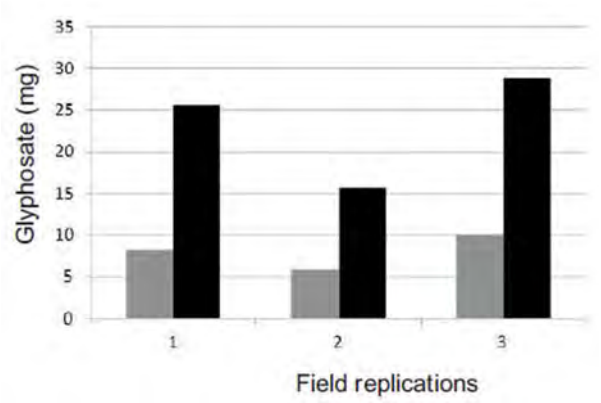


Fig. 2. Chernozem: total amounts of glyphosate in liquid run-off at the 3 field replication plots. Legend as in Fig. 1.

The Chernozem had a favourable crumby structure in the NT-plots, with no cracks, no preferential flow, and optimal conditions for water retention in the upper soil layers at the moment of the rainfall simulation experiment, so that more than 50% of the adsorbed glyphosate was retained in the first 5 cm of the soil. The fact that AMPA could already be detected 1 h after the Roundup Max® application underlines the quick glyphosate degradation in soil, as reported by Mamy *et al.*(2005) as well. The total (liquid and solid) amount of surface run-off in the Cambisol is shown in Fig. 5. The Cambisol had a dry, crusty, and very deeply cracky soil surface of the CT-plots before starting the rainfall simulation and therefore the first amount of the precipitation quickly infiltrated in the cracks, but very soon a splash process and loss of infiltration took place due to the fine sandy texture and low surface protection by weeds. This led to a higher surface run-off of the CT-plots than the NT-plots. Consequently, Figs 6 and 7a show that the contents and concentrations of glyphosate in the liquid run-off of the NT-plots of the Cambisol were much higher than in the CT-plots. In the dry and cracky soil surface of the CT-plots, it took some time before run-off started and glyphosate could easily enter deeper into the soil; on the other hand, the NT-plots had a nearly 100% weed cover, as reported also by Locke and Bryson (1997); consequently, this might buffer potential effects of glyphosate in the soil (Locke *et al.,* 2008).
In this study, most of the applied glyphosate adhered to the photosynthetically active plant organs (stem and leaves) immediately after application; consequently, glyphosate was literally washed out of the 2x2 m simulation plots with runoff and had less time to infiltrate the soil surface (Fig. 8a). Based on the high content of pedogenical Fe-oxides (15 000 mg Fe_d/kg, Table 1), high soil adsorption of glyphosate was expected for the Cambisol. The surprisingly high loss of glyphosate by surface run-off (in one of the 3 field replications about 47% of the applied glyphosate) measured in this study confirmed the crucial effect of soil structure and preferential flow on the dissipation of glyphosate after heavy erosive precipitations, which were also be observed by other scientists. The contents of glyphosate and AMPA in the solid phase of run-off at the Cambisol are shown in Figs 7b, c, respectively. The concentrations of glyphosate and AMPA in the solid phase of run-off at the Cambisol are similarly distributed to the

corresponding aqueous fractions of run-off; they are mostly higher in the NT-plots than in the CT-plots. Figure 8 shows the content of glyphosate and AMPA adsorbed by the soil immediately after the rain simulation experiments at the Cambisol. Immediately after the rain simulation experiment, a very clear distribution in the soil appears: glyphosate and AMPA are first adsorbed in the upper 0-2 cm of the soil and only a small amount reaches the next soil depth of 2-5 cm. In general, the NT-plots show a clearly lower content of glyphosate and AMPA as compared to the CT-plots. This is explained by the respectively higher glyphosate contents in run-off of NT-plots (Fig. 6). The soil losses of the Chernozem and Cambisol through erosion processes are shown in Fig. 9.

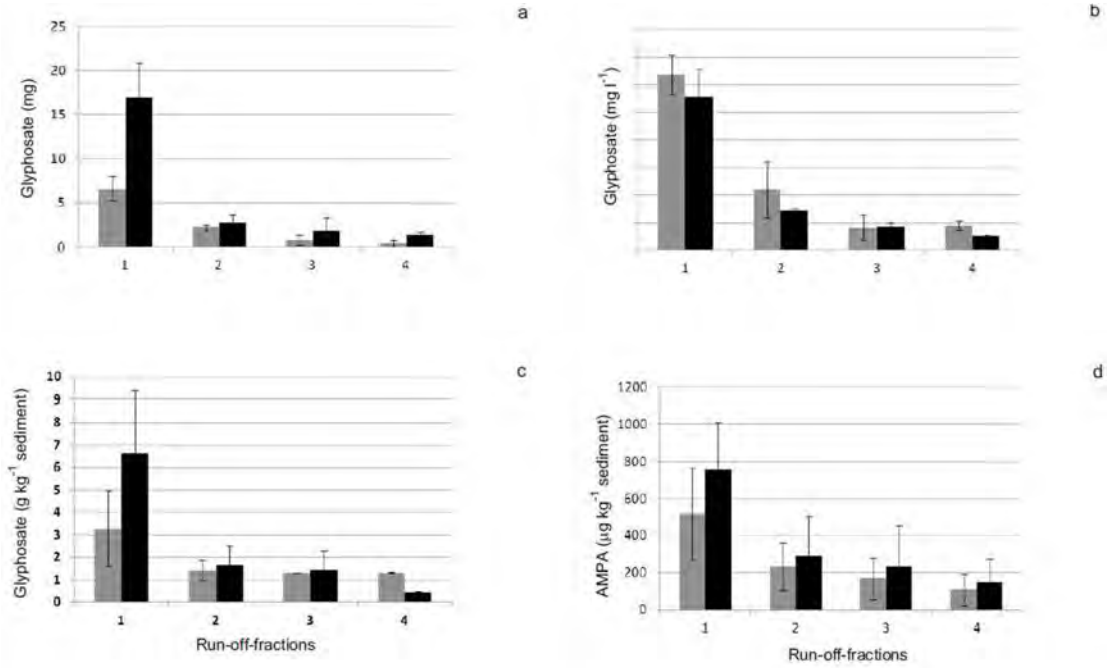


Fig. 3. Chernozem: a – glyphosate amount, b – glyphosate concentrations in liquid, and c – glyphosate contents, d –AMPA contents in the solid phase of run-off-fractions at 15-min intervals (average of 3 field replications). Legend as in Fig. 1.

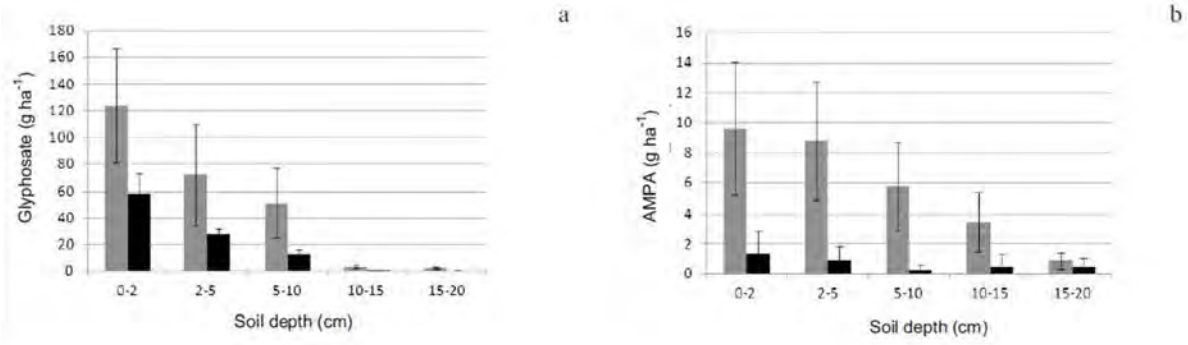


Fig. 4. Chernozem: a – glyphosate contents, and b – AMPA contents in the soil within the rain simulation plots (average value from the 3 field replications). Legend as in Fig. 1.

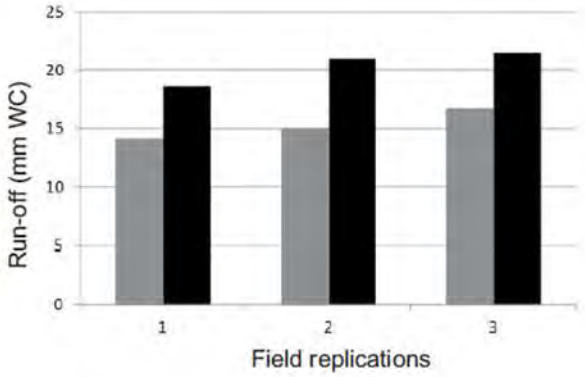


Fig. 5. Cambisol: total run-off of the CT- and NT-plots in the 3 field replications, mm WC – mm water column. Legend as in Fig. 1.

At both sites, the soil loss from the CT-plots, measured as sediment in the surface run-off, was higher than from the NT-plots because of the much lower vegetation cover before the simulation experiment, splash, and reduction of infiltration. The loss of the Cambisol soil was 10 times higher than that of the Chernozem. The reason for this is that the two experimented soils had a completely different soil structure and surface conditions before starting the rain simulation. The Chernozem had a very friable, crumby, permeable structure after the wheat yield. The Cambisol stood right after the corn yield, the soil surface was crusty and less permeable, except for shrinking cracks which swelled during the experiment.

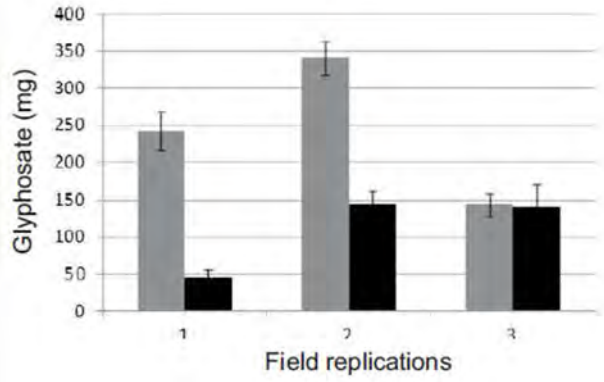


Fig. 6. Cambisol: total amounts of glyphosate in liquid run-off at the 3 field replication plots. Legend as in Fig. 1.

The Chernozem at Pixendorf and surroundings is generally known as a location with high erosion risk because of the high silt amount (> 60 mass %) and especially with corn crop, where deep gully erosion forms. The erosion rills discharge downslope to an artificial run-off retention basin at the footslope of the experimental field. This basin can run over and flow downwards on different paths and is collected through further toeslope retention basins. Water samples from both retention basins were analyzed and traces of glyphosate, which were surely not connected with rain simulation experiments, were found (Fig. 10). Moreover, soil percolation water samples at the footslope of the experimental field were collected at two depths from previously installed stations and analyzed for glyphosate and AMPA. Since the rainfall simulation experiment was conducted at the topslope whereas the percolation water samples were collected at the footslope (100 m distance) at the same time, it seems unlikely that the measured amounts of glyphosate and AMPA were influenced by the rain simulation, but are probably residual amounts from previous field application, confirming the possibility of dissipation through natural processes.

The results show that small amounts of glyphosate and its metabolite can dissipate through soil percolation, mainly depending on the physico-chemical adsorption and structural properties of soils, and

were found in collected soil solutions at two different depths far out of the 2x2 m precipitation plots (Fig. 11).

Conclusions

1. The rain simulation experiments clearly showed that even in a potentially high glyphosate adsorbing soil like the Cambisol, erosion and surface run-off can lead to severe glyphosate loss if the soil structure state *eg* compaction degree, crusting, infiltrability, pore size distribution, in the case of erosive precipitations shortly after Roundup Max® application, is unfavourable. In this study, in one of the NT-plot repetitions, up to 47% of the applied glyphosate amount were dispersed with run-off.
2. Traces of glyphosate in collected percolation soil water at Pixendorf, probably from previous conventional field application of Roundup Max®, confirmed the general low glyphosate adsorption capacity of Chernozems from Loess and the risk of transport towards groundwater.
3. Analysis of water from run-off retention basins in the landscape in the surroundings of the investigated Chernozem confirmed that through high erosion processes, especially in maize crop, glyphosate is partly transported outside the treated agricultural fields.

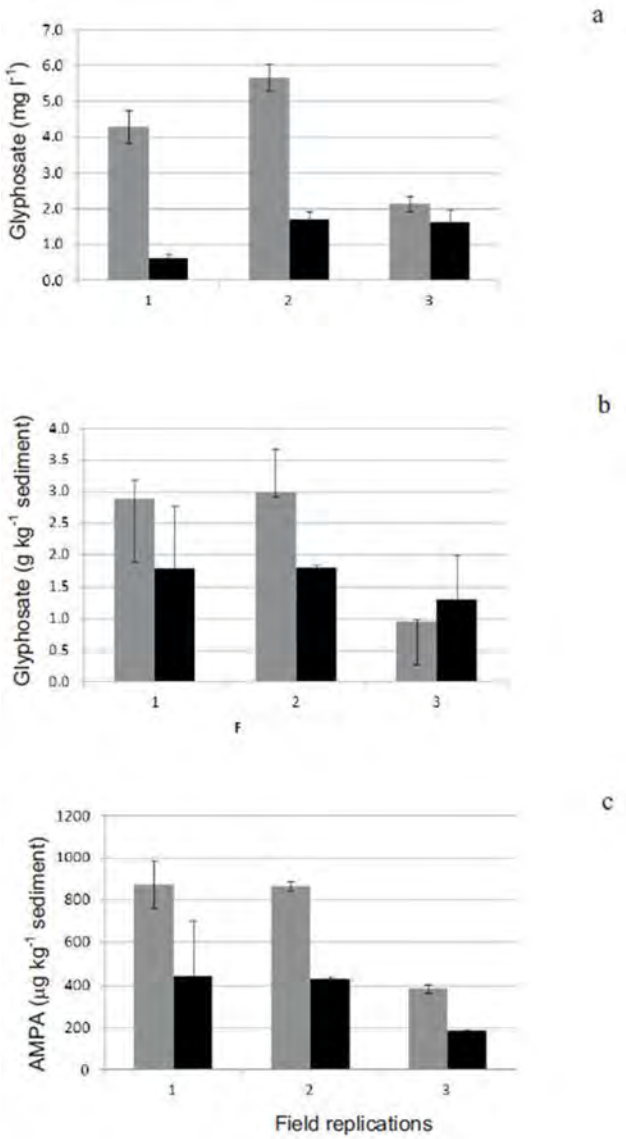


Fig. 7. Cambisol: a – glyphosate concentrations in liquid, b – glyphosate, and c –AMPA contents in the solid phase of run-off at the 3 field replications (average of the 60 min rain simulation). Legend as in Fig. 1.

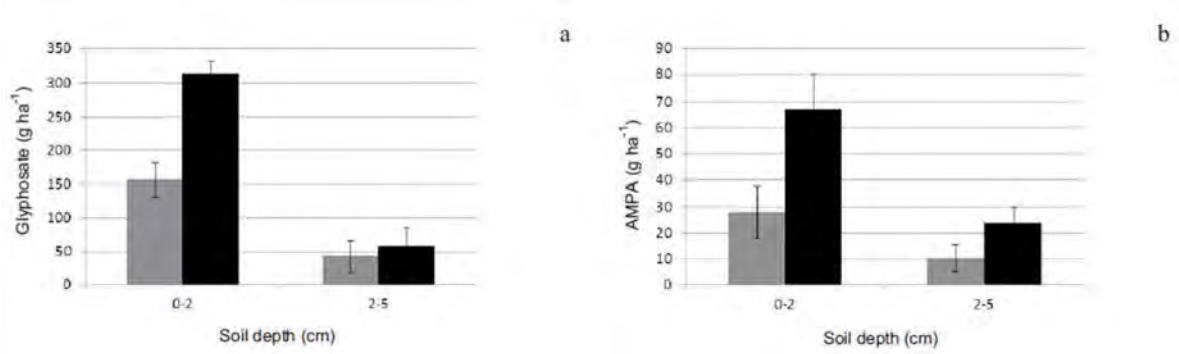


Fig. 8. Cambisol: a – glyphosate and b – AMPA contents in the soil within the rain simulation plots (average value from the 3 field replications). Legend as in Fig. 1.

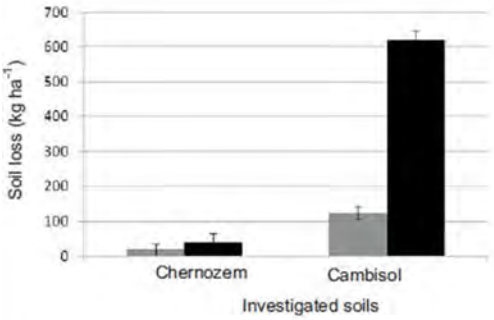


Fig. 9. Total soil loss of the investigated soils after the rain simulation experiments (averages of 3 field replications). Legend as in Fig. 1.

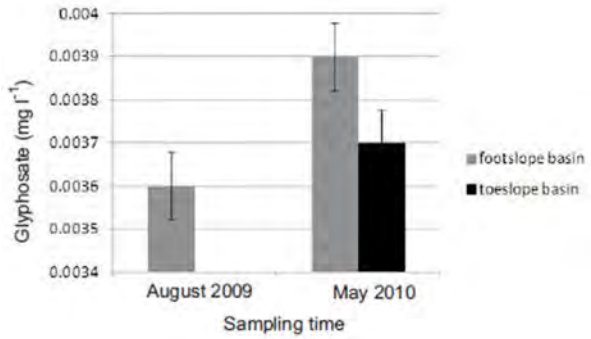


Fig. 10. Glyphosate concentrations in natural run-off retention basins outside the experimental fields.

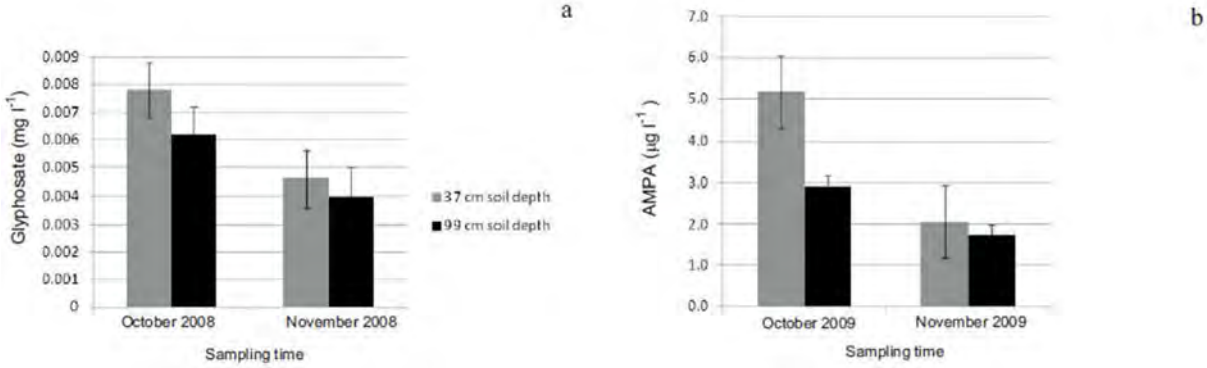


Fig. 11. Concentrations of: a – glyphosate, and b – AMPA in percolation water at 2 different times and soil depths.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes the runoff behavior of glyphosate and AMPA in two field experiments in two different European agricultural soils with artificial rainfall. No details on the description of the analytical method and of statistical analysis are provided.

The study is therefore classified as relevant to the data requirement but only as supplementary information (Category 2).

1. Information on the study

Data point:	KCA 7.1.4.3
Report author	Ulen, B., et al.
Report year	2014
Report title	Spatial variation in herbicide leaching from a marine clay soil via subsurface drains
Document No	Pest Management Science (2014)
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (essential parameters to derive endpoint missing)

2. Full summary of the study according to OECD format

BACKGROUND: Subsurface transport via tile drains can significantly contribute to pesticide contamination of surface waters. The spatial variation in subsurface leaching of normally applied herbicides was examined together with phosphorus losses in 24 experimental plots with water sampled flow-proportionally. The study site was a flat, tile-drained area with 60% marine clay in the topsoil in southeast Sweden. The objectives were to quantify the leaching of frequently used herbicides from a tile drained cracking clay soil and to evaluate the variation in leaching within the experimental area and relate this to topsoil management practices (tillage method and structure liming).
RESULTS: In summer 2009, 0.14, 0.22 and 1.62%, respectively, of simultaneously applied amounts of MCPA, fluroxypyr and clopyralid were leached by heavy rain five days after spraying. In summer 2011, on average 0.70% of applied bentazone was leached by short bursts of intensive rain 12 days after application. Peak flow concentrations for 50% of the treated area for MCPA and 33% for bentazone exceeded the Swedish no-effect guideline values for aquatic ecosystems. Approximately 0.08% of the glyphosate applied was leached in dissolved form in the winters of 2008/2009 and 2010/2011. Based on measurements of glyphosate in particulate form, total glyphosate losses were twice as high (0.16%) in the second winter. The spatial inter-plot variation was large (72–115%) for all five herbicides studied, despite small variations (25%) in water discharge.
CONCLUSIONS: The study shows the importance of local scale soil transport properties for herbicide leaching in cracking clay soils.

Materials and Methods

The field site is located in a flat valley with a clay soil of marine origin in eastern Sweden. The experimental field was tile-drained in 2006 to 0.9 m depth. Twenty-four of these plots were used in the present experiment. The plots are situated in two rows of 14 plots at varying distance from an open ditch that acts as the recipient of drainage water from the surrounding valley. Three management practices were randomly assigned to the plots: Conventional autumn ploughing, shallow autumn tillage and structure-liming (i.e. liming carried out to reduce phosphorus leaching and to improve crop yield by improving soil structure). Soil pH and total organic carbon (OC) content are given in Table 1. There were no significant differences ($P > 0.05$) in soil pH and OC between treatments.

Table 1

Mean values and standard deviations (SD) of soil pH and concentrations (%) of organic carbon (OC) at the start of the project in the autumn of 2007 and five years later in the spring of 2012 after repeated different tillage treatments and after structure liming first year

			Shallow tillage		Structure-limed		Conventional ploughing	
Sampling time	Property	Depth (cm)	Mean	SD	Mean	SD	Mean	SD
2007	pH	0–2	6.4	0.2	6.3	0.1	6.4	0.1
2012	pH	0–2	6.1	0.2	6.5	0.5	6.1	0.1
2007	OC (%)	0–23	2.4	0.5	2.5	0.5	2.5	0.5
2007	OC (%)	23–60	1.4	0.3	1.5	0.4	1.5	0.3
2007	OC (%)	60–90	0.6	0.1	0.6	0.1	0.6	0.1
2012	OC (%)	0–2	2.7	0.2	2.7	0.3	2.6	0.3

Pesticide leaching

We studied the leaching of seven different pesticides with contrasting properties (Table 2). It should be noted that both pesticide half-lives and adsorption partitioning coefficients are dependent on soil properties and the values presented in Table 2 may, therefore, not be representative for the clay soil at this site. Pesticide leaching was studied in two different crop rotations (Table 3), both with oats and peas during the last two years (2010–2011). In crop rotation I (20 plots), conventional autumn ploughing was compared with shallow autumn tillage and the effects of previous structure-liming in autumn 2007 were examined. Glyphosate was applied before sowing in spring 2008 to control couchgrass in eight shallow-tilled plots in crop rotation I (Table 4). In early summer the same year, the low-dose substances thifensulfuron-methyl and tribenuron-methyl were applied in both rotations. In autumn 2008 glyphosate was applied after harvest (four plots) in crop rotation II in order to control couchgrass and volunteer cereals. The three pesticides clopyralid, fluroxypyr and MCPA (all ingredients in the same commercial product) were sprayed for weed control on 9 June 2009 in crop rotation I (20 plots) and on 23 June 2010 in both rotations (24 plots). Glyphosate was applied after harvest in September 2010 and bentazone was applied on 11 June 2011 in both rotations (24 plots). Most applications were made in the evening, with no wind and always in the recommended dose. The total loads of herbicides applied (Table 4) were similar to those reported from agricultural catchments within the Swedish National Pesticide Monitoring Programme. Precipitation was measured at the site with unheated tilting bucket equipment and collected in a data logger.

Table 2

Herbicide properties and leaching potential data taken from the Pesticide Properties Database (PPDB, 2010)[11] Substance

DT_{50}lab[a](days)	DT_{50}field[b](days)	K_{oc}[c] ($cm^3\,g^{-1}$)	GUS[d]	pK_a[e]
Bentazone	13	14	55.3	2.30	3.28
MCPA	24	25	74[f]	2.94	3.73
Fluroxypyr	1	51	195[f]	0	2.94
Clopyralid	34	11	5.0	5.06	2.01
Glyphosate	12	12	1435	−0.49	2.34
Thifensulfuron-methyl	4	4	28.3	1.53	4.4
Tribenuron-methyl	14	14	35	2.88	4.7

[a] Degradation half-life for aerobic conditions measured in the laboratory.
[b] Degradation half-life for aerobic conditions measured in the field.
[c] Adsorption distribution coefficient to organic carbon.
[d] Groundwater ubiquity score.
[e] Acid dissociation constant.
[f] Freundlich adsorption coefficient to organic carbon.

Table 3

Year, crop, date and commercial brand name of herbicides applied in 2008–2011 in crop rotations I and II (number of conventionally ploughed plots/total number of treated plots)

Year	Rotation I Crop	Date	Herbicide (16/20 plots)	Rotation II Crop	Date	Herbicide (4/4 plots)
2008	Spring barley	24/4	Glypro Bio[a, b]	Winter wheat	26/6	Harmony 50T Plus[c]
	Spring barley	26/6	Harmony 50T Plus[c]	After W wheat	16/8	Glypro Bio[b]
2009	Spring barley	9/6	Ariane S[d]	Winter wheat	6/5	Harmony 50T Plus[c]
2010	Oats	23/6	Ariane S[d]	Oats	23/6	Ariane S[d]
	Oats	22/9	Glypro Bio[b]	After oats	22/9	Glypro Bio[b]
2011	Pea	11/6	Basagran[e]	Pea	11/6	Basagran[e]

[a] Only in eight shallow-tilled plots.
[b] Active ingredient glyphosate (49%).
[c] Active ingredients thifensulfuron-methyl (37%) and tribenuron-methyl (17%).
[d] Active ingredients MCPA (20%), fluroxypyr (4%) and clopyralid (2%).
[e] Active ingredient bentazone (87%).

Table 4

Year, date of application, substance analysed in drainage water, crop and applied dose of detected substance, together with the general dose (in g ha^{-1}) applied in Swedish monitored small catchments in 2008–2011

Year	Date	Substance	Crop	Dose (g ha^{-1})	General dose (g ha^{-1})
2008	24/4	Glyphosate	Before barley	707	748
2008	26/4	Thifensulfuron-methyl	Spring barley	4	6
		Tribenuron-methyl	Spring barley	2	3
2008	16/8	Glyphosate	After winter wheat	1060	1116
2009	26/5	Thifensulfuron-methyl	Winter wheat	6	6
		Tribenuron-methyl	Winter wheat	3	3
2009	9/6	Clopyralid	Spring barley	52	48
		Fluroxypyr	Spring barley	104	81
		MCPA	Spring barley	520	590
2010	23/6	Fluroxypyr	Oats	104	75
		MCPA	Oats	520	510
		Glyphosate	After oats	1060	1110
2011	11/6	Bentazone	Pea	475	500

Water sampling and analysis

Water discharge from each plot was measured with tilting vessels in an underground basement where sampling of drainage water also took place. The water was sampled flow-proportionally, with every subsample representing 0.003 mm discharge in summer and 0.04 mm discharge in the rest of the year. The bulk samples were collected weekly (or for the first flow events following application more frequently). The concentration of thifensulfuron-methyl and tribenuronmethyl (in 2008) was determined with solid-phase extraction followed by liquid chromatography and mass spectrometry (LCMS) and the concentration of clopyralid, fluroxypyr and MCPA (in 2009) by the same solid-phase extraction and by derivatisation and gas chromatography/mass spectrometry (GC/MS). Fluroxypyr and MCPA (in 2010) and bentazone (in 2011) were analysed by mass spectrometric determination (LC-MS/MS). Dissolved glyphosate (DissGly) and its main metabolite AMPA were analysed in winter 2008/2009 and 2010/2011, which involved ion exchange and derivatisation, followed by final identification and quantification by GC/MS. In winter 2010/2011, glyphosate analysis included particulate glyphosate (PartGly), which was trapped using a cellulose acetate filter with pore size 0.45 µm.

Table 5

Monthly precipitation (Prec) and total snow accumulation (Snow acc) in winter periods (October–April current year and January–April following year), water discharge (Flow) and ratio Flow/Prec for the experimental years and long-term (1988–2011) average

Year		May	June	July	August	September	October–April	Snow acc (cm)
2008	Prec (mm)	30	5	44	42	8	385	14
	Flow (mm)	10	1	0	4	5	395	
	Flow/Prec	0.33	0.20	0	0.03	0.63	0.99	
2009	Prec (mm)	45	95[a]	94	54	35	384	50
	Flow /mm)	3	43	3	1	0	370	
	Flow/Prec	0.07	0.45	0.03	0.02	0	0.85	
2010	Prec (mm)	53	39	155[b]	95	51	290	75
	Flow (mm)	16	3	8	87	42	310	
	Flow/Prec	0.30	0.08	0.05	0.92	0.82	0.85	
2011	Prec (mm)	40	70	26	138	72	358	3
	Flow (mm)	3	14	0	0	4	350	
	Flow/Prec	0.08	0.20	0	0	0.06	0.96	
1988–2011	Prec (mm)	40	67	82	69	63	338	32
	Flow (mm)	7	12	2	18	10	360	
	Flow/Prec	0.15	0.18	0.02	0.21	0.16	0.97	

[a] Maximum intensity 46 mm day^{-1} in the middle of the month.
[b] Maximum intensity 79 mm day^{-1} at the end of the month.

Results and Discussion

Concentrations of pesticides in drain water

The sulphonylureas (thifensulfuron-methyl and tribenuronmethyl) were not detected above LOD in 2008. Because of the fast dissipation of these substances, they were not analysed for in subsequent years. Unlike these low-dose substances, detectable levels of all other herbicides were found every year in drain flow in the first 1–2 months after early summer application. Detectable concentrations of fluroxypyr and MCPA were also observed 31 days after application (Table 6) in the samples taken after flooding of the measuring station. Detection of pesticides in the first few rainfall/drainage events after application is consistent with the flow was the simultaneous arrival of clopyralid and fluroxypyr on 14–16 June 2009, despite large differences in K_{oc} values (Table 2). However, since only five days had passed between application and rainfall, the substances might not have been in equilibrium with the soil solid material due to slow kinetics. Dissolved glyphosate was detected in consecutive events in autumn 2008. Both particle-bound glyphosate and dissolved glyphosate were detected in the discharge from all fast-flow events in autumn 2010. Levels above the $C_{\text{no effect}}$ concentrations were observed in 50% of the plots for MCPA and in 33% for bentazone (Table 6). Levels of glyphosate, AMPA, clopyralid and fluroxypyr were on all occasions below their $C_{\text{no effect}}$ concentrations. The coefficient of variation in the most important leaching event for the substances studied varied between 72 and 115% between all different plots (including different treatments) and increased in the order bentazone < clopyralid < fluroxypyr < PartGly < MCPA < DissGly. These highly variable pesticide concentrations were not significantly correlated to the basic soil factors pH value, clay content and organic matter content in the topsoil, which only showed minor variance (2, 17 and 10%, respectively).

Table 6

Year, date of application of substance (including glyphosate metabolite AMPA) and glyphosate in dissolved (diss) form and total glyphosate, numbers of plots (Plots), number of days (No. days) until major rain event, Swedish guideline values for no effect ($C_{no\ effect}$), maximum (Max) and mean concentration in the main drainage event, ratio of number of plots with concentration exceeding $C_{no\ effect}$ to total number of plots treated (Ratio $C_{no\ effect}$) and total period (days) after application when values exceeding $C_{no\ effect}$ were detected

Year	Date	Substance	No. days	$C_{no\ effect}$ (µg L^{-1})	Maximum (µg L^{-1})	Mean (µg L^{-1})	Ratio $C_{no\ effect}$	Period (days)
2008	16/8	Glyphosate diss.	47	100	1.2	0.48	0/4	—
		AMPA	47	500	0.3	—	—	—
2009	9/6	Clopyralid	5	50	5.5	2.2	0/20	—
		Fluroxypyr	5	100	1.7	0.67	0/20	—
		MCPA	5	1	5.5	2.0	10/20	5–14
2010	23/6	Fluroxypyr[a]	31	100	0.3	0.081	0/24	—
		MCPA[b]	31	1	0.04	0.007	0/24	—
2010	22/9	Glyphosate diss.	33	100	3.9	0.58	0/24	—
		Glyphosate total	33	100	9.4	2.2	0/24	—
		AMPA	33	500	0.7	—	—	—
2011	11/6	Bentazone	12	30	63	23.9	8/24	12–16

[a] Generally only analysed in dissolved form.
[b] Late collection of sample, as the measuring station was flooded.

Leaching losses of pesticides

The amount of pesticide leached in summer periods from conventionally ploughed plots sprayed simultaneously with the same herbicide in 2009–2011 varied between 0.2 and 3.3 g/ha (0.1–1.6% of amount applied) (Table 7). Leaching losses above 1% are generally associated with large rainfall amounts shortly after application. However, for our case the hydrological conditions did not represent 'worst-case' leaching conditions and hence the large leaching losses demonstrate the great potential for preferential transport in this soil. Losses exceeding 0.1% took place from 22 to 24 plots (92–100% of the experimental area) for clopyralid and bentazone, while the relative losses of MCPA exceeding 0.1% represented 42% of the area. The relative leaching losses of the substances studied here are presented in Table 7. Surprisingly, autumn application of glyphosate in 2008 and 2010 resulted in quite similar losses in dissolved form in the following winters (0.9 g/ha corresponding to 0.08% of applied amounts; Table 7), irrespective of whether the main discharge took place after autumn rain followed by a mild winter (2008) or in connection with snowmelt after a cold winter with continuous snow cover (2011). Due to slow degradation during the winter of 2010/2011 owing to long-lasting snow cover, glyphosate was available for leaching during the main snowmelt event, which was fast and probably resulted in preferential transport.

Table 7

Year, date, applied substance, including the sum of the three components in the commercial product Ariane S, mean losses from all ploughed plots with standard deviation (SD), mean losses relative to applied amount, range of the relative losses and area with relative losses exceeding 0.1 g ha^{-1}. Glyphosate was analysed in both dissolved (diss.) and particulate (part.) form in 2010

Year	Date	Substance	Mean (g ha^{-1})	SD	Relative losses (%)	Range of relative losses (%)	Area (%)
2008	16/8	Glyphosate diss.	0.89	0.64	0.084	0.02–0.17	25
2009	9/6	Clopyralid	0.84	0.70	1.62[c]	0.09–4.55	92
		Fluroxypyr	0.24	0.36	0.22	0.002–0.96	60
		MCPA	0.71	0.89	0.14	0.003–0.49	42
		Sum	1.81	3.03	0.34	0.02–1.36	60
2010	23/6	Fluroxypyr[a]	> 0.03	0.02	> 0.03	—	—
2010	22/9	Glyphosate diss.[b]	0.90	0.32	0.085	0.05–0.12	—
		Glyphosate part.	0.82	0.25	0.064	0.08–0.10	—
		Glyphosate total	1.72	1.47	0.15	0.12–0.23	100
2011	11/6	Bentazone	3.31	2.92	0.70[b]	0.42–2.16	100

[a] From late sample after flooding of the measuring station.
[b] Period 22 September–15 April and calculated from the same four plots as treated in 2008.
[c] Relative losses of clopyralid were significantly greater ($P < 0.01$) than losses of fluroxypyr applied simultaneously.

Herbicide correlation with particulate phosphorus and plot position

In spite of the small variation in the amounts of water discharge between plots, there was a large variation in herbicide losses for all substances resulting from the highly varying concentrations in drainage water. Similar relationships have previously been reported between total glyphosate and PP for the same field. Due to their strong sorption, both glyphosate and PP are considered to leach mainly through preferential transport in macropores. Our results suggest that preferential transport dominates leaching also for the weakly sorbed substances at this site. In addition, since the pesticides which were applied at the soil surface were leaching with a similar pattern as PP, this suggests that the topsoil was the major source of leached PP. We did not observe any surface runoff in the direction of the Recipient ditch during the experimental period. Lateral flows below the soil surface and e.g. on a plough pan were also unlikely to occur, since there was no distinct plough pan at the site. There was no correlation between the topsoil (0–23cm) pH and the plot position. However, topsoil OC clearly increased with decreasing distance between plot mid-point and the recipient ditch (R^2 = 0.70%, P < 0.001) and pH in the deeper subsoil (60–90 cm) decreased (R^2 = 78%, P < 0.001). The concentration of all pesticides tended to increase with decreasing distance between plot position and the recipient ditch. The relationship was significant for bentazone, and was also significant from a total ranking of all pesticides detected (Fig. 1).

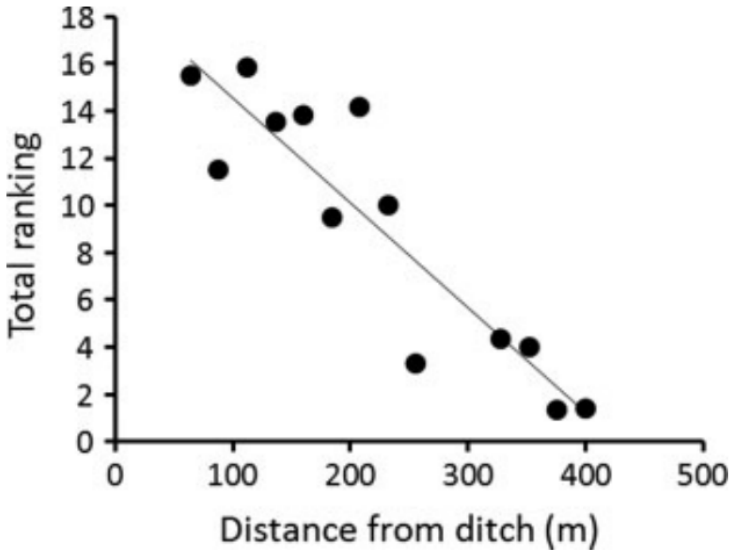


Fig. 1. Total ranking of mean concentration of clopyralid, fluroxypyr, MCPA, bentazone, dissolved glyphosate and particulate glyphosate related to the distance between the ditch and the centre of the respective plot. The estimates were made for the observed concentrations in the major event for every substance. The slope of the regression line is significantly different from zero (P < 0.001).

Effects of soil management, soil structure, pH and organic matter

There was a general tendency for larger losses of all substances from shallow-tilled plots than from ploughed plots, with or without previous structure liming (Table 8). The apparent differences, which were not significant for any single substance, increased in the order clopyralid < MCPA < bentazone < fluroxypyr < total glyphosate. However, estimated for all five substances lumped together (paired *t*-test), the difference between shallow-tilled and ploughed structure-limed plots was significant (P < 0.05), both before and after adjustment to the effect of plot position in relation to the ditch. From soils where preferential flow and transport are important, ploughing is generally considered to reduce pesticide leaching by interrupting continuous macropores. For our case the larger losses from the shallow-tilled plots may also have been an effect of shallow and uneven accumulation of crop residues in these plots which resulted in uneven infiltration and preferential herbicide transport along straw residues. At the study site, it has been demonstrated that structure liming (quicklime) significantly improves soil aggregate stability measured as a decrease in readily dispersed clay. Improved aggregate stability should influence the transport of glyphosate which adsorbs strongly to clay particles. However, the improved aggregate stability did not result in any significantly smaller losses of glyphosate from structure limed plots compared to conventionally tilled plots.

Table 8

Year of application, mean and standard deviation (SD) of transported masses of the applied substances (in g ha^{-1}) from tilled, structure-limed (+ ploughed) and conventionally ploughed plots

		Shallow tillage Mean			Structure-limed Mean			Conventional ploughing Mean		
Year	Substance	Un-adjusted	Adjusted	SD	Un-adjusted	Adjusted	SD	Un-adjusted	Adjusted	SD
2009	Clopyralid	1.16	1.14	0.77	1.07	1.13	0.95	0.73	0.71	0.59
2009	Fluroxypyr	0.36	0.35	0.29	0.25	0.26	0.28	0.22	0.21	0.25
2009	MCPA	1.11	1.09	1.03	0.86	0.88	1.25	0.63	0.61	0.82
2011	Bentazone	4.78	4.73	2.84	3.52	3.75	3.81	3.40	3.32	2.53
2010	Glyphosate[a]	3.81	3.77	2.58	0.85	0.90	1.13	1.59	1.56	1.56

Note: Mean transported losses are given both as unadjusted values and values adjusted for the distance to the ditch.
[a] Total glyphosate in both particulate and dissolved form in the period 22 September 2010–15 April 2011.

For ionisable pesticides, leaching is also affected by soil pH, with weaker sorption at higher pH. Based on the p*K*a values of the substances studied here and the small differences in pH between treatments (Table 8), any pH effects on leaching were probably minor. The topsoil OC content is often higher under long term shallow tillage than under conventional tillage, which has consequences for pesticide sorption and degradation. However, in our case the OC content was not significantly different between treatments and there were no significant differences in subsoil OC between plots with different management regimes. The coefficient of variation in relative leaching losses between all substances for the shallow-tilled plots varied between 40 and 92%. The coefficient of variation in the relative leaching losses from all plots and for all substances combined (92–156%) varied even more. In conclusion, the variation in relative leaching losses between plots within the same treatment was larger than that between different substances. This finding also demonstrates that the differences in transport pathways through the soil between plots have a larger effect on pesticide concentrations than the differences in pesticide properties.

Conclusions

Concentrations of the herbicides bentazone, clopyralid, fluroxypyr, MCPA and glyphosate were measured in subsurface drain discharge from a clay field during a four-year study. Despite hydrological conditions not representing a worst case scenario for leaching, the relative leaching losses of all herbicides studied were large compared to values reported in the literature. Measured concentrations of bentazone and MCPA exceeded Swedish guideline values based on predicted no effect on aquatic ecosystems for 50 and 33% of the plots for MCPA and bentazone, respectively. All substances studied (except sulphonyl ureas which were not detected), irrespective of sorption strength, showed similar leaching patterns. These observations clearly demonstrate that preferential transport in macropores is the dominant transport process at this site. The variation in relative leaching losses between plots within the same treatment was greater than that between different substances. Crack stabilisation by gyttja, especially in the deeper subsoil, was suggested as an important explanatory factor for this large spatial variation in pesticide leaching, although it was not possible to investigate differences in gyttja content between plots. Continuous macropores connecting the soil surface to the subsoil may be a factor contributing to the generally large pesticide losses observed after shallow tillage. However, careful studies of soil macropore systems, including topsoil and subsoil properties, are needed to explain the unpredictability in leaching at this site.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study describes a leaching experiment from a Swedish marine clay soil with agricultural land use. Glyphosate among other herbicides was considered in analysis. The study provides supportive information but not all parameters to derive endpoints are reported.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.1.4.3
Report author	Ulen, B., et al.
Report year	2012
Report title	Particulate-facilitated leaching of glyphosate and phosphorus from a marine clay soil via tile drains
Document No	Acta Agriculturae Scandinavica Section B - Soil and Plant Science, 2012; 62: Supplement 2, 241-251
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (Not enough information provided to check validity)

2. Full summary of the study according to OECD format

Losses of commonly used chemical pesticides from agricultural land may cause serious problems in recipient waters in a similar way to phosphorus (P). Due to analytical challenges concerning determination of glyphosate (Gly), transport behaviour of this widely used herbicide is still not well known. The objective of the present study was to quantify and evaluate leaching of Gly in parallel with P. Leaching losses of autumn-applied Gly (1.06 kg/ha) via drainage water were examined by flow-proportional sampling of discharge from 20 drained plots in a field experiment in eastern Sweden. Samples were analysed for Gly in particulate-bound (PGly) and dissolved (DGly) form. The first 10 mm water discharge contained no detectable Gly, but the following 70 mm had total Gly (TotGly) concentrations of up to 6 µg/L, with 62% occurring as PGly. On average, 0.7 g TotGly/ha was leached from conventionally ploughed plots, compared with 1.7 g TotGly/ha from shallow-tilled plots (cultivator to 12 cm working depth). Higher Gly losses occurred in snowmelt periods in spring, but then with the majority (60%) as DGly. All autumn concentrations of PGly in drainage water were significantly correlated ($p < 0.001$) to the concentrations of particulate-bound phosphorus (PP) lost from the different plots (Pearson correlation coefficient 0.84), while PP concentrations were in turn significantly correlated to water turbidity (Pearson correlation coefficient 0.81). Leaching losses of TotGly were significantly lower (by 1.3 g/ha; $p < 0.01$) from plots that had been structure-limed three years previously and ploughed thereafter than from shallow-tilled plots. Turbidity and PP concentration also tended to be lowest in discharge from structure-limed plots and highest from shallow-tilled plots. This difference in TotGly leaching between soil management regimes could not be explained by differences in measured pH in drainage water or amount of discharge. However, previously structure-limed plots had significantly better aggregate stability, measured as readily dispersed clay (RDC), than unlimited plots. The effects of building up good soil structure, with strong soil aggregates and an appropriate pore system in the topsoil, on mitigating Gly and P losses in particulate and dissolved form should be further investigated.

Materials and Methods

Experimental plots and soil characteristics

The experiment was done on 20 drained plots in an experimental field with a sub-surface drainage water collection system constructed on a flat plain close to the Lake Bornsjön reservoir. Drainage water flows to a sampling and measuring station and is recorded with tilting vessels and data logger. The data logger controls the flow proportional sampling by means of small tube pumps in the basement of the station. After a certain volume of water has passed, the suction tube is first cleaned by reverse pumping and thereafter a small volume is sampled. The flow-proportional (composite) sampling took place in dark

glass vessels at relatively cold temperature and in darkness for a maximum of one week prior to freezing the water samples and transport to the laboratory before analysis.
Clay content (60%), is high throughout the profile (Table 1), with small spatial variation in both topsoil and sub-soil (variance less than 0.5%). pH and soil concentration of P are uniformly distributed in the experimental area (variance less than 15%). In the soil profile, the pH (dry soil samples) varies between 5.2 and 6.9, with the lowest values occurring in the 70-100 cm layer, which includes the tile drains at approximately 90 cm depth. Under wet conditions the pH in the upper sub-soil is higher than that under dry conditions (6.9 compared with 6.6). Overall, the soil profile generally demonstrates a high ability to sorb P to the soil matrix.
The soil horizon has a strongly aggregated structure, especially in the deeper part, with approximately 10 cm wide and 10-20 cm prismatic aggregates in the layer 43-100 cm. Water retention is very high. In an adjoining field with an old drainage system, the deeper soil horizon is very wet, the aggregates similarly very prismatic and the structure is easily destroyed by digging.

Table 1. Selected physical and chemical properties of the soil at the study site.

Properties	Soil depth (cm)					Reference for the method
	0–10	10–30	30–50	50–70	70–100	
Particle size distribution						
<0.002 mm (clay) (%)	60	60	59	61	54	Eriksson et al. (1998)
0.002–0.02 mm (%)	31	30	30	31	34	Eriksson et al, (1998)
0.02–0.2 mm (%)	9	10	11	8	12	Eriksson et al. (1998)
Organic matter (%)	3.9	1.9	0.1	0.0	0.0	Eriksson et al. (1998)
pH_{H_2O}[a]	6.0	6.2	6.6	6.5	5.2	ISO (2005)
P_{Olsen} (mmol kg^{-1})[a]	0.59	0.53	0.13	0.17	0.68	Olsen and Sommers (1982)
P_{AL} (mmol kg^{-1})[a]	1.4	1.0	0.3	0.4	1.0	Egnér et al. (1960)
Al_{ox} (mmol kg^{-1})[a]	116	106	71	77	88	Schwertmann (1964)
Fe_{ox} (mmol kg^{-1})[a]	165	169	158	181	118	Schwertmann (1964)
Al_{AL} (mmol kg^{-1})[a]	10.3	9.9	9.8	9.6	16.1	Ulén (2006)
Fe_{AL} (mmol kg^{-1})[a]	9.4	10.1	8.8	9.4	12.5	Ulén (2006)
PSI_2 (mmol kg^{-1}).[a]	7.3	7.8	7.3	7.2	10.5	Börling et al. (2004)
P_{Olsen}/PSI_2[a] (%)	8.1	6.8	1.8	2.4	6.4	Börling et al. (2004)
DPS_{AL}[a] (%)	8.7	6.2	2.0	2.5	4.3	Ulén (2006)

[a]Data from Andersson et al. (2012).

Glyphosate application and cultivation practices.
No Gly had been applied to the actual experimental plots for the previous three years. Quicklime (CaO) had been applied in dry conditions on the stubble in four plots in 2007 Phosphorus fertilization was 11 kg/(ha year), always applied in mineral form in spring. This is a moderate load, since the area has special restrictions. When starting the experiment the aim was to avoid P limitation of the crop and therefore 20 kg/(ha year) were applied in 2007-2011 for all plots except four. Glyphosate was applied on 22 September 2010 as the commercial product Glypro Bio, at a rate equal to 1.06 kg/ha active substance. Twelve days later, the conventional and structure-limed plots were stubble-harrowed (Table 2) and eight plots were shallow-tilled (12 cm) twice and reconsolidated with a rib-roller. After a further 10 days, the conventionally ploughed plots (8) and the structure-limed plots (4) were mould-board-ploughed and the soil was inverted to a depth of 23 cm.

Table 2. Management regime in the different treatments (A-E) in 2010, where A+B (eight plots) represent regular conventional autumn ploughing, C (four plots) represents previous structure liming and D+E (eight plots) represent regular shallow tillage in autumn.

Treatment	Management	Date
A+B, C, D+E	Harrowing (0–5 cm)	16 May
A+B, C, D	Fertilization, seed drilling[a]	17 May
E	Fertilization (broadcasting)	17 May
A+B, C, D+E	Sowing (oats)	17 May
A+B, C, D+E	Harvesting	27 August
A+B, C, D+E	Glyphosate application (1.06 kg ha^{-1})	22 September
A+B, C	Stubble harrowing	4 October
D+E	Cultivation (8 cm) twice	4 October
A, B, C	Conventional ploughing (23 cm)	14 October

[a]No P fertilization to B plots.

Weather, discharge and water sampling procedure

Autumn 2010 was short, with permanent snow from the end of November (Fig. 1). Owing to the thickness of the snow cover, soil freezing was limited despite low air temperatures. The main snowmelt took place in late March and the first two weeks in April. The glass vessels with flow-proportional samples in the station basement were observed regularly (at least weekly) and when at least 300 mL turbid water had been collected from most plots, sub-samples were taken from every plot for Gly analysis. When there was a moderate amount of water or less turbid water in the glass vessel, sampling was performed only for analysis of P and turbidity for reasons of economy. Such sampling occurred in total on five sampling occasions. On 28 March, 186 days after glyphosate application in autumn, turbidity was observed once again in the flow-proportionally sampled water and additional water was collected for Gly analysis, which was performed on the 14 most turbid samples.

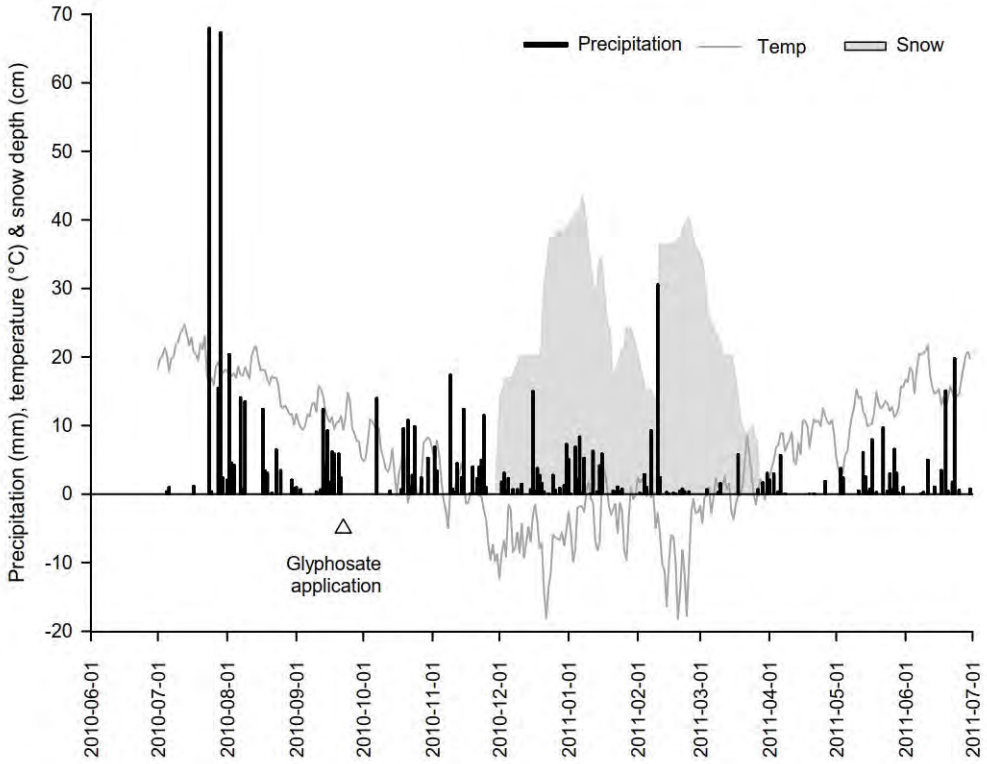


Fig. 1. Temperature (°C), precipitation (mm) and snow cover (mm) on the experimental field in 2010-2011.

Water analysis
Total P was analysed as soluble molybdate-reactive P after acid oxidation with $K_2S_2O_8$ (ECS, 1996). DRP was analysed after pre-filtration using filters with pore diameter 0.45. Particulate P (PP) is the absolute dominant P fraction, while non-mineral forms of dissolved P are very small, and accordingly the difference between TotP and DRP was taken as PP. The concentration of particles was analyzed from thawed samples as turbidity on a HACH 2100 turbidometer. Before analysing Gly, each thawed sample was thoroughly shaken by hand, centrifuged and filtered. The filtered water was used for analysis of DGly, including AMPA, after pH adjustment (pH 7-8) with either diluted HCl or NaOH. After a few more rounds of extraction, centrifugation and filtration, the pH of the samples was adjusted to 2 in order to precipitate any humic acids and to harmonize with the method used for stream and lake sediment. After dilution, the pH was readjusted to 7-8.
The same analytical procedure was used for both PGly and DGly and involved ion-exchange and derivatization, using a modified version of Mogadati et al. (1996), followed by final identification and quantification by gas chromatography-mass spectrometry (GC-MS).

Soil aggregate stability
Soil samples from plots with structure liming, conventional ploughing and reduced tillage were analysed in the laboratory for aggregate stability, expressed as readily dispersed clay (RDC). Slightly moist samples were collected from the topsoil (0-20 cm) on 27 August 2010, before post-harvest stubble cultivation, and gently transported to the laboratory. Four sub-samples representing 12 aggregates (8-10 mm) were prepared for each plot and gently wet-sieved (0.6 mm mesh opening) with a slow oscillating movement. After 4 hours sedimentation (to allow all particles larger than clay to settle; Sheldrick & Wang, 1993), the content of dispersed clay still in solution was determined by turbidometer.

Data calculations and statistical analyses
The mean and standard deviation were calculated for the experimental parameters determined in all flow-proportional samples (four or eight parallel samples) from replicate plots for the different treatments. If no residue of Gly or AMPA was detected in a given sample, the value 0 was used for calculating the mean. Pearson correlation and regression linear relationships were determined between the parameters total glyphosate (TotGly_PGly_DGly), TotP, PGly, PP and turbidity for the autumn period (27 September - 15 November) and between TotP and turbidity for the spring period (21 March - 11April). Any differences in glyphosate concentrations between the different soil treatments were analysed using Bonferroni post test assuming equal variance and a significance level of $p < 0.05$. Leaching losses from the different plots in the autumn period were calculated by multiplying discharge by measured flow-proportional concentrations in the periods between sample collections. In the spring period, transport of TotGly was estimated from measured values from 14 plots on 28 March.

Results and Discussion

Glyphosate and phosphorus concentrations in water
One week after Gly application in autumn, when 10 mm discharge had passed through the tile drainage system, no Gly or AMPA was present in detectable quantities in the discharge (Table 3). In the following 7-8 weeks, representing 70 mm water discharge, relatively high and quantifiable concentrations of both DGly and PGly were detected in practically all water samples and, in addition, dissolved AMPA was frequently observed. The concentrations varied greatly from plot to plot and TotGly concentrations of up to 5-6 µg/L were recorded for some plots. High PGly concentrations were generally associated with high DGly concentrations and the two forms of Gly were significantly correlated to each other (Pearson correlation 0.35; $p < 0.002$). Hence, more DGly seemed to leach with mobilized soil particles with high Gly content. Mean DGly concentration in discharge in the autumn (22/9-15/11) was 1.03 µg/L for plots with shallow tillage; 0.43 µg/L for plots with conventional ploughing and 0.36 µg/L for plots with structure liming (differences not statistically significant).

Table 3. Discharge, pH (in stored composite samples) and flow-proportional concentrations of dissolved glyphosate (DGly), AMPA, particulate glyphosate (PGly), dissolved reactive phosphorus (DRP), particulate P (PP) and turbidity (Turb) in five periods 2010 - 2011 (n.d. = not detected)

Period	22/9–27/9	28/9–25/10	26/10–8/11	8/11–15/11	21/3–28/3
Conventional ploughing					
Discharge (mm)	8.2±3.0	9.2±4.3	25.5±11.1	33.5±13.1	72.9±30.2
pH	6.5	7.2	7.0	6.7	6.6
DGly (μg L^{-1})	n.d.	0.43±0.32	0.43±0.34	0.39±0.21	0.31±0.34
AMPA (μg L^{-1})	n.d.	0.05	0.04	0.03	n.d.
PGly (μg L^{-1})	n.d.	0.67±0.63	0.61±0.67	0.60±0.34	0.22±0.43
DRP (mg L^{-1})	0.021±0.011	0.021±0.011	0.018±0.007	0.020±0.007	0.048±0.019
PP (mg L^{-1})	0.132±0.068	0.122±0.010	0.161±0.166	0.168±0.144	0.124±0.039
Turb (NTU)	64±26	36±7	62±24	60±20	30±16
Structure liming					
Discharge (mm)	10.4±4.1	13.5±5.4	29.1±11.4	30.1±5.1	74.4±25.5
pH	6.9	7.3	7.2	6.9	6.6
DGly (μg L^{-1})	n.d.	0.24±0.20	0.30±0.21	0.24±0.27	0.23±0.25
AMPA (μg L^{-1})	n.d.	0.03	e.d.	0.05	0.08
PGly (μg L^{-1})	n.d.	0.40±0.48	0.41±0.53	0.33±0.58	0.16±0.35
DRP (mg L^{-1})	0.018±0.007	0.017±0.008	0.015±0.005	0.020±0.006	0.047±0.027
PP (mg L^{-1})	0.075±0.066	0.066±0.074	0.093±0.131	0.100±0.106	0.078±0.032
Turb (NTU)	46±30	34±11	64±26	46±31	28±6
Shallow tillage					
Discharge (mm)	10.8±5.3	15.6±6.6	25.9±10.2	29.7±6.4	76.4±23.5
pH	6.8	7.2	7.1	6.8	6.6
DGly (μg L^{-1})	n.d.	1.15±0.89	1.28±1.42	0.99±0.64	0.82±0.93
AMPA (μg L^{-1})	n.d.	0.05	0.23	1.3	0.02
PGly (μg L^{-1})	n.d.	1.99±1.64	1.42±1.44	1.89±1.48	0.57±0.84
DRP (mg L^{-1})	0.024±0.007	0.024±0.007	0.023±0.008	0.027±0.007	0.047±0.021
PP (mg L^{-1})	0.142±0.078	0.236±0.181	0.411±0.355	0.275±0.151	0.136±0.029
Turb (NTU)	88±44	50±17	99±45	81±31	52±43

Similar to TotGly, the majority of TotP was lost in particulate form. The proportion of PP was higher (90%) than the proportion of PGly (60%). The present study site Gly was tilled down (10 or 23cm depth) in autumn after spraying which would facilitate the dispersion of Gly. A clear and positive correlation between TotGly and TotP concentrations and between PGly and PP concentrations was recorded (Fig. 2). In turn, PP concentrations could be quite well predicted from turbidity (Fig. 2). In contrast, DRP concentrations were generally low (0.018-0.027 mg/L) and DGly concentrations were more weakly correlated to DRP concentrations ($r = 0.65$; $p < 0.001$). Glyphosate is commonly suggested to compete with phosphate ions for adsorption sites, but at the present site, with high sorption capacity of the soil particles, this seemed not to be the case, since the correlation was positive. Mean PGly concentrations in the autumn were 1.73 μg/L in discharge from shallow-tilled plots; 0.62 μg/L for conventional ploughed plots; and 0.36 μg/L for structure-limed plots, all differences being statistically significantly different ($p < 0.001$). This implies that colloid P, colloid glyphosate and dissolved pesticides, although mobilized with different mechanisms (de Jonge et al., 2009), may be transported via macropore flow.

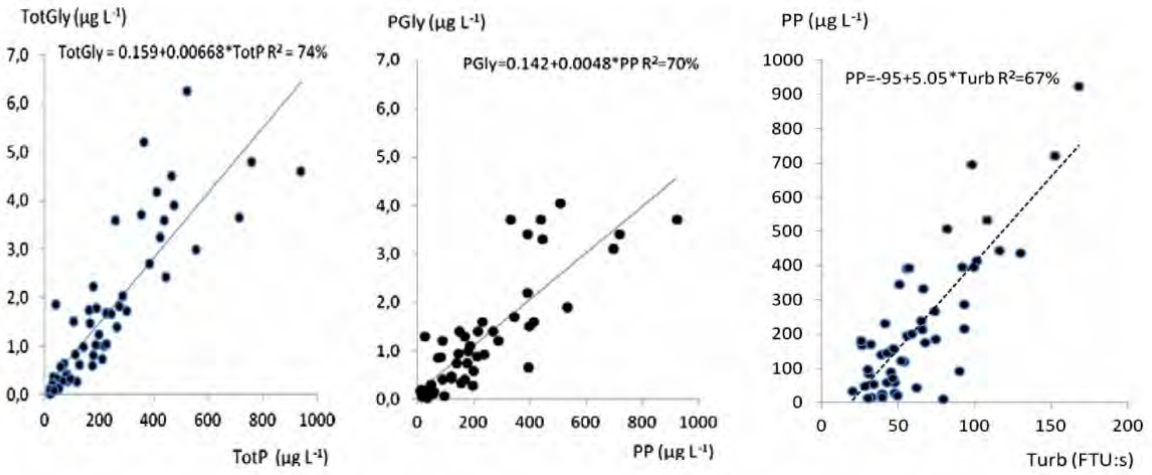


Fig. 2. Regression equation for the relationship between concentrations of: (a) total glyphosate (TotGly) and total phosphorus (TotP); (b) particulate glyphosate (PGly) and particulate P (PP); and (c) PP and turbidity (NTUs) in the period 27 September - 15 November 2010. Corresponding Pearson correlations (0.86, 0.84 and 0.82, respectively) were all significant ($p < 0.001$).

Glyphosate and phosphorus concentrations and losses in spring versus autumn period

As with Gly and P, pH was measured in the cumulative flow-proportionally sampled water and may have changed in the glass vessel. However, measured pH generally did not differ between the three treatments and pH in discharge from the previously structure-limed plots was similar to that in discharge from the unlimited plots (Table 3). The pH tended to be lower (6.6) in the snowmelt period (Table 3). The measured drop in (logarithmic-based) pH value is equal to 75% less H^+ ions, which may have influenced both the electrical charge of Gly and the hydrogen bonds of the minerals, and which may explain the high concentrations of DGly in snowmelt. The snowmelt water had low electric conductivity and DRP concentrations that were twice as high as those in the autumn discharge water. The PGly concentrations found in snowmelt in the present study were generally lower than the DGly concentrations and remained at nearly the same level as in autumn. Consequently, the relative proportions of DGly and PGly were reversed from autumn to spring (snowmelt) (Table 4). However, the latter case is based on a more limited number of analyses ($n = 14$).

Table 4. Number of samples analysed (n), relative proportions of dissolved glyphosate (DGly) and particulate glyphosate (PGly) in total glyphosate (TotGly) and relative proportions of dissolved reactive P (DRP) and particulate P (PP) in total phosphorus (TotP) in autumn (28/9 - 15/11 2010) and in a snowmelt period in spring (21 - 28/3 2011), based on flow-proportional concentrations.

Glyphosate			Phosphorus		
Fraction	Autumn	Spring	Fraction	Autumn	Spring
n	80	14	No	80	20
DGly/TotGly (%)	40	60	DRP/TotP (%)	10	32
PGly/ TotGly (%)	60	40	PP/TotP (%)	90	68

In practice, half-life degradation rate may be several months. However, as indicated here; ratio PGly/turbidity was only 20-40% lower in March than in November. Simultaneously, PGly/PP ratio decreased by 30% on average (from 0.54% in autumn to 0.15% in spring). Correspondingly the topsoil colloids may be more depleted of P in spring than in autumn, since the ratio PP to turbidity was lower and had a lower slope in snowmelt than in autumn.

Therefore, there may be similarities between Gly and P transport behavior in spite of the fact that P exists in a large P pool in topsoil and that yearly net P load to the soil in recent years has been six-fold higher than the glyphosate load.

Since the major water discharge took place during the snowmelt period, glyphosate losses tended to be higher in spring than in autumn (Tables 5 and 6). In relation to applied amount, losses were approximately 0.1% in spring and 0.05% in autumn for the conventionally ploughed plots. The main reason for the high spring discharge was the intensive snowmelt taking place after a winter with much snow accumulation. These results indicate the importance of such a snowmelt period for Gly losses, confirming findings by Laitinen et al. (2009). Snow accumulation also had great consequences for P losses.

Table 5. Discharge and transport of dissolved glyphosate (DGly), particulate glyphosate (PGly), total glyphosate (TotGly), dissolved reactive phosphorus (DRP), particulate P (PP) and total P (TotP) from conventionally ploughed, structure-limed (and ploughed) and shallow-tilled plots in the period 28/9 - 15/11 2010.

Period 28/9–15/11	Conventional	Structure-limed	Shallow-tilled
Discharge (mm)	69 ± 28	74 ± 23	72 ± 22
DGly (g ha^{-1})	0.25 ± 0.13	0.12 ± 0.10	0.65 ± 0.54
PGly (g ha^{-1})	0.45 ± 0.53	0.19 ± 0.19	1.01 ± 0.75
TotGly (g ha^{-1})	0.70 ± 0.60	0.31 ± 0.31**	1.65 ± 0.96
DRP (kg ha^{-1})	0.012 ± 0.004	0.012 ± 0.003	0.018 ± 0.007
PP (kg ha^{-1})	0.104 ± 0.082	0.048 ± 0.044	0.192 ± 0.111
TotP (kg ha^{-1})	0.117 ± 0.084	0.060 ± 0.044	0.209 ± 0.114

**Significantly lower ($p < 0.05$) than in shallow-tilled plots.

Table 6. Discharge (mm) and leaching losses of dissolved glyphosate (DGly), particulate glyphosate (PGly) and total glyphosate (TotGly) as a percentage of original amount applied from conventionally ploughed, structure-limed (and ploughed) and shallow-tilled plots based on measurements in autumn (28/9 - 15/11 2010) and more rough estimates in the most intensive spring snowmelt period (31/3 - 11/4).

	Conventional		Structure-limed		Shallow-tilled	
	Autumn	Snowmelt	Autumn	Snowmelt	Autumn	Snowmelt
Discharge (mm)	69	170	74	169	72	160
DGly (%)	0.024	–	0.011	–	0.061	–
PGly (%)	0.041	–	0.018	–	0.095	–
TotGly (%)	0.066	0.09	0.029	0.05	0.156	0.19

Glyphosate and phosphorus losses under different soil management regimes

In the autumn period, TotGly leaching losses were on average 0.70 g/ha from the conventionally ploughed plots (Table 6). TotGly losses from structure-limed plots were significantly lower ($p < 0.05$) than from shallow-tilled plots, expressed in absolute terms (Table 5), and also as a percentage of applied amount of Gly (Table 6). Fewer particles with attached Gly and P are expected to mobilize from soil aggregates that are less prone to dispersion. The structure-limed plots had significantly ($p < 0.05$) better aggregate stability (lower RDC values) in autumn than the conventionally ploughed and shallow-tilled plots (Fig. 3), which may explain the clear tendency for lower losses of both PGly and PP from this treatment (Table 5).

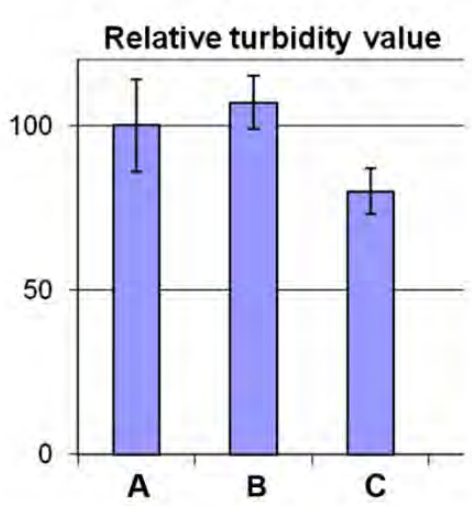


Fig. 3. Readily dispersed clay (RDC) in the topsoil from (A) conventionally ploughed, (C) structure-limed and (B) shallowtilled plots. The soil was sampled in September 2010, three years after structure liming

Leaching losses of both PGly and PP tended to be highest from the regular shallow-tilled plots (Table 5). Any enhanced amounts of stubble residues in the topsoil, combined with higher potential biological activity and organic matter content, did not seem to have improved aggregate stability from plots (Fig. 3). However, significantly higher organic matter content was not expected, since such major changes may take at least 10 years. Sorption of Gly is generally not increased in the presence of more straw residues as a consequence of reduced tillage. Therefore, the straw may have facilitated water transport rather than providing new sorption sites after the mixing and reconsolidation of the soil surface. In addition, shallow and uneven accumulation of crop residues on the shallow-tilled plots possibly resulted in uneven infiltration and rapid lateral water movement compared with annually ploughed plots. This is a factor that should be further investigated.

There was no major difference in amount of discharge between the different treatments (Table 6). Topsoil structure should be further explored in connection with topsoil susceptibility to preferential flow and transport under different agricultural management regimes. In addition, there was a great variation in concentrations between different plots. Both 'gyttja' (cohesive matter of organic origin settled in marine or lake sediment) and oxidized iron (rust) have been frequently observed in soils at the present site. Such material might strengthen the crack walls and make them into permanent pathways, which could explain the general fast transport of particulate-bound glyphosate and P at the present site.

The source of the Gly leaching in this study was the tilled topsoil (0-12 or 0-23 cm), which was possibly the main source of P leaching too. Besides the total amount applied, risk assessment of leaching is often based on the sorption/desorption properties of the actual substance. However, according to the results of the present study, factors such as soil structure, macropore topology and macropore flow may be of great importance.

Conclusion

This study has demonstrated that a significant proportion of glyphosate (Gly) leaching losses may occur in particulate form from clay soils with high amounts of sorption sites available. Crack stabilization by gyttja, especially in the deeper sub-soil, might be an important explanatory factor for fast vertical transport of Gly and phosphorus (P) at the study site. The crack might also be an important explanatory factor for the great spatial variability in Gly and P, in both particulate and dissolved form, at the study site. Structure liming of the topsoil was demonstrated to reduce total Gly leaching losses compared with unlimited soils, while shallow tillage may not be a suitable way to mitigate particle-facilitated transport of Gly and P via tile drains from this type of clay soil. Proper agricultural management and improved topsoil structure can counteract fast macropore flow in this type of clay soil.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The article describes a field leaching experiment with glyphosate in Sweden on an agriculturally used soil. The article provides no information to check the validity of the study against current standards.
The study is therefore classified as reliable with restrictions (Category 2).

1. Information on the study

Data point:	KCA 7.2.2.3
Report author	Wang, S. et al.
Report year	2016
Report title	(Bio)degradation of glyphosate in water-sediment microcosms - A stable isotope co-labeling approach
Document No	Water Research 99 (2016) 91-100
Guidelines followed in study	OECD guideline 308
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (water/sediment systems may have received inputs of glyphosate or AMPA within the previous 4 years; extremely high application rate)

2. Full summary of the study according to OECD format

Glyphosate and its metabolite aminomethylphosphonic acid (AMPA) are frequently detected in water and sediments. Up to date, there are no comprehensive studies on the fate of glyphosate in water sediment microcosms according to OECD 308 guideline. Stable isotope co-labeled $^{13}C_3{}^{15}N$-glyphosate was used to determine the turnover mass balance, formation of metabolites, and formation of residues over a period of 80 days. In the water-sediment system, 56 % of the initial $^{13}C_3$-glyphosate equivalents was ultimately mineralized, whereas the mineralization in the water system (without sediment) was low, reaching only 2 % of ^{13}C-glyphosate equivalents. This finding demonstrates the key role of sediments in its degradation. Glyphosate was detected below detection limit in the water compartment on day 40, but could still be detected in the sediments, ultimately reaching 5 % of $^{13}C_3{}^{15}N$-glyphosate equivalents. A rapid increase in $^{13}C_3{}^{15}N$-AMPA was noted after 10 days, and these transformation products ultimately constituted 26 % of the $^{13}C_3$-glyphosate equivalents and 79 % of the ^{15}N-glyphosate equivalents. In total, 10 % of the ^{13}C label and 12 % of the ^{15}N label were incorporated into amino acids, indicating no risk bearing biogenic residue formation from $^{13}C_3{}^{15}N$-glyphosate. Initially, glyphosate was biodegraded via the sarcosine pathway related to microbial growth, as shown by co-labeled $^{13}C_3{}^{15}N$-glycine and biogenic residue formation. Later, degradation via AMPA dominated under starvation conditions, as shown by the contents of ^{13}C-glycine. The presented data provide the first evidence of the speciation of the non-extractable residues as well as the utilization of glyphosate as a carbon and nitrogen source in the water-sediment system. This study also highlights the contribution of both the sarcosine and the AMPA degradation pathways under these conditions.

Materials and Methods

Chemicals

All the chemicals used were analytical or reagent grade and were obtained from the Carl Roth Company (Karlsruhe, Germany) if not specified otherwise. Resin for amino acid purification (Dowex 50W-X8, 50-100 mesh) was purchased from VWR/Merck (Darmstadt, Germany). Methanol and ammonium acetate for ultraperformance liquid chromatography-mass spectrometry (UPLC/MS) measurements were provided by Biosolve (Valkenswaard, Netherlands). Labeled $^{13}C_3{}^{15}N$-glyphosate was purchased from IsoSciences Company (Trevose, PA, USA). The isotopic enrichment of the labeled glyphosate was 99 at % for ^{13}C and 98 at % for ^{15}N; the chemical purity was 98 %.

Sediments and water

The sediments and associated water were collected from the Getel creek, Harz Mountains in Saxony-Anhalt, Germany. The catchment of this creek comprises agricultural lowlands with continuous

crop rotation and pesticide application. It is thus a high risk area for exposure to pesticides. The sediments contained 38 % (±0.7 %) sand (>0.05 mm), 62 % (±0.7 %) silt + clay (<0.05 mm), 85 mg/g (±2 mg/g) total organic carbon and 15 mg/g (±1 mg/g) total nitrogen. The pH of the sediments and creek water was 7.1 and 8.8, respectively. The content of total organic carbon of the suspended matter in the creek water was 8 mg/L (±1 mg/L), and the content of total nitrogen was 3 mg/L (±0.6 mg/L). Neither glyphosate nor AMPA were detected in the sediments or creek water. Sediments and associated water were taken from the upper layer (up to 5 cm) of the Getel creek sediment. The sediments were separated from the water by filtration, wet sieved and gently homogenized.

Incubation experiment

Degradation experiments were conducted according to the OECD guideline 308 in biometer flasks to address the transformation in aquatic sediment systems. Six incubations were performed: 1) water-sediment without glyphosate (non-amended control), 2) water-sediment with unlabeled glyphosate (unlabeled control), 3) water-sediment with labeled glyphosate (biotic system), 4) water with unlabeled glyphosate (unlabeled control), 5) water with labeled glyphosate and 6) sterilized water sediment with labeled glyphosate (abiotic system). The two controls without glyphosate and unlabeled glyphosate were used to correct for the natural abundances of ^{13}C (~1.1 at %) and ^{15}N (~0.37 at %) in the sediment, and water systems without sediment were prepared to test the effect of sediment on the microbial degradation of glyphosate. Abiotic controls were incubated to distinguish between abiotic and biotic degradation of $^{13}C_3{}^{15}N$-glyphosate. In these controls, sediment and water were sterilized by autoclaving three times at 120°C for 20 min prior to incubation.

50 g (dw) of sediment and 90 mL of creek water containing either unlabeled or labeled glyphosate were added to glass bottles. The initial concentration of glyphosate was 50 mg/L in water and water-sediment systems, except in the blanks containing no glyphosate. This concentration is well above environmentally relevant levels, but it was required to obtain reliable isotopic enrichment results in the water-sediment systems given the limited sensitivity of $^{13}C/^{15}N$ isotope analytical methods and the high background due to natural abundance of the heavy isotopes in the controls. To assess the overall fate and turnover at lower concentrations that were closer to environmentally relevant concentrations, additional water-sediment experiments at 3 mg/L (minimum ^{13}C and ^{15}N label detection limit) were prepared. Incubation experiments were conducted in the dark and at constant temperature (20°C) for 80 days. The bottles were sampled after 0, 5, 10, 20, 40 and 80 days (abiotic, blank, water and 3 mg/L systems only after 80 days). At each sampling time, the respective systems were destructively sampled, and the water and sediments were separated by filtration and subjected to further analyses. The CO_2 evolved from the mineralized glyphosate was trapped in 2 M NaOH; the NaOH solution was exchanged at regular intervals. Because the pH of the water was >7.0, a certain amount of CO_2 originating from the mineralization of glyphosate may partition into the water phase, which therefore has been analyzed in addition to the NaOH traps. Mineralization in biotic and abiotic systems includes $^{13}CO_2$ in both the sodium hydroxide and water phases.

Chemical analyses

A general mass balance of the ^{13}C and ^{15}N labels in the systems was set up based on the contents and isotopic compositions of CO_2, the extractable glyphosate and its metabolites and either ^{13}C or ^{15}N in the total NER. Proteins were hydrolyzed, and the amino acids (AA) were extracted and analyzed for their concentration and isotopic composition to estimate the extent that C and N from $^{13}C_3{}^{15}N$-glyphosate were incorporated into microbial biomass and ultimately into biogenic residues. Proteins are the main constituents of microbial biomass (50 % of cells); therefore, the quantification of biogenic residues formation was based on a factor of 2 for both ^{13}C and ^{15}N-amino acids (AA).

CO_2 measurements

The ^{13}C labeled CO_2 was quantified by measuring the total inorganic C in a 2 M NaOH solution on a total organic carbon analyzer. The isotopic composition of CO_2 (at % ^{13}C) was measured by

GC-combustion-isotope ratio mass spectrometry (GC-C-irMS; Finnigan MAT252 Thermo Electron coupled to a Hewlett Packard 6890 GC) with a Porabond Q-HT Plot FS column (50 m - 0.32 m - 5 µm).

Extractable glyphosate and AMPA

Glyphosate and AMPA were extracted with borate buffer (40 mM, pH 9.2) from sediments and derivatized with 0.5 mL of fluorenylmethyloxycarbonyl (Fmoc). The water samples were directly derivatized with Fmoc in borate buffer. The concentrations of glyphosate and AMPA were determined by UPLC-MS i-Class system (Waters, Manchester, UK) with an Acquity UPLC HSS T3 column (1.7 µm, 2.1 x 100 mm; Waters, Milford, MA, USA). The temperatures of the column and the autosampler were set at 60°C and 4°C, respectively. The injection volume was 10 µL. The eluents were 5 mM NH_4 acetate (pH 8) in water (eluent A) and methanol (eluent B). The flow rate was set to 0.6 mL/min. The gradient program was as follows: 0-3 min 5 % B, 7-8 min 95 % B, 8.1-10 min 5 % B. The MS analysis was performed using a Xevo TQ-S mass spectrometer (Waters, Manchester, UK) equipped with an ESI source in negative ion mode working in multiple reaction monitoring (MRM) mode. A capillary voltage of 2 kV and a desolvation temperature of 600°C were used. The flow of the desolvation gas was set at 1000 L/h. Unlabeled glyphosate and AMPA were used for calibration and as internal standards for correction of possible matrix effects which may occur during the measurement of glyphosate and AMPA concentrations. Transitions, cone voltages, and collision energies were automatically tuned for the compounds: $^{13}C_3{}^{15}N$-glyphosate (m/z 172 / m/z 154, cone: 58 V, collision energy: 10 V; m/z 172 / m/z 63, cone: 58 V, collision energy: 16 V) and $^{13}C_3{}^{15}N$-AMPA (m/z 112 / m/z 63, cone: 58 V, collision energy: 16 V; m/z 112 / m/z 79, cone: 58 V, collision energy: 10 V). The detection limit (LOD) of glyphosate was determined at 20 µg/L, and the LOD for AMPA was 30 µg/L based on the signal-to-noise method (signal >3 S/N). For the entire procedure, including the extraction of the sediment samples, the detection limits were 0.608 mg/kg (glyphosate) and 0.912 mg/kg (AMPA). The recovery of glyphosate and AMPA was >98 %. The values of the coefficient of determination (R^2) for all calibration curves were greater than 0.99. The relative error of UPLC-MS measurements was <10 %.

Non-extractable residues (NER)

After the extraction of glyphosate and AMPA, the sediment sample containing unextracted ^{13}C and ^{15}N label as NER was airdried. An aliquot of 4-5 mg was weighed and combusted using an elemental analyzer-combustion-isotope ratio mass spectrometer combination (EA-C-irMS; Euro EA 3000, Eurovector, Milano, Italy + Finnigan MAT 253, Thermo Electron, Bremen, Germany). Glyphosate-derived C and N were calculated as the excess ^{13}C and ^{15}N over the controls. The values of the coefficient of determination (R^2) for all calibration curves were greater than 0.99.

Amino acids (AA)

Amino acids were analyzed in the living microbial biomass AA fraction of sediment (bioAA) and in the total AA pool of the sediment fraction (tAA). Microbial biomass was extracted from the sediment with ion exchanger and sodium deoxycholate/polyethylenglycol solution. The sediment and microbial biomass pellets containing accordingly tAA and bioAA were hydrolyzed using 6 M HCl. Thereafter, the hydrolysate was purified over a cation exchange resin. The detailed extraction, purification and derivatization methods for bioAA and tAA were described previously. The identity and quantity of AA were measured using GC-MS, HP 6890 with a BPX-5 column. The isotopic composition of the respective AA (at % ^{13}C and at % ^{15}N) was determined by GC-C-irMS, Finnigan MAT 253 coupled to a Trace GC, with a BPX-5 column. The details on the analytical conditions for AA separation by GC-MS and GC-C-irMS are reported in Nowak et al. (2013). For quantification and identification of respective AA in samples, an external standard containing all detectable AA in the samples (alanine, glycine, threonine, valine, leucine, isoleucine, proline, aspartate, glutamate, phenylalanine and lysine) was used. The internal standard L-norleucine was added to each sample before hydrolysis to estimate the losses in soil AA analyses. The recovery of all measured AA was >90 %, except from threonine (>80 %). The measured isotopic compositions were corrected for shifts due to derivatization.

Data analyses and mass balance

All incubation experiments and chemical analyses were conducted in triplicate, and the data are presented as averages of three replicates. Mineralization, extractable and non-extractable $^{13}C_3$ ^{15}N-glyphosate residues were quantified for each sampling date in order to set up the full carbon and nitrogen mass balance, and to determine the compound degradation kinetics. The contents of the $^{13}CO_2$, ^{13}C and ^{15}N-NER, ^{13}C and ^{15}N-AA (bioAA + tAA) were based on quantitation of the total concentration of the respective carbon or nitrogen pool and on analyzing the excess of ^{13}C (^{15}N) over the controls (non-amended without glyphosate and unlabeled containing unlabeled glyphosate) as described by Lerch et al. (2009). The results were expressed as a percentage of ^{13}C or ^{15}N label relative to the initial $^{13}C_3$-glyphosate equivalents or ^{15}N-glyphosate equivalents. The total uncertainty of the carbon pool in CO_2 and of the carbon and nitrogen pools in NER was <10 %, whereas the total uncertainty of the determination of at % ^{13}C and at % ^{13}N isotope signatures was <0.5 % for unlabeled samples, but <3 % for the labeled ones. The relative average error of the label excess (based on Gaussian error propagation) was <10 % for CO_2 and NER.

The total uncertainty of carbon and nitrogen pool in tAA and bioAA was <15 %. The total uncertainty on the determination of at % ^{13}C and at % ^{13}N isotope analysis was <0.5 % for unlabeled samples, but <1 % for labeled ones. The relative average error of the label excess (based on Gaussian error propagation) was <10 % for tAA and bioAA.

The recovery of the ^{13}C and ^{15}N labels expressed as a percentage of the initially applied isotope label equivalents ranged from 93 to 110 % for C and from 86 to 110 % for N. Incorporation of the ^{13}C and ^{15}N labels into the microbial biomass and thus the total content of biogenic residues formed during degradation of $^{13}C_3{}^{15}N$-glyphosate in the water-sediment system were estimated from ^{13}C -tAA and ^{15}N-tAA, considering that AA constituted approximately 50 % of the total C and total N in the biomass. The recovery of microbial biomass extraction is estimated at 40 %. The bioAA results are presented both as the original data and the recalculated values based on 40 % extraction efficiency, but interpretation of bioAA was based on the original data.

Results and Discussion

Mineralization of $^{13}C_3$-glyphosate

Mineralization of $^{13}C_3$-glyphosate in the biotic water-sediment system consisted of three periods (Figure 1): an initial short lag-phase from day 0 to day 10 characterized by low mineralization rates (0.3 %/day), day 10-40 characterized by the highest mineralization rate (1.2 %/day), and day 40-80 characterized by decreasing mineralization rates to 0.4 %/day. At the end of incubation, a total of 56 % of the $^{13}C_3$-glyphosate had been mineralized. Abiotic processes played a minor role in the mineralization of $^{13}C_3$-glyphosate (<20 %). The mineralization rates of $^{13}C_3$-glyphosate in the water system (without sediment) were very low and increased slowly during the first ten days (~0.1 %/day). Thereafter, the mineralization rate decreased and only 2 % of $^{13}C_3$-glyphosate equivalents were mineralized at the end, demonstrating the key role of sediments in the mineralization of $^{13}C_3$-glyphosate. Mineralization of $^{13}C_3$-glyphosate at 3 mg/L was slightly higher (65 % of $^{13}C_3$-glyphosate equivalents) than at 50 mg/L. The acclimation period at 50 mg/L was longer (10 days vs. 5 days for 3 mg/L). The mineralization rate in the initial phase (0 – 10 days) was two-fold higher at 3 mg/L (0.6 %/day) than at 50 mg/L (0.3 %/day) and 1.3-fold higher in the second phase (10 – 40 days; 1.6 %/day compared to 1.2 %/day, respectively). In the third phase (40 – 80 days), the mineralization rate was 2-fold lower at 3 mg/L (0.2 %/day) than at 50 mg/L (0.4 %/day).To date, there are no reports on the mineralization of $^{13}C_3$-glyphosate in water-sediment systems. Although glyphosate was below the detection limit in the sediment and associated water used in the present experiments, prior exposure to this herbicide is very likely due to input from the agricultural area in the catchment, with major biodegradation occurring in the sediment phase. In contrast to the high $^{13}CO_2$ evolution from $^{13}C_3$-glyphosate equivalents, no or minimal mineralization of ^{15}N-glyphosate was found in the present study because the total recovery of the ^{15}N label ranged from 86 to 110 %.

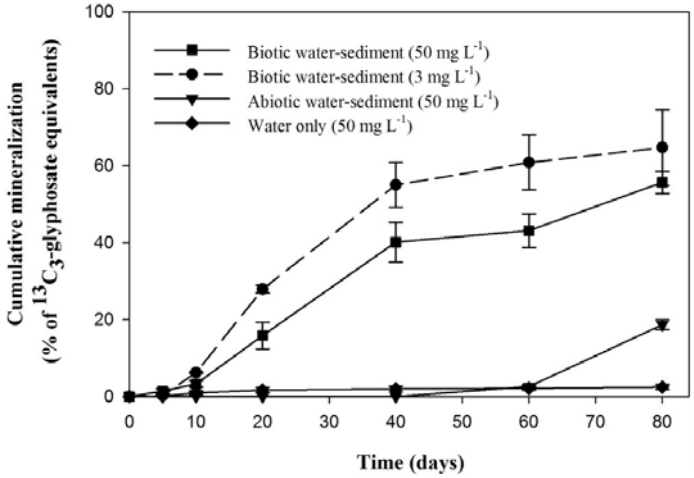


Figure 1. **Cumulative mineralization of $^{13}C_3$-glyphosate in water-sediment and water only systems over 80 days given as percentages of applied $^{13}C_3$-glyphosate equivalents.**

Turnover of $^{13}C_3{}^{15}N$-glyphosate

The content of $^{13}C_3{}^{15}N$-glyphosate in the biotic 50 mg/L water sediment system decreased rapidly until day 40 (Fig. 2A), indicating its low persistence reflected in its half-life (DT_{50}) of 15 days. In the water compartment, glyphosate dissipated rapidly during the first five days. Thereafter, elimination of this herbicide continued slowly until its ultimate removal by day 40. From day 40 onwards, $^{13}C_3{}^{15}N$-glyphosate was detected only in the sediment compartment although it was initially spiked in the water phase. This indicates that elimination from the water compartment was a combined process of sorption onto sediments and microbial transformation. A quick partitioning of glyphosate from the water compartment to the sediments had already been observed on day 0. At the initial sampling, which was performed 3 h after the addition of the glyphosate-spiked water to allow for particle sedimentation, 16 % of the initially added $^{13}C_3{}^{15}N$-glyphosate was already detected in the sediment phase. The high abundance of the silt + clay fraction (62 %), which is typically rich in oxides, of the sediments might explain the rapid elimination of glyphosate from the water by adsorption to the sediments. The turnover kinetics of $^{13}C_3{}^{15}N$-glyphosate in the sediment was much slower than in the water. A maximum amount of $^{13}C_3{}^{15}N$-glyphosate (51 % of the initially added $^{13}C_3{}^{15}N$ label) was detected in the sediments on day 5. Therefore, a potential risk by residual glyphosate in the sediment is given. Thereafter, elimination of $^{13}C_3{}^{15}N$-glyphosate from sediments was rapid (days 5-40), followed by a slower disappearance towards the end to ultimately result in 5 % of the initially added $^{13}C_3{}^{15}N$ label.

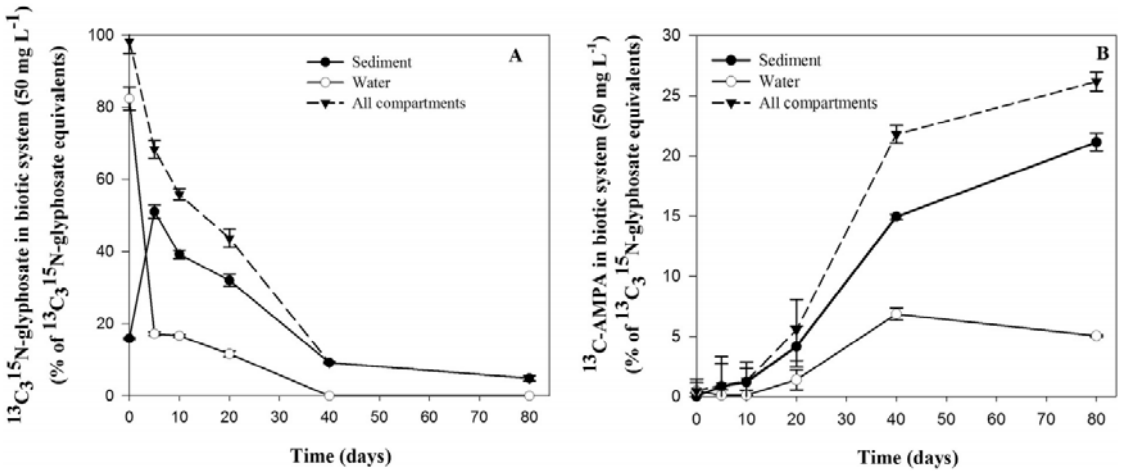


Figure 2. **Distribution of the extracted $^{13}C_3{}^{15}N$-glyphosate (A) and $^{13}C1$-AMPA (B) in biotic water-sediment systems (50 mg/L) over 80 days expressed as the percentage of applied $^{13}C_3{}^{15}N$-glyphosate. (Please note: ^{13}C-AMPA only contains one labeled carbon atom; the second metabolite glyoxylate contains the other two).**

The decrease in $^{13}C_3{}^{15}N$-glyphosate in both the water and sediment compartments during days 5-40 parallels the increasing mineralization of glyphosate. At the same time, a large amount of the recovered $^{13}C_3{}^{15}N$-glyphosate from the water-sediment system was associated with the sediments (~74 %), whereas only ~25 % was dissolved in the water. Finally, when $^{13}C_3{}^{15}N$-glyphosate was detected only in the sediment compartment (40 – 80 days), mineralization kinetics were slower indicating a limited bioavailability of glyphosate adsorbed onto sediment particles (Katagi, 2013). In the first ten days of the present experiments, only low contents of ^{13}C in AMPA (1 % of $^{13}C_3{}^{15}N$-glyphosate equivalents; Figure 2B) and ^{15}N in AMPA (4 % of $^{13}C_3{}^{15}N$-glyphosate equivalents) were observed. Thereafter, (10-40 days), a rapid increase in the $^{13}C_3{}^{15}N$-AMPA contents was noted and was accompanied by the rapid degradation of $^{13}C_3{}^{15}N$-glyphosate in the water-sediment system with concomitant $^{13}CO_2$ formation. From day 40 onwards, when glyphosate partitioned into the sediment and its mineralization rate decreased, the increase in the $^{13}C_3{}^{15}N$-AMPA contents slowed down. At the end of the experiment, ^{13}C in AMPA accounted for 26 % of the $^{13}C_3$-glyphosate equivalents. As only one of the three labeled ^{13}C atoms from the glyphosate, but all of the ^{15}N (one atom) is retained in AMPA (Figure 4) and the percentages are referred to the initial amount of labeled atoms (not molecules), the percentage of ^{15}N-AMPA was generally 3-fold higher than that of ^{13}C-AMPA and thus amounted to 79 % of the initially added ^{15}N-glyphosate. Similar to $^{13}C_3{}^{15}N$-glyphosate, the recovered $^{13}C_3{}^{15}N$-AMPA from the system was mostly associated to the sediment (70-90 %), whereas the residual (10-30 %) was dissolved in the water phase. In contrast to $^{13}C_3{}^{15}N$-glyphosate, $^{13}C_3{}^{15}N$-AMPA was more persistent; this was indicated by its continuous increase until the end of the experiment, indicating that $^{13}C_3{}^{15}N$-AMPAwas degraded more slowly than it was produced from glyphosate, as reported earlier (Mamy et al.,2005). Unfortunately, our data do not allow quantification of microbial AMPA degradation due to the simultaneous formation and degradation. Due to the continuing production of AMPA at a higher rate than degradation, a potential risk may be given by this metabolite. Compared to the biotic systems, the abiotic controls, water without sediment and biotic systems at 3 mg/L showed much lower formation of $^{13}C_3{}^{15}N$-AMPA from glyphosate.

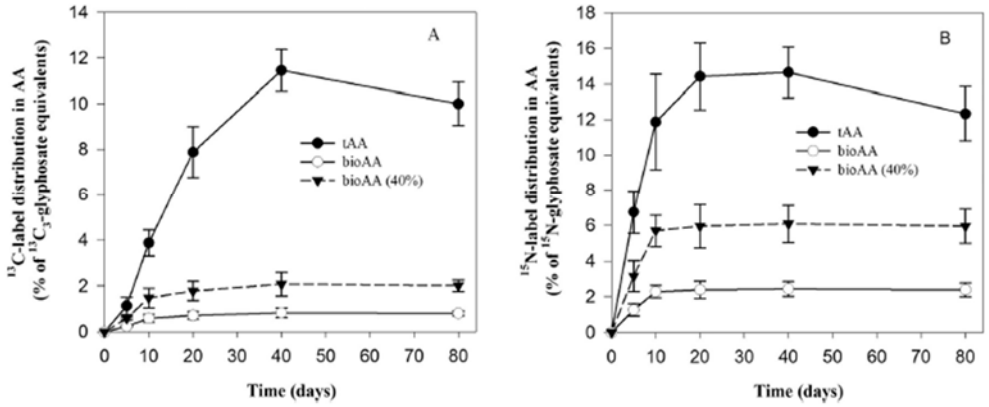


Figure 3. **Time dependent ^{13}C- (A) and ^{15}N-label (B) incorporation into tAA, bioAA and recalculated bioAA (40 % extraction efficiency) during microbial degradation of $^{13}C_3{}^{15}N$-glyphosate in biotic water-sediment system (50 mg/L) expressed as the percentage of applied $^{13}C_3{}^{15}N$-glyphosate equivalents.**

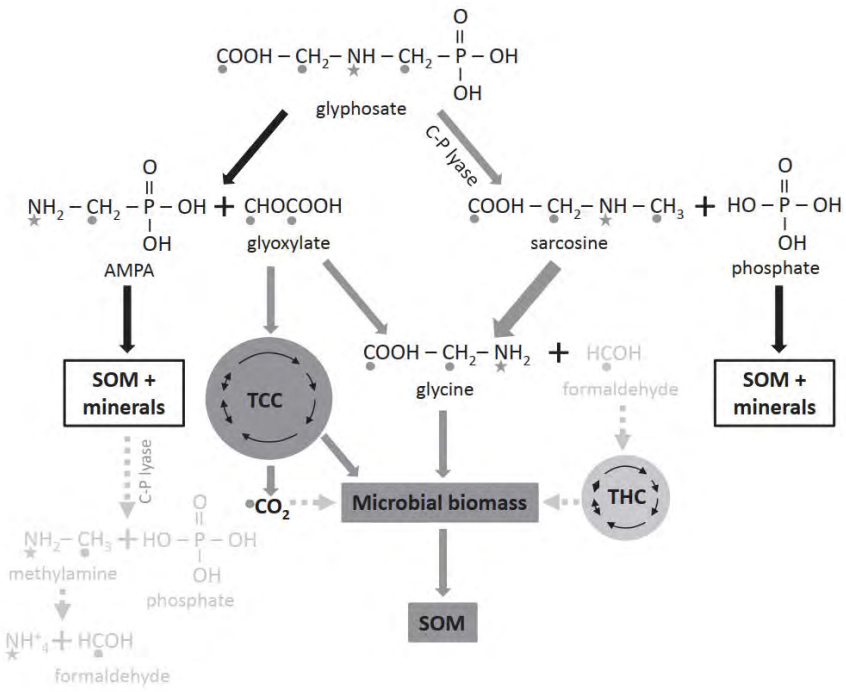


Figure 4. **Pathways of microbial degradation of glyphosate through sarcosine and AMPA in biotic water-sediment system (50 mg/L). Dark grey arrows: biogenic residue formation; black arrows: xenobiotic NER formation; TCC: tricarboxylic acid cycle; THC: tetrahydrofolate cycle; grey circles = ^{13}C label; grey stars = ^{15}N label; Light grey = presumed further degradation.**

Incorporation of the ^{13}C and ^{15}N labels into AA and biogenic residues

The total AA pool of sediment (tAA) includes the AA in the living biomass and in the dead and decaying necromass. Neither ^{13}C nor ^{15}N enrichment in AA was detected in the suspended particles in either the water compartment of the abiotic sediment-water systems or the water systems. In the biotic water-sediment systems, ^{13}C label incorporation into the bioAA fraction was observed in the first sampling, and the contents of ^{13}C-bioAA increased rapidly to a maximum on day 40 (0.83 % of $^{13}C^{3}$-glyphosate equivalents; Figure 3A). A continuous flux of ^{13}C--labeled AA from the living biomass to the non-living fraction OM was noted from day 5 onwards. ^{13}C-tAA initially increased sharply whereas from day 20 onwards, the ^{13}C-bioAA remained nearly constant, and approximately 92 % of the ^{13}C label in the tAA could be attributed to the non-living OM. In contrast to the ^{13}C bioAA, the ^{13}C-tAA contents slightly decreased after 40 days. At the end of the experiment, the contents of ^{13}C in tAA reached 10 % of the initially added $^{13}C_3$-glyphosate. Considering a protein content of 50 % in bacterial cells (Nowak et al., 2013), we arrive at a total of 20 % ^{13}C-biogenic residues at the end of the experiment. Similar to ^{13}CAA, incorporation of the ^{15}N label into bioAA and tAA was also observed starting from day 5 (Figure 3B). In contrast to ^{13}C-bioAA, ^{15}N-bioAA contents plateaued on day 10 (2.38 % of ^{15}N-glyphosate equivalents). The incorporation of ^{15}N-bioAA into the non-living OM fraction was also similar to that of ^{13}C-bioAA, starting rapidly (on day 5), and approximately 81 % of the ^{15}N-tAA was stabilized in the non-living OM at the end. The rapid initial increase in ^{15}N-tAA continued until day 20 and then remained stable until day 40. Analogous to ^{13}C-tAA, ^{15}N-tAA decreased slightly towards the end.

^{15}N-tAA amounted to 12 % of the initially added ^{15}N-glyphosate at the end (similar to ^{13}C-tAA), and 24 %was observed for ^{15}N-biogenic residues based on a conversion factor of two for biomass in general. The dominant incorporation of both the ^{13}C and ^{15}N labels into the glycine was observed throughout the experiment and was most pronounced in the initial incubation period (see Tables 1 and 2A, B). Various AA were progressively enriched in both isotopes over time. In general, incorporation of ^{15}N proceeded faster than that of ^{13}C (2A, 2B). In contrast to ^{13}C, the ^{15}N label disappeared from $^{13}C^{15}$N-glycine quickly and was distributed within different AA more rapidly than the ^{13}C label.

The results based on the ^{13}C- and ^{15}N-colabeling technique allowed comprehensive insight into the C and N fluxes from the colabeled $^{13}C_3{}^{15}N$ -glyphosate via microbial biomass to the non-living OM. Microorganisms assimilated the carbon and nitrogen from glyphosate to synthesize biomass compounds, as shown by the ^{13}C and ^{15}N-labeled bioAA. After death and cell lysis, their biomass constituents were progressively incorporated into the non-living OM fraction where they were stabilized and ultimately formed non-toxic biogenic residues.

Table 1. ^{13}C label distribution in diverse ^{13}C-bioAA (A) and ^{13}C-AA in the non-living SOM (B) during biodegradation of $^{13}C_3$-glyphosate in biotic water-sediment system (50 mg/L).

(A)

Incubation time (days)	^{13}C-bioAA (% of $^{13}C_3$-glyphosate equivalents applied)					
	0	5	10	20	40	80
Alanine	n.d.	0.06 (±0.01)	0.07 (±0.00)	0.07 (±0.02)	0.07 (±0.00)	0.06 (±0.01)
Glycine	n.d.	**0.17** (±0.02)	**0.18** (±0.01)	0.14 (±0.01)	0.15 (±0.03)	**0.11**↓ (±0.01)
Threonine	n.d.	n.d.	n.d.	0.07 (±0.00)	0.06 (±0.01)	0.06 (±0.02)
Valine	n.d.	n.d.	0.10 (±0.02)	0.10 (±0.01)	0.09 (±0.02)	0.09 (±0.00)
Leucine	n.d.	n.d.	n.d.	n.d.	0.14 (±0.00)	0.12 (±0.01)
Isoleucine	n.d.	n.d.	0.11 (±0.00)	0.14 (±0.00)	0.05 (±0.01)	0.04 (±0.02)
Proline	n.d.	n.d.	n.d.	0.05 (±0.03)	0.04 (±0.01)	0.09 (±0.00)
Aspartate[a]	n.d.	n.d.	0.02 (±0.00)	0.03 (±0.00)	0.04 (±0.02)	0.08 (±0.01)
Glutamate[b]	n.d.	n.d.	0.06 (±0.03)	0.07 (±0.03)	0.05 (±0.02)	0.05 (±0.00)
Phenylalanine	n.d.	n.d.	n.d.	n.d.	0.10 (±0.03)	0.07 (±0.01)
Lysine	n.d.	n.d.	0.04 (±0.01)	0.04 (±0.03)	0.04 (±0.06)	0.03 (±0.01)
Total	**n.d.**	**0.23 (± 0.03)**	**0.58 (± 0.07)**	**0.71 (± 0.13)**	**0.83 (± 0.21)**	**0.80 (± 0.10)**

(B)

Incubation time (days)	^{13}C-non-living AA (% of $^{13}C_3$-glyphosate equivalents applied)					
	0	5	10	20	40	80
Alanine	n.d.	0.4 (±0.1)	0.6 (±0.1)	1.3 (±0.3)	1.4 (±0.1)	0.7 (±0.1)
Glycine	n.d.	**0.5** (±0.2)	**1.0** (±0.1)	1.0 (±0.1)	0.5 (±0.1)	0.6 (±0.2)
Threonine	n.d.	n.d.	n.d.	0.4 (±0.1)	0.1 (±0.1)	0.5 (±0.1)
Valine	n.d.	n.d.	n.d.	1.0 (±0.6)	1.1 (±0.1)	1.3 (±0.3)
Leucine	n.d.	n.d.	n.d.	n.d.	1.6 (±0.3)	1.2 (±0.1)
Isoleucine	n.d.	n.d.	n.d.	0.7 (±0.1)	1.1 (±0.1)	0.9 (±0.3)
Proline	n.d.	n.d.	0.5 (±0.1)	0.5 (±0.1)	0.5 (±0.1)	0.3 (±0.1)
Aspartate[a]	n.d.	n.d.	0.5 (±0.1)	0.5 (±0.1)	1.1 (±0.2)	0.8 (±0.1)
Glutamate[b]	n.d.	n.d.	0.7 (±0.1)	0.5 (±0.1)	1.1 (±0.1)	1.6 (±0.2)
Phenylalanine	n.d.	n.d.	n.d.	n.d.	1.0 (±0.3)	0.6 (±0.1)
Lysine	n.d.	n.d.	n.d.	1.3 (±0.1)	1.3 (±0.4)	0.9 (±0.3)
Total	**n.d.**	**0.9 (± 0.3)**	**3.3 (± 0.5)**	**7.2 (± 1.6)**	**10.8 (± 2.0)**	**9.4 (± 1.9)**

[a] Incl. Asparagine.

[b] Incl. Glutamine; n.d. - not detectable; values are presented as averages ± standard deviation; values printed in bold show characteristic values; arrows illustrates increases or decreases of the respective AA compared to the preceding sampling event.

Table 2. ^{15}N label distribution in diverse ^{15}N-bioAA (A) and ^{15}N-AA in the non-living SOM (B) during biodegradation of ^{15}N-glyphosate in biotic water-sediment system (50 mg/L).

(A)

Incubation time (days)	^{15}N-bioAA (% of ^{15}N-glyphosate equivalents applied)					
	0	5	10	20	40	80
Alanine	n.d.	0.01 (±0.08)	0.09 (±0.1)	0.03 (±0.02)	0.15 (±0.02)	0.23 (±0.07)
Glycine	n.d.	**0.70** (±0.07)	**1.20**↑(±0.01)	**1.20** (±0.09)	**0.80**↓(±0.10)	**0.43**↓(±0.07)
Threonine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	n.d.	0.38 (±0.07)	0.28 (±0.03)	0.28 (±0.02)	0.30 (±0.05)	0.28 (±0.02)
Leucine	n.d.	0.17 (±0.02)	0.17 (±0.04)	0.14 (±0.07)	0.40 (±0.09)	0.38 (±0.03)
Isoleucine	n.d.	n.d.	0.30 (±0.01)	0.28 (±0.01)	0.09 (±0.07)	0.13 (±0.06)
Proline	n.d.	n.d.	0.04 (±0.04)	0.04 (±0.13)	0.05 (±0.04)	0.22 (±0.05)
Aspartate[a]	n.d.	n.d.	n.d.	0.13 (±0.06)	0.10 (±0.09)	0.22 (±0.10)
Glutamate[b]	n.d.	n.d.	0.10 (±0.03)	0.19 (±0.07)	0.19 (±0.08)	0.20 (±0.02)
Phenylalanine	n.d.	n.d.	0.06 (±0.03)	0.08 (±0.02)	0.21 (±0.04)	0.19 (±0.02)
Lysine	n.d.	n.d.	0.04 (±0.03)	0.01 (±0.01)	0.14 (±0.05)	0.10 (±0.08)
Total	**n.d.**	**1.26 (±0.24)**	**2.28 (±0.32)**	**2.38 (±0.5)**	**2.43 (±0.63)**	**2.38 (±0.52)**

(B)

Incubation time (days)	^{15}N-non-living AA (% of ^{15}N-glyphosate equivalents applied)					
	0	5	10	20	40	80
Alanine	n.d.	0.2 (±0.1)	0.6 (±0.1)	0.4 (±0.1)	0.8 (±0.2)	0.2 (±0.1)
Glycine	n.d.	**1.1** (±0.1)	1.2 (±0.2)	1.2 (±0.1)	**0.8**↓(±0.1)	**0.7**↓ (±0.1)
Threonine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	n.d.	0.8 (±0.1)	1.4 (±0.2)	1.5 (±0.5)	1.3 (±0.2)	1.0 (±0.2)
Leucine	n.d.	0.7 (±0.1)	1.2 (±0.8)	2.8 (±0.4)	1.8 (±0.1)	1.5 (±0.1)
Isoleucine	n.d.	n.d.	1.5 (±0.1)	1.2 (±0.2)	1.6 (±0.1)	1.4 (±0.3)
Proline	n.d.	n.d.	0.6 (±0.1)	0.8 (±0.1)	0.8 (±0.1)	0.4 (±0.1)
Aspartate[a]	n.d.	n.d.	0.1 (±0.2)	0.5 (±0.1)	1.0 (±0.1)	0.6 (±0.2)
Glutamate[b]	n.d.	**1.2** (±0.1)	1.0 (±0.1)	0.7 (±0.1)	1.4 (±0.1)	1.8 (±0.1)
Phenylalanine	n.d.	n.d.	0.7 (±0.1)	1.4 (±0.1)	1.4 (±0.1)	1.2 (±0.1)
Lysine	n.d.	n.d.	0.1 (±0.1)	1.6 (±0.1)	1.7 (±0.1)	0.9 (±0.1)
Total	**n.d.**	**4.0 (±0.5)**	**8.4 (±2.0)**	**12.1 (±1.8)**	**12.6 (±1.2)**	**9.7 (±1.4)**

[a] Incl. Asparagine.
[b] Incl. Glutamine; n.d. - not detectable; values are presented as averages ± standard deviation; values printed in bold show characteristic values; arrows illustrates increases or decreases of the respective AA compared to the preceding sampling event.

Indication of different (bio)degradation pathways of glyphosate in water-sediment systems

Based on the detailed glyphosate turnover mass balance and the patterns of ^{13}C and ^{15}N labeled AA over time, particularly of the dominant glycine, we could distinguish between two degradation pathways of this herbicide in water-sediment system. The dominance of co-labeled ^{13}C^{15}N-glycine especially in the first sampling event indicates its formation via the sarcosine pathway (Borggaard and Gimsing, 2008; Singh and Walker, 2006; see also Figure 4). However, the occurrence of the sarcosine pathway in soil or sediment has not yet been proven (Borggaard and Gimsing, 2008; Singh andWalker, 2006). We could not detect sarcosine in our experiment, but this compound is rapidly oxidized to glycine and thus does not accumulate. The formed glycine is directly incorporated into microbial biomass, resulting in the observed occurrence of co-labeled ^{13}C^{15}N-glycine in the living biomass AA. The negligible mineralization (3 % of ^{13}C equivalents initially applied) with high simultaneous removal of $^{13}C_3{}^{15}$N -glyphosate and the maximum contents of ^{13}C and ^{15}N glycine on day 10 also support the hypothesis that glyphosate is initially degraded via the sarcosine pathway. Hence, the sarcosine pathway was actually contributing at the beginning of glyphosate degradation, whereas the AMPA pathway dominated in the later degradation phase. A later decrease of co-labeled $^{13}C_3{}^{15}$N-glycine (10-20 days) was accompanied by a rapid increase in AMPA over time.

The risk potential of glyphosate residues in water-sediment systems

To date, there is no detailed information on the metabolic fate of glyphosate residues and their distribution in the water-sediment system. The present results provide detailed insight into the biodegradation processes of $^{13}C_3{}^{15}$N-glyphosate in the water-sediment system and into the transformation of this herbicide into AMPA, microbial biomass and NER. Since glyphosate is biodegraded and the NER are dominantly biogenic residues, the highest potential risk is provided by the significant concentrations of AMPA.

Non-extractable $^{13}C_3$-glyphosate residues were formed immediately (6 % of the initially added ^{13}C label, see Table 3). The NER contents increased until day 10 and then remained on a high level. From day 20 onwards, their contents decreased and ultimately reached 23 % of the $^{13}C_3$-glyphosate equivalents. The chemical composition of the NER formed during degradation of glyphosate is not yet known, and their

analyses are limited to quantification. In the present study, glyphosate was initially a source of xenobiotic ^{13}CNER formation that was dominant until day 10 (Figure 5A). However, immobilized glyphosate in the NER was microbially degradable, as shown by the continuous decrease of xenobiotic NER over time, specifically of ^{13}C-xenobiotic NER. Microorganisms used the carbon and nitrogen from $^{13}C_3{}^{15}$N-glyphosate to synthesize their biomass compounds, as shown by the ^{13}C and ^{15}N incorporation into microbial AA, leading to biogenic residues in OM after cell death and lysis. Based on the ^{13}C-tAA content, 20 % of the ^{13}C-biogenic residues were formed and constituted the major fraction of ^{13}C-NER (87 %, Figure 5A). These results agree with previous studies on biogenic residue formation during biodegradation of ^{13}C-labeled pesticides or pharmaceuticals.

Table. 3. **Mass balance of $^{13}C_3{}^{15}$N-glyphosate degradation in biotic and abiotic water-sediment systems and in water over 80 days (% of initially applied ^{13}C- and ^{15}N-label equivalents).**

		% of initial ^{13}C				
	Time (days)	**Mineralization**	**Glyphosate**	**AMPA**	**NER**	**Recovery**
	0	n.d.	98 (± 1)	0.2 (±0.1)	6 (± 1)	104 (± 2)
Biotic	**5**	1 (± 1)	68 (± 2)	1 (± 1)	28 (± 1)	98 (± 4)
	10	3 (± 1)	56 (± 2)	1 (± 1)	33 (± 5)	93 (± 7)
	20	16 (± 0)	44 (± 2)	6 (± 1)	35 (± 1)	101 (± 5)
	40	40 (± 1)	9 (± 1)	22 (± 1)	31 (± 1)	102 (± 4)
	80	**56 (± 3)**	**5 (± 1)**	**26 (± 1)**	**23 (± 2)**	**110 (± 6)**
Abiotic	**80**	19 (± 1)	41 (± 2)	11 (± 1)	26 (± 0)	97 (± 3)
Only water	**80**	2 (± 1)	90 (± 2)	2 (± 0)	n.d.	94 (± 3)

		% of initial ^{15}N			
	Time (days)	**Glyphosate**	**AMPA**	**NER**	**Recovery**
	0	98 (± 1)	1 (± 1)	5 (± 0)	104 (± 2)
Biotic	**5**	68 (± 2)	3 (± 1)	20 (± 2)	91 (± 6)
	10	56 (± 2)	4 (± 1)	26 (± 3)	86 (± 6)
	20	44 (± 2)	17 (± 3)	30 (± 1)	91 (± 6)
	40	9 (± 1)	65 (± 4)	31 (± 0)	105 (± 5)
	80	**5 (± 1)**	**79 (± 5)**	**26 (± 0)**	**110 (± 6)**
Abiotic	**80**	41 (± 2)	33 (± 1)	26 (± 3)	100 (± 6)
Only water	**80**	90 (± 2)	6 (± 1)	n.d.	96 (± 2)

n.d. - not detectable; values are shown as averages ± standard deviation

The kinetics of ^{15}N-NER formation showed a similar pattern to that of ^{13}C-NER and reached 26 % of the initially added ^{15}N-glyphosate (Table. S2). Analogous to the ^{13}C-biogenic residues, the ^{15}N-NER were primarily biogenic (Figure 5B); at the end of the experiment, the ^{15}N-biogenic residues amounted to 24 % of ^{15}N-glyphosate equivalents and constituted 92 % of the ^{15}N-NER. In contrast to the ^{13}C-biogenic residues, the ^{15}N-biogenic residues were formed rapidly, which is in line with the metabolization and mineralization of $^{13}C_3{}^{15}$N-glyphosate via the sarcosine pathway without N mineralization in the initial degradation phase. The contents of extractable $^{13}C_3{}^{15}$N-glyphosate residues (31 % of the $^{13}C_3$-glyphosate equivalents and 84 % of the ^{15}N-glyphosate equivalents) comprised a large proportion of the ^{13}C and ^{15}N-isotope mass balance at the end, with AMPA accounting for almost all of these residues (Table. S2). The percentage of ^{15}N-AMPA was 3-fold higher than that of ^{13}C-AMPA because only one out of three ^{13}C atoms, but all ^{15}N atoms from the co-labeled glyphosate are transferred to AMPA during metabolization (see Figure 4). In the sediment-water systems nearly all of the NER could be explained by biogenic residues bearing no potential risk. However, high contents of extracted AMPA were detected, which typically biodegrades slower than glyphosate. The detailed fate of AMPA needs to be investigated to assess the potential risks related to this degradation product of glyphosate. In contrast to previous studies in which biogenic residues remained constant, ^{13}C and ^{15}N biogenic

residues from glyphosate slightly decreased towards the end of the experiment. Total hydrolysable ^{13}C- and ^{15}N-labeled AA decreased progressively after 69 days in sediments incubated with ^{13}C-glucose and ^{15}N-labeled ammonium, which is in agreement with the present study.

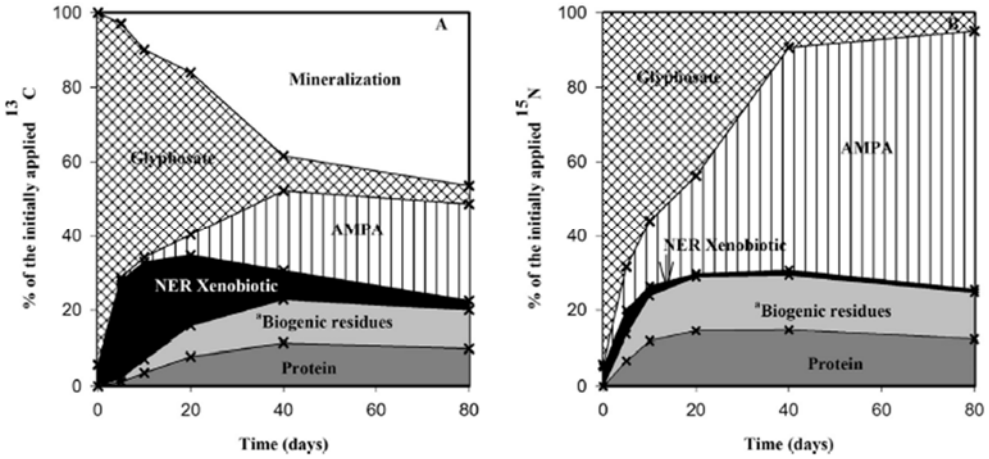


Figure 5. **Detailed mass balance including biogenic residue formation (A) of $^{13}C_3$-glyphosate and (B) of ^{15}N-glyphosate in biotic water-sediment system (50 mg/L). a: Biogenic residues were calculated based on a conversion factor of 2 for proteins.**

Conclusions

- This is the first detailed glyphosate turnover mass balance including NER speciation in water-sediment systems using stable isotope co-labeled tracers (^{13}C and ^{15}N).

- Sediment plays a key role in the microbial degradation of glyphosate via both the sarcosine and AMPA pathway.

- At the end, nearly all of the NER can be assigned to non-toxic biogenic residues after degradation of the parent compound.

- Accumulation of main metabolite of glyphosate, AMPA, may because for environmental concern; therefore, an additional investigation of the fate of AMPA in water-sediment systems is needed.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article reports the results from a water-sediment dissipation experiment with ^{13}C-^{15}N-labelled-glyphosate, conducted according to OECD guideline 308. The methods and results are generally well described and conclusive. However, the water and associated sediment were taken from a German small water body located in agricultural lowlands with continuous crop rotation and pesticide application, which is considered a high risk area for exposure to pesticides. Thus, it cannot be excluded that the water/sediment system received inputs of glyphosate or AMPA within the previous 4 years, as is required in OECD 308 guideline. Further, for the main experiment, the application rate was extremely high (50 mg/L, equivalent to an application rate of 150 kg/ha when assuming overspray of a 30 cm deep water body) while in OECD 308 guideline it is recommended that the test concentration should be close to application rate or environmental concentrations.

The article is therefore classified as reliable with restrictions (Category 2).

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
General criteria for reliability considered for all data requirements indicated by the corresponding EU data points as specified in EC Regulation (EU) No 283/2013	1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines met.	No
	2. Previous exposure to other chemicals is documented (where relevant).	No
	3. The test substance is dissolved in water or non-toxic solvent	Yes
	4. Glyphosate, when the test substance, is sufficiently documented - identity of the test material reported (i.e. purity, source, content, storage conditions)	Yes
	5. Only glyphosate is the tested substance (excluding mixture), and information on application of glyphosate is described	Yes
	6. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite)	Yes
	7. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes
	8. Analytical verifications performed in test media (concentration)/ collected samples, stability of glyphosate in test media documented	Yes
	9. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information	Yes
	10. Assessment of the statistical power of the assay is possible with reported data.	Yes
	11. If statistical methodology was applied for findings reported, then the data analysis applied is clearly reported (e.g., checking the plots and confidence intervals)	Yes
	12. Field locations relevant/comparable to European conditions. Soils not completely matching the OECD criteria but from Europe or to some extent representative for the European Agriculture.	Yes
	13. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5 %), bulk density, water retention, microbial biomass (~1 % of organic carbon)	No
	14. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass	No
	15. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year	No
	16. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	No
	17. Data on precipitation is recorded	No
	18. The temperature was in the range between 20-25°C and the moisture was reported	Yes
	19. The presence of glyphosate identified in samples collected from groundwater, soil, surface waters, sediments or air from European areas	Yes
	20. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate	Yes

E-Fate: Reliability criteria for the detailed assessment of full-text documents

Data requirements (indicated by the corresponding EU data point)	Criteria for "Reliable" articles	Criteria met? Yes / No / Uncertain
	21. Analytical methods clearly described and adequate Statement of specificity and sensitivity of the analytical methods is included	Yes
	22. Radiolabel characterization: purity, specific activity, location of label	Yes
	23. If degradation kinetics are included: expect to see data tables provided, model description. Statistical parameters for kinetic fit.	Yes
	24. Glyphosate monitoring data: description of matrix analysed, and analytical methods fully described as above.	No
	25. For environmental fate studies: clear description of application rate and relevance to approved uses.	Yes

1. Information on the study

Data point:	KCA 7.1.3.1.1
Report author	Zhelezova, A., et al.
Report year	2017
Report title	Effect of Biochar Amendment and Ageing on Adsorption and Degradation of Two Herbicides
Document No	Water Air Soil Pollut (2017) 228: 216
Guidelines followed in study	OECD 106 (2000)
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Reliable with restrictions (not all validity criteria reported for the sorption experiment, no guideline followed, no endpoint can be derived according to current guidance for the degradation experiment)

2. Full summary of the study according to OECD format

Biochar amendment can alter soil properties, for instance, the ability to adsorb and degrade different chemicals. However, ageing of the biochar, due to processes occurring in the soil over time, can influence such biochar-mediated effects. This study examined how biochar affected adsorption and degradation of two herbicides, glyphosate (N-(phosphonomethyl)-glycine) and diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) in soil and how these effects were modulated by ageing of the biochar. One sandy and one clayey soil that had been freshly amended with a wood-based biochar (0, 1, 10, 20 and 30% w/w) were studied. An ageing experiment, in which the soil-biochar mixtures were aged for 3.5 months in the laboratory, was also performed. Adsorption and degradation were studied in these soil and soil-biochar mixtures, and compared to results from a soil historically enriched with charcoal. Biochar amendment increased the pH in both soils and increased the water-holding capacity of the sandy soil. Adsorption of diuron was enhanced by biochar amendment in both soils, while glyphosate adsorption was decreased in the sandy soil. Ageing of soil-biochar mixtures decreased adsorption of both herbicides in comparison with freshly biochar-amended soil. Herbicide degradation rates were not consistently affected by biochar amendment or ageing in any of the soils. However, glyphosate half-lives correlated with the Freundlich Kf values in the clayey soil, indicating that degradation was limited by availability there.

Materials & Methods

Soil Sampling and Processing

The soil samples were collected in September 2015 from arable fields at two locations: Länna (L) (59° 52′ N, 17° 58′ E) and Ulleråker (U) (59° 49′ N, 17° 39′ E). Soil sampling at L was performed in two parts of the arable field: an untreated part (L) and a historically charcoal-enriched part (LB). Because of the long-term charcoal amendment, the latter soil was characterised by lower bulk density and higher loss on ignition and water-holding capacity (WHC) than the unamended soil from the same field, which leads to higher yields in dry years. In each soil, about 10 samples were taken from the upper layer (5–15 cm below surface) and pooled. After sieving, the Ø < 2 mm fraction was homogenised and stored at −20 °C in plastic bags until the start of the experiment. Moisture content and WHC were measured for all soil samples. Moisture content was determined by drying at 110 °C for 10 h, while WHC was defined as the moisture content after saturation of 30 g soil with distilled water for 10 h followed by 4 h of free drainage. Chemical and physical properties of the three soils studied (L, LB, U) were determined by a commercial laboratory and are presented in Tables 1 and 2.

Preparation and Ageing of Soil-Biochar Mixtures

The biochar used was the commercial product *Skogens kol*, which is produced from a mixture of about 80% hardwood, mainly birchwood (*Betula* sp.) and 20% wood from Norway spruce (*Picea abies*), by slow pyrolysis with a maximum process temperature of 380– 430 °C (Cederlund et al. 2016). Soil-biochar mixtures were prepared by mixing soil (L and U) with sieved biochar (Ø < 2 mm) at a rate of 1, 10, 20 and 30% biochar per unit soil dry weight (designated L1, L10, L20 and L30 and U1, U10, U20 and U30). WHC was determined as described above and pH for all mixtures was measured in a 1:2 slurry of soil and distilled water (*w*/*v*) after shaking and stabilisation for 10 h. Biochar ageing was performed with soil-biochar mixtures made from U soil. These mixtures were incubated in darkness at 20 °C for 3.5 months. The moisture content was adjusted to 55% of WHC and monitored and adjusted weekly by addition of deionised water.

Chemicals Used in Herbicide Adsorption and Degradation Experiments

Glyphosate (N-(phosphonomethyl)-glycine, CAS [1071-83-6], 98%) and diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), CAS [330-54-1], 99.0%) were provided by Dr. Ehrenstorfer GmbH, Augsburg, Germany. ^{14}C-labelled diuron ([ring-U-^{14}C], 96.4%, 5.71 MBq/mg) and glyphosate ([P- methylene-^{14}C], 4.87 MBq/mg) were provided by the Institute of Isotopes Co. Ltd., Budapest, Hungary.

Measurement of Herbicide Adsorption in Soils and Soil-Biochar Mixtures

Adsorption was determined in a batch-equilibrium system according to OECD guideline 106 (OECD 2000). A pre-study was performed to estimate the time when the equilibrium between adsorbed herbicide and herbicide in solution was reached (8, 24 and 32 h). In all cases, equilibrium was reached within 24 h. For high-percentage soil-biochar mixtures with U soil, an additional pre-study was performed to estimate an appropriate soil to solution ratio as defined in the OECD guideline. Soil and soil-biochar mixtures, corresponding to 1 g of soil or mixture dry weight, were weighed into tubes (15-mL glass tubes for diuron and 50-mL polypropylene tubes for glyphosate) and adjusted with 0.01 M $CaCl_2$ to reach the appropriate soil-solution ratio. This was 1:40 for all samples with glyphosate and for U20, U30, U20a and U30a with diuron and 1:4 for all other samples with diuron. The samples were shaken for 24 h (20 °C, 200 revolutions/min). After that, herbicides were added to reach concentrations of 1, 5, 10, 50 and 100 μg/g dry weight (dw) soil for glyphosate and 0.1, 0.5, 1, 5 and 10 μg/g dw soil for diuron, due to its lower water solubility. In addition, a fixed amount (10 μL for glyphosate and 20 μL for diuron) of ^{14}C- labelled herbicide was added to each tube to reach an activity of 2000 DPM (3.333×10^{-5} MBq) per sample. There were two replicate tubes of each concentration. After 24 h, the tubes were centrifuged (3000 revolutions/min for 30 min), samples of supernatant were transferred to scintillation vials (4 mL for diuron and 10 mL for glyphosate samples) and Quicksafe A (Scintvaruhuset, LAB-service, Uppsala, Sweden) was added directly before measurement of scintillation. ^{14}C activity was measured on a Beckman LS 6000TA liquid scintillation counter (Beckman Counter Inc., Fullerton, CA). Controls without herbicides were measured for all samples to exclude the level of background radioactivity. The data obtained were fitted using the linear form of the Freundlich isotherm.

Herbicide Degradation Experiment

The herbicides were dissolved in water (glyphosate) or methanol (diuron) and added dropwise to a fraction (10%) of the soils and soil-biochar mixtures. Water and methanol were allowed to evaporate from the samples for 10 h. The herbicide-treated part was then mixed with the rest of each sample to give an initial nominal concentration of 10 mg/kg soil dry weight. Portions of soil corresponding to 5 g of dry weight were weighed into 50-mL plastic tubes and the water content was adjusted to 60% of WHC and kept at this level for the duration of the experiment. The tubes were sealed with caps and were incubated at 20 °C in the dark. After 1, 2, 5, 8, 16, 23 (only for U samples) and 31 days of incubation, two replicate tubes from each treatment were placed in the freezer (−20 °C) for future extraction and analysis.

Table 1. Chemical properties of the soils studied

Soil	Code	HCl extracted K (mg 100 g^{-1})	HCl extracted P (mg 100 g^{-1})	Al-K[a] (mg 100 g^{-1})	Al-P[a] (mg 100 g^{-1})	Total C (%)	Total N (%)	pH
Charcoal-amended soil from Länna	LB	68.35	85.02	3.82	16.48	17.57	0.37	5.57
Untreated soil from Länna	L	229.36	78.77	37.28	16.67	4.86	0.34	5.27
Soil from Ullerâker	U	287.59	68.16	34.95	4.87	1.36	0.1	6.41

[a] Al–K/Al-P = ammonium lactate-extractable K and P—Swedish standard method for estimation of plant available K and P fractions (Otabbong et al. 2009)

Table 2. Physical properties of the soils studied

Soil code	Clay Ø < 0.002 mm	Fine silt 0.002–0.006 mm	Medium silt 0.006–0.02 mm	Coarse silt 0.02–0.06 mm	Fine sand 0.06–0.2 mm	Medium sand 0.2–0.6 mm	Coarse sand 0.6–2 mm	Loss on ignition %
LB	n.d.	n.d.	n.d.	n.d.	5.9	3.3	4.1	39.4
L	66.5	14.8	9.1	6.5	2.1	0.7	0.3	13.7
U	7.5	3.2	2.4	3.2	12.1	63.8	7.8	3.3

n.d. not determined

Data from the degradation experiment after recovery correction were used to estimate herbicide half-life. Recovery was calculated as:

$$\text{Recovery} = \left(\frac{C_0}{C_{\text{nominal}}}\right) \times 100$$

where *C0* is the herbicide concentration determined at day 0. Natural logarithms of remaining concentrations for days 0–31 were plotted against time, giving the first- order rate constant *k* as the slope of the linear regression line. Half-life (*T*½) was calculated as:

$$T^{1}/_{2} = \frac{\ln 2}{k}$$

Analysis of Diuron

For diuron extraction from soil and soil-biochar mixtures, the following protocol was used: 10 mL methanol were added using a Vogel pipette to the tubes with sample. The tubes were shaken at 200 revolutions/min for 60 min, centrifuged at 4000 revolutions/min for 10 min and the supernatant was filtered (OOH Whatman; 11 cm). Portions (1 mL) of filtrate were transferred to sample vials and HPLC analysis was performed according to the protocol in Cederlund et al. (2007). Standard solutions with concentration range 0.05–50 µg/mL were analysed with extracts from samples. The HPLC was equipped with a G1314A UV detector, a G1311A pump, a G1329A auto injector (Agilent Technologies AB; 1100 Series; Sweden) and a Zorbax SB-C18 column (12.5 × 4.6 mm, 5 mm; ChromTech AB, Sundbyberg, Sweden).

Analysis of Glyphosate

Extraction of glyphosate, derivatisation and measurement on GC-MS were performed using the same reagents for analytical standards, glyphosate extraction and internal standards as previously described (Bergström, Börjesson, and Stenström, 2011).

Results & Discussion

Effect of Biochar on Soil Water-Holding Capacity and pH

The studied soils had different physical texture: the dominant particle fractions in the L soil were clay and fine silt, while the U soil was dominated by medium and fine sand. The texture of the LB soil could not be fully determined due to its high organic matter content, as traces of organic C remained in the sample after digestion (oxidation by H_2O_2). Coming from the same field as L, it is likely that the LB soil was also dominated by clay. However, the proportion of sand was higher (Table 2). This agrees with Kihlberg et al. (unpublished), who also reported a coarser particle size distribution in LB compared with L soil, but also did not subdivide particles with Ø < 0.06 mm. The WHC of the clayey L soil (53%) was higher than in the sandy U soil, where it was only 27%, and was not affected by biochar addition. However, the LB soil, which was historically amended by charcoal, had a higher WHC (57%) than the L soil with or without fresh biochar amendment. In the sandy soil, the WHC increased from 27 to 42% with biochar addition and was correlated positively (r = 0.98) with the biochar percentage (Fig. 1). Biochar addition increased the pH from 5.27 to 6.07 in the L soil and from 6.41 to 7.69 in the U soil (Fig. 2). Ageing of the biochar led to a further pH increase in most of the soil-biochar mixtures (U10a-U30a). In the LB soil, the pH was higher (5.77) than in the L soil. The pH of soil-biochar mixtures was correlated with the percentage of biochar added in all cases (r = 0.99 for L soil-biochar mixtures; r = 0.99 for fresh U soil-biochar mixtures; r = 0.98 for aged U soil- biochar mixtures).

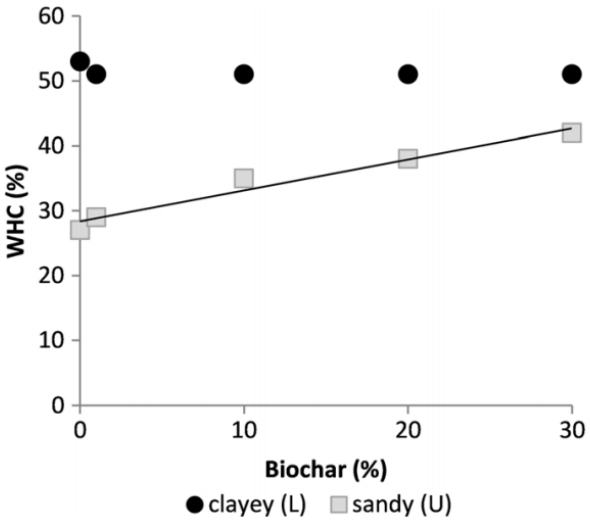


Fig. 1. Water-holding capacity (WHC) of the soil samples ± standard deviations plotted against biochar percentage added

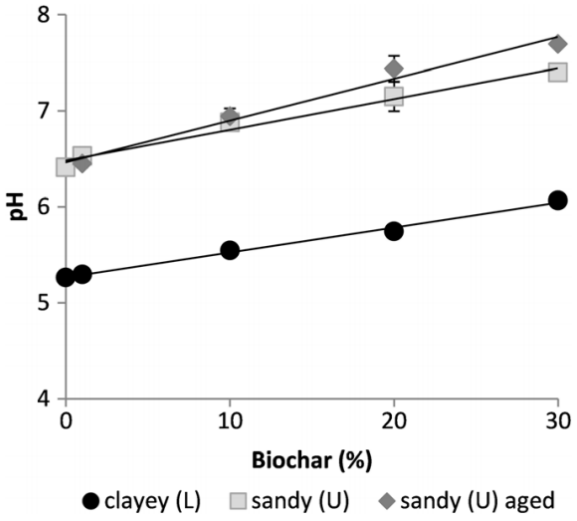


Fig. 2. pH of the soil samples (N = 2) ± standard deviations plotted against biochar percentage added

Adsorption of Diuron

Biochar amendment increased diuron adsorption in both the L and U soils (Fig. 3). In the LB soil, K_F was 364 $\mu g^{1-1/n}(mL)^{1/n} g^{-1}$, which is quite close to the K_F value of the L20 soil-biochar mixture. K_F values in the aged soil-biochar mixtures were lower than in mixtures with fresh biochar addition. There were positive correlations between the diuron K_F values and biochar percentage for L, U and aged U soils ($r = 0.96$, $r = 0.95$, and $r = 0.95$, respectively).

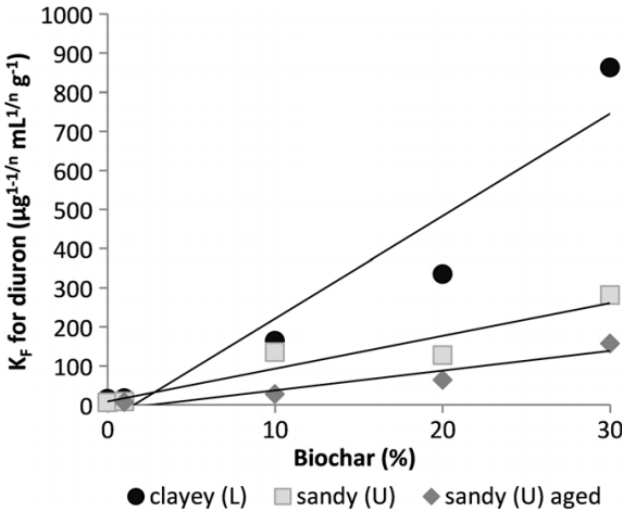


Fig. 3. Freundlich K_F values for diuron plotted against biochar percentage added in samples from Länna (L) and Ulleråker (U)

Adsorption of Glyphosate

Glyphosate was more strongly adsorbed in the L soil ($K_F = 1218$ $\mu g^{1-1/n}$ $mL^{1/n}$ g^{-1}) than in the U soil ($K_F = 146$ $\mu g^{1-1/n}$ $mL^{1/n}$ g^{-1}). No consistent effect of biochar amendment on glyphosate adsorption in L soil was observed (Fig. 4). A very high K_F value was observed for the sample with 1% biochar addition ($K_F = 1892$ $\mu g^{1-1/n}$ $mL^{1/n}$ g^{-1}), while the K_F values for the unamended L soil and the other soil-biochar mixtures varied between 1099 and 1294 $\mu g^{1-1/n}$ $mL^{1/n}$ g^{-1}. The LB soil had a much lower *KF* value (539 $\mu g^{1-1/n}$ $mL^{1/n}$ g^{-1}) than the L soil and soil-biochar mixtures. However, in the U soil, glyphosate adsorption was correlated negatively ($r = -0.99$) with the biochar percent- age (Fig. 4). Ageing of the biochar decreased adsorption further.

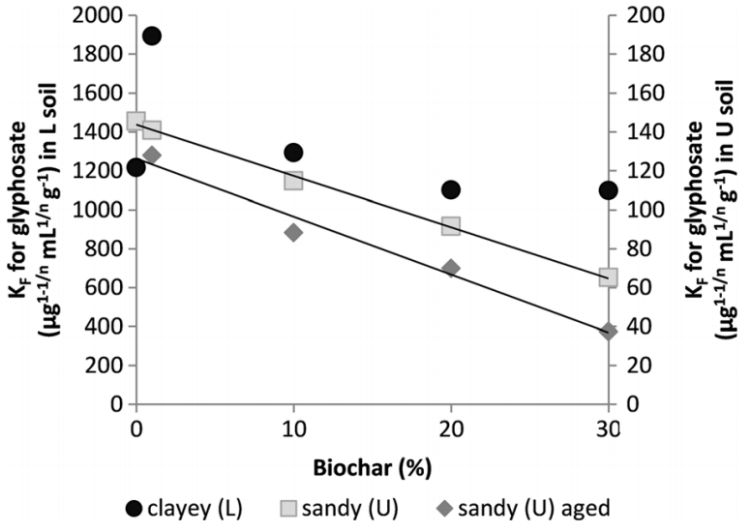


Fig. 4. Freundlich K_F values for glyphosate plotted against biochar percentage added in samples from Länna (L) and Ulleråker (U)

Table 3. Freundlich parameters (K_F, 1/n and R^2 value) for adsorption and half-life of diuron and glyphosate

Sampling site		Adsorption						Degradation			
		Diuron			Glyphosate			Diuron		Glyphosate	
		K_F[a]	1/*n*	R^2	K_F[a]	1/*n*	R^2	$T½$[b]	R^2	$T½$[b]	R^2
Länna	LB	364	0.859	0.99	539	0.890	0.99	36	0.965	17	0.97
	L	15.21	0.863	0.99	1218	0.842	0.99	40	0.963	87	0.606
	L1	17.10	0.807	0.99	1892	0.872	0.99	47	0.708	187	0.333
	L10	164	0.859	0.99	1294	0.806	0.99	42	0.853	151	0.385
	L20	335	0.822	0.99	1102	0.796	0.99	56	0.918	131	0.402
	L30	863	0.978	0.96	1099	0.780	0.98	45	0.86	51	0.945
Ulleråker	U	5.73	0.798	0.99	145.5	0.783	0.99	112	0.663	182	0.482
	U1	8.60	0.586	0.95	140.9	0.765	0.99	58	0.718	83	0.767
	U10	135	0.789	0.99	114.8	0.754	0.99	33	0.866	66	0.674
	U20	127	0.727	0.85	91.6	0.780	0.99	35	0.868	78	0.621
	U30	281	0.753	0.97	65.2	0.750	0.99	40	0.888	53	0.861
	U1a	6.44	0.760	0.99	127.9	0.761	0.99	37	0.71	51	0.716
	U10a	27.4	0.824	0.99	88.3	0.776	0.99	27	0.785	81	0.683
	U20a	64	0.547	0.94	70.0	0.788	0.99	29	0.849	49	0.917
	U30a	157	0.686	0.97	37.4	0.751	0.99	35	0.871	68	0.885

[a] The unit of K_F is $\mu g^{1-1/n}$ $mL^{1/n}$ g^{-1}

[b] The unit of $T½$ is days

Degradation of Diuron

In the L and U soils and soil-biochar mixtures, from 20 to 50% of the added diuron was degraded during the experimental period. Diuron half-life varied between 40 to 56 days in the L soil, was 36 days in the LB soil and varied between 26 to 112 days in the U soil (Fig. 5). No correlation was seen between the biochar percentage and diuron half-life in any of the soils. However, in the U soil, the half-life was shorter in all samples with biochar addition compared with the unamended soil. Here, it should be noted that the half-life of 112 days found for the U soil without biochar may be a less accurate estimation, since the dynamics of diuron degradation did not fit well with a first-order kinetic model in this sample. The degradation kinetics of all other samples followed first-order kinetics reasonably well, with R^2 values of 0.7–0.96. Ageing of the biochar consistently decreased diuron half-life in the U soil.

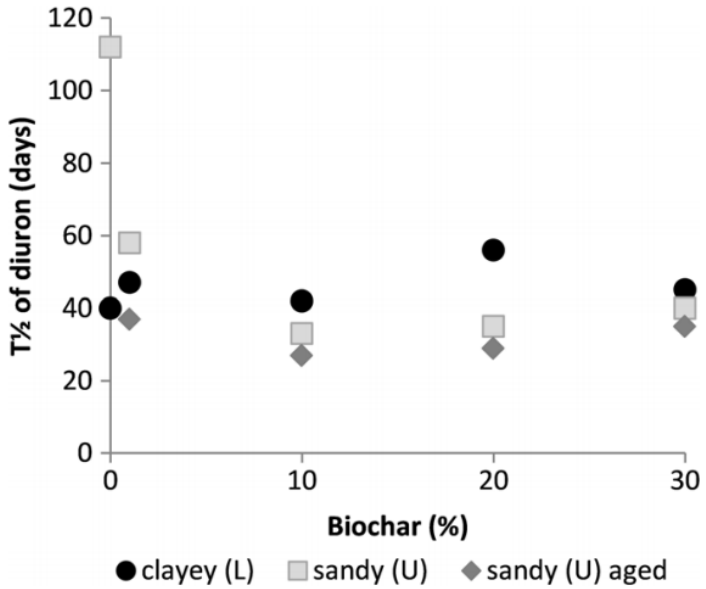


Fig. 5. Diuron half-life in the Länna (L) and Ulleråker (U) soils and soil-biochar mixtures

Degradation of Glyphosate

In the L and U soils and soil-biochar mixtures, 10–70% of the added glyphosate was degraded during the experimental period. Glyphosate half-life in the L soil varied between 51 and 187 days. However, in the L, L1, L10 and L20 samples, the data fitted poorly to the first-order kinetic model (R^2 = 0.33–0.61), mostly due to great variation in glyphosate concentrations during the first week of degradation. This fact can explain the some-what inconsistent pattern of half-life variation for the soil-biochar mixes. However, degradation in the LB and L30 samples followed first-order kinetics well (R^2 = 0.97 and 0.94). The shortest glyphosate half-life (19 days) was observed in the LB soil. Degradation of glyphosate was relatively slow in the unamended U soil, but was faster in all samples with biochar amendment. In the unamended U soil, the half- life of glyphosate was 182 days, while in the U soil- biochar mixtures, it varied between 49 and 83 days. However, as in the case of diuron, data from the un-amended U soil were a poor fit to the first-order model (R^2 = 0.48) and the degradation rate in the biochar-amended samples did not appear to be related to the biochar percentage added. The fastest degradation was observed in the U1a and U20a soil-biochar mixtures, but ageing of the biochar did not consistently affect degradation rates (Fig. 6). No correlations between glyphosate half-life and amount of added biochar were found for any of the L and U soils (Fig. 6). However, the half-life was correlated with the K_F value for glyphosate (r = 0.88) in samples of the L soil when the LB sample was included (Fig. 7). In the U soil and soil-biochar mixtures, the adsorption coefficient of glyphosate was generally lower and its half-life was not correlated with the K_F value (Fig. 7).

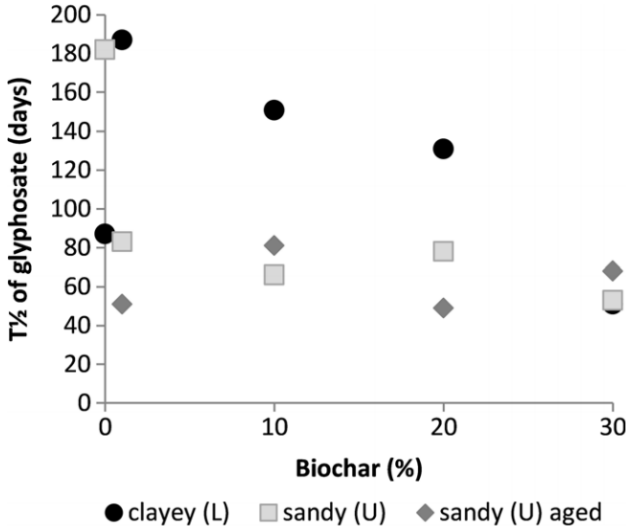


Fig. 6. Glyphosate half-life in the Länna (L) and Ullerâker (U) soils and soil-biochar mixtures

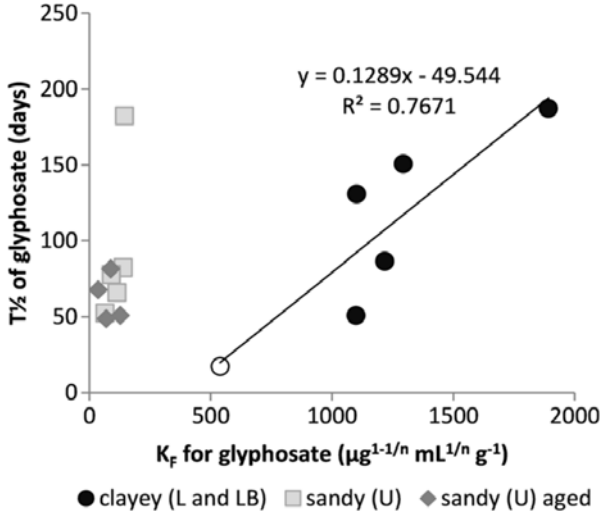


Fig. 7. Correlation between glyphosate half-life and adsorption coefficient (KF). The open circle is for the LB soil

Effects of Biochar on Herbicide Adsorption

Diuron adsorption increased after biochar amendment in both the L and U soils. This effect of biochar addition has been observed in previous studies for silty loam and sandy soil. Biochar contains many adsorption sites that can bind non-polar herbicides, so diuron adsorption increased with amount of biochar added, and the risk of it leaching is lower. The increased pH obtained with biochar addition is not likely to have contributed to the increased sorption since diuron is uncharged at relevant soil pH-levels. In a previous study, we also found that pH has no effect on diuron adsorption when studying this particular biochar without soil (Cederlund et al. 2016).

Biochar addition decreased glyphosate adsorption in the sandy U soil, but not in the clayey L soil. The difference in effects of biochar on glyphosate adsorption between the L and U soils may be explained by the different soil texture and physical properties of these soils. The decreased glyphosate adsorption in the U soil is likely to be related to the induced pH changes. According to several studies, soil pH is negatively correlated with glyphosate adsorption (Gimsing et al. 2004b; Mamy and Barriuso 2005; Vereecken 2005). Increased soil pH can increase the negative charge of both soil surfaces and glyphosate itself, which leads to enhanced repulsion. Glyphosate has a pH-dependent OH^- group with a pKa value of 5.7, so its charge is likely to have been affected in the pH range studied here. The same relationship with pH has been observed for glyphosate adsorption on pure biochar: Herath et al. (2016) studied the effect of pH on adsorption of glyphosate on a rice husk biochar and found that the adsorption percentage varied from 75 to 85% at pH 3–5, decreased to 75–65% at pH 6–8 and then significantly dropped to 55% at pH 9. However, in a previous study, we showed that glyphosate adsorption by the studied biochar was low at both low and high pH (Cederlund et al. 2016). In the L soil, there was no linear relationship between glyphosate adsorption coefficient and biochar amendment. The overall strong adsorption in this soil possibly contributed to masking the relatively minor effects of the biochar. It is known that inorganic components of soil, such as Al- and Fe-oxides, adsorb glyphosate effectively (Gimsing et al. 2004a) and that this herbicide is less available in soils with a high clay content. The induced pH changes in this soil also occurred over a different pH interval, which may have contributed to the less clear outcome.

Effects of Biochar Ageing on Adsorption

Short-term ageing of the biochar mixtures in the laboratory decreased adsorption of both herbicides. This suggests that processes that have the potential to reduce sorption, such as organo-mineral interactions with the biochar surface (Pignatello et al. 2006; Singh and Kookana 2009; Lin et al. 2012), were the dominant forces affecting the biochar during our ageing experiment. For diuron, our results are consistent with findings in a field study on biochar amendment of Australian ferrosols, in which diuron and atrazine adsorption to soils amended by poultry litter and paper mill biochar was significantly reduced after 32 months of ageing. For glyphosate, it is possible that the further increase in pH during the 3 months of ageing contributed to the additional decrease observed in sorption. Although we cannot know the original properties of the charcoal applied to the historically charcoal-enriched LB soil, it may be informative to compare the adsorption results from this soil. In LB, the K_F value for diuron was comparable to that determined in the 20% soil-biochar mixture (L20) and, considering that the total carbon content of the LB soil is about 18% (Table 1), this suggests limited effects of ageing. However, for glyphosate, the K_F value of the LB soil was only 539 $\mu g^{1-1/n}\ mL^{1/n}\ g^{-1}$, which is only about half the K_F value found for any of the fresh biochar mixtures or the unamended L soil (Table 3). Since the adsorption of glyphosate on the biochar itself is very weak, this low adsorption is difficult to explain in terms of reduced adsorptive affinity of the charcoal. It is more likely to reflect a reduced affinity for glyphosate of the soil itself. Kihlberg et al. (unpublished) suggest that the heat from the charcoal kilns in LB may have contributed to sintering the clay particles in the soil, causing a shift towards a coarser particle size distribution. Heating clay soils to 500 °C has been shown to change soil physical texture and increase the amount of silt and sand particles. Such a reduction in the proportion of clay would consequently reduce the amount of surfaces available for glyphosate adsorption. Heating may also cause other mineralogical changes in soil that affect adsorption, for instance de Santana et al. (2006) reported reduced interaction between glyphosate and Al_2O_3 and Fe_2O_3 in soil after burning. Our results for glyphosate differ somewhat from those of Kumari et al. (2016), who found that glyphosate sorption was

increased in a silty loam soil amended with the same wood-based biochar that we used (*Skogens kol*) after 7–10 months of ageing under field conditions. The application rates used in their study varied from 10 to 100 Mg biochar/ha added to the topsoil layer (0– 10 cm), which corresponds to about 0.8–8% of biochar per gramme dry weight assuming a bulk density of the soil of 1.3 g/cm³. Increases in glyphosate sorption occurred in plots amended with 10, 20 and 40 Mg/ha of biochar (i.e. corresponding to 0.8, 1.6 and 3.2% *w*/*w*), while the plot amended with 100 Mg/ha, where the glyphosate adsorption was the same as in the unamended plots, was considered to be an outlier (Kumari et al. 2016). In the present study, the clayey L soil with the lowest application rate was the outlier: the adsorption coefficient in the L1 soil-biochar mixture was much higher than in L soil without amendment, while the adsorption coefficient in the L10, L20 and L30 soil-biochar mixtures was the same or lower than in the unamended clayey L soil. However, we cannot offer an explanation for this pattern. In the sandy U soil, the adsorption of glyphosate was reduced after the ageing process, which can be explained by a further pH increase and low affinity to sorb glyphosate in both sandy soil and biochar itself.

Herbicide Degradation before and after Biochar Amendment

Microbial degradation of chemicals in soil has often been reported to be limited by strong sorption (Bergström et al. 2011; Gimsing et al. 2004a; Wu et al. 2011). Moreover, pesticide degradation is often inhibited after fresh biochar addition (Kookana 2010), which can be explained by a decrease in their bioavailability. In the present case, it seems that despite the fact that adsorption of diuron increased in both soils and that adsorption of glyphosate decreased in the sandy soil, biochar amendment had no clear effect on either diuron or glyphosate degradation. However, even though neither the K_F value nor the half-life of glyphosate was clearly correlated with the added biochar percentage in the clayey L soil, the half-life was correlated with the K_F value (Fig. 7). This indicates that in the case of glyphosate in the clayey L soil, which had K_F values >1000 $\mu g^{1-1/n}\ mL^{1/n}\ g^{-1}$, availability of glyphosate may have been a rate-limiting factor for its degradation, while in the other cases adsorption was too weak to have an effect.

Conclusion

As hypothesised, fresh biochar addition increased diuron adsorption in both clayey (L) and sandy (U) soils. However, glyphosate adsorption decreased only in the sandy U soil. These effects are most likely due to adsorption of diuron on the biochar itself, while in the case of glyphosate the decreased sorption may be explained by an increase in soil pH after biochar addition. No consistent effect of biochar amendment on herbicide degradation was observed in the studied soils, which contradicts our initial hypothesis. However, there was a positive relationship between adsorption and glyphosate half-life in the clayey soil-biochar mixtures, indicating that availability may be the rate-limiting step, but only where adsorption is strong. The consequences of biochar ageing under laboratory conditions were further increases in soil pH and a reduction in adsorption of both herbicides. Changes in biochar adsorptive properties during ageing in soil should be taken into consideration when planning its use in agriculture and for soil remediation purposes.

Assessment and conclusion by applicant:

The study describes the adsorption and degradation behavior of two pesticides in two different agricultural soils from Northern Europe after amendment of a biochar at different portions. Data on adsorption and degradation were evaluated for Glyphosate as well for the original soils without biochar amendment. The study design is well described and the adsorption parameters are sufficiently reported. The adsorption experiments was conducted according to the OECD guideline 106. However, not all validity criteria could be cross-checked as they are not reported. For the evaluation of the degradation behavior of Glyphosate, no information on the use of a specific guideline was reported. The results were only evaluated against Single First Order kinetics with partly poor fits. No information of the Glyphosate findings for the samples at different time points are reported, i.e. no additional kinetic evaluation is possible with the presented data. The study is therefore classified as reliable with restrictions (Category 2).

グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2010 年 1 月 1 日～2019 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	KCA 8.2.1
Report author	Antunes, A. M. *et al.*
Report year	2017
Report title	Gender-specific histopathological response in guppies *Poecilia reticulata* exposed to glyphosate or its metabolite aminomethylphosphonic acid
Document No	Journal of applied toxicology, 2017; 37:1098-1107
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Ecotoxicity of glyphosate (GLY) and its metabolite aminomethylphosphonic acid (AMPA) was investigated in guppies, *Poecilia reticulata*. The median lethal concentration after 96 hours of exposure (LC_{50}, 96 h) of both test item was determined in male and female guppies

Both genders showed similar median lethal concentration (LC_{50}) at 96 hours for glyphosate and AMPA. The acute 96 hour-LC_{50} of glyphosate obtained for male and female guppies *P. reticulata* were 68.78 mg/L (95 % C.I.: 64.59–73.24 mg/L and 70.87 mg/L (95 % C.I.: 65.91–76.26 mg/L), respectively. The 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L (95 % C.I.: 175.12–184.54 mg/L) and 164.3 mg/L (95 % C.I.: 160.6–168.54 mg/L), respectively.

Materials and methods

Tested products; GLY and AMPA 96% and 99%, respectively, were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). The stock solutions of GLY and AMPA were prepared in ultrapure water with a nominal concentration of 250 mg l^{-1}.

Animal collection and maintenance; *P. reticulata* used in the experiments was part of the animals group kept in the Aquatic Animal Biotery of the Cell Behavior Laboratory (Institute of Biological Sciences IV, Universidade Federal de Goiás, Goiânia, Brazil). All of them were 3-month-old F1 generation animals born in the biotery from a wild parental generation. 318 mature male and 318 mature female guppies (vitellogenic oocyte occurred) of an average weight of 252 ± 20 mg and 178.6 ± 14.4 mg, and total average length of 2.98 ± 0.3 cm and 2.47 ± 0.2 cm, respectively.

Toxicity test: LC_{50}; For each experimental condition, eight males or eight females guppies were maintained in 2 liter tanks (4 fish L) and exposed to different nominal concentrations of GLY (50, 55, 60.5, 66.5 and 73.2 mg/L) or AMPA (86.8, 104.2, 125, 150 and 180 mg/L) during 96 h in the static test under 12: 12 h light/dark cycles. These concentrations were determined in the preliminary tests. The control group that consisted of eight fish kept in dechlorinated water. All treatments were performed in a triplicate design and the fish were not fed during the experimental period (USEPA, 1993). The mortality was reported at different exposure times (2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h). Physical and chemical parameters of water were analyzed every morning and did not show any changes over the experimental period, such as temperature 24 ± 1 °C, dissolved oxygen 8 mg/L, ammonia 0.002 mg/L, pH 7.0 ± 1, nitrite 0.025 mg/L and nitrate 0.5 mg/L. Cumulative mortality data obtained at the end of the experiments (96 h) were analyzed by the trimmed Spearman–Karber method to estimate the LC_{50} of a 96 h exposure to GLY and AMPA.

Statistical analysis; All statistical analyses were performed using the Statistica 7.0 software (Statsoft Inc., 2005, Tulsa, OK, USA). The differences between the treatments of the analyzed variables were identified using parametric tests (two-way ANOVA, followed by the Tukey's test) and/or non-parametric tests (Kruskal–Wallis), depending on the distribution of the data and homogeneity of variances (Shapiro–Wilk and Levene's tests). Linear and non-linear regression analyses were also applied to verify the relationship between variables.

Results

Median lethal concentration (LC_{50}); No mortality was observed for both genders in the control group during the experimental period of 96 h. The LC_{50} results showed that the GLY is more toxic to the guppies than to its metabolite AMPA, whereas no significant difference was observed between the genders ($P > 0.05$). The GLY LC_{50} values obtained for male and female *P. reticulata* were, respectively, 68.78 mg/L (95% confidence interval = 64.59–73.24 mg/L) and 70.87 mg/L (95 % confidence interval = 65.91–76.26 mg/L). The AMPA LC_{50} in turn, were 180 mg/L (95 % confidence interval = 175.12–184.54 mg/L) and 164.3 mg/L (95 % confidence interval = 160.6–168.54 mg/L), respectively. The GLY and AMPA toxicity increased linearly with the increasing concentration for females (GLY: $y = 0.6281x – 30.141$, $r = 0.96$, $P < 0.05$; AMPA: $y = 0.150x – 11.193$, $r = 0.93$, $P < 0.05$) and males (GLY: $y = 0.666x – 30.653$, $r = 0.93$, $P < 0.05$; AMPA: $y = 0.168x – 11.898$, $r = 0.88$, $P < 0.05$).

Discussion

The results of the LC_{50} values of GLY (male 68.78 mg/L and female 70.87 mg/L) and AMPA (male 180 mg/L and female 164.3 mg/L) based on the mortality test indicated a low sensitivity of *P. reticulata* in comparison to the other teleost species, as reported by the USEPA. In addition, it was observed that the AMPA is less toxic to *P. reticulata* than GLY (male 2.6-fold, female 2.3-fold).

Conclusion

The present study determined the acute 96 hour-LC_{50} of glyphosate and AMPA. The glyphosate LC_{50} values obtained for male and female guppies *P. reticulata* were 68.78 mg/L (95 % C.I.: 64.59–73.24 mg/L and 70.87 mg/L (95 % C.I.: 65.91–76.26 mg/L), respectively.
The 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L (95 % C.I.: 175.12–184.54 mg/L) and 164.3 mg/L (95 % C.I.: 160.6–168.54 mg/L), respectively.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The acute 96 hour-LC_{50} values for male and female guppies *P. reticulata* after exposure to glyphosate were 68.78 mg/L and 70.87 mg/L, respectively. The acute 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L and 164.3 mg/L, respectively.
In the material and methods part some important is missing. No information on preparation of test solution and application is given. Source and composition of media are unclear. Furthermore, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.8
Report author	Daam M.A *et al.*
Report year	2019
Report title	Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae
Document No	Ecotoxicology (2019) 28:707–715
Guidelines followed in study	OECD (2015) Test No. 241: the larval amphibian growth and development assay ASTM (2013) Standard guide for conducting the frog embryo teratogenesis assay-Xenopus (FETAX). ASTM E1439-12
Deviations from current test guideline	Not reported
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of this study was to evaluate the acute toxicity of the active ingredient glyphosate to tadpoles of two tropical frog species: *Physalaemus cuvieri* and *Hypsiboas pardalis*. The calculated 96 h LC_{50} (median lethal concentration; in mg a.s./L) values for *P. cuvieri* and *H. pardalis* were 115 and 106 mg a.s./L, respectively.

Materials and methods

Test species

Three or more egg masses from different parents of *Physalaemus cuvieri* and *Hypsiboas pardalis* were collected from ponds at the Estação Biológica de Boracéia in Salesópolis, South-East Brazil (23°37′59”S, 45°31′59”W), which is located within a non-polluted, protected watershed. Egg masses were transported in sealed plastic bags containing water from the collection site to the laboratory of the School of Arts, Sciences and Humanities in the University of São Paulo. Hatched larvae were kept in 50 L plastic tanks filled with tap water filtered through an activated carbon granular filter. Tank water was renewed every other day. The temperature in the laboratory was controlled at 25 ± 2 °C with natural photoperiod. Larvae were fed daily with a 3:1 ground mixture of rabbit chow (Purina Mills, LLC, USA; ~16% protein) and Tetra Min Fish Flakes (Tetra Werke, Melle, Germany; ~45% protein) ad libitum until the beginning of the experiments. The bioassays were conducted with Gosner stage 25 tadpoles. Only healthy individuals, as judged by external morphology and behavior, were selected for the experiments.

Lethality tests

Acute (96 h) bioassays were conducted to evaluate the sensitivity of *P. cuvieri* and *H. pardalis* to the pure active ingredients glyphosate (CAS Number 1071-83-6; Purity 99.2%; Sigma-Aldrich). A semi-static design was adopted, in which test solutions were renewed 48 h after the start of the experiment. The tests were conducted under the same conditions as those described above, except that animals were not fed during the test. Based on the results of range-finding tests, five logarithmically-spaced test concentrations (all in mg a.i./L) were determined: Glyphosate: 84; 97; 112; 130; 150.

Test concentrations were prepared with stock solutions. Each treatment was conducted in quadruplicate, in which each replicate consisted of a glass jar containing 10 tadpoles in 1 L test solution. Every 24 h, water quality parameters (pH, temperature, conductivity, DO) were recorded using a multi-parameter meter (YSI 556), and dead individuals counted and removed.

Data analysis

The 96h LC_{50}, LOEC (lowest observed effect concentration) and NOEC (no observed effect

concentration) were calculated based on the % mortality rates in the different treatments using the statistical programs PROBIT 1.5 and TSK 1.5. In all cases, the most appropriate statistical test was defined depending on the experimental design and the nature of the available data, following the recommendations of EPA. To test for interspecies differences in sensitivity, LC50 values for each compound and species were compared with a Z test using the formula proposed by EPA. Analyses of Variance (ANOVA) followed by post hoc tests were employed to test for treatment effects on physical-chemical variables (mean values for each treatment over the experimental period) using the software PAST.

Results

Survival was 100% in all control treatments. Water quality parameters were comparable in control replicates with a cofficient of variation of less than 4% for all para- meters (pH, temperature, conductivity and DO).
The 96h LC_{50} values generated and is presented in Table 1, whereas the mortality levels of the individual treatments for *P. cuvieri* and *H. pardalis* are visualized in Figure 1 and 2, respectively.

Table 1: LC_{50} (median lethal concentration; in mg/L) and the 95% confidence interval as determined for larval *Physalaemus cuvieri* and *Hypsiboas pardalis* after 96-h exposure to glyphosate.

	Physalaemus cuvieri	Figure	*Hypsiboas pardalis*	Figure
Glyphosate	115 (112–119)[b]	1	106 (103–109)[b]	2

[b]Probit test

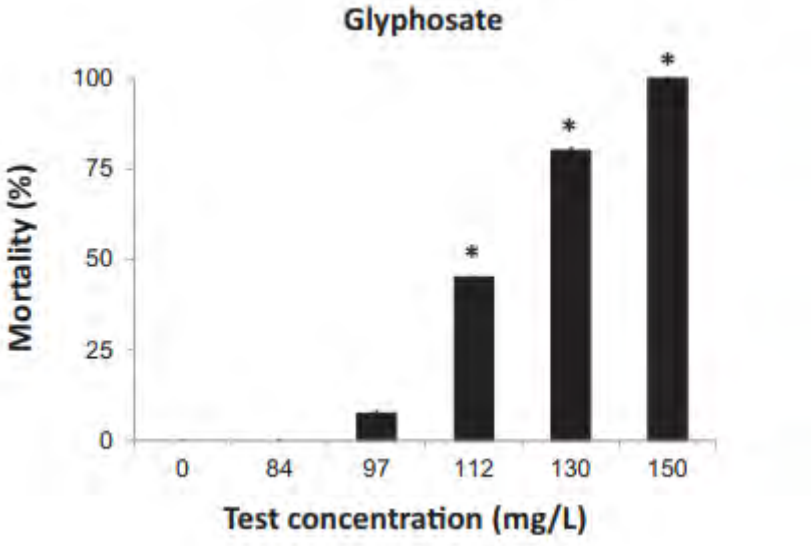


Figure 1: Mortality (in %) of *Physalaemus cuvieri* at the end of the 96 h laboratory tests evaluating the toxicity of glyphosate. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control.

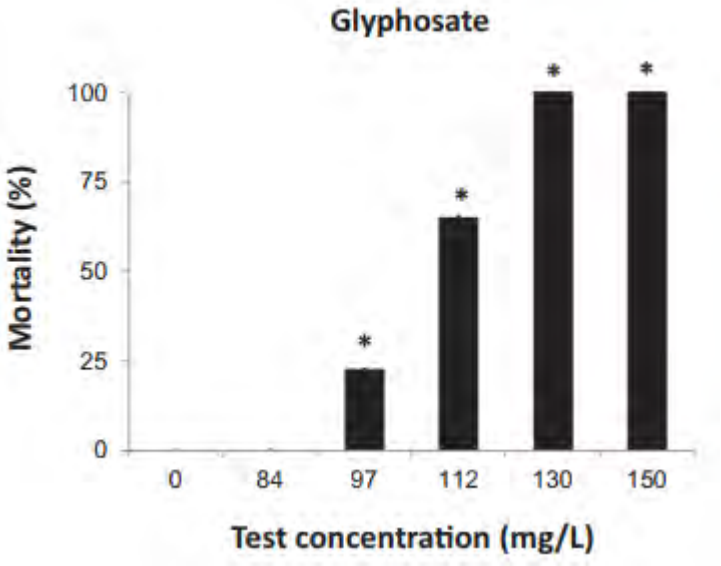


Figure 2: Mortality (in %) of *Hypsiboas pardalis* at the end of the 96 h laboratory tests evaluating the toxicity of glyphosate. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control.

Conclusion

The LC_{50} for *Physalaemus cuvieri* and *Hypsiboas pardali* was determined to be 115 mg a.s./L and 106 mg a.s./L, respectively.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study investigated the acute toxicity of glyphosate to larvae of *Physalaemus cuvieri* and *Hypsiboas pardalis.* The LC_{50} for *Physalaemus cuvieri* and *Hypsiboas pardali* was determined to be 115 mg a.s./L and 106 mg a.s./L, respectively.

The study was conducted according to portions of OECD 241. However, validity criteria were not reported. It is unknown if the larvae were exposed to any other chemicals as no analysis of watershed water was provided.There was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.2.1
Report author	Rodrigues L. B. *et al.*
Report year	2019
Report title	Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms
Document No	Mutat Res Gen Tox En 842 (2019) 94-101
Guidelines followed in study	OECD 236
Deviations from current test guideline	Not reported
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The present study assessed the acute toxicity of glyphosate, as well as the main metabolite aminomethylphosphonic acid (AMPA) on non-target aquatic organisms. The toxic effects of these chemicals were evaluated in a zebrafish (*Danio rerio*) embryo-larval toxicity test according to OECD Test Guideline 236 at 6 concentrations between 1.7 and 100 mg/L. Three replicates with 20 fertilized eggs per concentration were used.

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L).

Materials and methods

Test chemicals; Technical-grade glyphosate (GLY; Glyphosate PESTANAL®; purity 99%, CAS No. 1071-83-6) and aminomethylphosphonic acid (AMPA, purity 99%, CAS No. 106651-9) were purchased from Sigma-Aldrich.

Zebrafish maintenance and egg production; Adult male and female zebrafish (D. rerio) were provided by the zebrafish facility (ZebTec Tecniplast) at the Institute of Biology, University of Brasília and kept in separate tanks (ethical approval UFG Nº 102/2014). Fish were maintained in a Rack Hydrus (Alesco) recirculating system using water filtered by reverse osmosis, where water passes through several levels of filtration (activated carbon filters and biological filters), is then disinfected by ultraviolet (UV) light and automatically adjusted for pH and conductivity. The temperature was maintained at 26 ± 1 °C, conductivity at 750 ± 50 μS, pH at 7.5 ± 0.5 and dissolved oxygen of 8 ppm. Nitrate, nitrite and ammonia were regularly monitored. This water was used in preparing the test solutions of all assays performed. Adult organisms were fed with commercial dry flake food (TetraColor Flakes®) and live brine shrimp. On the day of the test, zebrafish eggs were collected about 30 min after natural mating, rinsed in water and examined under a stereomicroscope (Bel Photonics STM PRO). Unfertilized or damaged eggs were discarded. The fertilization success was checked, and only batches of eggs with a minimum fertilization rate of 90% were used.

Fish embryo acute toxicity (FET) test; The zebrafish embryo-larval toxicity test was carried out according to OECD Test Guideline 236. Twenty fertilized eggs per concentration were randomly selected and carefully distributed in a 24-well plate, filled with 2 mL of GLY, AMPA at 1.7, 5, 10, 23, 50 and 100 mg/L and controls (negative control – NC: maintenance water and positive control – PC: 3,4-dichloroaniline at 4.5 mg/L). Tests were performed in triplicates (three independent experiments) in a climate chamber at 26 ± 1 °C and 12 h light under static conditions. Neither food nor aeration was provided during the bioassays. Embryo development was assessed at 24, 48, 72 and 96 h post-fertilization (hpf), using a stereomicroscope (Bel Photonics STM PRO) with 3x magnification. The distinction between the normal and abnormal development of embryos was established according to the zebrafish development descriptions reported previously. Lethal (egg coagulation, no somite formation, nondetachment of the tail from yolk sac and no heart beating) and sublethal (effects on the eye and body pigmentation, absorption of the yolk sac, hatching rate, swimming bladder inflation, otolith, presence of edemas and blood accumulation, tail deformities) parameters were observed and reported.

Statistical analysis; The FET and Comet data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Each experimental value was compared to its corresponding negative control and the statistical difference was considered significant when $p < 0.05$. With respect to the FET, the toxicity was expressed as the lethal concentration (LC50), which was calculated using GraphPad Prism software (version 5.0, GraphPad Software, San Diego, CA, USA) with 95% confidence interval.

Results

Acute effects for zebrafish early-life stages; The present study investigated the effects of active ingredient GLY and its metabolite AMPA on the zebrafish embryonic development (survival and malformations) at 24, 48, 72 and 96 h of exposure. According to Fig. 1, no significant mortality was observed in zebrafish early-life stage after exposure to different concentrations (1.7–100 mg/L) of GLY and AMPA (Fig. 1), which presented survival rate ≥90% in all exposure periods.

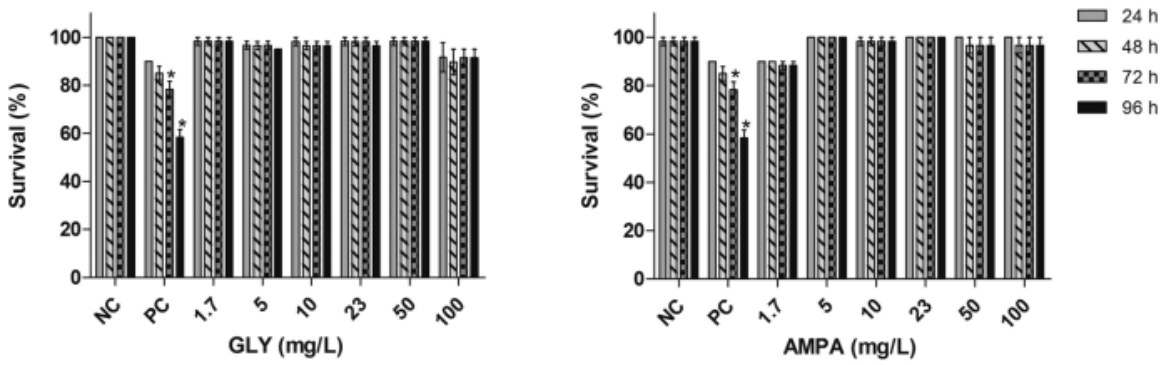


Fig. 1. Survival rate of zebrafish at different developmental stages exposed to GLY and AMPA for 24, 48, 72 and 96 h. Twenty fertilized eggs per experimental group were evaluated. Bars represent the mean ± standard error of the mean of three independent experiments. *$p < 0.05$ statistically different from the respective negative control (NC) based on one-way ANOVA and Dunnett's post hoc test. PC = positive control (3,4-dichloroaniline at 4.5 mg/L after 24, 48, 72 and 96 h of exposure).

In relation to sublethal effects, Fig. 2 shows that GLY induced some morphological abnormalities, however, these malformations were not statistically significant when compared to their respective negative control.

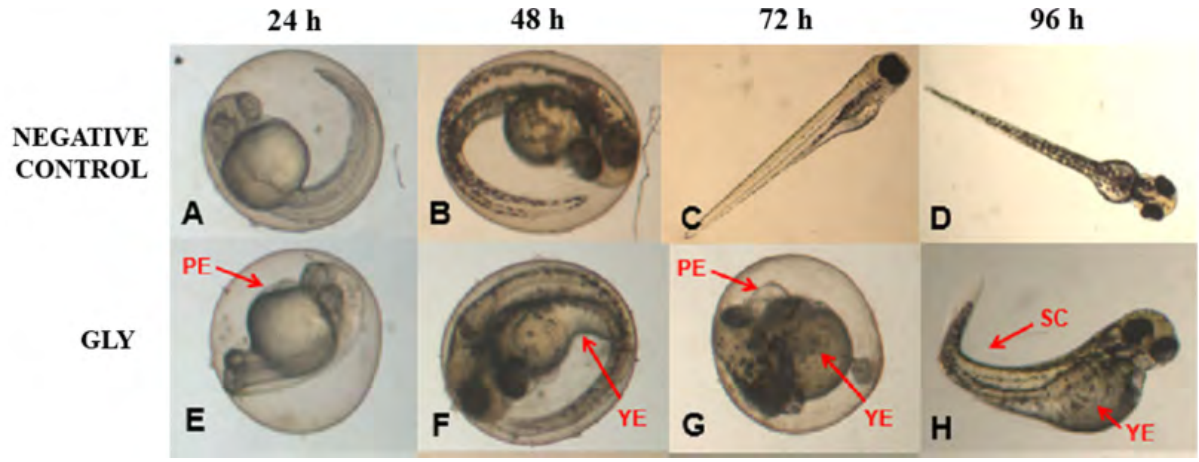


Fig. 2. Zebrafish embryos and larvae abnormalities after GLY exposure: pericardial edema (PE), yolk sac edema (YE), spinal curvature (SC). Embryos control after 24 h and 48 h of exposure, respectively (A–B); larvae control after 72 h and 96 h of exposure, respectively (C–D), embryos exposed to GLY at 23 mg/L and 100 mg/L for 24 h and 48 h, respectively (E–F); non-hatching embryo exposed to GLY at 10 mg/L for 72 h (G); larvae exposed to GLY at 100 mg/L for 96 h (H).

Discussion

The current results showed that glyphosate and AMPA did not induce acute toxicity in zebrafish early-life stage with LC_{50}-96 h > 100 mg/L. Similar effect was observed by researchers in assessing the acute effects of glyphosate (0.005; 0.05; 5; 10 and 50 mg/L) on early-life stages of zebrafish and common carp (Cyprinus carpio) for 120 h. The authors demonstrated that all tested concentrations, except the highest concentration (50 mg/L), induced cumulative mortality ≤ 10% after 96 h of exposure. Glyphosate at 50 mg/L caused the highest cumulative mortality, reaching 17.5% after 120 h of exposure while in this study, there were no significant differences between this group (glyphosate at 50 mg/L) and control with 1.7% of larvae mortality after 96 of exposure. It is worth noting that according to OECD 236, the survival of embryos in the NC must be ≥ 90% (validation criterion of the test), and therefore mortality ≤ 10% in the experimental groups is acceptable.

Conclusion

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L) in zebrafish.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The acute toxicity of technical glyphosate and its metabolite aminomethylphosphonic acid (AMPA) to zebrafish embryos was investigated.

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L) in zebrafish.

The study was stated to have been conducted according to OECD guideline 236, but there is no information on hatching rates in the treatment and control groups, so exposure of the embryo without a potential barrier function of the chorion cannot be confirmed.

Concerning the validity of the study, four of the six validity criteria from the test guideline are mentioned in the paper (fertilization rate of embryo batches used was >90%, survival in the negative control group was > 90%, temperature was maintained at 26 ±1ºC and dissolved oxygen was at an

acceptable level 8ppm). There is no information presented on the performance of the positive control group (3, 4-dichloroaniline) and no information provided on the hatching rates in the negative control group at 96 hours, which for the control group should exceed 80%. As these information are not presented and the fact that there was no analytical verification of test concentrations reported, this study considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.1 / KCP 10.2.1
Report author	Gabriel U. U. *et al.*
Report year	2010
Report title	Toxicity of roundup (a glyphosate product) to fingerlings of *Clarias gariepinus*
Document No	Animal Research International (2010) 7(2):1184-1193
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Acute static renewal bioassays were conducted on fingerling and adult of *Clarias gariepinus* (mean weight, 1.22 ± 0.6g; mean total length, 5.25 ± 1.25 cm) using the herbicide, Roundup (glyphosate). In the acute study, fingerlings were exposed in triplicate to 0.0, 14.0, 16.0, 18.0, 20.0 22.0, and 24.0 mg/l of the herbicide for 96 hours to determine general behavioural responses.

The 96 hour LC_{50} of Roundup on the fish was 19.58 mg/l.

Materials and methods

The fingerlings of *C. gariepinus* (mean weight 1.22 ± 0.6g; mean total length 5.25 ± 1.25 cm) were obtained from a private farm, Comsystem, Kpite, Rivers State and transported in 25 litre jerry can to the Wet Laboratory, Department of Fisheries and Aquatic Environment, Rivers State University of Science and Technology, where they were distributed 60 fish per aquarium in four rectangular aquaria filled with 20 litre borehole water (dissolved oxygen, 0.01 ± 0.05 mg/l, pH- 7.5 ± 1.3; conductivity, 410 ± 20.4 µS/cm; total dissolved solid 400 ± 10.25 ppm). They were fed at one percent biomass, half at 0900 and 1600 hours for a week. Cleaning of the tanks and water exchange were done daily. Mortality during acclimation period was less than one percent. Mucus accumulation on the skin as well as gills and skin pigmentation were recorded.
Range finding test and trial runs were done. Twenty litres of each of the following concentrations: 14, 16, 18, 20, 22 and 24 ppm of Roundup containing 360 g/l glyphosate (in the form of 480g/l isopropylamine salt) and a control were prepared in triplicate in glass aquaria. Ten fish was randomly distributed into each of the tanks. The general behaviours, opercular beat frequency, OBF, tail beat frequency, TBF and mortality (%) were recorded at 12, 24, 48, 72 and 96th hour, respectively. The exposure lasted for 96 hours. Data obtained from the experiments were subjected to ANOVA using Statistical Package for the Social Sciences, SPSS version 15 and differences among means were separated by Duncan Multiple Range test at 0.05%. The dependent variables in the trials (OBF, TBF and cumulative mortality) were regressed on concentration of the toxicant to obtain the regression lines of best fit for predicting the values of the dependent variables with changes in that of the independent with Microsoft Excel®. Correlation analysis was used to determine the degree of association among the dependent and independent variables. Lethal concentrations (LC50) values for the 24, 48, 72 and 96 hour and the median lethal times (MLT50) for the various concentrations of herbicide were done with Probit Analysis. Safe concentration of the herbicide at the various time intervals were obtained by multiplying the lethal concentration by a factor, 0.1. The interaction effects of the behavioural responses (TBF and OBF) with exposure duration and concentrations of the herbicides were presented graphically.

Results

On introduction into the toxicant the fish showed initial hyper-excitability, stress responses such as increased opercular ventilatory rate, dash and erratic swimming and gasping for air within the first two hours. As exposure time increased before death occurred they "hung" on the surface of the solution gulping air, fell steadily to the aquaria bottom. This was usually followed by dash swimming. This sequence was repeated several times before the fish lost balance, lay flat on the bottom (exertion), tail beat stopped, followed by cessation of opercular movement and then death (non-response to tactile stimuli).

Table 1: Tail and opercular beat frequency (TBF and OBF) and cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

Variable	Time of exposure (hours)				
	12	24	48	72	96
TBF/min.	5.45 ± 2.73^{b}	15.49 ± 4.41^{ab}	7.87 ± 4.41^{ab}	21.12 ± 5.22^{b}	4.49 ± 5.40^{b}
OBF/min.	113.50 ± 7.23^{a}	113.10 ±7.60^{a}	115.23 ±7.23^{a}	10.79 ± 8.99^{a}	65.15 ± 9.15^{b}
Cum. mortality	6.67 ± 6.86^{e}	37.227 ± 31.21^{d}	56.11 ± 36.16^{c}	63.89 ± 32.39^{b}	73.33 ±29.31^{a}
	Concentration of Roundup (mg/l)				
	0.0	4.0	16.0	18.0	20.0	22.0	24.0
TBF/min.	15.73 ±4.41^{a}	5.79 ±4.41^{a}	16.46 ±4.41^{a}	9.00 ±4.63^{a}	7.04 ±5.92^{a}	0.00 ±0.00	0.00 ±0.00
OBF/min.	96.71 ±7.60^{bc}	123.27 ±8.00^{ab}	108.58 ±8.00^{a-c}	100.93 ±8.00^{a-c}	73.42 ±10.19^{c}	124.72 ±16.99^{ab}	136.99 ±16.99^{a}
Cum. Mortality	0.00 ±0.00	22.67 ±12.80^{d}	24.00 ±24.43^{d}	32.67 ±19.81^{c}	50.67 ±37.70^{b}	77.33 ±36.15^{a}	77.33 ±37.89^{a}

Means with the same superscript in the row are not significantly different (p>0.05)

Table 2: Regression lines of best fit for the prediction of the values of OBF/min., TBF/min. and cumulative mortality of C. gariepinus exposed to acute levels Roundup for 96 hours

Dependent Variable	Independent variable	Prediction equation	Curve type	r^2
TBF	Time	$Y=2.8415e^{0.221x}$	Exponential	0.9828
TBF	Concentration	$Y=0.0017x^2 +0.0192x$	Power	0.9442
OBF	Time	$Y=23.314Ln(x) +16.325$	Logarithmic	0.9186
OBF	Concentration	$Y=25.117Ln(x) +19.269$	Logarithmic	0.9812
Mortality	Time	$Y=0.513x$	Linear	0.9922
Mortality	Concentration	$Y=0.0021x^2 + 0.2451x +4.4278$	Polynomial	0.9722

Where x = independent variable, y= dependent variable

Cumulative mortality of exposed fish was very variable relative to the concentration of the herbicide (Figure 1).

The cumulative mortality differed with the time of exposure ($p < 0.01$), concentration of toxicant ($p < 0.001$) and interactions between exposure duration and herbicide concentration ($p < 0.01$, Figure 1). Exposed fish produced copious amount of mucus on the gill and skin which appeared to be concentration-dependent in exposed fish with minimal amount on the control group. Pigmentation of the skin of the fish was not noticed in any of the exposure concentrations.

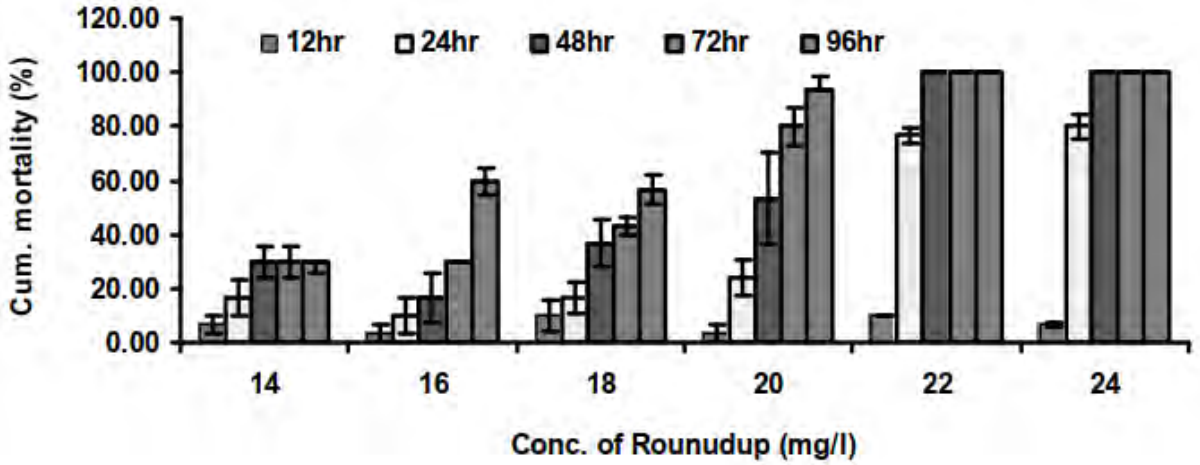


Figure 1: Percentage cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

The 24, 48, 72 and 96 hour LC_{50} and associated 95% confidence limits of the herbicide concentrations shown below indicated that the range of the values between the 24 hour and 96 hour LC_{50} (4.93 mg/l) as very narrow. Safe concentrations of Roundup to fingerlings of C. gariepinus were very low (2.08 mg/l for 24 hour and 1.59 mg/l for 96 hour). The time it took for half of the exposed fish to die at the various exposure concentrations decreased with time with the highest concentration (24 mg/l) killing half of the exposed fish at about one sixth the time it took for 14 mg/l of the herbicide.

Table 3: Lethal concentrations and associated 95% confidence limits of Roundup to *C. gariepinus* fingerling exposure to Roundup for 96 hours

Time (hours)	Lethal Concentration	Safe concentration	Probit model estimation equation
24	LC_{50}- 20.81 (19.58-22.48)	2.08	y= -4.73+0.23x
24	LC_{90}- 26.44 (24.45-31.47)	2.64	
48	LC_{50}-18.50 (16.67-19.40)	1.85	y= -5.18+0.29x
48	LC_{90}-22.54 (20.88-26..09)	2.25	
72	LC_{50}-17.11 (16.30-17.84)	1.71	y= -5.30+0.31x
72	LC_{90}-21.44 (20.59-23.31)	2.14	
96	LC_{50}-15.88 (14.99-16.64)	1.59	y= -5.06+5.06x
96	LC_{90}-19.91 (18.97-21.42)	1.20	

Where y=dependent variable, x= independable variable

Discussion

The threshold concentration causing 100% mortality in this study was 22 mg/l which is lower than that reported for other toxicants tested on any of the clariid species 7 suggesting that it may be more toxic than other tested toxicants. Half of the exposed fish (50%) were killed by 15.88 mg/l of herbicide in 19.69 hours, hence the herbicide can be classified as being slightly toxic. Besides, in the wild where the agro-chemical is indiscriminately used the impact of the exposure stress caused by the herbicide, may be protracted, following the survivors throughout life and may affect various aspects of their lives.

Conclusion

The 24, 48, 72 and 96 hour LC_{50} and associated 95% confidence limits indicated that the range of the values between the 24 hour and 96 hour LC_{50} (4.93 mg/l) as very narrow. Safe concentrations of Roundup to fingerlings of C. gariepinus were very low (2.08 mg/l for 24 hour and 1.59 mg/l for 96 hour). The 96 hour LC_{50} of Roundup on the fish was 19.58 mg/l.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effects of Roundup containing 360 g/l glyphosate (equivalent to 480g/L isopropylamine salt) were tested in an acute test with *C. gariepinus* fingerlings. The 96 hour-LC_{90} was determined to be 19.91 mg prod./L.

There is no analytical verification of test concentrations reported and thus the reliability of the endpoint is questionable. The appearance of mucus accumulation on the skin and gills and skin pigmentation recorded in fish in the holding / stock vessels is a clear indicator of stress. Therefore, the condition of the fish used in the test is questionable. The study was not conducted in accordance with a recognised test guideline and was not performed under conditions of GLP. Furthermore, the purity of the formulation roundup is not clearly given as the specification in the full text contains some typing errors. The study is considered reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.2 / KCA 8.2.5
Report author	Levine S.L. *et al.*
Report year	2015
Report title	Aminomethylphosphonic acid has low chronic toxicity to *Daphnia magna* and *Pimephales promelas*
Document No	Environmental Toxicology and Chemistry (2015), Vol. 34, No. 6, pp. 1382-1389
Guidelines followed in study	OECD 211, OECD 210
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

The purpose of the present study was to assess the potential for chronic toxicity of AMPA to fathead minnow (*Pimephales promelas*) and *Daphnia magna*. Chronic toxicity to *P. promelas* was evaluated in a fish early–life stage study. The primary endpoints were larval survival, growth, and development. The chronic toxicity to *D. magna* was evaluated in a *Daphnia* reproduction test. The primary endpoints were survival, growth, and reproduction.

The NOAEC for *P. promelas* was determined to be 12 mg/L, the highest concentration tested. The no-observed-effect concentration for *D. magna* was determined to be 15 mg/L.

Materials and methods

Test substance

Synthesis of AMPA was performed by Chemir, and it had a purity of 98.7%. The water solubility for AMPA is reported to be 10 500 mg/L (based on glyphosate acid solubility data [RMS Germany. 2013]); therefore, solvent (to aid the dissolution of AMPA into water) was not required for the aquatic exposures. Stock solutions for waterborne exposures were prepared in well water, appeared clear and colorless after mixing, and were stored under refrigerated conditions (~ 4 ± 1 °C).

For the *D. magna* reproduction study, primary stocks and test solutions were prepared every 2 d to 3 d during the test. A primary stock solution was prepared in ultraviolet sterilized dilution water at a nominal concentration of 120 mg AMPA/L, equivalent to the highest concentration tested. Proportional dilutions of the primary stock solution were made in dilution water to prepare test solutions at nominal concentrations of 7.5 mg AMPA/L, 15 mg AMPA/L, 30 mg AMPA/L, and 60 mg AMPA/L.

For the fish early–life stage study, stock solutions were delivered using syringe pumps into mixing vessels and mixed with diluent water in a continuous diluter system to prepare nominal test concentrations of 0.75 mg AMPA/L, 1.5 mg AMPA/L, 3.0 mg AMPA/L, 6.0 mg AMPA/L, and 12 mg AMPA/L. Delivery of the test solutions was started 7 d prior to the initiation of the test to achieve equilibrium of the test substance in the test chambers.

Daphnia magna reproduction study—Culturing, exposure, and observations

Daphnia magna are the required cladoceran test species under the Organisation for Economic Co-operation and Development (OECD) 211 guideline [OECD 2008]. *Daphnia magna* was tested because it is representative of an important group of freshwater invertebrates and has a long and successful history as a test organism in the laboratory. Neonates (juveniles) <24 h old were used to initiate the test and were obtained from established cultures. Parental daphnids were cultured in well water that was

filtered with a 0.45-µm filter and passed through an ultraviolet sterilizer. The source of well water was characterized as moderately hard water with an average specific conductance of 362 µS/cm, hardness of 132 mg/L as $CaCO_3$, alkalinity of 173 mg/L as $CaCO_3$, and pH of 8.2 during the 4-wk period immediately preceding the test.

During the 2-wk period preceding the test, culture temperatures ranged from 19.6 °C to 20.8 °C, pH from 8.1 to 8.7, and dissolved oxygen from 7.6 mg/L to 9.5 mg/L. During culturing and testing, daphnids were fed daily with a mixture of yeast, cereal grass medium, and trout chow, as well as a suspension of the freshwater green alga *Pseudokirchneriella subcapitata*. During the test, organisms in each test chamber were fed 0.5 mL of yeast–cereal–trout chow and 1.0 mL of algae, which represented 0.60 mg C/daphnid/d. Although this amount of feed exceeded the OECD guideline recommended amount of 0.1 mg C/daphnid/d to 0.2 mg C/daphnid/d, an excess amount was fed to maintain sufficient feed in the system to support acceptable reproduction rates, which is an acceptable deviation from the testing guideline.

The 4 adult daphnids used to supply neonates for the test were held for 19 d prior to collection of the juveniles for testing and had each produced at least 1 previous brood. Adult daphnids in the culture had produced an average of at least 3 young per adult per day over the 7-d period prior to the test. The adults showed no signs of disease or stress, and no ephippia were produced during the holding period. To initiate the test, juvenile daphnids were collected from the cultures and indiscriminately transferred 1 or 2 at a time into the transfer chambers that were impartially assigned to a control or treatment group until each transfer chamber contained 10 daphnids. All animals were released from the transfer chambers into the assigned test chambers below the water surface (to avoid air contact) using wide-bore pipettes to not harm the neonates.

We tested AMPA in a semistatic renewal design with the renewal of test solutions every 2 d or 3 d. Concentrations of AMPA were measured on 3 occasions during the test: at the beginning and end of the first renewal cycle, at the beginning and end of the longest renewal cycle during the second wk of the test, and at the beginning and end of the last renewal cycle. Test chambers were 250-mL glass beakers that contained approximately 200 mL of test solution and were loosely covered with plastic Petri dishes. Beakers were impartially positioned in an environmental chamber that was programmed to maintain the target water temperature (20 ± 1 °C) throughout the test period. A 16:8-h light:dark photoperiod was used with a 30-min transition period of low light intensity when lights went on and off to avoid sudden changes in lighting. Lighting was provided by fluorescent light bulbs that emit wavelengths similar to natural sunlight. At test initiation the light intensity at the water surface of 1 representative test chamber was 296 lux (measured with a SPER Scientific Model 840006C light meter).

Temperature was measured continuously in 2 replicate test chambers in each treatment group, and measurements rotated among replicates in each group. Dissolved oxygen and pH were measured in the newly prepared solutions for each treatment group at test initiation and on renewal days and in the old solutions from 2 replicate test chambers in each treatment and control group on renewal days and at test termination. When a first-generation daphnid was found dead, measurements of temperature, dissolved oxygen, and pH were taken in the replicate at that time and then discontinued. Hardness, alkalinity, and specific conductance were measured in batch solutions of the negative control; the highest test concentration at test initiation and on 1 renewal day each week (day 7 and day 14); and pooled replicate solutions at test termination. Total organic carbon (TOC) was measured in the dilution water at test initiation and termination using a Shimadzu model TOC-VCSH analyzer and following the *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association]. Hardness and alkalinity were measured by titration based on procedures in the *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association].

First-generation daphnids were observed daily during the test for immobility, the onset of reproduction, and clinical signs of toxicity. Following the onset of reproduction, the second- generation daphnids were counted 3 times per week and at test termination (day 21). Body lengths and dry weights of the surviving first-generation daphnids were measured at the end of the exposure period.

Fish early–life stage study—Culturing, exposure, and observations

Test methodology followed the procedure outlined in the OECD 210 test guideline for *P. promelas* with the exception of doubling the required level of replication [OECD 1992]. We selected *P. promelas* for the early–life stage study based on past use and ease of handling in the laboratory. Embryos (Chesapeake Cultures) were examined under a dissecting microscope to select healthy, viable specimens at approximately the same stage of development (<24 h). Embryos collected for use in the test were from 10 individual spawns and were <24 h old when the test initiated. Test chambers were 9-L glass aquaria filled with approximately 7 L of test solution and contained an embryo incubation cup attached to a reciprocating rocker arm (2 rpm) for water circulation during embryo incubation. To initiate the test, groups of 1 to 3 embryos were impartially distributed among incubation cups until each cup contained 20 embryos. A single incubation cup constructed from 50-mm-diameter glass cylinders with 425-µm nylon screen mesh attached to the bottom was placed into each test chamber. The incubation cup with the embryos was impartially assigned to each of the control and treatment groups.

The test was conducted in a temperature-controlled environmental chamber designed to maintain the target test temperature of 25 ± 1 °C throughout the test period. Temperature was measured in each test chamber at the beginning of the test, weekly during the test, and at the end of the test using a liquid-in-glass thermometer. Temperature also was monitored continuously in 1 negative control replicate using a Fulscope ER/C Recorder. Fluorescent light bulbs that emit wavelengths similar to natural sunlight were used on a 16:8-h light:dark photoperiod. A 30-min transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting.

The negative (dilution water) control and AMPA test concentrations were delivered in a continuous-flow diluter. Syringe pumps (Harvard Apparatus) delivered the stock solutions (at a rate of 30 µL/min) into mixing chambers and mixed with dilution water (at a rate of 125 mL/min) to achieve the target test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters that were calibrated prior to test initiation and verified at weekly intervals during the test. The flow of test water from each mixing chamber was split and directed into 4 replicate test chambers. The proportion of the test water that was split into each replicate was checked prior to the test and at approximately weekly intervals during the test to ensure that flow rates varied by no more than ± 10% of the mean for the 4 replicates. The diluter flow rate was adjusted to provide approximately 6 volume additions of test water in each test chamber per day. The general operation of the diluter was checked visually at least 2 times/d during the test and at least once at the end of the test.

According to the OECD guideline, concentrations above the 96-h lethal concentration for 50% of the population or 10 mg/L, whichever is lower, need not be tested. To assure that a mean measured concentration ≥10 mg/L was tested, the highest nominal test concentration of 12 mg/L and the lower concentrations of 6 mg/L, 3 mg/L, 1.5 mg/L, and 0.75 mg/L were selected. Stock solutions were stored under refrigerated conditions, and fresh aliquots were placed in the syringe pumps daily during the test. Water samples were collected from 1 test chamber of each treatment and control group 4 d prior to test initiation to confirm the operation of the diluter. Water samples were collected from alternating replicate test chambers of each treatment and control group on day 0, day 7, day 14, day 21, day 28, and day 33 (test termination) to determine concentrations of the test substance in the test chambers. All samples were collected at mid-depth in the test chambers, placed in glass vials, and processed immediately for analysis.

Dissolved oxygen and pH were measured in alternating replicates of each treatment and control group at the beginning of the test, weekly during the test, and at the end of the test. Hardness, alkalinity, and specific conductance were measured in alternating replicates of the negative control (dilution water) and the highest concentration treatment group at the beginning of the test, weekly during the test and at the end of the test. Hardness and alkalinity were measured by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association], and specific conductance was measured using an Acorn Series Model CON6 Conductivity-Temperature meter.

During the first day of exposure, embryos were observed twice for mortality and fungal infection. Thereafter, until hatching was complete, observations of embryo mortality and the removal of dead embryos were performed once daily. When hatching reached >90% in the control groups on day 5 of the test, the larvae were released to their respective test chambers and the posthatch period began. During the 28-d posthatch exposure period, the larvae were observed daily to evaluate the mortality and the numbers of individuals exhibiting clinical signs of toxicity or abnormal behavior. From these observations, time to hatch, hatching success, and posthatch growth and survival were evaluated. Hatching success was calculated as the percentage of embryos that hatched successfully. Posthatch survival was calculated from the number of larvae that survived to test termination as a percentage of the number of embryos that hatched successfully.

Newly hatched larvae were fed live brine shrimp nauplii (*Artemia* sp.) 3 times/d during the first 7 d of the posthatch period. Thereafter, they were fed live brine shrimp nauplii 3 times/d on weekdays and at least 2 times/d on weekends. Fish were not fed for approximately 48 h prior to the termination of the test to allow for clearance of the digestive tract before weight measurements were made. To ensure that the feeding rate per fish remained constant, rations were adjusted at least weekly. The test chamber loading rate (the total wet wt of fish per liter of water in the tank) at the end of the test was 0.32 g fish/L.

Posthatch growth of *P. promelas* was evaluated at the conclusion of the 28-d posthatch exposure period. Total length for each surviving fish was measured to the nearest 1mm using a metric ruler, with wet and dry weights measured to the nearest 0.1 mg using an analytical balance. Fish were placed in an oven at 60 °C for up to approximately 48 h to obtain dry weight data.

Analytical method for detection of AMPA

Samples were diluted, as appropriate, with freshwater. The 2.0 mL of diluted sample and/or external calibration standards were placed into the 15-mL test tube. Then, 1.0 mL of 0.37 M aqueous potassium tetraborate was added to each test tube, followed by 2.0 mL of 0.025 M NBD-C1 (methanolic) for derivatization. Solutions were capped, mixed, and heated at approximately 80 °C for 40 min. Next, 1.0 mL of 1.2 M HCl was added to each test tube, and samples were then left undisturbed for approximately 10 min prior to analysis. Samples (25 µL injection volume) were analyzed on an Agilent Series 1100/1200 high performance liquid chromatograph equipped with an Agilent Series 1100 variable wavelength detector at 500 nm. Chromatographic separations were achieved using a YMC-Pack ODS-AM (150 mm × 4.6 mm, 3 µm particle size) analytical column at a temperature of 40 °C and eluted over a gradient of 0.1% H_3PO_4 (solvent A) and CH_3CN (solvent B). The retention time for AMPA was approximately 6.5 min to 7.3 min, and the method limit of quantitation for these analyses was defined as 0.4 mg AMPA/L.

Statistical and power analyses

Test endpoints analyzed statistically in the *Daphnia* test for first-generation daphnids were survival, reproduction (the number of live young produced per 21-d surviving adult), and growth (length and dry wt). Neonates produced by those first-generation daphnids that did not survive the full 21 d were excluded from analysis of reproduction.

Test endpoints analyzed statistically in the fish early–life stage test were hatching success, larval survival, and growth (total length, wet wt, and dry wt). Data on time to hatch were evaluated by visual interpretation.

Discrete-variables data were analyzed using Fisher's exact test to identify treatment groups that showed a statistically significant difference ($p \leq 0.050$) from the negative control. All continuous-variable data were evaluated for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test ($p = 0.010$). When the data passed the assumptions of normality and homogeneity of variance, those treatments that were significantly different from the control means were identified using the 1-tailed Dunnett's test ($p \leq 0.050$). All statistical tests were performed using a personal computer

with SAS software. The results of the statistical analyses were used to aid in the determination of the no-observed–adverse effect concentration (NOAEC), defined as the greatest test concentration that produced no significant treatment-related adverse effects on survival, reproduction, or growth.

Results

Daphnia magna survival, growth, and reproduction

Water temperatures were maintained within the targeted range of 20 ± 1 °C, dissolved oxygen concentrations remained ≥76% of saturation (6.8 mg/L), and pH ranged from 7.1 to 8.6 during the test. Specific conductance, hardness, and alkalinity were similar between the control and treatment groups and did not appear to be influenced by AMPA. The TOC in the dilution water at test initiation and termination was <1 mg C/L.

Table 1: Means and ranges of water quality measurements taken during the 21-Day *D. magna* exposure to AMPA

Mean Measured Concentration (mg AMPA/L)	Mean ± Std. Dev. and Range of Measured Parameters					
	Temperature (°C)	Dissolved Oxygen [1] (mg/L)	pH	Hardness [2] (mg/L as $CaCO_3$)	Alkalinity [2] (mg/L as $CaCO_3$)	Conductivity [4] (µS/cm)
Negative Control	19.8 ± 0.55 (19.1 – 20.8)	8.2 ± 0.58 (7.2 – 9.1)	8.4 ± 0.10 (8.3 – 8.6)	135 ± 2 (132 - 136)	167 ± 5 (160 – 171)	339 ± 46 (280 – 391)
7.4	19.9 ± 0.48 (19.2 – 20.7)	8.0 ± 0.60 (7.1 – 9.1)	8.4 ± 0.09 (8.3 – 8.6)	--	--	--
15	19.9 ± 0.50 (19.1 – 20.8)	7.9 ± 0.65 (6.9 – 9.1)	8.4 ± 0.08 (8.2 – 8.6)	--	--	--
30	19.9 ± 0.54 (19.0 – 20.7)	7.9 ± 0.66 (6.8 – 9.1)	8.3 ± 0.10 (8.2 – 8.5)	--	--	--
57	20.0 ± 0.52 (19.1 – 20.6)	7.9 ± 0.60 (7.0 – 9.1)	8.2 ± 0.23 (7.7 – 8.5)	--	--	--
120	20.1 ± 0.54 (19.1 – 20.7)	8.0 ± 0.54 (7.1 – 9.1)	7.9 ± 0.48 (7.1 – 8.4)	139 ± 2 (136 – 140)	164 ± 6 (156 – 170)	340 ± 46 (274 – 381)

[1] A dissolved oxygen concentration of 9.1 mg/L represents 100% saturation at 20ºC in freshwater. Any recorded dissolved oxygen measurement greater than 100% saturation is reported as 9.1 mg/L.
[2] -- = no measurements scheduled.

Measured concentrations of AMPA for the *D. magna* study were close to nominal concentrations throughout the renewal periods. Concentrations of AMPA in the new test solutions prepared and sampled on day 0, day 9, and day 19 ranged from 92.5% to 106% of the nominal concentrations. Concentrations of AMPA in the old test solutions sampled immediately prior to renewal on day 2, day 12, and at test termination on day 21 ranged from 78.6% to 117% of the nominal concentrations. When the measured concentrations of the samples collected during the test were averaged for each treatment group, the mean measured test concentrations were 7.4 mg AMPA/L, 15 mg AMPA/L, 30 mg AMPA/L, 57 mg AMPA/L, and 120 mg AMPA/L.

There was no significant effect of AMPA on individually exposed first-generation daphnids across the treatments, and survival was ≥80%. A summary of adult survival is presented in Figure 1A. After 21 d of exposure, survival in the negative control, 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L groups was 75%, 80%, 100%, 70%, 100%, and 90%, respectively. Although survival in the negative control group was slightly below the 80% criterion in OECD guideline 211, it is not considered to have impacted the validity of the present study because of the small difference and with the final mortality occurring near the end of the study. In addition, there was ≥80% survival in all treatment groups. The surviving daphnids in the control replicates appeared normal and healthy through the end of the test, indicating that the mortality observed was attributed to incidental death and not the health of the organisms. In addition, the percentage survival of the control replicates was within the control criterion of 70% as specified in the American Society for Testing and Materials standard guide E 1193-97 [ASTM International. 1997]. Survival in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups at test termination did not follow a concentration–response pattern and was 80%, 100%, 70%, 100%, and 90%, respectively. No significant differences in survival were detected in any of the AMPA treatment groups in comparison

with the control ($p > 0.05$, Fisher's exact test). Consequently, the no-observed-effect concentration (NOEC) for survival was 120 mg AMPA/L.

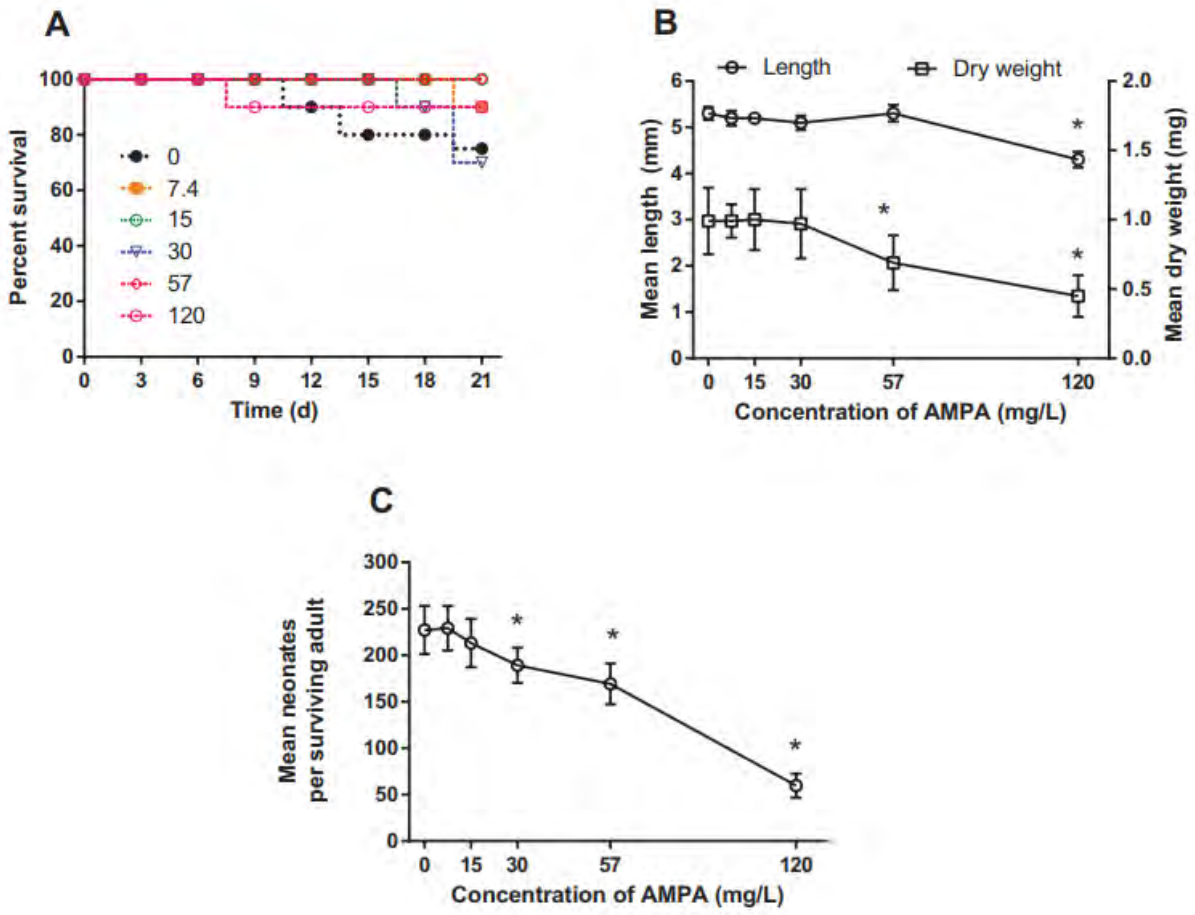


Fig. 1. (A) Survival (percentage) of *Daphnia magna* exposed to increasing concentrations of AMPA for 21 d. (B) Sublethal endpoints in 21-d chronic *D. magna*: body length and dry weight. (C) Reproductive endpoint neonates per surviving adults. * Statistically significant difference ($p < 0.05$) from the control (0 mg/L).

Daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, and 57–mg AMPA/L treatment groups that survived until test termination generally appeared normal. In the 120–mg AMPA/L treatment group, all surviving first-generation daphnids appeared pale in comparison with the control organisms from day 5 through the end of the test. Daphnids in this treatment group were also observed to be smaller than the control organisms from day 7 through the end of the test. All surviving daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, and 57–mg AMPA/L treatment groups were normal in appearance throughout the test and at test termination, with the exception of 1 daphnid in the 57–mg AMPA/L treatment group that appeared pale on day 6 of the test but appeared normal from day 7 to the end of the test.

A summary of production of neonates by surviving first-generation daphnids is presented in Figure 1C. The first day of brood production in the negative controls and in all AMPA treatment replicates was day 7, day 8, or day 9 of the test, indicating that there was no apparent delay in the onset of production at any concentration of AMPA tested. Immobile neonates were noted in the control, 7.4–mg AMPA/L, and 57–mg AMPA/L treatment groups. However, the mean number of immobile neonates per surviving adult in these replicates was less than 1. No aborted brood or aborted eggs were present in the control or any of the AMPA treatment replicates. No males or ephippia were produced during the test.

Summaries of the mean lengths and dry weights of surviving first-generation daphnids are presented in Figure 1B. Daphnids in the negative control group averaged 5.3 mm in length and 0.99 mg in dry weight. Daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups had mean lengths of 5.2 mm, 5.2 mm, 5.1 mm, 5.3 mm, and 4.3 mm, respectively, and mean dry weights of 0.99 mg, 1.0 mg, 0.97 mg, 0.69 mg, and 0.45 mg, respectively. There were significant decreases in length in the 30–mg AMPA/L and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$) but not in the 57–mg AMPA/L treatment group. There were significant decreases in dry weight in the 57–mg AMPA/L and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$). Consequently, the NOEC for growth was 30 mg AMPA/L.

Adult daphnids in the negative control group produced an average of 227 live young per surviving adult (coefficient of variance of 11.6%), well above the validity criterion of ≥60 live young per surviving adult. Adult daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/ L, and 120–mg AMPA/L treatment groups produced an average of 229, 213, 189, 169, and 59.6 live young per surviving adult, respectively. There was a significant decrease in mean neonate production in the 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$). Consequently, the NOAEC for reproduction is 15 mg AMPA/L.

Pimephales promelas embryo hatching success, growth, and survival

Samples of the test solutions collected during the test had measured concentrations that ranged from 82.5% to 117% of nominal concentrations. When the measured concentrations of test solution samples collected on day 0, day 7, day 14, day 21, day 28, and day 33 of the test were averaged for each treatment group, the mean measured test concentrations were 0.73 mg AMPA/L, 1.5 mg AMPA/L, 2.9 mg AMPA/L, 6.0 mg AMPA/L, and 12 mg AMPA/L, which represented 97%, 100%, 97%, 100%, and 100% of nominal concentrations, respectively. Therefore, the results of the present study have been based on mean measured concentrations. The analytical results are summarized in Supplemental Data, Table S4.

Hatching success of the *P. promelas* embryos is summarized in Figure 2A. Daily observations of the embryos indicated that there were no apparent differences in time to hatch between the negative control group and any of the AMPA treatment groups. All *P. promelas* embryos in the control and treatment replicates hatched by day 5 of the test. Hatching reached >90% in the control groups on day 5 of the test, at which time the larvae were released to their respective test chambers.

Hatching success in the negative control, 0.73–mg active ingredient (a.i.)/L, 1.5–mg a.i./L, 2.9–mg a.i./L, 6.0–mg a.i./L, and 12–mg a.i./L treatment groups was 99%, 100%, 100%, 100%, 100%, and 99%, respectively (Figure 2A). There were no statistically significant differences in hatching success in any of the AMPA treatment groups in comparison with the negative control ($p > 0.05$). Larval survival in the negative control, 0.73–mg a.i./L, 1.5–mg a.i./L, 2.9–mg a.i./L, 6.0–mg a.i./L, and 12–mg a.i./L treatment groups was 91%, 91%, 93%, 90%, 91%, and 92% (Figure 2A), respectively; and there were no statistically significant differences in hatching success in any of the AMPA treatment groups in comparison with the negative control ($p > 0.05$). In addition, there were no statistically significant reductions in total length, wet weight, and dry weight (Figure 2B) among fish in the AMPA treatment groups in comparison with the negative control ($p > 0.05$). Based on an evaluation of each of these endpoints, the NOAEC for growth was 12 mg a.i./L.

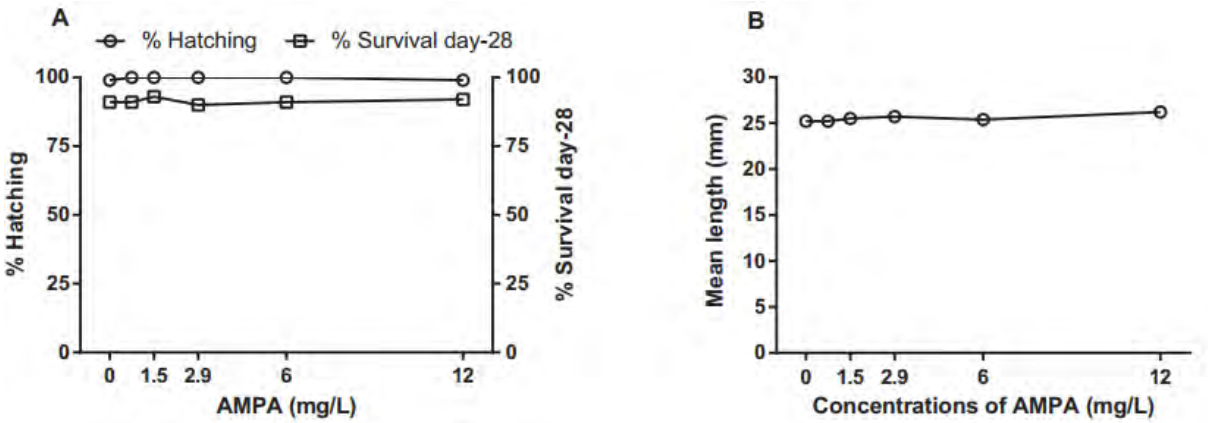


Fig. 2. (A) Hatching success (percentage) and survival (percentage) at day 28 of *Pimephales promelas* in an early–life stage study with aminomethylphosphonic acid (AMPA). (B) Body length (millimeters) of *P. promelas* exposed to AMPA.

Table 2. Means and Ranges of Water Quality Measurements Taken During the 33-Day *P. promelas* Exposure to AMPA

Mean Measured Concentration (mg AMPA/L)	Mean ± SD and Range of Measured Parameters					
	Temperature (°C)	DO[2] (mg/L)	pH	Hardness[2] (mg/L as $CaCO_3$)	Alkalinity[2] (mg/L as $CaCO_3$)	Conductivity[2] (µS/cm)
Assay Control	24.8 ± 0.27 (24.4 – 25.4)	7.9 ± 0.40 (7.4 – 8.2)	8.1 ± 0.10 (8.0 – 8.2)	136 ± 3 (132 – 140)	172 ± 5 (166 – 178)	378 ± 11 (361 – 393)
0.73	24.7 ± 0.59 (23.7 – 25.5)	7.9 ± 0.39 (7.4 – 8.2)	8.1 ± 0.09 (8.0 – 8.2)	-- --	-- --	-- --
1.5	24.9 ± 0.66 (23.9 – 25.7)	7.9 ± 0.39 (7.3 – 8.2)	8.1 ± 0.10 (8.0 – 8.2)	-- --	-- --	-- --
2.9	25.0 ± 0.52 (24.2 – 25.6)	7.9 ± 0.33 (7.5 – 8.2)	8.1 ± 0.12 (7.9 – 8.2)	-- --	-- --	-- --
6.0	25.0 ± 0.46 (24.3 – 25.7)	7.9 ± 0.34 (7.5 – 8.2)	8.0 ± 0.12 (7.9 – 8.2)	-- --	-- --	-- --
12	25.1 ± 0.48 (24.3 – 25.7)	7.9 ± 0.37 (7.4 – 8.2)	7.9 ± 0.13 (7.8 – 8.1)	138 ± 2 (136 – 140)	174 ± 4 (170 – 180)	379 ± 11 (365 – 395)

[1] A dissolved oxygen concentration of 4.9 mg/L represents 60% saturation at 25°C in freshwater. Any recorded DO measurement greater than 100% saturation is reported as 8.2 mg/L.
[2] -- = no measurements scheduled.

Conclusion

For *D. magna* exposed to concentrations ranging from 7.4 mg AMPA/L to 120 mg AMPA/L for 21 d, reproduction was the most sensitive endpoint with significant treatment-related effects noted at 30 mg AMPA/L, 57 mg AMPA/L, and 120 mg AMPA/L. Consequently, the NOAEC based on reproduction was 15 mg AMPA/L. No impact was noted on hatching success, survival, or growth in *P. promelas* embryos exposed to concentrations ranging from 0.73 mg AMPA/L to 12 mg AMPA/L for 33 d. Consequently, the NOAEC was 12 mg AMPA/L, the greatest concentration tested. These values exceed the worst-case water concentrations from conservative modelling and surface water monitoring data by 2 to 3 orders of magnitude, indicating no unacceptable chronic risk for vertebrate and invertebrate aquatic organisms from environ mental exposure to AMPA.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Chronic toxicity tests of the glyphosate environmental metabolite aminomethylphosphonic acid (AMPA) were performed with fathead minnow (*Pimephales promelas*) and *Daphnia magna*.
During a 21-d exposure period under semi-static test conditions the effects on survival, growth, and reproduction of the cladoceran *Daphnia magna* were determined resulting in a no-observed-effect concentration (NOEC) of 15 mg AMPA/L. During a 33-d exposure period under continuous renewal test conditions the effects on time to hatch, hatching success, posthatch growth and survival of the fish *Pimephales promelas* were assessed resulting in an NOAEC of 12 mg AMPA/L, the highest tested concentration. Test methodology followed the procedure outlined in the OECD 210 test guideline for *P. promelas*. For the chronic test on *Daphnia magna* the OECD 211 guideline is mentioned in the full text.

The study is well documented and all relevant information, e.g. information on the test item, test design, application method and implementation of the study, is available. In addition, a chemical analysis of test solutions was performed. All information for evaluation of the study is given. The study is considered as reliable.

1. Information on the study

Data point:	KCA 8.2.1
Report author	Schweizer M. *et al.*
Report year	2019
Report title	How glyphosate and its associated acidity affect early development in zebrafish (*Danio rerio)*
Document No	PeerJ, (2019) Vol. 7, pp. e7094
Guidelines followed in study	OECD Guideline 236
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Zebrafish (*Danio rerio*) embryos exposed to concentrations between 10 µM and 10 mM glyphosate (corresponding concentrations between 1.69 and 1690.7 mg glyphosate/L) in an unbuffered aqueous medium, as well as at pH 7, for 96 hours post fertilization (hpf). Furthermore, for investigations of the influence of pH, the test concentration 1 mM glyphosate (169.07 mg glyphosate/L) was tested at different pH values ranging between pH 3 and 8 vs. the respective pH controls. A total of 32 embryos were used per treatment with 8 replicates of 4 embryos each. The observed endpoints included mortality, the hatching rate, developmental delays at 24 hpf, the heart rate at 48 hpf, hatching success from 60 to 96 hpf and malformations at 96 hpf. $LC_{10/50}$, EC_{10} and, if reasonable, EC_{50} values were determined for unbuffered glyphosate.

In unbuffered glyphosate medium the lethal concentrations were calculated to be 385 mM (LC_{10}) and 582 mM (LC_{50}) at 96 hpf. Regarding heart rates the EC_{10} was 43 mM. Concerning the hatching rate, EC_{10} and EC_{50} levels at 96 hpf were 155 and 224 mM, respectively. For developmental delays at 24 hpf the EC_{10} was 126 mM.

Materials and methods

Glyphosate; Glyphosate (N-(phosphonomethyl)glycine, 96% pure substance, molecular weight: 169.07 g/mol, CAS: 1071-83-6; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used to prepare the test solutions. A stock solution with a concentration of 25 mM was prepared as follows: glyphosate was diluted in reconstituted water (0.23 g KCl, 2.59 g $NaHCO_3$, 4.93 g MgS_4O x 7 H_2O and 11.76 g $CaCl_2$ x 2 H_2O were dissolved separately in one L double-distilled water, then 25 mL of each stock solution was added to 900 mL double-distilled water). The stock solution was then diluted to the following test concentrations: 10, 50, 100, 250, 500, 750 mM, one and 10 mM glyphosate. All those concentrations were tested unbuffered and at pH 7. For pH adjustments, 1M HCl and NaOH solutions were used as recommended in the Organisation for Economic Co-operation and Development (OECD) 236 (2013) guideline. For investigations of the influence of pH, 1 mM glyphosate was tested at different pH values ranging between pH 3 and 8 vs. the respective pH controls. Due to preliminary results from the broad-scale pH testing, particular attention was paid to the range between pH 3 and 4. Measurements of pH were conducted with a pH meter (SevenCompactDuo; Mettler Toledo, Gießen, Germany) directly prior to the exposure.

Maintenance of zebrafish and test procedure; The embryos used in this study stem from our own breeding stock of the D. rerio West aquarium strain established in the Animal Physiological Ecology group, Tübingen University. Adult zebrafish were kept in 90 L aquaria filled with a 1:1 mixture of purified water and filtered tap water (AE-2L water filter with an ABL-0240-29 activated carbon filter, 0.3 mm; Reiser, Seligenstadt, Germany) at 26 ± 1 °C and an oxygen saturation of 100% ± 5%. Conductivity ranged from 260 to 350 mS/cm, nitrite and nitrate concentrations from 0.025 to 0.1 mg/L, one and five mg/L, respectively, and total water hardness from eight to 12 dH. Fish were subjected to

an artificial 12:12 h day/night cycle and fed three times daily with flake food (TetraMin; Tetra GmbH, Melle, Germany) supplemented with frozen black mosquito larvae and glass worms (Poseidon Aquakultur Freeze, Ruppichteroth, Germany) prior to spawning to ensure sufficient dietary protein. The day before the test, pre-exposure and test Petri dishes (90 and 30 mm in diameter) were filled with the respective solutions and stored at 26 ± 1 °C overnight to saturate the glass (the same was done with the Schott flask used for the stock solution, beforehand). On the morning of the test, Petri dishes were emptied and refilled with 70 mL (pre-exposure) and three mL (test Petri dishes) solution. For spawning, Plexiglas boxes 20 x 20 x 6 cm in size and covered with a mesh grid to keep zebrafish from feeding on their own eggs were used as breeding boxes. They were topped with artificial sea grass acting as an optical spawning stimulus and were placed into the fish tanks the evening before the start of the test. Zebrafish spawn at sunrise; therefore, spawning in the laboratory starts with the onset of light the next morning. Eggs were collected with a sieve, rinsed with tepid tap water, transferred into pre-exposure Petri dishes and incubated for 2 h at 26 ± 1 °C. Following the pre-exposure, eggs for the test were chosen with regard to their age and developmental stage (0 hours post fertilization (hpf) ≙ 8 a.m.), placed into the small 30 mm Petri dishes and stored in a heated cabinet at 26 ± 1 °C. A total of 32 individuals were used per treatment, that is, four per Petri dish and eight replicates each. Embryos were checked every 12 to 24 h. Endpoints investigated under a stereo microscope (Stemi 2000-C; Zeiss, Oberkochen, Germany) included mortality, developmental delays at 24 hpf, heart rate at 48 hpf, hatching success from 60 to 96 hpf and malformations at 96 hpf. Except for mortality, analysis of all endpoints, including hatching success, was based on living embryos/larvae at the respective time point of evaluation.

Table 1. Overview of observed lethal and sublethal endpoints at respective time points.

Endpoint	12 hpf	24 hpf	48 hpf	60 hpf	72 hpf	96 hpf
Mortality	✓	✓	✓	✓	✓	✓
Developmental delays		✓				
No somites		✓				
Non-detachment of the tail		✓				
No development of the eyes		✓				
Heart rate			✓			
Hatching success				✓	✓	✓
Malformations						✓
Oedema						✓
Eye/brain defects						✓
Deformation of the spine						✓
Light pigmentation						✓

Heart rates were determined from two out of four individuals per Petri dish for 20 s, and values were extrapolated to 1 min. Coagulated eggs, dead larvae and empty egg shells were removed from the Petri dishes to avoid depletion of oxygen due to biological degradation processes. The embryo test was run three times and conducted according to Organisation for Economic Co-operation and Development (OECD) 236 (2013). The compound 3,4-dichloraniline (98%, CAS: 95-76-1; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at a concentration of four mg/L served as a positive control and reconstituted water, as a negative control. According to the Directive 2010/63/EU of the European Parliament and the Council on the protection of animals for scientific purposes, D. rerio embryos and larvae that do not feed independently are not regarded as animals, thus regulations and permissions for animal testing do not apply. Nevertheless, all embryos in our tests were handled in the least stressful way possible and with the utmost care. After test termination embryos/larvae were euthanized with MS222.

Statistics; All statistical analyses were conducted in JMP 11.2.0 (SAS Institute Inc., Cary, NC, USA). Mortality, hatching success and the malformation rate at 96 hpf, as well as developmental delays at 24 hpf, were analysed with a likelihood-ratio w2 test, followed by Fisher's exact test. Finally, the sequential Bonferroni-Holm method was applied accounting for multiple testing. A Cox regression was used to assess mortality and hatching success over time. For the analysis of heart rate, the data were averaged

per Petri dish and checked for a normal distribution and homogeneity of variances. Subsequently an ANOVA with Tukey's HSD or Dunnett's test was conducted. If data did not meet the criteria for an ANOVA and transformation of the data did not lead to the desired result, a non-parametric Steel-Dwass test was conducted instead. Additionally, for assessing the pH range in which pH control and glyphosate treatments differed in heart rate across the whole span of tested pH, non-linear regression analysis, including calculation of 95% confidence intervals (TableCurve 2D v5.01; SYSTAT Software Inc., San Jose, CA, USA), was applied. Non-linear regression analysis by TableCurve was also used for determining LC10/EC10 and LC50/EC50 values of endpoints in unbuffered glyphosate treatments.

Results

After 96 hpf, mortality and hatching success were 0% and above 80%, respectively, in control embryos. The 3,4-dichloraniline positive control induced high mortalities, with rates consistently above 80% after 96 hpf. Thus, the validity criteria according to Organisation for Economic Co-operation and Development (OECD) 236 (2013), including sensitivity of zebrafish, were met.

Unbuffered glyphosate: At the two highest concentrations tested (1 and 10 mM), it was already difficult to select well-developed eggs after the 2 h pre-exposure period. The yolk sac, which usually has a regular spherical shape, was found to be asymmetric and partly oval, and the chorion fluid, which is naturally clear, was murky in some cases and contained indefinable streaks.

As early as 12 hpf, all individuals, without exception, in the 10 mM treatment died. Mortality in the 1 mM exposure experiment was beyond 85% at 12 hpf and reached 100% within the first 24 h. Within the 750 mM glyphosate treatment, only six out of a total of 96 individuals survived until the end of the test at 96 hpf, whereas concentrations of 250 mM and below resulted in negligible or no mortality (3.125%). Regarding mortality at 96 hpf, all treatments 500 mM were highly significantly different from the control (likelihood ratio w2, $p < 0.001$). Lethal concentrations were calculated to be 385 mM (LC10) and 582 mM (LC50) at 96 hpf. Heart rates showed a concentration-dependent relationship, decreasing with increasing glyphosate concentration. The mean heart rate was 149 beats per minute (bpm) for the control and between 130 and 140 bpm for low (10, 50 mM), 120 and 130 bpm for medium (100, 250 mM) and 110 and 120 bpm for the higher (500, 750 mM) concentrations. Thus, differences between the control and the 750 mM concentration ranged between 30 and 40 bpm. The treatments with the highest concentrations of glyphosate (one mM, 10 mM) could not be evaluated due to 100% mortality at that time point. Only two individuals out of those exposed to one mM glyphosate survived until 60 hpf and seemed to continue the observed relationship between glyphosate and heart rate by showing even lower rates (93 and 96 bpm). As single individuals, they were not included in the statistical analysis. All remaining treatments were significantly different from the control (ANOVA with Tukey's HSD, $p < 0.001$) and the relationship between glyphosate concentration and heart rate could be described by linear regression analysis ($R2 = 0.546074$, $p < 0.001$). The EC10 was 43 mM. Concerning the hatching rate, we observed a clear division between a cluster of treatments that comprised the control treatment and lower concentrations of glyphosate (10, 50, 100 mM) and another treatment cluster comprising higher concentrations (250, 500, 750 mM).

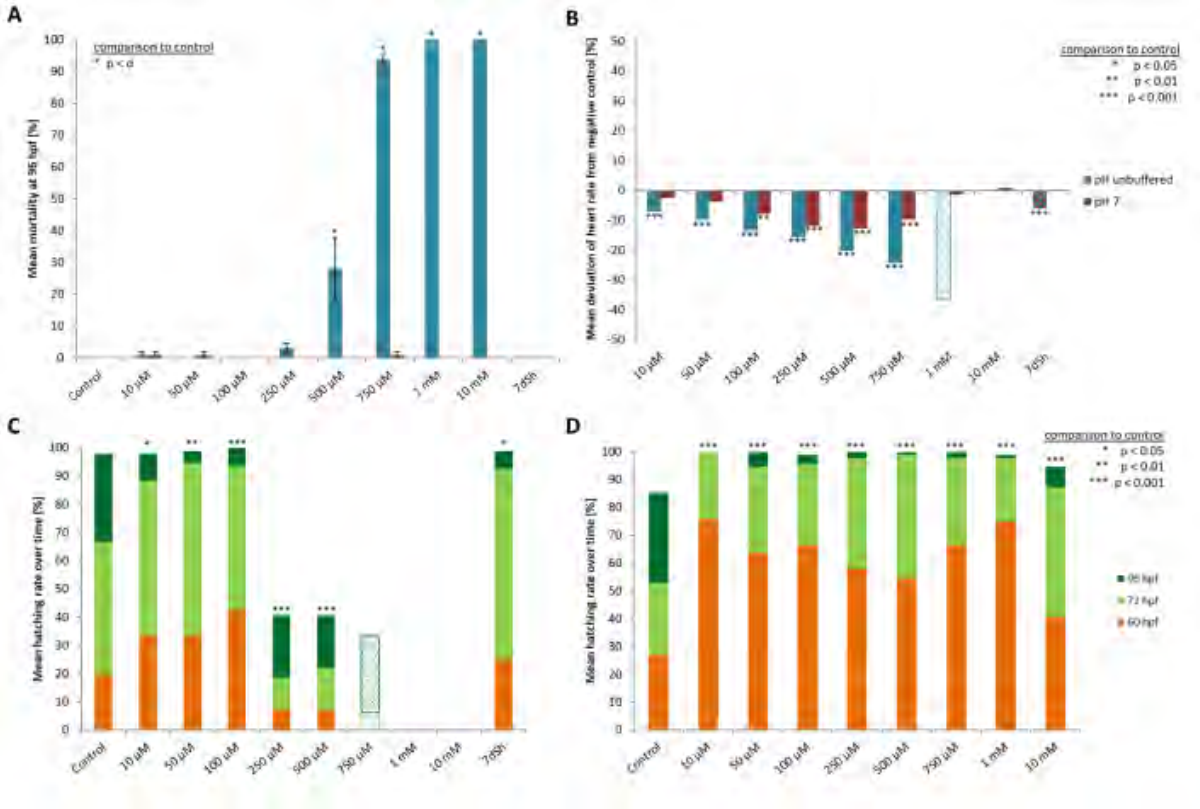


Figure 1. Mortality, heart rate and hatching success in percentage of unbuffered and pH 7 treatment. (A) Mortality after 96 hpf (likelihood ratio $\varkappa^2$, Fisher's exact test, Bonferroni-Holm, $p < \alpha$), (B) heart rate at 48 hpf relative to the negative control (Steel-Dwass, $p < 0.01$), (C) hatching rate over time in unbuffered treatments (Cox regression, $p < 0.05$), (D) hatching rate in pH 7 treatments over time (Cox regression, $p < 0.001$); shaded bars mark treatments with $n < 5$ that show tendencies but are not included in the statistical analyses.

Embryos exposed to lower concentrations hatched in 98–100% of cases, whereas hatching success in the experiments with 250 and 500 mM glyphosate was approximately 40%. All glyphosate treatments showed significant differences compared with the control (Cox regression, $p < 0.05$). EC10 and EC50 levels at 96 hpf were 155 and 224 mM, respectively. There were no developmental delays at 24 hpf for glyphosate concentrations between 10 and 100 mM, whereas in treatments with 250 to 750 mM, rates varied from 15% to 25%. The EC10 for this endpoint was 126 mM. Results for all concentrations 250 mM were highly significant (likelihood ratio w2, $p < 0.001$) compared with the control. A direct concentration dependency could not be observed. Rather, it seemed that a distinct concentration threshold had to be exceeded to induce those developmental delays and failures, which later approached the same level. Prevalent defects were a lack of tail detachment, sometimes combined with apically curved tails; a lack of somite formation and an impairment of eye development was not detected. Occasionally, embryos were fully developed but either the complete tail or just the posterior end of their tails remained attached to the yolk sac. Under normal conditions, movement begins after tail detachment. Yet, even the embryos in glyphosate treatments that lacked tail detachment, overall development had progressed to a point at which muscular contractions were already visible. But due to the undetached tails, embryos were unable to turn around and their movement was very limited. Additionally, some embryos had the posterior end of their tails detached but displayed severe spine deformations.

Those embryos could not move their tails in the same fluid manner as normally developed embryos could. Malformations could be found in embryos of all glyphosate treatments but with rates below 20%. All glyphosate treatments were significantly different from the control. Among the malformations recorded, lightly pigmented embryos and larvae were particularly frequen). Furthermore, reduced eye size occurred regularly, and some individuals suffered from cardiac or yolk sac oedemas. Two

individuals showed a notable shortening of the tail. Deformations of the spine at 96 hpf were observed surprisingly rarely, despite the high rates of tail and spine malformations at 24 hpf.

Table 2. Results for concentration-dependent glyphosate treatments, as well as for pH-dependent control and glyphosate treatments, as percentages.

	Mortality		Hatching		HR	D	M
	96 hpf (%)	Over time	96 hpf (%)	Over time	48 hpf (bpm)	24 hpf (%)	96 hpf (%)
Unbuffered							
Neg. control	0	–	97.92	–	148.75	0	0.26
10 µM	1.04	n.s.	97.92	*	**138.38*** **	0	**2.36***
50 µM	0	n.s.	98.96	*	**134.19*** **	0	**5.47***
100 µM	0	n.s.	100	***	**128.69*** **	0	**4.69***
250 µM	3.13	n.s.	**40.65***	***	**125.56*** **	**22.23***	**13.57***
500 µM	**28.13***	*	**40.58***	***	**118.63*** **	**20.36***	**16.06***
750 µM	**93.64***	***	**33.33***	n.a.	**94.50*** **	**19.04***	**18.06***
1 mM	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
10 mM	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
LC_{10}/EC_{10}	385 µM		155 µM		43 µM	126 µM	179 µM
LC_{50}/EC_{50}	582 µM		224 µM		–	–	–
7dSh	0	n.s.	98.93	*	**139.75*** **†**	0.35	**0.27†**
Neutral (pH 7)							
Neg. control	0	–	85.42	–	148.04	0	0.52
10 µM	1.04	n.s.	**100***	***	144.25	0	1.04
50 µM	1.04	n.s.	**100***	***	142.56	1.04	1.87
100 µM	0	n.s.	**98.96***	***	**136.57****	0	2.60
250 µM	0	n.s.	**100***	***	**130.31*** **	0	0.26
500 µM	0	n.s.	**100***	***	**129.19*** **	0	1.56
750 µM	1.04	n.s.	**100***	***	**133.50***	0	2.35
1 mM	0	n.s.	**98.96***	***	145.94	0.69	**6.56***
10 mM	0	n.s.	**94.79***	***	149.38	0	**7.29***
pH range—control							
Neg. control	**0**	–	94.69	–	160.38	0	0.26
pH 3	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	**84.03***	***	**61.11***	***	**140.19*** **	**9.36***	**4.17***
pH 3.4	**51.04***	***	**77.78*†**	***	**141.38*** **	**6.93***	**3.51***
pH 3.5	**8.33***	n.s.	**15.77*†**	***†	**144.25*** **	**8.32***	**2.0***
pH range—glyphosate							
pH 3	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	**72.57***	***	83.33	***	**136.46*** **	**13.33***	0
pH 3.4	**28.47***	***	**60.61*†**	***	**141.49*** **	**4.54***	**3.80***
pH 3.5	**17.36***	n.s.	**66.57*†**	***†	**143.50*** **	**10.31***	**2.04***

Notes:
Asterisks (*) and bold indicate statistically significant differences from the negative control (Cox regression, ANOVA. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Likelihood ratio χ^2, Fisher's exact test, Bonferroni-Holm: *$p < \alpha$).
Crosses (†) denote additional statistical significances between pH control and glyphosate within the same pH range or, in the case of 7dSh, differences from 1 mM glyphosate at pH 7. For unbuffered glyphosate concentrations, endpoint-related LC_{10}/EC_{10} and LC_{50}/EC_{50} values are given.
HR, heart rate; D, developmental delays; M, malformations; n.s, not significant; n.a., not available (no sufficient sample sizes for statistical analysis).

Glyphosate at pH 7; When the glyphosate solutions were adjusted to pH 7, almost no mortality or developmental delays occurred, and malformation rates were below 10% but were still significantly elevated in 1 and 10 mM treatments (likelihood ratio w2, $p < 0.001$). In the concentration range of 10 to 500 mM, heart rates showed a similar trend to those in unbuffered treatments but at a lower level: bpm decreased with increasing concentration. Still, treatments between 100 and 500 mM differed significantly from the negative control (Tukey's HSD, $p < 0.01$). At 750 mM, heart rates increased again, with a higher frequency than at 250 and 500 mM. At the two highest concentrations (1, 10 mM), heart rates were, on the one hand, marginally decelerated (1 mM) and on the other hand, marginally accelerated (10 mM) compared with the negative control. Thus, it seems that there is a turning point between 500 and 750 mM, at which the relationship between increasing concentration and heart rate shifts from deceleration to acceleration in comparison with the negative control. As already seen for

lower concentrations in unbuffered treatments, glyphosate tends to induce early hatching, even at the lowest concentration and independently of concentration. This effect unfolded to its true extent in the pH-neutral treatments. At least twice as much larvae had hatched across all glyphosate treatments at 60 hpf compared with the negative control. After 72 hpf, all larvae were hatched in glyphosate treatments, except for single individuals that hatched at 96 hpf or did not hatch at all, whereas in the negative control, only 53% of the embryos were hatched at 72 hpf and even about 15% remained unhatched at 96 hpf.

pH range , In a first step, one mM glyphosate was tested at pH 3, 4, 5, 6, 7 and 8 in comparison with negative controls at the respective pH but without the pesticide. Mortality was 100% for both treatments at pH 3, independent of the presence of glyphosate. Only a single individual survived the first 12 hpf. In contrast, only one individual died throughout all other exposures within 96 hpf. Morphological aberrations described for high glyphosate concentrations under unbuffered conditions also applied to low pH treatments, independent of glyphosate addition. Concerning sublethal endpoints, results between different acidities in the range of pH 4 to 8, as well as between control and glyphosate within the same pH range, were inconspicuous for the most part. Thus, the pH 3 to 8 series was tested just once, and subsequent testing concentrated on the range from pH 3 to 4. Thus, in the next step, pH 3, 3.25, 3.5, 3.75 and 4 were investigated in detail. As embryos exposed to pH 3.75 and 4 did not show any prominent effects, only a single run was conducted, and the final testing scheme was determined from pH 3 to 3.5 in 0.1 increments. Additionally, a test with unbuffered glyphosate at a one mM concentration (which resulted in a pH of 3.2 in the test solution) was included for direct comparison. Mortality decreased with increasing pH. Treatments with a pH of 3.2 and lower induced 100% mortality after 96 hpf. Whereas embryos exposed to pH 3 and 3.1 died within 48 hpf at the latest, embryos in pH 3.2 treatments survived considerably longer.

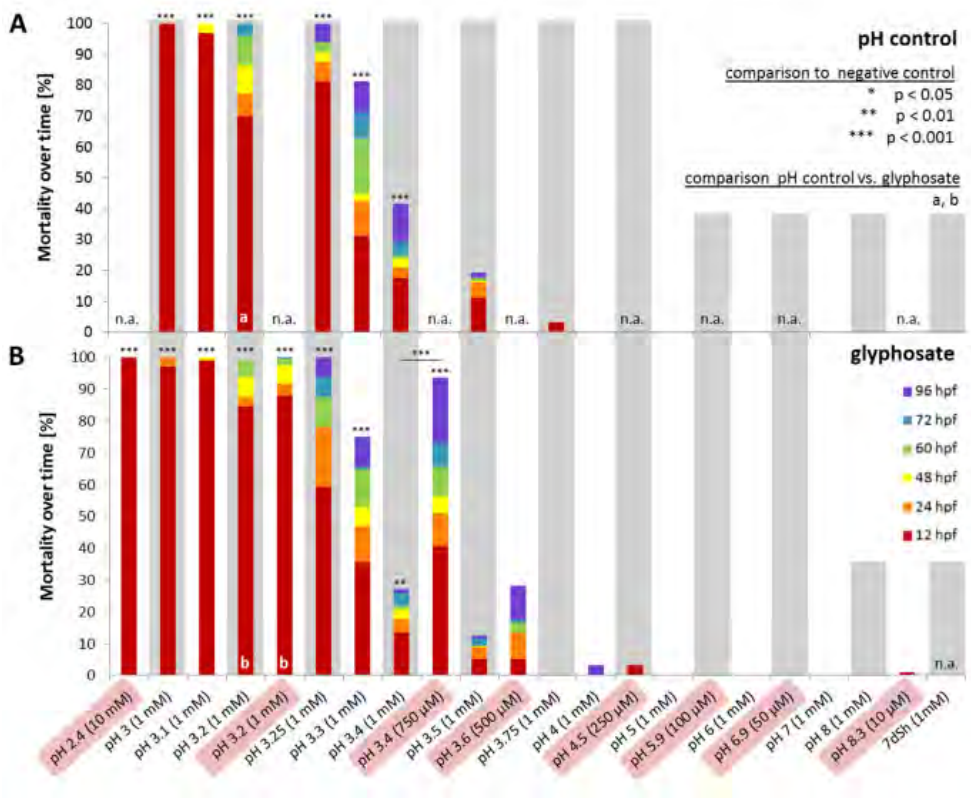


Figure 2. Mortality over time as percentages of embryos exposed to the pH control (A) and glyphosate (B). Respective concentrations of glyphosate are given in brackets. Results from unbuffered treatments (50 µM–10 µM glyphosate; highlighted in red) are combined with pH range results and positioned according to their measured pH. Treatments not conducted in the pH control or glyphosate scheme are labelled n.a. (not available). Significant differences from the negative control are marked with asterisks (*), except glyphosate pH 3.4 with an additional significant comparison between unbuffered and pH 7 treatment. Significances between pH control and glyphosate treatments within respective pH ranges are denoted with letters (a and b) (Cox regression, $p < 0.01$).

Apart from pH 3.5 without glyphosate, all treatments showed elevated mortality rates compared with the negative control (Cox regression, $p < 0.05$). There were no differences between control and glyphosate treatments with corresponding pH values, except for the elevated mortality in unbuffered glyphosate compared with the respective pH 3.2 control. Compared with the negative control, hatching was significantly delayed and also reduced in both glyphosate and pH control treatments (Cox regression, $p < 0.001$). Whereas 30% of the control embryos hatched at 60 hpf, in the pH control and glyphosate exposures, the hatching rate at 60 hpf was consistently below 5% (see Supplementary File, hatching rate). The tendency toward glyphosate-induced premature hatching at 60 hpf that was observed in pH-neutral treatments was not evident at low pH. Although not statistically significant (except for pH 3.5: Cox regression, $p < 0.001$), embryos exposed to glyphosate tended to hatch earlier and more frequently than embryos in the respective pH controls. Heart rates were significantly lowered by glyphosate at pH 3.3 to 3.5, as well as by the corresponding control pH treatments (Steel-Dwass, $p < 0.001$). Differences between glyphosate and the respective controls at the same pH value could only be detected when the full pH range dataset (including results for pH 3 to 8) was analyzed. At a pH between 5.55 and 6.02, glyphosate elevated the embryonic heart rate significantly compared with pH controls (TableCurve 2D v5.01). Developmental delays and malformations occurred in the low pH treatments, but they did not vary in a pH-dependent manner, and there was no detectable difference between glyphosate and the respective pH controls.

Comparison; When datasets for the unbuffered glyphosate treatment and the pH range were merged regarding mortality in relation to pH, interestingly, embryos exposed to unbuffered glyphosate showed higher mortalities at 500 and 750 mM compared with their 1 mM counterparts at pH 3.5 and 3.4, respectively. The unbuffered 750 mM treatment with a pH of 3.4, in particular, resulted in a mortality rate more than twice as high as that in the glyphosate pH 3.4 treatment (1 mM), mirroring mortality effects seen in treatments ranging rather between pH 3.25 and 3.3.

Conclusion

In unbuffered glyphosate medium the lethal concentrations were calculated to be 385 mM (LC_{10}) and 582 mM (LC_{50}) at 96 hpf. Regarding heart rates the EC_{10} was 43 mM. Concerning the hatching rate, EC_{10} and EC_{50} levels at 96 hpf were 155 and 224 mM, respectively. For developmental delays at 24 hpf the EC_{10} was 126 mM.

3. Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

For Zebrafish (*Danio rerio*) embryos acutely exposed to glyphosate at concentrations between 1.69 and 1690.7 mg glyphosate/L (10 µM to 10 mM) for 96 hours post fertilization (hpf) the LC_{10} and LC_{50} values (96 hpf) were calculated to be 65.1 mg a.s./L (385 µM) and 98.4 mg a.s./L (582 µM), respectively (in unbuffered glyphosate medium). Regarding heart rates the EC_{10} was 7.27 mg a.s./L (43 µM). Concerning hatching rate, 96 hpf -EC_{10} and EC_{50} values were 26.2 mg a.s./L (155 µM) and 37.9 (224 µM), respectively. For developmental delays at 24 hpf the EC10 was 21.3 mg a.s./L (126 µM). The test was conducted according to OECD 236 test guideline.

Concerning the validity criteria in the OECD 236, despite the stated > 80% mortality in the positive control (>30% required) there are no details presented to confirm the level of mortality. The fertilisation rate of the batch of eggs used was not reported. Finally, acute endpoints based on developmental delay and heart rate are not relevant to an EU level risk assessment for Annex I renewal purposes.

The test design is adequately described, however, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.1
Report author	Gholami Syedkolaei S.J. *et al*. (Seyed Jalil Gholami)
Report year	2013
Report title	Toxicity evaluation of Malathion, Carbaryle and Glyphosate in common carp fingerlings (Cyprinus carpio, Linnaeus, 1758).
Document No	Journal of Veterinary Research (2013), Volume 68, Number 3, pp. 257-267
Guidelines followed in study	OECD 203
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities.
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Fingerlings of the common carp (*Cyprinus carpio*, Linnaeus, 1758) are often exposed to a wide range of pesticides when they are released introduced into the sea at the estuaries of the rivers flowing into the Caspian Sea. The present study investigated effects of lethal concentrations (expressed as 96-hour LC_{50s}) and sublethal concentrations (determined by acetylcholinesterase assay) of glyphosate on these fingerlings.

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

Materials and methods

Chemicals: Sodium carbonate, sodium hydroxide, copper sulfate, potassium sodium tartrate, bovine serum albumin, phosphoric acid, tris and hydrochloric acid were purchased from the official representative of the German Company Merck in Iran. Glyphosate was purchased from Bazargan Kala (Iran). Absorbance was read using an ELISA Microplate Reader (ELx 808, BioTek).

Reactants: 0.1 mol phosphate buffer solution (PBS) (pH 7 with no Tritone), the Folin–Ciocalteu reagent (FCR) (diluted with an equal volume of distilled water), DTNB (dissolved in TRIS/HCl buffer) and acetylthiocholine iodide were used in the experiments.

Two thousand fingerlings with the mean weight of 2±0.4 g were obtained from the Shahid Rajaee Fish Breeding and Rearing Center, Sari, Mazandaran Province, and were transferred to the Fish Breeding and Rearing Research Center in the Department of Fisheries at the College of Agriculture & Natural Resources (UTCAN) in University of Tehran (Karaj). In order to adapt to the new environmental conditions, the fish were kept in two 1000-liter fiberglass tanks for 15-20 days. The physicochemical parameters of water were controlled as follows: pH=7, total water hardness ($CaCO_3$) =175 mg/l, dissolved oxygen=more than 7 ppm and temperature=20±2°C. The stock solution of glyphosate was prepared with the concentration of 10,000 ppm.

Lethal concentration experiments (bioassays): To perform bioassay, the range of concentrations of glyphosate and the logarithmic distances were determined in a pilot test and then the main experiment was carried out. Based on the results of this pilot test, the fingerlings were exposed to the following concentrations of glyphosate for 96 hours: 5.5, 6.5, 7.5, 8.5, and 9.5 mg/l. Effects and LC_{50s} were

determined in accordance with the OECD Guidelines for the Testing of Chemicals (No. 203) in static water. Bioassay for each pesticide was performed on 150 fingerlings (a total of 450) that were randomly and equally put in fifteen 100-N fiberglass tanks (three replicates for each concentration with 10 fish in each tank). The experimental conditions were close to those during the adaptation period. The fingerlings were not fed during the experiment. All experimental groups were monitored twice a day and the behavior of the fingerlings was studied. Moreover, the number of deaths was recorded at 24, 48, 72, and 96 hours after the toxin was added.

Sublethal toxicity experiment: The fingerlings were randomly placed in nine 100-N fiberglass tanks. Each tank contained 40 fingerlings, and the experimental conditions were the same as in the previous experiments. As in the rearing and adaptation periods, the subjects were fed 2% of their body weight and the feeding was stopped 24 hours before they were killed. In the sublethal toxicity experiment, the fingerlings were exposed to three different concentrations of glyphosate, each with three replicates for 15 days. The treatments were as follows: 0 (control), 0.6, and 1.2 mg/l of glyphosate. These concentrations were determined based on the LC_{50} values. About 10% of the water in each tank was siphoned off every day in order to remove waste materials and reduce ammonia levels in the water. To maintain the stability of experimental conditions, the removed water was replaced by an equal volume of water with the initial concentrations of the pesticide.

Sampling and extract preparation (upper layer): A number of fingerlings from each treatment were sampled on the fifth, tenth, and fifteenth days after their first exposure to pesticides. Because of the very small size of the fingerlings, it was not possible to take blood or tissue samples. Therefore, they were beheaded and both parts (head and trunk) were frozen at -70°C to be later used for extract preparation. The obtained tissues were manually homogenized in 0.1mmol PBS (pH 7 and containing 1% of Tritone X-100). The samples were centrifuged and the resulting extract (upper layer) was removed to be used as the enzyme source.

Total protein assay and ACHE activity measurement: Total protein concentration in the tissues was measured by using the Lowry method at 540 nm utilizing an ELISA microplate reader. In this method, FCR was used as the color reagent. Protein concentration in tissue samples was then determined using the resulting curve and its linear equation. The specific activity of cholinesterase (in µU/min/mg protein) was measured based on Ellman's method at 420 nm using a microplate reader. To this end, a mixture of the extract (upper layer), 0.1 mol PBS, DTNB (Ellman's reagent) and acetylthiocholine iodide were added to each tube. Finally, 100 ml of the final solution was poured into each well of the microplate and absorbance per minute (O.D. /min) was read.

Calculations and statistical analysis: The data obtained from the bioassay and mortality rate of the fingerlings determined by using the probit model were analyzed. The values obtained from bioassays were then estimated using the POLO-PC 2002 software (under license of the University of Tehran). The specific activity of the enzyme (in µU/min/mg protein) was calculated as the dependent variable. The data were statistically analyzed using two-way ANOVA. The concentrations of the pesticides and the durations of exposure to them were the independent variables. The difference between means was also evaluated using Duncan's test with type-I error level of 0.05.

Results

Bioassay results: No mortality was observed during the adaptation period of the fingerlings. The results showed that their mortality rate increased with at the higher concentrations. Based on the mortality rates in the bioassays, the mean LC_{10}, LC_{50}, and LC_{90} values of glyphosate for the fingerlings at 24, 48, 72, and 96 hours were calculated (α=0.95) (Table 1).

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

Table 1: The mean values obtained from bioassays in Caspian Sea common carp fingerlings

Chemical's name	Lethal concentration (mg/l)	24-hour	48-hour	72-hour	96-hour
Glyphosate	LC_{10}	5.995	5.976	5.865	5.548
	LC_{50}	7.202	7.172	6.985	6.753
	LC_{90}	8.651	8.606	8.319	8.168

Conclusion

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The acute 96 hours- LC_{50} for common carp fingerlings was determined to be 6.75 mg/L by static exposure to glyphosate at 5 test concentrations between 5.5 and 9.5 mg/L.

The test was conducted according to OECD 203, but validity criteria are missing. No information on the test item such as purity is given. The results for the control are not stated. Furthermore, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.3.1.3 / KCP 10.3.1.5
Report author	Thompson H. M. *et al.*
Report year	2014
Report title	Evaluating Exposure and Potential Effects on Honeybee Brood (*Apis mellifera*) Development Using Glyphosate as an Example
Document No	Integr Environ Assess Manag (2014), 10: 463-470
Guidelines followed in study	Oomen et al. 1992
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

This study aimed to develop an approach to evaluate potential effects of plant protection products on honeybee brood with colonies at realistic worst-case exposure rates. The approach comprised 2 stages. In the first stage, honeybee colonies were exposed to a commercial formulation of glyphosate applied to flowering *Phacelia tanacetifolia* with glyphosate residues quantified in relevant matrices (pollen and nectar) collected by foraging bees on days 1, 2, 3, 4, and 7 post-application and glyphosate levels in larvae were measured on days 4 and 7. Glyphosate levels in pollen were approximately 10 times higher than in nectar and glyphosate demonstrated rapid decline in both matrices. Residue data along with foraging rates and food requirements of the colony were then used to set dose rates in the effects study. In the second stage, the toxicity of technical glyphosate to developing honeybee larvae and pupae, and residues in larvae, were then determined by feeding treated sucrose directly to honeybee colonies at dose rates that reflect worst-case exposure scenarios. There were no significant effects from glyphosate observed in brood survival, development, and mean pupal weight. Additionally, there were no biologically significant levels of adult mortality observed in any glyphosate treatment group. Significant effects were observed only in the fenoxycarb toxic reference group and included increased brood mortality and a decline in the numbers of bees and brood. Mean glyphosate residues in larvae were comparable at 4 days after spray application in the exposure study and also following dosing at a level calculated from the mean measured levels in pollen and nectar, showing the applicability and robustness of the approach for dose setting with honeybee brood studies. This study has developed a versatile and predictive approach for use in higher tier honeybee toxicity studies. It can be used to realistically quantify exposure of colonies to pesticides to allow the appropriate dose rates to be determined, based on realistic worst-case residues in pollen and nectar and estimated intake by the colony, as shown by the residue analysis. Previous studies have used the standard methodology developed primarily to identify pesticides with insect-growth disrupting properties of pesticide formulations, which are less reliant on identifying realistic exposure scenarios. However, this adaptation of the method can be used to determine dose–response effects of colony level exposure to pesticides with a wide range of properties. This approach would limit the number of replicated tunnel or field-scale studies that need to be undertaken to assess effects on honeybee brood and may be of particular benefit where residues in pollen and nectar are crop- and/or formulation-specific, such as systemic seed treatments and granular applications.

Materials and methods

Technical grade glyphosate (62.27% w/w glyphosate isopropylamine [IPA] salt corresponding to 46.14% w/w glyphosate acid equivalent [a.e.]) and the soluble concentrate formulation of glyphosate (MON 52276) (30.68% glyphosate a.e. as the IPA salt, batch no GLP-0810-19515-A), supplied by Monsanto (St. Louis, MO) were used in the study. All honeybee colonies were obtained from National Bee Unit, FERA, (York, UK) apiaries and were confirmed as having low incidence of adult bee diseases, viruses, and varroa with no clinical signs of brood diseases.

Exposure assessment

Two 180 m^2 well-ventilated but insect-proof glasshouses were used for the study so as to be as representative as possible of the outdoor situation (e.g., polytunnel) but without direct rainfall. *Phacelia* was planted directly into the soil in the glasshouses and no pesticides were used during its cultivation. Application was performed when *Phacelia* flowers were at 100% of full bloom.

Three days before the application, 2 small honeybee colonies comprised of 4 to 6 frames of brood and 6000 to 12 000 adult bees were located on opposite sides of each glasshouse and allowed to fly freely. At the time of installation, each colony was fitted with a pollen trap and provided with a limited amount of stores to ensure that feeding on the crop was encouraged. This was done by removing as many frames as possible which contain only nectar or pollen, while ensuring survival and a maximum foraging activity. A supply of clean water, with provision to prevent bees from drowning, i.e., a sponge, was provided and replenished as required (it was removed during spray application).

To confirm that bees were foraging on the flowering *Phacelia*, foraging assessments were carried out each day during times when peak activity was expected. The assessments were performed by marking a 5 m × 1 m wide transect within the crop and counting the number of bees foraging within the marked area during a 1 min period once each day during the peak activity period (between 10.00–15.00 h in this study, based on previous experience). In addition, the number of bees returning to each hive and the number carrying pollen loads were counted during a 30 s period. These 2 counts provided information on the level of foraging activity of each hive within each glasshouse. Daily assessments of the crop were undertaken by visual assessment of the quality of the forage available, e.g., % plants with wilted flowers, wilted leaves.

The glyphosate formulation was applied at a rate equivalent to 8 L/ha (2.88 kg a.e./ha) in 400 L water/ha achieving an application efficiency of between 102% to 104% of the target rate, in both glasshouses. The application rate of 2.88 kg a.e./ha is the highest single application rate recommended for glyphosate, whereas the typical single application rate is 2.16 kg a.e./ha. The final treatment solution was prepared by adding the required quantities of test item—measured by weight, to measured volumes of tap water and thoroughly mixing in the field immediately before use to give the final treatment solution. The application was made during a period when the bees were actively foraging, using a 3 nozzle lunch box sprayer unit with a hand-held boom fitted with Lurmark 03 F110 nozzles. Direct spray drift onto the colonies was avoided by directing the spray away from the hives, and no direct overspray of the colonies occurred.

Pollen traps were activated 24 h before pollen collection, and the content of the pollen trap fitted to each hive was collected on days −1 (i.e., the day before application), 1, 2, 3, 4, and 7 after the application. The content of the traps was discarded on day 6 so as to only collect a sample from days 6 to 7. Each day and hive sample was kept separate unless they were too small for residue analysis, in which case samples from the same glasshouse were combined. All samples of pollen, nectar, and larvae were stored at −20° C.

On days 0 (before application), 1, 2, 3, 4, and 7 after the application samples of approximately 40 returning forager bees were collected from each colony by blocking the entrance of the hives with a foam bung and collecting returning foraging bees directly into collection jars. The nectar was collected from the honey stomachs of individual honeybees by removal of the stomach by dissection and placed in a preweighed tube. Samples were combined to produce samples large enough for residue analysis (minimum 200 mg).

On days 4 and 7 after the application, samples of 10 4–5-day-old larvae were taken from each colony using a forceps and stored at −20° C. Each day and hive sample was kept separate. On day 7, an additional sample of nectar was taken from the combs using a syringe in each colony and each hive sample was kept separate.

Residue analysis

Residues of glyphosate were extracted from larvae, pollen, nectar, and sucrose solution samples with acetonitrile/water (1:4, v/v). Recovery samples were fortified by spiking blank samples after weighing. For larvae, pollen, and nectar, the whole sample was accurately weighed into a single-use centrifugation tube. The sample was then homogenized, extracted with acetonitrile–water (1:4) with a high speed laboratory mixer, separated by centrifugation followed by solid-phase extraction of the supernate using a C18 column. All samples were then derivatized with fluorenylmethyl-chloroformate (FMOC-Cl). For derivatization, internal standard (1.0 µg/mL), borate buffer (0.2 mol/L sodium tetraborate decahydrate in water), and FMOC-Cl (5 g/L in acetonitrile) were added to the diluted extract. The samples were closed, mixed, and incubated at ambient temperature for at least 1 h. Finally, pH 3 water was added.

A second cleanup was carried out by applying the derivatized product to an Oasis HLB SPE column (equilibrated with dichloromethane followed by methanol and pH 3 water) and then rinsed with dichloromethane and the glyphosate-FMOC was eluted with methanol. The eluate was evaporated to dryness using a vacuum rotary evaporator. The residue was reconstituted in 5% acetonitrile solution and transferred into a glass vial for high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) analysis.

The samples were analyzed using high-pressure liquid chromatography (Shimadzu LC-System) coupled with a triple quadrupole mass spectrometry detector (Sciex API4000). A Phenomenex Synergi column 2.5 µm Max-RP, 20 × 2.0 mm, 2.5 µm (No. 00M-4372-B0-CE) + 4 mm guard column was used. The column temperature was 40°C and a 30 µL injection volume was used. The mobile phase comprised A: water + 0.1% acetic acid (80%), B: methanol + 0.1% acetic acid (15%), and C: 100 mM ammonium acetate solution in methanol (5%) with a linear gradient over 5 min to comprise A: water + 0.1% acetic acid (0%); B: methanol + 0.1% acetic acid (95%) and C: 100 mM ammonium acetate solution in methanol (5%). Glyphosate-FMOC was quantified using the transition 390.0 to 149.8 with an internal standard glyphosate 1,2-^{13}C2 15N-FMOC transition 393.0 to 152.8.

At the start of the analytical sequence, the detector linearity was confirmed over the calibration range of interest by constructing a calibration function of peak area versus concentration within the range from 2.0 ng/mL to 5000 ng/mL for larvae and nectar samples, 1.0 ng/mL to 3500 ng/mL for pollen samples, and from 2.0 ng/mL to 4000 ng/mL for sucrose solution samples. Injections of sample extracts were interspersed with injections of quality control standards after 2 to 4 samples to verify the detector response.

The methods were validated before use and showed 92%–102% recovery with relative standard deviation (RSD) <15% with sucrose samples spiked at 1 and 400 mg a.e./kg, larval samples spiked at 1 and 200 mg a.e./kg, pollen samples spiked at 1, 500 and 700 mg a.e./kg and nectar samples spiked at 1 and 500 mg a.e./kg. Calibrations were linear within the range. Unless otherwise specified the limit of detection (LOD) was 0.3 mg a.e./kg, denoted as not detected (n.d.), and the limit of quantitation (LOQ) was 1.0 mg a.e./kg. Where data were used to generate mean values residues less than the LOQ were ascribed a value of 0.6 mg a.e./kg.

Effects assessment

Two approaches were made to assess exposure levels to be used in the effects study: one based on generic published data on the requirements for nectar and pollen by larvae (generic data) and the other based on the observations made in the exposure study (study data).

Generic data. The calculations were based on a daily brood requirement of 30 mg nectar (based on 40% sugar in nectar) and 1mg pollen per worker larva (Rortais et al. 2005). Based on a brood frame being 3600 cells (British Standard frame) and 5 frames of brood (4–6 were used in this study), there are 18 000 brood cells. The brood is unsealed for 25% of the time (hatch day 3 to sealed day 8 with emergence day 21, empirically determined in this study) therefore 4500 larvae have a requirement for 135 g/d nectar

and 4.5 g/d pollen.

Study data. The second approach was to assess the amount of pollen and nectar returning to the hive over the time course of exposure using the data on the numbers of returning foragers in the study and the amounts of pollen and nectar collected from bees by using the pollen trap and individual bee samples.

The maximum pollen collected per colony was 2.9 g on day 1 and the traps were estimated to be approximately 50% efficient based on calculated pollen collection (Levin and Loper 1984; Delaplane et al. 2013). Thus 6 g of pollen per day was returned to the hive (the colony was using approximately 4.5 g of this based on the study by Rortais et al. [2005]).

The nectar collection was more difficult to directly assess but with a mean of 18 foragers returning to the hive per 30 s (observed in this study) and approximately 50 µL per load (max) this gives 18 trips/30 s × 60 s/min × 60 min/h × 12 h max foraging/d = 25 920 trips/d × 0.050 mL = 1296 mL/day (of which the colony was using 135 g, based on Rortais et al. [2005]). Because the assessment is brood exposure, the conservative collection estimate is justified. Therefore, as a worst case example considering the colony size used in the exposure study, the colony collected 6 g pollen and 1296 mL (i.e., 518 g sugar, assuming 40% sugar content) nectar and of this the brood consumes 4.5 g pollen and 135 g nectar (Rortais et al. 2005) that allowed the excess to be stored for later consumption.

Considering that bee colonies used in the brood study were up to 50% bigger than those used in the residue study, an additional calculation for the expected total daily intake of glyphosate residues was undertaken assuming that such colonies would collect 9 g pollen and 1944 mL nectar. Furthermore, the determined residue content based on a worst-case application rate of 2.88 kg a.e./ha for spot treatments in orchards and vines and was adjusted to reflect the more realistic maximum application rate of 2.16 kg a.e./ha for preplanting, preemergence of crops, and preharvest applications.

The brood feeding study was undertaken using glyphosate as the technical grade IPA salt. Three dose levels of the test item were used based on the residues identified in pollen and nectar in a glass house study performed before the initiation of the bee brood study. The lowest dose was based on the mean residue concentrations achieved over the first 3 days following the residue study spray application (75 mg glyphosate a.e./L). The mid-dose was based on the highest residue concentrations following the spray application (150 mg glyphosate a.e./L) and the highest dose was equivalent to twice this latter rate (301 mg glyphosate a.e./L). The test item was introduced into each hive in equivalent volumes of 50% sucrose (w/v) solution (1 L) for each treatment group. Hence, the range could also be expressed in terms of concentration in the introduced dosing solution (mg glyphosate a.e./L and mg glyphosate a.e./kg). Control colonies were supplied with 50% w/v sucrose solution in deionized water and the toxic reference, fenoxycarb, (750 mg a.s./L as the formulation Insegar WG 250 g a.s./kg, batch no SM01A406) reported to have significant adverse effects on honeybee brood, was used to ensure that the study had the ability to detect effects of the test substance if they occurred (de Ruijter and van der Steen 1987).

Twenty standardized honeybee colonies each consisting of a single wooden Smith hive with British Standard frames and a queen were used; each of the queens used in the study was of similar age and lineage. The colonies were divided into 5 groups of 4 colonies. Each colony had a dead bee trap fitted to the front and the contents were counted daily during the brood assessment period (Imdorf et al. 1987). The colonies contained a mean of 14 250 to 19 500 adult bees, 1.5 to 2.5 frames of brood, 1.0 to 1.9 frames of stores, and 0.2 to 0.7 frames of pollen. The test colonies were allowed to fly freely, there were no nearby flowering crops and few flowering weeds (clover). Colonies were assembled according to treatment and groups were placed at least 20 m apart from each other. Two colonies (one control colony and one of the highest exposure rate colonies) (301 mg glyphosate a.e./L) became queenless after dosing but were retained in the study as the marked brood was viable and this was therefore not considered to have a significant impact on the study. All colonies were generally assessed within 1 week before dosing and again within weeks 1, 2, and 3 after dosing (day 0). Each assessment was carried out on every frame within each colony, and included counts of the number of combs of adults, brood (sealed and unsealed), and stores (nectar and pollen) as well as any behavioral or physical abnormalities.

The processes during the study followed the method for honeybee brood feeding test with insect growth regulating compounds (Oomen et al. 1992). Up to 24 h before dosing, 100 brood cells containing eggs, 100 cells containing 1- to 2-day-old larvae and 100 cells containing 3- to 4-day-old larvae were selected in each colony and marked using the standard Oomen et al. (1992) acetate overlay sheet method.

On day 0, one group was an untreated control, i.e., fed 1 L 50% sucrose solution, 3 groups were treated with glyphosate IPA salt (added to 1 L of 50% sucrose to achieve doses of 301, 150 and 75 mg glyphosate a.e./L), and one group was treated with the toxic reference, fenoxycarb, dispersed in 1 L of 50% w/v sucrose (750 mg a.s./L). Doses were administered by removing frames of stores from the colonies and placing a 1 L glass container containing the treated or control sucrose within the brood chamber. The container contained a cork float to allow access to the sucrose solution. Samples of each concentration of test item treated sucrose solution were retained for analysis by subsampling 5 mL from each of the prepared solutions and combining to a single sample (total 4 samples; control and 3 doses of glyphosate). The uptake of each sucrose solution was checked daily and the container removed when empty or after 5 days whichever was later.

On day 7, the marked brood cells (eggs, young, and old larvae) were assessed for mortality and appearance in each test colony. The final assessment for each larval was undertaken at day 13 for brood cells marked as containing old larvae, day 15 for cells containing young larvae, and day 16 for cells containing eggs. The cells were uncapped, the bee removed carefully with forceps, and the age of the bee assessed, weighed, and any deformities noted.

On days 4 and 7 (when the marked brood cells were assessed), samples of ten 4- to 5-day-old larvae were sampled from each treated colony (not from an area in which marked brood cells were located) for residue analysis. For the purpose of this study, mortality was defined as the total number of cells in any one group at any one observation period that were empty (other than recently emerged), contained dead larvae or pupae or contained larvae or pupae that were considered unhealthy (sick) and unlikely to survive. Brood mortality was statistically analyzed using a generalized linear model linked to a logit distribution for the brood mortality data and an analysis of variance for pupae weight data to determine the no observed effect concentration (NOEC) (equivalent to the no observed adverse effect level [NOAEL]) statistically, using the software Genstat v12 (VSN International). The study was considered valid if there were significant effects of the toxic reference (>40% effects on all stages) during the detailed brood assessment when compared to the control. The performance of the colonies in the control group were comparable with historical control data for the testing facility (10%–30% larval mortality overall), and demonstrate that the control colonies had performed correctly.

Results

Exposure study

Daily assessments were made of the percentage of the plants that had wilted leaves or flowers. The crop started to show significant effects of the treatment from day 4 onward in both glasshouses and this coincided with the decreased foraging activity in glasshouse 2 although less pronounced effects on foraging were observed in glasshouse 1.

Foraging assessments showed foraging activity on the crop at the start of the study and this continued throughout the exposure period in glasshouse 1 with a peak on day 4; lowest foraging activity was on day 5 at 38% of the mean prespray activity. In glasshouse 2, the foraging activity declined throughout the assessment period and reached <10% of the mean prespray activity on days 5 to 7. The weights of pollen collected from the traps fitted to each hive ranged from 0.37 to 1.8 g per colony per day.

Samples of honeybee products (nectar and pollen) and larvae were analyzed for residues of glyphosate acid equivalents. Glyphosate residues in nectar samples taken from forager bees before the application were not detectable (<0.3 mg a.e./kg). Residues in nectar samples taken at various time points after the application and originating from forager honeybees ranged from 2.78 to 31.3 mg a.e./kg and declined

over time (Figure 1A). Residues in nectar samples taken from the colonies 7 days after the application ranged from below the LOQ (1.0 mg a.e./kg) to 1.30 mg a.e./kg.

Residues in pollen samples taken from the pollen trap before the application were not detectable (<0.3 mg a.e./kg). Residues in pollen samples taken at various time points after the application and originating from the trap ranged from 87.2 mg a.e./kg to 629 mg a.e./kg and declined over time (Figure 1B). Residues in larvae samples at 2 time points (day 4 and day 7) after the application ranged from 1.23 mg a.e./kg to 19.50 mg a.e./kg.

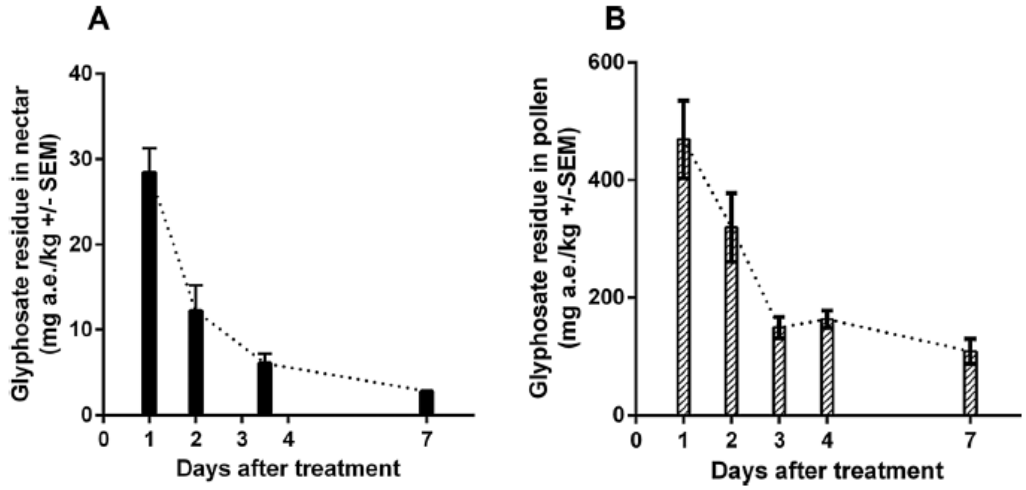


Fig. 1. Decline of glyphosate residues (mg a.e./kg ± SE). (A) Nectar collected from foragers. The nectar sample from days 3 and 4 were combined due to the small amount collected for analysis. (B) Pollen collected in pollen traps in mg a.e./kg matrix.

Effects study

Consumption of treated sucrose. Analysis of the dosing solutions showed they were within 11% of the nominal doses. The control colonies consumed between 0.63 and 1.0 L of untreated sucrose. In the glyphosate-treated colonies, at least 3 of the 4 colonies in each group consumed the total volume of treated sucrose fed to each of them. There was no statistically significant difference in sucrose consumption in comparison to control for the 301 mg a.i./L group ($p = 0.438$), 150 mg a.i./L group ($p = 0.212$), the 75 mg a.i./L group ($p = 0.054$), which was slightly higher than the control, and the positive control fenoxycarb ($p = 0.151$).

In the 301 mg glyphosate a.e./L group, one colony consumed 0.39 L and the other 3 each consumed 1.0 L resulting in mean exposure to 255 ± 26 mg glyphosate a.e. In the 150 mg glyphosate a.e./L group, one colony consumed 0.67 L and the other 3 each consumed 1.0 L resulting in mean exposure to 130 ± 12 mg glyphosate a.e. In the 75 mg glyphosate a.e./L group one colony consumed 0.90 L and the other 3 each consumed 1.0 L resulting in mean exposure to 73 ± 2 mg glyphosate a.e. In the fenoxycarb treated colonies, consumption rates ranged from 0.45 to 0.88 L resulting in mean exposure to 510 ± 72 mg fenoxycarb. Exposure at the 150 mg a.i./L dose was significantly lower than at the 301 mg a.i./L dose ($p = 0.049$) and exposure at the 75 mg a.i./L dose was significantly lower than at 150 mg a.i./L dose ($p = 0.002$).

Brood mortality. Figure 2 summarizes the survival of marked brood stages at day 7 after dosing and just before emergence. There were no significant treatment-related effects except in the fenoxycarb toxic reference treated colonies, in which overall survival of marked cells was 20% for marked eggs ($p < 0.001$), 0% for marked young larvae ($p < 0.001$) and 12% for marked old larvae ($p < 0.001$), meeting the established validity criterion for the toxic reference (>40% effects at all stages). This can be compared with overall survival of 85% for marked eggs, 96% for marked young larvae, and 96% for marked old larvae in controls and 82%–87% for marked eggs (300 mg a.i./L: $p = 0.435$, 150 mg a.i./L:

p = 0.310, 75 mg a.i./L: p = 0.250), 87%–94% for marked young larvae (300 mg a.i./L: p = 0.185, 150 mg a.i./L: p = 0.060, 75 mg a.i./L: p = 0.254), and 94%–95% for marked old larvae (300 mg a.i./L: p = 0.434, 150 mg a.i./L: p = 0.202, 75 mg a.i./L: p = 0.291) in the glyphosate-treated colonies. The control mortality is similar to historical levels in studies conducted at the Food and Environmental Research Agency (FERA) (10%–30%). Deformities were observed in the fenoxycarb-treated colonies where discolored heads, thorax, and abdomens were noted. No deformities were observed in of the control or any glyphosate-treated colonies. Additionally, there were no significant effects on the mean weight of the exposed pupae (Table 1) compared to controls in the 300 mg a.i./L group (p = 0.424), the 150 mg a.i./L (p = 0.207), or the 75 mg a.i./L (p = 0.292). The fenoxycarb-treated colonies showed significant effects on weight of surviving pupae marked as old larvae (p = 0.003). The only dead pupae observed in any significant number were those in the fenoxycarb treated group where a mean of up to 190 pupae/day was observed and a mean of 600 pupae were recovered from the colonies over the 17-day period after dosing compared with 2.0 pupae/d in the control and 1.3 to 1.8 pupae/d in the glyphosate-treated colonies. The only adverse effects on colony development were observed in the fenoxycarb-treated colonies where declines in the numbers of bees and brood were observed in the latter stages of the study compared to controls for the 300 mg a.i./L group (p = 0.401), the 150 mg a.i./L group (p = 0.414), the 75 mg a.i./L group (p = 0.360), or the positive control fenoxycarb (p = 0.070).

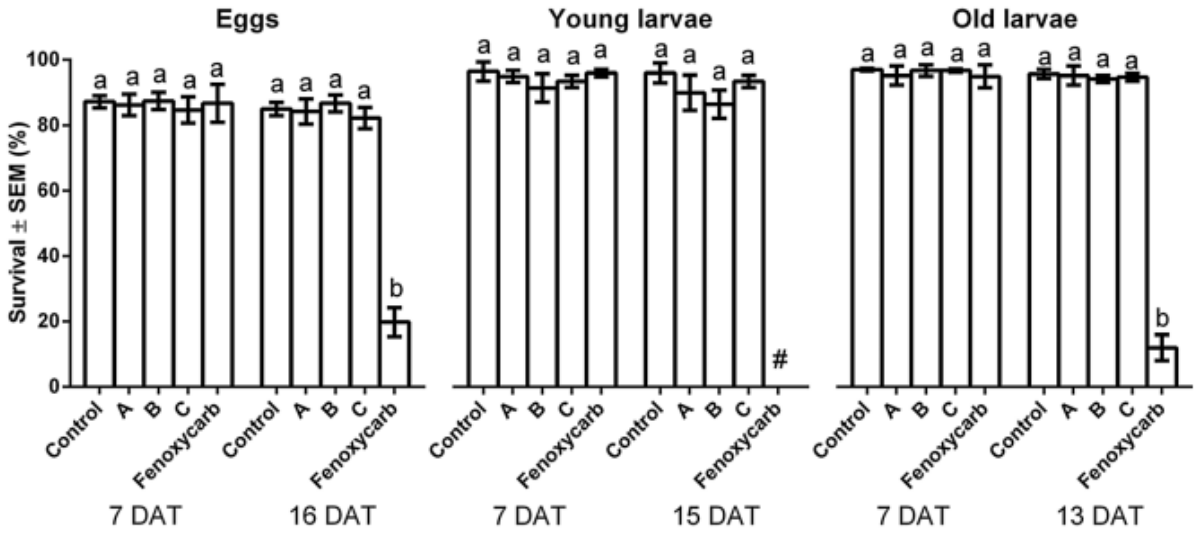


Fig. 2. Survival (% ± SE) of Eggs (7 and 16 Days After Treatment, DAT), Young Larvae (7 and 15 DAT) and Old Larvae (7 and 13 DAT) for treatment groups (mean consumption) Control (0 mg glyphosate a.e.), A (255 ± 46 mg glyphosate a.e.), B (138 ± 12 mg a.e.), C (73 ± 2 mg glyphosate a.e.), and Fenoxycarb (510 ± 72 mg). Different letters above the bars indicate statistical difference (p < 0.05) from the respective control. # no statistical analysis as no variance due to 100% mortality.

Table 1. Mean pupae weight with SE at final assessment including dead and sick in the fenoxycarb treatment

Treatment	Dose rate mg/L	Mean dose consumed mg (SE)	Weight-surviving pupae marked as eggs (mg)	Weight-surviving pupae marked as young larvae (mg)	Weight-surviving pupae marked as old larvae (mg)
Control	0	0	127.5 ± 0.7	128.4 ± 0.6	128.9 ± 0.4
Glyphosate	301	255 ± 46	135.7 ± 0.6	125.4 ± 0.6	125.6 ± 0.4
Glyphosate	150	138 ± 12	126.7 ± 0.6	124.4 ± 0.8	122.6 ± 0.5
Glyphosate	75	73 ± 2	124.7 ± 0.8	128.3 ± 1.0	121.2 ± 0.5
Fenoxycarb	750	510 ± 72	125.9 ± 0.9	128.8 ± 1.3	115.4 ± 1.0[a]

SE = standard error

[a] Statistically different effect ($p < 0.01$)

Adult bee mortality. No biologically significant adult mortality was observed in any treatment group with a mean total of 73 to 25 dead adult workers were recovered from dead bee traps over the entire 17-day period after dosing.

Residue analysis. The residues in larvae sampled at 2 time points (day 4 and day 7) after dosing of the colonies (Figure 3) ranged from below the LOQ (1.0 mg a.e./kg) to 82.1 mg a.e./kg (at the highest dose rate) confirming that larvae were exposed to test item provided in the sucrose solution and consumed it. There was a linear relationship between dose level and glyphosate levels in larvae on days 4 and 7. Levels of day 7 were considerably lower than on day 4 and are likely the result of larval growth and glyphosate exposure ending after 5 days of exposure. Notably, these residue levels are comparable with values from the exposure study which ranged from 2.9 to 19.5 mg a.e./kg with a mean of 11.5 mg a.e./kg on day 4 to 1.2 to 10.6 mg a.e./kg with a mean of 5.3 mg a.e./kg on day 7 after the glyphosate application.

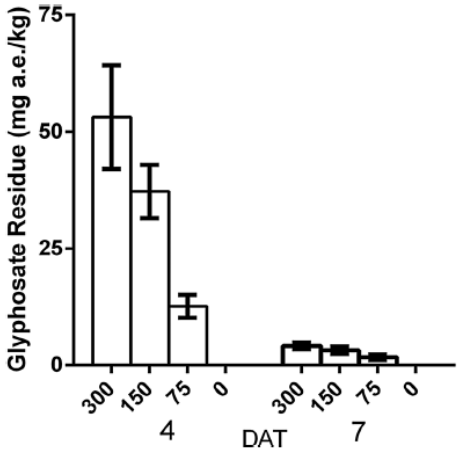


Fig. 3. Residues (mg a.e./kg ± SE) in larvae 4 and 7 days after treatment (DAT) for dose groups with dose rate of 300, 150, 75, and 0 mg a.e./kg sucrose solution.

Conclusion

There were no significant effects from glyphosate observed in brood survival, development, and mean pupal weight. Additionally, there were no biologically significant levels of adult mortality observed in any glyphosate treatment group. Significant effects were observed only in the fenoxycarb toxic reference group and included increased brood mortality and a decline in the numbers of bees and brood. Mean glyphosate residues in larvae were comparable at 4 days after spray application in the exposure study

and also following dosing at a level calculated from the mean measured levels in pollen and nectar, showing the applicability and robustness of the approach for dose setting with honeybee brood studies.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The Oomen et al. (1992) approach was used to quantify at residues in relevant matrices (pollen, nectar, and larvae) following application of glyphosate at 2.88 kg a.e./ha (400 L water/ha) to flowering *Phacelia tenacetifolia* in large glasshouses. Then brood feeding tests following the Oomen approach, were conducted by feeding 1 L treated sucrose solution at 75 / 150 and 301 mg glyphosate a.e./L directly to honeybee colonies.

The study is adequately described and all information to evaluate the study are available. At the time the study was conducted, there were no field level test guidelines adopted for use in the EU. The test did follow a recognised approach and is considered fit for purpose. The study is considered as reliable.

1. Information on the study

Data point:	KCA 8.2.7
Report author	Tian Y. *et al.*
Report year	2015
Report title	Growth inhibition of two herbicides on Spirodela polyrhiza
Document No	Nongyao kexue yu guanli (Pesticide Science and Administration), 2015, Vol. 36 (issue 3), pp. 61-65
Guidelines followed in study	OECD 221
Deviations from current test guideline	Not reported
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The inhibitory activities of glyphosate on the aquatic macrophyte *Spirodela polyrhiza*, were studied in the laboratory by using quantity of the thallus as test indicator. The effects of glyphosate were tested in a semi-static exposure of 7 days at concentrations between 8.4 and 20.902 mg/L. The results showed that glyphosate had remarkable effects on the growth inhibition of *Spirodela polyrhiza*, and the inhibitory rate increased with higher concentrations. The 168 hour-EC_{50} value was determined to be 12.817 mg/L.

Materials and Methods

Test materials and culture

The tested organism, *Spirodela polyrhiza*, was introduced from Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

The *Spirodela polyrhiza* was placed in a crystal dish with the volume of 300 mL (10 × 5cm). The Swiss standard (SIS) culture medium was added (Table 1). The light was 9000 - 10 000lx incandescent light, the temperature was 24 ± 2℃, and the culture medium was replaced every 7 days to maintain the stability of the concentration of the nutritional ingredients in the solution. It can only be used in the experiment after 14 days of continuous pre-culture. Before the experiment, enough 4-leaf *Spirodela polyrhiza* with good shape and similar shape and size were selected to carry out the experiment. The above experimental operations should be carried out in an ultra-clean work table to prevent culture medium pollution.

Main instruments and test reagents for the test

Main instruments and reagents

Intelligent artificial climate box PRX-350B (Ningbo Saifu Experimental instrument Co., Ltd.), super clean worktable VS-1300L-U (Sujing Antai), biosafety cabinet BHC-1300 II A/B3 (Suzhou Antai); Glyphosate 96.8% original drug (provided by Ministry of Agriculture Pesticide Inspection Institute), Dimethylformamide (Analytical Reagent, Beijing Chemical Plant), Twin 80 (Analytical Reagent, Beijing Chemical Plant).

Table 1: (SIS) culture medium component*

Storage solution serial No.	Reagent	Storage solution concentration (g/L)	Concentration of culture medium (mg/L)
A	$NaNO_3$	8.5	85
	KH_2PO_4	1.34	13.4
B	$MgSO_4\ 7H_2O$	15	75
C	$CaCl_2{\cdot}2H_2O$	7.2	36
D	Na_2CO_3	4	20
E	$Na_2EDTA{\cdot}2H_2O$	0.28	1.4
	$FeCl_3{\cdot}6H_2O$	0.17	0.84
F	H_3BO_3	1	1
	$CuSO_4\ 5H_2O$	0.005	0.005
	$ZnSO_4\ 7H_2O$	0.05	0.05
	$MnCl_2\ 4H_2O$	0.2	0.2
	$Na_2MoO_4\ 2H_2O$	0.01	0.01
	$Co(NO_3)_2\ 6H_2O$	0.01	0.01

* All storage solutions shall be kept in refrigerated and dark conditions, and the storage solution AE can be kept for 6 months, while the reserve liquid F can only be kept for 1 month. Prepare 1LSIS medium, take 10 mL stock solution A, 5 mL storage solution B~E, 1 mL stock solution F into volumetric flask, add 900 mL distilled water, adjust pH to 6~7 with 1 mol/L HCl, and then use distilled water to 1L.

Experimental Methods

Allocation of test mother liquid: 0.1 g glyphosate was obtained by dissolving it in aseptic water, and the volume was fixed to 100 mL capacity bottle. And then 1 000 mg/L glyphosate mother liquid could be obtained. After sealing the above liquid with sealing film, put it in the refrigerator at 4℃ for further test.

Experimental Design

On the basis of the pre-test, a series of concentration gradients are set according to the equal ratio difference. The concentrations of glyphosate were 8.4, 10.08, 12.096, 14.515, 17.418, 20.902 mg/L and solvent control group and blank control group. 200 mL (height > 2cm) culture solution containing different concentrations of glyphosate was added to the crystal dish with diameter 10 cm. Three selected ***Spirodela polyrhizas*** were put into the above toxic solution, sealed with an aseptic culture container ligated with a rubber band. 3 repeats were set up in each treatment, and finally they were randomly placed in an artificial climate box. The experimental conditions were consistent with the pre-culture conditions. In order to maintain the concentration of the test solution, semi-static culture was used in this experiment. PH was measured before replacing the culture test solution on the 3rd and 5th day, respectively. All the above operations should be operated under aseptic conditions to prevent culture medium pollution. The test period was seven days. After the experiment was over, the average specific growth rate μ of the blank control was calculated, and the growth inhibition percentage of each treatment group was also calculated.

Test Index

The number and growth condition of ***Spirodela polyrhiza*** in each treatment group were recorded every 2 days, and whether the culture medium was normal or not was also recorded. All clearly visible leaves should be counted. The increase of the number of ***Spirodela polyrhiza*** leaves indicated its growth, and the difference between each concentration group and the control group indicated the toxic effect.

Data Processing

The average specific growth rate (μ)

The average specific growth rate in a specific period is to calculate the growth variables (leaf number, total leaf area) during the logarithmic growth period, and the following formula is used to calculate each repetition of the control and treatment.

$$I = \frac{\mu_c - \mu_t}{} \times 100\%$$

μ_c

In this: I - Average specific growth inhibition rate, %;

μ_c - control group μ mean value;

μ_t - control group μ mean value

Results

In the process of effectiveness analysis, the solvent control group grew well and the solvent content was less than 100 μL/L; In addition, the pH variation range (0.6 - 1.2) was not more than 1.5 before and after the replacement of the *Spirodela polyrhiza* culture solution. The average specific growth rates of leaf bodies in each blank treatment group were calculated to be 0.294 d^{-1} and 0.317 d^{-1}, respectively, both > 0.275 d^{-1}. The average specific growth rate of leaves in each blank treatment group was 0.294 d^{-1} and 0.317 d^{-1}, respectively. The above test results meet the requirements of *Spirodela polyrhiza* growth inhibition test in OECD, and the test system is effective.

Effect of glyphosate on the growth of *Spirodela polyrhiza* can be seen from Figure 1. Within a certain range, the herbicide can inhibit the growth of *Spirodela polyrhiza*, and with the increase of the concentration of the test solutions, the inhibition effect is strengthened.

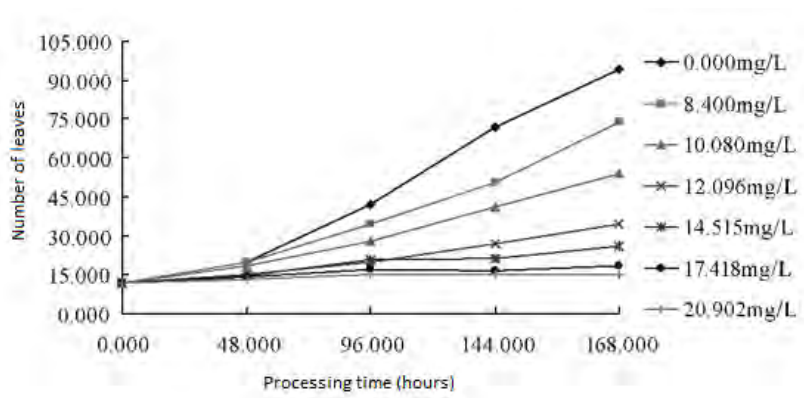


Figure 1: Inhibition of different concentrations of glyphosate on the growth of *Spirodela polyrhiza*

The growth inhibition rates of glyphosate on the leaves of *Spirodela polyrhiza* can be seen from Table 2. The coefficient of variation of each treatment group changed a bit, and the growth inhibition rates on the leaves of *Spirodela polyrhiza* showed significant differences at different concentrations of the test solution. Within a certain range, the growth inhibition rates on the leaves of the *Spirodela polyrhiza* increased with the increase of the concentration of the test solution.

Table 2: Inhibition rate of different concentrations of glyphosate on the growth of *Spirodela polyrhiza*

Treatment concentration (mg/L)	Coefficient of variation (%)	Inhibition rate of growth*I (%)
0.000	1.359	0.000 ± 0.231g
8.400	2.707	11.650 ± 0.406f
10.080	1.231	26.926 ± 0.153e
12.096	5.600	48.512 ± 0.489d
14.515	4.980	62.456 ± 0.317c
17.418	7.070	78.548 ± 0.257b
20.902	15.230	88.113 ± 0.307a

* indicates growth inhibition rate ± standard error. In the same column of data, the same letter indicates that there is no significant difference at 0.05 level (P = 0.05).

The EC_{50} of glyphosate on the leaves of duckweed was calculated by using "SPSS Statistics 17.0" software. The EC_{50}, 95% confidence interval and linear equation of glyphosate for *Spirodela polyrhiza* were calculated (Table 3). It can be seen from the correlation coefficient of the linear equation that the growth inhibition rate of the two herbicides on the *Spirodela polyrhiza* is a good linear relationship with the concentration of the test solution. The EC_{50} of glyphosate to the *Spirodela polyrhiza* was 12.817 mg/L.

Table 3: Inhibitory medium concentration of glyphosate

Test solution	EC_{50} (mg/L)	EC_{50} 95% confidence interval	Linear equation
Glyphosate	12.817	12.256 - 13.388	$y = 5.928x – 6.567$ $R^2 = 0.993$

Conclusion

The results showed that glyphosate had remarkable effects on the growth inhibition of *Spirodela polyrhiza*, and the inhibitory rate increased with higher concentrations. The 7 day-EC_{50} value was determined to be 12.817 mg/L.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effects of glyphosate to the aquatic macrophyte *Spirodela polyrhiza* was tested in a semi-static exposure of 7 days at concentrations between 8.4 and 20.902 mg/L. The 7 day-EC_{50} value was determined to be 12.817 mg/L.

This study was conducted to guideline but not to GLP. The test concentrations were not analytically verified and thus the exact exposure concentrations of the aquatic macrophyte are unknown. Therefore, the study should considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.4.1 / KCA 8.4.2.1 / KCA 8.5
Report author	von Mérey G. *et al.*
Report year	2016
Report title	Glyphosate and aminomethylphosphonic acid chronic risk assessment for soil biota
Document No	Environmental Toxicology and Chemistry (2016), Vol. 35, No. 11, pp. 2742-2752
Guidelines followed in study	OECD 222; OECD 226; OECD 232; OECD 216
Deviations from current test guideline	Earthworm cocoons were not counted, in accordance with OECD 222.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

The exposure risk from glyphosate and the primary soil metabolite aminomethylphosphonic acid (AMPA) on representative species of earthworms, springtails, and predatory soil mites and the effects on nitrogen-transformation processes by soil microorganisms were assessed under laboratory conditions based on internationally recognized guidelines. For earthworms, the reproductive no-observed-effect concentration (NOEC) was 472.8 mg glyphosate acid equivalent (a.e.)/kg dry soil, which was the highest concentration tested, and 198.1 mg/kg dry soil for AMPA. For predatory mites, the reproductive NOEC was 472.8 mg a.e./kg dry soil for glyphosate and 320 mg/kg dry soil for AMPA, the highest concentrations tested. For springtails, the reproductive NOEC was 472.8 mg a.e./kg dry soil for glyphosate and 315 mg/kg dry soil for AMPA, the highest concentrations tested. Soil nitrogen-transformation processes were unaffected by glyphosate and AMPA at 33.1 mg a.e./kg dry soil and 160 mg/kg dry soil, respectively. Comparison of these endpoints with worst-case soil concentrations expected for glyphosate (6.62 mg a.e./kg dry soil) and AMPA (6.18 mg/kg dry soil) for annual applications at the highest annual rate of 4.32 kg a.e./ha indicate very low likelihood of adverse effects on soil biota.

Materials and methods

Test substances

Glyphosate (N-phosphonomethylglycine) is an acidic substance, which is manufactured and formulated as a salt to increase the solubility in water and compatibility with other formulation components. In water, AMPA is highly soluble (56 g/L at 20 °C), whereas neither glyphosate nor AMPA is significantly soluble in common organic solvents. Therefore, no cosolvent was required, and both stock solutions of glyphosate and AMPA test items were prepared in deionized water (5 - 20 Mohm at 25 °C). Two batches of AMPA analytical reference standards with purity of 98.7% (synthesized by Chemir) and 99.7% (Acros Organics BVBA) were dissolved in deionized water. For soil nitrogen-transformation tests, stock solutions of glyphosate acid technical grade (96.59% purity; Monsanto Europe) were prepared by direct addition of test item to deionized water. For all other tests, glyphosate isopropylamine salt (nominal purity 62% w/w, measured purity 63.81 ± 0.29% w/w; MON 0139), corresponding to 45.9% w/w glyphosate a.e. (measured 47.28 ± 0.21% w/w; Monsanto Europe), were prepared in deionized water.

Earthworm reproduction tests

The earthworm reproduction test with glyphosate was conducted according to OECD guideline 222. For AMPA, an earthworm reproduction test was conducted according to the OECD 222. Both testing guidelines are equivalent in terms of the procedures employed during the tests (soil pH, temperature, lighting regime, soil composition and humidity, rearing, feeding quantities, test design, endpoints, number of replicates, growth stage of worms at test initiation, and so on). Therefore, to avoid repetition,

the procedures used in the glyphosate study only are described.

Glyphosate - Earthworm reproduction test. In the earthworm reproduction glyphosate study *Eisenia fetida* (Haplotaxida: Lumbricidae, Savigny, 1826) were used as the test species. Mature adult *E. fetida* (~3 mo old with clitellum), weighing between 300 mg and 600 mg, were obtained from an age-synchronized stock culture from the test facility and reared under ambient laboratory conditions in the test facility. The original breeding animals were purchased from W. Neudorff. A detailed description of earthworm culturing is provided in Annex 4 of OECD 222. The *E. fetida* were reared in the laboratory on standard breeding medium (1:1:1 mixture of straw, horse manure, and peat; straw and horse manure were purchased from farmers, and peat was purchased from Torfwerk Moorkultur Ramsloh); no exposure to the test item was allowed prior to use in testing. Testing was conducted in artificial soil, equivalent to the soil in which the worms were originally cultured. The test aims to evaluate effects on adult body weight and survival percentage (according to treatment) during an initial 4-wk adult exposure period. Effects on juvenile production were then assessed at the end of a 4-wk period that followed directly after adult removal from the test. Behavior (including feeding activity) and pathological symptoms (e.g., lethargy, morphological alterations) of adults and juveniles were also assessed.

On the day before the test start, earthworms (from aged-synchronized batches, to ensure that similar-sized earthworms were used) were acclimated to test conditions in a separate batch of artificial soil supplemented with pasteurized horse manure, purchased from farmers and collected from horses not treated with growth promoters, nematicides, or other veterinary products - also used as the food source during testing. On test start day, volumes of the test solution (prepared by direct addition of glyphosate isopropylamine to deionized water) were mixed into bulk samples of artificial soil, to achieve nominal glyphosate soil concentrations of 14.18 mg a.e./kg dry soil, 23.64 mg a.e./kg dry soil, 47.28 mg a.e./kg dry soil, 236.4 mg a.e./kg dry soil, and 472.8 mg a.e./kg dry soil. Glyphosate test concentrations were selected to cover the range and exceed field exposure concentrations. A toxic reference test was also performed in a separate test with carbendazim (Nutdazim 50 Flow, SC 500) at concentrations of 5 mg/kg dry soil and 10 mg/kg dry soil.

Test vessels were filled with the appropriate treated soil (810 g wet wt corresponding to 600 g dry wt). Groups of 10 individually weighed earthworms were randomly assigned to replicates within each treatment group, with a total of 40 earthworms used per treatment group divided equally between 4 replicates. For the control group (water only), 80 worms were used, divided equally between 8 replicates. Groups of 10 earthworms were placed onto the assigned replicate soil surface and closed with perforated transparent lids (following a brief burrowing period) to reduce evaporative water loss, allow gaseous exchange, and prevent worms from escaping the replicate vessels. Test vessels were then randomly positioned in an environmental test chamber under continuous light (to maintain worms in the soil). On day 1 and weekly thereafter for the 4-wk adult exposure period, 5 g of air-dried finely ground horse manure was scattered on the soil surface of each test vessel and wetted with 5mL of deionized water. The amount of manure applied each week (up to 5 g) was dictated by feeding activity.

After 4 wk, adult earthworms were removed from the vessels by emptying the contents of each replicate vessel onto a tray and removing the adult worms. Care was taken not to remove any cocoons from the soil. Cocoons were not counted, in accordance with OECD 222. It can be reasonably assumed that effects on cocoon numbers would lead to effects on numbers of juveniles; hence, the endpoint number of juveniles accounts for effects at earlier life stages of earthworm progeny. All worms were rinsed with deionized water and dried on filter paper before recording body weights (by replicate and by treatment). Behavioral (including feeding activity) and pathological symptoms were also recorded during the exposure period and at the time of adult removal. The adult worms were then discarded. The soil in each replicate vessel was then mixed carefully with 5 g of manure, and the mixture was returned to the vessels. The test continued for a further 4 wk. At test termination (8 wk after adult addition) the number of surviving juveniles in each test vessel was recorded on manual inspection of the substrate. Soil was emptied on the lower edge of a white tray (30 cm × 40 cm). Subportions of the soil were spread in the middle of the tray, resulting in a thin layer of soil of approximately 10 cm × 10 cm. The subportion was examined thoroughly for juvenile worms, after which it was moved to the upper edge of the tray. This procedure was repeated until the entire soil from a vessel was examined. The entire procedure was

repeated until there were no additional juvenile counts in 2 consecutive counting procedures, resulting in an average of 5 counting procedures per vessel. The counting tray and soil samples were illuminated using a fiber-optic light source connected with a double gooseneck light guide. The water content and pH of the artificial soil were determined. Adult body weights and the effects on reproduction (juvenile numbers) were analyzed using a lower-tailed Dunnett's multiple comparisons test ($\alpha = 0.05$). The Kolmogorov-Smirnov test and Cochran's test procedure were used, respectively, to test the biomass data for normality and homogeneity of variance. Survival was analyzed with a 1-sided Fisher's exact binomial test with Bonferroni correction ($\alpha = 0.05$).

AMPA - Earthworm reproduction tests. The procedures used during the AMPA earthworm study are considered equivalent to those employed in the glyphosate earthworm reproduction study described above in Glyphosate—Earthworm reproduction test. Mature adult *E. fetida* (~3mo old with clitellum), weighing between 300 mg and 600 mg, were obtained from an age-synchronized stock culture from the test facility and reared under ambient laboratory conditions in the test facility. A detailed description of earthworm culturing is provided in Annex 4 of OECD 222.

In the AMPA earthworm reproduction study, mature (clitellated) adult *E. fetida* were exposed to AMPA (99.7% purity; Acros Organics BVBA) mixed into artificial soil at nominal soil concentrations of 58.6 mg AMPA/kg dry soil, 87.8 mg AMPA/kg dry soil, 131.9 mg AMPA/kg dry soil, 198.1 mg AMPA/kg dry soil, 297.1 mg AMPA/kg dry soil, 445.5 mg AMPA/kg dry soil, 668.5 mg AMPA/kg dry soil, and 1002.5 mg AMPA/kg dry soil. A control group was prepared using deionized water only. A toxic reference test was also performed in parallel using earthworms from the same batch, exposed to carbendazim at concentrations of 1.0 mg active substance (a.s.)/kg dry soil, 2.2 mg a.s./kg dry soil, and 5.0 mg a.s./kg dry soil. For effects on biomass and production of juveniles, homogeneity was tested with the Brown-Forsythe and Bartlett tests. Dunnett's multiple comparison test was conducted using GraphPad Prism, Ver 6.03, because a continuous response could not be observed for all the test concentrations, as recommended by the OECD 222 test guideline and the OECD statistical guidance. The 50% effect rate on reproduction was calculated using GraphPad Prism.

Soil predatory mite reproduction test

The soil predatory mite reproduction tests for glyphosate and AMPA were both conducted according to OECD guideline 226 predatory mite (*Hypoaspis* [Geolaelaps] *aculeifer*) reproduction test in soil. The procedures used in the 2 studies were identical. Full details of the procedures are presented for glyphosate only. The *Hypoaspis* used in these studies were originally purchased from Katz Biotech and reared in the test facility under ambient conditions since June 2005.

Glyphosate - Soil predatory mite reproduction test. The glyphosate soil predatory mite reproduction test was conducted using glyphosate isopropylamine salt (MON 0139). Survival of mites (*H. aculeifer*) and their reproductive performance were evaluated at 4 nominal concentrations, equivalent to 50 mg MON 0139/kg dry soil, 100 mg MON 0139/kg dry soil, 500 mg MON 0139/kg dry soil, and 1000 mg MON 0139/kg dry soil (= 23.64 mg a.e./kg dry soil, 47.28 mg a.e./kg dry soil, 236.40 mg a.e./kg dry soil, and 472.80 mg a.e./kg dry soil, respectively). A negative control with deionized water only was also included. A toxic reference test was performed in parallel using dimethoate EC400 (422.4 g/L; Perfekthion) at concentrations of 4.1 mg active ingredient (a.i.)/kg dry soil, 5.12 mg a.i./kg dry soil, 6.4 mg a.i./kg dry soil, 8.0 mg a.i./kg dry soil, and 10 mg a.i./kg dry soil. Mites were reared in the laboratory under ambient conditions on a mixture of plaster of paris, activated charcoal, and deionized water (8:1:9). Adults with no more than a 3-d age difference were used at the start of the test. No exposure of the mites to glyphosate was allowed prior to the test. Each treatment group contained 40 mites divided equally between 4 replicate vessels, with the control group comprising 8 replicates, each containing 10 mites. In addition, 2 test vessels without mites were included with each test concentration and in the control group for soil pH measurements. Glass bottles (100mL nominal volume) with screw tops were filled with 20 g (dry wt) artificial soil at the required test concentrations. Cheese mites were added as a food source to the surface of the soil, and vessels were then covered to prevent mites from escaping. Bottles were opened every second day during the 14-d test for the addition of food and to allow aeration. At the end of the

test (day 14), the parental mites and juveniles were counted, after extraction using a MacFayden high-gradient extractor (heat/light extraction method). This was achieved by adding the soil substrate from each test vessel into a canister placed inverted onto the extraction system. Soil substrate was retained within the canister using a plastic net (2mm mesh size) on the bottom. Beneath the canister was a funnel attached to a collecting flask with 25mL of a fixing liquid. A temperature gradient was created between the upper and the lower parts of the system, by circulating heated air in the canister area and cooled air in the collection area. Over the 48-h extraction time, the following regime was applied: 25 °C for 12 h, 35 °C for 12 h, and 45 °C for 24 h. During this time, adults and juveniles moved down through the soil away from the heat source and fell through the funnel into the fixing liquid. Extraction efficiency was determined to be 95% in a separate extraction using vessels containing a known number of juvenile and adult mites in untreated substrate. Water content and pH were determined at test start and end. Statistical analysis was performed with the software ToxRat Professional 2.10. A 1-sided Fisher exact binomial test with Bonferroni-Holm correction for mortality and a 1-sided Dunnett multiple comparisons test for reproduction ($\alpha = 0.05$) were used to compare the control with independent test item groups. Abbott's formula was used to correct for control mortality.

AMPA - Soil predatory mite reproduction test. The soil predatory mite reproduction test with AMPA was conducted at 5 nominal application rates, equivalent to 40 mg test item/kg dry soil, 80 mg test item/kg dry soil, 160 mg test item/kg dry soil, 240 mg test item/kg dry soil, and 320 mg test item/kg dry soil. A negative control (deionized water only) group was also included. All procedures and observations in the test with AMPA were as described for the mite (OECD 226) test with glyphosate in Glyphosate—Soil predatory mite reproduction test. A reference test was performed with dimethoate EC400 (414.8 g/L) at test concentrations of 0 mg a.i./kg dry soil, 4.1 mg a.i./kg dry soil, 5.12 mg a.i./kg dry soil, 6.4 mg a.i./kg dry soil, 8.0 mg a.i./kg dry soil, and 10 mg a.i./kg dry soil.

Springtail reproduction tests

The springtail reproduction tests for glyphosate and AMPA were both conducted according to OECD guideline 232. The procedures used in the 2 studies were identical. Full details of the procedures are presented for glyphosate only. Springtails used in these studies were originally purchased from Biologische Bundesanstalt in May 2000 and reared in the laboratory of the test facility under ambient laboratory conditions.

Glyphosate - Springtail reproduction test. The springtail reproduction test conducted for glyphosate was conducted using glyphosate isopropylamine salt. Survival of springtails (*Folsomia candida*) and their reproductive performance were evaluated at 5 nominal application rates of 32 µL MON 0139/kg dry soil, 50 µL MON 0139/kg dry soil, 100 µL MON 0139/kg dry soil, 500 µL MON 0139/kg dry soil, and 1000 µL MON 0139/kg dry soil (= 15.1 mg a.e./kg dry soil, 23.6 mg a.e./kg dry soil, 47.3 mg a.e./kg dry soil, 236.4 mg a.e./kg dry soil, and 472.8 mg a.e./kg dry soil, respectively). A negative control with deionized water only was also included. In a reference toxicity test with Betosip (15.7% phenmedipham), concentrations of 50 mg/kg dry soil, 100 mg/kg dry soil, 200 mg/kg dry soil, and 400 mg/kg dry soil were tested. Each treatment group, including the control group, comprised 50 mites divided equally between 5 replicate vessels. For each treatment group and for the control group, 2 test vessels without springtails were provided for pH measurement purposes. Glass containers (150mL nominal volume) were filled with 30 g (wet wt) of the required treated or control soil. Springtails were reared in the laboratory under ambient conditions on a mixture of plaster for stucco, activated charcoal, and water (8:1:9). No exposure to the test item was allowed prior to testing. Juvenile springtails, 10 d to 12 d old and from a synchronized cohort, were added to each test vessel and then covered with a glass lid for 28 d, following which the surviving adults and juveniles were counted. Water content and pH were determined at test start and end. Adult and juvenile springtails were counted at test end. Statistical analysis was performed with the software ToxRat Professional 2.10. A 1-sided Fisher exact binomial test with Bonferroni correction ($\alpha = 0.05$) and Welch's t test ($\alpha = 0.05$), because of non-heterogeneity of variance, were used to compare the control with the independent test item groups for significance of parental mortality and reproductive reduction, respectively. Abbott's formula was used to correct for control mortality.

AMPA = Springtail reproduction test. The springtail reproductive test for AMPA was conducted with AMPA (98.7% purity) mixed into artificial soil at 5 nominal application rates, equivalent to 30 mg/kg dry soil, 54 mg/kg dry soil, 97.2 mg/kg dry soil, 175 mg/kg dry soil, and 315 mg/kg dry soil. The negative control used deionized water only. In a separate toxic reference test with 100% crystalline boric acid (BDH Prolabo) mixed with the soil, also included in the test design, the sensitivity of the population was determined with test concentrations of 0 mg/kg dry soil, 44 mg/kg dry soil, 67 mg/kg dry soil, 97.2 mg/kg dry soil, 150 mg/kg dry soil, and 225 mg/kg dry soil. The procedures used during the Springtail reproduction study were essentially equivalent to those used in the springtail test with glyphosate (described in Glyphosate - Springtail reproduction test) with the following exceptions. Each treatment group comprised 40 springtails (10 per test vessel), whereas the control group comprised 8 replicates. Statistical evaluation was performed with ToxRat Professional 2.10. A 1-sided Fisher exact binomial test with Bonferroni correction and a 1-sided Dunnett test were used to compare the control with independent test item groups. Mortality of adult springtails

Soil nitrogen-transformation tests

Soil nitrogen-transformation tests were conducted with glyphosate and AMPA according to OECD guideline 216 and performed according to good laboratory practice. The procedures used in the 2 tests were identical, although tested rates differed. Full details of procedures used are presented for glyphosate only.

Glyphosate - Soil nitrogen-transformation test. The soil nitrogen-transformation test for glyphosate was conducted using glyphosate acid (96.59% purity; Monsanto Europe) applied at 2 soil concentrations, 6.62 mg a.e./kg dry soil and 33.1 mg a.e./kg dry soil. The tested rates were equivalent to 1 and 5 times the maximum predicted environmental concentration in soil following a worst-case application of glyphosate to bare soil in the EU. Each treatment group and the control comprised 3 replicate test vessels. The control was treated with water only. Field-collected soil was used (LUFA standard soil, type 2.3). On collection, the soil was manually cleared of large objects, such as stones and parts of plants, and then moist-sieved to a particle size ≤ 2 mm. The soil was stored under aerobic conditions in the dark at 4 ± 2 °C until required for use.

Glyphosate was prepared in deionized water and then mixed into a bulk sample of soil at the start of the test. The soil moisture content was 40% (± 5%) of the maximum water holding capacity. During the test, the weight of a moisture control vessel maintained under the same test conditions was used as a guide to correct for test vessel water loss. Control and treated bulk samples of soil were amended with ground lucerne meal (0.5%) as a nitrogen source with a C to N ratio of 16:4:1. Bulk samples were then subsampled (~500 g) into replicate vessels and incubated at 20 ± 2 °C for 28 d. All containers were covered with a perforated lid to avoid evaporative water loss and stored in the dark. Soil (10 g) was taken from 1 replicate from each treatment for pH (water) determination at the start and end of the Glyphosate - Soil nitrogen-transformation study. An additional soil sample was taken from 1 replicate per treatment for moisture and dry matter content determination at the end of the study. As soon as possible after dosing (day 0) and after 7 d, 14 d, and 28 d, a 50-g soil sample (based on dry wt) was removed from each replicate to determine NH_4^+, NO_2^-, and NO^3. Soil extracts were prepared by adding 250mL of 2 M KCl, then shaking for 2 h and centrifuging for 15 min. The supernatant was analyzed using a Bran+Luebbe Autoanalyzer AA3 system. Effects below 25% deviation from control were not considered to be biologically significant.

AMPA - Soil nitrogen-transformation test. In the soil nitrogen-transformation test conducted for AMPA, the bulk samples of field-sampled soil were prepared at AMPA (98.7% purity) soil concentrations of 40 mg/kg dry soil, 80 mg/kg dry soil, 160 mg/kg dry soil, 320 mg/kg dry soil, and 640 mg/kg dry soil. In addition, a negative control (nontreated soil) was tested. Although conducting reference tests and use of positive controls are not guideline requirements, in a separate reference test with dinoterb (2-tert-butyl-4,6-dinitrophenol, 99.9% purity; Sigma-Aldrich Chemie), test concentrations of 6.8 mg/kg dry soil, 16 mg/kg dry soil, and 27 mg/kg dry soil were applied, in addition to the control (0 mg/kg dry soil), with 3 replicates per treatment group.

Results

Glyphosate - Earthworm reproduction test

There was 0% mortality of adult *E. fetida* at glyphosate concentrations of 14.18 mg a.e./kg dry soil, 236.4 mg a.e./kg dry soil, and 472.8 mg a.e./kg dry soil. Mortality (2.5%) was observed at 23.64 mg a.e./kg dry soil and 47.28 mg a.e./kg dry soil, which is considered incidental background mortality given the 10% validity criterion for adult mortality in the control ($p > 0.3$). No statistically significant differences were detected for adult biomass ($p > 0.05$; Figure 1A) and for the numbers of juveniles produced at each of the treatment groups when compared to the control ($p > 0.05$; Figure 1A). Adult and juvenile feeding behavior was also not adversely affected over the duration of the test (56 d). The resulting no-observed-effect concentration (NOEC) for effects on reproduction was determined therefore to be the maximum test concentration of 472.8 mg a.e./kg dry soil.

In the reference test with carbendazim, juveniles were reduced by 65% and 92% at 5 mg reference item/kg dry soil and 10 mg reference item/kg dry soil, respectively. The control treatment had a mean number of 143 juveniles, whereas 5 mg/kg dry soil and 10 mg/kg dry soil treatments with carbendazim had a mean number of 51 juveniles and 11 juveniles, respectively. These reference test values show that the test system was appropriate to detect toxic effects on earthworm reproduction. The validity criteria, namely adult mortality < 20% and number of juveniles per replicate > 30 in the control treatment, and coefficient of variance between control replicates < 30% were all met. The guideline requirements for water content, temperature, and pH were all met.

AMPA - Earthworm reproduction test

In the earthworm reproduction study with AMPA, there were no significant effects on *E. fetida* adult mortality across concentrations compared to the control ($p > 0.22$). In all treatment groups, all 10 adults survived the treatments, except for 1 mortality in a single replicate of the 668.5 mg/kg dry soil treatment (Figure 1B). Adult earthworm biomass was significantly lower compared to the control at the 445.5 mg AMPA/kg dry soil, 668.5 mg AMPA/kg dry soil, and 1002.5 mg AMPA/kg dry soil test concentrations ($p < 0.0001$; Figure 1B). Adult biomass at 198.1 mg AMPA/kg dry soil was also significantly lower than the control ($p = 0.007$), but at 297.1 mg AMPA/kg dry soil there was no significant difference ($p > 0.802$) because the biomass (in percentage of control) was 88.5% and 88.2% in the 131.9 mg AMPA/kg dry soil and the 297.1 mg AMPA/kg dry soil treatment groups, respectively. The effect at 198.1 mg AMPA/kg dry soil is therefore considered to not be treatment-related. Juvenile production was not significantly affected at concentrations up to 198.1 mg/kg dry soil ($p > 0.342$). At 297.1 mg AMPA/kg dry soil and higher concentrations juvenile *E. fetida* numbers decreased significantly compared to the control ($p = 0.0013$). The resulting NOEC for effects on reproduction therefore was concluded to be 198.1 mg/kg dry soil, with a reproductive lowest-observed effect concentration (LOEC) at 297.1 mg AMPA/kg dry soil. The calculated 50% effective concentration (EC50) value for AMPA on earthworm survival was > 1000 mg/kg dry soil. The reproduction EC50 value was calculated at 654.7 mg AMPA/kg dry soil (95% confidence interval 610.9 - 705.5 mg/kg dry soil). The resulting regression equation was $y = - 0.1108 (\pm 0.005)$ AMPA mg/kg + $122.6 (\pm 2.271)$, with an R^2 of 0.92. The reference test item carbendazim resulted in decreased biomass of 33.3% at 5.0 mg/kg dry soil and no reproduction, showing that the test system was sensitive to pesticide application. The validity criteria and guideline requirements were all met.

Glyphosate - Soil predatory mite reproduction test

No significant effects were observed on soil mite survival ($p > 0.3$) or reproduction ($p > 0.05$) up to and including the highest test concentration (472.8 mg a.e./kg dry soil; Figure 1C) after 14 d of continuous exposure. All validity criteria and guideline recommendations were met. In the reference test with dimethoate the EC50 on reproduction was determined to be 4.9 mg a.i./kg dry soil. The reference test demonstrated the sensitivity of the test system to detect reproductive toxicity in soil mites. The NOEC was therefore set at the highest test concentration.

AMPA - Soil predatory mite reproduction test

No significant effects were observed on soil mite survival ($p > 0.1$) or reproduction ($p > 0.05$) up to and including the highest test concentration (320 mg AMPA/kg dry soil; Figure 1D). All validity criteria and guideline recommendations were met. The reference test with dimethoate showed that the test was sensitive at detecting reproductive toxicity in soil mites. The NOEC for AMPA was therefore concluded to be at the highest test concentration, 320 mg/kg dry soil.

Glyphosate - Springtail reproduction test

No significant effects were observed on springtail survival ($p > 0.5$) or reproduction ($p > 0.05$) up to and including the highest test concentration (472.8 mg a.e./kg dry soil; Figure 1E). The validity criteria and guideline recommendations were all met. In the reference test with phenmedipham, the EC50 on reproduction was determined to be 28.4 mg phenmedipham/kg dry soil, which demonstrates that the test system was sensitive for reproductive toxicity. The NOEC for glyphosate was therefore concluded to be the highest test concentration.

AMPA - Springtail reproduction test

No significant effects were observed on springtail survival ($p > 0.5$) or reproduction ($p > 0.06$, $\alpha = 0.01$) up to and including the highest test concentration (315 mg AMPA/kg dry soil; Figure 1F). The validity criteria and the guideline recommendations were all met. In the reference test with boric acid, the EC50 for reproduction was determined to be 108.6 mg/kg dry soil, demonstrating sensitivity to reproductive toxicity of the test system. The NOEC for AMPA was therefore concluded to be the highest test concentration.

Glyphosate - Soil nitrogen transformation test

Nitrogen-transformation rates in the soil treated at glyphosate rates equivalent to 6.62 mg a.e./kg dry soil and 33.1 mg a.e./kg dry soil were - 0.13% and 2.13% different compared to the control between day 14 and day 28, respectively (Figure 1G). The validity criterion of < 15% variation between control treatments was met in the test. As the rates of nitrate formation between the control and the treated groups were < 25% on day 28, glyphosate can be evaluated as having no long-term influence on nitrogen transformation in LUFA soils at concentrations ≤ 33.1 mg a.e./kg dry soil. No reference test was conducted, in line with the OECD guideline.

AMPA - Soil nitrogen-transformation test

Stimulation of nitrogen-transformation rates was observed across all treatments on day 7 and day 14, which was possibly linked to the high levels of nitrogen and phosphorus released from the degradation of AMPA in the biologically active soil. Only in the 2 highest test concentrations did the increase exceed 25% compared to the control at 28 d. The test was therefore prolonged from 28 d to 56 d for the 2 highest test concentrations, 320 mg/kg dry soil and 640 mg/kg dry soil (Figure 1H). At 56 d, the deviation from the control was 26.7% at 320 mg/kg dry soil and 43.1% at 640 mg/kg dry soil. The reference test results with dinoterb showed increases of 37.6% at 6.8 mg/kg dry soil, 51.4% at 16.00 mg/kg dry soil, and 27.1% at 27 mg/kg dry soil. The validity criterion of < 15% variation between controls was met at all sampling intervals.

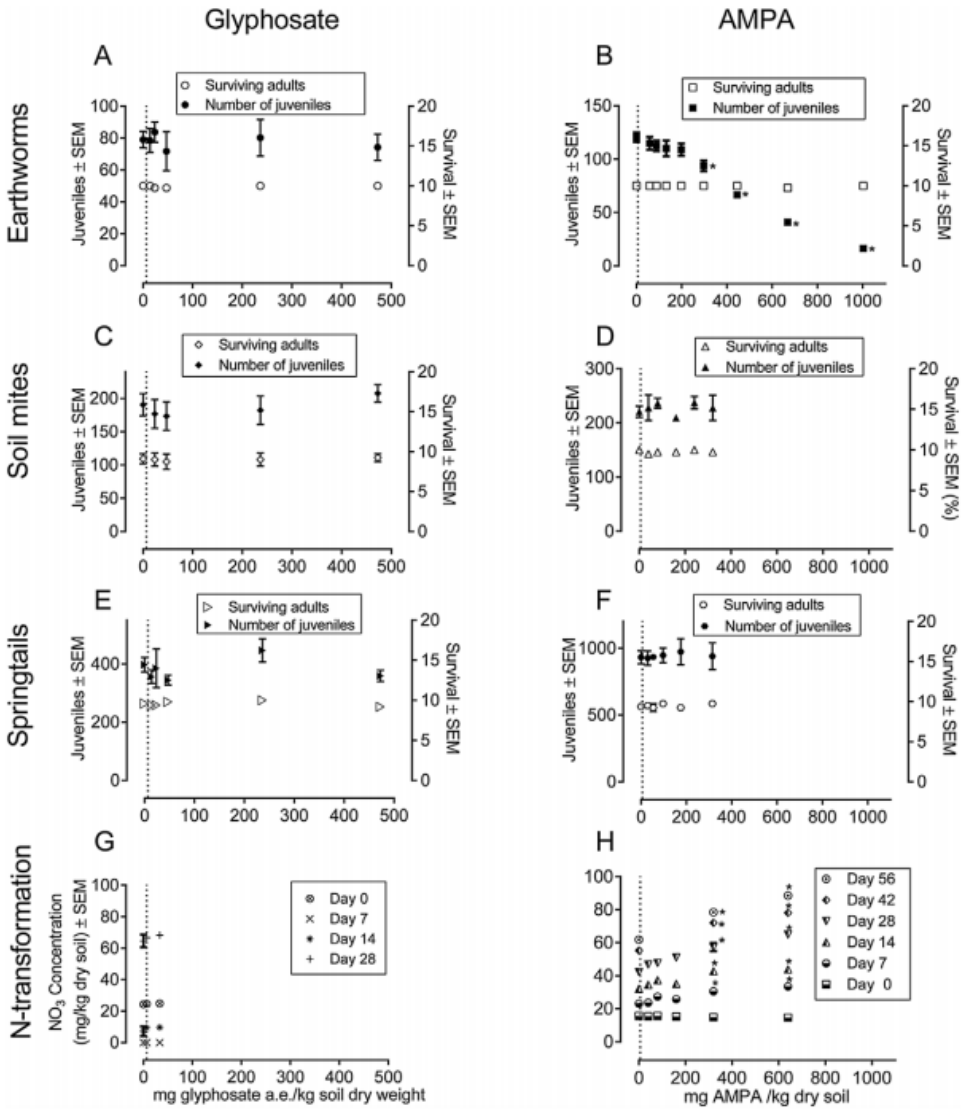


Fig. 1. Chronic risk assessment for glyphosate (left) and aminomethylphosphonic acid (AMPA; right) in soil. Number of surviving adults (28d) and number of juveniles (56d) in earthworms exposed to glyphosate (A) and AMPA (B), (* statistically significant effect [$p \leq 0.05$] compared with control treatment), in soil predatory mites (*Hypoaspis aculeifer*) exposed to glyphosate (C) and AMPA (D) for 14d, and in springtails (*Folsomia candida*) exposed to glyphosate (E) and AMPA (F) for 28 d. Effects on nitrogen transformation in soil treated with glyphosate (G) and AMPA (H) for 0 d, 7 d, 14 d,28 d, 42 d, and56 d(* > 25% effect compared with control treatment). Vertical dotted line in each graph indicates the worst-case predicted environmental concentration of glyphosate/AMPA. Vertical bars indicate standard error of the mean (SEM).

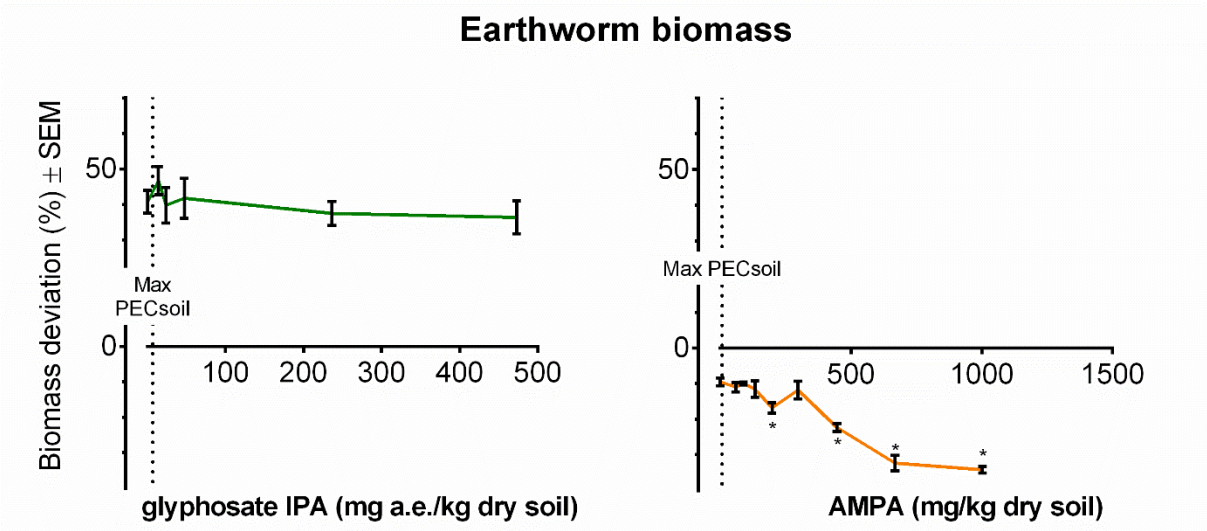


Fig. 2. Effects of glyphosate (A) and AMPA (B) on earthworm biomass after 28 d of exposure. The vertical dotted line indicates the predicted environmental concentration for AMPA and glyphosate. An asterisk next to a data point indicates a significant difference ($P < 0.05$) when compared against the control treatment.

Risk assessment

The chronic effects of exposure to glyphosate and the major soil metabolite AMPA to representative taxonomic groups of soil macroorganisms and nitrogen transformation were assessed following standard practices outlined under Annex VI Uniform Principles of the EU's Plant Protection Products Regulation (EC) No 1107/2009. At soil concentrations relevant to recommended glyphosate field application rates, no significant adverse effects were observed in any of the test species or systems exposed to glyphosate or AMPA. The risk assessment for soil macroorganisms in the EU compares the lowest NOEC achieved for each of the taxonomic groups with worst-case initial predicted soil concentrations (soil $PEC_{initial}$) achieved directly following a bare soil application and the potential for accumulation in soil following applications over multiple years to the same field (soil PEC_{accu}) The ratio of the endpoint to the predicted soil concentration is determined (toxicity exposure ratio = NOEC - $PEC_{initial}$) and compared against trigger values in accordance with Annex VI Uniform Principles of the EU's Plant Protection Products Regulation 1107/2009. Where trigger values are exceeded, a low exposure risk may be concluded. The long-term trigger value of 5 using NOECs derived from laboratory tests accounts for uncertainty related to interspecies sensitivity, predicted exposure estimates, and extrapolation from laboratory to field exposure.

For glyphosate and AMPA, the initial soil concentration ($PEC_{initial}$) at a soil depth of 5 cm has been determined based on a bare soil application (without foliar/crop interception), at the maximum cumulative annual application rate of 4.32 kg glyphosate a.e./ha for the EU. The risk of glyphosate and AMPA residues accumulating in soil over multiple years is considered by deriving the PEC_{accu} value. This is the sum of the $PEC_{initial}$ and plateau concentrations in soil, achieved in the top 5 cm (tillage depth for permanent crops) soil layer, following applications to bare soil at the maximum cumulative annual application rate (4.32 kg a.e./ha) each year for 10 yr.

It is important to mention that a single application rate of 4.32 kg glyphosate a.e./ha is not supported in the representative use rate but rather represents the recommended maximum cumulative (total) annual application rate for all uses and, therefore, a very conservative worst-case approach.

For exposure of soil mites, springtails, and earthworms to glyphosate in soil, the achieved chronic endpoints exceed the worst-case predicted glyphosate $PEC_{initial}$ and PEC_{accu} soil concentration by factors of 82 and 71, respectively.

For exposure of soil mites, springtails, and earthworms to AMPA in soil, the achieved chronic endpoints exceed worst-case AMPA $PEC_{initial}$ soil concentrations by factors of between 97 and 491, whereas the chronic endpoints exceed the PEC_{accu} soil concentrations by factors of between 32 and 162.

For soil nitrogen transformation, the endpoints achieved for glyphosate and AMPA (33.1 mg a.e./kg dry soil [glyphosate] and 160 mg a.e./kg dry soil [AMPA]) both achieved a < 25% effect on nitrogen-transformation rates following a 28-d soil exposure to either glyphosate or AMPA. These soil exposure rates exceed the worst-case predicted $PEC_{initial}$ soil concentrations by factors of 6 (glyphosate) and 78 (AMPA). The achieved endpoints also exceed the PEC_{accu} soil concentrations, by factors of 5 for glyphosate and 26 for AMPA.

For the soil mite, springtail, and earthworm reproduction chronic endpoints, the toxicity exposure ratio values exceed the EU Regulation No 546/2011 Annex VI trigger (5), indicating that for the ecotoxicologically relevant endpoints achieved for survival and reproduction, the use of glyphosate according to label recommendations is unlikely to result in adverse effects inside the treated area for soil biota - from exposure to both glyphosate and AMPA.

For the soil microbial community, relative to expected field application rates for exposure to glyphosate there is at least a 5-fold safety margin. For exposure to AMPA, a 26-fold safety margin applies. The observed increases in nitrate concentrations at the higher test concentrations are expected to be related to the large quantity of nitrogen and phosphate provided to the microbes via degradation of AMPA in the biologically active soil.

Table 1. Glyphosate and aminomethylphosphonic acid chronic risk assessment for soil organisms[a]

Test species	Test item	Test duration (d)	Endpoint type	NOEC (mg a.e. or AMPA/kg soil)	$PEC_{initial}$ (mg a.e./kg soil)	PEC_{accu} (mg a.e./kg soil)	$TER_{initial}$	TER_{accu}
Earthworm	Glyphosate IPA salt	56	Adult mortality	472.8	5.76	6.62	82	71
			Biomass	472.8			82	71
			Reproduction	472.8			82	71
	AMPA	56	Adult mortality	1002.5	2.04	6.18	491	162
			Biomass	297.1			146	48
			Reproduction	198.1			97	32
Soil mite	Glyphosate IPA salt	14	Adult mortality	472.8	5.76	6.62	82	71
			Reproduction	472.8			82	71
	AMPA	14	Adult mortality	320	2.04	6.18	157	52
			Reproduction	320			157	52
Springtail	Glyphosate IPA salt	28	Adult mortality	472.8	5.76	6.62	82	71
			Biomass	472.8			82	71
	AMPA	28	Adult mortality	315	2.04	6.18	154	51
			Biomass	315			154	51
N-transformation	Glyphosate acid	28	Effect <25%	33.1	5.76	6.62	6	5
	AMPA	28	Effect <25%	160	2.04	6.18	78	26

a.e. = acid equivalent; AMPA = aminomethylphosphonic acid; IPA = isopropylamine; NOEC = no-observed-effect concentration; PEC_{accu} = accumulative predicted environmental concentration, cumulative worst-case application of 4.32 kg a.e./ha of glyphosate for 10 yr; $PEC_{initial}$ = initial predicted environmental concentration, assuming single worst-case application of 4.32 kg a.e./ha of glyphosate; TER_{accu} = toxicity to exposure ratio (= NOEC - PEC_{accu}); $TER_{initial}$ = toxicity to exposure ratio (= NOEC - $PEC_{initial}$).

Conclusion

The risks from exposure to glyphosate and the primary soil metabolite AMPA at levels that exceed commercial application rates were evaluated against a battery of representative soil macroorganisms and microorganisms under controlled laboratory conditions. Results from the present studies demonstrate that the potential impact to beneficial soil macro-organisms and nutrient cycling soil microorganisms under environmentally relevant exposure scenarios is low.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The aim of the paper was to evaluate potential effects of Glyphosate, Glyphosate salt and AMPA on earthworm, soil mites, springtails and soil micro-organisms.

The studies have been conducted according to recognised guidelines and validity criteria were presented. Test substance information, test organism origin, study designs and toxicity effects were adequately described. The study is considered reliable.

グリホサートカリウム塩

要旨及び評価結果

（農作物及び畜産物への残留）

検索期間：2010 年 1 月 1 日～2019 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point	CA 6.10.1
Report author	Berg C.J. et al.
Report year	2018
Report title	Glyphosate residue concentrations in honey attributed through geospatial analysis to proximity of large-scale agriculture and transfer off-site by bees
Document No.	PLoS ONE 13(7): e0198876
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Executive Summary

Honey taken directly from 59 bee hives on the Hawaiian island of Kaua‘i was analyzed for glyphosate residue using ELISA techniques. Glyphosate residue was detected (> LOQ) in 27% of honey samples, at concentrations up to 342 ppb, with a mean = 118 ppb, S.E.M. 24 ppb. Of 15 honey samples store-purchased on Kaua‘i, glyphosate was detected in 33%, with a mean concentration of 41 ppb, S.E.M. 14. Glyphosate residue was not detected in two samples from the island of Molokai but was in one of four samples from the island of Hawai‘i. Presence and concentration of glyphosate residues were geospatially mapped with respect to Hawaiian land divisions. Mapping showed higher occurrence of glyphosate that was over LOQ (48%) and concentrations of glyphosate (mean = 125 ppb, S.E.M. 25 ppb; N = 15) in honey from the western, predominantly agricultural, half of Kaua‘i versus the eastern half (4%, mean = 15 ppb; N = 1). Geographic Information System analysis of land use percentage was performed within a circular zone of 1 km radius around each hive. Various land use types within each circular zone were transcribed into polygons and percent land use calculated. Only agriculture land use showed a strong positive correlation with glyphosate concentration. High glyphosate concentrations were also detected when extensive golf courses and/or highways were nearby. This suggests herbicide migration from the site of use into other areas by bees. Best management practices in use for curtailing pesticide migration are not effective and must be carefully re-assessed.

Materials and Methods

Sample collection

Honey samples were collected directly from hives by beekeepers on the island of Kaua‘i in three batches from 2013 through 2016 (**Table 1**). Samples were opportunistically obtained from all accessible parts of the island. Collections were constrained by lack of bee hives in the area or beekeepers’ unwillingness to provide samples. A strict confidentiality agreement was needed to get participation in the study. For some sites, sample batches were collected over time, to increase sample size. The timing was irrespective of seasonality of honey production by the bees. Each sample came from a single unique hive and its location was precisely recorded. Two other batches of honey were obtained from merchants and comprised honey from many hives under control of the manufacturing company.

In the fall of 2013 (Batch 1) two honey samples were collected by beekeepers, by scraping the honey comb with the open mouth of a clean glass mason jars and sealing the jars. These samples were stored at room temperature in a closed box, in a cabinet, until shipment to Microbe Inotech Laboratories, Inc., St. Louis, MO, for analysis of glyphosate concentration.

During the spring of 2015 (Batch 2) 36 samples of honey were collected directly from their unique hives by beekeepers of Kaua‘i, using only the certified pre-cleaned 40 ml amber borosilicate glass vials

provided to collect and store the honey. Vials were immediately sealed under a signed and dated custody seal by the collector and delivered directly to one of the authors (CJB, RK), along with a signed confidentiality statement containing contact information, date of collection, and hive location. Samples were stored at room temperature in a closed box, in a cabinet until shipment for analysis.

In fall of 2016 (Batch #3) 21 samples were collected by beekeepers and delivered to one of the authors (CJB), under the same procedures and stored for shipment as Batch #2.

In the winter of 2016 (Batch #4) 23 samples of honey were purchased from local famers' markets, produce stands, and stores. Honey was decanted into glass vials, sealed, and stored as above. Commercially produced honey is a composite from many hives. Source location was broadly determined from the label or from discussion with merchants. Date of honey collection is unknown. Samples were sent to Abraxis Inc, laboratory for analysis.

Batch #5 comprises three honey samples. Two samples were from the island of Molokai. One was purchased at a store on Molokai and the other was obtained from the beekeeper's bottled supplies. Both samples were a composite from hives at each beekeepers' farm. The farms' hives, which were located on Google Earth Pro™, were widely separated and thus represented different bee foraging sites. The third sample was purchased at a Kauai store and the source locality identified as from the island of Hawaii by its label.

Table 1: Honey collection data and laboratory where glyphosate was analyzed by ELISA.

Batch Number	Sample ID	Date Collected	ELISA analysis location
Hive Samples			
Batch #1	37, 38	Fall 2013	Micro Inotech Lab.
Batch #2	1 to 36	Summer 2015	Surfrider Lab.
Batch #3	39 to 59	Fall 2016	Surfrider Lab.
Merchant Samples			
Batch #4	91 to 23	Winter 2016	Abraxis Lab.
Batch #5	60, 61, 62	Winter 2016	Surfrider Lab.

Sample analysis

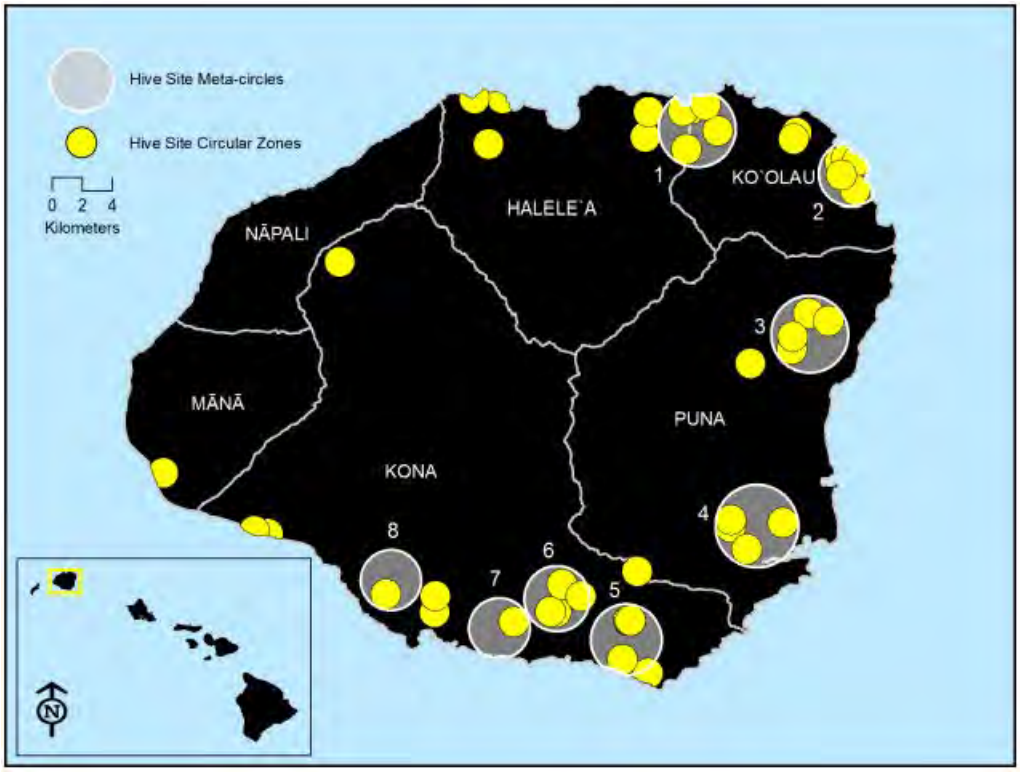
ELISA analysis was performed at each laboratory using the Abraxis method [1]. Abraxis test kit (cat. #500086) and microtiter equipment were used. The sample preparation method for honey followed published procedures [1, 17] (S1 Appendix). Samples were processed and read with a microplate reader Model 4303 [18] from Abraxis Inc. and analyzed using Molecular Devices Soft max pro evaluation program (4-Parameter). Results from Surfrider laboratory analysis were certified correct by Abraxis staff. Limit of quantitation (LOQ) was 15 ng/mL (15 ppb). Samples are stated as having detectable levels of glyphosate only if they are > LOQ.

Abraxis' ELISA methods for analysis of glyphosate have been compared to standard liquid chromatography and tandem mass spectrometry methods but not for honey. Therefore, 14 samples from Batch #2 were analyzed by both methods for validation. The results closely correlated with $R^2 = 0.99$ (S2 Appendix). Only ELISA derived data were used in this study.

Geospatial analysis

Presence and concentration of glyphosate residues were geospatially mapped with respect to general geography of the island and land use. Ancient Hawaiian biogeographical and management land divisions (Moku) (**Figure 1**) [19] were identified using the Google Earth Pro™ (GEP).

Figure 1: Distribution of 1 km radius circular zones (yellow) around hives on island of Kaua'i. Meta-circles of grouped circular zones are shaded in grey and numbered (N = 8). Moku divisions are indicated by white lines and each Moku is named.



Circular zones

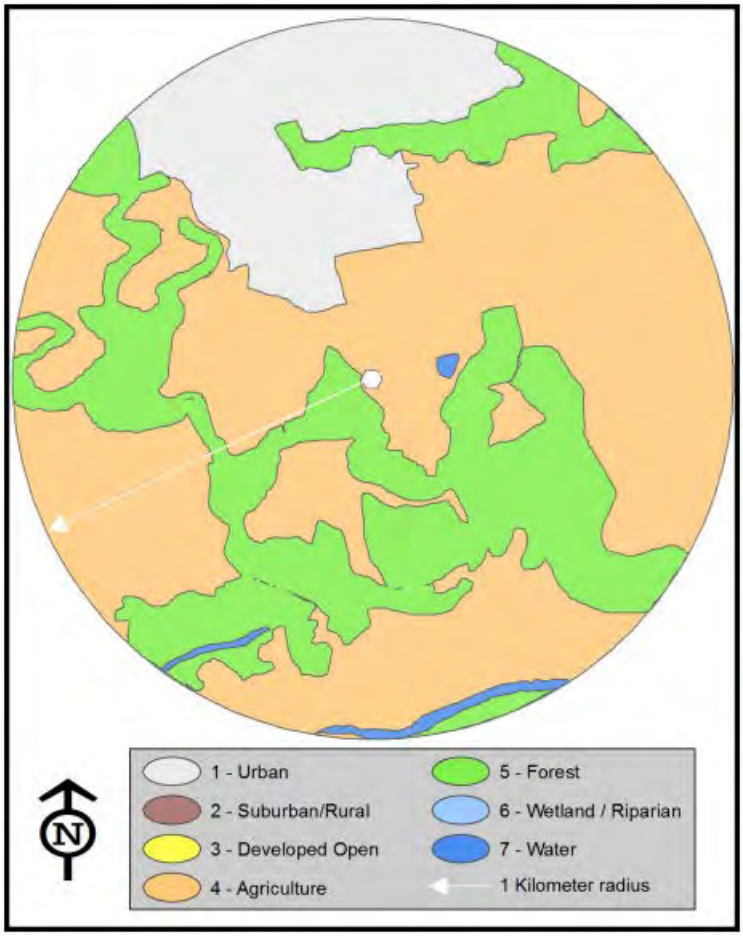
Bees have been reported to forage as far as 9.5 Km from the hive [20,21] with a mean distance closer to 1 km at times subject to patchiness of flowering resources [21]. Depending upon resource availability, the probability of plant visitation decreased non-linearly from the hive and > 85% probability of visitation was at less than 1 km [22]. Beekeepers note that bees forage as close to the hive as possible [23], especially on Kaua'i where naturally occurring plants and crops bloom year-round. Foraging on Kauai may also be constrained by discrete watersheds, bounded by mountainous ridges.

Based on this information, and to avoid overlapping of foraging sites, a 1 km radius was used to define the bees' foraging zone around each hive. Geospatial information analysis was applied using the GEP program with Digital Globe™ (DG) images to delineate circular zones 1 km in radius around each hive (**Figure 1**).

The land area within each circular zone was further sub-divided into discrete polygons, based upon land cover designations derived from NOAA C-CAP twenty-one classifications [24] (**Table 2**). Habitat codes were reclassified to seven land use categories.

Individual polygons were delineated in GEP using an optical mouse and area covered was calculated. The land area of each habitat type was then summed to provide a measure of the total land area (m^2) in each land use polygon (**Figure 2**). Each circular zone comprised 314.16 hectares, unless ocean area was excluded. A total of 18,872 hectares of land area were processed using the latest GEP images (years 2013–2014) and knowledge of current land use. Visual ground truthing was performed on sites known to differ from GEP images.

Figure 2: Circular zone around a central hive, drawn with 1 Km radius. Polygons represent different land uses categories. Site #16 provided as an illustration.



The percent of the current land use was calculated for each habitat type represented by the polygons within the hive sites' circular zones. These percentages were then correlated with the concentration of glyphosate residue from the hive in the circular zone. One hive (#48, Mānā Moku) was excluded from polygon land use calculations, as it had been moved among sites within the Moku.

A second independent geospatial analytical method for land use categorization used the NOAA Coastal–Change Analysis Program (C-CAP) [24] and ArcGIS Version 10.5 [25] (S3 Appendix). It derived area (m^2) within the 1 km radius circular zones using a program that automatically identified different types of ground cover (**Table 2**). A comparison of the two methods for accuracy in determining current land use patterns showed GEP preferable, so it was used in this study (S3 Appendix).

Table 2: Land use NOAA C-CAP classification descriptions.

This Study	Land use category	C-CAP	Land use classifications	Description of ground cover
		1	Unclassified	
1	Urban	2	High Developed	heavily built-up urban centers as well as large constructed surfaces in suburban and rural areas. Large buildings
		3	Medium Developed	constructed surface mixed with substantial amounts of vegetated surface. Small buildings
2	Suburban/Rural	4	Low Developed	class 3, with the addition of streets and roads with associated trees and grasses
3	Developed Open	5	Developed Open	parks, lawns, athletic fields, golf courses, and natural grasses occurring around airports and industrial sites
4	Agriculture	6	Orchard	herbaceous (cropland) and woody (e.g., orchards, nurseries, and vineyards) cultivated lands
		7	Pasture land	grasses, legumes or grass-legume mixtures planted for livestock grazing or the production of seed or hay crops
		8	Grassland	Grassland: grasses and non-grasses (forbs) that are not fertilized, cut, tilled, or planted regularly
		20	Bare land	bare soil, rock, sand, silt, gravel, or other earthen material with little or no vegetation
5	Forest	9	Deciduous forest	Deciduous Forest areas dominated by single stemmed, woody vegetation
		10	Evergreen forest	67 percent of the trees remain green throughout the year. Both coniferous and broad-leaved
		11	Mixed Forest	areas in which both evergreen and deciduous trees are growing and neither predominate
		12	Scrub/shrubs	woody vegetation: true shrubs,young trees, and trees or shrubs that are small
6	Wetland/ Riparian	13	Palustrine Forested Wetland	non-tidal wetlands dominated by woody vegetation >5m
		14	Palustrine Scrub/Shrub Wetland	non-tidal wetlands dominated by woody vegetation less than or equal to 5 meters
		15	Palustrine Emergent Wetland	non-tidal wetlands dominated by persistent emergents, emergent mosses, or lichens
		16	Estuarine Forest Wetland	tidal wetlands dominated by woody vegetation >5m, salinity >0.5ppt
		17	Estuarine Scrub/Shrub Wetland	tidal wetlands dominated by woody vegetation <5m, salinity >0.5ppt
		18	Estuarine Emergent Wetland	erect, rooted, herbaceous hydrophytes. Perennial plants usually dominate these wetlands
7	Water	19	Unconsolidated Shore	substrates lacking vegetation: beaches, bars, and flats
		21	Open water	open water with less than 25 percent cover of vegetation or soil.

Meta-circles

Analysis was done to determine if non-glyphosate using areas (e.g. containing forest, water, organic farms and residential) could be differentiated from areas of higher glyphosate use, as determined by conversations with the beekeepers. Eight meta-circles were made, comprising multiple 1-km circular zones that were grouped as having the same general land use description (**Table 2**, **Figure 1**) and situated in grouped watersheds. These meta-circles were encircled within a computer-generated circumference (mean 1707 hectares) that fully contained 3 to 9 circular zones of the same land-use practices (ranging from 1256 to 2365 hectares). In total, 41 samples were included within these eight meta-circles.

Large-scale divisions (East-West side of island, Moku)

The island of Kauai is divided by mountainous ranges and orographic rainfall in to two different biogeographical zones [16]. The drier leeward west-side of Kaua‘i comprises the Moku of Kona, Nāpali, and Mānā for approximately 73,710 hectares, 51.3% of the island’s area, while the wetter windward east-side comprises the Moku of Puna, Ko‘olau, and Halele‘a for approximately 70,049 hectares, 48.7% of the island’s area. Moku are identified by geological and biogeographic features [19] (**Figure 1**).

Statistical analysis

Data was analyzed with Microsoft Excel and Access (means, medians, S.D., S.E.M, t-tests, linear and exponential line fits). Analyse-it, a plug-in for Excel, was used for correlations and AICc line fits. TIBCO Spotfire Analyst® was used to produce the Trellis plots and non-parametric Kruskal-Wallis analysis.

Results

Island-wide

ELISA measured glyphosate concentrations in honey taken directly from the hive ranged from < LOQ to 342 ppb (**Table 3**). Sixteen (27.1%) of 59 samples had glyphosate concentrations detected over the

ELISA limit of quantitation (LOQ = 15 ppb).

Calculations of mean concentrations were done in two manners: using all sample ELISA data (N = 59, mean = 33.5 ppb, standard error of the mean, S.E.M. = 9.3) or for only those samples with ELISA values greater than the LOQ (N = 16, mean = 118.3, S.E.M. = 24.0).

Spatial and temporal variations at hive sites

Six separate sites had samples taken from multiple unique hives on those sites. At two of these six sites (Samples # 52, 53; 54, 56, 58), all hives had no glyphosate detected. At three of these six sites (Samples # 18, 59; 8, 14, 20, 21; 34, 35, 36), all hives had glyphosate > LOQ. At one site (Samples # 55, 57), only one hive had detectable glyphosate (Sample # 57) (27 ppb), while the other hive had none detected.

An extremely large feral beehive sampled in 2013 had 92 ppb glyphosate (**Table 3**, Sample # 37). In 2015, this site had four samples taken from widely spaced parts of the hive (Samples 8, 14, 20 & 21). Analysis yielded values ranging from 33 ppb to 342 ppb (mean = 147.7 ppb, S.E.M. = 69.7 ppb).

Two different sites were sampled in 2015 and again in 2016. Each of these two sites had multiple hives. Both sites showed an increase in concentration levels over time (0 ppb to 27 ppb for samples 55 & 57; 25 ppb to 95 ppb for Samples 18 & 59).

Of the store-bought samples (**Table 4** and Table A in S4 Appendix), 33.3% of those from Kaua‘i had glyphosate residue > LOQ (mean = 41 ppb, S.E.M. = 14.2)

East-West side of island

Presence and concentration of glyphosate residues were mapped with respect to ancient Hawaiian biogeographical and management land divisions (Moku) [19]. When all 59 samples were analyzed, there was a higher glyphosate concentration (mean = 61.6 ppb, N = 31, S.E.M. = 16.2) (**Table 5** and Tables B and C in S4 Appendix) in honey from the leeward western half of Kaua‘I versus the windward eastern half (mean = 2.4 ppb, N = 28, S.E.M. = 0.9). Mean values between the western and eastern sides are different (t-test, p = 0.001, df = 57) (Table D in S4 Appendix).

If only glyphosate values > LOQ are used (N = 16), the western Moku had 15 samples, 48.4% of which had glyphosate > LOQ (mean = 125.1 ppb). The eastern Moku had only 1 sample over the LOQ (3.6%). This sample value (15.2 ppb) is just greater than the LOQ.

A Trellis plot was made showing the glyphosate concentration across samples, grouped by side of island and by Moku. When all 59 samples are plotted, there is a clear pattern of the higher glyphosate concentrations in the western Moku vs the eastern Moku (Figure 3). No samples were collected from the remote western Moku of Napali.

Moku

Moku differed greatly in the and mean concentration of glyphosate in honey (Table B in S4 Appendix). Puna and Ko’olau Moku had no samples >LOQ and Halele’a had only one > LOQ. No samples were collected from remote Napali and only one sample from Mana. Concentrations from the west side Kona Moku were different from the three east-side Moku ($p < 0.003$) (Table E in S4 Appendix).

Since it is not known if these samples are from a normally distributed population, a non-parametric Kruskal-Wallis test was performed. This test confirmed the above parametric tests that glyphosate distributions were different depending upon the side of the island and the Moku (p = 0.0008 and 0.004, respectively) (Table F in S4 Appendix).

Source location of honey purchased from merchants on Kaua‘i was obtained from the label and discussions with vendors. Percentage of samples with glyphosate residue > LOQ and mean concentrations of glyphosate differed among Moku sampled (**Table 6** and Table A in S4 Appendix). Area with the greatest percentage of samples with glyphosate was in the agricultural district of Kona on the west side of the island. This is the same trend seen as with the hive samples (**Figure 3**).

Table 3: Glyphosate concentration and percent of land use (by category) within the circular zones surrounding the hives.

Google Earth Polygon Land Use Classification								[Glyphosate]
Sample #	% Urban	% Suburbs	% Open	% Ag	% Forest	% Wetland	% Water	ppb
1	71.4%	1.1%	6.6%	3.1%	0.0%	17.6%	0.2%	< LOQ
2	0.0%	30.1%	0.0%	0.0%	67.6%	2.4%	0.0%	
3	0.0%	13.5%	0.0%	70.9%	15.5%	0.0%	0.0%	
4	31.1%	0.0%	9.0%	30.0%	29.7%	0.0%	0.3%	
5	22.6%	0.0%	13.3%	21.0%	42.8%	0.0%	0.3%	< LOQ
6	19.8%	0.0%	3.3%	76.9%	0.0%	0.0%	0.0%	80
7	0.0%	10.4%	66.5%	3.2%	19.8%	0.0%	0.0%	
8	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	61
9	0.0%	46.6%	23.1%	1.1%	29.2%	0.1%	0.0%	
10	0.0%	6.5%	87.5%	4.4%	0.0%	0.0%	1.6%	< LOQ
11	0.0%	0.0%	4.2%	69.7%	26.1%	0.0%	0.0%	
12	0.0%	0.0%	48.2%	19.8%	32.0%	0.0%	0.0%	15
13	0.0%	30.9%	26.6%	8.6%	34.0%	0.0%	0.0%	
14	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	342
15	15.58%	13.8%	1.4%	43.6%	23.1%	0.0%	2.6%	
16	15.3%	0.0%	0.0%	53.8%	30.1%	0.0%	0.9%	
17	0.0%	4.0%	1.8%	0.0%	94.2%	0.0%	0.0%	
18	25.4%	0.0%	74.1%	0.1%	0.0%	0.0%	0.3%	25
19	52.9%	0.0%	44.6%	2.4%	0.0%	0.0%	0.0%	< LOQ
20	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	155
21	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	33
22	0.0%	45.8%	3.0%	33.9%	13.3%	0.0%	4.1%	
23	0.0%	50.2%	14.8%	0.4%	34.6%	0.0%	0.0%	
24	0.0%	1.5%	11.2%	64.3%	23.0%	0.0%	0.0%	
25	6.8%	10.1%	57.1%	2.3%	23.5%	0.0%	0.3%	
26	0.0%	1.0%	6.7%	68.6%	23.7%	0.0%	0.0%	
27	18.9%	0.0%	50.5%	0.0%	29.4%	0.0%	1.2%	
28	0.0%	0.0%	47.5%	11.5%	41.0%	0.0%	0.0%	< LOQ
29	0.0%	14.4%	0.5%	75.0%	10.1%	0.0%	0.0%	
30	0.0%	7.0%	30.2%	8.7%	54.1%	0.0%	0.0%	
31	0.0%	30.1%	0.0%	0.0%	67.6%	2.4%	0.0%	
32	0.0%	30.2%	8.3%	1.8%	57.7%	0.0%	2.0%	
33	22.2%	5.4%	61.4%	0.0%	7.8%	3.2%	0.0%	
34	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	187
35	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	178
36	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	172
37	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	92
38	36.2%	0.0%	2.8%	61.0%	0.0%	0.0%	0.0%	78
39	20.7%	4.2%	44.3%	3.5%	27.4%	0.0%	0.0%	
40	0.0%	49.0%	0.0%	12.9%	38.1%	0.0%	0.0%	< LOQ
41	17.1%	0.3%	0.0%	58.9%	23.8%	0.0%	0.0%	60
42	0.0%	0.0%	1.4%	67.9%	30.7%	0.0%	0.0%	
43	0.9%	1.2%	81.8%	4.7%	11.4%	0.0%	0.0%	
44	0.0%	28.2%	0.0%	25.3%	44.2%	0.0%	2.3%	< LOQ
45	0.0%	3.2%	0.0%	0.0%	95.5%	0.0%	1.3%	
46	6.8%	56.2%	0.0%	0.0%	37.0%	0.0%	0.0%	< LOQ
47	0.0%	0.0%	0.0%	19.5%	80.5%	0.0%	0.0%	
48	19.7%	0.0%	10.2%	16.3%	50.2%	0.0%	3.6%	292
49	20.0%	49.6%	0.0%	0.0%	29.0%	0.0%	1.3%	
50	0.0%	1.5%	0.0%	50.4%	48.1%	0.0%	0.0%	
51	0.0%	16.4%	0.0%	0.0%	83.6%	0.0%	0.0%	
52	19.0%	4.2%	2.2%	51.6%	21.4%	0.0%	1.6%	
53	19.0%	4.2%	2.2%	51.6%	21.4%	0.0%	1.6%	
54	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
55	18.0%	4.2%	2.2%	42.6%	31.2%	0.0%	1.8%	
56	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
57	15.6%	13.8%	1.4%	43.6%	23.1%	0.0%	2.6%	27
58	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
59	25.4%	0.0%	74.1%	0.2%	0.0%	0.0%	0.3%	95

Table 4: Concentration and percentage of glyphosate detected in store-bought honey. Samples originated from three Hawaiian islands and international blends. Samples categorized as Organic or Non-Organic.

Category			Samples N	> LOQ %	> LOQ Mean ppb
Location					
	Hawaii	Island:			
		Kaua'i	15	33.3	41.0
		Hawai'i	4	25.0	16.4
		Molokai	2	0	NA
	International		5	40.0	51.5
Type					
	Organic		5	20.0	30.6
	Non-Organic		21	33.3	42.0

Table 5: Glyphosate concentration by side of island and the six Moku. All 59 sample values used. Napali Moku had no samples ("ns").

Moku	Glyphosate Mean ppb	Median	S.D.	S.E.M.	Count
Windward:					
Koolau	0	0	0	0	10
Puna	2.5	0	5.1	1.7	9
Halelea	4.1	0	6.3	2.1	9
Totals	2.41	0	4.9	0.92	28
Leeward:					
Kona	53.9	11.7	80.9	14.8	30
Mana	292.2	292.2	na	na	1
Napali	ns	na	na	na	ns
Totals	61.61	13	90.3	16.2	31

Figure 3: Glyphosate concentrations across samples by side of island and within each Moku. Mean glyphosate (ppb) is shown by the horizontal line for each Moku. Side of the island and Moku names are listed at the top of the plot. Samples from the western Moku are shown as orange triangles and eastern Moku as blue circles.

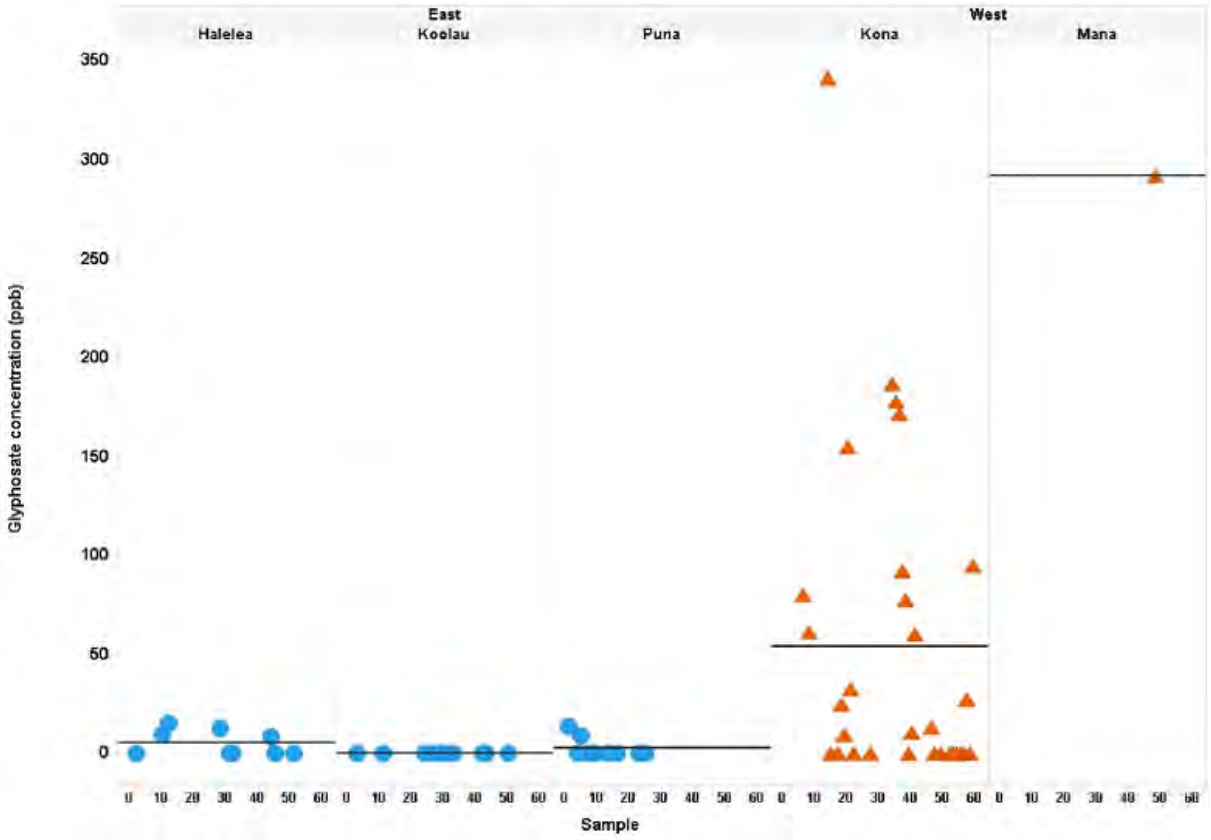


Table 6: Prevalence and concentration of glyphosate in Kauai honey from store-bought samples.

Moku	All samples N	> LOQ N	> LOQ % total	> LOQ mean	> LOQ SEM
Puna	6	1	16.7	15.0	na
Koolau	5	1	20.0	61.8	na
Kona	4	3	75.0	43.1	22.2

Circular zones and land use polygons

Land use within an area of 1 Km radius around each of the hives was determined using Google Earth Pro™ (GEP) (N = 59 hives from Kaua'i). These circular zones were divided into single land use polygons and the total meter^2 coverage for each of the seven land types was calculated. The percent of the total allocated to each of the seven land use types of each site was summarized with the glyphosate concentration found in the samples from that site (**Table 3**).

AICc analysis was performed to determine correlations between presence of glyphosate in honey and various land uses. Non-zero glyphosate data (N = 23) was used for these analyses. The exponential model for land use and glyphosate was chosen, as it has the highest correlation and strongest AICc values, compared with other line fits (Table G in S4 Appendix). Agriculture land use in the immediate 1 km radius vicinity of the hive showed the highest positive correlation with glyphosate concentration (**Table 7**, R^2 = 0.594) and the strongest AICc compared with the other land use categories. Open, Suburbs, Urban, and Forest land use all showed weak negative correlations (negative Parameter Estimates) between land use and glyphosate concentration. Wetland and Water land use showed very weak positive correlations. The negative correlations (e.g. Forest) is due to these land use types not being independent variables; rather, they are multicollinear (Figure A in S4 Appendix).

Concentration of glyphosate in honey was plotted versus the percent land use in agriculture. Samples with non-zero glyphosate were used (N = 23). **Figure 4** shows that the higher glyphosate concentrations are correlated with sites that have high percent agriculture land use (> 60% agriculture).

The hives in the western Moku (orange triangles) have a strong correlation with higher glyphosate when there is higher percent land use as agriculture. Hives in the eastern Moku (blue circles) had very low glyphosate, even with 60% to 80% of the area in agriculture (**Table 3**).

Table 7: Correlation of glyphosate concentration (ppb) in honey samples and the percent land use.

Land Use	R^2	AICc	SE of fit (RMSE)	Parameter estimate	95% CI	95% CI	SE	p-value	Exponential Equation
Agriculture	0.594	-8.664	0.784	2.552	1.594	3.511	0.461	0.000	$Y = 12.58 * 12.84^X$
Forest	0.326	2.967	1.010	-3.977	-6.572	-1.383	1.247	0.004	$Y = 65.24 * 0.01874^X$
Open	0.123	9.030	1.152	-1.465	-3.242	0.311	0.854	0.101	$Y = 50.03 * 0.231^X$
Suburbs	0.086	9.973	1.176	-2.276	-5.638	1.087	1.617	0.174	$Y = 46.98 * 0.1027^X$
Urban	0.049	10.897	1.200	-1.422	-4.274	1.430	1.371	0.311	$Y = 47.01 * 0.2412^X$
Wetlands	0.017	11.660	1.220	3.659	-9.110	16.427	6.140	0.558	$Y = 36.23 * 38.8^X$
Water	0.011	11.796	1.224	13.180	-44.017	70.377	27.504	0.637	$Y = 34.83 * 5.296e+05^X$

Meta-circles

In order to expand land use to watersheds or larger areas, meta-circle analysis was done on eight clusters of circular zones situated all around the island (**Figure 1**). They comprise similar environments. Discussions with beekeepers were used to develop a general description of each meta-circle (**Table 8**) as to predominant land use and glyphosate use.

The percent of each of the seven types of land use was calculated for each circular zone in each meta-circle (**Table 3** and Table H in S4 Appendix). Then the mean percent of each type of land use was calculated for each meta-circle. The highest percent land use was used to describe the meta-circle, if that land use type was at least 70%. If it was less than 70%, then a composite was used; the second highest type of land use was added to the highest land use type. This process was repeated until the composite land use designation comprised at least 70% of the meta-circle. This composite description is shown in

Table 8, in the column "Composite land use type".

The mean concentration of glyphosate in honey was calculated using all samples within each meta-circle (N = 48 samples total). The percentage of samples which had glyphosate > LOQ was also calculated (N = 16 total). Only three meta-circles had significant glyphosate residues and all were in areas on the western side of Kaua'i. The two meta-circles with the most glyphosate, Ag. 1 and Ag. 2, were in areas of large scale agriculture use. The Koloa meta-circle had some agricultural use and contained the circular zones with large amounts of golf courses and or highway present, as discussed below.

A Trellis plot was made to show glyphosate concentration across samples, grouped by meta-circle (**Figure 5**). Within each meta-circle, samples are plotted versus the percentage of agriculture for that sample. There is a clear pattern of the higher glyphosate concentrations for samples in the western meta-circles (orange) vs samples in the eastern meta-circles (blue). The samples with glyphosate > LOQ (triangles) are also all in the western meta-circles, while the eastern meta-circles all have glyphosate < LOQ (circles) (**Figure 5**).

Figure 4: Glyphosate concentration versus the percent land use in agriculture (N = 23). Samples from the western Moku are shown as orange triangles and eastern Moku as blue circles. Exponential fit is $Y = 12.6\ e^{12.8X}$, $R^2 = 0.594$.

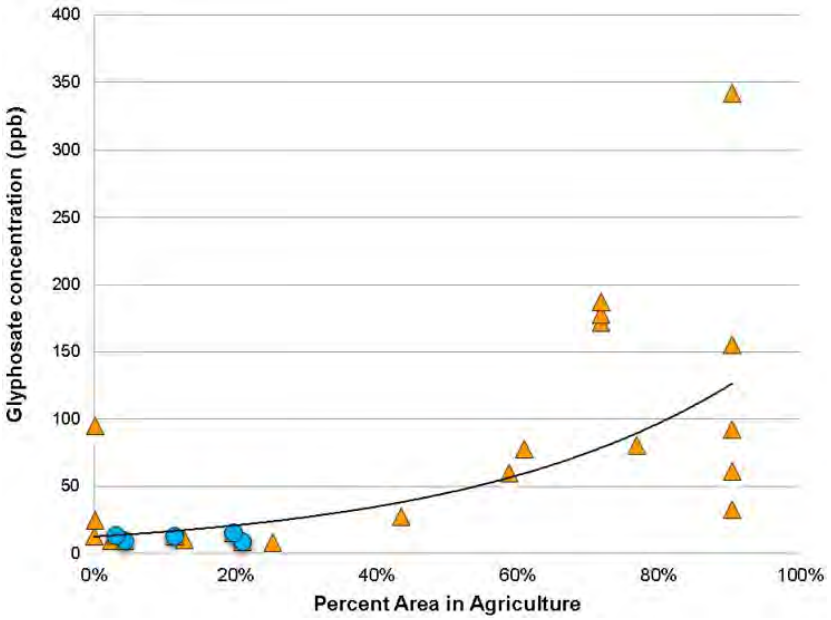
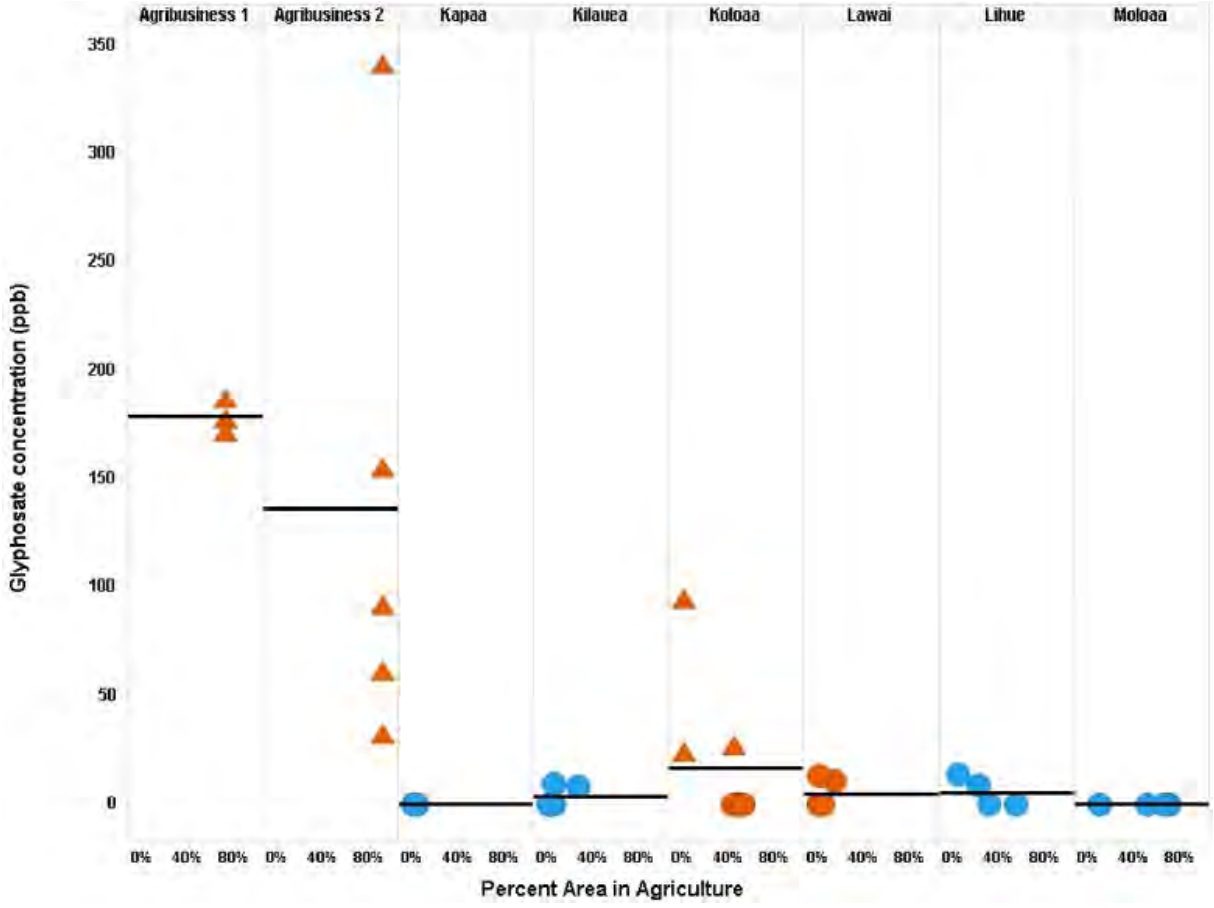


Table 8: Meta-circle composition, mean glyphosate concentration, and percent prevalence. Meta-circle # corresponds to Figure 1.

Meta-circle #	Meta-circle	Number of Circular Zones	General Description	Composite % land use	Composite land use type	Mean ppb	% > LOQ
1	Kīlauea	5	Rural, Suburbs	72.0%	Open, Forest	< LOQ	0%
2	Moloa'a	6	Organic farming	89.2%	Agriculture, Forest	< LOQ	0%
3	Kapa'a	4	Suburbs	82.0%	Open, Forest, Suburbs	< LOQ	0%
4	Līhu'e	4	Urban, open, agriculture	87.7%	Urban, Agriculture, Forest	< LOQ	0%
5	Kōloa	9	Suburbs, golf, resort	74.7%	Agriculture, urban, Forest	16.3	33%
6	Lāwa'i	5	Suburbs	82.9%	Forest, Suburbs, Open	< LOQ	0%
7	Ag. 1	3	Large scale agriculture	71.9%	Agriculture	179.0	100%
8	Ag. 2	5	Large scale agriculture	90.5%	Agriculture	136.6	100%

Figure 5: Glyphosate concentrations across samples within each meta-circle. Mean glyphosate (ppb) is shown by the horizontal line for each meta-circle. Meta-circle names are listed at the top of the plot. Samples from the western Moku are shown as orange and eastern Moku as blue. Samples with glyphosate > LOQ are shown as triangles, while those < LOQ are as circles.



Golf courses and highways

A smaller specific land use, golf course, was identified from GEP images, but was subsumed in the "Developed Open" C-CAP category (**Table 2**). There were only eight circular zones which encompassed golf course(s) and all had glyphosate residues in honey (**Table 9A**). Percent area in golf course varied from 1.2% to 16.2%. Three of those samples (samples #34, 35, 36) were from different hives on the same farm and were also associated with high percent (> 70%) agricultural land use. Two hives with the highest percent land use as golf course (samples # 18 and #59) were from the same residence with very low agricultural land use.

Major highways were identified as another small specific land use. These were subsumed under the Urban and Suburban/Rural categories (**Table 2**). Portions of highways were contained within 76% of the circular zones (Table I in S4 Appendix). Those in the top 10% of cumulative length of highway (> 4.6 km) had three samples with glyphosate > LOQ (25 to 95 ppb) (**Table 9B**). Frequent spraying of golf courses and highways may explain the presence of glyphosate (> LOQ) in samples # 18, 57, and 59.

Table 9: (A) 8 samples with highest % area Golf; (B) 6 samples with highest km highway present.

A				
Sample #	Glyphosate ppb	% Ag	% Golf	Km Highway
34	187	71.9%	1.2%	1.1
35	178	71.9%	1.2%	1.1
36	172	71.9%	1.2%	1.1
19	10	2.4%	1.6%	3.4
1	14	3.1%	4.8%	2.0
28	13	11.5%	13.7%	2.0
18	25	0.1%	16.2%	4.7
59	95	0.2%	16.2%	4.7
B				
55	0	42.6%	0.0%	4.6
57	27	43.6%	0.0%	4.6
59	95	0.2%	16.2%	4.7
18	25	0.1%	16.2%	4.7
52	0	51.6%	0.0%	4.7
53	0	51.6%	0.0%	4.7

Discussion

The presence of glyphosate residue in honey samples taken directly from the hive has been shown to correlate with areas that geospatial analysis has identified as comprised mainly of large-scale mono-crop agriculture. This suggests both a source and a pathway whereby pesticides migrate from site of use into other areas. Glyphosate residue >LOQ was found in 27.1% of the hives and 33.3% of store bought honey from Kauai, lower than the 59% in store bought honey from around the world [1]. With hive-collected honey, geospatial analysis was able to further identify: which side of the island (west), which Moku (Kona and Mana), which areas (agriculture meta-circles), and most specifically which land use (agriculture) had the greatest prevalence and greatest concentration of glyphosate in honey.

Purchased samples from the other Hawaiian islands had lower mean concentrations and a smaller percentage contaminated than those from Kauai. The mean concentration of glyphosate from international samples purchased on Kauai was 51.5 ppb, similar to the 64 ppb in Rubio [1]. Samples from Brazil and a sample from a blend of USA and Argentina approximated values reported earlier, while the blend from Brazil, Mexico and Uruguay did not [1].

One of five Kauai purchased samples (20%) labeled organic had glyphosate residues > LOQ (mean 30.6 ppb) compared to 45% (mean 50 ppb) reported elsewhere [1]. This supports supposition of some migration of pesticides from areas of application to organic farms. The twenty-one Kauai samples not labeled as organic had both a higher occurrence (33.3%) and higher mean concentration (42.0 ppb) of glyphosate than the organic labeled samples, suggesting application of glyphosate near the hives. Honey from traditional agriculture sites around the world had 62% with glyphosate > LOQ and mean 66 ppb [1], expressing widespread use of glyphosate in agriculture.

The actual process of how Kauai bees obtained, carried and processed glyphosate is not known and was not addressed in this study, but is discussed elsewhere [13,14]. As honey was obtained directly from the hive using clean vials, this eliminated the possibility of contamination occurring during processing. Each sample was unique to a single hive, not blended from various sites. A survey of beekeepers confirmed that their hives did not get sprayed with glyphosate. Uptake could have occurred if the bees themselves got sprayed while foraging, if flowers frequented by the bees contained glyphosate from either direct spraying or aerial drift, or if water that the bees drank on plants or on the ground was contaminated in some way. In all cases, contamination could have occurred at a distance from the hive. Geospatial analysis mallowed the determination that within a 1 km radius of the hive, glyphosate contamination was most closely associated with large scale agriculture. The proximity of golf courses and highways were also associated, but to a lesser degree. General land use changes and landscape composition may have indirect detrimental effect on bee fitness, although the association between pesticide and landscape composition was not investigated.

The presence of both restricted use pesticides and glyphosate in bee pollen and honey, even at very low

levels, identifies an important pathway whereby pesticides migrate from site of application to the hive and into the human food supply [12–14, 26]. Geospatial analysis can help honey producers estimate spatial pesticide exposure risks inherent in intensive agriculture. When bees are used for commercial large-scale crop pollination, hive placement can be optimized so that the bee colonies are not seriously impacted by pesticides that the bees must endure while foraging [26–27]. Linking spatial and temporal dynamics of flowering crops, agri-environmental schemes, and pesticide applications would lead to better understanding of environmental risk assessment, management of pollination services, and protecting biodiversity [26–28].

Supporting information

Supporting information with is available online:

S1 Appendix. Abraxis technical bulletin.
https://doi.org/10.1371/journal.pone.0198876.s001

S2 Appendix. ELISA verification with mass spectrometry.
https://doi.org/10.1371/journal.pone.0198876.s002

S3 Appendix. Geospatial analytical method comparison.
https://doi.org/10.1371/journal.pone.0198876.s003

S4 Appendix. Glyphosate data from Kauai hives and store-bought honey.
https://doi.org/10.1371/journal.pone.0198876.s004

This information is summarised at the end of this document.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication provides residue levels for glyphosate in honey produced in Hawaii (majority of samples) but also Argentina, Brazil, Canada, Mexico, Uruguay and USA (mainland). It is considered relevant to the setting of a suitable MRL for glyphosate in honey since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. As honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL.

The samples were analysed by means of an ELISA method which was validated indirectly by comparison with an LC-MS/MS method. A total of 14 honey samples were analysed with the two methods and the results were shown to be similar. The publication, however, does not provide validation data for the LC-MS/MS method (recovery rates from fortified samples).

The study showed a higher detection rate of glyphosate than in the EU-monitoring for 2016-2017. Besides the different origin of the samples, this may also be due to the use of different analytical methods with different LOQs. In line with the EU-monitoring the publication shows that glyphosate can occur in honey at levels > 0.05 mg/kg and that it is, therefore, appropriate to increase the existing EU-MRL. The highest measured residue level was 0.342 mg/kg, which is less than the maximum value found during the EU-monitoring for 2016-2017.

S1 Appendix. Abraxis Technical Bulletin

Glyphosate in Honey and Corn Syrup Sample Preparation

1. *Intended Use*

   For the detection of Glyphosate in honey and corn syrup.

2. *Sensitivity*

   0.015 ppm in matrix

3. *Materials and Reagents Required*

   Analytical balance
   Microcentrifuge tubes
   4 mL glass vials with Teflon-lined caps
   Disposable pipettes
   Micropipettes with disposable plastic tips
   Vortex mixer
   Microcentrifuge
   Timer
   Plate shaker or Micro-well plate holder with insert retainer for vortex mixer
   1 N Hydrochloric Acid (HCl)
   Glyphosate sample diluent
   Abraxis Glyphosate Plate ELISA Kit

4. *Notes and Precautions*

   This procedure is intended for use with honey and corn syrup (light and dark). Other matrices should be thoroughly validated before use with this procedure.

   Hydrochloric Acid must be handled with care. Wear appropriate protective clothing (gloves, glasses, etc.). Avoid contact with skin and mucous membranes. If contact occurs, wash with copious amounts of water and seek appropriate medical attention.

   Due to the viscous nature of the prepared samples, the microtiter plate should be placed on a plate shaker or vortex mixer fitted with a micro-well plate holder adapter for the incubations with the antibody and conjugate solutions. This will allow for the appropriate mixing of all reagents in the microtiter wells.

5. *Sample Preparation Procedure*

   5.1 Weigh 0.5 g of sample into an appropriately labeled microcentrifuge tube.

   5.2 Add 0.5 mL of 1 N HCl. Vortex for 2 minutes.

   5.3 Add 3.96 mL of Glyphosate Diluent to a clean, appropriately labeled 4 mL glass vial. Add 40 µL of the acid-treated sample (from step 5.2) to the Glyphosate Diluent in the vial (1:100 sample dilution). Vortex. This will then be analyzed as sample, see Derivatization of Standards, Control, and Samples in the Reagent Preparation section of the Glyphosate Plate ELISA Kit user's guide.

6. *Evaluation of Results*

   The Glyphosate concentration in the samples is determined by multiplying the ELISA results by a factor of 200.

   Samples showing a concentration lower than standard 1 (0.075 ppb) should be reported as containing < 15 ppb of Glyphosate. Samples showing a higher concentration than standard 5 (4.0 ppb) can be reported as containing > 800 ppb of Glyphosate or diluted further and re-analyzed to obtain an accurate quantitative result.

7. *Performance Data Recovery*

   Honey samples were spiked with various amounts of Glyphosate, prepared as described above,

and then derivatized and assayed using the Glyphosate Plate Assay. Average recovery was 113%.

Corn syrup samples (light and dark) were spiked with various amounts of Glyphosate, prepared as described above, and then derivatized and assayed using the Glyphosate Plate Assay. Average recovery was 104%.

S2 Appendix. ELISA verification with mass spectrometry

To verify ELISA techniques for measuring glyphosate in a honey matrix (LOQ of 15 ng/g, 15 ppb) honey remaining in 14 vials from the Batch 2 samples analysed with ELISA were sent to Quality Services International GmbH (QSI), (Bremen, Germany) for analysis of herbicide residue by gas chromatography/mass spectrometry (GC-MS/MS) and/or liquid chromatography mass spectrometry (LC-MS/MS) methods (QSI method # 88505) with a LOQ of 0.01 mg/kg (10 ppb) (Table A). All concentrations derived from ELISA were used in analysis, however QSI did not report readings for levels < 10 ppb, so a value of zero was assigned for data analysis.

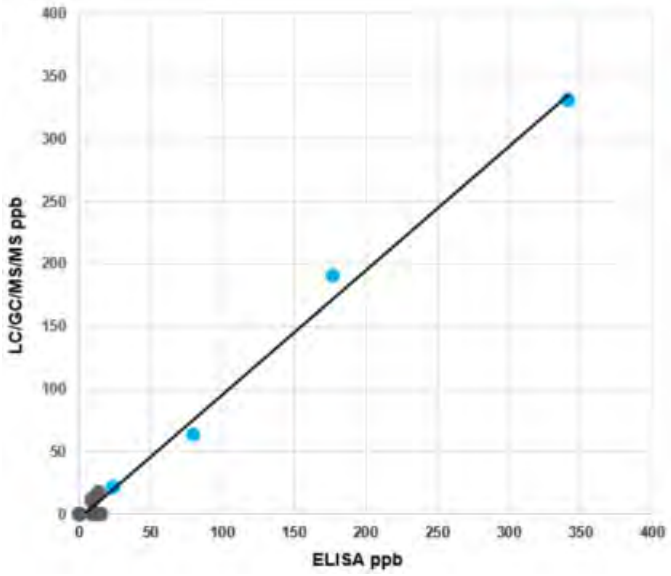
Table A: Glyphosate concentrations in honey matrix using either ELISA techniques or LC-GC-MS/MS techniques. Bold face numbers exceeded both techniques' LOQ and were plotted separately.

	Glyphosate ppb	Glyphosate ppb
Sample #	ELISA	QSI
1	13.6	17
5	8.8	10
6	**80.2**	**63**
7	0	0
10	9.2	0
12	15.2	0
14	**341.6**	**330**
16	0	0
18	**24.6**	**21**
19	9.6	12
25	0	0
27	0	0
28	12.6	0
35	**178.0**	**190**
LOQ=	15	10

Results for all 14 samples analysed by both methods correlated well (Figure A). Standard error of y for each x-value is 8.6 ppb. Only 4 samples had both the ELISA and LC/GC/MS/MS values over their respective LOQ, but the correlation coefficient remained high (Figure A).

Although sample size was small, a correlation coefficient $R^2 = 0.99$ supports the ELISA tests for accuracy, in addition to the use of blank and standards within each test run [1]. Comparison of ELISA techniques for monitoring glyphosate with chromatography-mass spectrometry have consistently found high correlations between the techniques in tests of various matrices, e.g. water [2,3], animal urine and animal tissues [4]. The use of Abraxis methods of ELISA determination of glyphosate in honey is well substantiated.

Figure A: Correlation of glyphosate concentration in honey split-samples using ELISA and LC-GC-MS/MS techniques. Linear fits: $Y = 0.99\ x - 3.1$, $R^2 = 0.993$ (N = 14; black and blue circles); $Y = 0.99\ x + 6.1$, $R^2 = 0.994$ (N = 4; blue circles).



S3 Appendix. Geospatial analytical method comparison

Development, application and comparison of two means for quantifying current land use practices within 1 km radius of a bee hive.

Geospatial analysis was performed in ArcGIS 10.5 on two separate Habitat datasets

1. Coastal Change Analysis Program (C-CAP) High Resolution Land Cover (1-4 meter resolution). Derived from high resolution imagery and analyzed according to the Coastal Change Analysis Program (C-CAP) protocol to determine land cover.
2. Vector polygons digitized in Google Earth Pro™ (GEP) using Digital Globe™ (DG) images (2013-2014) of 30-50 cm resolution as a base layer.

Coastal Change Analysis Program (C-CAP)

C-CAP analysis was conducted in January of 2016. Data downloaded was produced at a 1-4 meter resolution and utilized 35 full or partial WorldView2 multispectral scenes and the 2005 high-resolution Kauai C-CAP data set. The imagery and base classification were included in a multi-step semi-automated change detection process to extract land cover features in the 2010 imagery. Habitat within this dataset is classified into one of 21 different habitat classifications using a 2.5 meter cell size.

In order to extract out raster cells within the 2 kilometer boundary (1 Km radius) per hive site, the data set was masked using a vector dataset. This dataset was created by plotting each of the 38 hive sites in ArcGIS using their UTM location. Locations were converted into a point shapefile and then buffered by 1 km to create the 2 kilometer boundary polygon. Individual polygons were dissolved into one record to create the Mask to extract out pixels of the CCAP raster. Masking a raster using a vector is similar to the "Clip" geoprocessing routine done between two vector datasets. A vector representing an outline of the island was used to further mask the raster, removing pixels that were beyond the coastline, seemingly representing ocean (Figure A, B).

In order to quantify the percentage of habitat within each hive site boundary area (buffer a.k.a. circular zone), the raster pixels were converted into a polygon feature class (vector) for vector geoprocessing. This polygon conversion resulted in 26,176 records/polygons, representing 26,176 cells within the original Raster dataset residing in the hive site boundary area. The "Intersect" geoprocessing tool was

used next to assign to each record the corresponding hive site number it fell within. Habitat codes were reclassified, reducing the number of habitats considered by the analysis to seven land use categories. These were used in identifying the candidate habitats bees are believed to be foraging. Using the "Dissolve" geoprocessing tool, the habitat polygons were dissolved by Hive Site and reclassified Habitat Code, and the results stored in a geodatabase so that the area for each habitat could be reported using the Shape Area field. Totals for the amount of habitat polygon cells residing within each hive site boundary were then summed and the percentage for each habitat within the boundary calculated.

Figure A: ArcGIS 10.5 geoprocessing tools: Clip, Dissolve and Intersect.

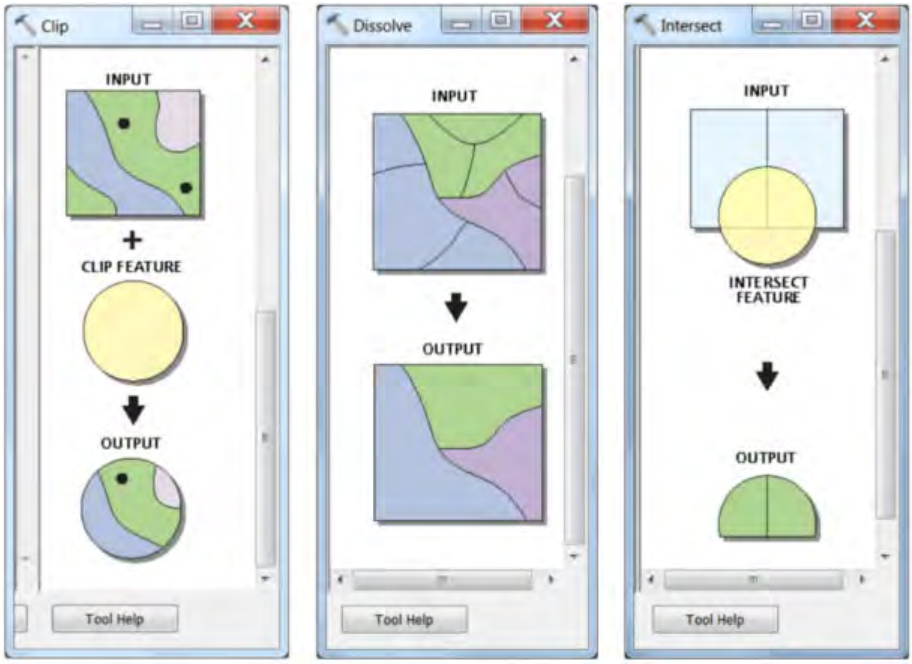
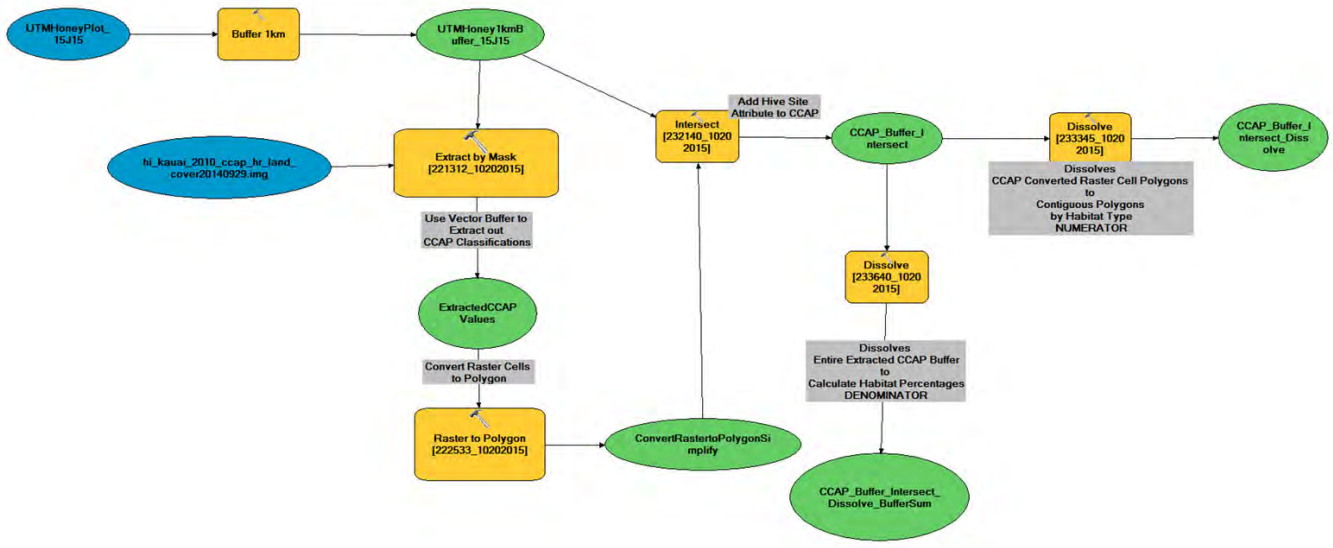


Figure B: Schematic of geoprocessing tools used to improve calculations of polygon areas for the C-CAP dataset.



Google Earth Pro™ (GEP)

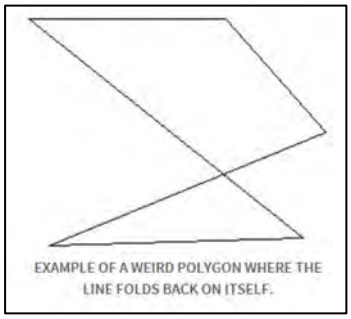
Digitizing in a Geographic Information System is the process of converting geographic data from a hard-

copy or scanned image into a vector dataset by tracing features; features are captured in coordinates and stored as either a point, line or polygon vector dataset. For this analysis, "heads up digitizing" in GEP was used to create discrete habitat polygons based on the reclassified habitats in the C-CAP analysis. Polygons created in GEP were stored as a KML/KMZ file, imported into ArcGIS 10.5 and converted into a feature class residing in a geodatabase so that areas of each habitat polygon could be calculated in square meters.

Upon importing the polygons from Google Earth, numerous topological errors were discovered in the polygons themselves, the most pervasive being knots, loops and slivers. These occur when "…the digitizer has an unsteady hand and moves the cursor or puck in such a way that the line being digitized ends up with extra vertices and/or nodes". Knots and loops result when a line forming a boundary of a polygon folds back on itself, creating small polygon like geometry known as "weird polygons".

Polygon features are enclosed areas created from a series of vertices that are connected with a continuous line traveling in one direction whereby the starting and ending point are coincident (Fig C). Because the depiction of the polygon begins with a start point and travels in one direction, the resulting geometry of the polygon means the GIS can interpret what area is 'right' as opposed to 'left' of the boundary, as well as what area is enclosed by the boundary of the entire polygon; when a knot or loop occurs, the topology of the polygon actually becomes confounded due to the extra node between them. As a result, right and left sides of the boundary violates the topological relationship of the polygon itself, preventing performance of common geoprocessing tasks (clip, intersect and dissolve).

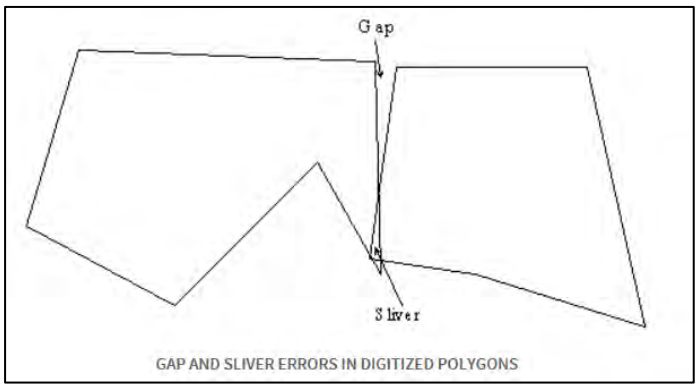
Figure C from https://www.gislounge.com/digitizing-errors-in-gis/



EXAMPLE OF A WEIRD POLYGON WHERE THE LINE FOLDS BACK ON ITSELF.

Another confounding topological error involves slivers. "Slivers are gaps in a digitized polygon layer where the adjoining polygons have gaps between them or where the two adjacent polygons overlap in error". This can inadvertently lead to areas among the polygons to have conflicting attributes as to what habitat the slivers represent (Fig D).

Figure D from https://www.gislounge.com/digitizing-errors-in-gis/



GAP AND SLIVER ERRORS IN DIGITIZED POLYGONS

Manual digitizing habitat polygons is time consuming and tedious. For this analysis, and to reduce anticipated issues related to slivers, it was decided early on in the digitizing process that the largest habitat within a circular zone could be left un-transcribed and the void filled utilizing geoprocessing tools in ArcGIS. Unanticipated topological inconsistencies related to knots and loops however prevented

these geoprocessing tools to be run and thus required that topology of all individual polygons to be inspected and corrected.

"Topology in GIS is generally defined as the spatial relationships between adjacent or neighboring features" Planar topology requires that intersections for lines and polygons in a digital data layer is enforced and that no two lines or polygons cross. This process involves removing twisted or self-intersecting polygons (i.e. knots and loops) so as to ensure that the "inside" of the polygon is on the correct side of the boundary. It also includes removing overlaps (i.e. slivers) found by intersecting each polygon with all other polygons.

Tools from ET Geowizards 11.3 were used to correct planar topology, rigorously testing and correcting for topological correctness and verifying the spatial relationships between neighboring polygons. Eight circular zone sites were chosen to validate the hand-drawn polygon designations and to determine if the process would improve calculations of polygon areas. Overestimation of the initial polygons varied by only 2.5% (n=51, t-test no significant difference in paired data p=.875)

Once the topology of the GEP dataset was reconciled, the "Intersect" geoprocessing tool was used to fill voids and assign a habitat code. The dataset was then "clipped" using an "island" polygon to remove those portions of the circular zone that extended past the coastline. Since there were multiple polygons representing a given habitat within a circular zone, the "dissolve" tool was used to consolidate records so that percent habitat calculations could be completed for each circular zone.

Total area of each habitat type for each 1 km hive site circular zone was summed and the percentage calculated (Table 2 in text). Each circular zone comprised approximately 314.16 hectares, unless ocean surface area was removed. A total of 18,872 hectares of land area was classified for the vector polygon dataset. Visual ground truthing was performed to ensure images in the GEP imagery matched images on the ground.

Comparing results between the C-CAP and GEP Datasets

C-CAP high-resolution land cover for 2010, produced at 2.4 m resolution, was applied to the 38 sites from the 2013 and 2015 sampling and compared to the same data grouped and processed using GEP polygons. For Agriculture and Urban land-cover categories, the two methods produced similar mean values, were not significantly different (t-test), and were well correlated (Table A). For Forest, Open and Water land-cover, the mean values were significantly different.

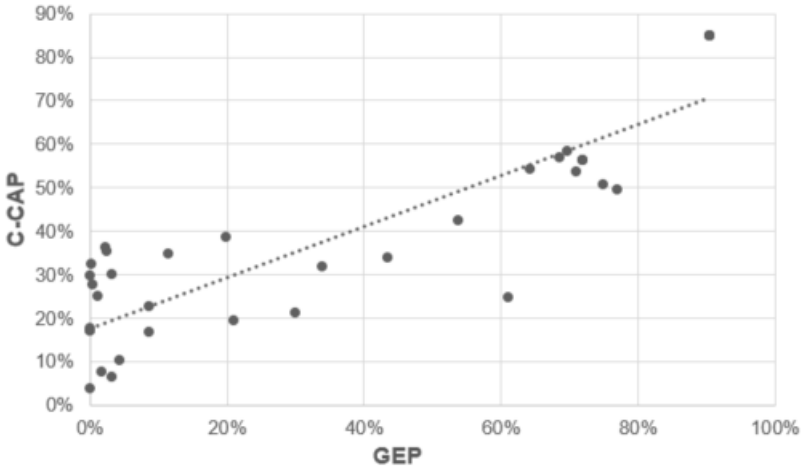
Table A: Glyphosate concentrations in honey matrix using either ELISA techniques or LC-GC-MS/MS techniques. Bold face numbers exceeded both techniques' LOQ and were plotted separately.

	C-CAP Mean	GEP Mean	t-test	correlation
% Ag	39.4%	36.9%	0.423	0.880
% Forest	36.4%	22.4%	0.000	0.836
% Open	12.4%	18.7%	0.078	0.584
% Urban	9.9%	9.6%	0.898	0.832
% Wetland	1.2%	1.2%	0.990	-0.034
% Water	1.0%	0.6%	0.041	0.532

The percentage coverage for Agriculture calculated with the C-CAP method was plotted versus the percentage coverage for Agriculture calculated with the GEP.

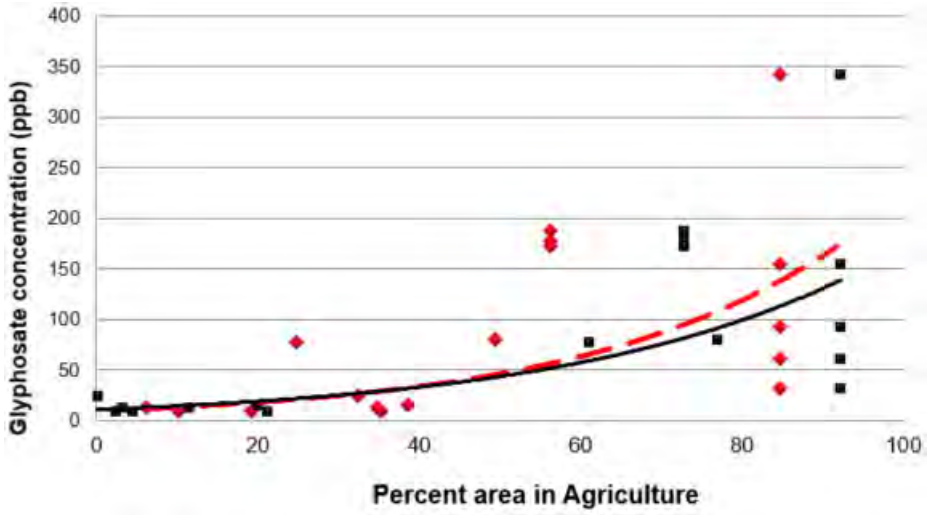
The plot illustrates the difference between GIS analyses of the two datasets and the general under-representation by C-CAP (Figure E).

Figure E: Correlation of % Agriculture in areas surrounding hive sites using C-CAP versus GEP analysis. Linear fit: Y = 0.586 x + 0.177, R^2 = 0.775.



When glyphosate concentrations are plotted against percent acreage in agriculture using the two methods (Fig F), the general trends as expressed by exponential curves are very similar, but the GEP polygon method produces a stronger correlation (R^2 = 0.71, AICc = -9.794).

Figure F: Correlation of % Agriculture and Glyphosate concentration surrounding hive sites using C-CAP and GEP analysis. Excel Analyse It software exponential fits produced Y = 9.648 $e^{0.23121x}$, R^2 = 0.48, AICc = 0.173 (C-CAP, red diamonds; dash line) and Y = 11.02 $e^{0.1628x}$, R^2 = 0.71, AICc = -9.794 (GEP Polygons, black squares; solid line).



There are many factors that would explain the differences in the land use designation and the choice of GEP polygons as the most accurate method for determining land use contemporary with honey production. These include:

- Cell size is 2.4 m for C-CAP vs Digital Globe has a 30-50 cm range. A smaller cell size allows for finer delineation and identification of objects.
- Date the image was accessed: 2010 for C-CAP but 2013-2014 for GEP with ground-truthing in areas in question.
- C-CAP would designate a ground cover as forest, but GEP showed it to be an orchard.
- C-CAP would identify open fields as "Open", but GEP showed that cattle are on it, so it is “Agriculture”.
- C-CAP does not recognize little streams or ponds but GEP resolution does.
- C-CAP sees "Forest" but Google Earth shows "Riparian"
- C-CAP see “Urban” but finer detail allows designation as “Rural/Suburban”

Conclusion

Although manually digitizing GEP polygon delineations is more tedious and time consuming, for the above stated reasons and the stronger correlation of the GEP derived curve, only the GEP polygon delineation method was used for final analysis of the relationship between land use and glyphosate concentration

S4 Appendix. Glyphosate data from Kauai hives and store-bought honey

Table A: Store-bought honey; sources and glyphosate concentration.

Sample Origin				Sample #	Glyphosate ppb
Hawaii	**Island:**	**Moku**	**Area**		
	Kauai	Kona	Waimea Valley	5	15.2
		Kona	Koloa	9	0
		Kona	Kalaheo	11	87
		Kona	Poipu	19	27.2
		Koolau	Waipake	3	5
		Koolau	North/Northeast Kauai	4	6.4
		Koolau	North Shore Kauai	6	60.8
		Koolau	Kilauea	8	0
		Koolau	Kilauea	12	11.2
		Puna	Puhi	1	15
		Puna	Hanamaulu	2	6.2
		Puna	Kapa'a	7	0
		Puna	Kapa'a	10	7
		Puna	Puhi	20	10.4
		Puna	Hanamaulu	21	6.4
	Hawaii Island		Hawaii Island	15	12
			Kealakekua, Big Island	16	7.4
			Kealakekua, Hawaii Island	60	16.4
			Big Island and Oahu	18	8
	Molokai		Molokai	61	0
			Molokai	62	0
Country		Product of Brazil and Canada		17	0
		Product of Brazil and Canada		22	30.6
		Product of Brazil and Canada		14	8.2
		Product of Mexico, Brazil and Uruguay		13	0
		Product of USA and Argentina		23	72.4

Table B: Kauai hive samples categorized by side of island and Moku with glyphosate concentration.

Side of island	Moku	Sample #	Glyphosate ppb	Count	Median	Mean	SD
WINDWARD							
	Halele'a	2	0				
		10	9.2				
		12	15.2				
		28	12.6				
		31	0				
		32	0				
		44	8.2				
		45	0				

Side of island	Moku	Sample #	Glyphosate ppb	Count	Median	Mean	SD
		51	0	9	0	5.0	6.3
	Ko'olau	3	0				
		11	0				
		24	0				
		26	0				
		29	0				
		30	0				
		33	0				
		42	0				
		43	0				
		50	0	10	0	0	0
	Puna	1	13.6				
		4	0				
		5	8.8				
		7	0				
		9	0				
		13	0				
		16	0				
		23	0				
		25	0	9	0	2.5	5.1
LEEWARD							
	Kona	6	80.2				
		8	61.4				
		14	341.6				
		15	0				
		17	0				
		18	24.6				
		19	9.6				
		20	155.2				
		21	32.6				
		22	0				
		27	0				
		34	187.2				
		35	178				
		36	171.8				
		37	92.2				
		38	77.6				
		39	0				
		40	10.4				
		41	60				
		46	13				
		47	0				
		49	0				
		52	0				
		53	0				
		54	0				
		55	0				
		56	0				
		57	27.4				
		58	0				
		59	95	30	11.7	53.9	80.9
	Mana	48	292.2	1	292.2	292.2	na
	Napali	None	None	None			

Table C: Summary statistics of glyphosate with Kauai hive samples categorized by side of island.

Windward	Count	28
	Median	0
	Mean	2.41
	SD	4.87
Leeward	Count	31
	Median	13
	Mean	61.61
	SD	90.34

Table D: t-test comparing glyphosate from Windward (Eastern) and Leeward (Western) sides of Kauai. Data from Table B1.

Windward-Leeward:	
t-test probability	0.001
degrees of freedom	57

Table E: t-test comparing glyphosate between Moku pairs. Mana Moku had only one sample, thus could not be compared.

Moku differences	**t-test p**
Kona -Koolau	0.001
Kona - Puna	0.002
Kona - Halelea	0.003
Koolau -Halelea	0.043
Puna -Koolau	0.180
Puna - Halelea	0.361

Table F: Kruskal-Wallis analysis of impact of side of island and Moku on glyphosate concentration.

Y (numerical)	**X (categories)**	**H-stat**	**DF**	**N**	**p-value**
Glyphosate	Side	11.3	1	58	0.00077
Glyphosate	Moku	13.3	3	58	0.0041

Table G: AICc analysis of fits for glyphosate concentration vs. % Agriculture.

	Exp.	**Power**	**Linear**	**Log**	**Polynomial**
R2	0.594	0.174	0.417	0.155	0.429
AICc	-8.664	7.662	194.88	195.232	197.055

Table H: Sample #'s included within Meta-circles and their glyphosate concentrations.

Meta-circle #	**Meta-circle Name**	**Sample #**	**Glyphosate ppb**	**Glyphosate ppb Mean**
1	Kilauea	10	9	
		32	0	
		33	0	
		43	0	
		44	8	3.5
2	Moloaa	11	0	
		24	0	
		26	0	
		30	0	
		42	0	
		50	0	0.0

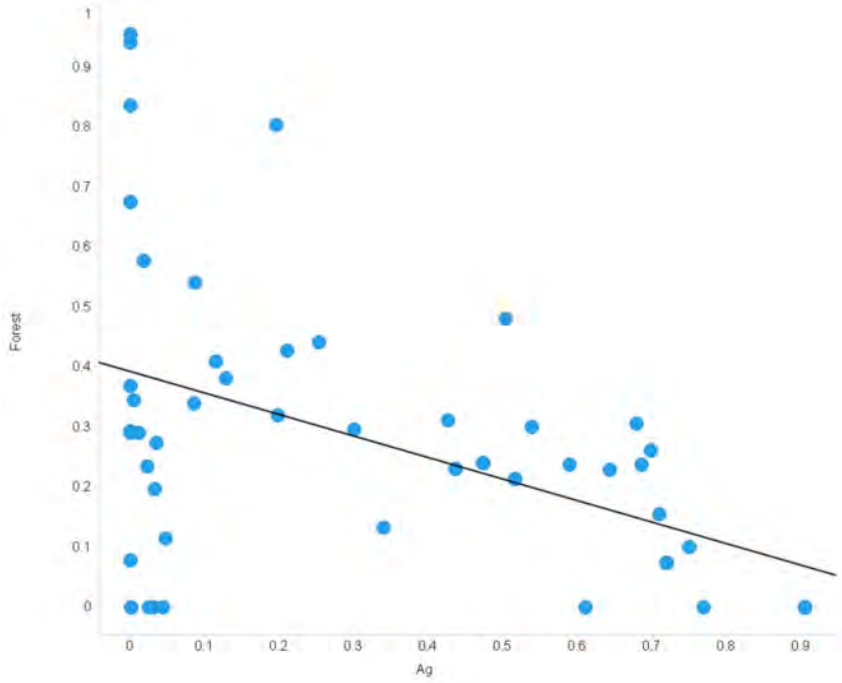
Meta-circle #	Meta-circle Name	Sample #	Glyphosate ppb	Glyphosate ppb Mean
3	Kapaa	7	0	
		9	0	
		23	0	
		25	0	0.0
4	Lihue	1	14	
		4	0	
		5	9	
		16	0	5.6
5	Koloa	15	0	
		18	25	
		52	0	
		53	0	
		54	0	
		55	0	
		56	0	
		57	27	
		59	95	16.3
6	Lawai	27	0	
		39	0	
		40	10	
		46	13	
		49	0	4.7
7	Agribusiness 1	34	187	
		35	178	
		36	172	179.0
8	Agribusiness 2	8	61	
		14	342	
		20	155	
		21	33	
		37	92	136.6

Table I: Samples by Side and Moku with % Agriculture, % Golf, Hiway Km, and Glyphosate concentrations.

Sample #	Side	Moku	Glyphosate ppb	% Agriculture	% Golf	Hiway Km
1	East	Puna	13.6	3.1%	4.8%	2.00
2	East	Halelea	0	0.0%	0.0%	2.39
3	East	Koolau	0	70.9%	0.0%	1.59
4	East	Puna	0	30.0%	0.0%	2.02
5	East	Puna	8.8	21.0%	0.0%	1.74
6	West	Kona	80.2	76.9%	0.0%	2.03
7	East	Puna	0	3.2%	0.0%	0.00
8	West	Kona	61.4	90.5%	0.0%	1.65
9	East	Puna	0	1.1%	0.0%	0.00
10	East	Halelea	9.2	4.4%	0.0%	2.04
11	East	Koolau	0	69.7%	0.0%	0.00
12	East	Halelea	15.2	19.8%	0.0%	0.00
13	East	Puna	0	8.6%	0.0%	0.00
14	West	Kona	341.6	90.5%	0.0%	1.65
15	West	Kona	0	43.6%	0.0%	4.53
16	East	Puna	0	53.8%	0.0%	0.00
17	West	Kona	0	0.0%	0.0%	0.00
18	West	Kona	24.6	0.1%	16.2%	4.66
19	West	Kona	9.6	2.4%	1.6%	3.36
20	West	Kona	155.2	90.5%	0.0%	1.65
21	West	Kona	32.6	90.5%	0.0%	1.65
22	West	Kona	0	33.9%	0.0%	1.44
23	East	Puna	0	0.4%	0.0%	0.00
24	East	Koolau	0	64.3%	0.0%	1.66
25	East	Puna	0	2.3%	0.0%	0.00
26	East	Koolau	0	68.6%	0.0%	1.10
27	West	Kona	0	0.0%	0.0%	2.29
28	East	Halelea	12.6	11.5%	13.7%	2.04
29	East	Koolau	0	75.0%	0.0%	2.03
30	East	Koolau	0	8.7%	0.0%	2.06
31	East	Halelea	0	0.0%	0.0%	2.36
32	East	Halelea	0	1.8%	0.0%	2.63
33	East	Koolau	0	0.0%	0.0%	1.98
34	West	Kona	187.2	71.9%	1.2%	1.08
35	West	Kona	178	71.9%	1.2%	1.08
36	West	Kona	171.8	71.9%	1.2%	1.08
37	West	Kona	92.2	90.5%	0.0%	1.65
38	West	Kona	77.6	61.0%	0.0%	2.18
39	West	Kona	0	3.5%	0.0%	0.32
40	West	Kona	10.4	12.9%	0.0%	0.52
41	West	Kona	60	58.9%	0.0%	0.00
42	East	Koolau	0	67.9%	0.0%	0.00
43	East	Koolau	0	4.7%	0.0%	1.51
44	East	Halelea	8.2	25.3%	0.0%	0.00
45	East	Halelea	0	0.0%	0.0%	0.00
46	West	Kona	13	0.0%	0.0%	1.20
47	West	Kona	0	19.5%	0.0%	0.00
48	West	Mana	292.2	16.3%	0.0%	0.00
49	West	Kona	0	0.0%	0.0%	2.10
50	East	Koolau	0	50.4%	0.0%	2.26
51	East	Halelea	0	0.0%	0.0%	2.25
52	West	Kona	0	51.6%	0.0%	4.73
53	West	Kona	0	51.6%	0.0%	4.73
54	West	Kona	0	47.3%	0.0%	4.35

Sample #	Side	Moku	Glyphosate ppb	% Agriculture	% Golf	Hiway Km
55	West	Kona	0	42.6%	0.0%	4.58
56	West	Kona	0	47.3%	0.0%	4.35
57	West	Kona	27.4	43.6%	0.0%	4.60
58	West	Kona	0	47.3%	0.0%	4.35
59	West	Kona	95	0.2%	16.2%	4.66

Fig A: Multicollinearity amongst land use types. Samples are plotted with their % Forest vs % Agriculture. $Y = 0.39 - 0.36*X$, $R^2 = 0.23$



1. Information on the study

Data point	KCA 6.10.1
Report author	Chiesa L.M. et al.
Report year	2019
Report title	Detection of glyphosate and its metabolites in food of animal origin based on ion-chromatography-high resolution mass spectrometry (IC-HRMS)
Document No.	Food Additives & Contaminants: Part A, 2019, Vol. 36, No. 4, 592-600
Guidelines followed in study	SANTE/11813/2017
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

Glyphosate and glufosinate are broad spectrum herbicides, widely used in agriculture and in inhabited or industrialised areas, and aminomethylphosphonic acid is a degradation product of glyphosate. In 2015, the International Agency for Research on Cancer reported that glyphosate is a probable carcinogenic. In 2017, however, a scientific opinion of the European Chemicals Agency concluded that glyphosate is not proven to be carcinogenic, mutagenic or to have negative effects on reproduction. Nevertheless, aminomethylphosphonic acid was not considered. Due to their chemical-physical characteristics, these molecules present difficulties that have not yet allowed routine monitoring to be carried out. For these reasons, we developed and validated a simple and versatile liquid extraction, before IC-HRMS analysis, of three different complex matrices: honey, bass fish and bovine muscle. Among the satisfactory validation parameters, the LOQs in the range of 4.30 – 9.26 ng/g demonstrated high method sensitivity, compared to the few works present in literature. Finally, the method was applied to real commercial samples, which showed no traces of the selected pesticides.

Materials and Methods

Chemicals and reagents

Glyphosate, glufosinate ammonium, aminomethyl-phosphonic acid (AMPA) and the internal standard Glyphosate-2-^{13}C,^{15}N were purchased from Merck (Sigma–Aldrich, Merck KGaA, Darmstadt, Germany). All solvents used were of LC-MS or analytical grade. Formic acid (98–100%) was obtained from Riedel-de Haën (Sigma–Aldrich). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

Standard solutions

Stock standard solutions (1 mg/mL) for each standard were prepared in water and kept at −20°C. Working solutions containing each of the studied analytes at a concentration of 100 ng/mL were prepared daily in methanol containing 1% of formic acid, as suggested by EU Reference Laboratory for pesticides (Anastassiades et al. 2016). Each working solution was maintained at 4°C during the method validation procedures. Plastic flasks and stoppers were used due to the fact that these pesticides tend to interact with glass surfaces.

Sample collection

Three different food matrices of animal origin were selected for the method validation: honey, fish (bass) and bovine muscle. Five commercial samples of each matrix were homogenized to create a pool to be used for the validation. After homogenization, the samples were stored at −20° C until analyses.

Ten real Italian commercial samples each of organic honey, bass and bovine muscle were also collected from different supermarkets of Milan for the application of the method.

Sample extraction

The extraction procedure was very simple and identical for the three different matrices. Homogenized samples (1 ± 0.05 g) of honey, or minced fish or bovine muscle were weighed into 15 mL polypropylene centrifuge tubes. Samples were spiked with the internal standard: 0.2 µg/g for honey and 0.4 µg/g for fish and bovine muscle samples. Three mL of methanol was added followed by 7 mL of acidified deionised water (1% formic acid). The samples were mixed for 1 min using a vortex and then sonicated for 15 min. After centrifugation (2500 × g, 4°C for 10 min), 1 mL of the supernatant was filtered through a mixed cellulose syringe filter (0.45 µm) directly into a plastic 2 mL vial, ready for determination by IC-MS/MS.

IC-HRMS orbitrap analyses

The analyses were performed by an Ionic Chromatography (IC) Dionex ICS-5000+ system (Sunnyvale, CA, USA) made up of Dual Pump (DP), a Conductivity Detector (EG), a Detector/Chromatography Module (DC) and an Autosampler (AS-AP). The ion chromatography separation column was a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a guard column Dionex IonPac AG19-4 µm (2 × 50 mm, 4 µm particle size) maintained at 30°C. The eluent flow rate was 0.30 mL/min with a gradient from 15 mM KOH (aq), held for 8 min, increased to 55 mM KOH (aq) at 20 min, held in these conditions for 4 min and back to 15 mM KOH (aq) at 24.1 min, with a cycle time of 30 min. The KOH eluent was neutralized using a Dionex AERS 500, 2 mm electrolytically regenerated suppressor (Thermo Scientific). The injection volume was 50 µL.

The detector was a Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), equipped with heated electrospray ionization (HESI) source. Capillary temperature and vaporizer temperature were set at 330°C and 280°C, while the electrospray voltage was set at 3.50 kV operating in negative mode. Sheath and auxiliary gas were set at 35 and 15 arbitrary units, with S lens RF level of 60.

Instrument calibration was done every analytical session with a direct infusion of an LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The Full Scan acquisition (FS) was combined with an Independent Data Acquisition mode (DIA), providing the MS2 spectra for confirmatory response, based on an inclusion list. The resolving power of FS was set at 70,000 Full Width at Half Maximum (FWHM). On the basis of our compound list, a scan range of m/z 50–250 was chosen; the automatic gain control (AGC) was set at 1×10^{-6} and the maximum injection time was 100 ms. The DIA segment operated in negative mode at 35,000 FWHM. The AGC target was set to 5×10^{-4}, with an auto maximum injection time. The precursor ions are filtered by the quadrupole which operates at an isolation window of 1 m/z. Fragmentation of precursors was optimised as three-stepped normalized collision energy (NCE) (10, 25 and 50 eV). Detection of analytes was based on the retention time of target compounds, on calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment. The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm glyphosate and its metabolites are reported in **Table 1**. Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system while Xcalibur™ 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HRMS system, determine the exact mass of the compounds, record and elaborate data.

Table 1: Main information (formula, retention time (tr), precursors, main products and polarity) about AMPA, glyphosate, glufosinate and the relative internal standard (IS) analysed by IC-HRMS.

Compound IC-HRMS	Formula	t_r (min)	Precursor (m/z)	Main products (m/z)			Polarity
AMPA	CH6NO3P	14.25	110.00125	62.96417	78.95904	80.97468	(-)
Glyphosate	C3H8NO5P	23.87	168.00673	62.96417	124.01687	149.99612	(-)
Glufosinate	C5H12NO4P	14.14	180.04312	85.02955	94.99042	136.05329	(-)
IS: Glyphosate-2-13C, 15N	[13]C2C[15]NH8O5P	23.85	171.01048	62.96415	80.97471	152.99988	(-)

Validation parameters

Validation was carried out following the European Commission (2017) SANTE/2017 Guidance document on method validation & quality control procedures for pesticide residues analysis in food & feed. The selectivity of the method was evaluated by injecting extracted blank honey, fish and bovine muscle samples. The absence of signal above a signal-to-noise ratio of 3 at the expected retention times of the target compounds was the parameter used to show the absence of interferences.

The matrix-matched calibration curves were obtained by spiking 1 g of the three different matrices with an appropriate volume of the standard working solution to cover the concentration range from 5 to 100 ng/g (five calibration points: 5, 10, 20, 50 and 100 ng/g). The limit of quantification (LOQ) of the methods was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120 % and an RSD ≤ 20% (European Commission. 2017. SANTE/11813/2017). The repeatability, evaluated as a coefficient of variation, CV %, was calculated by analysing six replicates at two fortification level (10 and 50 ng/g). Recoveries were calculated by comparing the concentrations of the extracted compounds, spiked before extraction, with those spiked at the end of the extraction procedure at two fortification level (10 and 50 ng/g) for all compounds. The matrix effect was also evaluated using the Matuszewski *et al.* (2003) approach by comparing the corresponding peak areas for standards, spiked after extraction into the extracts, to the peak areas obtained in neat solution standards, expressed as percentage.

Results and Discussion

Extraction procedure

During the preliminary phase, the QuPPe extraction method proposed by EU Reference Laboratories for Residues of pesticides (Anastassiades *et al.*, 2016) was followed, with good results for glyphosate and AMPA but found not suitable for glufosinate in our matrices after the IC-HRMS analysis. In particular, we observed a different and opposite extraction and chromatographic behaviour of the molecules (in particular for AMPA and glufosinate) on the basis of the different solvents used and injected during the ion chromatography separation. Moreover, the final dilution 1/10 suggested by the Anastassiades *et al.* (2016) or by others (Adams *et al.*, 2017) did not improve chromatographic problems when AMPA or glufosinate was hardly detectable.

So we decided to modify the method starting from a smaller amount of matrix (1 g instead of 5 g) to decrease interferences, investigating also the influences of the different tested extraction solvents compatible with our instrumentation: water, methanol and the related acidified solutions with 1% of formic acid. In particular, using only water (**Figure 1a**) or only methanol (**Figure 1b**) as extraction solvent we had poor results for AMPA, but very satisfactory chromatographic peaks for glyphosate and glufosinate. By using 1% of formic acid in water (**Figure 1c**) we had an improvement of AMPA signal, but it was not yet satisfactory, while with 1% of formic acid in methanol (**Figure 1d**) we observed the reverse situation, good chromatography for AMPA but not for glufosinate, which eluted with a too-jagged and wide peak. So after different trials, changing the percentage of formic acid and the composition of the methanol and acidified water mixture we reached the best compromise with 30% of methanol and 70% of acidified (1% formic acid) water as extraction solution. **Figure 2** reports the extracted parent ion chromatograms from full-scan IC-HRMS analysis and from data-independent acquisition mode with the relative fragmentation mass spectra of the three selected analytes after method optimisation.

Figure 1: Extracted parent ion chromatograms from full-scan IC-HRMS analysis of AMPA, glyphosate and glufosinate and influences of the different tested extraction solvents: water (a), methanol (b), 1% formic acid in water (c) and 1% formic acid in methanol (d).

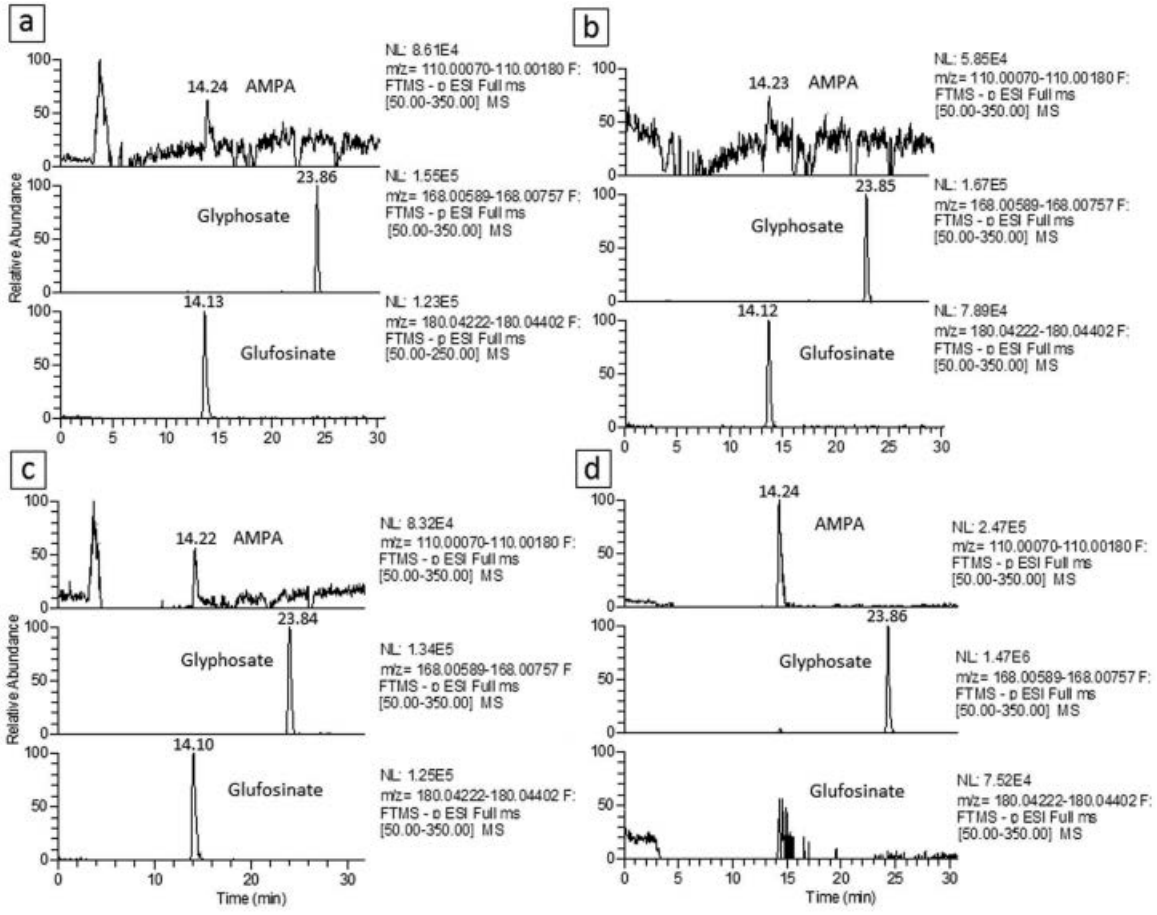
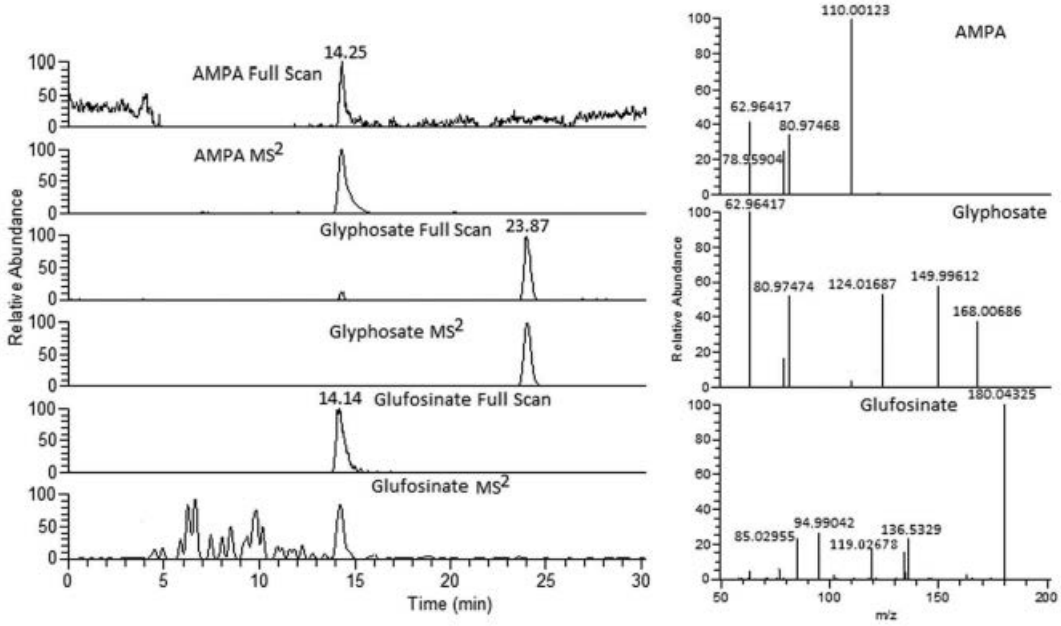


Figure 2: Extracted parent ion chromatograms from full-scan IC-HRMS analysis and from data-independent acquisition mode with the relative fragmentation mass spectra of the three selected analytes (concentration of 10 ng/g) after method optimisation.



IC-HRMS validation parameters

All instrument validation parameters are reported in **Table 2**. The method applied to the three different matrices (honey, bass fish, bovine muscle) showed high specificity, without any interference close to the retention time where the investigated compounds were expected to elute. The good selectivity of the method was demonstrated with a S/N ratio higher than 3 in presence of analytes at the lowest detectable concentrations. All identification criteria passed including retention time stability compared to the standard solution. The mean recoveries ranged between 75 and 112%, indicating the efficiency of the extraction protocol. Matrix validation curves demonstrated a good linearity over the working range with a good fit ($R^2 > 0.99$) for all compounds. Repeatability was calculated using one-way analysis of variance (ANOVA); the CVs were substantially lower than 20%, satisfying the criteria required by the European Commission (2017).

Table 2: Validation parameters about glyphosate, glufosinate and AMPA in the three different matrices analysed by IC-HRMS.

	LOQ (ng g^{-1})	Matrix effects %	CV % (at 2 Levels*)	Recovery % (at 2 Levels*)	Matrix calibration curve	Linearity R^2
HONEY						
AMPA	9.26	84	11, 4	91, 95	y = 0.15747x-0.514838	0.9996
Glyphosate	4.30	94	7, 7	99, 100	y = 0.295172x+0.858303	0.9975
Glufosinate	5.05	84	13, 12	100,101	y = 0.442863x+0.00858678	0.9957
FISH MUSCLE						
AMPA	5.38	95	4, 2	96, 95	y = 0.0897754x-0.231747	0.9951
Glyphosate	5.08	93	8, 8	80, 93	y = 0.072573x+0.112081	0.9985
Glufosinate	4.36	96	7, 6	112, 96	y = 0.127407x-0.176602	0.9976
BOVINE MUSCLE						
AMPA	6.44	99	12, 9	75, 79	y = 0.091067x-0.113588	0.9925
Glyphosate	6.47	107	13, 10	75, 80	y = 0.063115x+0.0920263	0.9922
Glufosinate	6.25	106	5, 2	76, 80	y = 0.120407x-0.107902	0.9962

*The two concentration levels were 10 and 50 ng g^{-1}.

Regarding the LOQs in the range from 4.30 to 9.26 ng/g, our satisfactory results showed high method sensitivity for glyphosate and its metabolites, when compared to the few reports present in the literature. In fact, Picò *et al.* (2007) reported LOQ of 0.05 mg/kg for glyphosate and AMPA in plant products, such as rice, wheat, vegetables, fruits and tea, pig and chicken muscles, aquatic products, chestnut, honey, etc. using High Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry; Krüger *et al.* (2014) reported validation parameters spiking at 100 µg/g of glyphosate in animal and human residues through ELISA followed by GC-MS/MS analysis. The overview of approximate LOQs reported by Anastassiades *et al.* (2016) in the range of 0.01–0.02 mg/kg obtained by the QuPPE extraction followed by LC-MS/MS analysis or those of Chamkasem *et al.* (2016) in the range of 4–26 ng/g using LC-MS/MS system are a little higher than our results. In **Table 3** we report all the LOQs and other information on the different studies presented in the literature about glyphosate, AMPA and glufosinate in food of animal origin. Matrix effects were modest in the three different matrices with a percentage variation lower than the 20% (from 84% to 107%) recommended by the European Commission (2017).

Based on our results the use of high-resolution mass spectrometry and hyphenation with ion chromatography has been demonstrated to be very effective for the analysis of these challenging analytes in very complex matrices of animal origin. Particularly, as stated by Rajski *et al.* (2018) in their analogous study on anionic pesticides in fruits and vegetables, the high ion-exchange capacity, the efficiency, the diameter reductions and the characteristic chemistry of bonded functional groups of IC columns are a major factor for the separation and identification of the highly polar pesticides, scarcely retained in reversed-phase LC, avoiding moreover any derivatisation step. The high-resolution mass spectrometry allowed obtaining low background matrix signals, improving the sensitivity in terms of LODs and efficient trapping and stability of low m/z ions, improving selectivity. The high MS resolving power and mass accuracy down to 1 ppm, combined with the rapid scan speed, also provide high specificity (Chiesa *et al.* 2018). The possibility to do retrospective analyses is an added value.

Table 3: Literature about glyphosate in products of animal origin.

Authors	Matrix	Analyte	LOQ	Analytical Instrumentation
Wang, Jaw, and Chen	Fish	Glyphosate	/	Beckman LS 1000C liquid scintillation counter.
Alferness and Iwata (1994)	Muscle, kidney, liver and fat beef, eggs and milk	Glyphosate, AMPA	0.01 μg g^{-1}	Capillary Gas Chromatography with Mass-Selective Detection
Picò et al. (2007)	Plant products, vegetables, fruits and tea, pig and chicken muscles, aquatic products, chestnut, honey	Glyphosate, AMPA	0.05 μg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Bo et al. (2007)	Vegetables, fruits, cereals, pig and chicken muscles, aquatic products, honey	Glyphosate, AMPA	0.05 μg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Krüger et al. (2014)	Liver, kidney, lung, spleen, muscle and intestine of cow	Glyphosate	100 μg g^{-1}	ELISA- Gas Chromatography-Mass Spectroscopy
Chamkasem et al. (2016)	Milk	Glyphosate, Glufosinate, and AMPA	100 μg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Anastassiades et al. (2016)	Foods of plant origin, cereals and honey	Glyphosate, Glufosinate, AMPA and others	4–26 ng g^{-1}	Liquid chromatography/ tandem mass spectrometry
Liao et al. (2018)	Milk-based baby foods and other milk products, infant formulae, yogurt	Glyphosate, Glufosinate	/	Liquid chromatography/ tandem mass spectrometry
Chamkasem et al. (2018)	Honey	Glyphosate, Glufosinate, and AMPA	0.25 μg mL^{-1}	Liquid chromatography/ tandem mass spectrometer

Application to real commercial samples

Finally, we applied the proposed method for the analysis of 30 real samples: 10 organic honeys, 10 beef muscle pools and 10 sea bass muscle pools, each thoroughly homogenised. All the samples were of Italian origin, taken from different supermarkets of Milan. None of the selected samples showed any traces of glyphosate or metabolites, ensuring the good quality of the samples, especially when it comes to organic products such as honey, demonstrating the absence of pesticide contamination both of the sample and of the production area.

Conclusions

In this study, we developed and validated a new and versatile IC-HRMS method for the detection of glyphosate, AMPA and glufosinate in three complex different matrices, honey, bass fish and bovine muscle. These results are of great importance and topical in the field of food safety because of the scarce data regarding this topic, the extractive and analytical difficulties related to these analytes in relation to complex matrices, and the legislative situation not yet outlined on the use of glyphosate and residues in consumer products. The application of the method to real commercial samples did not show any traces of the pesticides. Further studies of the method's application and statistical evaluation are necessary to form a more complete view on this matter.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The purpose of the publication is to describe and discuss the performance of a residue analytical method for glyphosate, AMPA and glufosinate in, food of animal origin. As such, the publication is not relevant to risk assessment. However, since it also reports residue levels for the investigated compounds in 10 honey samples and since according to SANTE/11956/2016 rev. 9 it is possible to derive EU MRLs in honey based on monitoring data, the publication may be considered relevant to risk assessment and MRL setting. Based on the provided validation results, the method is considered reliable. The LOQ (defined as the lowest fortification level yielding acceptable recoveries) was 0.010 mg/kg for both glyphosate and AMPA (although different values, presumably estimated from the signal to noise ratio, are stated in Table 2). None of the 10 analysed honey samples showed residues of glyphosate or AMPA above the LOQ. However, it is important to note that all the samples were from organic production and this may need to be taken into account in the evaluation.

1. Information on the study

Data point	CA 6.10.1
Report author	El Agrebi N. et al.
Report year	2020
Report title	Honeybee and consumer's exposure and risk characterisation to glyphosate-based herbicide (GBH) and its degradation product (AMPA): Residues in beebread, wax, and honey
Document No.	Science of the Total Environment 704 (2020) 135312
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

In order to assess bee and human exposure to residues of glyphosate-based herbicide (GBH) and its main degradation products aminomethylphosphonic acid (AMPA) and to characterise the risk posed by these substances, we analysed 3 different bee matrices; beebread (N = 81), wax (N = 100) and 10-paired samples of wax/honey collected in 2016/2017 from 379 Belgian apiaries. A high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS-MS) was used as analytical method. Limit of quantification and detection (LOQ and LOD) for GBH residues and AMPA in the 3 matrices was respectively of 10 ng/g and 1 ng/g. In beebread, 81.5% of the samples showed a residue concentration > LOQ and 9.9% of the samples a residue concentration < LOQ (detection without quantification); no significant difference in detection rate was found between the north and the south of the country. Glyphosate was detected in beeswax less frequently than in beebread (i.e. 26% >LOQ versus 81.5% >LOQ). The maximum GBH residues and AMPA concentration found in beebread (respectively 700 ng/g and 250 ng/g) led to sub-lethal exposure to bees. The Hazard Quotient (HQ) for beebread and beeswax (7 and 3.2, respectively) were far below the "safety" oral and contact thresholds for bees. For human health, the highest exposure to GBH residues in pollen corresponded to 0.312% and 0.187% of the ADI and of the ARfD respectively and, to 0.002% and to 0.001% for beeswax. No transfer of glyphosate from wax to honey was detected. Considering our results and the available regulatory data on the glyphosate molecule considered solely, not including the adjuvants in GBH formulation, the consumption of these three contaminated matrices would not be a food safety issue. Nonetheless, caution should be taken in the interpretation of the results as new studies indicate possible glyphosate/GBH residues toxicity below regulatory limits and at chronic sub-lethal doses.

Materials and Methods

Study areas

Three different bee matrices were sampled for the analysis of GBH residues and AMPA: (i) beebread (N = 179), (ii) wax from the brood chamber (N = 100) and additionally (iii) a combination of wax from the honey super and corresponding extracted honey (N = 10). We used 379 non-professional apiary sites located in Belgium, including 2,997 colonies of *Apis mellifera*. For beebread and wax sampling, apiaries were selected (193 for beebread, 186 for wax and honey) from the Federal Agency for the Safety of the Food Chain (FASFC) apiaries database that included 4,949 registered beekeepers in 2015. The apiaries were stratified by province (N = 20/province and 10 provinces in Belgium) and randomly distributed in Flanders (northern Belgium) and Wallonia (southern Belgium). All sampled bee colonies seemed healthy, with no clinical signs of infectious diseases or acute intoxication (Ravoet

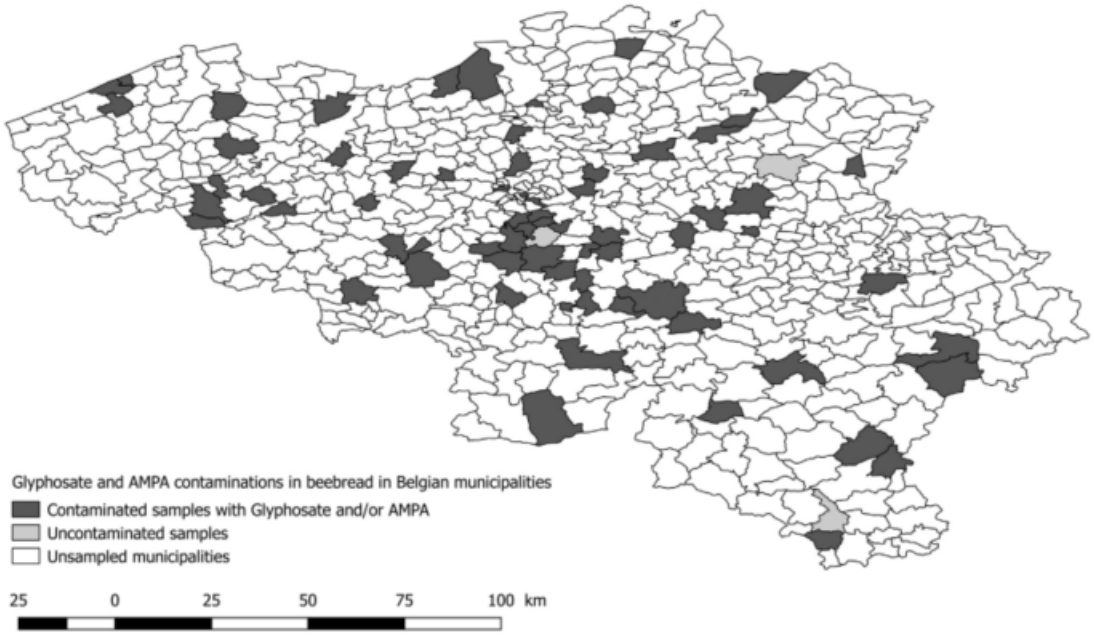
et al., 2015). Quantum GIS (QGIS Development Team, 2009; http://qgis.osgeo.org) was used to create the maps in **Figure 1** and **Figure 2**.

The risks posed by formulated products in the present study are restricted to the active ingredient glyphosate plus AMPA and the total risk of commercial products utilized by farmers is not the subject of this study.

Beebread collection

Beebread sampling (N = 179) was carried out by FASFC beekeepers and apiary technicians (Healthy Bee national monitoring program) between September and October 2016 from 193 apiaries including 865 colonies, out of 75 municipalities covering the entire Belgian territory (**Figure 1**). The samples were provided with a protocol defining sampling collection details and were personally instructed by expert beekeepers to improve the harmonization of the procedure across apiaries. At each apiary, one hive was sampled randomly by cutting a comb portion of 8 by 8 cm filled with beebread. The coded samples were kept in hermetic plastic bags and stored at -20°C the same day in order to be processed. A cool-box was used for shipment of samples from FASFC to Liège University to ensure that samples were maintained frozen (Tosi *et al*., 2018) until processing.

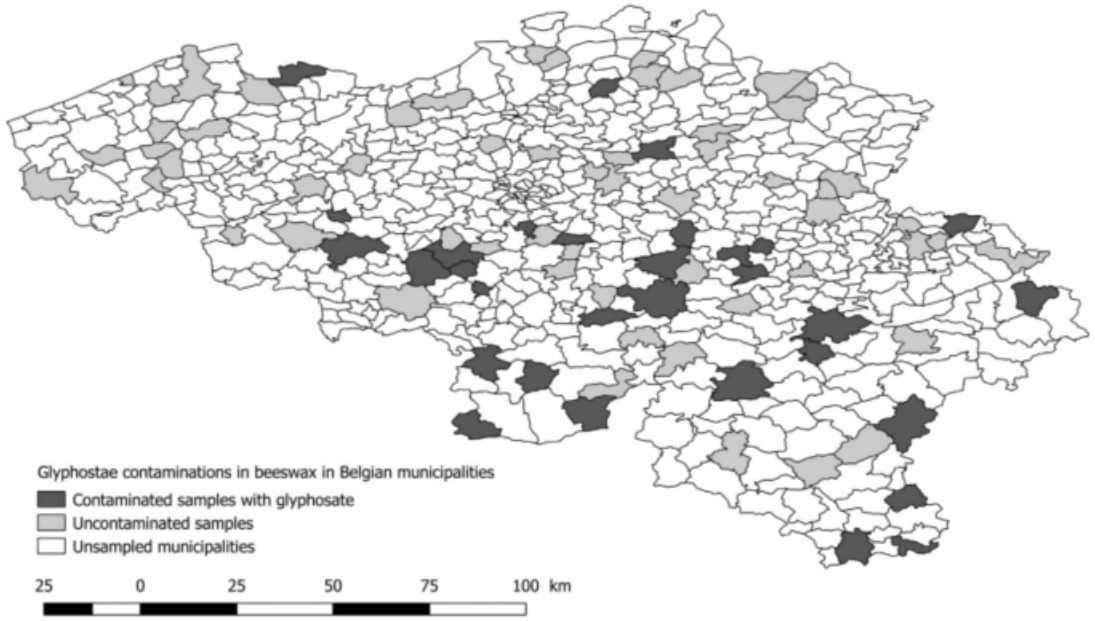
Figure 1: Glyphosate residues and AMPA contaminations in beebread across Belgium, in 2016.



Beebread extraction

For analyse purpose, 20 g of beebread were extracted manually from each comb sample using a disposable surgical blade (1 blade per sample). Cleaned beebread samples were stored in a 60 mL marked sterile polycarbonate containers with screw cap. Only 81 samples of beebread could be extracted from the 179 comb samples in adequate amounts for analysis.

Figure 2: Glyphosate residues and AMPA contaminations in beeswax across Belgium, in 2016.



Wax collection

Twenty grams (20 g) of wax from the brood chamber were sampled during spring 2016. Together with sampling, wax renewal rates were registered in a questionnaire (<50% and ≥50%). The coded samples were kept in hermetic plastic bags and stored the same day at -20°C until analysis. Financial limitations allowed us to randomly select only 100 wax samples out of the 186 original samples (2132 hives). These 100 samples were equally distributed between Flanders and Wallonia in 89 municipalities (**Figure 2**).

Honey/wax sampling

After wax analysis, out of the 32 beekeepers with the highest GBH residues contaminations in wax from the brood chamber, 10 beekeepers were randomly selected. Among these beekeepers, samples of 20 g of wax and of 50 g of honey harvested in summer 2017 were extracted both from the honey super (pairwise samples). The coded wax samples were kept in hermetic plastic bags, honey in polypropylene disposable containers and shipped the same day to the laboratory. Sampling and analysis of honey for GBH residues and AMPA were performed in September 2017 in the same laboratory and according to a similar method as for beebread and beeswax. Concentrations of GBH residues measured in honey were compared to the Maximum Residue Level (MRL) for human consumption (50 ng/g) (Regulation (EC) No 396/2005).

Glyphosate-based herbicide residues and AMPA detection

The GBH residues and AMPA analyses were carried out between May and June 2017 (September 2017 for the 10-paired samples of wax/honey) by the Phytocontrol laboratory (France) ISO 17,025 accredited under the number No 1–1904 for the analysis of bee products by the French competent authority. The analysis method used for the targeted matrices (beebread, beeswax, and honey) was a high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS-MS). The analytes were extracted using an aqueous solution followed by a simple clean up with a C18 solid-phase extraction (SPE) cartridge, and then glyphosate and AMPA were derivatised using 9-fluorenyl-methoxycarbonyl (FMOC-Cl) in borate buffer. For beeswax, an additional hexane treatment was used in order to defat the extract. The derivatives of glyphosate and AMPA were separated on a C18 column (105 x 4.6 mm; 5 µm) with gradient elution with the mobile phase of acetonitrile and 5 mmol/L ammonium acetate (pH 9), and finally detected with negative ion

electrospray ionization-mass spectrometry (ESI-MS) in multiple reaction monitoring (MRM) mode (drying gas flow at 15 mL/min, nebulizing gas flow at 3 L/min). Limits of quantification (LOQ) for both glyphosate and AMPA in the 3 matrices were 10 ng/g, while limits of detection (LOD) were 1 ng/g. Matrix effects were compensated by the addition of ^{13}C labeled glyphosate (used as internal standard) to the sample prior extraction, as well as in spiked samples used to set up the calibration curve. Three levels of spiking, including the LOQ, were performed on several matrices of different categories, which were analysed in condition of repeatability and intermediate fidelity. The mean spiked recoveries of glyphosate and AMPA at 3 spiked levels ranged from 72.2% to 112.9% with the relative standard deviations (RSD, n = 5) of 0.1% – 4.5%. The tolerance interval was plotted with a beta probability of 80%, which represents the proportion of future values that the routine method will produce over the entire field of application. This allows to ensure that the molecule of glyphosate is extracted correctly and to correct any matrix effects.

Exposure assessment and risk characterisation to honeybee health

We estimated the Hazard Quotient (HQ) for honeybees using the method described by (Stoner and Eitzer, 2013). The HQ is calculated as the exposure divided by the toxicity expressed, in this study, as the maximum residue concentration (ng/g or ppb) in beebread samples divided by the oral acute LD 50 (mg/bee) and multiplyed by 100. An adult bee that consumed 100 mg pollen with an HQ of 1000 would have consumed approximately 10% of the LD_{50} for the pesticide during this development stage (=10 days as nurse bee) (Calatayud-Vernich *et al.*, 2018). Assuming that 10% of the LD_{50} should never be exceeded (Atkins et al., 1981), the HQ value of 1000 would correspond to the limit of concern for bee health (Stoner *et al.*, 2013; Traynor *et al.*, 2016). For beeswax, we used a contact HQ of 5000 as threshold safety value, since residue concentrations are significantly higher in wax, and contact exposure routes are poorly understood in this matrix (Traynor *et al.*, 2016).

Then, we also assessed the risk posed by GBH residues and AMPA in beebread to honeybee health through the assessment of the honeybee exposure to these compounds through beebread consumption. To estimate the beebread consumption, we used published pollen consumption values. A nurse bee consumes between 13 and 120 mg of pollen during its first 10 days of life (OECD, 1998; Rortais *et al.*, 2005) with a mean value equal to 65 mg (Chauzat and Faucon, 2007). As a worst-case scenario, we took into account the maximum consumption level of 12 mg of pollen per day. Then, we multiplied this highest level of consumption with the highest GBH residues and AMPA concentrations. Finally, we compared the exposure levels with the oral acute LD_{50} of these compounds.

Until very recently, risk assessment procedures did not implement yet the side-effects of pesticides on developing brood and the chronic effects in general (OECD, 2017). We could only assess the acute risk for adult bees since the possible toxicity of GBH residues on bee larvae is currently not sufficiently characterized.

Risk to consumer's health

For human health, GBH residues toxicity has been redefined in 2015 (European Food Safety Authority, 2015); an acceptable daily intake (ADI) for consumers has been set to 0.3 mg/kg body weight/day and the acute reference dose (ARfD) at 0.5 mg/kg body weight/day. Concerning AMPA residues, only the ADI value is available (0.3 mg/kg body weight/day). ADI is the quantity of a chemical that can be ingested daily for a lifetime causing no harm (on the basis of all known facts) (Renwick, 2002). ARfD is the quantity of a chemical that can be ingested by a person at a single time causing no harm. MRL is the maximum concentration of pesticide residue legally permitted in or on food commodities or animal feeds (Food and Authority, 2017).

Then, we assessed the risk posed by GBH residues and AMPA in beebread and beeswax to consumer's health through the assessment of the consumer exposure to these compounds through pollen and beeswax consumption. Thus, we assumed that beebread contamination levels correspond to pollen contamination levels. To estimate the pollen and beeswax consumption, we used published consumption data. According to EFSA (EFSA, 2007), the 95th percentile of the daily consumption of beeswax corresponds to 1.29 g/person, which is 0.022 g/kg b.w. for a 60 kg individual. Concerning the daily consumption of pollen, the highest 95th percentile value recorded in the EFSA Comprehensive

European Food Consumption Database (EFSA, 2018) corresponds to 69.55 g/person, that is 1.35 g/kg b.w. for a 52 kg individual, in France (according to the second version of the FoodEx food classification system). Then, as a worst-case scenario, we multiplied these high levels of consumption with the highest GBH residues and AMPA concentrations. Finally, we compared the exposure levels with the reference toxicological values of these compounds (above mentioned) to characterise the risk.

Statistical analysis

Yearly wax renewal rates were divided into 2 categories: <50% and ≥50% of wax frames changed per year in the brood chamber. A Fisher's exact test was used to compare the annual renewal rate of wax frames between regions (Flanders versus Wallonia).

A Fisher's exact test was used for each pairwise comparison of frequency of detection of GBH residues and AMPA depending on the region/country and the matrix for GBH residues only (beebread versus beeswax). A two-sample Wilcoxon rank-sum (Mann-Whitney) (i.e. non-parametric test) test was used for each pairwise comparison of concentration of GBH residues and AMPA depending on the region/country and the matrix for GBH residues only (bee-bread versus beeswax).

A logistic regression (odds ratio's (OR) with 95% confidence intervals (95% CI)) was used to test a possible risk factor of GBH residues detection in beeswax and regions (Stata SE 14.1®, Stata-Corp LP, College Station, TX, USA). For all tests, a level of significance of 5% was used and divided, if needed, by the number of comparisons performed for the Bonferroni correction.

Results

Glyphosate-based herbicide residues and AMPA in beebread

In beebread, a high detection of GBH residues was registered (91.4% of positive samples overall) and AMPA (25.9% positive samples) in both Belgian regions. Glyphosate LOQ value (10 ng/g) was lower than the glyphosate median lethal doses LD_{50} for bees (10^6 ng/g). No significant difference of contamination prevalence in beebread between regions was confirmed by a one-tailed Fisher's exact test (1 degree of freedom; α =0.05) (N = 81; $p > 0.20$) (**Table 1**). GBH residues and AMPA were not detected in only 6 samples (7.4%), coming from 3 of the 75 sampled municipalities (**Figure 1**). Only 2 samples contained AMPA without GBH residue.

Table 1: Glyphosate and AMPA detection, residue levels and hazard quotient to bees in beebread, beeswax and honey samples in Flanders (North Belgium), Wallonia (South Belgium) and Belgium.

Matrix	Region	Sampling period	Nb. analysed samples	Nb. samples > LOQ	Nb. samples < LOQ	Nb. samples detected	% samples > LOQ	% samples < LOQ	% samples detected	Multi-test for detection	Average [] ng g^{-1}	S.D. [] ng g^{-1}	Multi-test for []	Max [] ng g^{-1}	Median [] ng g^{-1}	Max HQ
Beebread	**GBH**															
	Flanders	Fall 2016	39	34	3	37	87.2%	7.7%	94.9%	aa	58.44	133.28	aa	700	23	7
	Wallonia		42	32	5	37	76.2%	11.9%	88.1%	aa	52.41	39.70	aa	160	49.5	1.6
	Belgium		81	66	8	74	81.5%	9.9%	91.4%	aa	55.52	98.89	aa	700	26	7
	AMPA															
	Flanders	Fall 2016	39	5	3	8	12.8%	7.69%	20.5%	a-	39.8	25.16	a-	77	38	0.8
	Wallonia		42	10	3	13	23.8%	7.14%	30.9%	a-	80.8	78.09	a-	250	58.5	2.5
	Belgium		81	15	6	21	18.5%	7.4%	25.9%	a-	67.13	67.09	a-	250	44	2.5
	GBH															
Beeswax	Flanders	Spring	48	3	1	4	6.3%	2.08%	8.3%	ab	28.33	22.90	aa	54	21	0.5
	Wallonia	2016	52	23	5	28	44.2%	9.62%	53.8%	bb	66.43	84.01	aa	320	40	3.2
	Belgium		100	26	6	32	26%	6%	32%	cb	62.04	80.05	aa	320	36	3.2
Honey	Flanders	Summer	2	0	1	1	0%	50%	50%	/	/	/	/	/	/	/
	Wallonia	2017	8	1	0	1	12.5%	0%	13%	/	11	/	/	11	11	/
	Belgium		10	1	1	2	10%	10%	20%	/	11	/	/	11	11	/

GBH: Glyphosate based herbicide, Nb.: number; > LOQ: detection with quantification, <LOQ: detection without quantification, []: concentration; AMPA: aminomethylphosphonic acid; HQ beebread (oral) threshold value = 1000; HQ wax (contact) threshold value = 5000, + detection is the sum of samples > LOQ and < LOD; S.D.: standard deviation; /: not determined. Multi-testing: a Fisher's exact test and a two-sample Wilcoxon rank-sum (Mann-Whitney) test were respectively used for each pairwise comparison of frequency of detection and mean concentration of the compounds. Different letters were used for significant differences. The first position letter corresponds to the comparison of regions for a same matrix; the second position letter corresponds to the comparison of breebread and beeswax for the mean concentration of GBH. A level of significance of 5% was used, divided by the number of tests performed for the Bonferroni correction.

Exposure assessment and risk characterisation of GBH residues in beebread for honey bees

Based on the honeybee oral acute LD_{50} (48 h) of glyphosate (100 mg/bee = moderate toxicity for adult bees) (Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate 2015; Lewis *et al.*, 2016) and on the maximum concentration of GBH residues detected in beebread (700 ng/g), the estimated maximum HQ (oral) of GBH residues for beebread found in Belgium is equal to 7 (=700/100). Because the honeybee oral acute LD_{50} (48 h) of AMPA is currently unknown in published data, it was impossible to estimate its corresponding HQ.

Considering the maximum consumption level of 12 mg of pollen per day (Rortais *et al*., 2005) (worst-case) and the maximum concentration of GBH residues detected in beebread (700 ng/g), this would correspond to a dose of 84 ng of GBH residues ingested per nurse bee over 10 days (0.012 g x 700 ng/g x 10 days). This exposure level corresponds to about 0.08% of the oral glyphosate LD_{50}. As mentioned, in the open literature, no oral acute LD_{50} (48 h) for AMPA is available. To assess the risk of AMPA to bees, we used, therefore, the parent compound glyphosate LD_{50} (Traynor *et al*., 2016). AMPA detection in beebread (250 ng/g) would correspond to about 0.03% of the oral glyphosate LD_{50}. Cumulatively, GBH and AMPA maximal concentration would correspond to about 0.12% of oral glyphosate LD_{50}.

Glyphosate-based herbicide residues and AMPA in beeswax

GBH residues were found in 32% of Belgian beeswax samples (N = 100, T1). A significantly higher GBH residues prevalence was found in Wallonia (53.8% positive sample, **Figure 2**), as compared to Flanders (8.3% positive samples, one-tailed Fisher's exact test (1 degree of freedom; $\alpha = 0.05$), $p < 0.001$); confirmed by a logistic regression comparing contaminations in both regions (with Flanders as a reference): OR = 18.4, 95% CI = 4.66–72.60, $p < 0.001$). A two-sample Wilcoxon rank-sum (Mann-Whitney) test showed that the average GBH residue concentration observed in Wallonia is not significantly higher than in Flanders ($p = 0.33$) (**Table 1**).

Exposure assessment of GBH residues in beeswax

No trace of AMPA has been detected in beeswax. HQ (contact) of beeswax for the maximum GBH residues concentration in Belgium is equal to 3.2 (= 320/100).

Wax renewal rate in Flanders and Wallonia

Beekeepers should renew the wax foundation of their bee colonies periodically. This improves bee health reducing the disease and chemical load of beeswax and allowing bees to rear their brood in a freshly built environment.

Flemish beekeepers had a significant higher wax renewal rate ($\geq 50\%$ per year) as compared to Walloon ones (N = 98, one-tailed Fisher's exact test (1 degree of freedom; $\alpha = 0.05$) , $p = 0.017$) (data not shown).

Risk assessment for the consumer of contaminated beebread and beeswax

As shown in **Table 1**, GBH residues contaminated significantly more frequently beebread (87.2% >LOQ) than beeswax (26% >LOQ) (one-tailed Fisher's exact test (1 degree of freedom; a =0.05), N = 181; $p < 0.001$) but the average concentration found in beebread (55.52 ng/g) and wax (51.3 ng/g) were statistically comparable (two-sample Wilcoxon rank-sum (Mann-Whitney) test; $p > 0.05$).

A high consumption level (95th percentile) of the most contaminated pollen and beeswax by GBH residues, according to our results, leads to an exposure of respectively 0.936 and 0.007 mg GBH residues/kg b.w./day through beeswax and pollen consumption. Concerning AMPA, the highest exposure corresponds to 0.334 mg AMPA/kg b.w./day through pollen consumption).

Transfer of GBH residues and AMPA from beeswax to honey

We wondered if a transfer of GBH residues and AMPA from beeswax to honey was possible. Thus, to further test this hypothesis, we concomitantly collected both wax and honey from the bee colony honey supers of 10 apiaries out of the 32 beekeepers with the highest GBH residues contaminations in wax from the brood chamber. We found 1 out of 10 wax samples (10%) contaminated with GBH residues (concentration: 48 ng/g). In honey, 2 out of 10 samples were contaminated by GBH residues (20%; 11 ng/g for the first sample and a detection lower than the quantification limit [LOQ] < 10 ng/g for the second sample). These 3 positive GBH residues samples came from different bee colonies. No trace of AMPA was detected in any of the matrices. The highest GBH residues concentration detected

in honey was about 5 times lower than the MRL (50 ng/g).

Discussion

Beebread

Our study showed an extended presence of GBH residues in beebread (81.5% positive samples at the national level) in both Belgian regions. AMPA was found in 18.5% of beebread samples at the national level. Only 2 samples contained AMPA without GBH residue. The LOQ values for glyphosate and AMPA are of 10 ng/g, which makes the analysis method very sensitive. Simultaneous AMPA/GBH residues detection in beebread could be explained by the GBH residues degradation in the matrix or by their simultaneous occurrence in the environment. In soil, the primary pathway degradation of glyphosate residues is microbial action, which yields AMPA and glyoxylic acid (Roberts *et al*., 1999). The maximum GBH residues concentration found (700 ng/g) led to sublethal exposure (not acutely toxic to bees), corresponding to a dose of 84 ng/bee (0.08% of its LD_{50}), ingested over the first 10 days of life of a nurse bee. AMPA dose in beebread also corresponded to a sub-lethal exposure (to about 0.03% of oral glyphosate LD_{50}) alone or cumulated with GBH residues (about 0.12% of oral glyphosate LD_{50}). However, while the LD_{50} is measured as a one-time dose, bees could be exposed to GBH residues contaminated beebread for a longer period, when re-contamination occurs, since glyphosate degradation time DT_{50} ranges between 1.0 and 67.7 days. Therefore, the use of the LD_{50} as a single benchmark could underestimate the exposure risk to bees.

Bee and bee colony health is significantly impaired by doses that are lower than those we found through sub-lethal effects. Helmer *et al.* (Helmer *et al*., 2015) orally exposed bees to sub-lethal field-realistic doses of GBH residues (1.25, 2.50, and 5.00 ng/bee) and showed a significant decrease ($p < 0.05$; $n = 40$) of beta-carotene and protein levels in their bodies after 10 days. Our results confirm Helmer's field-realistic doses (lower than 700 ppb, corresponding to 84 ng/bee). Other studies (Herbert *et al*., 2014), showed that adult *A. mellifera* workers exposed orally to 2.5 and 5 mg/L of GBH residues (field-realistic doses equivalent) presented reduced sucrose sensitivity leading to loss and difficulty in establishing associative memories, which, in turn, could cause inefficient collection of nectar and pollen for the colony and, finally, compromise its survival. Oral exposure to GBH residues concentrations (2.5, 5.0, and 10.0 mg/L, corresponding to a dose of 0.125, 0.25, and 0.5 µg/bee) affects honeybee cognitive abilities, with potential long-term negative consequences for colony foraging success (Balbuena *et al*., 2015). Exposures to 5 and 10 mg/L of GBH residues (dose of 0.25 and 0.5 µg/bee) perturb the gut microbiota of honeybees. Bee gut symbionts influence bee development, nutrition, and defence against natural enemies (Motta *et al*., 2018). Perturbations of these gut communities may affect bee susceptibility to environmental stressors, including poor nutrition (Tosi *et al*., 2017) and pathogens (Motta *et al*., 2018). Moreover, in evaluating the effect of Roundup® on the royal jelly-producing glands, Faita *et al*. (2018) showed that exposure to GBH residues resulted in the alteration of these glands that can trigger damage to the development and survival of bee colonies.

Regarding AMPA, no trace was found in honey and beeswax. In beebread, the maximum AMPA concentration was 250 ng/g. Because no information on AMPA toxicity to bees is available yet in the open literature, we were not able to assess its risks to bees. Nevertheless, Blot *et al*. (2019) confirmed that glyphosate have sub-lethal effects on the honeybee microbiota, while AMPA did not induce any significant change.

Beeswax

Measured GBH residues concentrations should not cause acute lethal effects since the estimated HQ for beebread and beeswax (7 and 3.2, respectively) were far below the "safety" oral and contact thresholds (1000 and 5000, respectively). Since beebread can be stored in the hive for months after collection in the field, glyphosate degradation have likely reduced its concentration over time. Furthermore, bees typically collect multiple chemicals simultaneously (Tosi *et al*., 2018). Because bees are bio-indicators of environmental health and pollution, residues found in bee products provide valuable information on environmental punctual contamination or accumulation which, nevertheless, might be underestimated (i.e. residue degradation, dilution of highly-concentrated samples, technical

limitations such as LOD) or overestimated (i.e. accumulation of contaminated pollen) (Tosi *et al.*, 2018).

Due to glyphosate high water solubility and a very low octanol/water partition coefficient (Log P (= Log Kow) at pH 7 and at 20°C = –3.2), GBH residues were expected to be found only in beebread but not in wax (a very hydrophobic matrix). Beeswax samples contamination rate was of 26% at the national level. The addition of surfactant in the formulation of end-use pesticide products is at the origin of the phenomenon allowing glyphosate, which is water-soluble, to penetrate lipid-based structures (Shokri *et al.*, 2001). Nevertheless, the risk assessment for honey bees and the consumer has been evaluated for glyphosate molecule solely without the concomitant formulation ingredients and adjuvants, nor other possibly concurring pesticides (Tosi *et al.*, 2018). The use of the glyphosate/AMPA molecule solely does not render the combined toxic effects of the formulation constituents nor the synergetic potential effects of pesticide combinations.

Wallonia had both a higher GBH residue detection rate (53.8%) and a significantly lower rate of wax foundation renewal rate, as compared to Flanders ($p = 0.017$). This supports our hypothesis that the beekeeping management practice of renewing wax foundation can protect bees from the accumulation of pesticide residues inside the hive. No trace of AMPA could be detected in beeswax, probably because the matrix is not suitable for microorganism growth due to its rich hydrophobic protective properties (Fratini *et al.*, 2016), resulting in no degradation of glyphosate in AMPA. Beeswax's conservative properties for pesticide residues combined with the beekeeping practice of wax recycling (Perugini *et al.*, 2018), may be at the origin of the unequal detection of GBH residues in Flanders and Wallonia. This result highlights the importance of replacing at least 50% of wax frames per year, the current recommendation being the yearly replacement of 25 to 33% of the wax from the brood chamber (ITSAP, 2017; Vergaert, 2017).

For human health, the highest exposure to GBH residues in pollen corresponds to 0.312% and 0.187% respectively of the ADI and of the ARfD, and this through the pollen consumption (69.55 g/day/person of contaminated pollen with 700 ng of GBH residues/g). The exposure to GBH residues through the beeswax consumption (1.29 g/day/person of contaminated beeswax with 320 ng of GBH residues/g) corresponds to only 0.002% and 0.001% respectively of the ADI and of the ARfD. Concerning AMPA, the highest exposure to this compound corresponds to 0.111% of the ADI, and this through the pollen consumption (69.55 g/day/person of contaminated pollen with 250 ng of AMPA/g).

Honey

The honey analysis resulted in a maximum GBH residues concentration of 11 ng/g, not exceeding the EU MRL (50 ng/g) for honey and theoretically meaning no risk for the consumer. In a survey on GBH residues in honey samples originating from different countries (Brazil, Canada, China, Germany, Greece, Hungary, India, Korea, Mexico, Uruguay, New Zealand, Spain, Taiwan, Ukraine, Vietnam and USA), GBH residues were found in fifty nine percent (59%) of analysed samples, with concentrations ranging between 17 and 163 ng/g (mean = 64 ng/g) (Rubio *et al.*, 2014).

Our concomitant analyses of wax and honey in samples (N = 10) from honey supers resulted in one wax sample being contaminated (48 ng/g). The low contamination in honey supers suggests that GBH residues are mostly stored in the brood chamber, where pollen and nectar are stored and where most bee activity occurs. This preliminary study showed no transfer from wax to honey. Because our results on the concomitant honey/wax contamination are based on limited data (N = 10), they should be confirmed with further studies.

For human health, considering our results and the assumptions we made with the available regulatory data, the consumption of these three contaminated food matrices (pollen, beeswax, and honey) would not be a food safety issue, nonetheless, caution should be taken in the interpretation the results as new studies confirmed glyphosate toxicity below regulatory limits (Mesnage *et al.*, 2015), and the genotoxicity of AMPA (Mañas *et al.*, 2009).

Bees are major pollinators in agricultural systems. Beebread, beeswax, and honey pesticide residue contamination can impact the viability of a colony when larvae develop on highly contaminated

beeswax and feed with contaminated food (Orantes-Bermejo *et al*., 2010). Even a low concentration of pesticide residues can have amplified toxic effects on animals, including bees, through interactions with other chemicals (Zhu *et al*., 2017) or environmental stressors. The pesticide risk to bees can synergistically amplify the adverse effect of non-chemical stressors too and conversely, nutritional stress can synergistically increase the toxicity of pesticides (Tosi *et al*., 2017).

Conclusions

Our study gives a glimpse of bees and human exposures to GBH residues. At this stage, glyphosate is analysed alone, even though it is never used in this form but only as part of a mixture with adjuvants in commercial formulations. Clarifications and further research are needed to estimate the risk of the herbicide alone and in formulations (i.e. with the adjuvants), especially at levels below the regulatory safe limits and over longer durations. More studies are needed to assess synergies with other pesticides, and longer term exposures at sub-lethal doses. More transparency is needed regarding the commercial formulation products.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a survey of pesticide residues (glyphosate/AMPA) in various bee-related matrices (beebread, wax, honey) from Belgium. While the representativeness of the sampling procedures may be questioned and although the results of the analytical method validations are not provided in a high level of details, the results are considered reliable. A considerable number of samples of beebread/pollen (n = 82) and beeswax (n = 100) were analysed for parent glyphosate and its metabolite AMPA. However, according to the guideline SANTE/11956/2016 rev. 9 the intake of pollen and wax by consumers is negligible and, therefore, it is not a regulatory requirement to investigate the residue levels in these commodities. The publication also provides analytical results for 10 honey samples. Only one of these samples was found to contain residues of parent glyphosate above the LOQ of 0.010 mg/kg (at 0.011 mg/kg). None of the honey samples showed detectable residues of AMPA (i.e. these residues were < 0.001 mg/kg). Since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data, these results are deemed relevant.

The publication concludes that, based on the observed residue levels, the intake of pollen, beeswax and honey by consumers does not cause any health issue. While this conclusion is certainly correct some of the details of the risk assessment are questionable. For instance, the considered ADI of 0.3 mg/kg bw/day for parent glyphosate is obsolete (and was already obsolete at the time when the publication was issued). Furthermore, the long-term residue intakes were calculated based on maximum residue levels and high percentile consumption figures, which does not correspond to the standard approach.

The publication also includes extensive considerations on bee safety, which, however, are not relevant to this section of the dossier and, therefore, are not discussed here.

1. Information on the study

Data point	CA 6.10.1
Report author	Karise R. *et al.*
Report year	2017
Report title	Are pesticide residues in honey related to oilseed rape treatments?
Document No.	Chemosphere 188 (2017) 389-396
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Executive summary

Pesticide treatments before and during the flowering of honey bee forage crops may lead to residues in honey. In northern regions oilseed rape belongs to the main forage crops that is mostly cultivated by means of intensive agriculture, including several pesticide treatments. However, in addition to the focal forage crops, pesticides from non-forage crops can spread to wild flowers around fields, and thus the residues in honey would reflect the whole range of pesticides used in the agricultural landscape. The aim of our study was to clarify which currently used pesticides are present in honey gathered from heterogeneous agricultural landscapes after the end of flowering of oilseed crops.

Honey samples (N = 33) were collected from beehives of Estonia during 2013 and 2014, and analysed for residues of 47 currently used agricultural pesticides using the multiresidue method with HPLC-MS/MS and GC-MS and a single residue method for glyphosate, aminopyralid and clopyralid. Residues of eight different active ingredients with representatives from all three basic pesticide classes were determined. Although no correlation was detected between the cumulative amount of pesticide residues and percent of oilseed crops in the foraging territory, most of the residues are those allowed for oilseed rape treatments. Among all pesticides, herbicide residues prevailed in 2013 but not in 2014. Despite the relatively small agricultural impact of Estonia, the detected levels of pesticide residues sometimes exceeded maximum residue level; however, these concentrations do not pose a health risk to consumers, also acute toxicity to honey bees would be very unlikely.

Materials and methods

Study location

Honey samples were gathered from Eastern and Southern Estonia (Ida-Viru, Tartu, Polva and Valga Counties) in 2013 (N = 14) and 2014 (N = 19). This area is representative of typical agricultural landscapes in Estonia with mostly intensively managed fields, forested areas and human settlements. Among other field crops, both winter and spring oilseed rape are often grown in Estonia, and both belong to the common forage crops of honey bees. Within a 2 km radius of each hive there is on average 34.6 ± 20.7% cultivated land (min. 0.81%, max. 70.2%), 48.1 ± 20.6% forest, 5.3 ± 7.6% waste and vacant land, 7.6 ± 5.0% grassland and 2.1 ± 3.6% garden. The average oilseed crop coverage within the foraging territory remained between 0 and 12.9%.

Pesticide selection

The 47 active ingredients analysed were selected for the survey as being the most commonly used in Estonian fields according to the pesticide ordering lists of the Tartu County Farmers Association for the year 2013-2014. These include the most commonly used contemporary herbicides (21), fungicides (15) and insecticides (10), and plant growth regulator and retardant (1). The active ingredients

searched for were: 2,4D, alpha-cypermethrin, amido-sulphuron, aminopyralid, azoxystrobin, clopyralid, cypermethrin, cyproconazole, deltamethrin, dicamba, dimethachlor, dimethoate, ethyl trinexapac, fenoxaprop-p-ethyl, fenpropidin, florasulam, fludioxonil, fluoxastrobin, flutriafol, fuberidazole, glyphosate, imazalil, imidacloprid, indoxacarb, iodosulfuron-methyl-sodium, lambda-cyhalotrin, MCPA, mefenpyr-diethyl, pencycuron, picloram, pinoxaden, prochloraz, propaquizafop, propiconazole, propoxycarbazone-sodium, prothioconazole, pymetrozine, pyroxsulam, quizalofop-p-ethyl, spiroxamine, sulfosulfuron, tau-fluvalinate, tebuconazole, thiacloprid, triadimenol, triasulfuron and tribenuron-methyl.

Sample collection and handling

A total of 33 honey samples were collected from beehives in the eastern and southern part of Estonia (Tartu County and its near vicinity) during 2013 and 2014 for analysis of pesticide residues. Each honey sample originated from a different apiary, each of which consisted of 10-20 honey bee hives. The sampled hive was selected randomly for testing. The distance between sampled apiaries was at least 4 km in 2013 and at least 8 km in 2014 to preclude overlapping of the main forage area. The samples were gathered from honeycombs within beehives during the honey harvest in mid-July after the end of oilseed rape flowering. Due to the funding allocated for this study, it was decided to concentrate only on honey samples, and in order to cover more apiaries from the largest possible territory, we sampled only one hive per apiary. The honey was extracted from the comb wax and thereafter kept at 5°C until analysis.

Chemicals and materials

The reference standards of pesticides were purchased from AccuStandard (New Haven, USA) and Dr. Ehrenstorfer (Germany). HPLC grade acetonitrile and methanol were purchased from Merck-Millipore (Darmstadt, Germany). ACS grade formic acid (≥ 96.0%), acetic acid (glacial, ≥ 99.85%), and ammonium formate (99%) were purchased from SigmaeAldrich (St. Louis, MO, USA). Ultrapure deionised water was generated by a Millipore Milli-Q™ system (Billerica, MA, USA). A buffer-salt mixture (1 g trisodium citrate dihydrate, 1 g sodium chloride, 0.5 g disodium hydrogen citrate sesquihydrate and 4 g of anhydrous magnesium sulphate) and a mixture of dSPE (900 mg anhydrous magnesium sulphate, 150 mg PSA and 150 mg C18E) were obtained from Phenomenex (Torrance, CA, USA).

Stock solutions of approximately 1000 mg/L concentration were prepared by weighing 10 mg of standard in a 10 mL graduated flask and dissolving it in acetonitrile. The purity of the standard was taken into account in the preparation of standard solutions of final concentration. The mix of working standard solution with a concentration of 0.01 mg/L was prepared by diluting the appropriate volume of stock solution in acetonitrile. The stock and working standard solution were stored at – 20°C.

Sample preparation

Different sample extraction and detection procedures were used for analysis of the selected pesticides. Most compounds were analysed using QuEChERS extraction methodology followed by detection using GC-MS and UHPLC-MS/MS. Analysis of glyphosate, aminopyralid and clopyralid was performed as single analyses using extraction with methanol.

For analysis of glyphosate, aminopyralid and clopyralid, 5.0 ± 0.1 g of samples were weighed into a 50 mL polypropylene centrifuge tube, then 10 mL of water and 10 mL of methanol were added for extraction. The samples were shaken for 20 min and centrifuged for 10 min at 4500 rpm. An aliquot of extract was transferred to an autosampler vial for analysis by UHPLC-MS/MS.

UHPLC-MS/MS analysis

An Acquity UHPLC system (Waters, USA) coupled to QTrap 5500 (AB SCIEX, USA) equipped with an electrospray ionisation source was used for the analysis of pesticides in honey. The chromatographic conditions for analysis of glyphosate residues in honey are summarised in Table 1 below.

Table 1: Chromatographic conditions for analysis of glyphosate in honey

UHPLC system and conditions

Column:	Thermo Scientific, Hypercarb, 100 x 2.1 mm, 5 µm
Column temperature:	40°C
Injection volume:	10 µL
Mobile phase:	1% acetic acid in water
Column flow:	0.3 mL/min

MS system and conditions

	Quantification	*Confirmation*
Scan type:	MRM	MRM
Ionisation mode:	ESI negative	ESI negative
Ion source temperature:	500°C	500°C
Ion spray voltage [V]:	-4500	-4500
Curtain gas nebulizer [psi]:	45	45
Ion source gas 1 [psi]:	40	40
Ion source gas 2 [psi]:	60	60
Declustering potential [V]:	-50	-50
Collision energy [V]:	-20	-16
Mass transition for evaluation [*m/z*]:	168 → 63	168 → 150

Results

Performance of the method

The performance of the method was evaluated according to the EC guidance document SANCO/12571/2013. The method showed good linearity with the determination coefficients, higher than 0.990 for all compounds included in the study. The mean variation of coefficients for repeatability of the method ranged from 3.0% to 16%, and the recovery ranged from 78% to 115%.

The limit of quantification (LOQ) for which the S/N ratio exceeds 10 was assumed at a concentration level of 0.01 mg kg for all pesticides with the exception of aminopyralid, clopyralid, glyphosate, dicamba and picloram for which the LOQ was 0.05 mg/kg.

Analysis of the honey samples

The amounts and composition of pesticide residues found in the honey samples differed between years. The residues of glyphosate in honey samples are summarised in Table 2. The agricultural practices generally do not vary so much, but the need for different kinds of pesticides can vary widely from year to year.

Table 2: Concentrations of glyphosate residues found in honey samples in Estonia 2013-2014

Honey sample	**Year**	**% of oilseed rape in foraging range**	**Glyphosate residues [a] [µg/kg]**
1	2013	3.4	n.d.
2	2013	5.7	14
3	2013	6.2	**56**

Honey sample	**Year**	**% of oilseed rape in foraging range**	**Glyphosate residues [a] [µg/kg]**
4	2013	12.1	n.d.
5	2013	10	n.d.
6	2013	9.2	n.d.
7	2013	12.9	**62**
8	2013	9.2	n.d.
9	2013	9.1	n.d.
10	2013	14	n.d.
11	2013	8.6	n.d.
12	2013	5.1	n.d.
13	2013	9.2	n.d.
14	2013	9.3	n.d.
Average	*2013*	*8.86*	***44***
% of samples	*2013*		*21*
15	2014	0	n.d.
16	2014	8.6	n.d.
17	2014	1	n.d.
18	2014	3.8	n.d.
19	2014	0	n.d.
20	2014	2.8	n.d.
21	2014	8.8	n.d.
22	2014	2.7	n.d.
23	2014	11.6	(9)
24	2014	12.3	n.d.
25	2014	8.9	n.d.
26	2014	13.3	n.d.
27	2014	11.6	n.d.
28	2014	1.8	n.d.
29	2014	5.9	n.d.
30	2014	5.4	n.d.
31	2014	8.7	n.d.
32	2014	3.5	n.d.
33	2014	4.9	n.d.
Average	*2014*	*6.08*	*9*
% of samples	*2014*		*16*

(a): The numbers in parenthesis represent values under the limits of detection (LOD). The numbers in bold represent values above the maximum residue limits (MRL).

Honey as a product contains surprisingly few pesticide residues compared to bee bread or pollen (Thompson et al., 2014). Pesticide residues in different matrixes differ in their chemical composition and physical characteristics. Fat or lipid soluble compounds tend to contaminate wax, whereas water-

soluble compounds are more readily found in nectar or honey. Besides contaminated nectar, honey contamination may also occur via translocation of the compounds from comb wax to honey (Kochansky et al., 2001; Tremolada et al., 2004).

The relatively large areas with natural vegetation, and the low amounts of pesticides used in Estonian agriculture (Eurostat, 2015) has shaped the notion that the bee forage environment should be unpolluted in Estonia and probably also in other Nordic countries. Our results, however, suggest the situation may be of concern.

Despite the general low input of pesticides compared to the average usage over the European countries (Eurostat, 2015), some compounds found in honey samples exceeded the MRL. On the background of landscape characteristics, this might arise from relatively homogeneous land cover type – in Estonia, as in Ireland and the United Kingdom, the landscape in 2015 is dominated by larger areas composed of the same land cover type, also the number of structural green elements in the landscape is small (Eurostat, 2015). Larger forest areas may serve as barriers for bees, for instance. Forests have been shown to negatively affect bumble bees with larger foraging territories (Diaz-Forero et al., 2011). Such barriers may concentrate bees on other land, thus increasing the risk of forage on polluted plants. Honey bees prefer to forage in larger open areas rich in flowers, and flowering crops make up an important part of the forage. Since it is one of the most profitable crops, oilseed rape crops are common in crop rotations: covering 15% and 11% of total cultivated land in 2010 and 2015 accordingly (Statistics Estonia, 2012).

In northern regions, the most common group of pesticides sold are herbicides: these comprise more than 70% of pesticides sold in Estonia (Eurostat, 2015). The higher amounts of herbicide active ingredients needed for effective treatments compared to insecticides, for instance, may also be one reason why herbicide residues in particular were higher in our samples. The amounts of herbicides used on fields may differ from year to year depending on the weather conditions throughout the spring and summer. The amounts of herbicides sold in Estonia were higher in 2013 compared to 2014 (Eurostat, 2015) and this appears to have been reflected in our honey samples. Although pesticide residues may be retained in soils from the previous year or even from treatments made decades ago (Hilber et al., 2008; Lozowicka et al., 2016), the authors believe this probably did not affect our results because the samples with higher concentrations in 2013 did not show higher residue level in 2014. Most of the locations sampled in 2013 were also sampled in 2014. We suppose that in those cases where we found herbicide residues higher than the MRL, the bees must have foraged on recently treated fields. For instance, glyphosate residues may remain very high in nectar for up to seven days after treatment, as demonstrated by Thompson et al. (2014). Glyphosate-based herbicides are the most common herbicides worldwide. Moreover, its usage nowadays has gone beyond pest control purposes – being more of an agricultural instead of a pest management tool (Steinmann et al., 2012). We believe that this is something to consider for reducing the levels of pesticide residue found in food: by excluding the routine spray applications and retaining the weed management purpose of glyphosate, one could facilitate a less polluted environment.

The concentrations of all residues found from honey samples in this study remained below the lethal dose to honey bees. LD50 is measured for 2,4D was 0.0115 mg/bee (Extension Toxicology Network, 1996), clopyralid > 100 mg/bee (Dow AgroSciences, 2007) and glyphosate 100 mg/bee (Thompson et al., 2014), tebuconazole 83 mg/bee, azoxystrobin 200 mg/bee, dimethoate 0.11 mg/bee, thiacloprid 27.89 mg/bee, and tau-fluvalinlate 45 mg/bee (Sanchez-Bayo and Goka, 2014). This means that the concentrations found are definitely below acute lethal dosages, although sub-lethal effects cannot be excluded when considering that at least nurse bees consume the contaminated food until they produce the royal jelly, and also larger larvae are fed with nectar and pollen collected by foragers.

Conclusion

Our results demonstrate that intensively treated oilseed rape fields can be a source for pesticide residue contamination in honey, however no direct correlation was found. We believe that pesticides escape from fields over larger neighbouring areas with wild vegetation and contaminate the nectar of wild plants. Our study indicates that most of the agrochemical residues in Estonian honey can originate from oilseed treatments, however the same active ingredients are used for different crops, which is

why no direct references can be made. The compounds that were represented in the highest amounts belonged to herbicides, the most frequently used pesticide group in Northern European climatic conditions. In the context of honey as human food, the concentrations of pesticide residues do not pose any health risk to consumers, although in some cases the levels detected exceeded the MRLs. Concerning the health of bees, the residues remained below acute lethality, however some sub-lethal effects cannot be excluded.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication is considered relevant to the setting of a suitable MRL for glyphosate in honey since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. Although only limited information is given about the validation of the method for the determination of glyphosate residues, the analytical results are most likely reliable. The residue levels found for glyphosate are consistent with the EU-monitoring data published by EFSA for 2016-2017 in that: 1. Most of the samples do not show quantifiable residues of glyphosate. 2. Some samples show residues > 0.05 mg/kg, which indicates that it is appropriate to increase the existing MRL. 3. The measured residue levels are far below the levels found in the tunnel residue study.

1. Information on the study

Data point	CA 6.10.1
Report author	Rubio, R. et al.
Report year	2014
Report title	Survey of glyphosate residues in honey, corn and soy products
Document No.	J Environ Anal Toxicol 2014, Vol 5(1): 249
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

Samples of honey (sixty nine), pancake and corn syrup (twenty six), soy sauce (twenty eight), soy milk (eleven), and tofu (twenty) purchased in the Philadelphia, US metropolitan area were analyzed for glyphosate residue using ELISA. The limit of quantification (LOQ) and range of the method were determined for honey, pancake syrup, and corn syrup to be 15 to 800 ppb; soy sauce, soy milk, and tofu 75 to 4000 ppb. Glyphosate residues above the limit of quantification were not found in pancake and corn syrup, soy milk, and tofu. Of the sixty-nine honey samples analyzed, forty-one samples, or fifty-nine percent (59%), had glyphosate concentrations above the method LOQ (15 ppb), with a concentration range between 17 and 163 ppb and a mean of 64 ppb. Eleven of the tested honey samples were organic; five of the organic honey samples, or forty-five percent (45%), contained glyphosate concentrations above the method LOQ, with a range of 26 to 93 ppb and a mean of 50 ppb. Of the fifty-eight non-organic honey samples, thirty-six samples, or sixty-two percent (62%), contained glyphosate concentrations above the method LOQ, with a range of 17 to 163 ppb and a mean of 66 ppb. In addition to comparison of production method (organic *vs.* conventional), the honey results were evaluated according to pollen source and by country of origin, grouped by GMO usage (prohibited, limited, or permitted). Glyphosate concentrations above the method LOQ (75 ppb) were also found in ten of the twenty-eight soy sauce samples evaluated (36%), with a concentration range between 88 and 564 ppb and a mean of 242 ppb; all organic soy sauce samples tested were below the method LOQ.

Materials and Methods

Chemicals and reagents

Chemicals were of reagent grade and were purchased from Sigma Chemical Company, St. Louis MO, USA, except as indicated. Glyphosate (> 98% purity), Chem Service, West Chester, PA, USA. Glyphosate micro titer plate ELISA, Abraxis PN 500086; Glyphosate sample diluent, PN 500082, Abraxis LLC, Warminster, PA, USA. Glyphosate stock solution was prepared in deionized water to 1.0 mg/mL; spiking solutions were prepared from the working solution using deionized water.

Samples and sample preparation/extraction

In total, 153 representative samples were purchased from markets in the Philadelphia metropolitan area (69 honey, 26 corn and pancake syrup, 28 soy sauce, 11 soy milk, and 20 tofu products).

Honey, corn and pancake syrup samples: A 0.50 g aliquot of sample was weighed into a micro centrifuge tube and 0.50 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, then diluted by adding 40 µL of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/

extraction described above produced a 1:200 sample dilution.

Soy sauce: A 0.10 mL aliquot of sample was transferred into a micro centrifuge tube and 0.90 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, then diluted by adding 40 µL of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Soy milk: A 0.10 mL aliquot of sample was transferred into a micro centrifuge tube and 0.90 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, and then centrifuged at 6000 x g for 5 minutes. The sample was then diluted by adding 40 µL of the middle layer of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Tofu: A 1.0 g aliquot of sample was weighed into a 20 mL vial and 10.0 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, and then allowed to separate for 2 minutes. Approximately 1 mL of the mixture was transferred into a micro centrifuge tube and centrifuged at 6,000 x g for 5 minutes. The sample was then diluted by adding 40 µL of the middle layer of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Determination of glyphosate in samples

The instructions provided in the ELISA kit user's guide were followed, in brief, glyphosate calibrators provided in the kit and the samples to be tested are derivatized for ten minutes and then added, along with an antibody specific for glyphosate to micro titer wells coated with goat anti-rabbit antibody and incubated for thirty minutes with shaking. A glyphosate horseradish peroxidase (HRP) enzyme conjugate is then added. At this point a competitive reaction occurs between the glyphosate, in the calibrators or samples, and the enzyme labeled glyphosate for the antibody binding sites on the micro titer well. The reaction is allowed to continue for sixty minutes. After a washing step an enzyme substrate (hydrogen peroxide) and the chromogen (3,3',5,5'-tetramethylbenzidine) are added. The enzyme-labeled glyphosate bound to the glyphosate antibody catalyzes the conversion of the substrate /chromogen mixture to a colored product. After an incubation period, the reaction is stopped and stabilized by the addition of diluted acid and read in a Molecular Devices micro titer plate reader (450 nm). Since the labeled glyphosate (conjugate) was in competition with the unlabeled glyphosate (sample) for the antibody sites, the color developed is inversely proportional to the concentration of glyphosate in the sample.

Data analysis

The evaluation of the assay was performed using Molecular Devices Soft max pro evaluation program (4-Parameter). The program calculates the mean absorbance value for each of the standards (Bi) and calculates the %Bi /B0 for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance (B0). The program then constructs a non-linear regression model of a standard curve by plotting the % Bi/B0 for each standard on the vertical linear (y) axis versus the corresponding glyphosate concentration on the horizontal logarithmic (x) axis. The % Bi/B0 for samples is interpolated using the standard curve yielding sample concentration levels of glyphosate from the standard curve. Correlation coefficients of the assays were >0.995 and standard deviation between standard replicate analysis were < 10%.

Validation, performance and quality control

Specificity had been previously determined (ELISA user's guide), (**Table 1**). Recovery, limit of quantitation, range and limit of quantification were determined to test the validity of the dilution/ extraction procedures of each of the matrices used in combination with the glyphosate ELISA.

Table 1: Cross-reactivity table. The reactivity of glyphosate to various related compounds expressed as LOD and as the dose required for 50% absorbance inhibition (50% B/Bo).

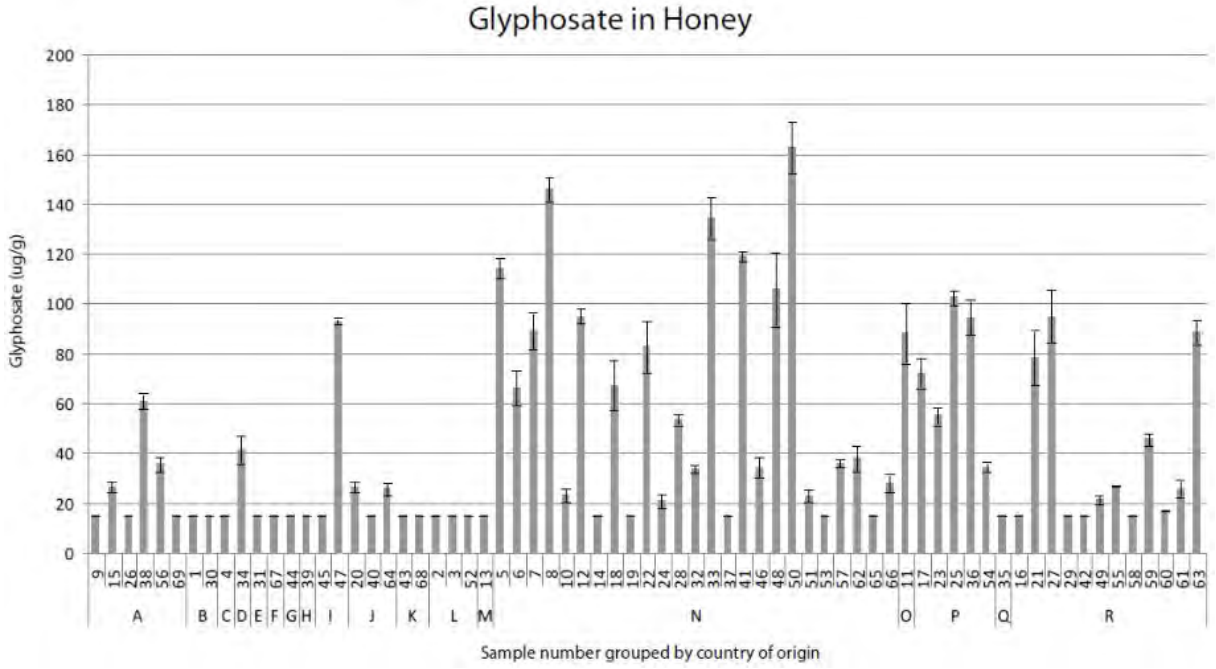
COMPOUND (B/Bo)	LOD(ng/mL)	50% B/Bo(ng/mL)
Glyphosate	0.05	0.5
Glyphosine	50	3,000
Glufosinate	2,000	70,000
AMPA	35,000	>1,000,000
Glycine	>10,000	>1,000,000

Results and Discussion

The method performance for glyphosate analysis was determined by conducting recovery tests on each of the matrices. To determine the accuracy of the glyphosate analysis for the sample matrices analyzed in this study, matrix samples that were glyphosate negative and positive (positive samples were not encountered with tofu, soy milk, pancake and corn syrup) were spiked as follows: 15, 40, 100, 200 and 400 ng/ mL (honey, pancake and corn syrup); 75, 200, 500, 1000 and 4000 ng/mL [soy sauce, soy milk and tofu (ng/g)]. Analysis was performed in duplicate for all unspiked and spiked samples at all levels. Average recovery obtained for glyphosate negative honey samples fortified with glyphosate was 119%, (SD = 10). Average recovery for glyphosate positive honey (unspiked contained 44 ng/g glyphosate) after fortification was 116% (SD = 10). Average recovery for negative soy sauce was 94% (SD = 5), and for positive fortified soy sauce (unspiked contained 417 ng/mL) was 86% (SD = 5). The limit of quantification and range of the method were determined for honey, pancake and corn syrup to be 15 to 800 ng/g; soy sauce, soy milk, and tofu 75 to 4000 ng/ mL or ng/g, respectively.

In this study, the first sample matrix analyzed for the presence of glyphosate was honey; 69 samples were analyzed and classified into 18 groups depending on the country of origin listed on the bottles: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. The glyphosate concentrations obtained are shown in (**Figure 2**). Forty-one out of the sixty-nine honey samples analyzed, or fifty nine percent (59%), had glyphosate concentrations above the method LOQ (15 ng/g) with a concentration range between 17 and 163 ng/g and a mean of 64 ng/g.

Figure 2: Concentration of glyphosate (ng/g) in honey samples listed by honey origin: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. Dashed line represents LOQ of method (15 ng/g). Error bars represent concentrations obtained during duplicate analysis.



The glyphosate concentration in honey grouped by flower (pollen) source is shown in (**Figure 3**). The pollen types listed on the bottles were: clover (12 samples), exotic (11 samples), wildflower (11 samples), unknown (35 samples). (**Figure 4**) depicts the concentration of glyphosate in honey samples grouped by growing method of source pollen: organic (11 samples) and traditional (58 samples); 5 of the 11 organic samples had glyphosate concentrations above the method LOQ with a range of 26 to 93 ng/g and a mean of 50 ng /g. Of the fifty-eight non-organic honey samples, thirty-six samples, or sixty-two percent (62%), contained glyphosate concentrations above the method LOQ, with a range of 17 to 163 ppb and a mean of 66 ppb.

Figure 3: Concentration of glyphosate (ng/g) in honey samples by flower (pollen) source. Dashed line represents LOQ of method (15 ng/g). Exotic flowers were sophora, manuka, orange, cactus, summer flower, lychee, alfalfa, acacia). Error bars represent concentrations obtained during duplicate analysis.

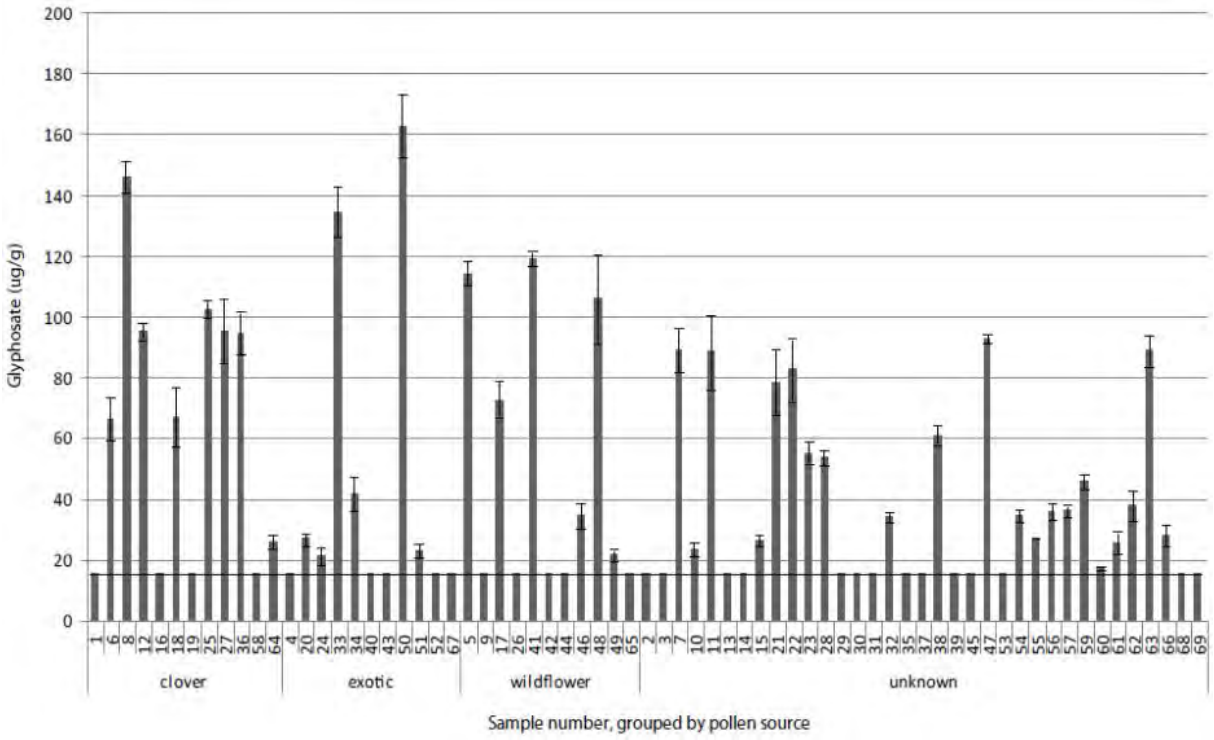


Figure 4: Concentration of glyphosate (ng/g) in honey samples by growing method of source pollen (Organic *vs.* Traditional). Dashed line represents LOQ of method (15 ng/g).) Error bars represent concentrations obtained during duplicate analysis.

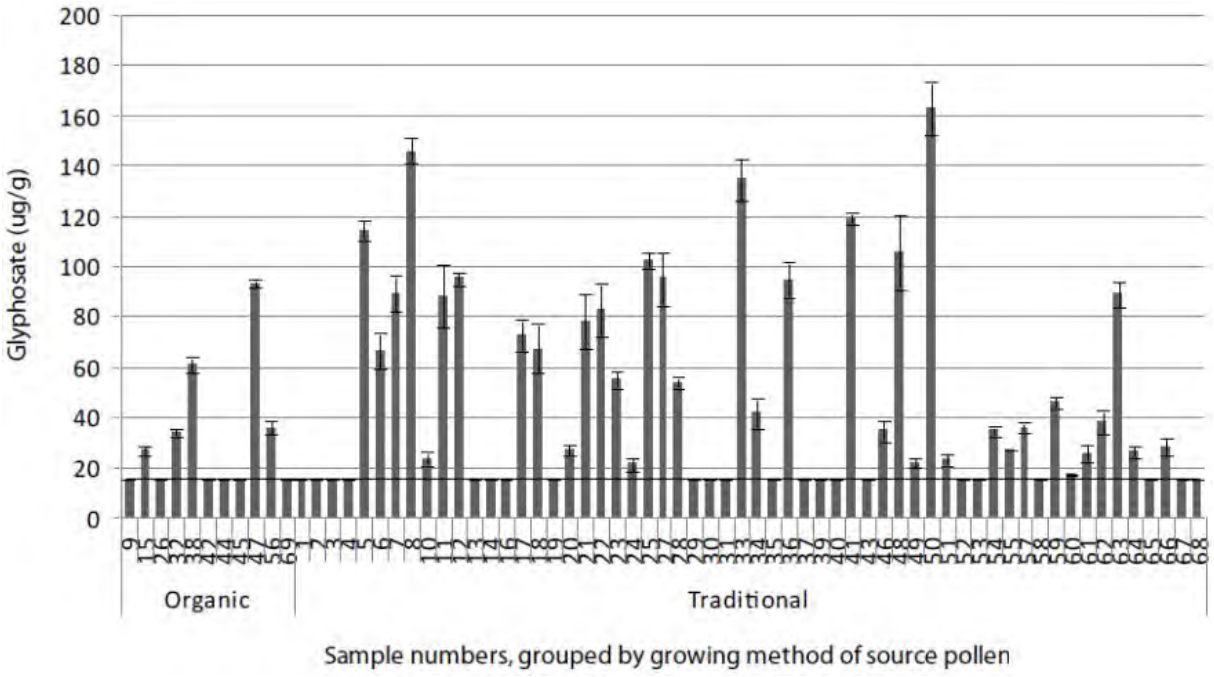
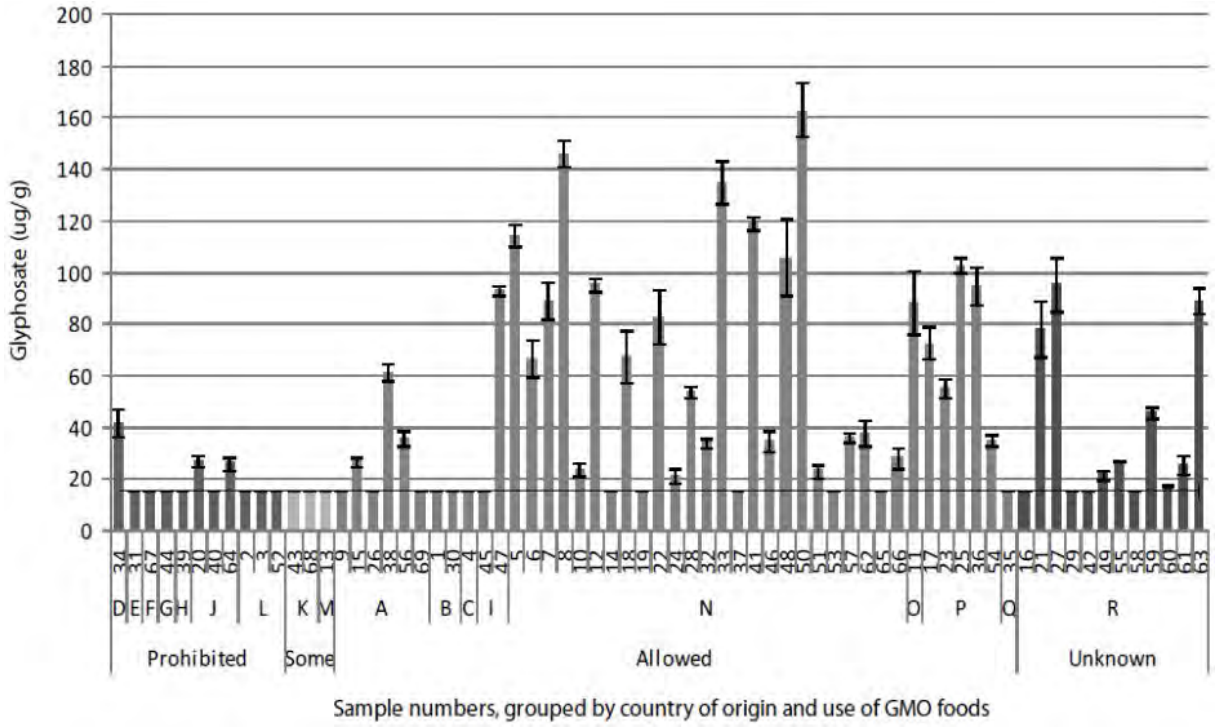


Figure 5 depicts the concentration of glyphosate in honey by country and whether the use of genetically modified organisms (GMO) seeds is prohibited or permitted. The graph also shows where some minimum uses of GMO traits are allowed (Spain, and blend of Vietnam/Ukraine). The glyphosate

concentration in honey originating in countries that do not allow or allow limited GMO traits (3 out of 14 samples above the LOQ) ranged from 26 to 41 ng/g with a mean of 31 ng/g. The glyphosate range for those countries that allow GMO (30 out of 43 samples above LOQ) was 21 to 163 ng/g with a mean of 71 ng/g. Samples of unknown origin (8 out of 12 samples above LOQ) ranged from 17 to 95 ng/g with a mean of 50 ng/g.

Figure 5: Concentration of glyphosate (ng/g) in honey samples listed by honey origin and the allowance of GMO use: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. Dashed line represents LOQ of method (15 ng/g). Error bars represent concentrations obtained during duplicate analysis.



The second matrix group analyzed for glyphosate was soy sauce. The analysis consisted of 28 samples, (**Figure 6**). Ten out of 28 samples (36%) had glyphosate concentrations above the method LOQ (75 ng/mL) with a concentration range between 88 and 564 ng/mL and a mean of 242 ng/mL. (**Figure 7**) shows the concentration of glyphosate in soy sauce by method of soy bean growing (organic vs. traditional). The recent report from the Chinese Academy of Medical Science and the Beijing Union Hospital [20] reported an average glyphosate concentration in soy sauce of 133 ng/mL in samples that did not specify on the bottle whether or not the raw material was GM soybean. In our study, the small subset of organic labeled samples (three) was all below the limit of quantitation of the test.

Figure 6: Concentration of glyphosate (ng/mL) in soy sauce samples. Dashed line represents LOQ of method (75 ng/mL). Error bars represent concentrations obtained during duplicate analysis.

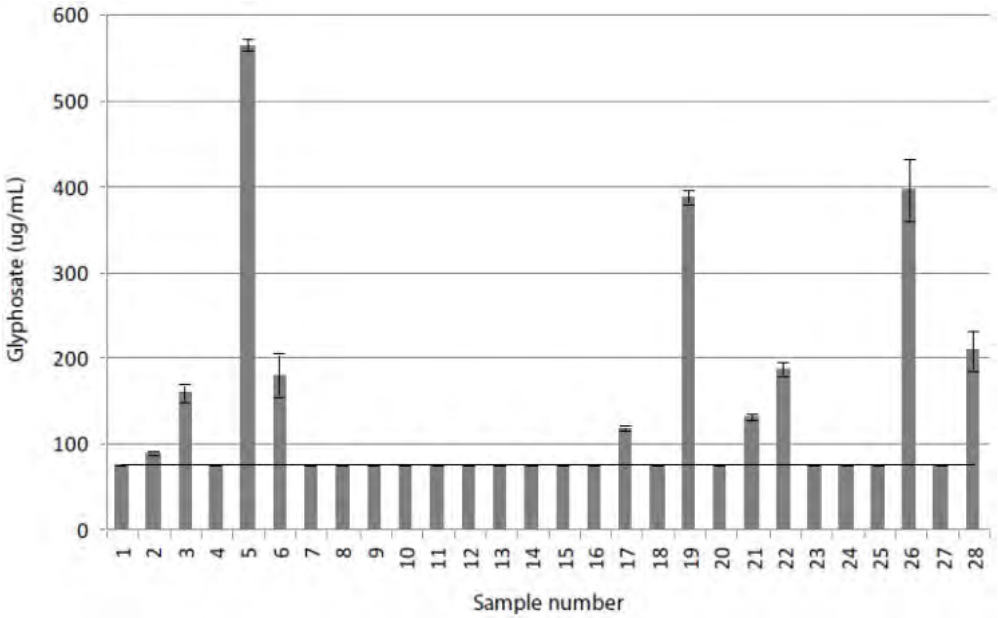
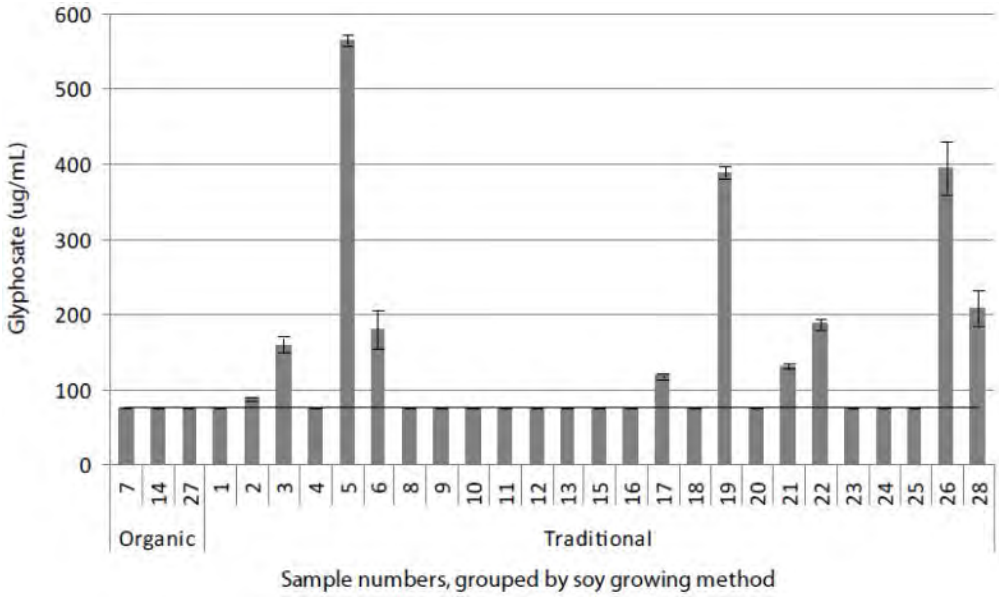


Figure 7: Concentration of glyphosate (ng/mL) in soy sauce samples by growing method of soy beans (Organic *vs.* Traditional). Dashed line represents LOQ of method (75 ng/mL). Error bars represent concentrations obtained during duplicate analysis.



Corn and pancake syrup (26 samples), soy milk (11 samples), and tofu (20 samples) tested were negative for glyphosate at the LOQ of the method (15 ng/g for pancake and corn syrup, and 75 ng/mL or ng/g for soy milk and tofu, respectively).

Studies on glyphosate residues in food are scarce. Among the few studies found was a recent report published on the incidence of glyphosate in soy sauce, conducted by the Chinese government [20]. Searches were conducted by the authors using various scientific databases on the concentration and incidence of glyphosate in honey, but these failed to provide any information. The honey samples analyzed in the present study show that 59% of all samples contained glyphosate residues (ranging from 17 to 163 ng/g, mean 64 ng/g); the residue concentration does not seem to depend on pollen source or growing method, even organic honey contained glyphosate residues (5 out 11 samples, or 45%, mean

glyphosate concentration 50 ng/g). Comparing the concentration of glyphosate in honey by countries that use GMO extensively with countries that allow the use of some GMO traits and those that do not allow GMO, shows that, in general, glyphosate levels are lower in samples from countries that do not allow or allow limited use of some GMO traits, such as Spain and Vietnam/ Ukraine blend (mean 31 ng/g), compared to those countries that allow planting of GMO traits (71 ng/g). It should be noted, however, that some residues of glyphosate (although < 50 ng/g) were found in honeys originating from Germany and New Zealand, countries where no GMO planting is allowed.

The European Union has specific guidelines for the labeling of organic honey [25,26]. According to those guidelines, the location of apiaries is strictly controlled and states that "Nectar and pollen sources available over a three-kilometer radius around the apiary sites must consist essentially of organically produced crops or crops treated with low-environmental-impact methods. Apiaries must also be far enough away from any non-agricultural production source that could lead to contamination (*e.g.* urban centers, waste dumps, waste incinerators, etc.). Member States have the option of prohibiting the production of organic honey in certain regions or areas that do not meet these conditions. Organic honey must not contain chemicals residues (synthetic pesticides, etc.)." The United States has no such guidelines for the organic production of honey, but uses organic farming certification for honey labeling purposes; one reason is that it is practically impossible to regulate without testing all honey for residues since bees can fly up to 3 miles in search of nectar and it is difficult to be certain that they do not feed on nectar contaminated by crop spraying or industrial sources. In the EU, glyphosate residues in non-organic honey regulatory limits are 50 ng/g [27], the United States does not have a limit in honey. The limit in drinking water in the United States is 700 ng/mL; the reference dose is 1.75 mg/kg/day; the One-Day Health Advisory level is 20 mg/L [28]. Also, it is widely known that like milk and olive oil, honey is one of the foods that is most commonly mislabeled and adulterated [29] providing yet another source of glyphosate contamination in honeys that, according to the bottle label, originated in non-GMO countries.

Bee colony collapse disorder (CCD) is a growing threat to the efficient production of food around the world. Honey bees pollinate nearly 130 species of plant life [30], such as fruits, vegetables, nuts, and seed crops. Honeybees are therefore indirectly responsible for an estimated one-third of the world food supply [31]. Although several factors are involved in CCD, including numerous pathogens and parasites, the extensive use of pesticides [32,33] such as neonicotinoids have provided evidence that these products are harmful to honey bees and have lead to a recent ban or restriction in the use of three neonicotoids by the European Union [34]. Although glyphosate is not acutely toxic to bees, it is chronically toxic to animals and is reported to disrupt the endocrine system [35,36] and a recent study indicates that honey bees exposed to increasing sub-lethal concentrations of glyphosate exhibit a decrease in acetyl cholinesterase (AChE) activity [37]. The high rate of glyphosate use creates the potential for wide-spread contamination of our food chain. Glyphosate is used throughout the bee foraging period in high amounts and is found in the air, water, and in plant parts frequented by bees, such as flowers and buds, potentially contaminating the nectar collected by bees from contaminated plants [38]. Based on its prevalence in the environment, as well as our findings in honey samples, we propose that future studies should be conducted to determine if glyphosate is in fact a contributing factor in CCD.

Conclusions

This study indicates the presence of glyphosate residues in honey and soy sauce, but not in pancake and corn syrups or soy based products such as soy milk and tofu. Forty one out of sixty nine (59%) honey samples analyzed contained glyphosate at a concentration above the method LOQ (15 ng/g) with a range between 17-163 ng/g and a mean of 64 ng/g. Ten out of twenty eight (36%) soy sauce samples contained glyphosate at a concentration above the method LOQ (75 ng/mL) with a range between 88-564 ng /mL and a mean of 242 ng /mL. Future studies should be conducted on many other food products to determine the extent of glyphosate residue contamination.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a survey of glyphosate residues in honey (n = 69), pancake and corn syrup (n = 26), soy sauce (n = 28), soy milk (n = 11) and tofu (n = 20) purchased in USA, but originating from various countries around the globe. In the context of the dossier for the renewal of the EU approval of glyphosate and with regard to the supported representative uses, the residue data for pancake and corn syrup, soy sauce, soy milk and tofu are not considered relevant. However, the residue data for glyphosate in honey are potentially relevant since according to the guideline SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. Only few of the analysed honey samples originated from Europe but, as honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL.

The samples were analysed by means of an ELISA method which was validated by determining the recovery rates from fortified samples. The validation results are not provided in detail, but the average recoveries and relative standard deviations were satisfactory, although the validation was not conducted exactly in accordance with EU or OECD guidelines (i.e. with at least 5 replicates at the LOQ and 5 replicates at a higher level). The limit of quantification was estimated at 0.015 mg/kg. The specificity of the method was investigated by assessing the response of the ELISA test to a series of substances chemically related to glyphosate and it was shown that the response of these substances was at least 1000 times less than that of glyphosate. While this experiment allows to exclude some possible sources of false-positive results, it does not allow to completely rule out that other (not tested compounds) may yield false positive results. Despite these limitations, the obtained analytical results are considered fairly reliable.

59% percent of the 69 honey samples contained glyphosate residues above the method LOQ (0.015 mg/kg) with a concentration range between 0.017 and 0.163 mg/kg and a mean of 0.064 mg/kg. While the individual results are not provided, it seems that about 31% of the samples (22 from 69) showed residues of glyphosate above the EU MRL of 0.05 mg/kg. The samples originating from the EU all showed residues < 0.05 mg/kg. Overall, the findings reported in the publication are in line with the results of the EU-monitoring since the publication shows that glyphosate can occur in honey at levels > 0.05 mg/kg and that it is, therefore, appropriate to increase the existing EU-MRL. The highest measured residue level was 0.163 mg/kg, which is less than the maximum value found during the EU-monitoring for 2016-2017.

1. Information on the study

Data point	CA 6.4.2
Report author	Schnabel K. *et al.*
Report year	2017
Report title	Effects of glyphosate residues and different concentrate feed proportions on performance, energy metabolism and health characteristics in lactating dairy cows
Document No.	Archives of Animal Nutrition, 2017, Vol. 71, No. 6, 413-427
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

The aim of this study was to examine the influence of glyphosate (GL) residues in feedstuffs on performance, energy balance and health-related characteristics of lactating dairy cows fed diets with different concentrate feed proportions. After an adaption period, 64 German Holstein cows (207 ± 49 d in milk; mean ± SD) were assigned to either groups receiving a GL contaminated total mixed ration (TMR) (GL groups) or an uncontaminated TMR (CON groups) during a 16 weeks trial. Contaminated feedstuffs used were legally GL-treated peas and wheat (straw and grain). GL and CON groups were subdivided into a "low concentrate" group (LC) fed on dry matter (DM) basis of 21% maize silage, 42% grass silage, 7% straw and 30% concentrate and a "high concentrate" group (HC) composed of 11% maize silage, 22% grass silage, 7% straw and 60% concentrate for ad libitum consumption. Body condition score, body weight, DM intake and milk performance parameters were recorded. In blood serum, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and glucose were measured and energy balance was calculated. Milk was analysed for GL residues.

At week 0, 7 and 15, general health status was evaluated by a modified clinical score. The average individual GL intake amounted for Groups CON_{LC}, CON_{HC}, GL_{LC} and GL_{HC} to 0.8, 0.8, 73.8 and 84.5 mg/d, respectively. No GL residues were detected in milk. GL contamination did not affect body condition score, body weight, DM intake, nutrient digestibility, net energy intake, net energy balance or BHB, glucose, NEFA and milk performance parameters; whereas concentrate feed proportion and time did affect most parameters. The clinical examination showed no adverse effect of GL-contaminated feedstuffs on cows' health condition. In the present study, GL-contaminated feedstuffs showed no influence on performance and energy balance of lactating dairy cows, irrespective of feed concentrate proportion.

Materials and Methods

Experimental design

Sixty-four German Holstein cows (207 ± 49 d in milk; mean ± SD) were used in a 17 weeks trial. The experiment was designed as a 2×2 factorial design with GL contamination and concentrate proportion in feed as the main factors. At the start of the experiment (week 0), all animals were fed with an energetically adequate total mixed ration (TMR), based on the recommendations of the Society of Nutrition Physiology (GfE 2001) consisting of 30% maize silage, 30% grass silage and 40% concentrate on a dry matter (DM) basis. To provide equal conditions in the following 16 weeks of trial, 48 cows and 16 heifers were assigned to four different feeding groups by considering number of lactation (2.8 ± 0.7) and data that had been collected prior to the trial, presenting a 3-d mean of body weight (BW, 645 ± 21 kg), daily feed intake (40.9 ± 0.2 kg fresh matter of the ration) and fat corrected

milk (FCM, 4% fat; 29.1 ± 0.5 kg). Half of the animals received in their ration GL contaminated peas and wheat kernels, processed in concentrate and GL contaminated straw (Groups GL). As control group, the other half of cows received a non-contaminated ration (Groups CON). Both groups were subdivided into one group receiving a diet with a low concentrate proportion (LC) composed on DM basis of 21% maize silage, 42% grass silage, 7% straw and 30% concentrate, and another group receiving a diet with high concentrate proportion (HC) composed of 11% maize silage, 22% grass silage, 7% straw and 60% concentrate . TMR and water were provided *ad libitum*. Cows were kept in a free stall-barn, Groups GL and CON were separated by the feed alley, and within each group subgroups LC and HC were separated by fences inside the barn.

Feedstuff production, animal measurements and sample collection

Maize, grass, peas and wheat were grown on the acreage of the experimental station of the Friedrich-Loeffler-Institut (FLI), in Braunschweig, Germany, to generate equal growth and soil conditions. The acreage had not been treated before with GL for at least 3 years. Maize and grass were grown without GL-application. For GL contamination Roundup Record® (007525–60/MOT), Monsanto, Agrar Deutschland GmbH (Düsseldorf, Germany) was used as water-soluble granulate, containing as active ingredient 720 g GL per kg GL solution. A part of wheat and peas was treated with Roundup Record®, in pre-harvest application with 2.5 l/ha for wheat and 2 l/ha for peas, according to the legal regulations [Regulation (EC) No. 396/2005 of the European Parliament and of the council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC] while another part remained untreated and served for uncontaminated control feedstuffs. GL contaminated and non-contaminated feedstuffs were harvested and stored separately to avoid cross contamination.

During the trial, samples of maize and grass silage were taken twice a week, while samples of straw and concentrate were taken once a week and pooled over 4 weeks. Water and DMI were recorded daily by computerised feeding bins (type RIC; Insentec B.V., Marknesse, The Netherlands). Every second week the body condition score (BCS) was evaluated using a 5-point scale (Edmonson et al. 1989). Cows were milked twice daily beginning at 05:30 h and at 15:30 h and milk yield was recorded using automatic milk counters (Lemmer Fullwood GmbH, Lohmar, Germany). Morning and evening milk samples were collected twice a week. In week 0 and 16, an additional morning and evening milk sample was taken and pooled according to their proportion of total daily milk yield and frozen at −20°C. BW was recorded automatically by a scale after leaving the milking parlour. Blood samples were taken after morning milking from a jugular vein in serum tubes at week 0, 4, 8, 12 and 16.

Analyses

Feed samples were dried at 60°C before analysis for chemical composition according to the methods of the VDLUFA (1993) applying method number 3.1 (DM), 8.1 (crude ash), 4.1.2 (crude protein), 5.1.1 (ether extract), 6.1.1 (crude fibre), 6.5.1 (neutral detergent fibre without ash, amylase treated) and 6.5.2 (acid detergent fibre without ash). The TMR of each treatment group was tested for the apparent digestibility of crude nutrients and net energy for lactation (NE_L) content by using German Blackhead/SKF wethers according to the regulations published by the Society of Nutrition Physiology (GfE 1991).

GL and aminomethylphosphonic acid (AMPA) concentration in feed samples were measured by an accredited laboratory (Wessling GmbH, Altenberge, Germany). Samples were extracted with formic acid (0.1%) and methylene chloride. Derivatisation was conducted with fluorenylmethoxycarbonyl chloride. After solid-phase extraction, GL and AMPA were determined by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). GL and AMPA were quantified using internal standards containing 1.2-$^{13}C_2{}^{15}N$ GL (1 µl/ml) and $^{13}C^{15}N$ AMPA (1 µl/ml). The limit of detection (LOD) and the limit of quantification (LOQ) for each substance were calculated from the signal-to-noise ratio amounting to 3 for the LOD and 10 for the LOQ, whereby the LOQ and LOD of the feed samples were 0.02 and 0.007 mg/kg for GL and AMPA, respectively. The recoveries for GL and AMPA analyses in feed samples were 70–120% using an internal standard concentration of 0.625 mg/kg for feed analyses.

Milk samples were analysed for fat, protein, lactose and urea using an infrared milk analyser (Milkoscan FT 6000®; Foss Electric, Hillerød, Denmark). Somatic cell count (SCC) was detected by flow cytometric measurement (Fossomatic 500®, Hillerød, Denmark). GL was determined in milk samples by Federal Office of Consumer Protection and Food Safety (BVL, Marienfelde, Berlin). Based on QuPPe-Method (Anastassiades et al. 2015), the samples were homogenised, water content adjusted to 100% and glyphosate $^{13}C_2{}^{15}N$ was added as internal standard. Afterwards, samples were extracted with MeOH/Cyclohexan. and purified with acetonitrile. After degreasing by freezing, derivatisation was conducted with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl). GL was analysed by LC-MS/MS equipped with electrospray ionisation source (negative mode). Confirmation was performed by diagnostic ions (precursor ion (*m/z*): 390, 167.9; production (*m/z*): 390, 149.9. The LOQ was 0.01 mg/kg (recovery rate 104%) which is according to (SANTE/11945/2015) the lowest spike level of the validation with recoveries between 70 and 120% and a within laboratory reproducibility $RSD_{wR} \leq 20\%$.

Blood samples were analysed for serum concentrations of β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and glucose, after centrifugation, using an automatic analysing system, based on photometric measurement (Eurolyser®, Type VET CCA, Salzburg, Austria).

Clinical examination

At weeks 0, 7 and 15, the general health status of all cows was evaluated by a modified clinical score according to Seyboldt et al. (2015); Dirksen et al. (2012). Scoring was performed by two veterinarians who were unaware of the treatment group at the examination. The score system for most parameters was 0–2 (0, no symptom; 1, moderate symptom; 2, severe symptom); however, the locomotion system got a score of 0–3 (0, no symptom; 1, mild symptom; 2, moderate symptom; 3, severe symptom). Those parameters leading to yes or no answers were scored only 0–1 (0, no symptom, 1, symptom). For evaluation, the data were summarised to four different symptom complexes, namely respiration and cardiovascular system including 22 tested parameters (max score of 35), gastrointestinal tract including 13 tested parameters (max score of 30). Furthermore, udder and locomotion system score were counted separately for each quarter of the udder and each leg. Consequently, udder health included five tested parameters (max score of 29), and locomotion system included 20 tested parameters (max score of 238). If a cow reached the maximal symptom score in all parameters of one symptom complex, we postulated 100% illness in that complex. For the total cumulative health score, each of the individual complexes then counted as 25%. For example, if one cow would reach 100% in one complex, the total illness would result in 25% whereas a cow with 0% in each complex would be considered as completely healthy.

Calculations

The energy content of the experimental TMR was calculated based on nutrient digestibility measured with wethers (GfE 1991) and on equations for calculation of energy content in feedstuffs published by the Society of Nutrition Physiology (GfE 2001), as well as net energy requirement for maintenance (NE M), feed content and requirement for NE_L and milk energy:

$$NE_M\,[\text{MJ } NE_L/\text{d}] = 0.293 \times BW^{0.75}\,[\text{kg}]$$

$$NE_L\,\text{content}\,[\text{MJ/kg feed}] = 0.6 \times [1 + 0.004 \times (\text{q - } 57)] \times \text{ME}\,[\,\text{MJ/kg}\,]$$

$$NE_L\,\text{requirement}\,[\text{MJ } NE_L/\text{d}] = [\text{Milk energy output}\,[\text{MJ } NE_L/\text{d}\,] + 0.086] \times \text{Milk yield}\,[\,\text{kg/d}\,]$$

$$\text{Milk energy}\,[\text{MJ } NE_L/\text{kg}] = 0.38 \times \text{Milk fat}\,[\%] + 0.21 \times \text{Milk protein}\,[\,\%] + 0.95$$

FCM (4% fat) was calculated based on the equation of Gaines (1928):

$$\text{FCM}\,[\text{kg/d}] = [(\text{Milk fat}\,[\%] \times 0.15) + 0.4] \times \text{Milk yield}\,[\,\text{kg/d}\,]$$

Energy-corrected milk (ECM) was calculated based on the equation of Sjaunja et al. (1990):

$$\text{ECM [kg/d]} = \text{Milk yield [kg/d]} \times$$
$$[(38.3 \times \text{Milk fat [g/kg]} + 24.2 \times \text{Milk protein [g/kg]} + 16.54 \times \text{Milk protein [g/kg]} + 20.7) / 3140]$$

Net energy (NE) balance was calculated as follows:

$$\text{NE balance [MJ NE}_L\text{/d]} = \text{Energy intake [MJ NE}_L\text{/d]} - \{\text{NE}_M\ [\text{MJ NE}_L\text{/d}] + \text{NE}_L\ [\text{MJ NE}_L\text{/d}]\}$$

Statistical analyses

Before data analyses, data of DMI, BW, NE balance and milk performance were condensed to a 14 d mean. Variables were all tested for normal distribution via visual histogram plot, only the number of cell counts had to be given as decimal logarithmic value. All statistical analyses were performed using the Software SAS (Version 9.2; SAS Institute Inc., Cary, North Carolina, USA). Parameters were analysed using the MIXED procedure for repeated measures (Littell et al. 1998). In case the variable showed significant effect between the groups in week 0, week 0 of that variable was set as covariable. For each variable covariance, structure was tested for compound symmetry (CS), autoregressive (1) AR (1) and unstructured (UN), and the model which proved the best Akaike information criterion for a finite sample size (AICC) was chosen. The model contained GL contamination (GL), concentrate feed proportion (CFP) and time (t) measured in trial weeks as fixed effects and the interaction between GL and CFP, GL and t, CFP and t and GL, CFP and t. Effects were declared as a trend if *p*-values were ≤ 0.10 and as significant if *p*-values were ≤ 0.05 after Tukey's test. Results are presented as Least Square (LS) Means ± standard error (SE) of LS means unless otherwise stated.

Results

In total, 61 out of the initial 64 cows completed the entire trial. Two cows were excluded because of diseases not related to the experimental treatments. In the first week of the trial, one cow of group GL HC had an abomasal displacement. A cow of Group GL LC developed a general peritonitis in week 8. Another cow of Group GL LC became dry in trial week 11 and was excluded from the trial.

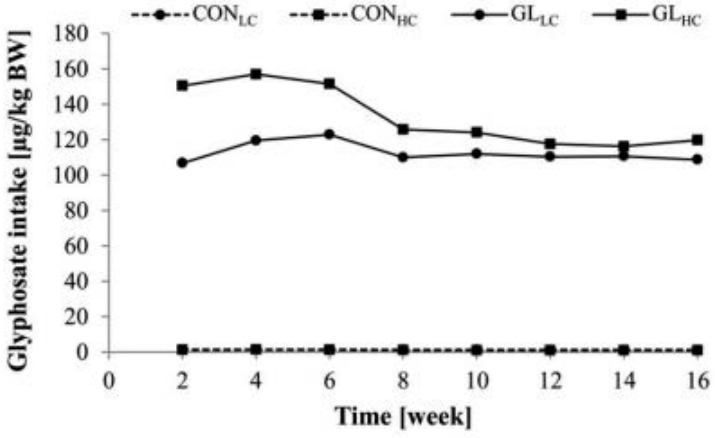
The chemical composition of the cows' ration components (Table 1) was within the normal range for the respective feedstuffs (DLG 1997). The average daily GL intake in Groups CON LC and CON HC was 0.8 and 0.8 mg/d, respectively, and in Groups GL LC and GL HC 73.8 and 84.5 mg/d, respectively. Figure 1 presents an overview of the cows' daily intake in the particular trial weeks in µg per kg BW).

Table 1: Ingredients of concentrate and chemical composition of the feedstuffs used in the total mixed ration (TMR).

	Concentrate composition[#]				Roughage[†]			
	Group CON_{LC}	Group CON_{HC}	Group GL_{LC}	Group GL_{HC}	Straw CON	Straw GL	Grass silage	Maize silage
Ingredients [% DM]								
Peas	29	29	-	-				
Peas GL treated	-	-	29	29				
Wheat	36	36	-	-				
Wheat GL treated	-	-	36	36				
Corn	26.8	30.8	26.8	30.8				
Urea	1.3	0.6	1.3	0.6				
Calcium carbonate	1.5	0.7	1.5	0.7				
Soybean oil	1	1	1	1				
Vitamin/mineral premix[§]	4.4	1.9	4.4	1.9				
Chemical composition								
Dry matter (DM) [g/kg]	881	884	885	881	899	895	324	345
Nutrients [g/kg DM]								
Crude ash	70	44	70	44	58	57	118	41
Crude protein	160	143	163	144	25	32	140	71
Ether extract	34	40	34	40	11	11	35	32
Crude fibre	34	34	34	35	436	440	298	230
$aNDF_{om}$[‡]	106	116	111	115	806	808	550	402
ADF_{om}[•]	43	45	42	46	504	507	326	237
Starch	589	610	591	612				322
Sugar	38	38	38	38				
Herbicide agent residue [mg/kg DM]								
Glyphosate	0.03	0.00	0.37	0.43	0.57	61.81	0.00	0.00
AMPA*	0.00	0.00	0.00	0.00	0.00	0.59	0.00	0.00

[#]CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR); [†]Composition (on DM basis of the TMR) in LC groups: 21% maize silage, 42% grass silage and 7% straw (GL or CON); in HC groups: 11% maize silage, 22% grass silage and 7% straw (GL or CON); [§]Provided per kg concentrate feed (according to manufacturer specification) for LC groups: 6.16 g Ca, 5.28 g Na, 3.52 g P, 2.2 g Mg, 0.31 g Zn, 0.21 g Mn, 0.06 g Cu, 4.4 mg I, 1.76 mg Se, 1.32 mg Co, 35 200 IU vitamin A, 4 400 IU vitamin D_3, 66 mg vitamin E; HC groups: 2.66 g Ca; 2.28 g Na; 1.52 g P; 0.95 g Mg; 0.13 g Zn; 0.09 g Mn; 0.02 g Cu; 1.9 mg I; 0.76 mg Se; 0.57 mg Co; 15 200 IU vitamin A; 1 900 IU vitamin D_3; 28.5 mg vitamin E. [‡]$aNDF_{om}$, neutral detergent fibre without ash, amylase treated, [•]ADF_{om}, acid detergent fibre without ash; *AMPA, aminomethylphosphonic acid (degradation product of GL). Values are presented as means.

Figure 1: Average daily glyphosate intake of the experimental groups per kg body weight (BW) (Values are presented as means). CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR).



BCS, BW, water intake, DMI, NE intake and NE balance are shown in Table 2. These variables were not affected by GL treatment, no matter which CFP, while an interaction for CFP and t was observed ($p < 0.001$). The interactions were driven by the concentrate proportion in the ration presented with DMI in Figure 2. The mentioned performance parameters increased in HC groups and decreased in LC groups over the experimental time. This is also illustrated by the data of measured energy content, which was lower in LC groups (in Groups CON_{LC} and GL_{LC}, 6.6 and 6.6 NE_L MJ/kg DM, respectively) than in HC groups (CON_{HC}, and GL_{HC}, 7.1 and 7.2 NE_L MJ/kg DM, respectively). No interactions between GL and CFP were detected.

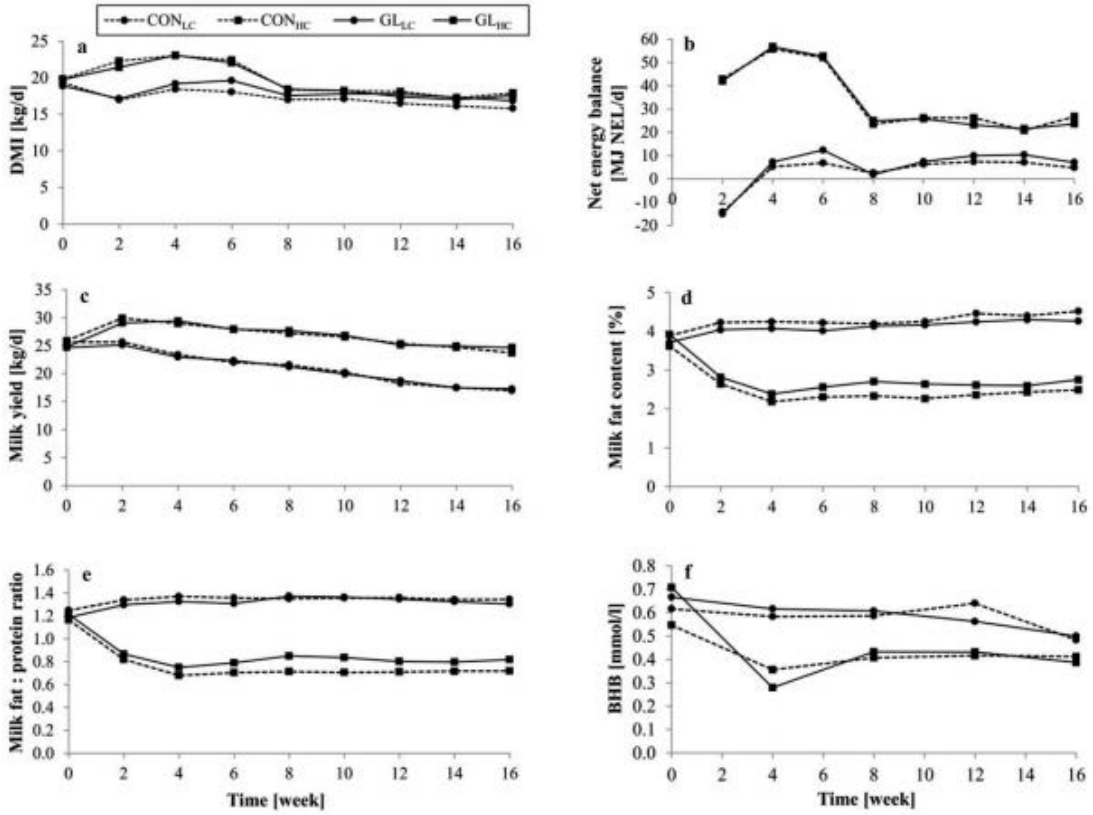
Table 2: Effects of glyphosate residues and concentrate feed proportion (CFP) in total mixed rations (TMR) on body condition score, body weight, dry matter intake (DMI), net energy intake and net energy balance.

	Control (CON)		Glyphosate (GL)		*p*-Value[2]					
	LC[1] (n = 16)	HC[1] (n = 16)	LC (n = 14)	HC (n = 15)	GL	CFP	t[3]	CFP × GL	CFP × t	GL × t
Body condition score	2.8 ± 0.1	3.2 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	0.469	0.018	0.021	0.673	<0.001	0.689
Body weight [kg]	646 ± 5	675 ± 5	641 ± 5	669 ± 5	0.232	0.870	<0.001	0.240	<0.001	0.070
DMI [kg/d]*	17.3 ± 0.3	19.7 ± 0.3	18.0 ± 0.3	19.4 ± 0.3	0.434	<0.001	<0.001	0.094	<0.001	0.707
Water intake [kg/d]	73.8 ± 2.7	82.3 ± 2.7	78.4 ± 2.9	84.1 ± 2.8	0.469	0.018	<0.001	0.619	0.007	0.281
Net energy intake [MJ NE_L/d]	112 ± 4	145 ± 4	112 ± 4	144 ± 4	0.990	<0.001	< 0.001	0.915	<0.001	0.845
Net energy balance [MJ NE_L/d]	3.3 ± 2.6	34.3 ± 2.6	5.1 ± 2.8	33.8 ± 2.7	0.769	<0.001	<0.001	0.631	<0.001	0.853
BHB[4] [mmol/l]	0.58 ± 0.02	0.43 ± 0.02	0.59 ± 0.02	0.45 ± 0.02	0.545	<0.001	<0.001	0.805	<0.001	0.222
Glucose [mg/dl]	59.6 ± 1.13	61.0 ± 1.13	60.8 ± 1.21	59.9 ± 1.17	0.987	0.837	<0.001	0.333	0.030	0.418
NEFA[5] [mmol/l]	0.22 ± 0.02	0.20 ± 0.02	0.22 ± 0.02	0.29 ± 0.02	0.012	0.175	<0.001	0.013	0.052	0.696

[1]LC, low concentrate proportion (30% concentrate in TMR); [1]HC, high concentrate proportion (60% concentrate in TMR); [3]t, time effect of trial week; [2]GL × CFP × t ($p > 0.05$) for all variables; *analysed with DMI week 0 as covariance factor; [4]BHB, β-hydroxybutyrate; [5]NEFA, non-esterified fatty acids. Values are presented as LS means ± SE (standard error).

The BHB concentrations in blood decreased in HC groups (CFP × t; $p < 0.001$), as presented in Figure 2. Glucose showed the same interaction (CFP × t; p = 0.030) but less pronounced, as presented in Table 2. NEFA concentrations in blood showed a trend for an interaction between CFP and t (p = 0.052). The higher NEFA concentrations in GL groups at the beginning of the trial and in Group GL_{HC} at the end of trial should not be interpreted due to the interaction between CFP and GL (p = 0.013) for this variable.

Figure 2: Effects of glyphosate residues and concentrate feed proportion in total mixed rations on dry matter intake (DMI) (A), net energy balance (B), milk yield (C), milk fat content (D), milk fat: protein ratio (E) and β-hydroxybutyrate (BHB) (F) during 16-weeks trial in established lactation period (Values are presented as LS means). CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR).



Measurements of pooled milk samples revealed virtually no incidences of GL in milk (LOQ < 0.01 mg/kg). The time-dependent increase of milk yield in the HC groups and decrease in the LC groups resulted in an interaction (CFP × t; $p < 0.001$) presented in Figure 2. In contrast, LC groups increased and HC decreased in milk fat content and milk fat yield, milk protein yield, milk lactose yield, milk fat:protein ratio, milk urea (CFP × t; $p < 0.001$) and slightly in FCM (CFP × t; $p = 0.007$) and ECM (CFP × t; $p = 0.076$); all milk variables are presented in Table 3. The data of milk protein content was affected by t ($p < 0.001$) and CFP ($p = 0.036$) for all groups, while milk lactose content displayed a significant time-effect and a trend for an interaction between CFP and GL (CFP × GL; $p = 0.090$). An interaction between GL, CFP and t was found for milk yield and FCM ($p = 0.011$ and $p = 0.023$). Milk urea showed an interaction (GL × t; $p = 0.004$) between t and GL; this was due to slight differences between Groups CON_{HC} and GL_{HC} at the beginning of the experiment and disappeared in the course of the experiment. These differences were not significant but an impact on the detected interactions cannot be excluded.

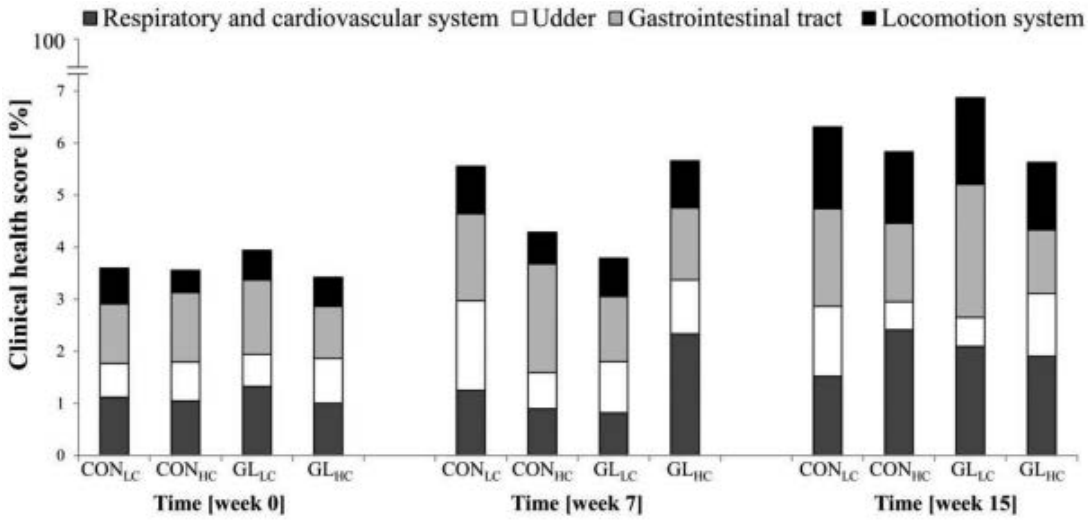
Table 3: Effects of glyphosate residues and concentrate feed proportion (CFP) in total mixed ration (TMR) on milk performance parameters.

	Control (CON)		Glyphosate (GL)		*p*-Value						
	LC[#] (*n* = 16)	HC[†] (*n* = 16)	LC (*n* = 14)	HC (*n* = 15)	GL	CFP	t[$]	CFP × GL	CFP × t	GL × t	GL × CFP × t
Milk yield [kg/d]	21.2 ± 1.0	26.7 ± 1.0	21.1 ± 1.1	26.7 ± 1.0	0.933	<0.001	<0.001	0.932	<0.001	0.855	0.011
Fat-corrected milk [kg/d]	22.6 ± 1.0	21.3 ± 1.0	21.9 ± 1.1	21.8 ± 1.0	0.905	0.523	<0.001	0.566	0.007	0.366	0.023
ECM[‡] [kg/d]	22.2 ± 0.9	22.1 ± 0.9	21.5 ± 1.0	22.7 ± 1.0	0.999	0.549	<0.001	0.484	0.076	0.696	0.179
ECM/dry matter intake [kg/kg][*]	1.26 ± 0.03	1.14 ± 0.03	1.22 ± 0.03	1.19 ± 0.03	0.945	0.036	<0.001	0.173	<0.001	0.931	0.980
Milk fat content [%]	4.27 ± 0.15	2.52 ± 0.15	4.11 ± 0.16	2.78 ± 0.15	0.749	<0.001	<0.001	0.170	<0.001	0.384	0.955
Milk fat yield [kg/d]	0.92 ± 0.04	0.69 ± 0.04	0.88 ± 0.05	0.74 ± 0.05	0.889	<0.001	<0.001	0.204	<0.001	0.612	0.910
Milk protein content [%]	3.19 ± 0.04	3.27 ± 0.04	3.13 ± 0.05	3.25 ± 0.05	0.343	0.036	<0.001	0.699	0.107	0.784	0.399
Milk protein yield [kg/d]	0.69 ± 0.03	0.88 ± 0.03	0.68 ± 0.03	0.87 ± 0.03	0.646	<0.001	<0.001	0.952	<0.001	0.633	0.240
Milk lactose content [%]	4.72 ± 0.05	4.69 ± 0.05	4.73 ± 0.05	4.86 ± 0.05	0.067	0.313	<0.001	0.090	0.249	0.194	0.221
Milk lactose yield [kg/d]	1.04 ± 0.05	1.27 ± 0.05	1.03 ± 0.05	1.31 ± 0.05	0.853	<0.001	<0.001	0.668	<0.001	0.709	0.509
Milk urea [mg/kg]	162 ± 7	77 ± 7	157 ± 7	88 ± 7	0.689	<0.001	<0.001	0.282	<0.001	0.004	0.453
Somatic cell count [log10/ml]	2.13 ± 0.10	2.31 ± 0.10	2.19 ± 0.10	2.28 ± 0.10	0.888	0.195	<0.001	0.649	0.289	0.770	0.911
Milk fat:protein ratio	1.34 ± 0.05	0.77 ± 0.05	1.31 ± 0.05	0.86 ± 0.05	0.520	<0.001	<0.001	0.230	<0.001	0.200	0.990

[#]LC, low concentrate proportion (30% concentrate in TMR); [†]HC, high concentrate proportion (60% concentrate in TMR); [$]t, time effect of trial week; [‡]ECM, energy corrected milk; [*]analysed with value of week 0 as covariance factor. Values are presented as LS means ± SE (standard error).

The average values of the total health score in Groups CON_{LC}, CON_{HC}, GL_{LC} and GL_{HC} were 5.2 ± 0.4, 4.5 ± 0.4, 4.9 ± 0.5 and 4.9 ± 0.5, respectively (LS means ± SE). The total health score (Figure 3) showed for all groups a time effect (t; $p < 0.001$) and an interaction between all three tested values (CFP × t × GL; $p = 0.010$).

Figure 3: Average daily glyphosate intake of the experimental groups per kg body weight (BW) (Values are presented as means). CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR).



Discussion

GL is worldwide the most used active substance in non-selective herbicides in agriculture (Duke and Powles 2008). According to von Soosten et al. (2016), Krüger et al. (2013); Krüger et al. (2014a)) and Rulff et al. (2016) dairy cows are exposed to 0.08–0.9 mg GL per day due to GL contamination in common dairy cow rations. Up to now, the effects of GL on health of dairy cows was solely deduced from field observations and in vitro studies. Therefore, the present exact-feeding experiment on dairy cows in practical conditions was designed to investigate the effects of GL-contaminated feedstuffs which were generated by a legal application and represent a worst-case scenario. Here, the daily exposure was approximately four-fold higher than the maximum observed under average feeding conditions as outlined above.

The cows were fed two different crude fibre and concentrate proportions in their rations with the intention to investigate whether the overall effects of GL depend on different ruminal conditions as

triggered by different concentrate feed proportions. The average daily GL intake in GL groups amounted to 79.1 mg and in both groups straw formed the major GL source. GL was used by spray application, so that the plants' surface was the most contaminated part. This may explain the high straw contamination and the rather small contamination of peas and wheat kernels which are protected by their husks and pods. In CON groups, a small daily GL intake with an average value of 0.8 mg/d was observed. This corresponds to the average value of GL concentration in usual dairy rations (von Soosten et al. 2016). The half-life of GL soils residues varies between 2.8 and 500.3 d DT_{50} (50% dissipation time) (EFSA 2015). Therefore, GL contamination in plants might be originated from soil residues. Overall, GL exposure in GL groups was about 100 times higher than in CON groups. In this study, 121 milk samples were analysed for GL and AMPA. No positive findings above the validated LOQ could be reported. These findings correspond to the study of von Soosten et al. (2016), who reported that milk was virtually free from GL and AMPA, while 8% of daily consumed GL were excreted in urine and 61% passed the digestive tract of dairy cows unmetabolised and were excreted with faeces. The high proportion of GL excreted with faeces also indicates a high concentration of unmetabolised GL in the gastrointestinal tract and the possibility for GL to interact with microorganisms within the ingesta passage time. The chemical composition of the cows ration offered the aimed components within the normal range for the respective feedstuffs with a high fibre content and low energy in LC groups and a low fibre content and high energy in HC feed groups (DLG 1997). Therefore, our experimental feeding design offered adequate conditions to test the effect of GL in rations with different concentrate parts on performance, energy metabolism and health characteristics of dairy cows.

Performance and health

In the present trial, the drop of DMI and the negative energy balance in LC groups at the beginning might be caused by the required adaptation to the experimental ration. The ME and NE_L concentrations of feed confirm the intended differences in energy supply between LC and HC groups, GL showed no influence on both of them and no differences between GL and CON groups were detectable.

Consequently, BW and BCS, NE intake and NE balance were affected by the concentrate feed proportion of the ration, but GL contamination had no influence on the parameters in both rations. The results are in accordance with the results of Donkin et al. (2003), who found a similar DMI of GL-tolerant RoundupReady corn sprayed with Roundup Ultra® (Monsanto Company, St. Lois, MO) and non-transgenic control corn.

Milk yield differed in accordance to the concentrate proportion of the ration and dropped slightly over time due to the advanced lactation period. Based on field observations, Krüger et al. (2014b) postulated milk yield decrease in GL fed cows; this could not be proven by our feeding trial. There was no change in the amount and composition of milk provable in GL groups compared to CON groups. Donkin et al. (2003) could not detect any influence of GL on milk components and on dairy cow performance. The lack of influence of GL on milk components might be related to the absence of GL residues in milk, demonstrating that milk is no major excretion pathway of GL. Consequently, direct effects of GL residues on synthesis of milk components in the mammary gland can be most probably excluded.

On the contrary, different dietary energy levels exhibited significant effects on concentrations and amounts of milk protein, milk fat content, milk lactose, milk urea, SSC and milk fat/protein ratio.

General energy metabolism was not adversely influenced by dietary treatments as blood NEFA, BHB and glucose values were in the normal reference range (Kraft and Dürr 2005). However, BHB levels were significantly influenced by dietary energy level. The overall blood BHB levels might result either from ketogenesis or from ruminal nutrient metabolism. Thus, the higher BHB levels in cows fed the LC diets might reflect a diet induced higher ruminal release of butyrate and/or a slight energy deficit compared to their HC-fed counterparts. Both dietary energy levels were not influenced by GL.

Regarding the putative health effects of GL, Rulff et al. (2016) considered GL being a part of pathogenesis of downer cow syndrome. Furthermore, Krüger et al. (2013, 2014b) and Ackermann et al. (2015) related possible symptoms of *C. botulinum* disease (drop of milk production, mobility disorders, retracted abdomen and forced respiration) to a forced production of BoNT, probably as a result from a

decline of enterococci population in gastrointestinal tract. This effect should be more pronounced in high fibre rations. Krüger et al. (2013) termed GL reasonable for the imbalance of the microorganisms, whereas Riede et al. (2016) could not show any effect of GL on microorganisms in their RUSITEC study. It should be noted that both were in vitro studies which are not able to represent realistic exposure conditions. But similar results were found in a study about the effect of GL contaminated feed on wethers, where GL showed no indication of an impairment of rumen bacteria or a shift in rumen microbial population, neither the group of cellulolytic bacteria, nor the group of amylolytic bacterial species (Hüther et al. 2005).

In our study, the general health status including the previously mentioned symptoms were evaluated. The general health status of cows is, among other factors, related to the health of the rumen microbiome (Zebeli et al. 2015). In our study, the cows showed less than 10% symptoms in total clinical health score. Symptoms occurred without discernible pattern, for instance both GL groups were scored less than CON_{LC} but more than CON_{HC}. Despite the clear influence of the different concentrate proportions of the rations on several parameters, which probably caused very different gastro-intestinal microbial conditions, an effect of GL-residues in feed on performance, metabolism and health characteristics of dairy cows could not be observed in the present trial.

3. Assessment and conclusion

Assessment and conclusion by applicant:

About 30 cows (distributed in two subgroups) were fed with glyphosate-treated commodities for 17 weeks. During this period the exposure of these cows to parent glyphosate residues via feed was about 0.110-0.120 mg/kg bw/day (Figure 1). None of the analysed milk samples (presumably about 60 pooled samples from the two subgroups fed with glyphosate-treated commodities) showed residues of parent glyphosate or AMPA above the limit of quantification of 0.01 mg/kg. This is fully in line with the results of the GLP cow feeding studies submitted in the dossier, which also show that the transfer (if any) of glyphosate-derived residues in cow milk is extremely low. Although the residue analytical method and residue analyses are not reported with a high level of detail, the results are considered reliable since the general principle of the described analytical procedures is well known and the validity of the residue determination was obviously demonstrated by suitable fortification trials. The publication, therefore, is considered relevant and reliable.

However, the main objective of the publication was to investigate the impact of glyphosate residues in feed on health and performance of dairy cows. No significant effects were identified but this part of the publication is not considered relevant to the residue section.

1. Information on the study

Data point	CA 6.4.1
Report author	Shehata A.A. *et al.*
Report year	2014
Report title	Distribution of Glyphosate in Chicken Organs and its Reduction by Humic Acid Supplementation
Document No.	J. Poult. Sci., 2014, 51: 333-337
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/ reliable with restrictions

2. Full summary of the study according to OECD format

Executive Summary

Glyphosate (N-(phosphonomethyl) glycine) is a most popular herb**icide in agricultural practices** throughout the world. It is possible that glyphosate spread in the ecosystems can reach plants, animals. The present work was directed to investigate the glyphosate residue in different organs of broiler chickens using ELISA and to study the possibility of its neutralisation using humic acid, *Chlorella vulgaris* and *Saccharomyces boulardii*. Results showed that glyphosate residues could be detected in the animal feed and different organs as liver, spleen, lung, intestine, heart, muscles and kidney. Humic acid, *Chlorella vulgaris* and *Saccharomyces boulardii* showed neutralization of the antimicrobial effect of glyphosate in vitro. Also, feed supplementation of commercial broiler with humic acid (0.2%) leads to a significant decrease in the glyphosate content, i.e. by 53%, 28%, 44%, 50%, 56%, 16%, 63% and 0% in serum, liver, spleen, lung, gastro-intestinal tract, heart, muscles and kidney, respectively. There were no significant effects of humic acid on the production parameters. This enlightenment will help to overcome the negative effect of glyphosate residues on gastrointestinal microbiota and protect consumers from glyphosate residues in chicken meat.

Materials and Methods

Distribution of Glyphosate in Feed and Tissues

A total of one hundred commercial broiler chickens collected from different farms were slaughtered at 30-day-old. Different organs as liver, spleen, lung, intestine, heart, muscles and kidney were collected and tested for presence of glyphosate using ELISA. Briefly, samples were collected from 10 chickens per farm at 39-day-old after slaughtering and cut to small pieces. In relation to its ability to retain water specimens were suspended in aqua distilled (Braun, Germany) at the rate of 1:1 (low water retention), 1:5 or 1:10 (high water retention). The specimens were heated at 100°C for 10 min, homogenized with ULTRA-TURRAX® (IKA, Wilmington, Germany) and frozen at minus 80°C for eight hours. Homogenized specimens were thawed at 40°C and centrifuged at 10000 x g for 10 min. The supernatant was filtered with an ultracentrifugal filter (3000 Da) to remove proteins and peptides. Filtrates were centrifuged again at 10000 x g for 10 min and the supernatant was tested for glyphosate concentration by ELISA using Glyphosate ELISA kits (Abraxis, Warminister, PA, USA) according to the manufacturer's protocol. Test validation was done with Gas Chromatography-Mass Spectroscopy (GC-MS) by Medizinische Labor (Bremen, Germany), the correlation coefficient between the two tests was 98%.

In vitro Neutralization of Glyphosate

The minimal inhibitory concentration (MIC) of glyphosate (Roundup UltraMax®, Monsanto, USA) on

E. faecalis, *Bacillus badius* (isolated from algae *Chlorella vulgaris*, Ökologische Produkte Altmark Co., Germany) and *Bifidobacterium adolescentis* (isolated from chickens), as indicators, was determined according to the National Committee for Clinical Laboratory Standards (NCCLS). Briefly, the lowest concentration of glyphosate which shows bactericidal or bacteriostatic effects was determined in a 24-well micro-titre plate. Serial dilutions of glyphosate (5, 2.5, 1.2, 0.6, 0.3, 0.15 and 0.075 mg/ml) were made in reinforced clostridial medium (RCM, Sifin, Germany). Tested bacteria was added at a final concentration of 10^4 CFU/ml and the test plates containing diluted glyphosate and tested bacteria were incubated overnight at 37°C. The MIC value was evaluated by quantitative analysis of bacterial growth on Citrat-Azid-Tween-Carbonat Agar (CATC, Oxoid, Germany). The neutralizing effect of humic acid RB4, composed of different molecular weights molecules ranged from 1500 Da to 200000 Da, (WH Pharmawerk Weinböhla GmbH, Weinböhla, Germany), was tested. The MIC value of glyphosate on *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of humic acid RB4 (1 mg/ml), *Chlorella vulgaris* extract (Ökologische Produkte Altmark Co., Germany) at a concentration of 1 mg/ml and *Saccharomyces boulardii* at a concentration of 10^9 CFU/ml (UCB Pharma GmbH, Monheim, Germany) determined.

In vivo Neutralization of Glyphosate Using Humic Acid

The experiment was performed in two chicken broiler barns, designated A and B, each barn accommodated for 22000 broiler chicks. Chickens kept in house A were fed the basic diet without supplementation of humic acid, while chickens kept in house B were fed the same diet with humic acid RB4 (WH Pharmawerk Weinböhla GmbH, Weinböhla, Germany) supplementation (0.2%) from the first day till slaughtering. The ration was formulated as follow: starter (21% corn, 40% wheat, 29% soya bean and 4.5% fat), grower (22% corn, 47% wheat, 19% soya bean and 5% fat), and finisher (17% corn, 48% wheat, 17% soya bean and 4.9% fat). Chickens were allowed to have free access to feed and water until the end of experiment. All chickens were vaccinated against infectious bronchitis (IB) at 12-day-old, Newcastle disease (ND) and infectious bursal disease at 18-days-old. The total mortality and body weight (BW) were calculated at the end of the experiments. Glyphosate residues were determined in serum, liver, spleen, lung, GIT, heart, muscles and kidney using ELISA as mentioned above.

Statistical Analysis

The statistical analysis was carried out with GraphPad Prism 4 (GaphPad Software, La Jolla, USA). Two-way analysis of variance followed by unpaired Student t-test was used to identify significant differences between means.

Results

Distribution of Glyphosate in Feed and Tissues

The glyphosate residues could be detected in feed, liver, spleen, lung, intestine, heart, muscles and kidney using ELISA in the concentrations of 370, 9.8, 21.1, 24.2, 98.3, 20.4, 5.0 and 16.0 ng/gm, respectively (Table 1).

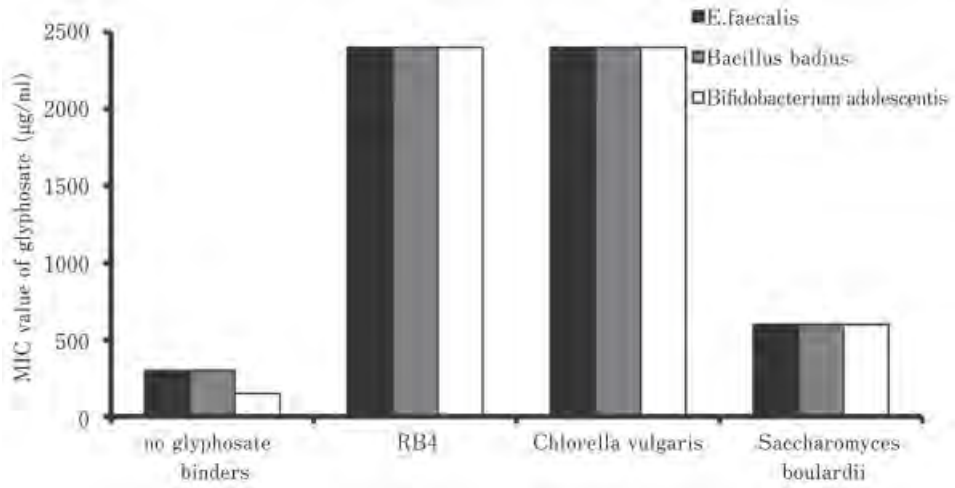
Table 1: Distribution of glyphosate in feed and chickens tissues.

Sample N=30	Glyphosate (ng/gm)		
	Minimum	Maximum	Mean±SD
Feed	190.0	400.0	370.0±92.0
Liver	6.0	13.6	9.8±3.0
Spleen	11.8	25.0	21.1±17.0
Lung	12.0	25.0	24.2±9.0
Intestine	20.0	120.0	98.3±42.0
Heart	17.0	20.0	20.4±0.6
Muscles	3.6	4.9	5.0±0.3
Kidney	0.4	17.6	16.0±13.0

Neutralisation of Glyphosate in vitro

The MIC value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* were 300, 300 and 150 µg/ml, respectively. The RB4 and *Chlorella vulgaris* in concentrations of 1 mg/ml showed the higher neutralization of the antimicrobial effect of glyphosate. The MIC-values of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of humic acid or *Chlorella vulgaris* were 2400 µg/ml (Fig. 1). However, the MIC-value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence Saccharomyces boulardii was 600 µg/ml (Fig. 1).

Figure 1: Changes in the MIC values of glyphosate on *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* using different glyphosate binders.



In vivo Neutralization of Glyphosate Using Humic Acid

In untreated chickens, the glyphosate concentrations in serum, liver, spleen, lung, GIT, heart, muscles and kidney were 2, 14, 21, 24, 101, 20, 6 and 6 ng/gm, respectively, however, in humic acid treated chickens, glyphosate residues were 0.88, 9.78, 11.79, 12.20, 43.6, 17.4, 1.9 and 6. 2 ng/gm, respectively. Supplementation of humic acid caused a significant decrease in the glyphosate content, i.e. by 53%, 28%, 44%, 50%, 56%, 16%, 63% and 0% in serum, liver, spleen, lung, GIT, heart, muscles and kidney, respectively (Fig. 2). At 30-day-old, there is no significant improvement of body weight and total mortalities between humic acid-treated and untreated chickens (Table 2), the average body weight of both was 1.69 Kg. However at 39-day-old, the average body weight of 2.456 Kg while it was 2.339 Kg in untreated chickens (Table 2).

Figure 2: Effect of humic acid supplementation on glyphosate accumulation in chickens. Glyphosate was measured using ELISA and expressed as ng/gm. Asterisks denote significant decrease of glyphosate in humic acid treated chickens (* $P=0.05$, ** $=P<0.001$).

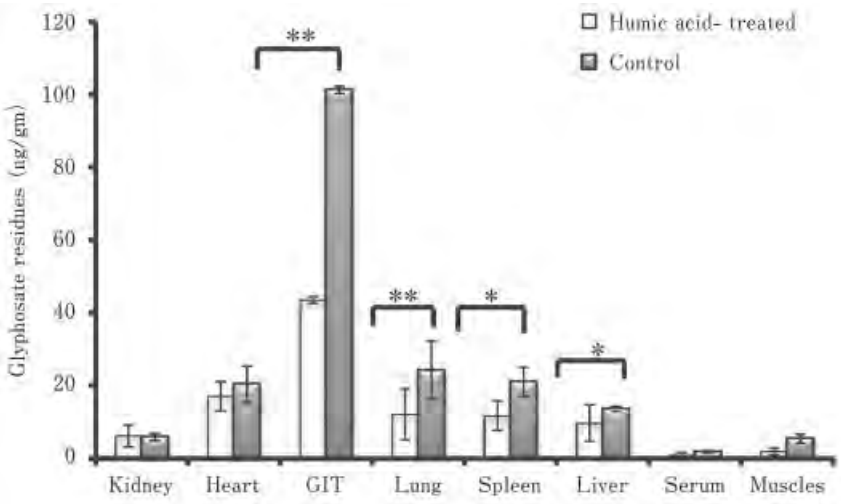


Table 2: Effect of humic acid supplementation on the production parameters.

Parameter	Humic acid-treated chickens	Non-treated chickens
Total number	22500	23100
Slaughtered number at 30-day-old	6509	6509
Slaughtered number at 39-day-old	14581	15573
Body weight at 30-day-old (average/kg)	1.69	1.69
Body weight at 39-day-old (average/kg)	2.453	2.339
Total feed intake (kg)	76530	78690
Food conversion ratio	1.64	1.66

Discussion

Distribution of Glyphosate in Feed and Tissues

Glyphosate residues in food and feed have been on the rise, due to higher rates and frequency of application, which in turn is due to increasing weed resistance (Samsel and Seneff, 2013). In the present study glyphosate residues could be detected in liver, spleen, lung, intestine, heart, muscles, kidney and animal feed (Table 1). The maximum residue levels (MRLs) of glyphosate in soya bean, maize, cereal grains, cotton seed, alfalfa, hay, sorghum straw, wheat and wheat straw were agreed by the United Nations Food and Culture Organization's to be 20, 5.0, 30, 40, 500, 500, 50, 200 and 300 mg/kg (WHO, 1994). Data on the real presence of glyphosate and its metabolite in feed from glyphosate sprayed crops are sparse. A now common practice of crop desiccation through herbicide administration shortly before the harvest assures an increased glyphosate residues in food sources as well (Baig et al., 2003; Ellis et al., 1998). Also, the maximum daily intake (MDI) of glyphosate depends on the ration composition and the percent of each component in the ration. Glyphosate residues concentrate in approximately 80% genetically modified plants grown for food and feed up to 400 ppm, maximal residual levels.

Neutralisation of Glyphosate in vitro

Many studies have reported that glyphosate can be sorbed to humic acids (Piccolo et al., 1996; Banta et al., 2009; Mazzei and Piccolo, 2012). In the present study the humic acid RB4 neutralized the antimicrobial effect of glyphosate in vitro. The MIC-value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of RB4 humic acids or Clorella vulgaris were 2.4 mg/ml.

Chlorella has also useful detoxifying properties. The use of oral supplements of *Chlorella pyrenoidosa*

has been reported to significantly reduce dioxin levels in breast milk of 35 nursing women in Japan (Nakano et al., 2007). Also Chlorella supplementation significantly reduced liver toxicity and cadmium-accumulation in cadmium poisoned rats (Shim et al., 2008).

Yeast has been used as general performance promoter in poultry feeds and has been shown to have beneficial effects against mycotoxins exposure (Celyk et al., 2003, Santin et al., 2003, Baptista et al., 2004). The absorbent ability of yeast to mycotoxins could be attributed to the presence of innumerable sites on its surface for physical adsorption of molecules (Shetty and Jespersen 2006). In the present study *Saccharomyces boulardii* showed a low absorbent ability to glyphosate (Fig. 1).

Neutralisation of Glyphosate by Humic Acid Supplementation in vivo

The use of humic acids and their sodium salt for the oral treatment of all animals on food production farms is currently permitted. Supplementing animal feeds with non-nutritive adsorbents as humic acid has proven to substantially reduce mycotoxicosis (Sabater-Vilar et al., 2007) and improved the performance, carcass, GIT and meat quality traits (Ozturk et al., 2011). In our study, the mortality was negligible with no difference between control and humic acid-treated group. Also the humic acid-treated chickens showed no improvement in feed conversion in birds and body weight at 30-day-old (Table 2). Kocabagli et al. (2002) reported an improvement in feed conversion in birds that were given 0.25% humic acid either from 0 to 42 d or during grow-out periods only, between d 21 to 42. A similar conclusion was drawn by Yoruk et al. (2004), who showed a better feed conversion in hens supplemented with 0.1-0.2% humic acid, and it did not affect body weight. On the contrary, Rath et al. (2006) found that humic acid-treated chickens showed a reduction in body weight, and the feed conversion ratio was numerically higher.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication provides information about the levels of parent glyphosate residues in feed and tissues of broiler chicken (including edible tissues such as muscle and liver). This may allow to estimate residue transfer factors from poultry feed to poultry meat. Therefore, the publication is considered relevant. The authors further investigated the impact of a feed supplementation with humic acid on the transfer of glyphosate residues in poultry tissues. It was concluded that the supplementation with humic acid allows to significantly decrease the residues of glyphosate in poultry tissues (-63% in muscle and -28% in liver). Thus, the control group (which received feed without humic acid supplementation) represents a worst case in terms of residues and is more relevant from a regulatory perspective. The highest residues found in chicken muscle and liver were extremely low (ca. 0.005 mg/kg and 0.018 mg/kg, respectively). This is consistent with the results of the submitted poultry feeding studies (which were conducted at dose levels far above the dietary exposure of the broiler chickens in the publication). However, both the experimental procedures and the obtained results are not described with a sufficient level of accuracy and it is difficult to figure out exactly what was done and how the presented results were generated. The sample preparation procedure (with consecutive steps at 100°C and -80°C) is quite unusual and no method validation data are presented. Because of that, the publication is reliable with restrictions.

1. Information on the study

Data point	CA 6.4.2
Report author	Shelver W.L. *et al.*
Report year	2018
Report title	Distribution of chemical residues among fat, skim, curd, whey, and protein fractions in fortified, pasteurized milk
Document No.	ACS Omega 2018, 3, 8697 − 8708
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

The distribution of 12 environmental contaminants or metabolites with diverse polarities (2,2′,4,4′,5-pentabromodi-phenyl ether; bisphenol A; estrone; glyphosate; β-hexabromocyclo-dodecane; imidacloprid; 2,3′,4,4′,5-pentachlorobiphenyl; 3′-methylsulfone 2,2′,4,5,5′-pentachlorobiphenyl; 1,2,7,8-tetrachlorodibenzo-*p*-dioxin; 2-hydroxy-1,3,7,8-tetrachlorodibenzo-*p*-dioxin; tetrabromo-bisphenol A; and triclocarban) among skim milk, fat, curd, whey, whey retentate, and whey permeate was characterized. Analysis of these compounds along with 15 drugs previously studied provided a robust linear model predicting the distribution between skim and fat and the chemical's lipophilicity (log *P*, $r^2 = 0.71$; log *D*, $r^2 = 0.79$). Similarly, distribution between curd and whey was correlated with lipophilicity (log *P*, $r^2 = 0.63$; log *D*, $r^2 = 0.73$). Phenolic compounds had less predictable distribution patterns based on their lipophilicities. Within the whey fraction, chemicals with greater lipophilicity are associated with whey proteins more than hydrophilic chemicals. The resultant model could help predict the potential distribution of chemical contaminants among milk products in cow milk, if present.

Materials and Methods

Selection of drugs and concentrations

Chemicals selected for study had to be potential environmental contaminants, encompass a wide range of lipophilicities, and be available with radiolabel (^3H or ^{14}C) incorporation. The chemicals selected had a log *P* range of − 3.3 to 7.3. Chemical structures, site of radiolabel, specific activity (SA), and physio-chemical properties are provided in **Table 1**.

To detect potential concentration-dependent distribution, chemical concentrations spanning 3 orders of magnitude (*i.e.*, 20 − 2000 nM) were generally used. The lowest concentration (usually 20 nM) was typically relevant to possible contamination scenarios with sufficient activity to allow radiochemical detection. Higher concentrations were used to determine whether concentration influenced overall xenobiotic distribution. In some instances, concentrations were adjusted because of limited solubility or if the SA of the radiolabeled compound was inadequate for the sensitivity of the analysis (**Table 1**). As a result of adding unlabeled chemical (typically 9:1 parts) for the highest dose, SA was lowered, relative to low concentration.

Table 1: Drug Structures and Physicochemical Properties.

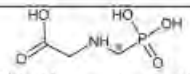
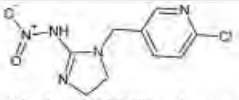
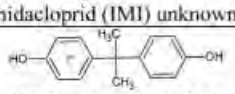
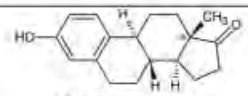
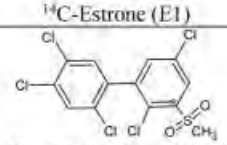
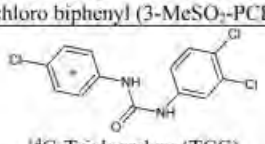
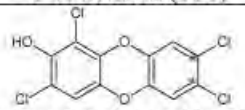
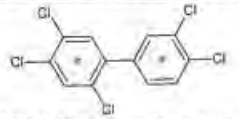
[a] Compound radioactively labeled with a directed label and specified on the structure with a red asterisk. An asterisk within a ring indicates a uniform label on the ring. Exceptions: IMI and *β*-HBCD carbon labels are unknown.

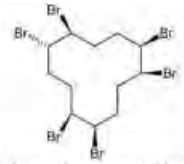
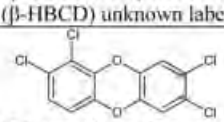
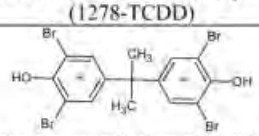
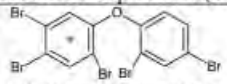
[b] SAs were adjusted depending on dose, as indicated. Values in parentheses are nominal concentrations

for initial fortification.

[c] Average log *P* calculated from literature log *P* values accessed from www.chemspider.com, www.drugbank.ca, www.ebi.ac.uk/chembl/, and pubchem.ncbi.nlm.nih.gov/ on 7/14/2017 using the predicted and experimental values were available.

[d] Values for log *D* at pH 6.8 were calculated using log *P* values from above sources and pK_a's from www.drugbank.ca, www.ebi.ac.uk/chembl/, www.druginfosys.com, pubchem.ncbi.nlm.nih.gov/, Johansson and Anlér [11] accessed on 7/14/2017.

Compound[a]	Class/ Use	M.W. S.A. (nCi/nmol)[b]	log P[c]	pKa[d]	log D[d]
 ^{14}C-Glyphosate (GLY)	Herbicide/ pesticide	169.07 g/mol 50 (20 nM/ 200 nM) 5.0 (2000 nM)	-3.26 ± 1.53	5.89 ± 0.40	-4.24 ± 1.49
 ^{14}C-Imidacloprid (IMI) unknown label	Insecticide/ pesticide	255.66 g/mol 25.3 (20 nM/ 200 nM) 2.5 (2000 nM)	0.39 ± 0.59	5.28	-0.38 ± 0.59
 ^{14}C-Bisphenol A (BPA)	Plasticizer	228.28 g/mol 53.5 (20nM/ 200nM) 6.0 (2000nM)	3.60 ± 0.27		3.60 ± 0.27
 ^{14}C-Estrone (E1)	Hormone	270.37 g/mol 51.3 (20nM/ 200nM) 5.7 (2000nM)	3.62 ± 0.45		3.62 ± 0.45
 ^{14}C-3'-methylsulfone-2,2',4,5,5'-pentachloro biphenyl (3-MeSO$_2$-PCB-101)	PCB Metabolite	404.52 g/mol 53 (20/100/500nM)	4.62		4.62
 ^{14}C-Triclocarban (TCC)	Antibacterial / disinfectant	315.58 g/mol 30 (20 nM/ 200 nM) 3.0 (2000 nM)	5.39 ± 0.45		5.39 ± 0.45
 ^{14}C-2-hydroxy-1,3,7,8-tetrachloro dibenzo-*p*-dioxin (2-OH-1378-TCDD)	TCDD Metabolite	337.97 g/mol 64.6 (20 nM/ 100 nM) 12.8 (500 nM)	6.15 ± 0.32		6.15 ± 0.32
 ^{14}C-2,3',4,4',5-pentachlorobiphenyl (PCB-118)	Coolants/ plasticizers/ hydraulic fluids/ pesticides/ flame retardant	326.43 g/mol 10.3 (50 nM/ 200 nM) 2.5 (2000 nM)	6.78 ± 0.35		6.78 ± 0.35

 [14]C-β-hexachlorocyclododecane (β-HBCD) unknown label	Flame Retardant	641.69 g/mol 2 (200/500/2000 nM)	7.22 ± 0.65		7.22 ± 0.65
 [14]C-1,2,7,8-tetrachloro dibenzo-*p*-dioxin (1278-TCDD)	Industrial and incineration byproduct	321.97 g/mol 67.8 (20 nM/ 200 nM) 6.8 (2000 nM)	6.22 ± 0.72		6.22 ± 0.72
 [14]C-Tetrabromo Bisphenol A (TBBPA)	Flame Retardant	543.87 g/mol 25 (20 nM/ 200 nM) 3.7 (2000 nM)	6.69 ± 0.58		6.69 ± 0.58
 [14]C-2,4',4,5,5'-pentabromo diphenyl ether (BDE-99)	Flame Retardant	564.69 g/mol 49 (20 nM) 8.76 (200 nM) 0.98 (2000 nM)	7.31 ± 0.62		7.31 ± 0.62

Chemicals, supplies, and equipments

Raw (unpasteurized, nonhomogenized) cow milk was obtained from the bulk milk tank located at the North Dakota State University (Fargo, ND) Dairy farm within 48 h of milking. Non-radiolabeled chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO), U.S. Pharmacopeia (Rockville, MD), or other common vendors. Radiolabeled E1, GLY, PCB-118, and *β*-HBCD were procured through American Radio-labeled Chemicals, Inc. (ARC, St. Louis, MO). A mixture of the *β*- and *γ*-diastereoisomers of [^{14}C]-HBCD was identified in the ARC product. Flash chromatography on a silica gel column eluted with hexane containing increasing amounts of methylene chloride (0 − 50%) was used to isolate [^{14}C]-*β*-HBCD. [^{14}C]-BPA and [^{14}C]-TCC were purchased from Moravek Inc. (Brea, CA). [^{14}C]-IMI was a gift from Bayer Crop Science (Research Triangle Park, NC). [UL-7,8-ring^{14}C]-1278-TCDD was purchased from ChemSyn Science Laboratories (Lenexa, KS). [^{14}C]-2,2′,4,4′,5-pentabromodi-phenyl ether (BDE-99) was synthesized using published methods [23]. 2-OH-1378-TCDD was prepared in-house from [UL-7,8-ring^{14}C]-1278-TCDD by *in vitro* oxidation with human CYP1A1R Baculosomes (Cypex Ltd., Dundee, UK) and a glucose-6-phosphate dehydrogenase regenerating system according to manufacturer's instructions. [^{14}C]-2,2-bis(4-hydroxy-3,5-dibromophenyl)propane (TBBPA) was synthesized by brominating bis[^{14}C]-phenol A with 4.2 equivalents of bromine in 1:1 methanol/water; bis[^{14}C]-phenol A was prepared in-house from [UL-^{14}C]-phenol (2.0 mCi, 25 mCi/mmol) and acetone according to a published method [24]. 3′-[^{14}C]-MeSO$_2$-PCB-101 was synthesized de novo by Cadogan coupling as described in Haraguchi *et al*. [25] using sodium [^{14}C]-methyl thiolate for label introduction.

Silica gel plates were purchased from Analtech (Newark, DE). Scintillation cocktails were purchased from MP Biochemicals, LLC, (Ecolite; Solon, OH) or PerkinElmer (Waltham, MA; Carbosorb, and Permafluor). Amicon Ultra-15 centrifugal filters were purchased from Millipore (Billerica, MA). An Allegra X-14R centrifuge was obtained from Beckman-Coulter (Brea, CA). Liquid milk product fractions were mixed with scintillation fluid and assayed using a Tri-Carb 1900 liquid scintillation counter (LSC, Packard, Meriden, CT). Solid milk product samples were combusted using a Packard model 307 tissue oxidizer (Meriden, CT), trapped into Carbosorb, diluted with Permafluor, and then assayed by LSC. Sample purity was assessed by TLC and radioassay using a Bioscan AR-2000 Imaging Scanner for TLC (Washington, DC).

Determination of chemical purity and confirmation of test article stability

TLC analyses were used to assess chemical purities before and after the experiments, although for GLY, high-performance liquid chromatography instead of TLC was employed. Initial analyses were used to evaluate dose purity, whereas post-incubation analyses were used to evaluate whether chemical

degradation occurred during milk processing. TLC conditions and results are included in Table S3. GLY radiochemical purity (98.0 ± 0.4%, n = 4) was determined based on Nagatomi *et al*. [26] using a Waters 2695 HPLC, a radiometric detector (Packard LFA 515TR, PerkinElmer, Waltham, MA), and a Dionex IonPac AS 12 column (4 × 200 mm, 9 µ m, Dionex Company, Sunnyvale, CA). The mobile phase was isocratic 0.2% aqueous formic acid/acetonitrile (5/95, v/v), and the flow rate was 1 mL/min.

Milk processing and radiochemical analysis

The milk processing experiments consisted of three sequential phases. Specific details pertaining to preparation of phases are reported in Hakk *et al*. [7] and Shappell *et al*. [8]. Briefly, 12 tubes of raw milk (50 mL) were pasteurized at 63°C for 30 min. Triplicate tubes were fortified with each level of radiolabeled chemicals using three working solutions or with the appropriate solvent for blank milk, as described in **Table 2**. In phase 1, the fortified, pasteurized, whole milk samples were separated into milk fat and skim milk by centrifugation after equilibration; the partitioning of chemical between these phases was then determined by radiochemical detection methods. In phase 2, the skim milk originating from phase 1 was partitioned into curd and whey (enzymatically with rennet) and the distribution of the target chemical between these phases determined by radiochemical detection. In phase 3, the residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (> 10 kD), retentate (~ 5 mL) and permeate (~ 10 mL) fractions using ultracentrifuge filters. To determine if degradation occurred during processing, milk fat, curd, and whey from the highest dose concentration were extracted and analyzed by TLC side by side with radiolabeled standards with the exception of GLY because no satisfactory TLC method was found. The main difference in the current study compared to the cited research [7-9] was that here the radiolabeled compounds were fortified only once into whole milk and not anew at the beginning of each phase (**Figure 5**), resulting in lower initial chemical concentrations in skim and whey fractions.

Table 2: Compound Associated with Casein or Whey Protein (nmol/mg Protein and Percent Association Based on Whole Milk).

[a] SA of some compounds required different doses, as indicated by bold text. Each fortified level contains three replicates.
[b] These data were derived from phase 2 data and have whey associated drug subtracted, using "0% moisture curd" as described in text.
[c] These data were derived from phase 3 data as described in the text.
[d] Less than limit of quantitation (<LOQ). LOQ for PCB-118 is 1.92 nmol/L and for β-HBCD was 9.87 nmol/L.
[f] Inconsistent with other doses. No explanation.

compound	nominal conc. of whole milk[a] (actual) nM	nmol/mg casein protein[b]	nmol/mg whey protein[c]	conc. in casein/conc. in whey protein	mean % casein association based on whole milk[b]	mean % whey association based on whole milk[c]
GLY	20 (22)	0.08	0.15	0.53	7.92	3.68
	200 (217)	0.71	1.40	0.51		
	2000 (2059)	6.52	14.09	0.46		
IMI	20 (20)	0.21	0.11	1.91	15.43	3.19
	200 (201)	2.20	1.13	1.95		
	2000 (2066)	22.33	12.29	1.82		
BPA	20 (22)	0.44	0.23	1.91	45.76	6.68
	200 (216)	4.54	2.25	2.02		
	2000 (1992)	42.43	20.88	2.03		
E1	20 (20)	0.21	0.10	2.10	17.86	3.52
	200 (200)	1.66	0.96	1.73		
	2000 (1796)	15.89	8.70	1.83		
3-$MeSO_2$-PCB-101	20 (24)	0.06	0.04	1.50	4.25	0.99
	100 (70)	0.18	0.12	1.50		
	500(628)	1.60	1.08	1.48		
TCC	20 (19)	0.05	0.10	0.50	5.92	3.62
	200 (193)	0.54	0.95	0.57		
	2000 (1938)	5.66	9.72	0.58		
2-OH-1378-TCDD	20 (22)	0.16	0.64	0.25	17.85	16.79
	100 (107)	**0.77**	**3.26**	0.24		
	500 (556)	**4.71**	**16.16**	0.29		
PCB-118	**50 (60)**	**0.07**	**<LOQ**[d]		3.42	0.70
	200 (221)	0.35	0.24	1.46		
	2000 (2203)	2.90	2.37	1.22		
β-HBCD	**200 (229)**	0.38	**<LOQ**[d]		2.95	0.59
	500(656)	1.09	0.59	1.85		
	2000 (2067)	3.21	2.11	1.52		
1278-TCDD	20 (27)	0.11	0.06	1.83	4.14	1.29
	200 (184)	0.46	0.34	1.35		
	2000 (1784)	5.07	3.58	1.42		
TBBPA	20	0.27	0.79	0.34	18.01	22.96
	200 (234)	3.53	9.36	0.38		
	2000 (1815)	21.63	76.47	0.28		
BDE-99	20 (20)	0.12	0.03	4.0	6.66	1.20
	200 (178)	1.05	0.38	2.76[f]		
	2000 (1890)	18.33	3.35	5.47		

Calculation of chemical associated with casein and whey Protein

The percentage of chemical associated with whey proteins was calculated according to Shappell *et al.* [8]. Briefly, the amount of free chemical measured in permeate (calculated by concentration and volume) was subtracted from the total amount of chemical present in retentate. The difference was assumed to be the amount of chemical associated with whey protein. Residual radioactivity on ultrafilters (measured by combustion analysis) was considered nonspecific binding and was subtracted from the fortified whey results; however, radioactivity present in filter washes was included with retentate radioactivity.

Averaged Kjeldahl protein concentrations in curd from Shappell *et al*. [8] and Lupton *et al*. [9] and the resultant 0% moisture curd radioactivity (see below) along with its SA were used to calculate nanomole per milligram casein protein association. Similarly, averaged Kjeldahl protein concentration in retentate from Shappell *et al*. [8] and Lupton *et al*. [9] and the protein associated radioactivity and its SA in retentate was used to calculate nanomole per milligram whey protein association.

Statistical analyses

Standard statistical methods were used to calculate means and variability and make inferences with respect to the significance of differences between means. Linear regression was used to assess dose dependence of the observed drug distribution log ratio of [chemical] milk fat /[chemical] skim milk or 0% moisture [chemical] curd /[chemical] whey. Dose dependency was based on instances when the slope differed ($P < 0.05$) from zero. Because curd is 70% moisture and contains a small quantity of entrained whey, a 0% moisture curd radioactivity value was calculated by subtracting entrained whey-associated radioactivity (calculated based on the percent moisture) from curd. The value representing entrained whey was added back to the whey fraction.

Coefficient of variation with respect to measured partition values across doses was typically much less than 10%, whereas literature values for log *P* for a given chemical could sometimes differ by an order of magnitude or greater. Therefore, distribution data were modeled using mean log P values ± SD for each chemical. Mean log P values were calculated from predicted and measured entries included in Chemspider, DrugBank, ChemBL, and Pubchem databases. For 3′-$MeSO_2$-PCB-101, the log *P* value was derived from using conversion of chlorocyclohexatriene into *p*-chlorophenyl methyl sulfone as a model, which has log differences of 1.76. By using PCB-101 log *P* of 6.38 and subtracting 1.76, the log *P* of 3′-$MeSO_2$-PCB-101was derived as 4.62. Log *D* values were calculated as described by Scherrer and Howard [16] using a pH of 6.8 (reflecting the pH of milk); to obtain a theoretical range of log *D* values for each compound, the range of log P values derived from the above sources was used in conjunction with the range of pK_a values obtained from the same sources; log *D* values were averaged and SDs calculated. Relationships between the log distribution ratios and lipophilicity (log *D* and log *P*) were performed using linear function and included the 99% CI and prediction interval by GraphPad Prism Version 7.03 (Graph-Pad Software, La Jolla, CA).

Results and Discussion

Chemical distribution from whole milk into milk fat and skim milk.

Milk partitioning into lipid was highly reproducible, with typical coefficient of variance (CV) values of ≤ 5%; exception was GLY with CV up to 19% (Tables S5−S16). The high CV of GLY was due to its low partitioning into milk fat (Table S5). Similarly, CV of partitioning into skim milk was ≤ 5%; exceptions were BDE, *β*-HBCD, 3′-$MeSO_2$-PCB-101, PCB, and TCC because of low amounts in the skim milk. Recoveries (sum of total radioactivity in skim milk and milk fat) were > 90%, ranging from ~ 91% (for chemicals with log $D \geq 6.7$) up to 100% for GLY (Figure 1 and Tables S5−S16). Distribution of chemical residues was not dose-dependent over the range of doses used (linear regression slope $P > 0.05$), suggesting that a chemical's distribution between skim milk and milk fat would be constant regardless of the concentration. In the absence of overt physiologic effects such as toxicity or effects on blood flow to the mammary gland, such results suggest that whole milk composition (*i.e.*, across species or breed types) would influence a chemical's presence in milk to a greater extent than the dose received.

For the 12 chemicals tested, distribution into milk fat ranged from < 3% (0.95% for GLY and 2.5% for IMI) to > 80% of the total amount added (3′-$MeSO_2$-PCB-101, TCC, PCB-118, *β*-HBCD, 1278-TCDD, and BDE-99). Intermediate distributions into milk fat occurred for phenolic compounds (BPA, 39%; TBBPA, 46%; 2-OH-1378-TCDD, 54%; and E1, 74%) (Tables S5−S16, **Figure 1**).

As would be anticipated, the data indicated that nonpolar chemicals concentrate into high lipid milk fractions. The concentration ratios in milk fat relative to whole milk for moderately polar phenolic compounds were about 10 (BPA, 8.2; TBBPA, 10.5; 2-OH-1378-TCDD, 11.2; and E1, 15.8) and were ~ 18−20 for highly nonpolar persistent environmental contaminants (BDE-99, *β*-HBCD, 3′-$MeSO_2$-PCB-101, PCB-118, TCC, and 1278-TCDD; Figure 1). Also as expected, polar chemicals partitioned to

a large degree into skim milk, resulting in milk fat/whole milk concentration ratios of < 1 (GLY was 0.2, and IMI was 0.5; Figure 1). For the phenolic compound BPA, substitution of four phenyl hydrogens with bromines to form TBBPA (Table 1) increased lipophilicity (log D = 3.60 vs 6.69) and was reflected by TBBPA's milk fat/whole milk concentration ratio of 10.5 compared to that of 8.2 for BPA (**Figure 1**). Hydroxylation of a molecule decreases its relative lipophilicity with respect to its non-hydroxylated analogue, as is commonly observed during oxidative metabolism. Although 1278-TCDD and 2-OH-1378-TCDD have very similar log *D* values (6.15 and 6.22, respectively) hydroxylation resulted in reduced lipid solubility and a ~ 30% reduction in milk fat distribution. However, the addition of a more polar functional group onto a pentachloro biphenyl molecule to form 3′-$MeSO_2$-PCB-101 did not shift the milk fat distribution pattern when compared to PCB-118. One possible explanation may be due to the change of chlorine substitution pattern.

Figure 1: Chemical distribution and relative concentration ratios from whole milk into skim milk and milk fat fractions. Bars represent percent mean of all concentrations (n = 3 concentrations, 3 replicates per concentration, replicate exceptions are n = 2 replicates each for 1278-TCDD 20 and 200 nM and n = 2 replicates for BDE-99 2000 nM) ± SD of the three dose means based on disintegrations per minute (dpm) of skim milk and milk fat fractions compared to whole milk dpm. Values on graph represent the mean ratio of the drug concentration in the fraction (milk fat or skim milk) to the initial drug concentration in whole milk ± SD of means between doses (n = 3 mean dose ratios). Sum of stack plot represents total chemical recovery. log *D* values given for each compound at bottom of plot.

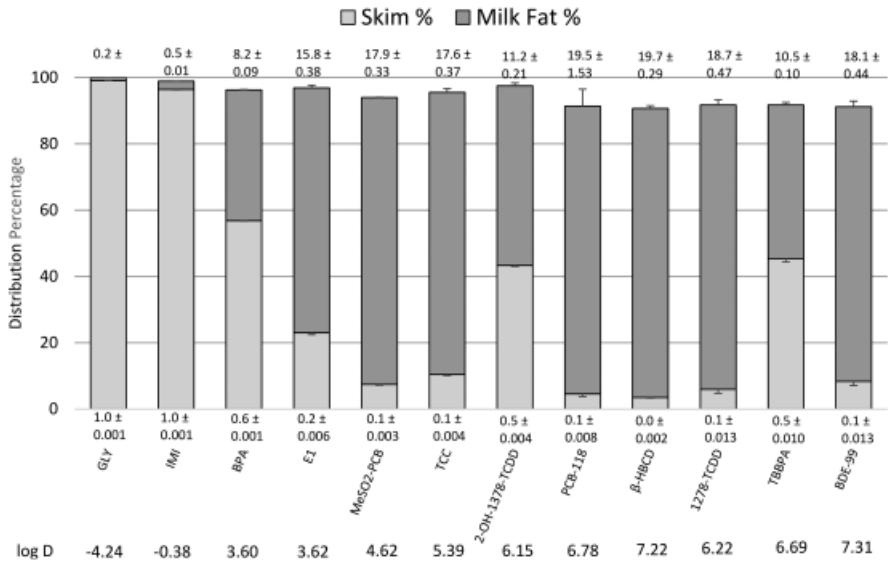


Table S5: GLY Phase 1 Average Distribution Data and Ratios.

Phase 1	Whole			Skim Milk				Milk Fat				Percent Recovery	Obs. Ratio
Mean	Spike DPM	Volume (mL)	Initial nM	Total DPM	Skim Milk Volume (mL)	Final nM	% GLY Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% GLY Dose	Total % based on Whole	[milk fat]/ [skim milk]
0nM		48.83			46.51				2.33				
20nM	117,903	48.90	21.72	116,815	46.58	22.59	99.10	948	2.31	3.69	0.80	99.90	0.16
200nM	1,178,074	48.90	217.04	1,167,088	46.52	226.03	99.07	11,398	2.38	43.21	0.97	100.04	0.19
2000nM	1,117,710	48.90	2,059.36	1,107,061	46.48	2,145.80	99.05	11,973	2.42	444.70	1.07	100.12	0.21
St Dev													
0nM		0.08			0.03				0.05				
20nM	1,658.	0.00	0.31	1,951	0.02	0.39	2.58	140	0.02	0.51	0.13	1,658.35	0.02
200nM	5,136	0.01	0.99	10,791	0.02	2.18	1.07	973	0.04	3.22	0.09	5,135.79	0.02
2000nM	11,354	0.01	21.18	4,072	0.08	7.45	1.11	2,330	0.07	73.05	0.20	11,354	0.03
% RSD													
0nM		0.16			0.06				2.07				
20nM	1.41	0.01	1.41	1.67	0.05	1.72	2.60	14.82	0.85	13.95	15.56	2.70	12.15
200nM	0.44	0.02	0.46	0.92	0.05	0.97	1.08	8.54	1.65	7.45	8.86	1.05	8.16
2000nM	1.02	0.03	1.03	0.37	0.17	0.35	1.12	19.46	3.01	16.43	18.54	0.92	16.51

Although literature describing the milk partitioning of the exact compounds studied here has not been found, there are several relevant studies available for comparison. For example, Jensen and Hummel [10] administered 2,4,5-trichlorophenoxy-acetic acid containing 2,3,7,8-TCDD to lactating dairy cows and found that 2,3,7,8-TCDD residues in cream exceeded those in milk by a factor of about 10. Although this is much lower than our reported ratio of ~ 19 for 1,2,7,8-TCDD (**Figure 1**), the difference could originate from the "medium heavy cream" used in the Jensen and Hummel study [10] which would have a fat content < 36%. On the basis of our previous reports by Hakk *et al.* [7] and Lupton *et al.* [9], our milk fat had an average fat content of 82%. Regardless, our data confirmed those of Jensen and Hummel [10] in that the majority of dioxin residues would be associated with milk fat.

Compounds with a log *D* or *P* value of about 6 consistently concentrated in milk fat (or cream as cited in references). Concentrations of dichlorodiphenyltrichloroethane (DDT, Table S4) (log *D* 6.22 and log *P* = 5.92) in raw whole milk (5% lipid), skim milk, and cream (70% lipid) were reported as 7.5, 0.2, and 67.2 ppm, respectively, with a cream/whole milk ratio of 9.0 [12]. Pasteurization produced a slight increase of the cream/whole milk distribution ratio, as pasteurized whole milk contained 6.0 ppm and cream contained 70.2 ppm DDT resulting in a cream/whole milk ratio of 12 [12]. Langlois *et al.* [13] reported the identical ratio of cream/whole milk for DDT in spite of a fat content for cream of only 37%. Relative to the Mann [12] and Langois *et al.* [13] reports, higher milk fat/whole milk concentration ratios were found in this study for compounds having log *P* = ~ 6 (TCC, log *P* = 5.39, ratio 17.6; 1278-TCDD, log *P* = 6.22, ratio 18.7; PCB-118, log *P* = 6.78, ratio 19.5; **Figure 1**), which is also consistent with IVR (log *P* = 6.61, ratio 18.4) as reported by Hakk *et al.* [7]. The exception was 2-OH-1378-TCDD (log *P* = 6.15) which had a milk fat/whole milk concentration ratio of 11.2 in this study (**Figure 1**). These lower concentration ratios reported in the literature versus the current findings may be a reflection of differences in composition of the milk fat prepared here and the cream prepared in the cited reports.

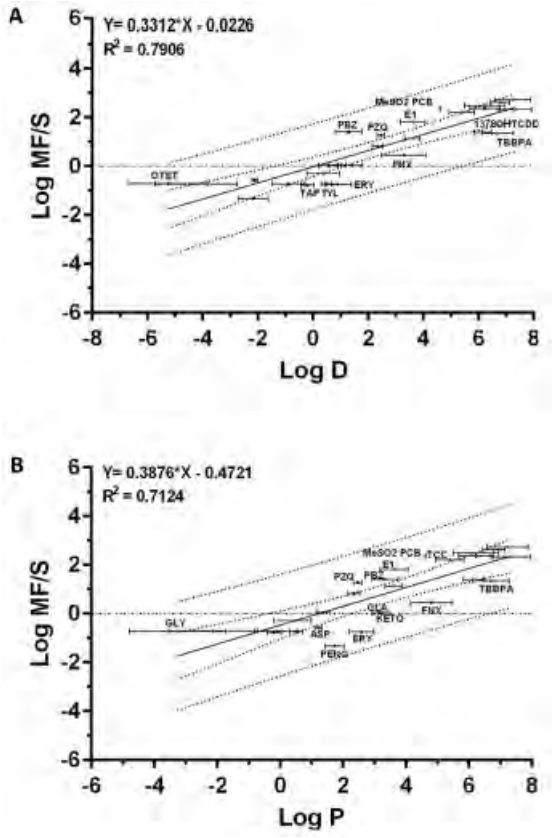
A compound with a log *P* value similar to that of BPA (log *P* = 3.60) is the organophosphate cruformate (log *P* = 3.33, Table S4), which was fed to cows [14]. Similar to BPA, which concentrated eightfold in fat relative to whole milk, cruformate concentrated about fi vefold into cream [14]. If values were adjusted to reflect lipid mass yield (15% of whole milk in their study, 10% in ours) the fivefold concentration would increase to ~ 7.6-fold, in close agreement with the eightfold concentration found for BPA. For fenthion (log *P* = 3.21, Table S4), an organothiophosphate insecticide, the concentration ratio of fat/whole milk was ~ 5, with 80 − 90% of the fenthion found in the fat fractions [15]. In the current work, the E1 (log *P* = 3.62) milk fat/whole milk concentration ratio was ~ 16 and 3′-$MeSO_2$-PCB (log *P* = 4.62, calculated) was ~ 18. Thus, the present results and those of O'Keeffe *et al.* [14, 15] suggested that factors in addition to log *P* also govern chemical disposition in milk.

Similar to the studies done by Hakk *et al*. [7] and Lupton *et al*. [9], GLY and IMI (this study) distributed predominantly into the skim milk; thus, the concentration ratio between skim milk/whole milk was ~ 1, whereas the ratio of milk fat/whole milk was ~ 0.2 (Figure 1). Hakk *et al.* [7] observed similar distributions for compounds with low log *D* values, for example, OTET, PENG, and ERY, as did Lupton et al. [9] for ASP, CIPR, TAP, and TYL despite the diversity of chemical structures.

Using literature values of log *P* and pK_a for each chemical (**Tables 1**, S1, and S2), mean and standard deviation (SD) log *D* values were calculated for ionizable compounds [16]. Relationships between log *D* or log *P* values and log [milk fat]/[skim milk] distributions, including 99% confidence interval (CI) and prediction interval, are shown in **Figure 2A** (log *D*) and **2B** (log *P*). There are apparent uncertainties with respect to log *D* or log *P* for many of the studied compounds (**Figure 2A,B**). In general, distribution uncertainties with regard to log *D* or log *P* are much greater than the error associated with measurements of milk fat or skim partitioning. By combining the log [milk fat]/[skim milk] data of the current set with results obtained from those of Hakk *et al*. [7] and Lupton *et al*. [9], the linear regression with log *D* had a regression coefficient of 0.79 and with log *P*, the resulting linear regression had an $r^2 = 0.71$ (**Figure 2A,B**). The slightly better regression using log *D* data reinforces the conclusions of Hakk *et al.* [7] and Lupton *et al.* [9] that log D was a better predictor of the distribution between milk fat and skim milk than log *P*. Nevertheless, **Figure 2A** indicates that based on the 99% CI for log *D*, numerous outliers were present when all 27 compounds were modeled. Outliers with respect to the 99% CI for the log *D* plot (**Figure 2A**) included ERY, FNX, TAP, TBBPA, 2-OH-1378-TCDD, and TYL, compounds which distributed more toward skim than predicted. 2-OH-1378-TCDD likely would fall within the 99% CI based on the SD of the calculated log *D*. Conversely, E1, 3′-$MeSO_2$-PCB-101, OTET, PBZ, and PZQ distributed more toward milk fat than predicted. Overall, the greatest limitation to predicting the behavior of any one chemical contaminant in milk seems to be the uncertainty associated with literature log *P* and pK_a values used to calculate log *D* values in the model derivation.

Slopes of the linear log *D* and log *P* models were not 1, but 0.33 and 0.39, respectively (**Figure 2**). There was no reason to expect a 1:1 relationship between log *D* or *P* values of a chemical and its distribution between milk fat and skim milk. The lower slopes do indicate modeled chemicals that typically distribute to a greater extent into skim milk than merely reflected by their log *D* or *P* values. Distribution data were not affected by the presence of degradates because none were detected by thin-layer chromatography (TLC) (Table S3). The model slopes highlight the differences between the simple, ideal, octanol/water partition system and the complex milk matrix which consists of water, lipid, protein, sugar, minerals, and micelles. We hypothesize that the presence of these additional milk components could account for the enhanced distribution into skim milk. For instance, milk proteins (casein, *β*-lactoglobulin, and lactalbumin) enhanced the solubilization of DDT in water [17].

Figure 2: Regression analyses of log[chemical]$_{milk\ fat}$ /[chemical]$_{skim\ milk}$ (log *F/S*) vs log *D* and log *P* (pH 6.8). Plot A is the regression analysis of log *F/S* vs log *D*. Plot B is the regression analysis of log *F/S* vs log *P*. Error bars on the log *D* and log *P* for the chemicals reflect the variability of values reported in the literature. Compounds outside the 99% CI but within 99% of the prediction interval are labeled. Regressions are based on data from 27 chemicals. Red dots are chemicals of the current study, whereas black dots are chemicals published in Hakk *et al.* [7] and Lupton *et al.* [9].



Chemical distribution from skim milk into curd and whey.

Recoveries of radioactivity across tested chemicals were ≥ 95% (sum of whey and curd), with the highest mean recovery (106.5%) occurring for *β*-HBCD and the lowest recovery occurring for PCB-118 (90.7%). The CVs for within dose replicates in whey and curd were generally < 4% for the majority of chemicals tested; however, the CVs for the most lipophilic persistent organic pollutants, that is, 1278-TCDD, BDE-99, *β*-HBCD, 3′-MeSO$_2$-PCB-101, and PCB-118, were considerably higher, exceeding 3% for whey (range 3.9 − 16.0%) and 4% for curd (range 4.3 − 10.0%; **Figure 3** and Tables S17−S28). Higher CVs for these lipophilic chemicals in whey are to be expected, especially at lower concentrations, because of the small percentage of each compound that distributed into whey. Chemical distributions were generally not dose-dependent for 0% moisture curd/whey ratios across the starting concentrations present in skim milk, although a dose dependency was apparent for BDE-99 ($p < 0.05$). An ~ 8% increase in association with the curd fraction was measured with BDE-99 with each 10-fold increase in dose, that is, from 73% to 80% to 92%, respectively. Initial concentrations in skim milk were 1.7, 13, and 204 nM (Table S28).

For the 12 compounds tested in the current study, chemicals retained in the curd fraction ranged from approximately 16.5% for GLY to 86% for *β*-HBCD when related to residual chemical in the skim milk of phase 1 (Tables S17 − S28). Distribution into curd was largely proportional to a chemical's lipophilicity. Of the most lipophilic compounds tested, ~ 80% of chemical was distributed into curd (1278-TCDD, BDE-99, *β*-HBCD, and PCB-118). Compounds having moderate lipophilicity, that is, TBBPA, 3′-MeSO$_2$-PCB-101, 2-OH-1378-TCDD, and TCC, were more evenly distributed into both curd (40 − 60%) and whey (35 − 60%). Highly polar compounds had the lowest affinity for curd, for example, GLY (16.5%) followed by IMI (23.7%; **Figure 3**, Tables S17−S28).

Figure 3: Drug distribution and relative concentration ratios from skim milk into whey and curd fractions. Bars represent percent mean of all concentrations (n = 3 concentrations; n = 3 replicates per concentration, replicate exceptions are n = 2 replicates each for PCB-118 50 and 200 nM, n = 2 replicates each for β-HBCD 200 and 500 nM, n = 2 replicates each for 1278-TCDD 20 and 200 nM, and n = 2 replicates for BDE-99 20 nM) ± SD of all three dose mean percentages based on dpm of whey and curd (at 70% moisture) fractions compared to forti fi ed skim milk dpm. Numerical values on the graph represent the mean ratio (n = 3) of the drug concentration in the fraction (curd or whey) to the initial drug concentration in skim milk ± SD. BDE-99 distribution was dose-dependent ($P < 0.05$). Sum of stacked plots represents total, unadjusted drug recovery values.

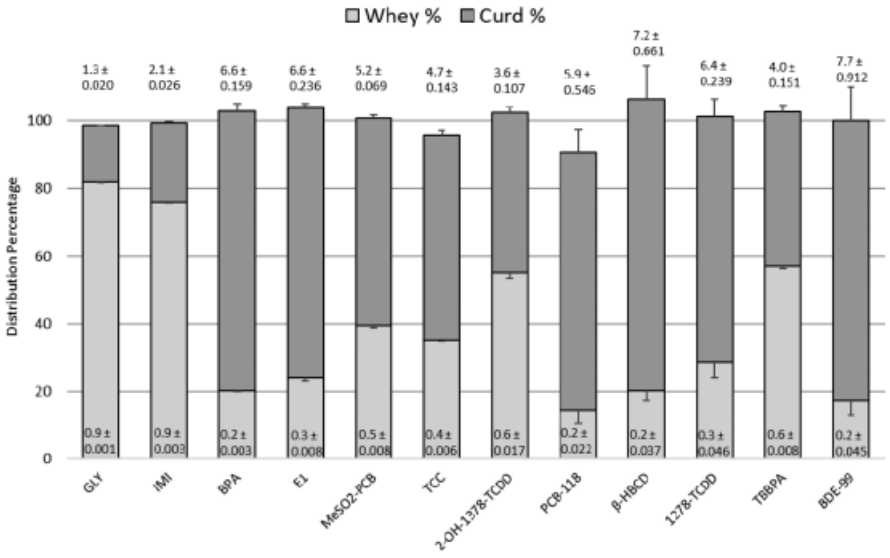


Table S17: GLY Phase 2 Average Distribution Data and Ratios.

Phase 2	Skim Milk			Whey Fraction					Curd Fraction					Percent Recovery	Obs. Ratio	Adj. Ratio
Average	Initial DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% GLY Dose	% GLY Dose (Corrected)	Total DPM	Mass (g)	Final nM (nmole/kg)	% GLY Dose	% GLY Dose (0% moisture)	Percent Recovery based on Skim Milk	$[GLY]_{curd}/[GLY]_{whey}$	$[GLY]_{0\%curd}/[GLY]_{whey}$
0nM		45.33			39.59					6.01						
20nM	114,206	45.54	22.59	93,858	40.05	21.11	82.20	90.69	18,869	5.76	29.51	16.52	8.03	98.72	1.40	2.63
200nM	1,139,999	45.44	226.03	936,093	39.85	211.60	82.12	90.65	189,618	5.88	290.74	16.63	8.10	98.75	1.37	2.45
2000nM	1,081,531	45.41	2,145.80	885,328	39.83	2,002.63	81.86	90.37	177,067	5.87	2,715.70	16.37	7.86	98.23	1.36	2.38
St Dev																
0nM		0.08			0.37					0.29						
20nM	1,882	0.04	0.39	907	0.16	0.28	1.41	1.55	189	0.15	0.49	0.19	0.05	1.59	0.04	0.30
200nM	10,494	0.02	2.18	3,990	0.12	0.45	0.95	1.04	728	0.13	7.32	0.10	0.02	1.05	0.04	0.21
2000nM	3,918	0.07	7.45	2,693	0.02	5.56	0.06	0.07	4,651	0.09	38.25	0.45	0.44	0.50	0.02	0.07
% RSD																
0nM		0.18			0.94					4.74						
20nM	1.65	0.08	1.72	0.97	0.40	1.33	1.71	1.70	1.00	2.54	1.67	1.13	0.65	1.61	3.01	11.24
200nM	0.92	0.05	0.97	0.43	0.31	0.21	1.16	1.15	0.38	2.23	2.52	0.61	0.30	1.07	2.56	8.45
2000nM	0.36	0.15	0.35	0.30	0.05	0.28	0.08	0.08	2.63	1.52	1.41	2.74	5.59	0.51	1.54	2.94

When curd data (normally 70% moisture) were expressed on a dry matter basis, the concentration ratios of 0% moisture curd to whey (Tables S17−S28) were > 100 for the most lipophilic compounds, that is, 1278-TCDD (115), BDE-99 (327), β-HBCD (152), and PCB-118 (136), and for two of the phenolics, BPA (111) and E1 (104). Other phenolic compounds, that is, TBBPA and 2-OH-1378-TCDD, had much lower concentration ratios of 32 and 18, respectively, whereas 3′-$MeSO_2$-PCB-101 (56) and TCC (46) were also lower than the most lipophilic compounds. The 0% moisture curd/whey concentration ratios

for the most polar compounds ranged from 9.2 for IMI to 2.5 for GLY (Tables S17−S28).

Results for TBBPA were unexpected based on its structural similarity to BPA. The fire-retardant TBBPA is identical in the base structure to the plasticizer BPA with the exception that the 4-ortho hydrogens, with respect to the phenolic hydroxyls, are replaced by bromines. Bromination of the ortho-protons enhanced lipophilicity (log *P*) of TBBPA compared to BPA. In the 0% moisture curd/whey, however, the concentration ratio decreased from 111 for BPA to 32 for TBBPA (Tables S17−S28). Based solely on lipophilicity (log *P*), the curd/whey concentration ratio would have been expected to increase for TBBPA relative to BPA. One possibility for the lower concentration ratio for TBBPA is that the much larger atomic radius of bromine (compared to hydrogen) resulted in steric hindrances for potential casein − TBBPA interactions.

Hydroxylation and methylsulfonation of chemicals altered distribution patterns in milk. Aromatic hydroxylation decreased lipophilicity slightly and thus increased distribution into skim milk for phase 1 and into whey for phase 2. For example, 2-OH-1378-TCDD had a greater distribution into the whey (~ 30% greater) compared to 1278-TCDD. Comparison of PCB-118 and 3′-MeSO_2-PCB-101 also indicated that a methyl sulfone group decreased lipophilicity (log *D* 6.38 vs 4.62, respectively) and increased (> 25%) distribution into whey. Despite a different chlorine substitution pattern between this pair of chemicals, the presence or absence of a methyl sulfone functional group likely plays a more important role in determining the effect on curd versus whey distribution. The full nature of this partitioning difference is undoubtedly based on more than hydrophobic interaction, for example, possible chemical/protein interactions or sequestration.

Published reports related to the partitioning of chemicals tested in this study into whey and curd are scant, but structures and characteristics of chemicals cited for comparison are provided in Table S4. For example, concentrations of the aromatic, chlorinated insecticide DDT (log *D* = 6.22 and log *P* = 5.92) were greater in cheddar cheese than in whey after milk processing, with cheese and whey concentrations of 47 and 0.5 ppm, respectively [12]. Similarly, Swiss-type cheese made from milk produced by dairy cows fed DDT contained ~ 8 times the original DDT concentration of whole milk, though DDT was not reported in whey [13]. In other studies, however, DDT was unstable during processing and 27 − 53% of the starting DDT degraded to DDE and DDD [18] during the manufacturing of cheese. While DDT was not identified in whey at the dipping stage, it was measured in the whey pressed from curd [19]. Whey produced during the processing of raw whole milk had levels of DDE and DDD that increased twofold when measured at acidification, and concentrations were the same in the cheese product [19]. Similar concentrations of DDT were reported for whole milk and cheddar or Monterey cheese, indicating some net loss of DDT, as total cheese mass would be less than the original milk mass. No changes in DDT concentration were observed during storage.

Lipophilic compounds in this study concentrated in the curd to a greater extent than whey, but the lipophilic pesticide lindane (log *P* = 3.99), a cyclo-chlorinated structure with similarities to *β*-HBCD, did not concentrate in cheese or yogurt (produced from curd) made from contaminated raw milk [20]. The authors attributed the lack of concentration to heat treatment during pasteurization which resulted in phenolic metabolite formation. Pasteurization resulted in a 65 − 73% reduction in lindane, with more losses during refrigeration of yogurt (1.4 − 8% over 3 days) and cheeses (36.7% in Ras cheese during 6 months in storage). Although the effects of pasteurization and storage were not investigated in the current study, similar losses in *β*-HBCD might occur. Contrary to Abou-Arab [20], Langlois *et al*. [13] found that lindane concentration in curd (4.3 ppm) was approximately 12 times that measured in whey (0.34 ppm). In a second study, Langlois *et al*. [21] determined that curd concentrations of endrin (log *D* and *P* = 4.9) were about eight times those in whole milk (5.48 vs 0.7 ppm), whereas whey concentration was only 0.06 ppm (curd/whey concentration ratio = 91). Surprisingly, heptachlor (log *P* = 5.46), with higher lipophilicity than endrin, was present in whey (0.17 ppm) at approximately 1/20 the concentration measured in curd (3.77 ppm) (curd/whey concentration ratio = 22) [21]. Cruformate (log *P* = 3.33), which has a log *P* similar to BPA (3.60) and E1 (3.62), had a dose-dependent distribution. At a starting milk concentration of 0.07 ppm, cruformate was 22 times more concentrated in curd than that in whey (0.43 vs 0.02 ppm, respectively), but with a starting milk concentration of 0.16 ppm, the curd/whey concentration ratio was 31 (0.92 and 0.03 ppm, respectively) similar to that of BPA (29) and E1 (24) [14].

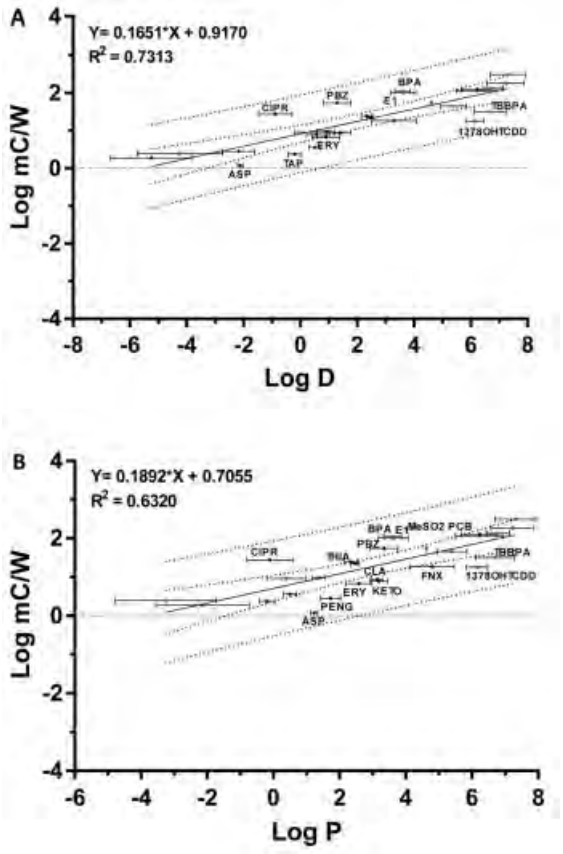
Hydrophilic compounds distributed more evenly between curd and whey. For example, the curd/whey

concentration ratio for GLY (log $D = -4.24$) was 1.4 and for IMI (log $D = -0.38$) was 2.4, similar to SDMX (ratio 3.2), PENG (ratio 1.2), OTET (ratio 1.4), ERY (ratio 2.4), and KETO (ratio 2.4) as previously reported [8]. Given the diversity of chemical structures tested, the log *D* value of hydrophilic compounds does provide some predictive measure for curd and whey distribution. Similarly, TAP and TYL possessed fairly low curd/whey concentration ratios, that is, 1.3 and 1.5, respectively [9].

Figure 4A (log *D*) and **4B** (log *P*) shows the relationships between log D or log P values and log[0% moisture curd]/[whey] concentration ratios, including 99% CI and prediction interval. By combining the log[0% moisture curd]/[whey] data of the current set with those of Shappell et al. [8] and Lupton *et al.* [9] the regression with log D had an $r^2 = 0.73$, whereas the log *P* regression had an $r^2 = 0.63$ (**Figure 4A,B**). The higher regression coe ffi cient obtained using log D data reinforces the previous conclusion that log *D* is a better predictor of the distribution between curd and whey than log *P* [8,9].

On the basis of the 99% CIs for the log *P* regression, numerous outliers were present when all 27 compounds were modeled. Outliers for the curve fit on a log *D* basis (Figure 4A) included ASP, ERY, 2-OH-1378-TCDD, TAP, and TBBPA compounds which distributed more toward whey than predicted. Conversely, BPA, CIPR, E1, and PBZ distributed more toward curd than predicted. In the log *P* model (Figure 4B), four additional chemicals (CLA, KETO, FNX, and PENG) fell outside of the 99% CIs.

Figure 4: Regression analyses of log[chemical]$_{0\%\text{moisture curd}}$ /[chemical]$_{\text{whey}}$ (log *mC/W*) vs log *D* and log *P* (pH 6.8). Plot A is the regression analysis of log *mC/W* vs log *D*. Plot B is the regression analysis of log *mC/W* vs log *P*. Error bars on the log *D* and log *P* for the chemicals reflect the variability of values reported in the literature. Compounds in between the 99% CI and 99% of the prediction interval are labeled. Red dots are chemicals of the current study, whereas black dots are chemicals published in Shappell *et al.* [8] and Lupton *et al.* [9]. Regressions are based on data from 27 chemicals.



Chemical distribution from whey into retentate and permeate.

In order to assess the percent of drug associated with the whey proteins, ultra filtration in conjunction with centrifugation was performed (phase 3, **Figure 5**). The expected volume of retentate was 33% of the applied sample volume based on centrifugation time and speed, with the actual measured mean for

all compounds being 37 ± 3.3%. Mean recovery of radioactivity across all compounds was 100 ± 4.5%. Mean non-specific binding of compounds to filters ranged from 0.2% for GLY to 22.5% for E1. Compounds with > 3% filter binding include PCB-118 (6.4%), BDE-99 (7.1%), β-HBCD (8.2%), BPA (13.4%), and E1 (22.5%) (Tables S29 − S40). Although compounds with high log *D* values could be expected to be "sticky" in the aqueous medium, four compounds with high log *D* values [TCC (log *D* = 5.39), 2-OH-1378-TCDD (log *D* = 6.15), 1278-TCDD (log *D* = 6.22), and TBBPA (log *D* = 6.69)] had filter binding of ≤ 2.4%.

The associations of the 12 xenobiotics with whey protein, as determined by the percentage of compound measured in the retentate, revealed three groupings (**Figure 6**). The first was represented by GLY and IMI that have negative log *D* values (− 4.24 and − 0.38, respectively), where there was essentially no association with the whey protein (< 5%) occurred (Tables S29 and S30). The second grouping was composed of BPA, E1, 3′-MeSO$_2$-PCB-101, 2-OH-1378-TCDD, and 1278-TCDD, which had moderate associations with whey protein, ranging from 33 to 76% (Tables S31−S33, S35, and S38). Similar to our findings of ~ 64% association of E1 with whey protein, Wolford and Argoudelis [22] reported 48 and 53% of 17 β-estradiol and E1, respectively, associated with whey protein. The third grouping was composed of those compounds that were almost totally associated with retentate whey proteins (84 − 98%, one outlier of 107% for PCB-118 due extremely low starting radiocarbon in the whey). Chemicals in this grouping included BDE-99, β-HBCD, PCB-118, TBBPA, and TCC (Tables S34, S36, S37, S39, and S40). If present in whey, these compounds would concentrate in whey-derived protein products.

The percent of whole milk dose associated with either casein or whey proteins is reported in **Table 2**. About 25% of TBBPA and 2-OH-1378-TCDD from whole milk distributed to whey, yet ~ 90 and 70% (TBBPA and 2-OH-1378-TCDD, respectively) of that were associated with whey protein.

Figure 5: Scheme of milk partitioning processes that yielded cream and milk fat from whole milk (phase 1) curd and whey from skim milk (phase 2) and retentate and permeate from whey (phase 3).

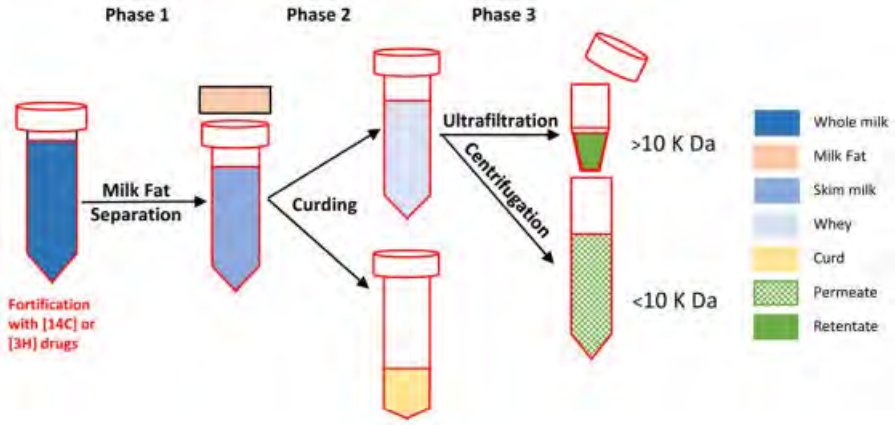


Figure 6: Drug distribution from whey into permeate, retentate, and filter fractions. Bars represent percent mean of all concentrations (n = 3 concentrations, concentration exceptions are PCB-118 and *β*-HBCD n = 2 concentrations; n = 3 replicates per concentration, replicate exceptions n = 2 replicates for TCC 20 nM, n = 2 replicates each for 1278-TCDD 200 and 2000 nM, n = 2 replicates for BDE-99 200 nM) ± SD of all three dose mean percentages based on dpm of permeate and retentate fractions compared to fortified whey dpm. Horizontal lines on each bar represent the actual retentate and permeate volume percentage after centrifugation. Sum of stacked plots represents total, unadjusted drug recovery values).

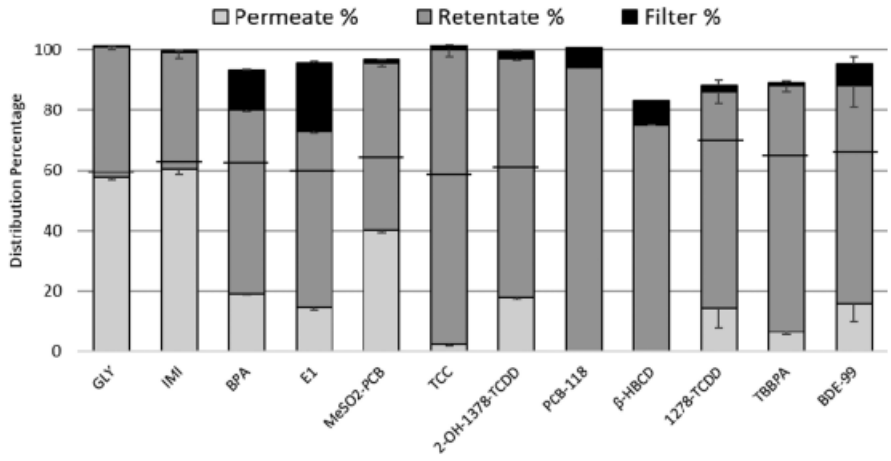


Table S29: GLY Phase 3 Average Distribution Data and Ratios.

Phase 3	Whey			Retentate Fraction				Permeate Fraction				Filter		Total Dose Recovery	Protein Association
Mean	Initial DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% GLY Dose	Total DPM	Volume (mL)	Final nM	% GLY Dose	Total DPM	% GLY Dose	Percent Recovery based on Whey	% Associated
0nM		14.86			5.82				9.12						
20nM	34,827	14.86	21.11	15,225	6.16	22.25	43.73	19,722	8.78	20.24	56.62	57	0.16	101.12	4.6
200nM	349,549	14.88	211.60	148,612	6.00	223.16	42.52	202,487	8.96	203.51	57.93	490	0.14	101.20	4.4
2000nM	330,961	14.89	2,002.63	142,417	6.02	2,132.92	43.03	192,264	8.96	1,934.18	58.09	484	0.15	101.91	4.7
St Dev															
0nM		0.01			0.17				0.17						
20nM	458	0.01	0.28	231	0.10	0.06	1.19	371	0.10	0.23	0.33	11	0.03	0.85	0.6
200nM	864	0.01	0.45	4,001	0.14	1.20	1.24	3,503	0.14	1.02	0.91	26	0.01	0.71	0.3
2000nM	1,160	0.01	5.56	1,910	0.08	6.83	0.73	1,738	0.09	5.50	0.33	26	0.01	0.44	0.2
% RSD															
0nM		0.0			2.85				1.85						
20nM	1.31	0.1	1.33	1.52	1.70	0.25	2.72	1.88	1.13	1.13	0.58	18.57	18.79	0.84	12.4
200nM	0.25	0.04	0.21	2.69	2.26	0.54	2.92	1.73	1.59	0.50	1.57	5.37	5.52	0.71	6.7
2000nM	0.35	0.08	0.28	1.34	1.29	0.32	1.69	0.90	1.00	0.28	0.57	5.37	5.61	0.44	4.8

Chemical concentration based on protein mass for casein and whey proteins

Using 0% moisture curd data from phase 2, the amount of chemical associated with caseins was calculated based on proteins present in curd and largely result from agglutination of casein (**Table 2**). Similarly, using phase 3 data, the amount of chemical associated with whey proteins can be calculated (**Table 2**). Chemical saturation of casein or whey protein was not observed because the mass of chemical per milligram protein increased as the concentration increased. In some instances, the initial expected fortification concentrations in whole milk differed from measured concentrations, as seen with 3′-MeSO$_2$-PCB-101 and *β*-HBCD. Whey protein association values for the lowest dose of BDE-99 are questionable because the starting skim milk contained < 2 nM and whey 0.3 nM. However, confidence in casein/whey protein association results is enhanced by the agreement found across doses (**Table 2**),

exception was BDE-99, where ratios ranged from 2.8 to 5.5.

For the majority of chemicals tested (BDE-99, BPA, E1, *β*-HBCD, IMI, 3′-MeSO$_2$-PCB-101, PCB-118, and 1278-TCDD), the association with caseins was greater than that for whey proteins (ratio > 1, **Table 2**). The importance of methodology is evident when comparing ourfindings to those of Wolford and Argoudelis [22] that used equilibrium dialysis with E1 and the slightly more hydrophilic compound E2. They reported that E1 and E2 were largely (> 84%) bound to protein when incubated in skim milk, and > 50% of the bound estrogens was associated with whey proteins. These data are in contrast to our findings for E1, in which the association (nmol/mg protein) ratio was approximately 2 for casein/whey.

The difference between the results of the two studies was most likely the precipitation of curd caseins in the present work versus the presence of soluble caseins used for dialysis by Wolford and Argoudelis [22] (1979).

Other chemicals that preferentially associated with caseins relative to whey protein (ratio > 1) include THIA (2.5), IVR (2.0) [8], TYL (1.4), CIPR (2.0), and PZQ (1.5) [9]. Although the current work used a majority of chemicals with log *D* greater than 3.4, our previous reports described only one such chemical (IVR). The casein/whey protein association ratio of IVR was more similar to BPA (2.0), E1 (1.9), and IMI (1.9) (**Table 2**).

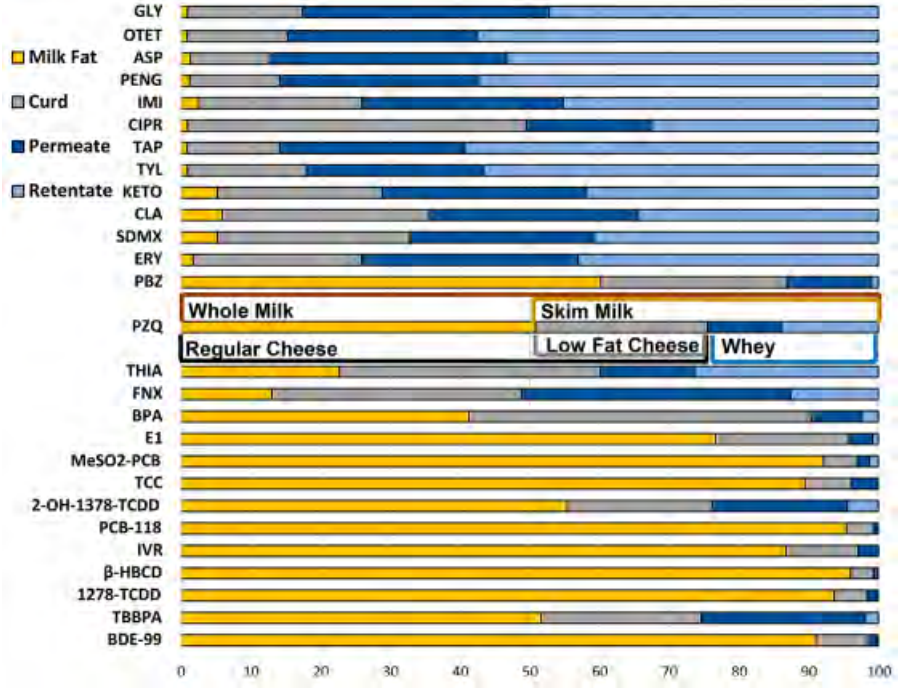
In spite of higher distribution of GLY into whey than curd (**Figure 3**), there was in fact very little preferential retention of GLY associated with whey protein (Figure 6). Similarly, TCC, 2-OH-1378-TCDD, and TBBPA also had casein/whey protein ratios <1. Although most of the total TCC dose was partitioned with milk fat (mean 85%), the remainder distributed almost equally between whey and 0% moisture curd (57% curd, Table S22). TCC remaining in the whey was concentrated almost exclusively in the retentate (98%) during ultracentrifugation (Table S34). The log *D* values of 2-OH-1378-TCDD (6.15) and TBBPA (6.69) did not predict the respective mean casein/whey protein ratios of 0.26 and 0.33. Both chemicals also distributed to a lesser extent than predicted into milk fat. The common feature of both compounds is a hydroxyl moiety between two halogens (chlorines for 2-OH-1378-TCDD and bromines for TBBPA).

Previously studied chemicals that had higher association for whey proteins versus caseins were PENG (casein/whey ratio = 0.2), ERY (0.5), KETO (0.4), SDMX (0.8) [8]; TAP (0.5), CLA (0.4), and FNX (0.25) [9]. Although the distribution between lipid and aqueous phases was markedly dependent on the property of proteins, namely lipophilicity, small-molecule binding to proteins seems to be more dependent on specific functional groups within the protein. Identifying the specific functional groups and binding domains that can associate with studied chemicals within a plethora of whey and casein proteins lies outside the scope of the present research.

Relation to consumer products

To determine how the distributions of these compounds, if detected in whole milk, related to consumer products, the percent distributions intomilk fat, curd, retentate, and permeate were calculated in relation to the starting concentration in whole milk. **Figure 7** includes the experimentally derived percentages of each compound in high-fat products which would include butter, cream, and cheese; low-fat products would include skim milk, low fat cheese, yogurt, and low-fat derived whey protein products such as whey protein powders and baby formulas. Comparable to compounds previously tested [8,9], higher log *D* compounds (i.e., E1, 3 ′ -MeSO 2 -PCB-101, TCC, PCB-118, *β*-HBCD, 1278-TCDD, and BDE-99) generally distributed to high-fat products such as butter and cream. High-fat products that contain protein (*i.e.*, cheese) will concentrate both mid- to high-range log *D* molecules such as BPA, 2-OH-1378-TCDD, and TBBPA along with the higher log *D* compounds. Two compounds with low log *D*'s, that is, GLY and IMI, will primarily distribute into aqueous products, such as skim milk and whey.

Figure 7: Normalized percentages of chemicals calculated from whole milk to be in the milk end-products of milk fat, curd, permeate, and retentate based on data generated from the current studies as well as those reported in Hakk *et al.* [7], Shappell *et al.* [8], and Lupton *et al.* [9]. The PZQ bar has additional information on which milk end products comprise whole milk, skim milk, curd, low-fat curd, and whey, as a guide to where drug may partition during commercial milk processing. For percentage of chemical associated with whey protein see supplemental information tables S29 − S40).



Determining where a compound would concentrate in consumer products will also depend on the processing steps involved and what specific end product is being manufactured. For example, whole milk processed into skim milk and cream would generally have compounds with high log *D* values concentrated in butter and cream, whereas compounds with low log *D* values will be in skim milk. Compounds with mid-range log *D* values will be split between the higher fat products and skim milk. However, if whole milk is processed directly into cheese, then the mid-range and high-range log D value compounds will mainly concentrate in the cheese.

Conclusions

The partitioning of 12 environmental contaminants or metabolites into milk fractions was assessed. Partitioning between milk fat and skim milk and between 0% moisture curd and whey was usually governed by the compound's lipophilicity. If a chemical was found in whey, the more nonpolar the compound the more likely it would be found in whey protein products. Phenolic compounds were the main chemicals that fell outside of the 99% CIs of the models' regression analyses. These models provide a tool using log *D* as the primary chemical property to predict the distribution of chemicals into various milk products.

Supporting information

Supporting information with (Tables S1−S40) is available online:

http://pubs.acs.org/doi/suppl/10.1021/acsomega.8b00762/suppl_file/ao8b00762_si_001.pdf.

Only the tables relevant to glyphosate (S5, S17, S29) are shown in this summary.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The purpose of the described work was to investigate the partitioning of 12 environmental chemicals of diverse polarities into various milk fractions. One of the tested chemicals was glyphosate. The experiments were conducted with radio-labelled test materials which were fortified to raw (unpasteurized, non-homogenized) cow milk (3 fortification levels were investigated for each compound). Thereafter, the milk was processed into skim milk, milk fat, curd, whey, whey retentate and whey permeate. A linear model predicting the distribution of chemicals between skim milk and milk fat based on their lipophilicity was established. The distribution between curd and whey was also correlated with lipophilicity. Phenolic compounds had less predictable distribution patterns based on their lipophilicities.

During processing of whole milk to skim milk and milk fat, glyphosate partitioned essentially to skim milk (> 99%). Only about 1% of the glyphosate fortified to whole milk was recovered in milk fat. Following curding of the skim milk, most glyphosate remained in the whey fraction (> 80%). The associations of glyphosate with whey protein (calculated by subtracting the amount present in permeate from the amount present in retentate) was very low (< 5%). As expected due to its hydrophilicity, glyphosate primarily distributes into aqueous products, such as skim milk and whey. The distribution pattern between the various milk fractions was similar for the various amounts of glyphosate fortified to whole milk (range of ca. 0.004 mg/L to 0.348 mg/L).

Although the distribution of residues between skim milk and milk fat is not a data requirement for hydrophilic compounds like glyphosate, this information is considered relevant to risk assessment. Overall, the publication is deemed reliable. Normally, the distribution of residues between skim milk and milk fat should be investigated with raw milk containing incurred residues (in the context of metabolism or feeding studies) and not by (artificially) fortifying raw milk. However, due to the very low transfer of glyphosate-derived residues in milk, the approach used in the publication seems to be the best option to determine the distribution of parent glyphosate residues between skim milk and milk fat.

1. Information on the study

Data point	CA 6.10.1
Report author	Thompson T.S. et al.
Report year	2019
Report title	Determination of glyphosate, AMPA, and glufosinate in honey by online solid-phase extraction-liquid chromatography-tandem mass spectrometry
Document No.	Food Additives & Contaminants: Part A, 2019, Vol. 36, No. 3, 434-446
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

A simple method was developed for the simultaneous determination of glyphosate, its main degradation product (aminomethylphosphonic acid), and glufosinate in honey. Aqueous honey solutions were derivatised offline prior to direct analysis of the target analytes using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry. Using the developed procedure, accuracies ranging from 95.2% to 105.3% were observed for all analytes at fortification levels of 5, 50, and 150 µg/kg with intra-day precisions ranging from 1.6% to 7.2%. The limit of quantitation (LOQ) was 1 µg/kg for each analyte. Two hundred honey samples were analysed for the three analytes with AMPA and glyphosate being most frequently detected (99.0% and 98.5% of samples tested, respectively). The concentrations of glyphosate were found to range from < 1 to 49.8 µg/kg while those of its degradation product ranged from < 1 to 50.1 µg/kg. The ratio of glyphosate to AMPA was found to vary significantly amongst the samples where both analytes were present above the LOQ. Glufosinate was detected in 125 of 200 samples up to a maximum concentration of 33.0 µg/kg.

Materials and Methods

Reagents and standards

Reagent water (>18 MΩ resistivity) was produced using a Barnstead NANOPure reverse osmosis system. Acetonitrile (ACN; HPLC grade) was purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Ammonium carbonate (ACS reagent grade), sodium carbonate (ACS reagent grade), 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl), and neat reference materials of glyphosate, AMPA, and glufosinate ammonium were obtained from Sigma Aldrich Canada (Oakville, ON, Canada). Isotopically labelled forms of the analytes, specifically $^{13}C_2$,^{15}N-glyphosate, ^{13}C,^{15}N-AMPA, and D_3-glufosinate hydrochloride, were purchased from Toronto Research Chemicals (North York, ON, Canada).

Individual stock standard solutions of glyphosate, AMPA, and glufosinate were prepared by dissolving 10 mg of each analyte in 10 mL of reagent water). A mixed working spike solution containing 1 µg/mL of each analyte in water was prepared from the stock standard solutions. A second working spike solution containing 0.1 µg/mL of each analyte was prepared by diluting the 1 µg/mL solution ten-fold with water. Stock standards of the isotopically labelled internal standards were likewise prepared in water but at a concentration of 100 µg/mL. A working solution containing 1 µg/mL of each internal standard compound was prepared by mixing 0.1 mL of each stock standard solution and diluting to a final volume of 10 mL.

A 0.1 M solution of sodium carbonate, used to adjust the pH of the honey solutions prior to derivatisation, was prepared in reagent water. A 0.05% (w/v) solution of FMOC-Cl in ACN was prepared fresh for use in derivatising the analytes and their corresponding internal standards.

Sample preparation

Two gram portions of individual honey samples were weighed into 15-mL polypropylene centrifuge tubes (VWR Canada, Edmonton, AB, Canada). The samples were fortified with 50 µL of the working internal standard solution and allowed to sit for 10 min prior to the addition of 5 mL of reagent water. The centrifuge tubes were capped and mixed on a mechanical shaker until the honey was completely dissolved.

Due to difficulties encountered in obtaining a honey sample which did not contain traces of glyphosate, calibration standards were prepared in reagent water. To compensate for the final volume of the honey solution obtained by dissolving 2 g of honey in 5 mL of water, the volume of reagent water added to each 15-mL centrifuge tube was 6.5 mL. A series of 9 calibration standards were prepared by spiking the reagent water aliquots with equivalent analyte concentrations of 0, 1, 5, 10, 20, 50, 75, 100, and 200 µg/kg. Each calibration standard was also spiked with 50 µL of the working internal standard solution. Replicate spiked honey samples for method validation were prepared by fortifying portions of a honey sample which was found to be free of all three analytes at the LOQ values (1 µg/kg) of the proposed method. The levels of fortification for the spiked replicates were chosen at equivalents of 5, 50, and 150 µg/kg).

Prior to analysis by LC-MS/MS, 0.5 mL aliquots of all honey solutions and calibration standards were pipetted into a 2-mL polypropylene microvial to which 0.5 mL of 0.1 M sodium carbonate solution was added. The tubes were capped and mixed by inverting several times. A 0.2 mL portion of the FMOC-Cl in ACN solution was added to each microvial which was then recapped and mixed using a high-speed orbital shaker (Bead Ruptor 12, Omni International Inc., Kennesaw, GA, USA) for two 90 s cycles at maximum speed. Next, the micro-vials were mixed for an additional 60 min using a rocking bed mixer. After derivatisation, the honey mixtures were filtered using 25 mm nylon filters (0.25 µm pore size) directly into polypropylene LC vials (Chromatographic Specialties, Brockville, ON, Canada).

Instrumental analysis

The configuration of the online SPE-LC-MS/MS setup is illustrated in **Figure 1**. The Shimadzu liquid chromatograph system included a SIL30AC autosampler, two LC30AD solvent delivery pumps, and a CBM20A module controller. A six-port, two-position, electronically actuated switching valve (Rheodyne MXT715, Scientific Products and Equipment, Oshawa, ON, Canada) was used to incorporate the online SPE cartridge within the LC-MS/MS system via contact closure through the LC module controller. An Oasis HLB extraction cartridge, 20 × 3.9 mm with 5 µm particles (Waters Ltd., Mississauga, ON, Canada) was employed for the online SPE step. The extraction cartridge was protected by a 4 × 2 mm i.d. RP-1 polymeric guard cartridge (Phenomenex, Torrance, CA, USA). The analytical column was an Agilent Zorbax Extend-C18 column (50 × 2.1 mm, 1.8 µm) preceded by a guard column with similar stationary phase material (5 × 3.0 mm). A binary gradient elution programme employing 10 mM ammonium carbonate in water and ACN as the two mobile phases was used for the online SPE step and the final chromatographic separation. The parameters for the gradient elution programme including the switch positioning of the six-port valve are listed in **Table 1**. The LC was re-equilibrated at initial conditions for 4 min prior to the next injection. The injection volume for all analyses was 50 µL.

A Sciex 4500 quadrupole tandem mass spectrometer was interfaced to the LC using an electrospray ionisation (ESI) probe. The MS/MS was operated in the negative ESI mode with the following general parameters: probe temperature = 700°C; ion spray voltage = −3.5 kV; curtain gas = 20 units; source gases 1 and 2 at 70 units each; collisionally activated dissociation (CAD) gas value = 8 units. All analyses were performed using multiple reaction monitoring (MRM) with the analyte-specific parameters provided in **Table 2**. The dwell time for each MRM transition was 50 ms. A programmable six-port switching valve incorporated into the MS/MS was used to divert flow from the analytical column to the MS/MS only from 6.5 to 9 min during the LC gradient elution programme in order to minimise contamination of the MS ion source.

Figure 1: Schematic diagram of online solid-phase extraction coupled to LC-MS/MS showing solvent flow with switching valve in (a) position #1 for flushing bulk matrix to waste and (b) position #2 for elution and chromatographic separation of analytes prior to MS/MS detection.

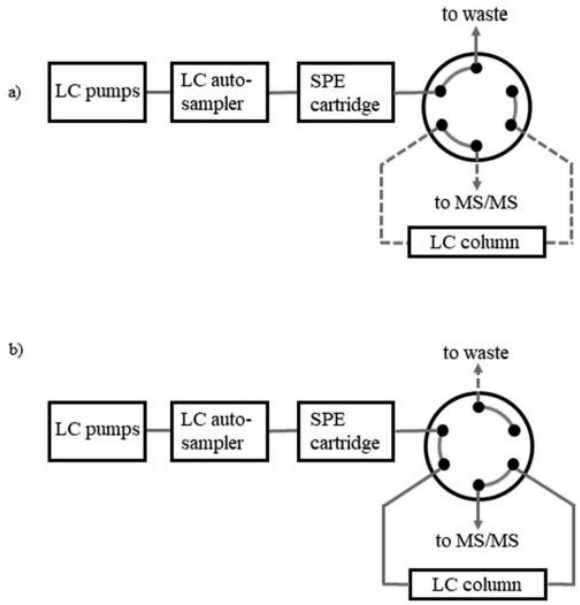


Table 1: LC gradient elution program and six-port switching valve position.

Time (min)	%A (10 mM $(NH_4)_2CO_3$)	%B (ACN)	Flow rate (mL min^{-1})	Valve position
0	100	0	1.00	to waste
2.9	100	0	1.00	to waste
3.0	100	0	0.35	to waste
5.5	*73.6**	*26.4**	0.35	to analytical column
12.0	5	95	0.35	to analytical column
12.5	5	95	0.35	to analytical column
13.0	5	95	0.60	to analytical column
15.5	5	95	0.60	to analytical column
16.00	5	95	0.35	to analytical column
17.00	98	2	0.35	to analytical column
19.00	98	2	0.35	to waste

*Estimated composition based on programmed gradient from 100% A at 3.0 min to 5% A at 12.0 min.

Table 2: MRM parameters for analytes and corresponding internal standards.

Compound	Precursor > product ions*	DP (V)	CE (eV)
glyphosate	***390 > 168***	−40	−16
	390 > 150	−40	−34
$^{13}C_2$,^{15}N-glyphosate	***393 > 170***	−40	−16
AMPA	***332 > 110***	−40	−16
	332 > 136	−40	−20
^{13}C,^{15}N-AMPA	***334 > 112***	−40	−16
glufosinate	***402 > 180***	−45	−14
	402 > 206	−45	−20
D_3-glufosinate	***405 > 183***	−45	−14

*Transition in bolded italics used for quantitation.

Results and Discussion

Considerations for proposed analytical method

There were two main considerations which dictated the direction taken for the development of the method to determine trace residues of glyphosate, AMPA, and glufosinate in honey. The first

consideration was the desired LOQ which was established based on the maximum residue limit (MRL) for glyphosate and glufosinate in honey. While Health Canada has not established an MRL for either glyphosate or glufosinate in honey, the EU has set the maximum acceptable concentration at 50 µg/kg for each compound (European Union: Pesticides database 2016). It was decided that the targeted LOQ value should not exceed one-tenth of this MRL value (in other words be 5 µg/kg or lower). The main reason for this targeted LOQ was to have a method which would permit its application to a general survey to establish baseline residue levels rather than determine compliance with existing MRL values.

The second consideration was the necessity to isolate the analytes from the honey matrix which is comprised mainly of the monosaccharides fructose and glucose as well as lower amounts of disaccharides and various other carbohydrates (Bell 2007). On the basis of weight, water typically accounts for less than 20% of the honey matrix with the majority of the remaining components consisting of simple sugars. The challenge of separating the highly polar analytes of interest from the relatively large quantities of highly polar carbohydrates prior to MS/MS analysis was a significant factor in the development of the proposed testing method.

One of the major advantages of LC over GC is the amenability of the former for the determination of analytes with polar functional groups without the necessity of performing derivatisation. There are, however, still two inherent benefits to performing derivatisation of highly polar analytes such as glyphosate, AMPA, and glufosinate prior to analysis by LC-MS based techniques. Derivatisation of highly polar analytes can result in increased retention using reversed phase stationary phases and increased sensitivity in electrospray ionisation MS (Toss *et al.* 2017). While direct determination of non-derivatised analytes is desirable in that it simplifies the analytical method, there has been mixed success in the development of such procedures. Ibanez *et al.* (2005) attempted to determine glyphosate, AMPA, and glufosinate without derivatisation but encountered difficulties including reduced sensitivity and lack of robustness of their proposed hydrophilic interaction liquid chromatography (HILIC) method. This ultimately resulted in their decision to employ derivatisation with FMOC-Cl. Similarly, Ehling and Reddy (2015) explored the direct analysis of glyphosate and AMPA using a variety of chromatographic stationary phases but also reported problems with lack of ruggedness, poor chromatographic peak shapes, and inadequate ESI-MS/MS sensitivity. Liao *et al.* (2018) stated that direct determination of glyphosate did not provide adequate sensitivity and selectivity to permit its analysis in baby food samples at concentrations as low as 10 µg/kg. For these reasons, derivatisation with FMOC-Cl has remained a popular procedure in numerous LC-MS-based methods (Arkan and Molnar-Perl 2015).

Based on initial investigations in our lab, it was observed that the sensitivity obtained for FMOC-Cl derivatives of the target analytes was significantly greater than for the non-derivatised compounds under negative electrospray ionisation conditions. A further complication of the direct determination of non-derivatised glyphosate, AMPA, and glufosinate in honey is the fact that the highly polar analytes of interest are difficult to separate from the polar carbohydrates which comprise the bulk of the honey matrix (approximately 80% by weight simple sugars). While ion exchange solid-phase extraction (SPE) remains an option for isolating glyphosate, AMPA, and glufosinate from the sugars, the inclusion of an offline SPE clean-up step was undesirable due to the additional associated increases in labour, cost, and time. Derivatisation with FMOC-Cl increases the retention of glyphosate, AMPA, and glufosinate on reversed phase stationary phases making it possible to separate the derivatised analytes from highly polar carbohydrates which constitute the bulk of the honey matrix.

Numerous groups have employed online solid-phase extraction methods for the determination of one or more of glyphosate, AMPA, and glufosinate in water samples after offline derivatisation using FMOC-Cl (Vreeken *et al.* 1998; Meyer *et al.* 2009; Sanchis *et al.* 2012; Poiger *et al.* 2017). The advantages of online SPE versus offline SPE are three-fold: firstly to automate the clean-up procedure thereby reducing labour and preparation time; secondly to permit the direct transfer of the analytes of interest from the extraction column/cartridge to the analytical column; and thirdly to facilitate the refinement of the conditions under which the analytes are trapped and subsequently eluted for direct determination. The capability to monitor the chromatographic behaviour of the analytes during online SPE coupled to LC-MS/MS simplifies method development. It was therefore decided to investigate an analytical procedure employing offline derivatisation of glyphosate, AMPA, and glufosinate followed by online SPE separation of the derivatives from the bulk honey matrix with subsequent direct determination by LC-MS/MS.

Derivatisation using FMOC-Cl

Two challenges were encountered in establishing the derivatisation procedure. Firstly, derivatisation of the analytes using FMOC-Cl was discovered to not work efficiently when sodium tetraborate was used in the presence of the honey matrix. Honey is quite acidic in a relatively concentrated solution (2 g of honey plus 5 mL of water) and the borate solution did not have enough buffering capacity to permit the pH of the resulting mixture to be approximately 9 as commonly established in the derivatisation reaction employing FMOC-Cl (Arkan and Molnar-Perl 2015). Sodium carbonate has been used in the derivatisation reaction with FMOC-Cl with aminophosphonic acids (Huber and Calabrese 1985) while a carbonate buffer was used in conjunction with FMOC-Cl and tertiary amphetamines (Herraez-Hernandez and Campins-Falco 2000). Descombes *et al.* (1991) reported that borate and carbonate buffers both worked well in providing alkaline conditions (pH = 9.5) under which the derivatisation of catecholamines and amphetamines could be achieved with FMOC-Cl. Upon switching to 0.1 M sodium carbonate for pH adjustment, it was observed that the derivatisation step proceeded smoothly.

The second challenge was realised when it became obvious that relatively dilute solutions of FMOC-Cl in ACN (e.g. 1 to 10 mg/mL) were not adequate to fully derivatise the analytes in the presence of the honey matrix. Nedelkoska and Low (2004) noted that excessive amounts of FMOC-Cl relative to the quantities of glyphosate present in the sample are required for complete derivatisation of the target analyte due to the reactivity of FMOC-Cl with matrix compounds containing primary and secondary amine functional groups. According to Ehling and Reddy (2015), concentrations of FMOC-Cl solutions used to derivatise glyphosate and AMPA have been previously reported to range from 1 to 28 mg/mL. Toss *et al.* (2017) used 0.14 mL of a 30 mg/mL solution of FMOC-Cl in acetonitrile to derivatise glyphosate and AMPA in surface water samples containing high levels of organic matter.

Honey is a complex matrix which may contain up to 1% (w/w) of free amino acids and 0.2–1.6% protein (Santos-Buelga and Gonzalez-Paramas 2017) which will potentially react with the FMOC-Cl. It was determined that increasing the FMOC-Cl concentration to 50 mg/mL in CAN and utilising 0.2 mL of this solution was necessary to provide the successful derivatisation of the analytes and their corresponding internal standards in the presence of the honey matrix.

Development of online SPE-LC-MS/MS method

The major sugars present in honey were poorly retained by the HLB extraction cartridge and could be flushed to waste without ever reaching the analytical LC column. The derivatised analytes were retained by the extraction cartridge and switching the position of the six-port valve allowed them to be subsequently eluted onto the analytical LC column for further chromatographic separation.

Figures 2 and **3** show the reconstructed MRM ion chromatograms obtained for the determination of a nominally blank honey (*i.e.* all analytes below the LOQ of 1 µg/kg) and the same honey fortified with 5 µg/kg each of glyphosate, AMPA, and glufosinate. During initial method development work it was discovered that it was virtually impossible to find a honey sample which was completely free of all three analytes. Each pair of ion chromatograms for the unspiked and spiked honey samples have been plotted on the same scale for each analyte. While there are additional peaks present in the chromatograms for both glyphosate and glufosinate in the blank honey, these peaks elute after the target analytes and therefore do not interfere in their analysis .

Figure 2: Reconstructed ion chromatograms for blank honey fortified with 25 µg/kg of each isotopically labelled internal standard. The quantitation and confirmatory MRM transitions (respectively) are: (a)+(b) glyphosate; (d)+(e) AMPA; and (g)+(h) glufosinate. The quantitation MRMs for the internal standards are: (c) 13C2,15N-glyphosate; (f) 13C,15N-AMPA; and (i) D3-glufosinate.

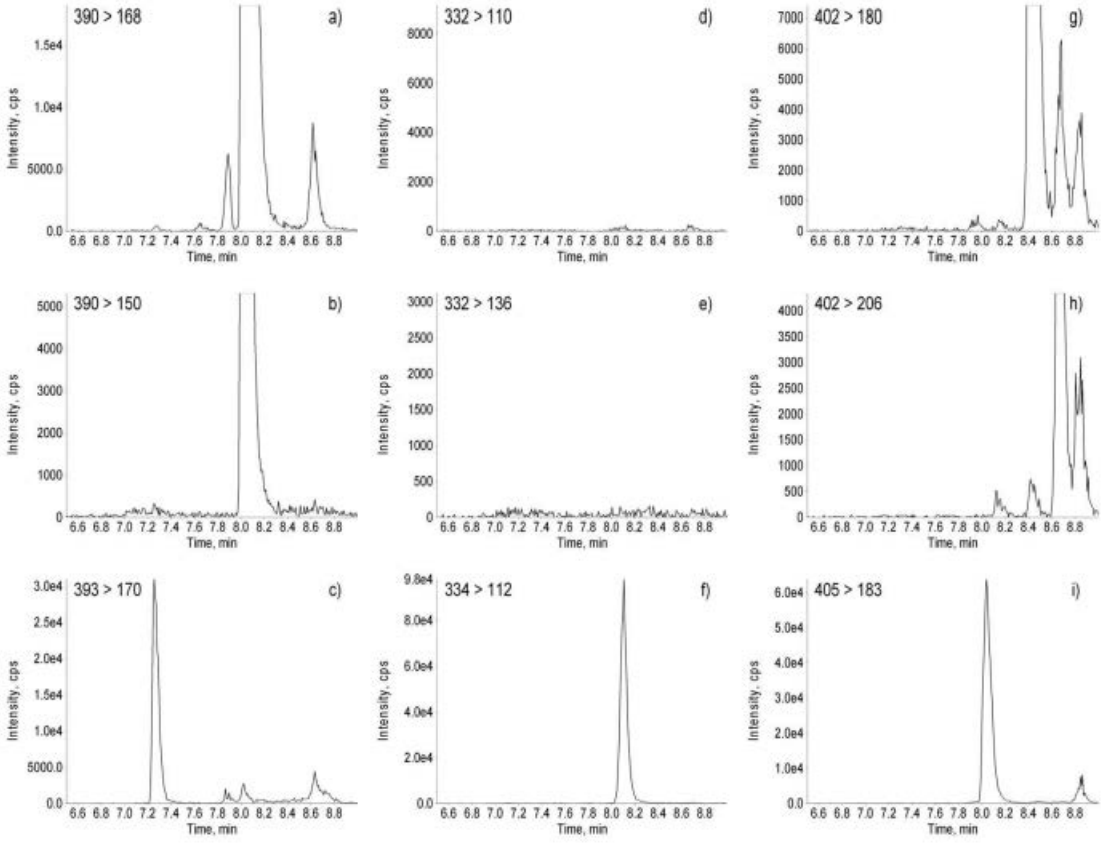
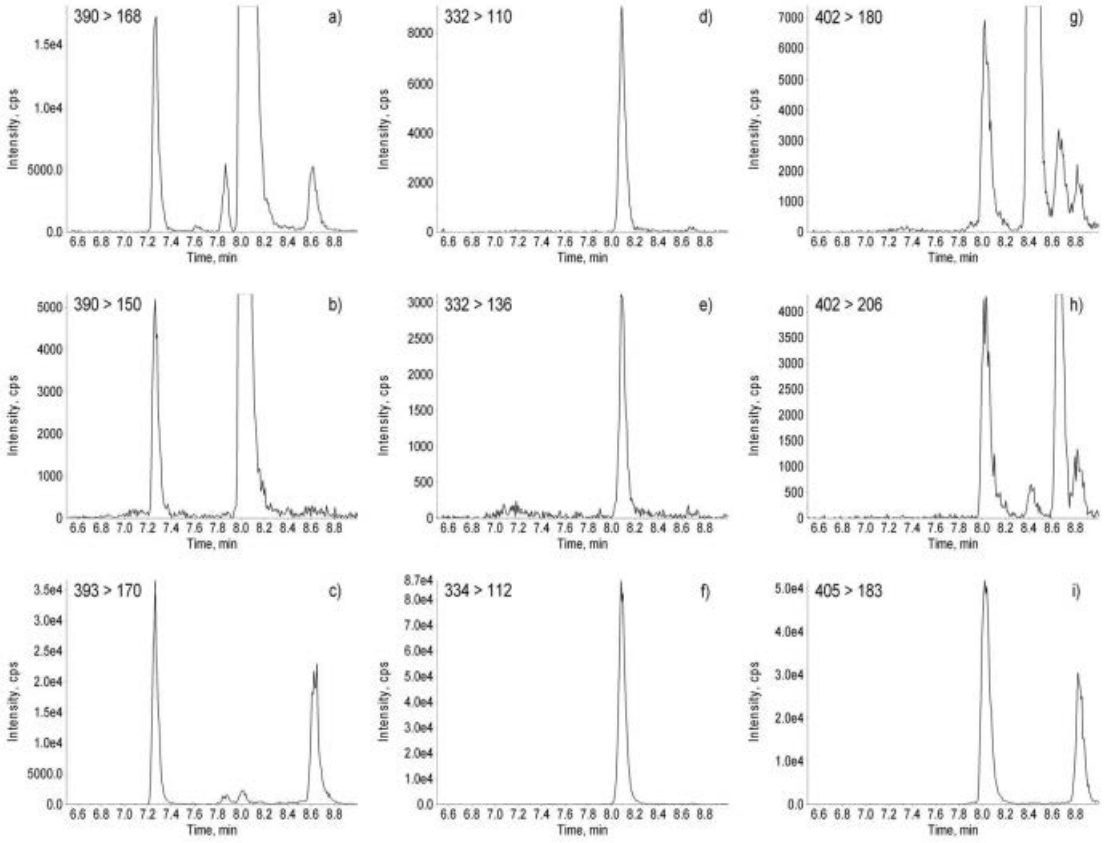


Figure 3: Reconstructed ion chromatograms for blank honey fortified with 5 µg/kg each of glyphosate, AMPA, and glufosinate as well as 25 µg/kg of each isotopically labelled internal standard. The quantitation and confirmatory MRM transitions (respectively) are: (a)+(b) glyphosate; (d)+(e) AMPA; and (g)+(h) glufosinate. The quantitation MRMs for the internal standards are: (c) 13C2,15N-glyphosate; (f) 13C,15N-AMPA; and (i) D3-glufosinate.



Criteria for confirmation of analyte identity

Two MRM transitions were monitored for each incurred analyte in order to permit confirmation of compound identity. A chromatographic peak must be present in both reconstructed ion traces within ± 0.05 min of the retention time of the associated isotopically labelled internal standard. The ratio of the peak areas for the quantitation and confirmation reconstructed MRM traces must be within ± 30% relative to that obtained for authentic reference material analysed under the same set of operational parameters within the same analytical batch.

Evaluation of matrix effects

Matrix effects were evaluated by comparing calibration curves obtained for standards prepared in reagent water and honey solutions. Unfortunately it was extremely difficult to find a truly blank honey and it was decided that a set of calibration standards would be prepared using a nominally blank honey which did not contain any of the analytes above the LOQ of 1 µg/kg. The results of the calibration curves obtained for standards prepared in either water or honey are given in **Table 3**. The calibration curves were determined using two techniques: firstly by external standardisation and secondly by internal standardization using each analyte's respective isotopically labelled analogue. The matrix effect (ME) was calculated based on the ratio of the slopes obtained for the calibration curves in matrix versus reagent water:

ME = 100 x (slope of calibration curve in honey) / (slope of calibration curve in reagent water)

where ME = 100 would indicate no matrix effect while ME < 100 or ME > 100 would indicate ionization suppression or enhancement, respectively. When the calibration is performed using external standardisation, there is minor ionisation enhancement (ME > 100) observed for glyphosate where

ME = 109%. The opposite ionisation effect (suppression) is observed for both AMPA and glufosinate which have ME values of 51% and 54%, respectively. However, when the calibration curves are established using internal standardisation by isotope dilution, the ME values are all within 100 ± 10%. Based on these results it was concluded that the use of isotopically labelled internal standards for quantitation would adequately overcome the ionisation effects observed because of the honey matrix. Reagent-based calibration standards were subsequently used for all method validation experiments.

Table 3: Comparison of calibration standards prepared in reagent water and honey.

Compound	Standardisation	Equation of curve prepared in reagent water (r^2)	Equation of curve prepared in honey solution	Matrix effect (ME*)
glyphosate	external	$y = 6540.7x + 780.0$ ($r^2 = 0.99944$)	$y = 7134.5x + 13125.5$ ($r^2 = 0.99988$)	109%
AMPA	external	$y = 105014.4x - 121.9$ ($r^2 = 0.99972$)	$y = 5317.0x + 1117.5$ ($r^2 = 0.99912$)	51%
glufosinate	external	$y = 9029.6x + 386.2$ ($r^2 = 0.99976$)	$y = 4901.0x + 9180.4$ ($r^2 = 0.99954$)	54%
glyphosate	internal	$y = 0.07890x + 0.02175$ ($r^2 = 0.99826$)	$y = 0.08481x + 0.01717$ ($r^2 = 0.99924$)	107%
AMPA	internal	$y = 0.02129x + 0.00153$ ($r^2 = 0.99866$)	$y = 0.02059x + 0.00457$ ($r^2 = 0.99930$)	97%
glufosinate	internal	$y = 0.02574x + 0.00171$ ($r^2 = 0.99856$)	$y = 0.02480x + 0.03888$ ($r^2 = 0.99818$)	96%

*ME = 100 × (slope of curve in honey)/(slope of curve in water).

Method validation

The analytical method was validated by analysing a series of spiked replicate honey samples. A honey sample which had no analytes at a concentration above the LOQ of 1 µg/kg was found after a large number of honeys were screened using the proposed methodology. A set of spiked replicates fortified at three different concentrations were analysed in order to determine the accuracy and precision of the proposed method. The results of these analyses are summarised in **Table 4.** The inter-day reproducibility was also evaluated by carrying out the analysis of replicate samples over three separate days. The calculated accuracies obtained for the daily analysis of six spiked replicates at each of three concentration levels (5, 50, and 150 µg/kg) ranged from 95.2% to 105.3% for all three compounds. The daily precision (standard deviation) for all three analytes at all fortification levels ranged from 1.6% to 7.2%. The inter-day accuracy and precision for all three compounds at the three different levels studied over three separate days (a total of 18 replicates at each concentration level) were calculated to be between 97.7% to 103.1% and 2.1% to 5.4%, respectively. Based on these results, the method was deemed to be fit for purpose.

Table 4: Method validation data.

		Accuracy ± SD			
Compound	Fortification level (µg kg^{-1})	Day 1 ($n = 6$)	Day 2 ($n = 6$)	Day 3 ($n = 6$)	Inter-day ($n = 18$)
glyphosate	5	105.3 ± 5.4	102.9 ± 4.5	101.2 ± 6.3	103.1 ± 5.4
	50	100.4 ± 2.1	96.2 ± 4.4	104.5 ± 2.8	100.4 ± 4.6
	150	100.2 ± 4.3	96.1 ± 2.2	101.8 ± 3.1	99.3 ± 4.0
AMPA	5	98.9 ± 6.4	101.5 ± 4.4	96.0 ± 3.1	98.8 ± 5.1
	50	103.6 ± 4.4	103.0 ± 3.1	96.8 ± 3.5	101.1 ± 4.7
	150	100.1 ± 1.7	99.1 ± 3.5	96.6 ± 2.5	98.6 ± 2.9
glufosinate	5	101.8 ± 3.8	99.3 ± 3.4	97.3 ± 7.2	99.5 ± 5.1
	50	99.8 ± 3.4	99.2 ± 1.6	95.2 ± 2.5	98.0 ± 3.2
	150	97.7 ± 1.7	98.8 ± 2.4	96.6 ± 1.8	97.7 ± 2.1

The measurement uncertainty for each analyte was estimated using in-house method validation data according to the procedure described in the Codex guidelines on estimation of uncertainty of results (Codex Alimentarius Commission 2011). Method validation data obtained for the analysis of spiked replicates at the three different concentration levels covering a range from 5 to 150 µg/kg was used to calculate an expanded uncertainty (U′) with a coverage factor of 2 (95% confidence interval) for each analyte. The expanded uncertainties were estimated as U′ = 14% for glyphosate, 13% for AMPA, and 11% for glufosinate.

Application to honey samples

Two hundred randomly chosen honey samples, which were submitted to our laboratory for other testing, were analysed using the online SPE-LC-MS/MS method to obtain information regarding baseline levels of glyphosate, its main degradation product AMPA, and the other acidic herbicide, glufosinate. The results of these analyses are summarised in **Table 5**. Glyphosate was detected in almost all honey samples analysed with 197 out of 200 samples (98.5%) having residues equal to or above the LOQ of 1 µg/kg. The maximum concentration of glyphosate residue in the honey samples analysed was 49.8 µg/kg. AMPA was also frequently detected (198 or 99.0% of 200 samples tested) up to a maximum concentration of 50.1 µg/kg. There were no samples where both glyphosate and AMPA were below the LOQ value.

Table 5: Concentrations of glyphosate, AMPA, and glufosinate in incurred honey samples.*

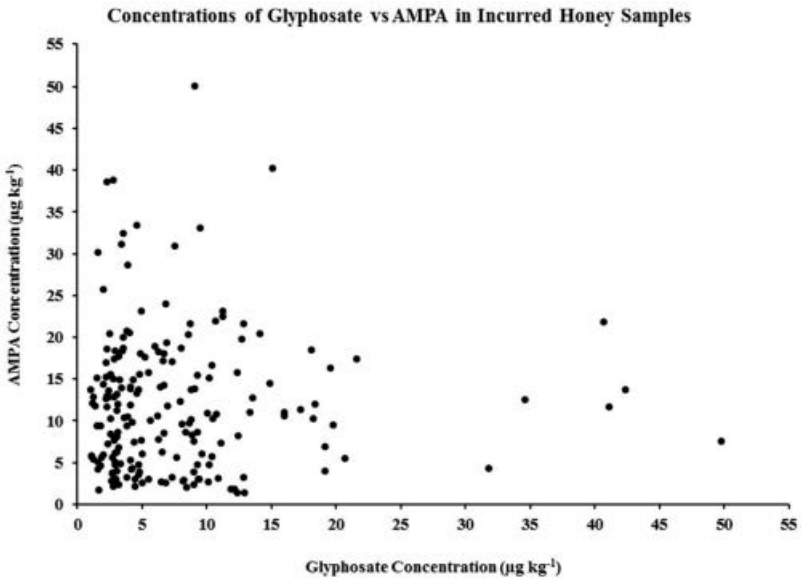
Compound	# of Detections	Median (µg kg^{-1})	90th Percentile (µg kg^{-1})	95th Percentile (µg kg^{-1})	Maximum (µg kg^{-1})
glyphosate	197	4.9	14.2	19.2	49.8
AMPA	198	10.3	20.8	28.7	50.1
glufosinate	125**	1.4	6.1	9.9	33.0

*n = 200 samples analysed.
**One sample did not meet the required ion ratio criterion for confirmation of compound identity and was not included in this value.

The third analyte, glufosinate, was detected much less frequently than either glyphosate or AMPA and also at lower levels in general. Glufosinate was found to be present in 125 of 200 samples analysed with the maximum concentration detected being 33.0 µg/kg. It must be noted that there was a single honey sample where the ratio of the two precursor > product ion MRM transitions for glufosinate was not within the acceptable relative ratio of ±30% (average ion ratio for calibration standards = 59.3% while the ion ratio for the sample was 7.0%). Assuming that there was an interference in the quantitative MRM transition (thereby giving the unacceptably low relative ion ratio), if the confirmatory MRM transition was used for quantitation, the glufosinate concentration was estimated to be just above 1 µg/kg. All samples of honey containing either glyphosate or AMPA at concentrations above the LOQ of 1 µg/kg were successfully confirmed based on the criteria established for compound identification.

Interestingly, the ratio of glyphosate to AMPA was found to vary considerably in samples that contained both analytes. In some cases the two analytes were roughly equal in concentration while in others one of the pair was significantly higher than the other compound. This is illustrated by the scatter plot shown in **Figure 4** where the concentration of glyphosate is plotted versus the concentration of AMPA in the 200 honey samples which were analysed (note that only samples containing both glyphosate and AMPA at or above the LOQ were included in this plot). There are multiple factors which may influence the relative amounts of glyphosate and its degradation product AMPA. Differences in the chemical composition of the honeys tested as well as their age and handling/storage conditions prior to receipt by the laboratory may be important factors. The long-term stability of glyphosate and AMPA in honey has not been established. Other factors which may influence the relative ratios of the two compounds may include agricultural practices such as the timing of herbicide application relative to honey bee foraging, environmental decomposition of the targeted analytes, and differences in crops treated and subsequently pollinated by the bees. The contribution of glyphosate and AMPA residues present in the ambient environment to contamination of plant nectar and subsequently honey itself is further complicated by the variations in the levels of these compounds in environmental matrices such as soil and surface water. No conclusions can be drawn regarding any trend in the relative amounts of these glyphosate and AMPA in honey. The ratio of the concentration of glyphosate to that of AMPA present in samples containing both analytes at ≥1 µg/kg (195 samples) ranged from 0.05 to 9.16. It should also be noted that there were two samples containing glyphosate ≥1 µg/kg (7.7 and 8.8 µg/kg where the concentration of AMPA was below the LOQ. Conversely, there were three samples with AMPA concentrations ≥1 µg/kg (2.7, 9.0, and 10.6 µg/kg) where the glyphosate concentration was below 1 µg/kg. The concentration of glyphosate exceeded that of AMPA in 63 out of 200 honey samples tested.

Figure 4: Scatter plot of glyphosate versus AMPA concentrations in samples containing both analytes at or above the limit of quantitation (LOQ = 1 µg/kg).



Comparison of residue levels in honey to other reported studies

Table 6 provides a comparison between the residues of glyphosate present in honey samples analysed in this study and those previously reported by other research groups. Bo *et al.* (2007) developed an analytical method for the determination of glyphosate and AMPA residues in a variety of foods including honey. Their reported LOQ was 50 µg/kg and while the method was employed for the analysis of several different food types it does not appear that it was actually applied to honey samples. In several subsequently reported studies, LOQ values were in the range of 10–50 µg/kg (Rubio *et al.* 2014; Chamkasem and Vargo 2017; Karise *et al.* 2017; Berg *et al.* 2018) which permitted frequent detection of glyphosate residues in honey. Zoller *et al.* (2018) and our work both achieved LOQ values of 1 µg/kg and also each had greater than 90% of tested honey samples containing quantifiable residues of glyphosate. None of the honey samples in either our baseline study or in the survey of honey sold on the Swiss market (Zoller *et al.* 2018) had glyphosate residues above the EU MRL of 50 µg/kg. In a study of honey from numerous countries around the world (Rubio *et al.* 2014), 22 out of 69 samples tested contained glyphosate residues above the MRL of 50 µg/kg up to a maximum of 163 µg/kg. Glyphosate levels in honey samples mainly from the USA and a small number from other countries exceeded the MRL of 50 µg/kg in only 4 of 28 samples tested but with one sample containing 653 µg/kg (Chamkasem and Vargo 2017). Only 2 out of 33 honey samples from Estonia had glyphosate residues above the MRL of 50 µg/kg with a maximum of 62 µg/kg being detected (Karise *et al.* 2017). Berg *et al.* (2018) obtained 59 honey samples from Hawaiian beehives as well as 26 samples from commercially available products. A total of 8 of the 26 merchant samples had detectable residues, three of which were above the MRL of 50 µg/kg. A total of 16 of the 59 samples collected directly from beehives were determined to contain glyphosate residues above the LOQ of 15 µg/kg with 12 samples above the MRL of 50 µg/kg. The maximum concentrations of glyphosate detected in the merchant and hive samples were 87 and 342 µg/kg, respectively. John and Liu (2018) measured glyphosate residues in water, various food matrices, and human urine using an ELISA method. Only one honey was tested amongst the samples and was found to contain 22 µg/kg of glyphosate. In the 2016 EU report on pesticide residues in food (EFSA (European Food Safety Authority) 2018a), 18 of 220 honey samples were found to have detectable residues of glyphosate. The report does not include specific details regarding either the analytical methods used by the reporting laboratories or their LOQs for glyphosate in honey. Six honey samples contained glyphosate residues above the EU MRL of 50 µg/kg with levels ranging from 90 to 610 µg/kg.

Table 6: Glyphosate residues in honey from various studies.

	Country of study					
	USA (Rubio et al. 2014)	USA (Chamkasem and Vargo 2017)	Estonia (Karise et al. 2017)	Switzerland (Zoller et al. 2018)	USA (Berg et al. 2018)	Canada (this study)
Testing method	ELISA	LC-MS/MS	LC-MS/MS	LC-MS/MS	ELISA	LC-MS/MS
Source of honeys	Various countries of origin	Mainly from USA	Estonia	Not specified	Mainly from USA (Hawaii)	Mainly western Canada
# Samples tested	69	28	33	16	85	200
# Positives (%)	41 (59.4%)	17 (60.7%)	3 (9.1%)	15 (93.8%)	24 (28.2%)	197 (98.5%)
LOQ (μg kg^{-1})	15	10 to 16	50 (LOD = 10)	1	15	1
Maximum (μg kg^{-1})	163	653	62	15.9	342	49.8

Neither AMPA nor glufosinate were detected, with LOQs of 16 and 18 μg/kg respectively, in 19 honey samples analysed by direct determination of the underivatised analytes using LC-MS/MS (Chamkasem and Vargo 2017). None of the 16 honey samples analysed by Zoller *et al.* (2018) contained AMPA residues above the LOQ of 2.5 μg/kg. Considering the low levels of glyphosate found in these samples (median concentration of 3.0 μg/kg), it is entirely plausible that AMPA could be undetected since its LOQ was 2.5 times higher than for glyphosate.

It should be noted that the LC-MS/MS methods employed by Chamkasem and Vargo (2017) as well as by Karise *et al.* (2017) both involved the determination of glyphosate residues without derivatisation or subsequent extract clean-up. The combination of FMOC-Cl derivatisation and online SPE coupled directly to LC-MS/MS as performed in our method made it possible to achieve LOQ values which were at least one order of magnitude lower by comparison. The analytical method used by Zoller et al. (2018) did not employ a derivatisation step but did carry out an offline SPE clean-up step followed by extract dilution prior to LC-MS/MS analysis. Their LOQ values for glyphosate and AMPA were equal to and just slightly higher, respectively, than those obtained with our procedure.

Considerations for future studies

It should be noted that the current EU MRL for glyphosate in honey only includes the parent compound as the marker residue (EU 2016). A recent review by the European Food Safety Authority (EFSA) indicates that there is a proposal to include other related analytes in the residue definition for glyphosate in different foods (EFSA 2018b). While there is no specific mention of honey, it has been proposed that the residue definition for numerous other commodities be expanded to include the sum of glyphosate, AMPA, and the metabolite N-acetyl-glyphosate for enforcement purposes. It has also been recommended that residue analysis for risk assessment include glyphosate, AMPA, N-acetyl-glyphosate, and N-acetyl-AMPA. While several studies to date, including the work described herein, have reported residues of glyphosate and AMPA in honey, there is a need for the N-acetylated metabolites of these compounds to be considered for addition in future studies. The current EU MRL for glufosinate in honey includes the sum of the parent compound plus its metabolites 3-[hydroxyl(methyl)phosphinoyl]propionic acid (MPP) and N-acetyl-glufosinate (NAG) (European Union: Pesticides database 2016). While glufosinate was not detected in honey according to a single previously reported study (Chamkasem and Vargo 2017), its presence in honey samples analysed in our survey suggests the need to investigate MPP and NAG residues in future work.

Conclusions

A relatively simple method was developed for the determination of glyphosate, AMPA, and glufosinate residues in honey with an LOQ of 1 μg/kg for each analyte. A key component of the method was the utilisation of isotopically labelled internal standards to overcome matrix effects associated with the samples. Following a simple derivatisation step, it was possible to use online solid-phase extraction for the isolation of the derivatised analytes from the bulk of the honey matrix with subsequent direct determination of the residues by LC-MS/MS. A survey of honey samples from western Canada indicated the widespread contamination of these samples by glyphosate, AMPA, and glufosinate, albeit at low concentrations. While Health Canada has not currently established an MRL for either glyphosate or glufosinate in honey, in consideration of the EU MRLs of 50 μg/kg for each compound the risk to consumer health appears to be quite low based on the residues detected.

Chromatographic conditions

Chromatograph:	Shimadzu 30 LC System (SIL30AC autosampler, two LC30AD solvent delivery pumps, CBM20A module controller)
Column:	Agilent Zorbax Extend-C18 (50 mm x 2.1 mm, 1.8 µm)
Column oven temperature:	Not provided
Injection volume:	50 µL
Mobile phases:	(A) 10 mM ammonium carbonate in water (B) Acetonitrile

Gradient (linear transitions):

Time (Min)	Eluent A (%)	Eluent B (%)	Flow rate (mL/min)	Valve position
0.0	100	0	1.00	to waste
2.9	100	0	1.00	to waste
3.0	100	0	0.35	to waste
5.5	73.6	26.4	0.35	to column
12.0	5	95	0.35	to column
12.5	5	95	0.35	to column
13.0	5	95	0.60	to column
15.5	5	95	0.60	to column
16.0	5	95	0.35	to column
17.0	98	2	0.35	to column
19.0	98	2	0.35	to waste

Retention time:	Glyphosate: ~ 8.1 min 13C2,15N-glyphosate (IS): ~ 8.1 min AMPA: ~ 8.1 min 13C,15N-AMPA (IS): ~ 8.1 min Glufosinate: ~ 8.1 min D3-glufosinate (IS): ~ 8.1 min
Detector:	Sciex 4500 quadrupole tandem mass spectrometer
Scan type:	MRM
Ion source:	ESI negative

Source gas:	70 units	Source temperature:	700°C
CAD gas:	8 units	Source voltage:	−3500 V
Curtain gas:	20 units		

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Declustering potential (V)	Collision energy (eV)	Scan time (ms)
Primary transition (quantification)					
Glyphosate	390	168	−40	−16	50
Glyphosate (IS)	393	170	−40	−16	50
AMPA	332	110	−40	−16	50
AMPA (IS)	334	112	−40	−16	50
Glufosinate	402	180	−45	−14	50
Glufosinate (IS)	405	183	−45	−14	50
Secondary transition (confirmation)					

Glyphosate	390	150	−40	−34	50
AMPA	332	136	−40	−20	50
Glufosinate	402	206	−45	−20	50

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes the development and validation of a method for the analysis of glyphosate, AMPA, and glufosinate in honey. Aqueous honey solutions were derivatised offline prior to direct analysis of the target analytes using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Method validation fulfil EU requirements. The method showed good performance for all analytes with a LOQ of 1 µg/kg for each analyte.

The method can be considered valid for monitoring purposes and has been applied for the analysis of two hundred randomly chosen honey samples from Canada. Virtually all the samples were found to contain measurable residues of glyphosate and/or AMPA, which is at least in part due to the extremely LOQ (1 µg/kg). The ratio between parent glyphosate and AMPA was very variable, which is also in contrast to the findings of the EU monitoring (where no measurable residues of AMPA were found) but may also be accounted for by the very low LOQ. In spite of the large number of samples analysed, none showed residues of parent glyphosate exceeding the current EU MRL of 0.05 mg/kg.

According to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. As honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL. Therefore, the publication is considered relevant and reliable. It also includes a useful discussion of the residue levels of glyphosate in honey reported by other authors.

1. Information on the study

Data point	CA 6.4.2
Report author	Von Soosten D. *et al.*
Report year	2016
Report title	Excretion pathways and ruminal disappearance of glyphosate and its degradation product aminomethylphosphonic acid in dairy cows
Document No.	J. Dairy Sci. 99 :5318–5324
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

From 6 balance experiments with total collection of feces and urine, samples were obtained to investigate the excretion pathways of glyphosate (GLY) in lactating dairy cows. Each experiment lasted for 26 d. The first 21 d served for adaptation to the diet, and during the remaining 5 d collection of total feces and urine was conducted. Dry matter intake and milk yield were recorded daily and milk and feed samples were taken during the sampling periods. In 2 of the 6 experiments, at the sampling period for feces and urine, duodenal contents were collected for 5 d. Cows were equipped with cannulas at the dorsal sac of the rumen and the proximal duodenum. Duodenal contents were collected every 2 h over 5 consecutive days. The daily duodenal dry matter flow was measured by using chromium oxide as a volume marker. All samples (feed, feces, urine, milk and duodenal contents were analyzed for GLY and aminomethylphosphonic acid (AMPA). Overall, across the 6 experiments (n = 32) the range of GLY intake was 0.08 to 6.67 mg/d. The main proportion (61 ± 11%; ± SD) of consumed GLY was excreted with feces; whereas excretion by urine was 8 ± 3% of GLY intake. Elimination via milk was negligible. The GLY concentrations above the limit of quantification were not detected in any of the milk samples. A potential ruminal degradation of GLY to AMPA was derived from daily duodenal GLY flow. The apparent ruminal disappearance of GLY intake was 36 and 6%. In conclusion, the results of the present study indicate that the gastrointestinal absorption of GLY is of minor importance and fecal excretion represents the major excretion pathway. A degradation of GLY to AMPA by rumen microbes or a possible retention in the body has to be taken into account.

Materials and Methods

Six balance experiments with collection of total urine and feces were conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut, Brunswick, Germany. The experiments were approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany

Animals, Feeding, and Design of the Experiments

Overall, the 6 experiments included 32 lactating dairy cows of the German Holstein breed. For experiments 1 to 6, we used 5, 6, 5, 4, 6, and 6 animals per experiment, respectively. The animals were, on average, 90 DIM and in their second to fifth lactation. All cows were fitted with rumen and duodenum cannulas and were housed in a tiestall barn. Milking took place twice daily at 05:30 and 15:30 h. The animals were fed at the milking times. In all experiments the diet was based on maize silage (single forage component) and concentrates in different proportions (Table 1). The composition

of the concentrates as well as the GLY and AMPA concentrations in the concentrates are shown in Table 2. Each balance experiment lasted 26 d. The first 21 d were allowed for equilibration to the experimental diet and the remaining 5 d were the sampling period. In experiments 1 and 2, the quantitative collection of urine and feces was followed by the quantification of daily duodenal dry matter flow (DMF) for 5 consecutive days

Table 1: Forage-to-concentrate ratio of the diet during the experiments.

Experiment	Maize silage (%)	Concentrate (%)
1	60	40
2	60	40
3	70	30
4	55	45
5	70	30
6	70	30

Measurements and Sample Collection

During the sampling period, DMI and milk yield were recorded in each individual animal daily. Feed samples for maize silage were taken twice and concentrate samples once during the sampling period. Milk samples were taken once at morning and evening milking in the sampling periods. Total collection of feces and urine was conducted over 5 consecutive days. Cows were equipped with urine devices for separated drain of urine. The device was manufactured of artificial leather and was fitted and agglutinated around the vulva and pins. A polyvinylchloride tube drained the urine into a canister. The feces were collected in a stainless steel tub, which was positioned below a perforated floor at the end of the tiestall. The urine canister and feces tub were emptied once per day at the same time. Urine and feces were weighed and homogenized. Two percent of the daily fecal amounts were sampled and given into a pooled sample over the 5 consecutive days. A urine sample of 100 mL was taken from total urine volume each day. Urine and feces samples were stored at −20°C until analysis.

In experiments 1 and 2 a chromium oxide (Cr_2O_3) marker (19.8% Cr_2O_3, 79.1% wheat flour and 0.67% aluminum sulfate) was introduced into the rumen via the rumen cannula and was used as a marker for quantitative measurement of the daily duodenal DMF. The administration of Cr_2O_3 was started 11 d before collection of duodenal chyme. Two portions of 50 g of Cr_2O_3 were administered every 12 h. During the duodenal chyme sampling period and 1 d before, 4 portions of 25 g of Cr_2O_3 were given every 6 h. Samples of duodenal contents were taken every 2 h during the 5 consecutive days of sampling. At each sampling, 100 mL of duodenal contents were collected and pooled over 24 h. The samples were stored at −20°C. The individual animal DMI was recorded and samples of the feedstuffs were retained according to the same pattern during the total collection period of urine and feces. Body weight was recorded before the start and after the end of an experiment.

Analyses

Samples of maize silage were dried at 60°C for 72 h. Duodenal contents and feces samples were freeze-dried for determination of DM. The feedstuffs and duodenal and fecal samples were ground through a 1-mm sieve. Aliquots of the morning and evening milk samples were pooled according to their proportion of total daily milk yield. The urine samples were thawed and pooled over the 5 consecutive sampling days according to their daily proportion of the total urine amount over the sampling period. In the daily duodenal samples the chromium concentrations were measured using an inductively coupled plasma optical emission spectrometer (Quantima, GBC Scientific Equipment Pty Ltd., Victoria, Australia) after sample preparation according to Williams et al. (1962). The chromium concentration was used to calculate the daily duodenal DMF. According to the daily duodenal DMF on the 5 sampling days, one aliquot pooled sample was generated per cow per 5 sampling days.

All samples were analyzed for GLY and AMPA in accredited laboratories. Feed and milk samples were analyzed by Wessling GmbH (Altenberge, Germany) and feces, urine, and duodenal chyme by

Medizinisches Labor Bremen (Bremen, Germany). In milk and feed samples GLY and AMPA were extracted with formic acid (0.1%) and methylene chloride. Derivatization was conducted with fluorenylmethoxycarbonyl chloride. After solid phase extraction, GLY and AMPA were determined by using LC-MS/MS.

In feces, urine, and duodenal chime, GLY and AMPA were extracted with water. Derivatization was conducted with trifluoroacetic anhydride and trifluoroethanol. Glyphosate and AMPA were determined by GC-MS/MS.

For all GLY and AMPA analyses an internal standard containing 1,2–$^{13}C_2$ ^{15}N GLY (1 µg/mL) and ^{13}C ^{15}N AMPA (1 µg/mL) was used. The limit of detection (LOD) and the limit of quantification (LOQ) for each substance was calculated form the signal-to-noise ratios. This ratio was 3 for the LOD and 10 for the LOQ. For both GLY and AMPA in feed samples, the LOQ and LOD was 0.02 and 0.007 mg/kg, respectively. For all other matrices the LOQ was 0.01 mg/kg and the LOD was 0.003 mg/kg. The recoveries for GLY and AMPA analyses in feed and milk samples were 70 to 120% using an internal standard concentration of 0.625 mg/kg for feed analyses and 0.25 mg/kg for milk analyses. Recoveries for GLY in feces, urine, and duodenal content were 80 to 90, 98 to 101, and 96 to 102%, respectively. Recoveries for AMPA analyses in feces, urine, and duodenal content were 80 to 95, 80 to 101, and 94 to 106%, respectively. For determination of the recoveries in feces, urine, and duodenal content the internal standard concentration was 0.1, 0.001, and 5 mg/kg, respectively.

Calculations

Apparent GLY and AMPA retention was calculated with the following equation:

Apparent GLY/AMPA retention (mg/d) = GLY/AMPA intake (mg/d) – fecal excretion of GLY/AMPA (mg/d) – urinary excretion of GLY/AMPA (mg/d) – milk excretion of GLY/AMPA (mg/d).

Daily duodenal DMF and duodenal GLY/AMPA flow were calculated as follows:

DMF (kg/d) = [chromium application (mg/d)/duodenal chromium concentration (mg/g of DM)]/1000, and

Daily duodenal GLY/AMPA flow (mg/d) = DMF (kg/d) × duodenal GLY/AMPA concentration (mg/kg DM).

Ruminal disappearance of GLY and AMPA was calculated with the following equation:

Ruminal disappearance of GLY/AMPA (mg/d) = GLY/AMPA intake (mg/d) – duodenal GLY/AMPA flow (mg/d).

Results

The determined GLY and AMPA concentrations of the individual sample matrices differed between the experiments. Only in experiment 4 could GLY be detected in the maize silage (0.035 mg/kg of DM). Maize silage in all other experiments contained GLY lower than the LOQ; AMPA was not detected in any of the maize silages. The GLY concentration in the concentrates ranged from 0.02 to 0.95 mg/kg of DM and AMPA concentrations ranged from a value lower than the LOQ to 0.65 mg/kg of DM. Therefore, the concentrates were the main source for exposure of GLY and AMPA in all experiments (Table 2). In urine and feces GLY concentrations ranged from 0.20 to 75.1 µg/L and 0.01 to 0.88 mg/kg of DM, respectively.

Table 2: Composition and concentrations of glyphosate and aminomethylphosphonic acid in the concentrates of the different experiments.

	Experiment					
Component (%, unless noted)	1	2	3	4	5	6
Soybean meal	20	20	25	15		
Rape seed meal			13			
Barley grain	22	22		14.7		
Wheat grain	22	22	36.5	29		
Wheat gluten					10	10
Maize grain	18	18		29	35	35
Sugar beet pulp, dried	15	15	18.3	8.4	48	48
Urea	2	2	2.5	1	3	3
Calcium carbonate/dicalcium phosphate			2.5	1.5	1.5	1.5
Sodium chloride			0.2	0.2	0.2	0.2
Mineral and vitamin-mix	1	1	2.0	1.2	2.3	2.3
Glyphosate (mg/kg of DM)	0.95	0.48	0.82	0.08	0.02	0.03
AMPA[1] (mg/kg of DM)	0.65	0.43	0.46	<LOQ[2]	<LOQ	0.02

[1]Aminomethylphosphonic acid (degradation product of glyphosate).
[2]<LOQ = AMPA concentrations in the samples were lower than the limit of quantification (LOQ).

In experiment 1 the highest GLY intake (6.7 mg/d) was observed. The lowest GLY intake (0.08 mg/d) was found in experiment 6 (Table 3). In accordance to the GLY intake, the excretion of feces (4.3 mg/d) and urine (0.44 mg/d) were highest in experiment 1. In experiment 5 the excretion of feces and urine were lowest, at 0.02 and 0.08 mg/d, respectively. In all milk samples the GLY concentration was below the LOQ.

Table 3: Intake and fecal and renal excretion of glyphosate in animals during the sampling period (means ± SD).

Experiment (animals)	Intake in feed (mg/d)	Excretion in feces (mg/d)	Excretion in urine (mg/d)
Experiment 1 (n = 5)	6.67 ± 0.02	4.34 ± 0.35	0.44 ± 0.08
Experiment 2 (n = 6)	3.36 ± 0.01	2.04 ± 0.24	0.26 ± 0.10
Experiment 3 (n = 5)	3.36 ± 0.00	1.69 ± 0.41	0.12 ± 0.02
Experiment 4 (n = 4)	0.53 ± 0.02	0.39 ± 0.05	0.04 ± 0.01
Experiment 5 (n = 6)	0.15 ± 0.21	0.02 ± 0.03	0.08 ± 0.10
Experiment 6 (n = 6)	0.08 ± 0.07	0.04 ± 0.06	0.14 ± 0.14
Experiment 1–6 (n = 32)	2.36 ± 2.61	1.42 ± 1.67	0.18 ± 0.15

The results for AMPA intake and excretion are presented in Table 4. The AMPA intake was on a lower level compared with GLY intake. The highest AMPA intake was observed in experiment 1 (4.57 mg/d) and the lowest in experiment 5 (lower than the LOQ; Table 4). Considerable excreted amounts of AMPA with feces and urine were only observed in experiments 1, 2, and 3. The excretion with feces was lower than the LOQ in experiments 4, 5, and 6. In the same experiments the excretion with urine (0.01 mg/d) was marginal; AMPA concentrations in milk were below the LOQ in all experiments.

Table 4: Intake and fecal and renal excretion of AMPA[1] in animals during the sampling period (means ± SD).

Experiment (animals)	Intake in feed (mg/d)	Excretion in feces (mg/d)	Excretion in urine (mg/d)
Experiment 1 (n = 5)	4.57 ± 0.21	2.25 ± 0.23	0.41 ± 0.05
Experiment 2 (n = 6)	3.03 ± 0.05	1.51 ± 0.21	0.36 ± 0.09
Experiment 3 (n = 5)	1.89 ± 0.00	0.83 ± 0.19	0.15 ± 0.03
Experiment 4 (n = 4)	<LOQ[2]	<LOQ	<LOQ
Experiment 5 (n = 6)	<LOQ	<LOQ	<LOQ
Experiment 6 (n = 6)	0.05 ± 0.04	<LOQ	<LOQ
Experiment 1–6 (n = 32)	1.59 ± 1.77	0.77 ± 0.89	0.16 ± 0.18

[1]AMPA = aminomethylphosphonic acid.
[2]<LOQ = AMPA concentrations in feed, feces or urine samples were lower than the limit of quantification (LOQ), and therefore the intake as well as the excretion with feces and urine was considered as zero.

The duodenal flows of GLY and AMPA in experiments 1 and 2 (measurement subsequent to total collection of feces and urine) are shown in Table 5. The intakes of GLY and AMPA during the duodenal sampling period were different in the 2 experiments, with highest intakes in experiment 1. However, the duodenal flows of GLY and AMPA were in a similar range. In both experiments an apparent ruminal disappearance occurred for both substances; 2.27 mg/d disappeared in experiment 1 and 0.19 mg/d of GLY disappeared in the rumen in experiment 2.

Table 5: Glyphosate and AMPA[1] intake and flow at the duodenum (means ± SD) during times of duodenal sampling followed after the balance experiment 1 and 2.

Experiment (animals)	Intake in feed (mg/d)	Flow at the duodenum (mg/d)	Apparent ruminal disappearance (mg/d)
Glyphosate			
Experiment 1 (n = 5)	6.24 ± 1.61	3.97 ± 0.83	2.27 ± 1.66
Experiment 2 (n = 6)	3.38 ± 0.14	3.20 ± 0.48	0.19 ± 0.34
AMPA			
Experiment 1 (n = 5)	3.54 ± 0.81	2.16 ± 0.57	1.38 ± 1.00
Experiment 2 (n = 6)	2.66 ± 0.11	2.32 ± 0.22	0.34 ± 0.18

[1]AMPA = aminomethylphosphonic acid.

Fecal, renal, and mammary excretion of GLY and the apparent retention of GLY, expressed as percentage of intake, are presented in Table 6. Due to very low intakes of GLY in experiments 5 and 6 these variables were not calculable. Overall, the ratio of GLY intake to excretion of GLY via feces or urine remained independent from the level of GLY intake and averaged 61 ± 11% (fecal; mean ± SD) and 8 ± 3% (renal). The mammary excretion was 0% and the apparent retention 31 ± 13%.

Table 6: Fecal, renal, and mammary glyphosate excretion as well as apparent retention expressed as proportion of glyphosate intake (means ± SD).

Experiment (animals)	Fecal (% of intake)	Renal (% of intake)	Mammary (% of intake)	Apparent retention (% of intake)
Experiment 1 (n = 5)	65 ± 5	7 ± 1	<LOQ[1]	28 ± 5
Experiment 2 (n = 6)	61 ± 7	8 ± 1	<LOQ	31 ± 10
Experiment 3 (n = 5)	50 ± 12	4 ± 1	<LOQ	46 ± 13
Experiment 4 (n = 4)	73 ± 10	8 ± 1	<LOQ	19 ± 10
Experiment 5 (n = 6)	NC[2]	NC	NC	NC
Experiment 6 (n = 6)	NC	NC	NC	NC
Experiment 1–4 (n = 20)	61 ± 11	8 ± 3	<LOQ	31 ± 13

[1]<LOQ = GLY concentrations in milk samples were lower than the limit of quantification (LOQ) and therefore the transfer into milk was considered as zero.
[2]NC = not calculated due to very low glyphosate intake in experiment 5 and 6 (<0.15 mg/d).

For AMPA the fecal, renal, and mammary excretion as well as apparent retention were not calculable

for experiments 4, 5, and 6. In experiments 1, 2, and 3, the average fecal and renal excretion were 48 ± 8 and 10 ± 3%, respectively. The mammary excretion was 0% and the apparent retention was 42 ± 9% (Table 7).

Table 7: Fecal, renal, and mammary AMPA[1] excretion as well as apparent retention expressed as proportion of AMPA intake (means).

Experiment (animals)	Fecal (% of intake)	Renal (% of intake)	Mammary (% of intake)	Apparent retention (% of intake)
Experiment 1 (n = 5)	50 ± 6	9 ± 1	0	42 ± 6
Experiment 2 (n = 6)	50 ± 6	12 ± 3	0	38 ± 9
Experiment 3 (n = 5)	44 ± 10	8 ± 2	0	48 ± 11
Experiment 4 (n = 4)	NC[2]	NC	NC	NC
Experiment 5 (n = 6)	NC	NC	NC	NC
Experiment 6 (n = 6)	NC	NC	NC	NC
Experiment 1–3 (n = 16)	48 ± 8	10 ± 3	0	42 ± 9

[1]AMPA = aminomethylphosphonic acid.

[2]NC = not calculated due to very low AMPA intake in experiment 4, 5, and 6 (<0.06 mg/d).

Discussion

The data from the present study represent the first results on GLY balance data in lactating cows and are therefore of high scientific relevance. In the present study a broad range of GLY exposition (0.08–6.7 mg/d) of the cows was measured. On average, cows were exposed daily to 4 µg of GLY/kg of BW. The maximum exposure of the cows was observed in experiment 1 (11 µg of GLY/kg of BW), and the minimum exposure was in experiment 6 (0.1 µg of GLY/kg of BW). The highest GLY contamination was observed in the concentrates of experiments 1 to 4. The average proportion of soybean meal in these concentrates was 22% and the average GLY concentration was 0.58 mg/kg. If the greatest extent of GLY originated from soybean meal, the concentration of GLY in this ingredient should be 4.5 times higher than in the complete concentrate; this would result in values of approximately 3 mg/kg for soybean meal. This value is in the range of GLY concentrations (0.4–8.8 mg/kg) observed in genetically modified soybeans (Bøhn et al., 2014) and leads to the assumption that GLY in the present investigation originated mainly from soybean meal.

In the present study 61 ± 11% of the ingested GLY was excreted in feces and passed the gastrointestinal tract of the dairy cows unmetabolized. The excretion with urine (8 ± 3% of daily intake) was the second important excretion pathway. In studies with rats, the elimination of ingested GLY with urine was approximately 30% (Brewster et al., 1991; Chan and Mahler, 1992). The difference in urinary elimination of GLY between species might be explained by a possible higher gastrointestinal degradation of GLY to AMPA in dairy cows compared with rats. Gerlach et al. (2014) observed GLY concentrations of approximately 5 to 20 µg/L in urine samples of dairy cows. However, in Gerlach et al. (2014), neither the urine volume nor the GLY intake was measured and GLY excretion was not determined quantitatively. The GLY concentrations in urine of the present study ranged between values lower than the LOQ and 75.1 µg/L and suggest a representative range for conventional feeding conditions in dairy cows. A dietary intake lower than 10 mg/d as measured in the present study did not result in GLY excretion via milk. In all milk samples the GLY concentrations were below the LOQ. Under the conditions of the present study milk was no excretion pathway for GLY, but these results should be verified by further investigations, especially with higher daily GLY intakes.

For the remaining 31 ± 13% of GLY that was not excreted with feces and urine, degradation by rumen microbes could be relevant. For experiments 1 and 2, with the highest GLY intake per day, the duodenal flow of GLY was 36 and 6% lower compared with the daily intake, respectively. These results suggest that GLY might have been degraded in the rumen. Jacob et al. (1988) and Heitkamp et al. (1992) described microbes originating from soil (Pseudomonas sp. strain LBr) with the ability to degrade GLY to AMPA. In contrast to dairy cows, metabolism of GLY in rats was 7% (Anadón et al., 2009) or less than 1% (Brewster et al., 1991). The relevance of rumen microbes for degradation of GLY has to be clarified in further studies.

The fact that a high proportion of GLY (61 ± 11%) passes the rumen and intestine unmetabolized is important regarding potential effects of GLY on microbes in the gastrointestinal tract. In recently

published studies, a relationship of GLY to the development of chronic visceral botulism in dairy cows was hypothesized (Krüger et al., 2013; Gerlach et al., 2014). For ruminants, only a few studies are available regarding the effects of GLY on microbial community and ruminal fermentation parameters. Riede et al. (2014) found no effects of GLY on ruminal fermentation and microbial community in vitro. These results agreed with results of a study in wethers by Hüther et al. (2005), which showed no effects of GLY on pH value and concentration of VFA in rumen fluid. Further research is necessary to clarify whether the unmetabolized GLY in the gastrointestinal tract may affect rumen microbes.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication describes a series of 6 experiments in which dairy cows (n = 4-6 per experiment) were fed with glyphosate-treated feed for 26 days and where the excretion of parent glyphosate and AMPA residues via feces, urine and milk was investigated during the last 5 days of the experiments (i.e. at a time when steady state can be assumed). The intake of parent glyphosate residues ranged between < 0.001 mg/kg bw/day (experiments 4, 5 and 6) and 0.011 mg/kg bw/day (experiment 1) while the intake of AMPA residues ranged between < 0.001 mg/kg bw/day (experiments 4, 5 and 6) and about 0.008 mg/kg bw/day (experiment 1). These intake levels are far below the dose levels investigated in the goat metabolism studies and cow feeding studies submitted in the dossier (since the applicable guidelines require that the dose levels be higher) but are likely to reflect "typical" intake levels of dietary cows. In the experiments it was found that 50-73% of ingested glyphosate was excreted in feces and 4-8% in urine. Similarly, 44-50% of ingested AMPA was excreted in feces and 8-12% in urine (these figures assume that no glyphosate is metabolized to AMPA in the cows). These results are consistent with the results of the submitted goat metabolism studies which show that 47-78% of the administered radioactivity is excreted via feces and 4.7-23% via urine. The residues of parent glyphosate and AMPA in milk were below the limit of quantification of 0.01 mg/kg, which is consistent with the results of the GLP cow feeding studies submitted in the dossier. Although the residue analytical method and residue analyses are not reported with a high level of detail, the results are considered reliable since the general principle of the described analytical procedures is well known and the validity of the residue determination was obviously demonstrated by suitable fortification trials. The publication, therefore, is considered relevant and reliable.

1. Information on the study

Data point	CA 6.9
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2. Full summary of the study according to OECD format

Executive Summary

A total of 243 samples of diverse foodstuffs were analysed for glyphosate and aminomethylphosphonic acid (AMPA) using a liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) method with a relatively low limit of quantification in the range of 0.0005 – 0.0025 mg/kg. Main contributors for dietary glyphosate and AMPA intake were cereals and pulses. The results suggest that pasta is a very important foodstuff for dietary glyphosate residue intake in Switzerland. Interestingly all samples of wine, fruit juice and nearly all samples of honey tested positive for glyphosate although at very low levels. A dietary risk assessment was conducted. Food products for analysis were not selected purely at random, rather products were selected for which high levels of glyphosate residues were suspected. However, even in samples where high residue levels were expected, no exceedances of maximum residue levels were found. Consequently, human exposure did not exceed neither acceptable daily intake nor acute reference dose. Therefore, glyphosate residues found in the sampled foodstuffs from the Swiss market were of no concern for human health.

Materials and Methods

Samples

In total, 243 samples were analysed. All samples were bought in retail stores with the aim to represent a wide range of food products. Usually a single consumer package of 500 – 2000 g was sampled, irrespective of the lot size. When necessary, samples were homogenised using different mills and mixing devices to a particle size of about 0.1 mm before further processing.

Chemicals, reagents, and consumables

All solvents were obtained in LC-MS grade (Chromasolv ®) from Sigma-Aldrich (Buchs, Switzerland), as well as formic acid. Ultrapure water, further referred to as water, was obtained from an Elga Purelab ultra-water purification system (Labtec Services, Villmergen, Switzerland). Glyphosate standards and AMPA were obtained from Sigma-Aldrich; glyphosate internal standard (IS) $^{13}C_3$-D_2-Glyphosate from Alsachim (Illkirch-Graffenstaden, France); AMPA IS ^{13}C-^{15}N-AMPA from Dr. Ehrenstorfer (LGC Standards, Teddington, UK). All dilutions of standard solutions were prepared in water except the last dilution for standards ready for injection where dilution solvent was used. These dilutions were made in 20 mL vials, which were rinsed with water and methanol before use.

The extraction solvent was a water/methanol 1/1 (v/v) mixture with 0.5% formic acid; the dilution

solvent was a water/acetonitrile 1/1 (v/v) mixture with 0.2% formic acid; the glyphosate IS and the AMPA IS solutions were 5000 ng/mL in water; the glyphosate and the AMPA stock solutions were 250 ng/mL in water; the calibration working solutions were 0.004 mL each of glyphosate IS and of AMPA IS solutions, ranging 0 – 0.060 mL of both stock solutions, respectively and extraction solvent up to 0.500 mL. The calibration injection solutions for solid samples were 0.100 mL of calibration working solutions diluted with 0.400 mL of dilution solvent. Similar for liquid samples, but dilution with 0.200 mL of dilution solvent.

The applied consumables were 2 and 50 mL centrifuge vials, polypropylene (PP) tubes, high density polyethylene (PE) screw caps (Eppendorf, Hamburg, Germany); 20 mL super PE vials for liquid scintillation (PerkinElmer, Waltham, MA, USA); 0.6 mL PE autosampler vials (06-PESV, Chromacol, Thermo Fisher Scientific Inc., Waltham, MA, USA); PP pipet tips for microman (Gilson Inc., Middleton, WI, USA); solid-phase extraction (SPE) cartridges Oasis HLB, 3 cc, 60 mg sorbent (Waters, Milford, MA, USA).

Sample preparation

Solid samples

Five gram of the homogenous or homogenised sample was weighed (rounded to the next 10 mg) into a 50 mL centrifuge vial and 20 mL of extraction solvent and 0.160 mL each of IS solutions were added. The tube was vigorously shaken by hand, then treated for 10 min in an ultrasound bath and shaken for 30 min on a shaker (Innova 2000, Eppendorf, Hamburg, Germany) at 400 rpm. The mixture was then centrifuged for 10 min at 2500 relative centrifugal force (RCF) and 10°C. Two times 1.5 mL of the supernatant was transferred into a 2 mL centrifuge vial and centrifuged for 10 min at 20,000 RCF. The combined supernatants were the final extract. Clean-up was performed on a SPE cartridge, which was first activated with 2 mL of methanol, conditioned with 2 mL of extraction solvent and pre-rinsed with 0.5 mL of extract. The eluate was discarded up to this step. A further 0.4 mL of extract was loaded onto the cartridge, the eluate collected in a 2 mL centrifuge vial and 0.100 mL of this eluate was diluted with 0.400 mL of dilution solvent in an autosampler vial.

Liquid samples

Five millitre of degassed (20 s in an ultrasound bath) beverage was transferred into a 50 mL centrifuge vial and 5 mL of extraction solvent and 0.080 mL each of IS solutions were added. The tube was shaken by hand. The SPE cartridge clean-up was performed as described above, only differing in the last step where 0.100 mL of the final eluate was diluted with 0.200 mL of dilution solvent in an autosampler vial.

Calibration

A 6-point calibration curve, corresponding to a range of 0 – 0.120 mg/kg for solid samples and a range of 0 – 0.060 mg/L for liquid samples, was constructed. If a sample contained a higher concentration, an extract using a lower amount of sample was prepared or further calibration points were introduced..

LC/MS/MS conditions

LC-system and conditions

A Symbiosis-System (Spark Holland B.V., Emmen, The Netherlands) was used with the following parameters: injection volume 10 µL; column BioRad Micro-Guard Cation H Refill Cartridge 30 × 4.6 mm (BioRad, Hercules, CA, USA); column oven at 40°C; elution solvent A: water; elution solvent B: acetonitrile with 0.2% formic acid; program: 0:00 flow rate 0.5 mL/min 60% A; 1:00 flow rate 0.5 mL/min 60% A; 1:30 flow rate 0.5 mL/min 99% A; 3:30 flow rate 0.5 mL/min 99% A; 3:35 flow rate 0.8 mL/min 99% A; 7:50 flow rate 0.8 mL/min 99% A; 8:00 flow rate 0.8 mL/min 60% A; 10:00 flow rate 0.5 mL/min 60% A; 10:10 flow rate 0.5 mL/min 60% A. The use of a specific rinsing procedure was important to minimise carryover and contamination. Needle rinsing was performed as follows: 500 µl water/methanol/acetonitrile 8/1/1 (v/v) followed by 700 µl water/methanol 1/1 (v/v) with 0.1% phosphoric acid 85% and finishing with 500 µl water/acetonitrile 6/4 (v/v) with 0.1 % formic acid.

After each sample, a blank run was carried out.

MS/MS-system and conditions

An API 5000 (AB Sciex Netherlands B.V., Nieuwerkerk aan den Ijssel, The Netherlands) with electrospray ionisation in negative mode was used and scheduled multiple reaction monitoring was applied. The eluent in the first 1.5 min was diverted into waste. The optimized ionisation source parameters were source temperature, 650°C; ionisation voltage − 4500 V; curtain gas, 25 units; collision gas, 5 units; gas 1, 60 units; gas 2, 50 units; Dwell time, 50 ms. The transitions measured were the following (quantifier in bold): glyphosate, 168 → 150, 168 → 124, 168 → 79, **168 → 63**; glyphosate IS, 173 → 128, 173 → 81, **173 → 63**; AMPA, 110 → 81, 110 → 79, **110 → 63**; AMPA IS, 112 → 81, 112 → 79, **112 → 63**..

Method validation

The applied anion exchange method was based on the methods published by Guo *et al.* (2016) and Jensen *et al.* (2016). Validation of the analytical method was based on repeated experiments verifying limit of detection (LOD), LOQ, repeatability, and recovery in different matrices. Internal reference materials were used in each run. For the LOQ, the signal-to-noise threshold was set at 10 for the quantifier and at 7 for the two qualifiers. In addition, two external reference materials of wheat flour and rapeseed and the respective blank materials were analysed on a regular basis: reference material P1601-RMWh, wheat flour spiked with glyphosate, AMPA, glufosinate; blank material P1601-BLWh, wheat flour; reference material P1601-RMRape, rapeseed spiked with glyphosate, AMPA, glufosinate; blank material P1601-BLRape, rapeseed; all from PROOF-ACS GmbH (Hamburg, Germany). Further details of these reference materials are given in the explanation to **Table 1**. A Food Analysis Performance Assessment Scheme (FAPAS 2017) proficiency test on oat test material with chlormequat, mepiquat, and glyphosate was also completed, of which only glyphosate was analysed.

Results and Discussion

Method quality assurance

The method showed to be very robust and can be applied for nearly all kind of foodstuffs. It turned out that it is not necessary to use matrix-matched calibration. The absolute recovery was estimated using the absolute peak area of the IS. The absolute recovery was always better than 70% for liquid samples and for solid samples it was always better than 50% and in most cases also better than 70%. Dilution experiments with naturally contaminated samples with concentrations above 0.05 mg/kg showed identical quantitative results. There was no indication for disturbing matrix effects in the undiluted sample. The LOQ for solid samples was generally 0.001 and 0.0025 mg/kg for glyphosate and AMPA, respectively. For liquid samples (i.e. beverages like wine and beer), the LOQ was 0.0005 mg/kg for glyphosate and 0.0005 – 0.001 mg/kg for AMPA. Details of the performance data of the method are given in **Table 1**. The FAPAS proficiency test (2017) was successfully passed with a z-score of 0.9 at the assigned value for glyphosate of 0.483 mg/kg. This level was appropriate for the validation of the higher levels that were measured, for instance in durum wheat and pasta, but not optimal for the lower levels around and below 0.05 mg/kg. For these levels, the wheat and rapeseed reference materials (PROOF-ACS GmbH) with assigned values for glyphosate of 0.034 and 0.086 mg/kg, respectively, were more appropriate. In **Table 1** it is shown that our measurements were in good agreement with the assigned values and also with the spiked values. In the FAPAS 09109b, oats blank material, 0.0057 mg/kg of glyphosate was measured.

The measurement uncertainty which is indicated in the supporting information is an estimate for the expanded uncertainty with a confidence level of 95%. The values are roughly estimated with the help of the method performance data given in **Table 1**. Twenty percent is set as minimum value for the uncertainty. A more conservative approach would be to take the uncertainty from the proficiency tests of the mentioned FAPAS test and PROOF-ACS reference materials. The range of ±2 for z-scores is a good estimate for the confidence interval of 95%. In this case, the uncertainty would generally be set at 45% as the uncertainty for all values from the PROOF-ACS materials were between 43.3% and 44.7%. The respective uncertainty for glyphosate in the FAPAS test was 35.6%.

In a few cases where it was suspected that the sample might not be sufficiently homogeneous, another two subsamples were analysed. In all cases, the difference to the first result was well below 10%. In the case of the gram flour with a concentration of 2.756 mg/kg of glyphosate, which is discussed further down in the text, a package of the same lot could be purchased 6 months later. The measured concentration in the second package differed less than 2% from the first result.

Table 1: Method performance data.

Analyte	Matrix	LOD [mg kg^{-1}]	LOQ [mg kg^{-1}]	concentration [mg kg^{-1}]	Repetitions (n)	Recovery (%)	RSD (%)	Comments and applied reference materials
Glyphosate	Wheat, white flour	0.0003	0.001	0.001	5	94	9.5	s, st
AMPA	Wheat, white flour	0.001	0.0025	0.005	5	101	6.5	s, st
Glyphosate	Beer	0.0002	0.0005	0.001	5	103	2.2	s, st
AMPA	Beer	0.0005	0.001	0.001	5	97	6.6	s, st
Glyphosate	Beer	0.0002	0.0005	0.010	3	98	7.1	s, st, d
AMPA	Beer	0.0005	0.001	0.010	3	102	0.6	s, st, d
Glyphosate	Wine	0.0002	0.0005	0.010	2	92	9.2	s, st, d
AMPA	Wine	0.0005	0.001	0.010	2	99	5.0	s, st, d
Glyphosate	Milk	0.0002	0.0005	0.004	2	96	1.8	s, st, d
AMPA	Milk	0.0005	0.001	0.004	2	111	1.6	s, st, d
Glyphosate	Honey	0.0003	0.001	0.005	5	92	13.9	s, st, d
AMPA	Honey	0.001	0.0025	0.005	5	115	3.5	s, st, d
Glyphosate	Vegetable oil	0.0004	0.001	0.010	2	102	2.8	s, st, d
AMPA	Vegetable oil	0.001	0.0025	0.010	2	92	6.1	s, st, d
Glyphosate	Smoked salmon	0.0004	0.001	0.010	1	95	N/A	s
AMPA	Smoked salmon	0.001	0.0025	0.010	1	97	N/A	s
Glyphosate	Poultry meat	0.0003	0.001	0.050	3	102	1.3	s, st, d
AMPA	Poultry meat	0.001	0.0025	0.050	3	100	1.3	s, st, d
Glyphosate	Red wine	0.0002	0.0005	0.0132	7	N/A	3.6	nc, lt
AMPA	Red wine	0.0005	0.001	<0.001	7	N/A	N/A	nc, lt
Glyphosate	Whole meal flour	0.0003	0.001	0.051	5	N/A	3.7	nc, st
AMPA	Whole meal flour	0.001	0.0025	0.0036	5	N/A	8.4	nc, st
Glyphosate	Whole meal flour	0.0003	0.001	0.051	22	N/A	5.4	nc, lt
AMPA	Whole meal flour	0.001	0.0025	0.0024	22	N/A	12.5	nc, lt
Glyphosate	Wheat	0.0003	0.001	<0.001	19	N/A	N/A	P1601-BLWh, lt
AMPA	Wheat	0.001	0.0025	<0.0025	19	N/A	N/A	P1601-BLWh, lt
Glyphosate	Wheat	0.0003	0.001	0.0376	21	N/A	8.4	P1601-RMWh, lt
AMPA	Wheat	0.001	0.0025	0.0577	21	N/A	9.1	P1601-RMWh, lt
Glyphosate	Rapeseed	0.0003	0.001	<0.001	3	N/A	N/A	P1601-BLRape, lt
AMPA	Rapeseed	0.001	0.0025	<0.0025	3	N/A	N/A	P1601-BLRape, lt
Glyphosate	Rapeseed	0.0003	0.001	0.0925	3	N/A	2.2	P1601-RMRape, lt
AMPA	Rapeseed	0.001	0.0025	0.0778	3	N/A	3.1	P1601-RMRape, lt

N/A: not applicable; s: spiked; nc: naturally contaminated; st: repetitions within 1 day; lt: repetitions over a time period of 7 months; d: different products; P1601-BLWh: wheat blank material; P1601-RMWh: wheat reference material, spiked level for glyphosate 0.037 mg kg^{-1} and assigned value by proficiency test 0.034 mg kg^{-1}, spiked level for AMPA 0.055 mg kg^{-1} and assigned value by proficiency test 0.050 mg kg^{-1}; P1601-BLRape: rapeseed blank material; P1601-RMRape: rapeseed reference material, spiked level for glyphosate 0.098 mg kg^{-1} and assigned value by proficiency test 0.0859 mg kg^{-1}, spiked level for AMPA 0.088 mg kg^{-1} and assigned value by proficiency test 0.0739 mg kg^{-1}.

Another peak showing quite similar ion transitions as glyphosate, eluting just after glyphosate, was often observed. This peak was identified as 2-amino-3-phosphonopropionic acid, a substance with identical sum formula and similar functional groups as glyphosate. This compound seems to occur in many products in the range of 0.001 – 0.5 mg/kg. For this reason, it can be recommended to check if 2-amino-3-phosphonopropionic acid is properly distinguished from glyphosate in the chromatograms, as to avoid the risk of too high results when analysing glyphosate. 2-Amino-3-phosphonopropionic acid was analysed semi-quantitatively and seems to occur in many products, especially in cereals, in the range of 0.001 – 0.9 mg/kg. There was no correlation between the concentration of 2-amino-3-phosphono-propionic acid and glyphosate. From the chemical structure point of view, it seems unlikely that 2-amino-3-phosphonopropionic acid is a metabolite of glyphosate. 2-Amino-3-phosphonopropionic acid may be a natural compound. Its occurrence in the ciliate *Tetrahymena pyriformis* is described by Horsman and Zechel (2017); however, no reference on the occurrence in higher plants is available. This issue will be examined in more detail in the context of another project.

Table 2: Concentrations of glyphosate and AMPA in different food categories.

	Glyphosate								AMPA					
Food category	Number of samples	Number of samples above the LOQ	Proportion of samples above the LOQ	LOQ (mg kg^{-1})	Min (mg kg^{-1})	Median (mg kg^{-1})	Arithmetic mean (mg kg^{-1})	Max (mg kg^{-1})	Number of samples above the LOQ	Proportion of samples above the LOQ	LOQ (mg kg^{-1})	Min (mg kg^{-1})	Median (mg kg^{-1})	Arithmetic mean (mg kg^{-1})	Max (mg kg^{-1})
Beer	15	2	13%	0.0005	<0.0005	<0.0005	0.0006	0.0068	0	0%	0.001	<0.001	<0.001	<0.001	<0.001
Wine	21	21	100%	0.0005	0.0006	0.0031	0.0048	0.0189	4	19%	0.0007	<0.0007	<0.0007	0.0005	0.0034
Mineral water	2	0	0%	0.0005	<0.0005	<0.0005	<0.0005	<0.0005	0	0%	0.0005	<0.0005	<0.0005	<0.0005	<0.0005
Milk	3	0	0%	0.0005	<0.0005	<0.0005	<0.0005	<0.0005	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Fruit juice	11	11	100%	0.0005	0.0005	0.0016	0.0019	0.0035	2	18%	0.0006	<0.0006	<0.0006	0.0002	0.0006
Baby food	11	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Potatoes and vegetables	10	3	30%	0.001	<0.001	<0.001	0.0013	0.0077	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Honey	16	15	94%	0.001	<0.001	0.0030	0.0046	0.0159	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Eggs	1	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Meat and fish	13	3	23%	0.001	<0.001	<0.001	0.0008	0.0049	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Pulses	41	21	51%	0.001	<0.001	0.0012	0.1733	2.948	10	24%	0.0025	<0.0025	<0.0025	0.0031	0.025
Oilseeds and vegetable oil	6	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Pseudo cereals	3	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Breakfast cereals	10	8	80%	0.001	<0.001	0.0036	0.0508	0.291	3	30%	0.0025	<0.0025	<0.0025	0.0025	0.010
Durum wheat	18	16	89%	0.001	<0.001	0.139	0.1349	0.421	15	83%	0.0025	<0.0025	0.0107	0.0110	0.0247
Pastry and snacks	11	4	36%	0.001	<0.001	<0.001	0.0037	0.0179	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Bread	10	7	70%	0.001	<0.001	0.0019	0.0069	0.0458	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Flour and baking mixtures	28	8	29%	0.001	<0.001	<0.001	0.0106	0.133	2	7%	0.0025	<0.0025	<0.0025	0.0007	0.0027
Other cereal products	13	2	15%	0.001	<0.001	<0.001	0.0012	0.0124	1	8%	0.0025	<0.0025	<0.0025	0.0007	0.0052

Description of categories: *pulses*: including products thereof like tofu and soy sauce, etc.; *breakfast cereals*: processed breakfast cereals like corn flakes, pops, etc. Rolled oats are placed in the category of other cereal products; *durum wheat*: all products with durum wheat as main ingredient as for instance pasta; *pastry and snacks*: all dry bake goods, sweet or salty, and also tortilla chips and potato chips (crisps); *bread*: also special bread that may contain minor amounts of oilseeds or pulses; *flour and baking mixtures*: flour and baking mixtures for bread making, the main ingredients are bread cereals like wheat, rye, and spelt, but they may also contain minor amounts of other cereals, oilseeds, and pulses; *other cereal products*: category with a wide variety of products like rolled oats, popcorn, semolina of maize (polenta), pasta with wheat instead of durum wheat, etc.; for beverages as beer, wine, milk, fruit juices, and mineral water, the measurement unit is mg L^{-1} instead of mg kg^{-1}. For the calculation of the arithmetic mean, all samples below the LOD were taken as zero; for samples between the LOQ and the LOD, the estimated value was used.

Concentrations in foodstuffs

Food products were sampled with the aim to determine the relevant foodstuffs for glyphosate intake. Samples with higher residue concentrations are probably over-represented to some extent, because categories like pulses and durum wheat were more frequently sampled, since these were suspect to reveal more glyphosate positive results. Additionally, every time when food samples turned out to contain more than 0.01 mg/kg, a few similar food items were collected. All together survey results are probably not representative for the residue levels in all foodstuffs on the market, as to achieve this goal analysis of a few thousand samples would have been necessary. The results for glyphosate and AMPA are summarised in **Table 2** and grouped into different food categories. Detailed data is available as supporting information.

For cereals and pulses, the contamination rate for glyphosate on the level above 0.1 mg/kg is comparable with data from Germany (Scherbaum *et al.* 2012) and a bit lower as in the United Kingdom (Stephenson and Harris 2016). The two samples with the highest glyphosate concentration were chickpeas originating from Canada with 2.948 mg/kg and gram flour (chickpea flour) with 2.756 mg/kg produced in the United Kingdom with unknown origin of the processed chickpeas. In 24 samples, glyphosate was measured above 0.1 mg/kg, but all AMPA values were below 0.1 mg/kg and usually much lower than the respective glyphosate values. Thirteen of 24 samples were durum wheat products like pasta and semolina, 8 samples were pulses and products thereof, 2 further samples were breakfast cereals and the last product was a bread baking mix containing seeds. It could be shown that the main contributor for glyphosate residue in this mix was linseed. There was no hint that 1 of these 24 products contained relevant ingredients of Swiss origin. Pulses are not consumed very often in Switzerland; however, pasta is an important dish of the regional diet. As nearly 100% of durum wheat for the production of pasta is imported, this might be an important commodity regarding glyphosate residues. All samples of wine and fruit juice and all except one sample of honey were positive for glyphosate but all in the low ng/g range.

Of all analysed samples, 38 were clearly indicated as made of Swiss ingredients. The product with the highest glyphosate concentration of this category was a red wine containing 0.0132 mg/kg. All cereal products of this category contained undetectable or low amounts. The highest value found was 0.0025 mg/kg glyphosate in a wholegrain wheat flour. The number of 38 samples with ingredients of Swiss origin is not large enough as to guarantee that Swiss regulations on the use of glyphosate in agricultural practice are not violated, but at least do not indicate unregistered use of glyphosate, since not one single high contamination was found in food items containing raw products originating from Switzerland.

Also, all products labelled as organic had no or only low residues. In 37 of totally 43 organic samples, the concentration was below the LOQ and only 6 samples showed quantifiable amounts. In three of these six samples the concentration was just above the LOQ and only one sample showed a concentration above 0.01 mg/kg. This organic sample with the highest glyphosate concentration was a pasta product (spaghetti) containing 0.0123 mg/kg of glyphosate and 0.0024 mg/kg of AMPA. On the label, it was indicated that the durum wheat originated from North America, Europe and the eggs from Europe. Carryover during transport and production is conceivable. No detailed data are available to what extent such a contamination is avoidable by using adequate practices. As far as we know there is not yet a binding agreement on how low the residues in organic products should be, but a value of 0.01 mg/kg is at least under discussion or maybe already partially implemented.

Risk assessment

Based on the measured residues (**Table 2**), simple exposure estimates were derived (**Table 3**) and compared to the ARfD and the ADI, both amounting to 0.5 mg/kg bw/day, as recently established by EFSA's revaluation (EFSA 2015). Food consumption values applied in the exposure estimation were chosen at a level to overestimate actual daily average consumption. It seems plausible that these amounts of the respective food items are consumed at least occasionally during a single day. Risk assessments, i.e. comparison of estimated residue intake with the ADI and ARfD, were conducted for both the measured median and MRLs found per food item.

None of the median residues found in any food item resulted in an exposure greater than 0.5% of the

ADI/ARfD and virtually all are significantly below 0.5% of the ADI/ARfD. If measured MRLs were applied, substantial exposures (ca. 5% of ADI/ARfD in adults and ca. 10% of ADI/ARfD in children) resulted for pulses, exclusively. All other MRLs resulted in exposures that were mostly significantly lower than 1% of the ADI/ARfD. It is concluded that none of the residue levels identified in any of the food categories are of any health concern. This is not surprising, as none of the measured residue levels exceeded the legally tolerated MRL.

Table 3: Exposure to median and maximum glyphosate residue levels and expected urine glyphosate concentrations (nr: not relevant).

	Child of 15 kg body weight				Adult of 60 kg body weight			
		Exposure as % of ADI or ARfD				Exposure of % of ADI or ARfD		
Food category	Consumption (kg or L/day)	At median residue level	At maximum residue level	Expected urine concentration (µg L^{-1})	Consumption (kg or L per day)	At median residue level	At maximum residue level	Expected urine concentration (µg L^{-1})
Beer	nr	nr	nr	nr	0.50	0.0008	0.0113	0.340
Wine	nr	nr	nr	nr	0.25	0.0026	0.0158	0.473
Mineral water	1.00	0.0067	0.0067	0.067	2.00	0.0033	0.0033	0.100
Milk	0.50	0.0033	0.0033	0.033	1.00	0.0017	0.0017	0.050
Fruit juice	0.50	0.0107	0.0233	0.233	1.00	0.0053	0.0117	0.350
Potatoes and vegetables	0.25	0.0033	0.0257	0.257	0.50	0.0017	0.0128	0.385
Honey	0.03	0.0010	0.0053	0.053	0.05	0.0005	0.0027	0.080
Eggs	0.10	0.0013	0.0013	0.013	0.20	0.0007	0.0007	0.020
Meat and fish	0.25	0.0033	0.0163	0.163	0.50	0.0017	0.0082	0.245
Pulses	0.25	0.0033	9.8267	98.27	0.50	0.0017	4.9133	147.4
Oilseeds	0.05	0.0007	0.0007	0.007	0.10	0.0003	0.0003	0.010
Pseudo cereals	0.10	0.0013	0.0013	0.013	0.20	0.0007	0.0007	0.020
Breakfast cereals	0.10	0.0048	0.3880	3.880	0.20	0.0024	0.1940	5.820
Durum wheat	0.25	0.4633	1.4033	14.03	0.50	0.2317	0.7017	21.05
Pastry and snacks	0.05	0.0007	0.0119	0.119	0.10	0.0003	0.0060	0.179
Bread	0.25	0.0063	0.1527	1.527	0.50	0.0032	0.0763	2.290
Flour and baking mixtures	0.25	0.0033	0.4433	4.433	0.50	0.0017	0.2217	6.650
Other cereal products	0.10	0.0013	0.0165	0.165	0.20	0.0007	0.0083	0.248

Exposure per kg body weight is calculated by multiplying the residue concentration in food by the assumed food consumption and dividing the result by body weight (15 kg for children and 60 kg for adults). Risk is expressed by calculating exposure as per cent ADI or ARfD (both amounting to 0.5 mg kg^{-1} bw). Maximally expected urine concentrations are calculated by multiplying maximum residue concentrations in food by the assumed consumption and by the fraction of orally ingested glyphosate excreted by the urine (20%). The obtained result is divided by an assumed daily urine volume of 1.5 L for a child and 2 L for an adult. If residues were below LOQ, the LOQ value was used for risk assessment.

The exposure estimates for maximum residues derived as described above were also used to predict probable urine concentrations. It was assumed that the amount indicated in Table 3 of the respective food item was ingested and this food item contained the measured MRL of glyphosate (**Table 2**). Based on toxicokinetic studies, the amount of an orally ingested single dose of glyphosate excreted with the urine was assumed to equal 20% (EFSA 2015). Further, it was assumed that daily urine volumes of 1.5 and 2.0 L are excreted by children and adults, respectively. For glyphosate residues at the maximally measured levels, predicted urine concentrations would be greater than 0.5 µg/L only for a few commodities. Again, only for the maximum residues found in pulses substantial amounts were predicted in urine of adults (ca. 147 µg/L). Overall, the predicted urine concentrations correspond very well with actually measured glyphosate urine levels in samples of the human population: Conrad *et al.* (2017) reported median levels well below 0.5 µg/L in samples of the German population, while maximum values slightly exceeded 0.5 µg/L. Also Niemann *et al.* (2015) concluded that urine concentrations of glyphosate corresponded well with levels in food; however, urine levels of AMPA were somewhat too high and not in good agreement with reported levels in foodstuffs. In a report of glyphosate urine levels in a small, not representative survey of the Swiss population, values in the range of 0.1 – 1.5 µg/L (RTS 2015) were measured.

Conclusion

In this market survey, food products for analysis were not selected purely randomly, rather products were selected for which measurable levels of glyphosate residues were suspected. However, even in samples where high residues were expected, no exceedances of MRLs were detected. Consequently,

exposures did not exceed neither ADI nor ARfD. Therefore, glyphosate residues found in the sampled foodstuffs from the Swiss market are of no health concern for the consumer. This conclusion may be valid for all food products on the Swiss food market, considering that products for which high residue levels were suspected were over-represented in this survey.

Chromatographic conditions

Chromatograph:	Symbiosis			
Column:	BioRad Micro-Guard Cation H Refill Cartridge (30 mm x 4.6 mm)			
Column oven temperature:	40°C			
Injection volume:	10 µL			
Mobile phases:	(A) Water (B) Acetonitrile with 0.2% formic acid			
Gradient:	Time (Min)	Eluent A (%)	Eluent B (%)	Flow rate (mL/min)
	0:00	60	40	0.5
	1:00	60	40	0.5
	1:30	99	1	0.5
	3:30	99	1	0.5
	3:35	99	1	0.8
	7:50	99	1	0.8
	8:00	60	40	0.8
	10:00	60	40	0.5
	10:10	60	40	0.5
Retention time:	Not provided			
Detector:	Sciex API 5000 triple quadrupole mass spectrometer			
Scan type:	MRM			
Ion source:	ESI negative			

Source gas 1:	60 units	Source gas 2:	50 units
Collision gas:	5 units	Source temperature:	650°C
Curtain gas:	25 units	Source voltage:	−4500 V

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Declustering potential (V)	Collision energy (eV)	Scan time (ms)
Primary transition (quantification)					
Glyphosate	168	63	−	−	50
Glyphosate (IS)	173	63	−	−	50
AMPA	110	63	−	−	50
AMPA (IS)	112	63	−	−	50
Secondary transition (confirmation)					
Glyphosate	168	79	−	−	50
Glyphosate (IS)	173	81	−	−	50
AMPA	110	79	−	−	50
AMPA (IS)	112	79	−	−	50

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes the results of monitoring analyses for residues of glyphosate and AMPA in food conducted by Swiss authorities between 2012 and 2017. A total of 243 samples of diverse food commodities were analysed for glyphosate and AMPA using an LC-MS/MS method that was developed specifically by the Swiss monitoring laboratory. According to the authors the method has a limit of quantification of 0.001 mg/kg for parent glyphosate and 0.0025 mg/kg for AMPA in solid matrices and 0.0005 mg/kg and 0.001 mg/kg, respectively, in liquid matrices (beer, fruit juice, wine). While it seems that these LOQs were established according to recognized procedures, details are missing and it is, therefore, difficult to evaluate the reliability of the provided analytical results. This would be especially important since the reported LOQs are far below the LOQs achieved by most of the other official monitoring laboratories.

As stated by the authors the publication is not intended to provide a representative picture of the residues of glyphosate and AMPA in food commodities placed on the market in Switzerland since the commodities showing high residues were over-represented. In spite of that, the samples relevant to the uses supported in the renewal dossier (e.g. fruits, vegetables, fruit juice, wine, food of animal origin) all showed residues of glyphosate and AMPA far below 0.05 mg/kg (LOQ of most enforcement method so far).

In total, 16 honey samples from Europe and the Americas were analysed. They showed residues of parent glyphosate between < 0.001 mg/kg and 0.0159 mg/kg while the residues of AMPA were always < 0.0025 mg/kg (details are provided as supplementary data). Since according to SANTE/11956/2016 rev. 9 it is possible to derive EU MRLs in honey based on monitoring data and since honey marketed in Switzerland is likely to be also marketed in the EU, these results are deemed relevant to the setting of an EU MRL for glyphosate in honey. The fact that all the samples showed residues of AMPA < 0.0025 mg/kg is in contrast to another publication in which the analyses were also conducted with a very sensitive analytical method and where the residues of AMPA were often found at levels comparable to or even greater than the levels of parent glyphosate residues.

グリホサートカリウム塩

要旨及び評価結果

（ヒトに対する毒性）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 5.5/026
Report author	Crump, K. et al.
Report year	2020
Report title	Accounting for Multiple Comparisons in Statistical Analysis of the Extensive Bioassay Data on Glyphosate.
Document No	https://doi.org/10.1093/toxsci/kfaa039
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	-/Reliable without restrictions

2. Full summary of the study according to OECD format

Abstract

Glyphosate is a widely used herbicide worldwide. In 2015, the International Agency for Research on Cancer (IARC) reviewed glyphosate cancer bioassays and human studies and declared that the evidence for carcinogenicity of glyphosate is sufficient in experimental animals. We analyzed ten glyphosate rodent bioassays, including those in which IARC found evidence of carcinogenicity, using a multi-response permutation procedure that adjusts for the large number of tumors eligible for statistical testing and provides valid false-positive probabilities. The test statistics for these permutation tests are functions of p-values from a standard test for dose-response trend applied to each specific type of tumor. We evaluated three permutation tests, using as test statistics the smallest p-value from a standard statistical test for dose-response trend and the number of such tests for which the p-value is less than or equal to 0.05 or 0.01. The false-positive probabilities obtained from two implementations of these three permutation tests are: smallest p-value: 0.26, 0.17, p-values ≤ 0.05: 0.08, 0.12, p-values ≤ 0.01: 0.06, 0.08. In addition, we found more evidence for negative dose-response trends than positive. Thus, we found no strong evidence that glyphosate is an animal carcinogen. The main cause for the discrepancy between IARC's finding and ours appears to be that IARC did not account for the large number of tumor responses analyzed and the increased likelihood that several of these would show statistical significance simply by chance. This work provides a more comprehensive analysis of the animal carcinogenicity data for this important herbicide than previously available.

Materials and methods

Analysis of bioassay data - Of the bioassays identified by U.S. EPA, EFSA, or IARC, 10 glyphosate cancer studies of sufficient quality were selected which allowed the analysis of individual animal data. The characteristics of the selected studies are summarized in Table 1.

Table 1. Characteristics of the bioassays selected for analysis.

Bioassay	Species	Strain	# Dose Groups/ Sex	Animals/Dose	Maximum Dose[a] (mg/kg/d)		Maximum Weeks on test	Sites Where Histopathology was Conducted in All Dose Groups[b]
					Males	Females		
Atkinson et al 1993b[c]	Mouse	CD-1	4	50	988	1000	105	Kidney, Liver, Lung, Vascular System
Knezevich and Hogan 1983[c]	Mouse	CD-1	4	50	4841	5873	102	(all)
Wood et al 2009b	Mouse	CD-1	4	51	810	1081	81	Kidney, Liver and Lung
Sugimoto 1997	Mouse	CD-1-ICR	4	50	4348	4116	78	(all)
Atkinson et al 1993a[c]	Rat	SD[d]	5	50	1007	1018	105	Kidney, Liver, Lung and Salivary Glands: Parotid, Mandibular and Sublingual
Lankas 1981[c]	Rat	SD	4	50	31.49	34.02	111	(all)
Stout and Ruecker 1990[c]	Rat	SD	4	60	940	1183	105	(all)
Brammer 2001[c]	Rat	Wistar	4	64	1214	1498	104	(all)
Suresh 1996	Rat	Wistar	4	50	595.2	886	107	(none)
Wood et al 2009a	Rat	Wistar	4	51	1077	1382	105	Kidney, Liver, Lung and Bone Marrow

[a] All doses in each bioassay are listed in Table 2.
[b] Systemic tumors are assumed to have been searched for if at least one tissue in an animal was given a histopathological examination.
[c] These six studies were evaluated by IARC. IARC (2015) also reviewed two additional studies in which they identified shortcomings, but which they did not claim were "inadequate": Chruscielska et al (2000) and JMPR (2006). No glyphosate-related tumor responses were noted in either of these studies.
[d] SD = Sprague Dawley.

Eight of the 10 studies were assigned a Klimisch code 1 (reliable without restrictions). The Knezevich and Hogan (1983) study was assigned a Klimisch code 2 (reliable with restrictions) mainly because this study was initiated before the implementation of the OECD test guidelines and GLP, and the study of Lankas (1981) also a pre-GLP and pre-guideline study was assigned a Klimisch code 3 (not reliable) mainly because of the low power of the study due to the low dose range selected.

Statistical tests - The statistical tests applied in the analysis were functions of p-values obtained from conventional continuity-corrected poly-3 tests (survival-adjusted Cochran-Armitage test) for trend applied to each type of tumor or combination of tumor types in each bioassay. In the present analysis, the continuity-corrected version of the poly-3 test used was copied from a key portion of the computer program used by the NTP. Direct comparisons have shown that the author's implementation gives the same results as the version used by the NTP. Throughout this paper, all implementations of the poly-3 test, are one-sided, as are the NTP implementations of the test.

Results from 3 multi-response permutation tests are presented. In the simplest test, referred to as the "min-test", the test statistic is the smallest p-value obtained from applying the poly-3 test to all tumor types in all of the 10 bioassays. In the simplest implementation of this test, animals are randomly reassigned to dose groups (permuted among dose groups) in a Monte Carlo analysis, keeping the total numbers of animals in each dose group equal to the number in the original data. The tumors in each such reassignment are analyzed using the poly-3 test in exactly the same way as in the original data. Males and females are permuted separately. The false positive rate is the proportion of random reassignments that result in a smallest poly-3 p-value that is smaller than or equal to the smallest poly-3 p-value obtained from the original data. In addition to the min-test, two additional permutation tests were computed. The test statistics for these tests were the number of poly-3 tests of tumors in the original data for which the p-value is less than or equal to the critical value of 0.05 (05-test) or 0.01 (01-test). The false positive rates for these tests are the proportion of random permutations of the data for which the number of poly-3 p-values from the permuted data that are less than or equal to the critical value equal or exceed the number from the original data. The min-test could have enhanced power in a situation in which a test agent causes cancer at a single site, whereas the 05-test could have enhanced power when a test agent causes detectable cancer of several types. The permutation tests described above are conditional, not just on the total number of tumors, but also on the patterns of tumors occurring in individual animals.

In addition to conducting conventional poly-3 tests on specific types of tumors, tests were also conducted on combinations of tumor types thought to have a common origin such as liver adenomas and carcinomas. Since including these combinations resulted in the same tumors being present in multiple analyses, it was decided to perform two analyses, one (primary analysis) that included all of the individual tumors and combinations, and one (reduced analysis) in which individual tumors and combinations of tumors were removed from the analysis if they were part of a more inclusive tumor combination (e.g. the individual tumor categories of liver adenoma and liver carcinoma were removed and only the combination was used in the analysis).

For 5 of the glyphosate bioassays, all tissues listed for histopathological examination were scheduled for a histological examination in all animals of all dose groups. In the remaining 5 bioassays, control and high dose animals were all given a complete histopathological examination, along with the animals that died before the final sacrifice in the intermediate dose groups. In addition, certain tissues in all animals were scheduled for a histopathological examination regardless of when they died.

Simple randomization suffers from a potential bias due to dose-related differential survival, and, for studies with incomplete histopathology, a problem of data comparability.

In each of the 10 bioassays, dose-related effects on survival were tested using a Cochran-Armitage test for negative trend on the proportions of animals surviving to final sacrifice in the various dose groups. Regardless of the outcome of this test, to control for potential dose-related differences in survival each randomization of the data maintained the same number of survivors and non-survivors in each group as was seen in the actual data.

For other tissues than the mandatory tissues in the studies with incomplete histopathology, only the non-survivors in the intermediate dosed groups could be used in the trend analyses. For mandatory tissues, the survivors and non-survivors were separately permuted. Mandatory tissues and other tissues had to be separately randomized in studies with incomplete histopathology to make sure that all pathological information routinely collected in this type of studies is included.

In all applications of the poly-3 test, the test is applied only to data from one sex in a single study and the p-values from the poly-3 tests of all the studies are combined to create the “global” tests (min-test, 05-test and 01-test) to give the correct false positive rates. In addition to the randomization procedures for testing for positive dose-response trends in tumor incidence, the same procedures were repeated after reconfiguring the poly-3 test for negative trends.

Results

When the frequency of poly-3 p-values for positive trend computed from all tumors in all 10 bioassays in which at least two tumors occurred are considered there is an excess of large p-values (close to 1.0) compared to small p-values (close to 0.0). Since the version of the poly-3 trend test applied is a one-sided test for a positive trend, p-values close to 1.0 would translate into p-values near 0.0 for one-sided trend tests for anti-carcinogenicity.

Results of tests for a dose-related decrease in survival in each study show that in none of the bioassays analyzed this test was statistically significant. Moreover, 4 of the datasets had p-values in excess of 0.95 which indicates a significant positive trend in survival with increasing dose.

The 24 tumors for which the poly-3 test for a positive dose-related trend was significant at the 0.05 level in the primary analysis are shown in Table 2. Pancreatic islet-cell adenoma in male rats reported in the Stout and Ruecker (1990) study is not listed because it did not have a significant dose-related trend. In an identical analysis with the poly-3 test configured to test for a negative dose-related trend, there were 26 tumors for which the dose-response trend was significantly negative at the 0.05 level.

Table 2. Tumors with a significant positive trend (poly-3 p ≤ 0.05)

Bioassay	Species/ Sex	Tumor	Summary Tumor Incidence					Poly-3 p-value	Cited by IARC[a]
Atkinson et al. 1993b	M/M	Haemangiosarcoma	0/50	0/50	0/50	4/50		0.0013	IARC
Lankas 1981	R/F	Thyroid: C-cell Carcinoma	1/47	0/49	2/50	6/47		0.0015	
Sugimoto 1997	M/F	Hemangioma	0/50	0/50	2/50	5/50		0.0028	
Sugimoto 1997	M/F	Hemangioma, Hemangiosarcoma	0/50	0/50	3/50	5/50		0.0062	
Stout and Ruecker 1990	R/F	Adrenal: Cortical Carcinoma	0/60	0/60	0/60	3/60		0.0072	
Sugimoto 1997	M/F	Osteoma, Osteosarcoma	0/50	0/50	0/50	3/50		0.0074	
Wood et al. 2009b	M/M	Lymphoma	0/51	1/51	2/51	5/51		0.0076	
Brammer 2001	R/M	Liver: Hepatocellular Adenoma	0/64	2/64	0/64	5/64		0.014	
Lankas 1981	R/M	Testis: Interstitial Cell Tumor	0/50	3/50	1/50	6/50		0.021	
Stout and Ruecker 1990	R/M	Liver: Hepatocellular Adenoma	3/60	2/60	3/60	8/60		0.022	IARC[b]
Atkinson et al. 1993a	R/F	Lipoma	0/50	0/50	0/50	0/50	2/50	0.022	
Wood et al. 2009b	M/M	Lung: Adenocarcinoma	5/50	5/51	7/51	11/51		0.025	
Knezevich and Hogan 1983	M/M	Kidneys: Renal Tubal Adenoma	0/49	0/49	1/50	3/50		0.034	IARC
Lankas 1981	R/F	Lipoma	0/50	0/50	0/50	2/50		0.036	
Sugimoto 1997	M/M	Malignant Lymphoma	2/50	3/50	0/50	6/50		0.038	
Knezevich and Hogan 1983	M/F	Lymphoblastic Lymphosarcoma	0/50	1/50	0/50	3/50		0.041	
Sugimoto 1997	M/F	Osteosarcoma	0/50	0/50	0/50	2/50		0.041	
Sugimoto 1997	M/M	Kidney: Adenoma	0/50	0/50	0/50	2/50		0.042	
Sugimoto 1997	M/M	Hemangiosarcoma	0/50	0/50	0/50	2/50		0.043	
Stout and Ruecker 1990	R/M	Neurofibroma, Neurofibrocarcinoma	0/60	0/60	0/60	2/60		0.045	
Sugimoto 1997	M/F	Harderian Gland: Adenoma	1/50	3/50	0/50	5/50		0.046	
Stout and Ruecker 1990	R/F	Thyroid Gland: C-cell Adenoma	2/60	2/60	6/60	6/60		0.047	IARC
Suresh 1996	R/M	Lymphoma	0/50	0/50	0/50	2/50		0.049	
Stout and Ruecker 1990	R/F	Thyroid Gland: C-cell Adenoma or Carcinoma	2/60	2/60	7/60	6/60		0.049	

[a] Indicates tumor responses cited by IARC (2015) as evidence of carcinogenicity. Pancretic islets in male rats in Stout and Ruecker (1990) was also cited by IARC (1/58, 8/57, 5/60 and 7/59) but this response did not give a p-value ≤ 0.05 by the Poly-3 trend test.

[b] IARC (2015) resported tumor responses of 2, 2, 3, 7.

The most significant poly-3 trend in all 10 bioassays was for hemangiosarcoma in male mice in the Atkinson *et al*. (1993) study with a p-value of 0.0013. The actual significance of this smallest p-value, which is the false positive rate for the min-test, was 0.26 based on the primary analysis, rather than the naive value of 0.0013. This means that 26 % of the randomizations of the 10 datasets gave a smallest p-value less than or equal to the smallest p-value obtained from the original data.

The false positive rate for the 05-test was 0.08, which means that 8 % of randomizations of the 10 datasets found at least 24 sites for which the poly-3 p-value was ≤ 0.05. The results from all permutation tests based on the reduced data were similar to those based on the primary data. The false positive rate for the 01-test was 0.06 in the primary analysis and 0.08 in the reduced analysis. Overall, these findings suggest that, after accounting for the number of statistical tests performed, there was no clear evidence of a positive dose-related trend in tumor occurrence.

The evidence for negative trends is greater than that for positive trends in all analyses. The smallest poly-3 p-value for a negative trend was 0.0008 whereas the smallest p-value for a positive trend was 0.0013. The 01-test for a negative trend was highly significant in both the primary and reduced analyses with a p-value of 0.002 for each. These findings suggest stronger evidence for negative rather than positive dose-response trends in tumor occurrence.

Discussion and conclusions

The highest doses given to any animal groups in the 10 bioassays analyzed were 5,873 mg/kg bw/day and 4,841 mg/kg bw/day in female and male mice, respectively. Despite the extremely high doses, there was no evidence of reduced survival in this study. On the contrary, there was a statistically significantly enhanced survival in male mice in this study, as well as in male animals in several other bioassays. The use of the individual animal data allowed the authors to distinguish between an adenoma and a carcinoma occurring in separate animals and both tumors occurring together in a single animal.

Knowledge of the age at death of each individual animal is required for the conduct of the poly-3 test. In addition to the application of the poly-3 test, which is an age-adjusted Cochran-Armitage test, age was also controlled by keeping the numbers of animals surviving to final sacrifice in each dose group the same in all permutations as in the original data.

In the primary analysis of all bioassays 525 poly-3 analyses were conducted of individual tumor responses, of which a total of 174 were on combinations of individual tumor types that may have similar etiologies. In the primary analysis, individual tumors can appear in more than one poly-3 analysis. Since this will happen in the original data and the permuted data with equal frequency, it will not bias the analysis. Also a reduced analysis was conducted in which individual tumors and combinations of tumors were removed from the analysis if they were part of a more inclusive tumor combination. This reduced analysis involved 304 poly-3 analyses. Results from these two analyses were quite similar.

The smallest poly-3 p-value (0.0013) found in the analysis of all the datasets was that for hemangiosarcoma in male mice in the Atkinson *et al*. study. The analysis showed that the actual false positive rate for this finding after accounting for multiple comparisons was 0.26 in the primary analysis and 0.17 in the reduced analysis. Neither the 05-test nor the 01-test gave a false positive rate that was clearly less than 0.05 in the primary and reduced analysis. The statistically significant ($p = 0.0013$) response of 8 % in CD-1 males in the Atkinson *et al*. study resulted from 4 hemangiosarcomas at a dose level of 1,000 mg/kg bw/day, with no hemangiomas or hemangiosarcomas reported at the 3 lower doses. The CD-1 male mice in the Knezevich and Hogan study were exposed to 4,831 mg/kg bw/day, a dose nearly 5 times that used in the Atkinson *et al*. study with no hemangiomas or hemangiosarcomas. Moreover, the incidence of 8 % was still within the historical control range reported for male CD-1 mice by Atkinson *et al*. (0-8 %) and Giknis and Clifford (2005) (0-12 %). The 10 % incidence in hemangioma/ hemangiosarcoma in female CD-1 mice reported in the Sugimoto (1997) study is within the historical control range of 0-12 % of hemangiosarcoma reported by Giknis and Clifford (2005). The lack of a consistent dose response in either males or females suggests that finding significant responses in hemangioma and hemangiosarcoma in both sexes of mice may be attributable to chance, especially considering that this represents the “worst case” of more than 100 tumor sites/types in these bioassays that could have shown evidence of carcinogenicity.

The only other tumor in the mouse studies that the IARC regarded as being clearly related to glyphosate exposure was the marginally significant increase (0/49, 0/49, 1/50, 3/50) in kidney adenoma in male mice observed in the Knezevich and Hogan study. However, additional step sectioning of kidneys in the dosed and control groups revealed one kidney adenoma in the control group, but no additional kidney tumors in the dosed groups. Since the new data provided did not identify this control animal, this additional tumor could not be taken into account in the analysis of this paper. When this tumor-bearing control animal is taken into account in the trend analysis the trend is not anymore statistically significant, adding to the evidence that the tumor increases reported in the glyphosate studies are due to chance.

Comparing the results of negative dose-related trends with those testing for a positive trend, the evidence for an effect was stronger for negative than for positive trends. The smallest p-value for a positive trend was 0.0013 versus 0.0008 for a negative trend, although the corresponding false positive rates after correcting for multiple comparisons were 0.26 and 0.11 demonstrating how adjusting for multiple comparisons can change the interpretation of analyses of individual tumors. The only clearly significant results for any of the 3 permutation tests were the highly significant 01-tests for negative trend in both the primary and reduced analysis.

In all 10 bioassays, the analysis made in this paper identified 24 tumors that exhibited a poly-3 positive trend with a p-value ≤ 0.05. Nevertheless, after accounting for the multitude of statistical tests this analysis did not find that number statistically significant ($p = 0.08$). The statistical analysis of 10 glyphosate bioassays presented in this paper found no strong statistical evidence that glyphosate is carcinogenic. The main cause for the discrepancy between the analysis made by IARC and that of the authors appears to be that IARC failed to consider the large number of statistical tests performed in the multiple bioassays they reviewed and the resulting multiple comparison problem. IARC and other organizations involved with interpreting results from large data sets to which a large number of statistical

tests have been applied should consider applying analyses of the type used in this paper to make informed and reasonable decisions. The present analysis provides new information on the potential carcinogenicity of glyphosate by being the first to provide results from statistical tests with correct false positive rates. These tests found no strong or convincing evidence that glyphosate is an animal carcinogen.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Ten cancer bioassays of sufficient quality and which allowed the analysis of individual animal data was selected for the application of a multi-response permutation procedure that adjusts for the large number of tumors eligible for statistical testing and provides valid false-positive probabilities. The statistical tests applied in the analysis were functions of p-values obtained from conventional continuity-corrected poly-3 tests for trend applied to each type of tumor or combination of tumor types in each bioassay. Results from 3 multi-response permutation tests are reported and discussed: the "min-test", the "05-test" and the "01-test". In the min-test, the test statistic is the smallest p-value obtained from applying the poly-3 test to all tumor types in all bioassays investigated. Animals are randomly reassigned to dose groups in a Monte Carlo analysis, keeping the total numbers of animals in each dose group equal to the number in the original data. The tumors in each such reassignment are analyzed using the poly-3 test in exactly the same way as in the original data. Males and females are permuted separately. The false positive rate is the proportion of random reassignments that result in a smallest poly-3 p-value that is smaller than or equal to the smallest poly-3 p-value obtained from the original data. The test statistics for the 05-test and the 01-test are the number of poly-3 tests of tumors in the original data for which the p-value is less than or equal to the critical value of 0.05 or 0.01, respectively. In all applications of the poly-3 test, the test is applied only to data from one sex in a single study and the p-values from the poly-3 tests of all the studies are combined to create the "global" tests (min-test, 05-test and 01-test) to give the correct false positive rates. In addition to the randomization procedures for testing for positive dose-response trends in tumor incidence, the same procedures were repeated after reconfiguring the poly-3 test for negative trends. When the frequency of poly-3 p-values for positive trend computed from all tumors in all 10 bioassays in which at least two tumors occurred are considered there is an excess of large p-values (close to 1.0) compared to small p-values (close to 0.0). Since the version of the poly-3 trend test applied is a one-sided test for a positive trend, p-values close to 1.0 would translate into p-values near 0.0 for one-sided trend tests for anti-carcinogenicity. Results of tests for a dose-related decrease in survival in each study show that in none of the bioassays analyzed this test was statistically significant. Moreover, 4 of the datasets had p-values in excess of 0.95 which indicates a significant positive trend in survival with increasing dose. The most significant poly-3 trend in all 10 bioassays was found in the Atkinson *et al.* (1993) study for hemangiosarcoma in male mice with a p-value of 0.0013. The actual significance of this smallest p-value, which is the false positive rate for the min-test, was 0.26 based on the primary analysis, rather than the naive value of 0.0013. This means that 26 % of the randomizations of the 10 datasets gave a smallest p-value less than or equal to the smallest p-value obtained from the original data. Besides, the incidence in hemangiosarcomas (8 %) remained within the historical control range and no such tumors were identified in another mouse study at a dose level nearly 5 times of that used in the Atkinson *et al.* (1993) study. Overall, these findings suggest that, after accounting for the number of statistical tests performed, there was no clear evidence of a positive dose-related trend in tumor occurrence. The 01-test for a negative trend was highly significant with a p-value of 0.002. These findings suggest stronger evidence for negative rather than positive dose-response trends in tumor occurrence. In all 10 bioassays investigated, the analysis made in this paper identified 24 tumors that exhibited a poly-3 positive trend with a p-value of less than or equal to 0.05. Nevertheless, after accounting for the multitude of statistical tests this analysis did not find that number statistically significant ($p = 0.08$). The statistical analysis of 10 glyphosate bioassays presented in this paper found no strong statistical evidence that glyphosate is carcinogenic. This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because state-of-the-art statistical methods were employed to a selected set of cancer bioassays to demonstrate false-positive probabilities.

Reliability criteria for *in vivo* toxicology studies

Publication: Crump *et al*., 2020.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N.A.	
Study performed according to GLP	N.A.	
Study completely described and conducted following scientifically acceptable standards	Y	Statistical re-analysis of 10 selected bioassays for which individual animal data are available.
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Provided in the original bioassays.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	Provided in the original bioassays.
Test conditions clearly and completely described	Y	Provided in the original bioassays.
Route and mode of administration described	Y	Oral *via* the diet.
Dose levels reported	Y	Provided in the original assays. The maximum doses reported are 5,873 mg/kg bw/day and 4,841 mg/kg bw/day in female and male mice, respectively.
Number of animals used per dose level reported	Y	50 – 64 animals per dose group.
Method of analysis described for analysis test media	N	Should be provided in original bioassays.
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	N.A.	Statistical re-analysis of all tumor sites.
Statistical methods described	Y	Application of a multi-response permutation procedure providing valid false-positive probabilities.
Historical control data of the laboratory reported	Y	For some tumors.
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because state-of-the-art statistical methods were employed to a selected set of cancer bioassays to demonstrate false-positive probabilities.		

1. Information on the study

Data point:	CA 5.6
Report author	Ganesan S. *et al*.
Report year	2020
Report title	Absence of glyphosate-induced effects on ovarian folliculogenesis and steroidogenesis
Document No	Reproductive Toxicology, (2020) 96, 156-164 DOI: 10.1016/j.reprotox.2020.06.011
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate (GLY) is an herbicidal active ingredient and both in vitro and in vivo studies suggest that GLY alters ovarian function. To determine if a chronic GLY exposure model affected steroidogenesis or folliculogenesis in vivo, postnatal day 42 C57BL6 female mice were orally delivered vehicle control (saline) or GLY (2 mg/Kg) from a pipette tip five days per week for either five or ten weeks. Mice were euthanized at the proestrus stage of the oestrous cycle. GLY exposure did not impact body weight gain, organ weights, or healthy follicle numbers. In addition, GLY exposure did not affect abundance of ovarian mRNA encoding kit ligand (*Kitlg*), KIT proto-oncogene receptor tyrosine kinase (*c-Kit*), insulin receptor (*Insr*), insulin receptor substrate (*Irs1* or *Irs2*) and protein thymoma viral proto-oncogene 1 (AKT) or phosphorylated AKT. Ovarian mRNA or protein abundance of Star, 3β-hydroxysteroid dehydrogenase (*Hsd3b1*), *Cyp11a1* or *Cyp19a* were also not altered by GLY. Circulating 17β-estradiol and progesterone concentration were unaffected by GLY exposure. In conclusion, chronic GLY exposure for five or ten weeks did not affect the ovarian endpoints examined herein.

Materials and methods

Reagents

Glyphosate (CAS # 1071-83-6), 2-β-mercaptoethanol, Tris base, Tris HCL, Sodium chloride, EDTA, SDS, NaF, HEPES and Tween -20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Ponceau S, Invitrogen iBlot 2NC regular stacks, Pierce BCA protein assay kit, Glycerol, Citric acid, Saline, DAPI nuclear stain and Sodium citrate were obtained from Thermo Fisher Scientific. Mini-PROTEAN TGX™ precast protein gels and protein size markers were purchased from BioRad. RNeasy Mini kit, QIA shredder kit, RNeasy Min elute kit, and Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility (Ames, IA). Antibodies directed against AKT, phosphorylated AKT, CYP11A1, anti-mouse Alexa flour 488 and SignalFire ECL plus chemical luminescence detection kit were from Cell Signaling (Danvers, MA). Antibodies directed against ERα, STAR and donkey anti-rabbit IgG-FITC were from Abcam. Novus Biologicals (Centennial, CO) was the source of antibodies directed against HSD3β and CYP19A1. ELISA kits to measure E_2 and progesterone (P_4) were from DRG International Inc (Springfield, NJ).

Animals

The Iowa State University Institutional Animal Care and Use Committee approved all animal experiments in accordance with NIH guidelines. Postnatal day (PND) 42 C57BL/6 J female mice received vehicle control (saline; n = 20) or glyphosate (GLY; 2 mg/Kg/day; n = 20) per os for five days per week for a duration of 5 (n = 10 control; n = 10 GLY) or 10 weeks (n = 10 control; n = 10 GLY). Mice were housed in groups of 3-4 and were provided with ad libitum access to food and drinking water. Body weight was obtained weekly. Vaginal cytological analysis was performed for 10 days in the 5-week exposure study and for 21 days in the 10-week study prior to euthanasia and the length spent in each stage of the oestrous cycle calculated as a percentage of the time analysed. Mice were euthanized at the proestrus phase of their oestrous cycle. Weights of the ovaries, uterus, liver, kidneys and heart were recorded. Blood samples were collected post-euthanasia by cardiac puncture.

Histological analysis

One ovary per animal (n = 10 per treatment) was fixed in 4 % paraformaldehyde for 24 h and transferred to 70 % ethanol and paraffin embedded. Ovaries were sectioned (5 μM thickness) and every 6th section was mounted and stained by hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 12th section. Unhealthy follicles were distinguished from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Follicles were classified and enumerated as previously described (Flaws *et al*., 1994).

Quantitative reverse transcriptase polymerase chain reaction

Approximately 25 % of each ovary was used for RNA isolation and two ovarian samples were combined (n = 10 ovaries; 5 RNA samples). RNA was isolated using a RNeasy Mini kit and the RNA concentration determined using a Nano Drop spectrometer (λ = 260/280 nm; ND 1000; Nanodrop Technologies Inc., Wilmington, DE). RNA (200 ng) was reverse transcribed to cDNA, followed by quantitative PCR. Primers for specific genes of interest were designed by Primer 3 Input Version (0.4.0) (listed in Table 1). The regular cycling program consisted of a 15 min hold at 95 °C and 45 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s at which point data were acquired. Each sample was normalized to 18 s RNA before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Table 1: Primer sequences

Gene	Forward Primer	Reverse Primer
c-Kit	ttctccttaggaagcagccc	cctgcttgaatgttggcctt
Kit-lg	tcagtcatagattggagtttgca	tgtatcaaaagggtcgggaca
Akt	tttgttgctgtgtcccatgc	caagtgctaggagaagggct
Star	atgttcctcgctacgttcaag	cccagtgctctccagttgag
Hsd3b1	gctggaaactgtgagcttcc	tgcttcctcccagttgacaa
Cyp11a1	aggtccttcaatgagatcccctt	tccctgtaaatggggccatac
Cyp19a1	atgttcttggaaatgctgaaccc	aggacctggtattgaagacgac
ERa	aattctgacaatcgacgccag	gtgcttcaacattctccctcctc
Erb	tgctccaagggtaggatggac	ctgtgcctcttctcacaagga
Insr	tgtcatcaatgggcagtttg	atcaggttccgaacagttgc
Irs1	ctatgccagcatcagcttcc	ggaggatttgctgaggtcat
Irs2	gaagcggctaagtctcatgg	gacggtggtggtagaggaaa

Western blot analysis

Ovaries (n = 10 per treatment) were homogenized in tissue lysis buffer (containing protease and phosphatase inhibitors) and centrifuged at 10,000 rpm twice for 15 min. The protein concentration of the supernatant was measured using a bicinchoninic acid assay and stored at -80 °C until further use. Protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5 % milk in Tris-buffered saline containing tween 20. Membranes were incubated in primary antibodies directed against protein kinase B (AKT; primary - 1:200; secondary - 1:500), phosphorylated AKT (pAKT; primary - 1:200; secondary - 1:200), estrogen receptor alpha (ERα; primary - 1:500; secondary - 1:500), estrogen receptor beta (ERβ; primary - 1:500; secondary - 1:200), steroidogenic acute regulatory protein (STAR; primary - 1:500; secondary - 1:200); 3-β hydroxysteroid dehydrogenase (HSD3β; primary - 1:1000; secondary - 1000), cytochrome P450 isoform 11A1 (CYP11A1; primary - 1:500; secondary - 1:500) and cytochrome P450 isoform 19A1 (CYP19A1; primary - 1:500; secondary - 1:500) overnight at 4 °C with rocking. Following three washes in TTBS (1X) membranes were incubated with species-specific secondary antibody for 1 h at room temperature. Autoradiograms were developed on X-ray films in a dark room following 10 min incubation of membranes with 1X SignalFire™ ECL reagent. Densitometry of the appropriate-sized bands was measured using Image Studio Lite Version 3.1 (LI-COR Biosciences, Lincoln, NE) which eliminates background noise. The sum of the gray values of all the pixels in the selection divided by the number of pixels, or mean grey value was quantified for each membrane using ImageJ software. Membranes were normalized to Ponceau S protein staining in which the entire lane of the transferred protein was quantified to account for loading variation. To ensure antibody specificity, negative control blots for each antibody used were performed in which the membranes were incubated with primary antibody only, secondary antibody only, or normal IgG in place of primary antibody with the inclusion of the appropriate secondary antibody. No protein bands were observed on these control blots indicating the specificity of the protein bands detected and analysed.

Immunofluorescence staining

Paraffin embedded ovaries (n = 5 per treatment) from the 10 week exposure group were serially sectioned (5 μM thick) and every 10^{th} section was mounted. Sections were deparaffinised in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1 M, pH 6.1). Sections were blocked in 5 % BSA for 1 h at room temperature. Sections were incubated with primary antibodies directed against pAKT (1:50 dilution), STAR (1:500 dilution), HSD3β (1:500 dilution), CYP11A1 (1:100 dilution), CYP19A1 (1:100 dilution) and ERα (1:200 dilution) overnight at 4 °C. After washing in 1 % PBS, sections were incubated with the appropriate donkey anti-rabbit IgG-FITC (1:200 to 1:500 dilutions) or anti-mouse Alexa flour 488 (1:200 dilution) secondary antibody for 1 h. Slides were counterstained with 4-6-diamidino-2-phenylindole (DAPI) for 5 min. Negative technical controls incubated the ovarian sections omitting both the primary and secondary antibody to ensure specificity of the antibodies. Images were captured using a Leica DMI3000 B fluorescent microscope. Raw internal density of staining was analysed using Image J software. For analysis, 5 ovaries per treatment were assessed with three sections per ovary and 7-10 large follicles per slide analysed.

Steroid hormone quantification

Serum (25 μL; n = 10 per treatment) was added in duplicate per sample to an ELISA plate to measure E_2 or P_4. Plates were incubated for 60-90 min after adding the enzyme conjugate (100-200 μl). The wells were rinsed with wash solution three times, substrate solution added (100-200 μL) and incubated for 15-30 min. The enzymatic reaction was stopped by adding stop solution (50-100 μL) and the signal determined within 10 min using a plate reader at 450 nm absorbance.

Sample identity blinding

The identity of samples used for ELISA and follicle counting were not known to the investigator performing the analysis. In addition, mRNA for PCR and protein samples from mice treated for 10-weeks for western blotting were double blinded - the identity of the samples was unknown to the investigator and after western blots were completed, they were then identified by group for the purpose of statistical analysis. Only after the statistical analysis was completed was group identity revealed.

Statistical analysis
Raw data were analysed by unpaired t-test. All statistical analysis was performed using Prism 5.04 software (GraphPad Software). Statistical significance (*) was defined as $P < 0.05$. A tendency for a statistically meaningful difference was considering if the *P*-value was between 0.05-0.1

Results

Impact of GLY exposure on body weight gain
Body weight gain was determined from the onset to the completion of the dosing period which was either a duration of 5 or 10 weeks. As anticipated, mice gained weight with aging, however, there was no impact of GLY exposure on body weight gain at either time point (Figure 1).

Effect of GLY exposure on the estrous cyclicity
In mice exposed to GLY for either duration, no impact of GLY exposure on the percentage time spent at any stage of the oestrous cycle was observed, though there was a tendency ($P < 0.1$) for increased time spent in estrus and reduced time spent in metestrus/diestrus after 5 weeks of GLY exposure (Figure 2).

Endocrinological impact of GLY exposure
Mice were euthanized at the proestrus stage of the oestrous cycle, thus E_2 (Figure 3A) levels were higher than P_4 (Figure 3B). There was no effect of 5 or 10 weeks of GLY exposure on circulating E_2 (Figure 3A). No impact of GLY exposure on serum P_4 was noted at either timepoint (Figure 3B).

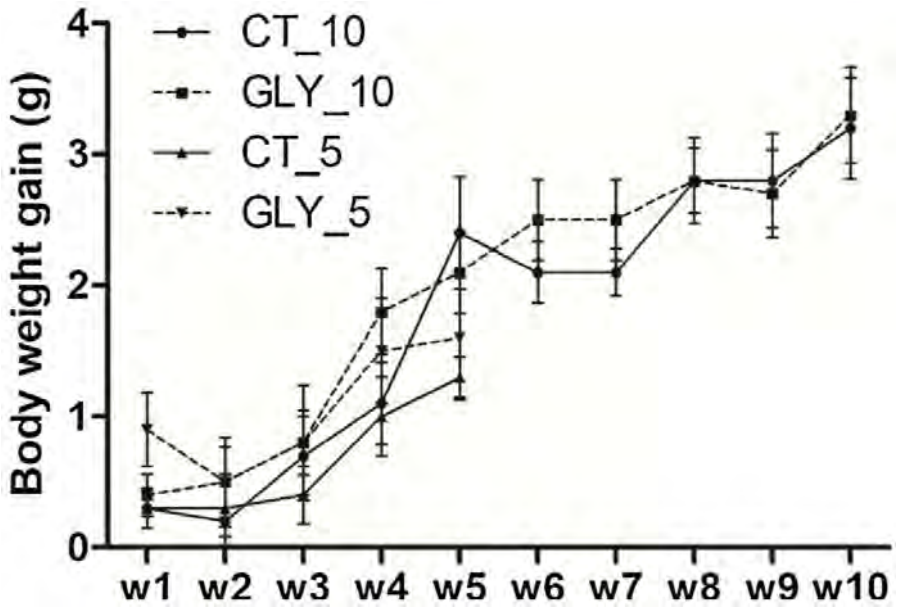
Relative organ weight effects of GLY exposure
There was no effect of either duration of GLY exposure on the relative weight of the heart (Figure 4A,F), liver (Figure 4B,G), kidneys (Figure 4C,H), or uterus (Figure 4D,I). In addition, neither 5 weeks (Figure 4E) or 10 weeks of GLY exposure (Figure 4J) affected ovarian weight, although a tendency ($P < 0.1$) towards a reduction in ovarian weight after 10 weeks of exposure was observed.

Impact of GLY exposure on ovarian follicle number
GLY exposure for 5 (Figure 5A) or 10 weeks (Figure 5B) did not influence the number of ovarian primordial, primary, secondary or antral follicles. There was also not a different between CT and GLY treated mice at either 5 or 10 weeks in total follicle number or the percentage of follicles per developmental stage within the ovary. Corpora lutea were evident, thus, there were not a defect in ovulation noted and there was no different in the number of CL per ovary due to GLY exposure (data not shown and Figure 5C-F).

Effect of GLY exposure on ovarian mRNA encoding genes involved in ovarian folliculogenesis and steroidogenesis
GLY exposure did not impact abundance of ovarian mRNA encoding the PI3K members kit ligand (*Kitl*), KIT proto-oncogene receptor tyrosine kinase (*c-Kit*), or protein kinase b (*Akt*) after 5 (Figure 6A) or 10 (Figure 6D) weeks. GLY exposure also did not alter ovarian mRNA abundance of steroidogenic acute regulatory protein (*Star*), 3β-hydroxysteroid dehydrogenase (*Hsd3b*), cytochrome P450 (*Cyp*) *11a1* and *Cyp19a* after 5 (Figure 6B) or 10 weeks (Figure 6E) of exposure. In addition, there was no effect of GLY exposure on mRNA encoding the ovarian insulin receptor (*Insr*), insulin receptor substrate 1 (*Irs1*) or *Irs2*, estrogen receptor (*Er*) alpha (*Erα*) or *Er* beta (*Erβ*) mRNA abundance at either 5-weeks (Figure 6C) or 10-weeks (Figure 6F) post-exposure.

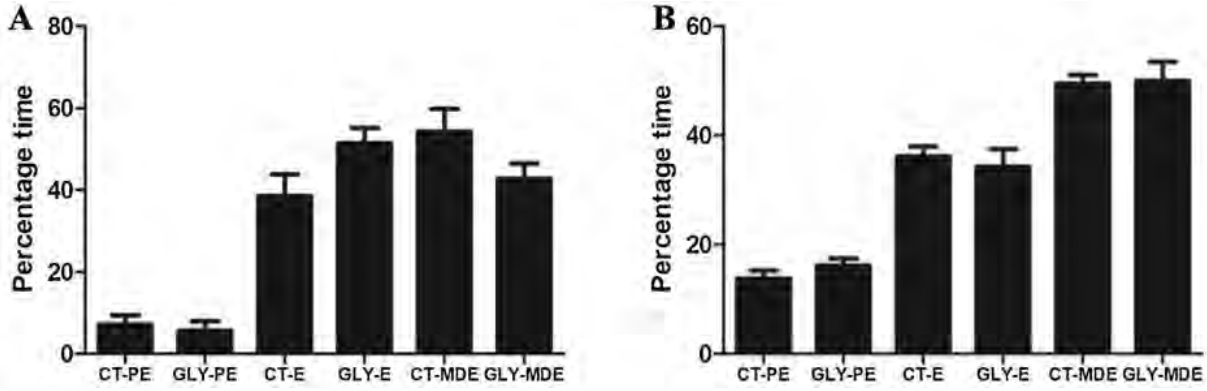
GLY exposure impact on ovarian folliculogenesis and steroidogenesis protein abundance
Using western blot analysis to interrogate changes to ovarian proteins involved in folliculogenesis or steroidogenesis, no impacts of 5 (Figure 7A,B) or 10 (Figure 7C,D) weeks of GLY exposure on ERα, ERβ, AKT, pAKT, STAR, HSD3β, CYP11A1, or CYP19A1 protein abundance were detected. Use of immunofluorescence staining in ovaries from the 10-week exposure group identified localization of pAKT (Figure 8A-C), STAR (Figure 8D-F), HSD3β (Figure 8G-I), CYP11A1 (Figure 8J-L), CYP19A1 (Figure 8M-O) and ERα (Figure 8 P–R) but, with the exception of a tendency for a reduction in HSD3B and CYP19A1 ($P < 0.1$); Figure 9A,B). there was no difference between saline- and GLY-treated mice in the internal density of immunostaining for these proteins.

Figure 1: Effect of glyphosate on body weight



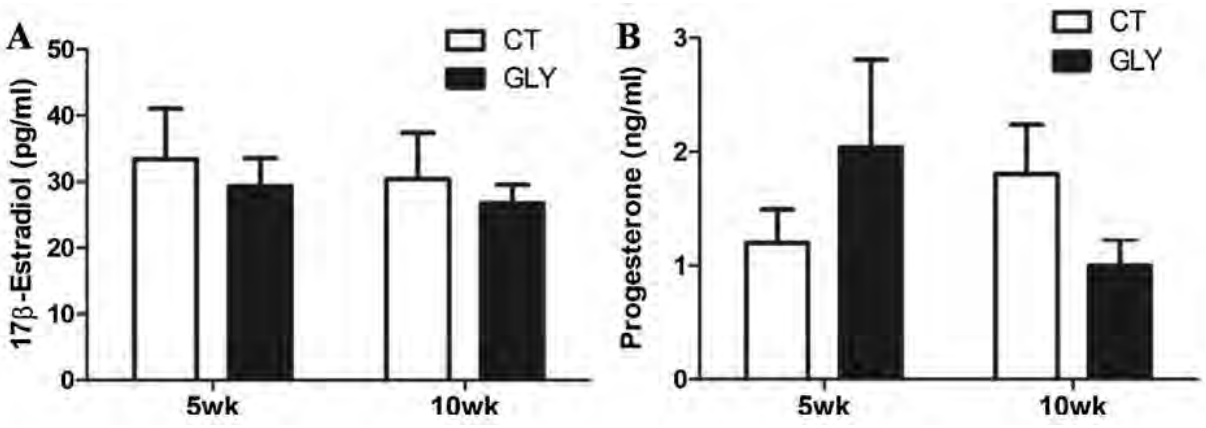
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for 5 or 10 weeks. Body weight was measured from day 0 of dosing to end of 5 or 10 weeks of dosing. Values are expressed as average body weight gained every week ± SEM; n = 10. Mice exposed to saline (CT) for 5 weeks indicated by solid line with triangle data point marker; Mice exposed to GLY for 5 weeks indicated by dashed line with triangle data point marker; Mice exposed to saline (CT) for 10 weeks indicated by solid line with circle data point marker; Mice exposed to GLY for 10 weeks indicated by dashed line with square data point marker.

Figure 2: Effect of GLY exposure on percentage time spent at stages of oestrous cycle



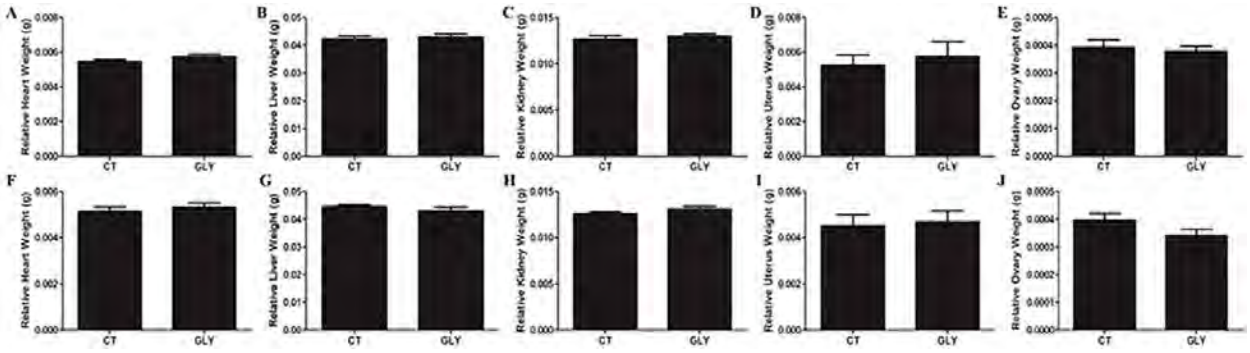
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A) 5 or (B) 10 weeks. The percentage time spent at stages of oestrous cycle were calculated: proestrus = PE; oestrus = E; metestrus and diestrus = MDE. Data points represent mean +/− SEM. Statistical analysis was performed on raw data.

Figure 3: GLY exposure effect on circulating E_2 and P_4



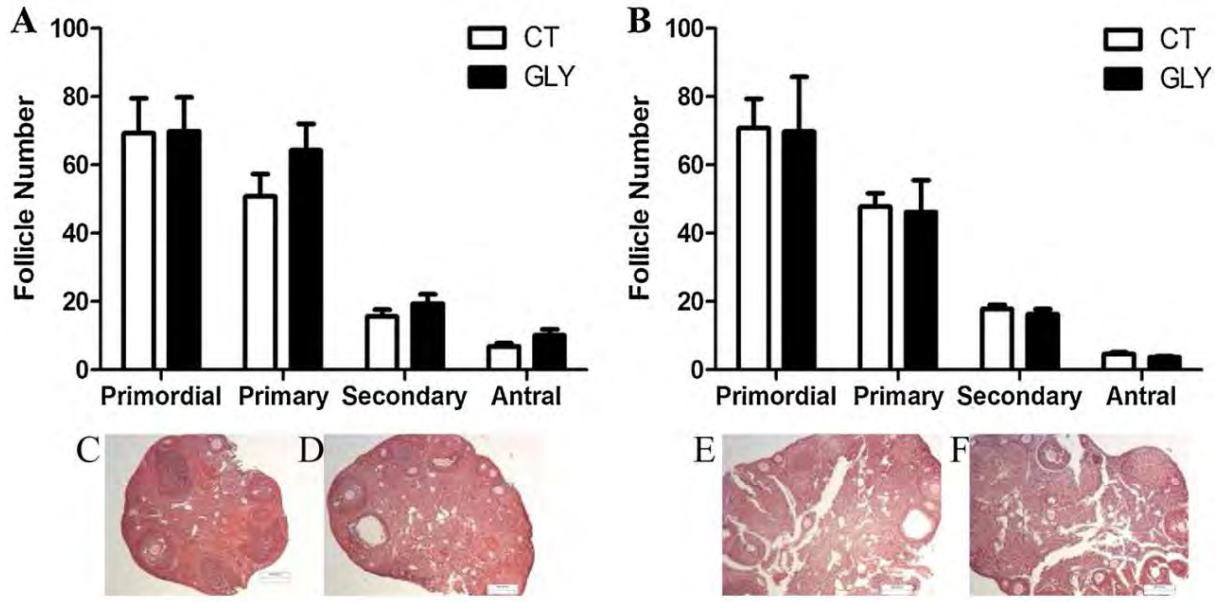
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for 5 or 10 weeks. Circulating (A) E_2 and (B) P_4 were measured by ELISA. Data points represent mean +/- SEM.

Figure 4: Relative organ weight impacts of GLY exposure



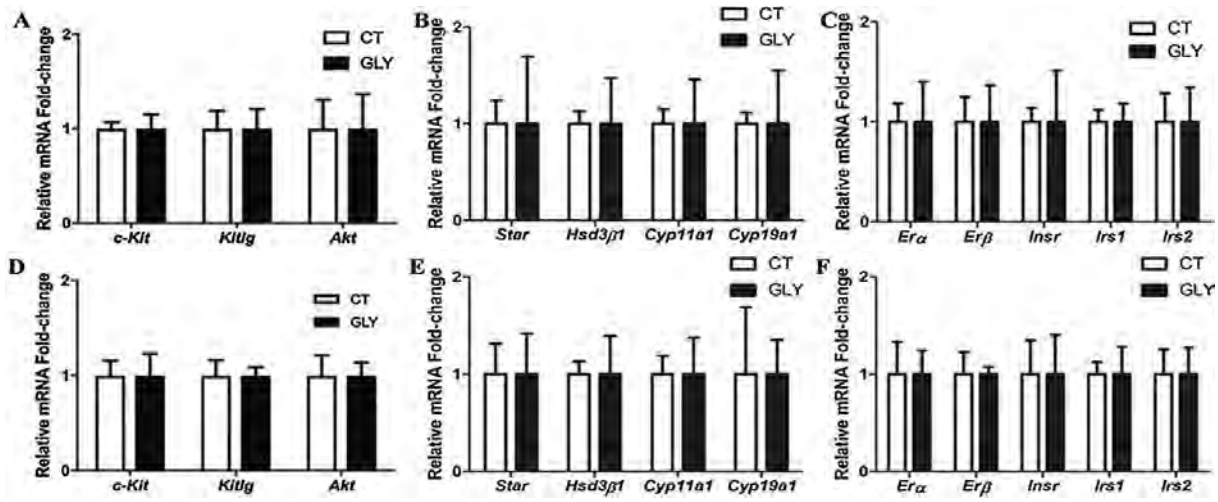
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A-E) 5 or (F-J) 10 weeks. Organ weights (g) were collected post-euthanasia and normalized to body weight: (A,C) Heart, (B,G) Liver, (C,H) Kidney, (D,I) Uterus and (E,J) Ovary weight. Data points represent mean +/- SEM.

Figure 5: Effect of GLY exposure on ovarian follicle number



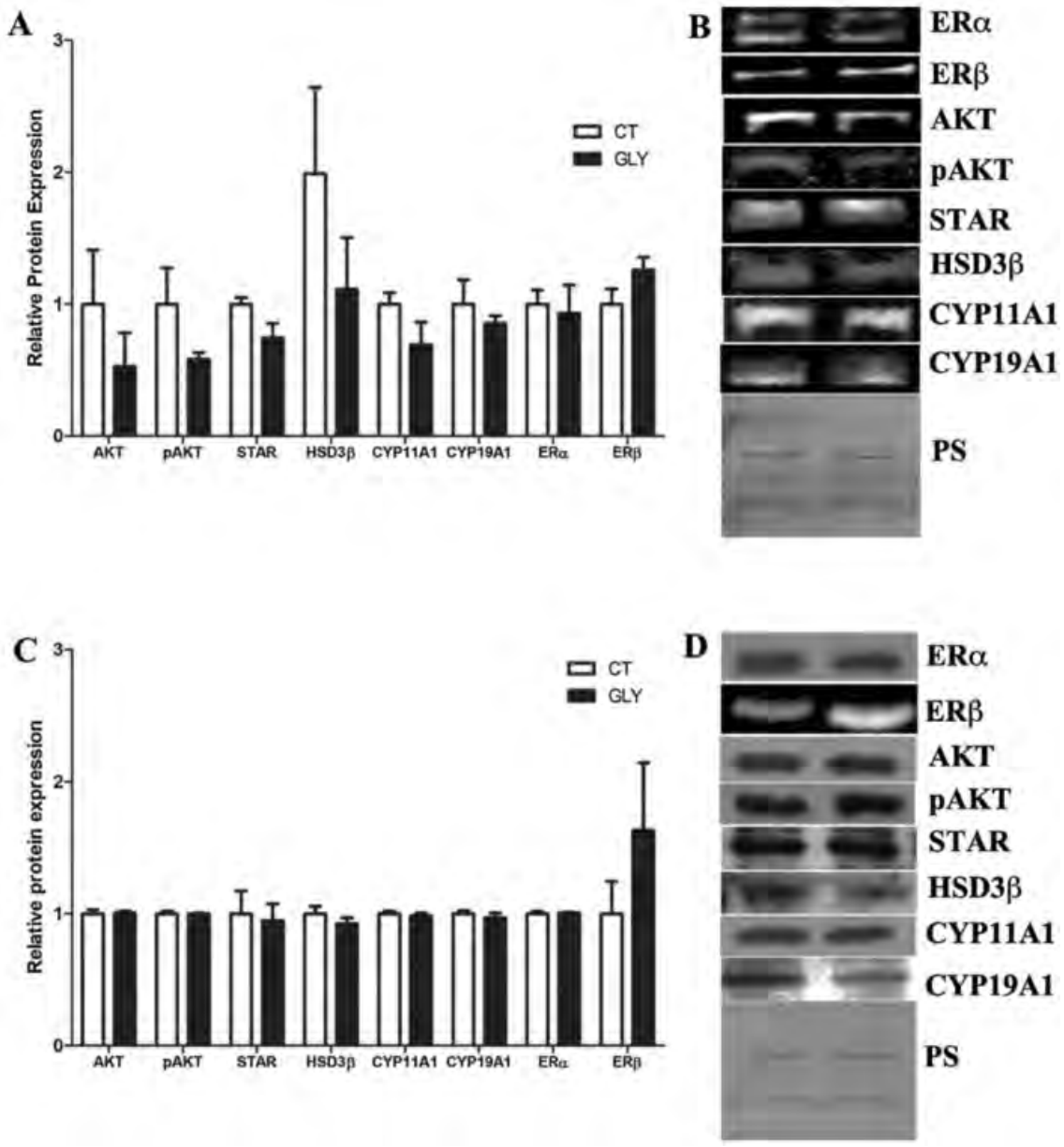
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A) 5 or (B) 10 weeks. Ovaries were sectioned, stained with haemotoxylin and eosin and follicular stages classified and counted as primordial, primary, secondary, and antral. Data points represent mean +/- SEM. Representative (C) CT 5 week, (D) GLY 5 week, (E) CT 10 week and (F) GLY 10 week ovaries are provided.

Figure 6: Impact of GLY exposure on ovarian mRNA abundance



Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A-C) 5 or (D-F) 10 weeks. Ovaries were homogenized and mRNA isolated for qRT-PCR to analyse relative abundance of genes involved in (A,D) PI3K signaling; (B,E) Steroidogenesis; and (C,F) Endocrine signaling. Data points represent fold change relative to CT treated ovaries +/- SEM.

Figure 7: Consequence of GLY exposure on ovarian protein abundance



Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A,B) 5 or (C,D) 10 weeks. Ovaries were homogenized and western blotting performed to quantify protein abundance of AKT, pAKT, STAR, HSD3B, CYP11A1, CYP19A1, ERA, and ERB. (A,C) Bars represent mean +/- SEM. (B,D) Representative images of western blots for each protein with ponceau S staining represented as PS.

Conclusions

Taken together, this study determined absence of GLY-induced alterations to the reproductive endpoints measured. After both 5 and 10 weeks of GLY exposure, relative to the saline vehicle control treated mice, any impacts of GLY on endpoints measured was largely absent. This study involved sample deidentification to remove any unintentional or perceived bias, was chronic in nature and performed at a conservative dosage (relevant to human exposure). In summary, the findings of the current study do not support that chronic, oral GLY at a dose considered non-hazardous alters oestrous cyclicity, follicle number, circulating ovarian steroid hormone levels and ovarian intracellular signalling in adult female mice.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In vivo study on post natal day 42, glyphosate administered to C57BL/6 J female mice at 0 or 2 mkd (10 mice/dose). Cyclicity, follicle number, circulating ovarian steroid hormone levels and ovarian intracellular signaling parameters were tested in adult female mice during 5 or 10 weeks. Glyphosate exposure for five or ten weeks did not affect the ovarian and endocrine endpoints examined.

The article is classified as reliable with restrictions for the following reasons: only 1 dose tested (no dose relationship can be evaluated), purity of glyphosate is not clear, method of analysis for analysis test media & no validation of the analytical method was described, no GLP status stated, no OECD guideline followed. Although no HCD were available in order to compare with the equivalent concurrent controls and test groups results, the results were negative and therefore did not require HCD to interpret or provide context to any findings.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies		
Publication: Ganesan S. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	Not stated
Study completely described and conducted following scientifically acceptable standards	Y	Use of control group, 10 female mice/group, 1 dose + 1 negative control, statistical analysis performed, no randomisation of animals mentioned, no HCD
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate No Purity, no storage condition stated
Only glyphosate acid or one of its salts is the tested substance	N	Glyphosate
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	C57BL/6 J mice, age, origin stated but initial BW not included
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Per os
Dose levels reported	Y	0 and 2 mg/kg/d; 5 days/week for 5 and 10 weeks
Number of animals used per dose level reported	Y	10 mice/group 5 weeks (n = 10 control; n = 10 GLY) and 10 weeks (n = 10 control; n = 10 GLY)
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	

Reliability Criteria: *In Vivo* Toxicology Studies		
Publication: Ganesan S. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	Only 1 dose tested
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	only 1 dose tested (no dose relationship can be evaluated), purity of glyphosate is not clear, method of analysis for analysis test media & no validation of the analytical method was described, no GLP status stated, no OECD guideline followed. Although no HCD were available in order to compare with the equivalent concurrent controls and test groups results, the results were negative and therefore did not require HCD to interpret or provide context to any findings.
Not reliable	N	

1. Information on the study

Data point:	CA 5.8.3
Report author	Gastiazoro M.P. *et al.*
Report year	2020
Report title	Glyphosate induces epithelial mesenchymal transition-related changes in human endometrial Ishikawa cells via estrogen receptor pathway
Document No	Molecular and cellular endocrinology, (2020), Vol. 510, Art. No. 110841
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

Ishikawa cells were exposed to glyphosate (Gly) (0.2 μM and 2 μM) or 17β-estradiol (E2: 10^{-9} M). Gly increased cell migration and invasion ability compared to vehicle, as did E2. Moreover, a down regulation of E-cadherin mRNA expression was determined in response to Gly, similar to E2-effects. These results show that Gly promotes epithelial mesenchymal transition (EMT)-related changes in Ishikawa cells. When an ER antagonist (Fulvestrant: 10^{-7} M) was co-administrated with Gly, all changes were reversed, suggesting that Gly might promote EMT-related changes via ER-dependent pathway. The results are interesting evidences of Gly effects on endometrial cancer progression via the ER-dependent pathway.

Materials and methods

Substances

All reagents and chemicals were of analytical grade. Glyphosate (CAS N° 1071-83-6) used as the PESTANAL® analytical standard (purity grade ≤ 100%) and 17β-estradiol (E2; purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Fulvestrant (ICI 182,780; purity ≥ 99%) was purchased from Tocris Bioscience (Minneapolis, Minnesota, USA).

Cell culture

The human endometrial adenocarcinoma cell line Ishikawa was provided by Masato Nishida, Department of Obstetrics and Gynecology, University of Tsukuba. These cells were obtained from an endometrial adenocarcinoma of a 39-year-old woman in 1985 by Nishida, and established as ER- and PR-positive cell line. The cells were cultured in Dulbecco's modified Eagle's medium F12 (DMEM/F12) (Biowest, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% Insulin-Transferrin-Selenium A (ITS) (Gibco-BRL, Grand Island, NY, USA) and maintained in culture 75 cm^2 flask at 5% CO2 and 37 °C. The medium was replaced every 48 h. Ishikawa cells were grown to 80% of confluence and enzymatically detached by trypsin and (0.05%) EDTA at 37 °C.

Treatment conditions

The concentrations in the experiment were chosen based on the reference dose (RfD) of 1 mg of glyphosate/kg b.w. per day established by U.S. EPA and it was in a range between 0.2 μM and 200 μM (0.2 μM, 2 μM, 20 μM and 200 μM), an interval of concentrations that include the RfD. A 1 mM Gly stock solution was prepared with ethanol and different volumes were added during the treatment to achieve the corresponding Gly concentration. In detail, to final Gly concentration 0.2 μM in a 6-well plate with 2 ml media per well, 0.4 μl of Gly stock solution was added; to final Gly concentration 2 μM was added 4 μl; to

final Gly concentration 20 µM was added 40 µl of Gly stock solution and to final Gly concentration 200 µM was added 400 µl of Gly stock solution. As a positive control estradiol (E2) at 10^{-9} M was used. ER antagonist Fulvestrant was tested at concentration of 10^{-7} M. Dimethylsulfoxide (DMSO) was used as vehicle for E2 and Fulvestrant and added in a way that the DMSO concentration in the test did not exceed 0.1%. Ethanol was used as vehicle for Gly and added in a way that ethanol concentration in the test did not exceed 2%. No difference between DMSO and ethanol was detected among assays. For that reason, vehicle is shown as a unique condition in the results. To carry out the treatments, the cells were seeded in a 6-well plate for 24 h to establish adherent monolayer after that the corresponding drug-treatment was added depending on the assay. The medium with the corresponding amount of Gly, E2, or Fulvestrant was renewed every 24 h.

Trypan blue dye exclusion assay

The authors determined the viability of Ishikawa cells after treatment with selected concentrations of Gly by trypan blue dye exclusion assay. The aim was to determine whether the assessed concentrations altered cell viability in a significant way. Four concentrations of Gly were tested, Gly 0.2: 0.2 µM, Gly 2: 2 µM, Gly 20: 20 µM and Gly 200: 200 µM. The exclusion criterium was to reject the concentrations that cause a viability ≤80% and show statistical significant difference respect to vehicle.

Scratch-wound healing assay

The Ishikawa cells were seeded in 6-well plates at density of 200,000 cells/well with DMEM/F12 - FBS 10% - ITS 1% medium and incubated at 5% CO_2 and 37 °C with vehicle, E2 or different Gly concentrations with/without addition of Fulvestrant for 24 h. Then the monolayers were scratched using a sterile 200 µl micropipette tip, and washed with PBS twice to remove the detached cells. The different treatments were renewed and the DMEM/F12 - FBS 10% - ITS 1% medium was replaced with DMEM/F12 - FBS 1% - ITS 1% medium. The cells were incubated during a 72-h period, renewed the media and treatments every 24 h. The 72-h period was selected based on the frame time of incubation on which only under optimal condition (positive control) be close to closure the scratch. In order to measure the percentage of wound closure area by migrating cells, 8–10 images (from each treatment group) of the wounded cell monolayers were taken at 0 and 72 h after scratching at 40X using an Olympus CK40 Inverted Microscope (Olympus, Japan) coupled with a camera Canon Power Shot G9 (Canon, Japan). The average area of wound covered was calculated as percentage of wound closure after 72 h = (uncovered area at 0 h – uncovered area at 72 h)/uncovered area at 0 h x 100. The uncovered areas were determined using Image J software. The results were expressed as % wound closure after 72 h relative to vehicle.

Transwell invasion assay

The invasion assay was conducted using Falcon cell culture insert 8 µm pore size placed into a 24-well plate. The inserts were uniformly coated with Matrigel® basement membrane matrix (Corning Life Science, U.S.) for 1 h at 37 °C before cells were added. Cells were treated with vehicle, E2 or different Gly concentrations with/without addition of Fulvestrant for 24 h and then, harvested by trypsinization. Cells (1 × 105 cells/well) were seeded into the upper chamber in 200 µl of DMEM/F12-FBS 1% - ITS 1% medium, while the bottom of the chamber was incubated with 750 µl DMEM/F12-ITS 1% medium containing FBS 20% as a chemoattractant. After 48 h of incubation, cells migrating from the top chamber to the lower surface of the insert, as a result of cell invasion through Matrigel®, were fixed 3 min with formaldehyde 4% and 20 min with methanol. Finally, the cells were stained 20 min with crystal violet 0.1%. To quantify the number of invasive cells, all the slides with stained cells were photographed at 40X using an Olympus CK40 Inverted Microscope (Olympus, Japan) coupled with a camera Canon Power Shot G9 (Canon, Japan). Images were analysed using the cell counting tool in Image J software. The number of invasive cells after 48 h was determined. The results were expressed as number of invasive cells after 48 h relative to vehicle.

RNA extraction, reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

The Ishikawa cells were seeded in 6-well plates with DMEM/F12 - FBS 10% - ITS 1% medium and

incubated at 5% CO_2 and 37 °C with vehicle, E2 or different Gly concentrations with/without addition of Fulvestrant for 24 h. Total RNA from cultured cells was isolated after treatment using Tri-Fast™ (Peqlab VWR, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed by enzymatic digestion (RQ1 DNase, Promega, U.S.) and checked by PCR. First-strand cDNA synthesis was performed by mixing 2 µg of digested RNA with MMLV reverse transcriptase (Promega, U.S.) and Oligo (dT) 12–18 primers (Eurofins MWG Operon, Germany). Quantitative real-time PCR was applied for cDNA amplification with SybrGreen I as the detection dye using the iCycler iQ™ Real-Time PCR Detection System (BioRad, U.S.). Product purity was confirmed by dissociation curves and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, yielding no consistent amplification. To quantify the expression relative to the vehicle-treated cells, the ΔΔCT method was used. The expression of E-cadherin and Vimentin were normalized to the housekeeping gene ribosomal protein S18 (Rps 18). All PCR reactions were conducted from three independent cell culture experiments.

Statistical analysis

Data are presented as the mean ± SEM of three independent experiments. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls comparison test using the Graph-Pad Prism software Version 5.03 (San Diego, CA, USA). P-values < 0.05 were regarded as statistically significant.

Results

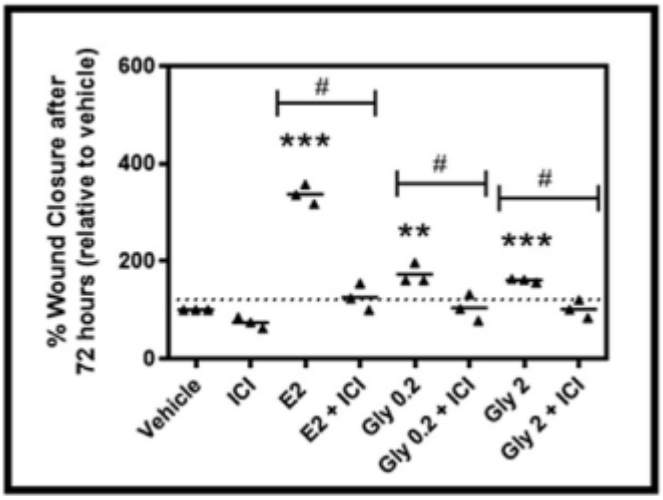
Effects of Glyphosate on cell viability of Ishikawa endometrial cancer cell

The two highest tested concentrations (20 µM and 200 µM) showed statistical significant difference respect to vehicle and the cell viability was lower than 80%. For that reason, these two concentrations were excluded. Contrary, 0.2 µM and 2 µM did not show differences respect to vehicle and the cell viability was kept above 80%. Based on these results, it was ensured that the cell survival is not altered by the two lower concentrations and it was decided to perform all the experiments with 0.2 µM and 2 µM.

Glyphosate induced Ishikawa endometrial cancer cell migration via estrogen receptor pathway

Migratory activity of Ishikawa cells was measured by scratch-wound healing assay. Fig. 1 shows the effects of Gly exposure on the percentage of wound closure after 72 h (relative to vehicle) evidencing that Gly 0.2 µM and Gly 2 µM promoted the migration of Ishikawa cells as did E2. In addition, there was no significant difference between Gly 0.2 µM and Gly 2 µM, for that reason migratory effect no dose dependency could be revealed. After Ishikawa cells were treated with E2 plus Fulvestrant or Gly plus Fulvestrant, the percentage of wound closure after 72 h did not change Fig. 1. In other words, the E2 or Gly effects were reversed by Fulvestrant.

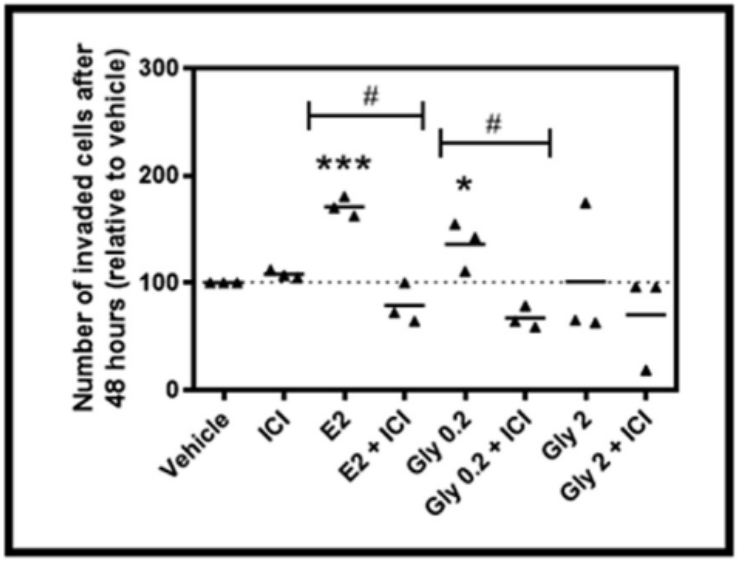
Figure 1: Effects of Gly and Fulvestrant treatment on migration ability of Ishikawa endometrial cancer cell. Results are presented as percentage of qound closure after 72 h relative to the vehicle control. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.



Glyphosate induces Ishikawa endometrial cancer cell invasion via estrogen receptor pathway

To investigate the invasiveness of Ishikawa cells, transwell invasion assay was conducted. Fig. 2 shows the effects of Gly exposure on the number of invaded cells compared to vehicle, evidencing that Gly 0.2 µM promoted the invasion of Ishikawa cells as did E2. Gly 2 µM did not show statistical difference. After Ishikawa cells were treated with E2 plus Fulvestrant or Gly plus Fulvestrant, the number of invasive cells after 48 h did not change, indicating that Fulvestrant attenuated the Gly 0.2 µM and E2 effects.

Figure 2: Effects of Gly and Fulvestrant treatment on invasion ability of Ishikawa endometrial cancer cell. Invasion ability was evaluated by the Transwell Invasion Assay with Matrigel®. The results are expressed as number of invasive cells after 48 h and presented as a percentage of the vehicle. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

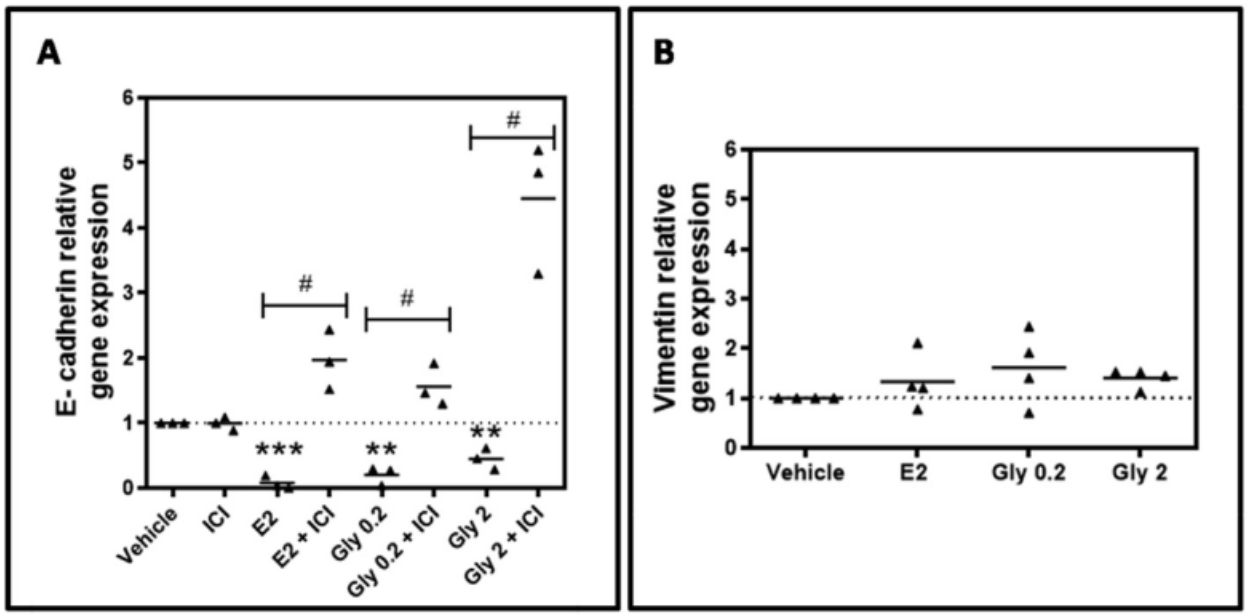


Glyphosate down-regulates E-Cadherin mRNA expression in Ishikawa endometrial cancer cells via estrogen receptor pathway

E-cadherin and vimentin mRNA expression was determined 24 h after the application of different concentrations of Gly or E2 (10^{-9} M). Fig. 3A shows that Gly down regulated E-cadherin mRNA expression

of Ishikawa cells, as did E2. Gly 0.2 μM produced a more pronounced effect on down regulation of E-cadherin mRNA expression in comparison with Gly 2 μM (Fig. 3A). Vimentin mRNA expression showed no changes with any of the treatments (Fig. 3B). After combinatorial treatment with E2 plus Fulvestrant or Gly plus Fulvestrant, E-cadherin mRNA expression was restored and even up regulated respect to the vehicle (Fig. 3A). This up regulation has higher potency to Gly 2 μM than E2 and Gly 0.2 μM.

Figure 3: Effects of Gly and Fulvestrant treatment on E-Cadherin gene expression (A) of Ishikawa endometrial cancer cell. Gly treatment effects on Vimentin gene expression (B) in Ishikawa endometrial cancer cell. Relative gene expression was quantified by RT-qPCR. The samples were normalized to the housekeeping gene RPS18. Data expressed as mean ± SEM. *p<0.05, **<0.01, ***p<0.001.



Conclusions

The results show that Gly promotes EMT process through the down regulation of E-Cadherin and increase cell migration and invasion abilities. Moreover, all changes could be prevented by the application of Fulvestrant (ER antagonist). According to our knowledge, these are the first pieces of evidence showing Gly effects on endometrial cancer cell progression via the ER-dependent pathway. The findings, in accordance with others, suggest that Gly might increase the risk of aggravating the disease for cancer patients.

Further studies are needed to shed light on the in vivo effects of Gly on the EMT process and cancer metastasis to contribute to the knowledge about carcinogenic potential of the herbicide.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Ishikawa endometrial cancer cells were treated with glyphosate at 0.2 µM and 2 µM. Glyphosate caused cell migration, invasion ability and down regulated E-cadherin mRNA expression. 17ß-estradiol, which was included as a positive control caused similar epithelial mesenchymal transition related changes, while treatment with fulvestrant (estrogen receptor antagonist) reversed the effects caused by glyphosate. The findings suggest that glyphosate has the ability to trigger the estrogen receptor-dependant pathway.

The relevance to human health risk assessment of this unvalidated *in vitro* research model in an immortal adenocarcinoma cell line containing estrogen and progesterone receptors is not clear. The results contradict a number of higher tier studies conducted across a variety of test systems,

The article is classified as reliable with restrictions for the following reason : glyphosate was tested at two different concentrations only, no test guideline was used and no historical control data were provided in order to compare with the equivalent concurrent controls and test groups results. Further, the study was not performed according to GLP.

Assessment and conclusion by RMS:

Reliability criteria for *in vitro* toxicology studies		
Publication: Gastiazoro M.P. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	Not stated
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate (purity ≤ 100%)
Only glyphosate acid or one of its salts is the tested substance	N	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Ishikawa cells
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	Not necessary
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.2 µM and 2 µM
Cytotoxicity tests reported	Y	Viability was assessed with tryptan blue dye exclusion assay
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	Y	
Dose-effect relationship reported	Y	

Reliability criteria for *in vitro* toxicology studies		
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	Glyphosate was tested at two different concentrations only, no test guideline was used and no historical control data were provided in order to compare with the equivalent concurrent controls and test groups results. Further, the study was not performed according to GLP.
Not reliable	N	

1. Information on the study

Data point:	CA 5.2.6
Report author	Lindberg T. *et al.*
Report year	2020
Report title	An integrated transcriptomic- and proteomic-based approach to evaluate the human skin sensitization potential of glyphosate and its commercial agrochemical formulations
Document No	Journal of proteomics, (2020) Vol. 217, pp. 103647
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

The objective of this study was to perform *in vitro* sensitization testing with a parallel MS-based proteomic approach to investigate the underlying cellular mechanisms induced. The experimental setup of the *in vitro* assay GARD™skin was used to stimulate cells of a human DC-like cell line with different materials, including glyphosate. As a first step, the DC maturation marker CD86 was investigated. While the positive control PPD led to a significant upregulation of CD86, glyphosate induced a significant downregulation of CD86. Furthermore, the skin sensitizing capacity of the test materials was determined. Glyphosate was predicted as a non-sensitizer.
Furthermore, the transcriptomic data were complemented with a proteomic analysis. The assembled protein groups, deduced from the peptides obtained from the MS analysis resulted in a clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate).

Materials and methods

Chemical and reagents

Dimethyl sulfoxide (DMSO), p-phenylene-diamine (PPD), HyClone™ minimum essential medium alpha modification with L-glutamine, ribo- and deoxyribonucleosides (MEM-α) and TRizol® were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was purchased from PeproTech (Rocky Hill, NJ, USA). Propidium iodide (PI), FITC-conjugated anti-human [CD86 (FUN-1), HLA-DR (L243), CD34 (581) and isotype control anti- IgG1 (MOPC-21)] and PE-conjugated anti-human [CD54 (HA58), CD80 (L307) and isotype control anti-IgG1 (MOPC-21)] antibodies were acquired from BD Biosciences (San Jose, CA, USA). FITC-conjugated anti-human CD1a (NA1/34) and PE-conjugated anti-human CD14 (TÜK4) antibodies were purchased from Dako (Santa Clara, CA, USA). Direct-zol™ RNA MiniPrep column purification kit and trypsin were acquired from Zymo Research (Irvine, CA, USA) and Promega Biotech AB (Madison, WI, USA), respectively. Hybridization buffer, reporter co-deset, capture probeset and nCounter® cartridges were purchased from NanoString® Technologies (Seattle, WA, USA). Protease inhibitor tables EDTA-free and Silica C18 UltraMicro spin columns were acquired from Roche Diagnostics GmbH (Mannheim, Germany) and The Nest group (Southborough, MA, USA), respectively. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

MUTZ-3-derived cells (DSMZ, Braunschweig, Germany) were cultured in MEM-α supplemented with 20% (v/v) fetal bovine serum (FBS) and rhGM-CSF (40 ng/mL), and maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cell viability was analyzed using LUNA™ automated cell counter (Logos Biosystems, Annandale, VA, USA), according to manufacturer's instructions. A cell viability value > 90% was considered satisfactory to carry out the experiments, which were performed using three independent batches of cells. Moreover, quality control analysis was performed for each cell batch using BD FACSCanto II flow cytometer (Biosciences, San Jose, CA, USA). For that, the following antibodies were used: FITC-conjugated anti-human CD1a, CD34, CD86 or HLA-DR, and PE-conjugated anti-human CD14, CD54 or CD80; FITC- or PE-anti-IgG1 were used as isotype controls, while staining with PI (1 μg/mL) was performed for cell viability analysis.

Cell exposure with the test materials

Cell exposures and selection of input non-cytotoxic concentration of each test material (e.g. test material concentration inducing 90% relative viability, when compared to unexposed cells, namely RV90 value) were performed as follows: test materials were diluted in appropriate vehicles (Table 1) and the cells (2 × 105 cells/mL) were exposed to different concentrations for 24 h. After that, flow cytometer analysis was conducted to evaluate the cell viability using PI staining and then to obtain the RV90 values. In the case of non-cytotoxic materials, an input concentration of 500 μM was used. After that, cells were exposed to each test material at input concentration (Table 1) to carry out the flow cytometric analysis of CD86 expression and transcriptional analysis. Cell exposures were performed in triplicate cell batch reactions, i.e. a new cell batch was used each exposure round, resulting in a total of 18 samples. Total RNA was collected by lysing 2× 105 cells/exposure in TRIzol® and stored at −20 °C until RNA purification and further processing for transcriptional analysis and GARD™ skin predictions.

GARD™skin assay

The *in vitro* assay GARD™skin (SenzaGen AB, Lund, Sweden) was used to predict the ability of chemical substances to induce skin sensitization. Total RNA was isolated from the cells lysed in TRIzol® reagent using the Direct-zol™ RNA MiniPrep column purification kit (Zymo Research), according to manufacturer' instructions. For each sample, concentration and RNA integrity was determined with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). The following gene expression analysis was performed on NanoString® GEN2 nCounter analysis system (NanoString® Technologies) using the GARD™skin assay. In brief, 5 μL RNA sample were mixed with 8 μL master mix, comprising hybridization buffer and reporter CodeSet, and 2 μL Capture ProbeSet followed by 24 h hybridization at 65 °C using a 3Prime thermal cycler (Bibby Scientific, Staffordshire, UK). Samples were then processed and transferred to a 12-well nCounter® cartridge in the GEN2 Prep station 5s set at high sensitivity followed by data collection on the Digital Analyzer 5s with an imaging resolution of 555 fields of view. The Reporter Code Count files (RCC) files were then downloaded from the Digital Analyzer and processed using SenzaGen software version 1.0 (SenzaGen AB), which conducts both NanoString® quality control and data normalization. A support vector machine (SVM) algorithm was used to assign a so-called decision value (DV) to each test material replicate. A test material is classified as skin sensitizer when the SVM median output value is > 0 of the three independent replicates, while a median DV < 0 classifies the material as a non-sensitizer.

Proteomic analysis

Protein extractions and concentration determination

For proteomic analysis, cells were also exposed to each test material at input concentration (Table 1). After 24 h, cells were harvested and processed for MS analysis by pelleting the chemically exposed cells in a maximum recovery Eppendorf tube followed by washing with phosphate buffered saline (PBS) before flash freezing of the pellet in liquid nitrogen. Cell pellets were stored at −80 °C before further MS processing. Each cell batch of chemical exposure was performed in duplicates of 2 × 106 cells/exposure, and thereafter

pooled to ensure a sufficient protein amount for MS analysis. In addition, triplicates of unexposed (water) samples were run for MS analysis. Following cellular exposure proteins were extracted from each sample by lysing the cells in 150 µL lysis buffer (8 M urea, 39 mM Tris, 5 mM MgAc, 4% CHAPS, protease inhibitor tablet) through three freeze/thaw cycles and separating supernatant and cell debris by centrifugation at 16000 ×g for 30 min. Protein extracts were stored in −80 °C until further processing. Protein concentration determination was performed using the Total Protein Micro-Lowry kit (Sigma-Aldrich) according to manufacturer's instructions. The concentration was determined by measuring the absorbance at 650 nm and 50 µg per sample were used for further processing.

Table 1: Test materials used for cell exposures.

Material	Abbreviation	CAS no.	Vehicle	Classification	Input concentration
Double-distilled water	H_2O	na	na	Unexposed	0.1% (v/v)
Dimethyl sulfoxide	DMSO	67-68-5	na	Vehicle control	0.1% (v/v)
p-Phenylenediamine	PPD	106-50-3	DMSO	Positive control	75 µM
Glyphosate	GLY	1071-83-6	H_2O	na	500 µM

na: not applicable

In gel trypsin digestion and mass spectrometry

Proteins were separated on an SDS-PAGE gel and digested into peptides using trypsin. Trypsinated peptides were then de-salted using UltraMicro spin columns (Silica C18, SUM SS18 V, Nest group) as described in Chawade et al.. Peptides were thereafter resuspended in 0.1% formic acid (FA) and loaded into an EASY-nano liquid chromatograpjy (LC) system 1200 (Thermo Fisher Scientific). Peptides were inected directly into the analytical column, a 15 cm long fused silica capillary (75 µm × 16 cm Pico Tip Emitter, New Objective, Woburn, MA, USA) packed in-house with C18 material ReproSil-Pur 1.9 µm (Dr Maisch GmbH, Germany). Peptides were separated using an 80 min gradient from 5% to 90% solvent B (80% acetonitrile, 0.1% FA, v/v) at a constant flow rate of 250 nL/min. The nLC system was coupled with a Q-Exactive™ HF-X (Hybrid Quadrupole-Orbitrap™) (Thermo Fisher Scientific) operated in a positive mode for data-dependent acquisition (DDA). The Orbitrap acquired the full MS scan with an automatic gain control (AGC) target value of 3 × 106 ions and a maximum fill time of 50 ms. The 20 most abundant peptide ions were selected from the MS for higher energy collision-induced dissociation (HCD) fragmentation (collision energy: 40 V). The instrument was scanning at a target MS1 resolution of 120,000 between 375 and 1500 m/z window with 15,000 MS/MS resolution for a target of 1 × 105 and a maximum injection time of 20 ms using an isolation window of 1.2 m/z.

Results

GARD™skin ASSAY

Following 24 h of exposure with pure glyphosate, cytotoxicity analysis was performed using PI staining to define the input concentration for each test material (Table 1). Cells were then exposed to the test materials at input concentrations for 24 h and the maturity state of the cells was assessed by measuring levels of cell surface expression of the co-stimulatory marker CD86. Only the positive control PPD induced a significant upregulation of CD86 expression ($p < 0.05$) in comparison to unexposed control. Cell exposure with pure glyphosate resulted in a modest downregulation compared to control ($p < 0.05$). No significant difference between DMSO and unexposed controls was observed.

Furthermore, skin sensitization hazard predictions were performed with the GARD™skin assay. Transcriptomic analysis of the 200 bio-markers from the GARD™skin prediction signature was carried out using the NanoString® nCounter™ System. The skin sensitizing hazard was predicted using an SVM algorithm where each test material replicate was given a DV. As expected, PPD and DMSO were correctly classified as skin sensitizer and non-sensitizer, respectively. Also, glyphosate was classified as a non-sensitizer with a mean SVM DV of −1.45.

Profiling of agrochemical-induced changes in the cellular proteome

Initially, peptide data, generated from the trypsin-digested cells exposed with test materials for 24 h were normalized and assembled to protein groups. PCA evaluation was used to identify patterns, i.e. which samples exhibit a similar protein expression profile. As a first step, unsupervised clustering of all protein groups was performed. A clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate) is observed. Additionally, by applying a multi-group comparison (FDR = 0.05) based on all the treatments, i.e. clustering of the individual treatments most alike based on their protein expression, a similar pattern was observed as when unsupervised clustering was applied.

Conclusions

The objective of this study was to perform *in vitro* sensitization testing with a parallel MS-based proteomic approach to investigate the underlying cellular mechanisms induced. The experimental setup of the *in vitro* assay GARD™skin was used to stimulate cells of a human DC-like cell line with different materials, including glyphosate. As a first step, the DC maturation marker CD86 was investigated. While the positive control PPD led to a significant upregulation of CD86, glyphosate induced a significant downregulation of CD86. Furthermore, the skin sensitizing capacity of the test materials was determined. Glyphosate was predicted as a non-sensitizer.
Furthermore, the transcriptomic data were complemented with a proteomic analysis. The assembled protein groups, deduced from the peptides obtained from the MS analysis resulted in a clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate).

3. Assessment and conclusion

Assessment and conclusion by applicant:
Investigation of molecular mechanisms in the skin sensitization process specifically focusing on DC activation using an integrated transcriptomic- and proteomic approach.
First, Mutz-3-derived cells were exposed to PPD, DMSO, unexposed sample and glyphosate. No cytotoxicity was observed for glyphosate and glyphosate was classified as non-sensitising.
Second, PPD, DMSO, unexposed sample and glyphosate were assembled to protein groups. A clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate) was observed. Data on glyphosate are consistent with other available validated assay results.

The article is classified as reliable with restrictions for the following reason: This is a non-validated test system. The purity and origin of glyphosate is unclear. only 1 dose tested (no dose relationship can be evaluated), no HCD were available in order to compare with the equivalent concurrent controls and test groups results.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vitro* Toxicology Studies		
Publication: Lindberg T. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Not applicable because there is no OECD guideline for this type of test
Study performed according to GLP	N	

Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	The only information is that glyphosate's input concentration was 500 μM. The purity, origin etc. is unclear.
Only glyphosate acid or one of its salts is the tested substance	N	Glyphosate alone and EU non-representative formulations were tested (summary above is provided only for glyphosate)
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	n.a.	Not applicable as no metabolic activation system used
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	N	Not applicable as only 1 concentration was used
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	This is a non-validated test system. The purity and origin of glyphosate is unclear. Only 1 dose tested (no dose relationship can be evaluated). No HCD were available in order to compare with the equivalent concurrent controls and test groups results.
Not reliable	N	

1. Information on the study

Data point:	CA 5.5/027
Report author	Portier, C.J.
Report year	2020
Report title	A comprehensive analysis of the animal carcinogenicity data for glyphosate from chronic exposure rodent carcinogenicity studies
Document No	Environ Health (2020) Vol. 19, 18. https://doi.org/10.1186/s12940-020-00574-1
Guidelines followed in study	Not applicable
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2. Full summary of the study according to OECD format

Abstract

Since the introduction of glyphosate-tolerant genetically-modified plants, the global use of glyphosate has increased dramatically making it the most widely used pesticide on the planet. There is considerable controversy concerning the carcinogenicity of glyphosate with scientists and regulatory authorities involved in the review of glyphosate having markedly different opinions. One key aspect of these opinions is the degree to which glyphosate causes cancer in laboratory animals after lifetime exposure. In this review, twenty-one chronic exposure animal carcinogenicity studies of glyphosate are identified from regulatory documents and reviews; 13 studies are of sufficient quality and detail to be reanalyzed in this review using trend tests, historical control tests and pooled analyses. The analyses identify 37 significant tumour findings in these studies and demonstrate consistency across studies in the same sex/species/strain for many of these tumours. Considering analyses of the individual studies, the consistency of the data across studies, the pooled analyses, the historical control data, non-neoplastic lesions, mechanistic evidence and the associated scientific literature, the tumour increases seen in this review are categorized as to the strength of the evidence that glyphosate causes these cancers. The strongest evidence shows that glyphosate causes hemangiosarcomas, kidney tumours and malignant lymphomas in male CD-1 mice, hemangiomas and malignant lymphomas in female CD-1 mice, hemangiomas in female Swiss albino mice, kidney adenomas, liver adenomas, skin keratoacanthomas and skin basal cell tumours in male Sprague-Dawley rats, adrenal cortical carcinomas in female Sprague-Dawley rats and hepatocellular adenomas and skin keratoacanthomas in male Wistar rats.

Materials and methods

Bioassay data - The animal carcinogenicity data analyzed in this publication were derived from the published literature, the EPA review, the addendum to the EFSA review prepared by the German Institute for Risk Analysis, the JMPR review, the review of the carcinogenicity of glyphosate by a panel of scientists on behalf of industry, and full laboratory reports. The 13 cancer bioassays considered acceptable for this evaluation are presented in Table 1.

Table 1: Characteristics of the cancer bioassays of glyphosate analyzed in this study

Study Reference	Duration (months)	Strain		Dietary exposure dose levels (mg/kg/day)	Animals per Group	Purity (%)	Comments on survival and weight
		Mouse	Rat				
A: Knezevich and Hogan (1983) [11]	24	CD-1		M: 0, 157, 814, 4841 F: 0, 190, 955, 5874	50	99.8	No survival differences, slight weight reduction in high dose (M)
B: Atkinson et al. (1993) [12]	24	CD-1		M: 0, 98, 297, 988 F: 0, 102, 298, 1000	50	> 97.0	No survival differences, no weight differences
C: Sugimoto (1997) [13]	18	CD-1		M: 0, 165, 838.1, 4348 F: 0, 153.2, 786.8, 4116	50	94.6–95.7	No survival differences, slight weight reduction in mid (F) & high dose (M + F)
D: Wood et al. (2009) [14]	18	CD-1		M: 0, 71.4, 234.2, 810 F: 0, 97.9, 299.5, 1081.2	51	95.7	No survival differences, no weight differences
E: Takahashi (1999a) [15]	18	CD-1		M: 0, 167.6, 685, 7470 F: 0, 93.2, 909, 8690	50	97.5	Reduced survival high dose (M), slight weight reduction in mid (M) & high dose (M + F). This study was only mentioned by JMPR [7] and provides limited tumor data.
F: Kumar (2001) [16]	18	S-A[a]		M: 0, 85.5, 285.2, 1077.4 F: 0, 104.5, 348.6, 1381.9	50	> 95.0	No survival differences, no weight differences
G: Lankas (1981) [17]	26		SD[b]	M: 0, 3.05, 10.3, 31.49 F: 0, 3.37, 11.22, 34.02	50	98.7	No survival differences, no weight differences
H: Stout and Ruecker (1990) [18]	24		SD[b]	M: 89, 362, 940 F: 0, 113, 457, 1183	50	98.7	No survival differences, slight weight reduction in high dose (F)
I: Atkinson (1993) [19]	24		SD[b]	M: 0, 11, 112, 320, 1147 F: 0, 12, 109, 347, 1134	50	98.9	No survival differences, slight weight reduction in high dose (M + F)
J: Enemoto (1997) [20]	24		SD[b]	M: 0, 104, 354, 1127 F: 0, 115, 393, 1247	50	95.7	Reduced survival high dose (M), slight weight reduction in high dose (M + F)
K: Suresh (1996) [21]	24		W[c]	M: 0, 6.3, 59.4, 595.2 F: 0, 8.6, 88.5, 886	50	96.8	No survival differences, no weight differences
L: Brammer (2001) [22]	24		W[c]	M: 0, 121, 361, 1214 F: 0, 145, 437, 1498	53	97.6	High-dose survived longer (M), reduced weight highest dose (M + F)
M: Wood et al. (2009) [23]	24		W[c]	M: 0, 165, 838.1, 4348 F: 0, 153.2, 786.8, 4116	51	94.7–97.6	No survival differences, no weight differences

[a]Swiss Albino mouse; [b]Sprague-Dawley rat; [c]Wistar rat

For twelve of these studies full study reports were available and most of them were conducted in accordance with the appropriate regulatory guidelines. A full study report was not available for the Takahashi (1999) study but the data on kidney tumours in males and malignant lymphomas in females could be obtained from a JMPR review.

Statistical tests - Individual tumour counts for the individual studies were re-analyzed using the exact Cochran-Armitage one-sided linear trend test in proportions. Re-analyses were conducted on all primary tumours where there were at least 3 tumours in all of the animals in a sex/species/strain combination. In addition, any tumour where a significant positive trend ($p \leq 0.05$) was seen in at least one study was also evaluated in all studies of the same sex/species/strain, regardless of the number of animals with the tumour. When adenomas and carcinomas were observed in the same tissue, a combined analysis of adenomas and carcinomas was also conducted. Pairwise comparisons between individual exposed groups and the control group are conducted using Fisher's exact test. To evaluate the consistency of a tumour finding across multiple studies using the same sex-species-strain combinations, logistic regression with individual background responses and dose trends are fit to the pooled data using maximum likelihood estimation. A common positive trend is seen in the pooled analysis when the null hypothesis that the slope is 0 is rejected (statistical p-value ≤ 0.05 using a likelihood-ratio test) in favor of the alternative that the slope is greater than 0. Heterogeneity is seen in the pooled analysis when the null hypothesis that the slopes are equal is rejected (statistical p-value ≤ 0.05 using a likelihood-ratio test) in favor of the alternative that at least one of the slopes is different. For CD-1 mice analyses were conducted separately for 18 month and 24 month studies and then a combined analysis was performed. Similar grouped analyses were conducted for SD rats with one study of 26 months and 3 studies of 24 months. Only the combined analysis over all study durations is provided. The same methods of analysis were used to evaluate the incidence of non-neoplastic toxicity in tissues where tumours were observed. In cases of rare tumours where the increase in incidence didn't reach statistical significance the test proposed by Tarone (1982) was applied using an appropriate historical control group. All analyses were done using MATLAB, version R2017b.

Results

The purpose of this analysis is to understand the potential of glyphosate to produce tumours across all studies and not one study at a time. Thus, rather than presenting the results of each study separately, this review focuses on the tumours that are seen as positive in any one study and compares the findings across all studies of the same tumour in the same sex/species/strain combination.

Re-analysis of the data from CD-1 mice - From Table 2 it can be derived that a significant ($p \leq 0.05$) positive trend was found in males for kidney adenomas and kidney adenomas/carcinomas in the Takahashi study, malignant lymphomas in the Sugimoto and the Wood studies, hemangiosarcomas in the Atkinson study, and alveolar-bronchiolar carcinomas in the Wood study. When Tarone's test was applied using historical control data then a significant increase was found for kidney adenomas in the Sugimoto study, kidney carcinomas in the Knezevich and Hogan study, kidney adenomas/carcinomas in the Knezevich and Hogan and the Sugimoto study, and hemagiosarcomas in the Sugimoto study. In females a significant ($p \leq 0.05$) positive trend was found for hemangiomas, harderian gland adenomas and adenomas/carcinomas in the Sugimoto study, alveolar-bronchiolar adenomas/carcinomas in the Atkinson study, and malignant lymphomas in the Takahashi study. A significant common trend was found for kidney adenomas, carcinomas and adenomas/carcinomas, hemangiosarcomas in males and hemangiomas and malignant lymphomas in females.

Table 2: P-values for the exact Cochran-Armitage trend test and pooled logistic regression analysis for tumours with at least one significant trend test ($p \leq 0.05$) or Fisher's exact test ($p \leq 0.05$) in male and female CD-1 mice.

Tumor	Individual study *p*-values for trend[a]					Common Trend	Heterogeneity Test
Males	A	B	C	D	E		
Kidney Adenomas	0.442 (0.138)[d]	0.938	0.062 (**0.009**)[d]	—[b]	**0.019**	**0.006**	0.268
Kidney Carcinomas	0.063 (**< 0.001**)[d]	0.938	—[b]	—[b]	0.250	**0.031**	0.546
Kidney Adenomas and Carcinomas	0.065 (**0.008**)[d]	0.981	0.062 (**0.009**)[d]	—[b]	**0.005**	**< 0.001**	0.106
Malignant Lymphomas	0.754	0.087	**0.016**	**0.007**	ND[c]	0.093	0.007
Hemangiosarcomas	0.505	**0.004**	0.062 (**0.005**)[d]	—[b]	ND[c]	**0.033**	0.007
Alveolar-Bronchiolar Adenomas	0.294	0.231	0.513	0.924	ND[c]	0.384	0.409
Alveolar-Bronchiolar Carcinomas	0.918	0.456	0.148	**0.028**	ND[c]	0.407	0.083
Alveolar-Bronchiolar Adenomas and Carcinomas	0.576	0.231	0.294	0.336	ND[c]	0.346	0.826
Females	A	B	C	D	E		
Hemangiomas	0.631	—[b]	**0.002**	0.438	ND[c]	**0.031**	0.155
Harderian Gland Adenomas	0.877	ND[c]	**0.040**	0.155	ND[c]	0.155	0.052
Harderian Gland Carcinomas	—[b]	ND[c]	—[b]	1.000	ND[c]	0.500	1.00
Harderian Gland Adenomas and Carcinomas	0.877	ND[c]	**0.040**	0.372	ND[c]	0.184	0.110
Alveolar-Bronchiolar Adenomas	0.999	0.144	0.800	0.656	ND[c]	0.996	0.211
Alveolar-Bronchiolar Carcinomas	0.183	0.110	0.623	0.601	ND[c]	0.268	0.544
Alveolar-Bronchiolar Adenomas and Carcinomas	0.985	**0.048**	0.842	0.688	ND[c]	0.982	0.241
Malignant Lymphomas	0.070[e]	0.484	0.294	0.353	**0.050**	**0.012**	0.995

[a] – Study A is Knezevich and Hogan [11] (Additional file 2: Table S1), Study B is Atkinson et al. [12] (Additional file 2: Table S2), Study C is Sugimoto [13] (Additional file 2: Table S3), Study D is Wood [14] (Additional file 2: Table S4), Study E is Takahashi [15] (Additional file 2: Table S5); [b] – three dashes "—" indicates all tumor counts are zero; [c] – ND indicates there is no data available for this tumor in this study; [d] – using historical control data (see text for details) and Tarone's test; [e] – Spleen composite lymphosarcomas (malignant lymphomas) are also significantly increased in female mice in this study (see Additional file 2: Table S1)

Re-analysis of the data from Swiss albino mice - The single study with Swiss albino mice (Kumar, 2001) shows a significant increase in hemangiomas in female mice ($p = 0.004$).

Re-analysis of the data from SD rats - From Table 3 it can be derived that a significant ($p \leq 0.05$) positive trend was found in males for testicular interstitial cell tumours in the Lankas study, hepatocellular adenomas and adenomas/carcinomas in the Stout and Ruecker study, kidney adenomas in the Enemoto study, skin keratoacanthomas in the Stout and Ruecker, Atkinson and Enemoto studies, and skin basal cell tumours in the Enomoto study. In females a significant ($p \leq 0.05$) positive trend was found for thyroid C-cell adenomas in the Stout and Ruecker study, C-cell carcinomas in the Lankas study, and adrenal cortical carcinoma in the Stout and Ruecker study. When Tarone's test was applied using historical control data then a significant increase was found for pancreatic islet cell adenomas in males in the Stout and Ruecker study, and thyroid C-cell adenomas/carcinomas in females in the Lankas study. A significant common trend was found for hepatocellular adenomas, kidney adenomas, skin keratoacanthomas and skin basal cell tumours in males. In females the common trend was statistically significant for adrenal cortical carcinoma.

Table 3: P-values for the exact Cochran-Armitage trend test and pooled logistic regression analysis for tumours with at least one significant trend test or Fisher's exact test ($p \leq 0.05$) in male and female Sprague-Dawley rats

Tumor	Individual study *p*-values for trend[a]				Common Trend	Heterogeneity Test
Males	G	H	I	J		
Testicular Interstitial Cell Tumors	**0.009**	0.296	0.580	0.594	0.461	0.105
Pancreas Islet Cell Adenomas	0.512	0.147 (**0.007**)[c]	0.974	0.859	0.849	0.143
Pancreas Islet Cell Carcinomas	0.251	1.000	–	0.500	0.731	0.166
Pancreas Islet Cell Adenomas or Carcinomas	0.316	0.206	0.974	0.844	0.875	0.185
Thyroid C-cell Adenomas	0.743	0.089	0.278	0.631	0.210	0.532
Thyroid C-cell Carcinomas	0.505	0.442	0.495	0.565	0.322	0.898
Thyroid C-cell Adenomas and Carcinomas	0.748	0.097	0.197	0.642	0.175	0.526
Thyroid Follicular-cell Adenomas	0.122	0.408	0.067	0.966	0.464	0.055
Thyroid Follicular-cell Carcinomas	---[b]	0.255	0.443	1.000	0.448	0.137
Thyroid Follicular-cell Adenoma and Carcinoma	0.122	0.232	0.099	0.986	0.446	0.031
Hepatocellular Adenomas	0.471	**0.015**	0.325	0.500	**0.029**	0.664
Hepatocellular Carcinomas	0.062	0.637	0.760	0.642	0.803	0.269
Hepatocellular Adenomas and Carcinomas	0.173	**0.050**	0.480	0.690	0.144	0.428
Kidney Adenomas	0.938	0.813	1.000	**0.004**	**0.039**	0.002
Skin Keratoacanthomas	---[b]	**0.042**	**0.047**	**0.029**	**< 0.001**	0.998
Skin Basal Cell Tumors	0.251	0.249	1.000	**0.004**	**< 0.001**	0.009
Females	G	H	I	J		
Thyroid C-cell Adenomas	0.679	**0.049**	0.207	0.912	0.287	0.150
Thyroid C-cell Carcinomas	**0.003 (< 0.001)**[c]	0.500	---[b]	---[b]	0.385	0.041
Thyroid C-cell Adenomas and Carcinomas	0.072 (**0.037**)[c]	0.052	0.207	0.912	0.275	0.071
Adrenal Cortical Adenoma	0.851	0.603	---[b]	0.626	0.713	0.750
Adrenal Cortical Carcinoma	0.386	**0.015**	0.493	---[b]	**0.031**	0.199
Adrenal Cortical Adenoma and Carcinoma	0.801	0.090	0.493	0.626	0.195	0.520

[a] – Study G is Lankas [17] (Additional file 2: Table S7), Study H is Stout and Ruecker [18] (Additional file 2: Table S8), Study I is Atkinson et al. [12] (Additional file 2: Table S9) and Study J is Enemoto [20] (Additional file 2: Table S10); [b] – three dashes "---" indicates all tumor counts are zero; [c] – using historical control data (see text for details) and Tarone's test

Re-analysis of the data from Wistar rats - From Table 4 it can be derived that a significant ($p \leq 0.05$) positive trend was found in males for hepatocellular adenomas and adenomas/carcinomas in the Brammer study, pituitary adenomas in the Wood study, skin keratoacanthomas in the Wood study, and adrenal pheochromocytomas in the Suresh study. In females a significant ($p \leq 0.05$) positive trend was found for mammary gland adenocarcinomas and adenomas/adenocarcinomas, pituitary adenomas and adenomas/carcinomas all in the Wood study. A significant common trend was found for hepatocellular adenomas and adenomas/carcinomas and skin keratoacanthomas in males. No statistically significant common trend was found for the tumours in females.

Table 4: P-values for the exact Cochran-Armitage trend test and pooled logistic regression analysis for tumours with at least one significant trend test or Fisher's exact test ($p \leq 0.05$) in male and female Wistar rats

Tumor	Individual study *p*-values for trend[a]			Common Trend	Homogeneity Test
Males	K	L	M		
Hepatocellular Adenomas	0.391	**0.008**	0.418	**0.048**	0.156
Hepatocellular Carcinomas	0.418	---[b]	1.000	0.492	0.242
Hepatocellular Adenomas and Carcinomas	0.286	**0.008**	0.610	**0.029**	0.194
Pituitary Adenomas	0.376	0.277	**0.045**	0.057	0.664
Pituitary Carcinomas	0.692	---[b]	1.000	0.771	0.956
Pituitary Adenomas and Carcinomas	0.454	0.277	0.059	0.073	0.700
Skin Keratoacanthomas	---[b]	0.387	**0.030**	**0.032**	0.823
Adrenal Pheochromocytomas	**0.048**	0.721	0.306	0.273	0.210
Females	K	L	M		
Mammary Gland Adenomas	0.539	0.941	0.062	0.448	0.015
Mammary Gland Adenocarcinomas	1.000	0.271	**0.042**	0.071	0.008
Mammary Gland Adenomas and Adenocarcinomas	0.729	0.590	**0.007**	0.113	0.064
Pituitary Adenomas	0.967	0.261	**0.014**	0.105	0.023
Pituitary Carcinomas	1.000	-	0.750	0.748	0.491
Pituitary Adenomas and Carcinomas	0.976	0.261	**0.017**	0.129	0.019

[a] – Study J is Suresh [21] (Additional file 2: Table S11), Study K is Brammer [22] (Additional file 2: Table S12), and Study L is Wood et al. [14] (Additional file 2: Table S13); [b] – three dashes "---" indicates all tumor counts are zero

False positive errors - The evaluation of any one animal cancer study involves a large number of statistical tests that could lead to false positives. To evaluate this issue, the probability that all of the results in any sex/species/strain could be due to false positive results is calculated. Overall, a total of 496 evaluations were done for these 13 studies including the few evaluations done against historical controls. There are 41 evaluations at 37 tumour/site combinations with a trend test $p \leq 0.05$. The probability that all of these are due to false positives is 0.001. Similarly, looking at the evaluations resulting in $p \leq 0.01$, the probability that all of the findings are due to false positives is < 0.001. The strongest evidence was found for male CD-1 mice where the probability for 11 positive findings to occur at $p \leq 0.05$ and 8 positive findings at $p \leq 0.01$ are both below 0.001.

Conclusions

Oral exposure of rats and mice to glyphosate *via* the diet in 13 separate carcinogenicity studies demonstrates that glyphosate causes a variety of tumours that differ by sex, species, strain and length of exposure. To summarize the results of the strength-of-evidence analysis, each tumour is placed in any of the following categories: Clear evidence (CE) when there is a causal link between glyphosate exposure and the tumour; Some evidence (SE) when there is a causal link between glyphosate exposure and the tumour but chance, although unlikely, cannot be ruled out; Equivocal evidence (EE) when there is a causal link between glyphosate exposure and the tumour but chance is as likely an explanation for the association as is exposure to glyphosate; No evidence (NE) indicates that any causal link between glyphosate exposure and the tumour is almost certainly due to chance. The factors used to place tumours into these categories include the analyses of the individual studies, the consistency of the data across studies (pooled analyses), the analyses using historical control data, the analyses of non-neoplastic lesions, mechanistic evidence and the associated scientific literature.

The weight-of-evidence analysis conducted in this study indicates that there is clear evidence (CE) that oral exposure to glyphosate *via* the diet produces adrenal cortical carcinoma in the female SD rat, hemangioma in the female mouse (CD-1 and Swiss albino), hemangiosarcoma in the male CD-1 mouse, kidney tumours in the male CD-1 mouse and SD rat, liver adenoma in male rats (SD and Wistar), malignant lymphoma in the male and female CD-1 mouse, skin basal cell tumour in the male SD rat and skin keratoacanthoma in male rats (SD and Wistar). Some evidence (SE) for a causal relationship was found for kidney tumours in male Swiss albino mice, mammary tumours in female Wistar rats, malignant lymphoma in male and female Swiss albino mice, pituitary adenoma in the male and the female Wistar rat, and testicular interstitial cell tumours in the male SD rat. The results of the analyses conducted in this study are supportive of the conclusions of IARC that there is sufficient evidence to consider glyphosate as a rodent carcinogen.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Thirteen glyphosate cancer bioassays considered acceptable for this re-analysis were selected from the published literature, the EPA review, the review from the German Institute for Risk Analysis, the JMPR review, and full laboratory reports. For twelve of them full study reports were available. Individual tumour counts for the individual studies were re-analyzed using the exact Cochran-Armitage one-sided linear trend test. Re-analyses were conducted on all primary tumours where there were at least 3 tumours in all of the animals in a sex/species/strain combination. In addition, any tumour where a significant positive trend ($p \leq 0.05$) was found in at least one study was also evaluated in all the other studies of the same sex/species/strain combination, regardless of the number of animals with the tumour. Pairwise comparisons between individual exposed groups and the control group were conducted using Fisher's exact test. To evaluate the consistency of a tumour finding across multiple studies using the same sex-species-strain combinations, logistic regression with individual background responses and dose trends are fit to the pooled data using maximum likelihood estimation. The same methods of analysis were used to evaluate the incidence of non-neoplastic lesions in tissues where tumours were observed. In cases of rare tumours where the increase in incidence didn't reach statistical significance the test proposed by Tarone (1982) was applied using an appropriate historical control group. To summarize the results of the strength-of-evidence analysis, each tumour is placed in any of the following categories: Clear evidence (CE), some evidence (SE), equivocal evidence (EE), and no evidence (NE). The factors used to place tumours into these categories include the analyses of the individual studies, the consistency of the data across studies (pooled analyses), the analyses using historical control data, the analyses of non-neoplastic lesions, and mechanistic evidence with the associated scientific literature. The author's weight-of-evidence analysis indicates that there is clear evidence (CE) that oral exposure to glyphosate *via* the diet produces adrenal cortical carcinoma in the female SD rat, hemangioma in the female mouse (CD-1 and Swiss albino), hemangiosarcoma in the male CD-1 mouse, kidney tumours in the male CD-1 mouse and SD rat, liver adenoma in the male rat (SD and Wistar), malignant lymphoma in the male and female CD-1 mouse, skin basal cell tumour in the male SD rat and skin keratoacanthoma in male rats (SD and Wistar). Some evidence (SE) for a causal relationship was put forth for kidney tumours in the male Swiss albino mouse, mammary tumours in the female Wistar rat, malignant lymphoma in the male and female Swiss albino mouse, pituitary adenoma in the male and the female Wistar rat, and testicular interstitial cell tumours in the male SD rat.

After thorough analysis and considering all factors that are important in the interpretation of cancer studies none of the tumours identified by the author as indicating clear evidence (CE) or some evidence (SE) of carcinogenicity were found relevant for reconsideration under the necessary due diligence of the European AIR5 review of glyphosate. Most of the tumours selected by the author were previously dismissed by the EU experts as not relevant even before the last review of glyphosate in 2017, and the applicant believes that there is no solid toxicological evidence for glyphosate exposure related carcinogenicity in the mouse and the rat that warrants any science-based concerns for human health. The discussion of each of the suspect tumours is given below.

Clear evidence (CE) for carcinogenicity:

Adrenal cortical carcinoma in the female SD rat (Stout and Ruecker study):
The tumour incidences were 0/60, 0/60, 0/60, 3/60 at 0, 113, 457, and 1183 mg/kg bw/day, respectively. This tumour has not been considered treatment related by the authors of the study. There is no dose-related increase in adrenal cortical adenoma (1/60, 3/60, 2/60, 1/60), no dose-related increase in pre-neoplastic lesions and this tumour was not found in the males of the same study or in other rat studies. Therefore, this tumour has been considered not relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

Hemangioma in female CD-1 mice (Sugimoto study):
In the Sugimoto study hemangiomas were observed in different tissues:
- In liver with an incidence of 0/50, 0/50, 1/50, 1/50;
- In the ovary with an incidence of 0/50, 0/50, 0/50, 1/50;
- In the uterus with an incidence of 0/50, 0/50, 1/50, 2/50;
- In the spleen with an incidence of 0/50, 0/50, 1/50, 0/50;
- In the abdominal cavity with an incidence of 0/8, 0/9, 0/9, 1/9;

At 0, 153.2, 786.8, and 4116 mg/kg bw/day, respectively. Taken together as systemic tumours a significant positive trend is obtained. However, hemangiomas have also been observed in males (liver and testes) but without any dose-response relationship and the highest incidence found was in the control group (1/50). These tumours have not been confirmed in the other carcinogenicity studies in the CD-1 mouse. Moreover, the dose level (4116 mg/kg bw/day) at which the incidence was statistically significantly increased when compared against the control, is more than 4-fold the limit dose for the testing of carcinogens in rodent species. If that dose is ignored there is no significative positive trend. Therefore, this tumour has been considered not relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

Hemangioma in female Swiss albino mice (Kumar study):
In the re-analysis of the tumour data of the Kumar study by K. Weber (report submitted in 2017) no statistically significant trend was found for systemic neoplasms using the Peto test. When analyzed using the Fischer's exact test no statistically significant increase in incidence was found in pair-wise comparisons of all dose groups with the control group. It is important to emphasize that this study was compromised by non-identified ecto-and endoparasites in a large number of animals. Therefore, this tumour has been considered not relevant for the assessment of glyphosate.

Hemangiosarcoma in the male CD-1 mouse (Atkinson study):
The tumour incidences were 0/50, 0/50, 0/50, 4/50 (3 in liver and 1 in prostate) at 0, 98, 297, and 988 mg/kg bw/day, respectively. The incidence at the highest dose (8 %) was still within the historical control range of the test laboratory (0-8 %, 300 male mice in 6 studies up to 1993). This tumour was not confirmed in other mouse studies of which one (Knezevich and Hogan study) with a dose level nearly 5-fold that of the Atkinson study (4841 mg/kg bw/day). Therefore, this tumour has been considered not relevant for the assessment of glyphosate.

Kidney tumours in the male CD-1 mouse (Takahashi study, as reported by JMPR, 2016):
Renal cell adenoma (3/50) and renal cell carcinoma (1/50) were observed in males at 7470 mg/kg bw/day, but, according to the authors, there was no statistically significant difference with the control group. It is of note that the high dose considered in this study for males is extraordinarily high, more than 7-fold the limit dose for the testing of carcinogens in rodent species. If this dose is ignored there is no significant positive trend. These tumours were re-examined by the original study pathologist in 2012 because the Pesticide Expert Panel of the Food Safety Commission of Japan requested more information on historical control data and association with the non-neoplastic renal findings. After re-examination, the incidences for renal cell adenoma were 1/50, 1/50, and 1/50 at 167.6, 685, and 7470 mg/kg bw/day, showing no dose-response relationship. The incidence for renal cell carcinoma was confirmed to be 1/50 at 7470 mg/kg bw/day. The historical control data for the Takahashi study were not available, but the historical control values described in the re-examination document for renal cell carcinoma were 1/725 (0.13 %) in males and 0/725 (0 %) in females and for renal cell adenoma were 3/564 (0.53 %) in males and 0/564 (0 %) in females. The re-examination report also

provides reference data of 0-1.8 % in males and 0 % for all doses in females for renal cell carcinoma, and 0-1.8 % in males and females for renal cell adenoma. The results of the re-examination revealed also that the tubular epithelial cell hypertrophy was localized with an incidence in each treatment group that did not significantly differ from that in the control group. There was no association between the tubular epithelial cell hypertrophy and the development of renal tumours. The renal cell tumours observed in this study are thus not relevant for the human risk assessment of glyphosate because (1) the incidence of renal tumours in males at 7470 mg/kg bw/day did not significantly differ from that in the control group upon re-evaluation; (2) none of the females had neoplastic or non-neoplastic lesions; and (3) the high dose considered in this study for males is more than 7-fold the limit dose for the testing of carcinogens in rodent species. Therefore, this tumour has been considered not relevant for the assessment of glyphosate.

Kidney tumours in the male SD rat (Enemoto study):
The incidences of kidney adenoma were 0/76, 0/75, 0/80, 4/78 at 0, 104, 354, and 1127 mg/kg bw/day. An increasing trend in the incidence of adenomas in the kidney was observed in males of the high dose group (animal 193: killed in extremis at week 92, animal 167: found dead at week 94, animal 159: found dead at week 101, and animal 169: killed by design after 104 weeks of treatment) and this incidence was greater than the historical control range referred to in the study report (0-2.9 %). However, according to the authors of this study, the increase observed was not statistically significant. No kidney tumours were found in the females and nearly all male rats at all dose levels suffered from chronic nephropathy (62/76, 63/75, 56/80, 67/78). This tumour in this study was not considered relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

Hepatocellular adenomas in the male SD rat (Stout and Ruecker study):
The tumour incidences for adenomas were 3/60, 2/60, 3/60, 8/60 and of carcinomas were 3/60, 2/60, 1/60, 2/60 at 0, 89, 362, and 940 mg/kg bw/day, respectively. The incidence of adenomas at the high dose (13.3 %) is still within the historical control range of the test laboratory (1.4-18.3 %). Foci of cellular alteration were observed at all dose levels without any dose-response relationship and there were no signs of hepatocellular hypertrophy, a prerequisite for hepatocellular carcinogenesis. Beside the Brammer study no increase in hepatocellular adenomas was noted in the other rat studies. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Hepatocellular adenomas in the male Wistar rat (Brammer study):
The tumour incidences were 0/64, 2/64, 0/64, 5/64 at 0, 121, 361, 1214 mg/kg bw/day, respectively. The positive trend is significant and the incidence at the high dose is significantly different from the control. However, the incidence at the high dose (7.8 %) is still within the historical control range of the test laboratory (0-11.5 %, 26 studies in 1984-2003). There were no histopathological signs of liver enzyme induction or pre-neoplastic lesions. The high dose animals in this study survived longer when compared to the other groups. This may also influence the spontaneous tumour rate. Beside the Stout and Ruecker study no increase in hepatocellular adenomas was noted in the other rat studies. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Malignant lymphoma in the male CD-1 mouse (Sugimoto study):
The tumour incidences were 2/50, 2/50, 0/50, 6/50 at 0, 165, 838, 4348 mg/kg bw/day, respectively. The positive trend is significant but the incidence at the high dose is not significantly different from the control. Moreover, the incidence at the high dose (12 %) is still within the historical control range of the test laboratory (3.6-19.2 %, 458 male mice in 12 studies in 1993-1998). The trend has been found significantly positive because of the elevated incidence at a dose level that is over 4-fold the limit dose for carcinogenicity studies in rodents. If this dose is ignored the trend is not positive. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Malignant lymphoma in the female CD-1 mouse (Takahashi study):
The tumour incidences were 3/50, 1/50, 4/50, 6/50 at 0, 93.2, 909, and 8690 mg/kg bw/day, respectively. The increased incidence of lymphoma at the high dose was statistically significant in the trend test but not in a pairwise comparison. The trend has been found significantly positive

because of the elevated incidence at an extraordinarily high dose level, more than 8-fold the limit dose for carcinogenicity studies in rodents. If this dose is ignored the trend is no longer significant. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Skin basal cell tumour in the male SD rat (Enemoto study):
The tumour incidences were 0/78, 0/75, 0/80, 3/78 for adenoma and 0/78, 0/75, 0/80, 1/78 for carcinoma at 0, 104, 354, and 1127 mg/kg bw/day. No increased incidence of this tumour was observed in the females or other rat studies and may be associated with other skin lesions (follicular hyperkeratosis and/or folliculitis/follicular abscess) observed in this study. Although there is a significant positive trend for the adenomas, the increase in incidence at the high dose level was not considered relevant for the risk assessment of glyphosate by the authors of this study. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male SD rat (Stout and Ruecker study):
The tumour incidences were 1/60, 3/60, 4/60, 5/60 at 0, 89, 362, and 940 mg/kg bw/day. Although there is a significant positive trend the incidence at the high dose was not statistically significantly different from the control and considered not related to treatment. Skin keratoacanthoma is one of the most common spontaneous benign neoplasms in male Sprague Dawley rats. Therefore, this tumour was not considered relevant for the risk assessment of glyphosate by the authors of this study. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male SD rat (Atkinson study):
The combined incidences of intracutaneous cornifying epithelioma (keratoacanthoma) were 1/50, 2/25, 0/19, 0/21, 5/50 at 0, 11, 112, 320, and 1147 mg/kg bw/day. Although the trend was significant, the incidence at the high dose was not statistically significantly different from the control and considered not related to treatment by the authors of this study. Skin keratoacanthoma is one of the most common spontaneous benign neoplasms in male Sprague Dawley rats. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male SD rat (Enemoto study):
The incidences of the tumour were 4/76, 3/75, 0/80, 7/78 at 0, 104, 354, and 1127 mg/kg bw/day. The increased incidence of this skin tumour at the high dose may be associated with other skin lesions (follicular hyperkeratosis and/or folliculitis/follicular abscess) observed in this study. Although there is a significant positive trend for this tumour, the increase in incidence at the high dose level was not statistically significantly different from the control. Skin keratoacanthoma is one of the most common spontaneous benign neoplasms in male Sprague Dawley rats and considered by the authors of this study not relevant for the risk assessment of glyphosate. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male Wistar rat (Wood study):
There were no treatment-related conditions seen in the skin or in subcutaneous tissues, but several spontaneous lesions were observed. Epidermal ulceration and scab formation, inflammatory lesions, abscess formation, focal acanthosis, focal mineralisation, focal dermal thickening, and focal necrosis were seen, occasionally or rarely and without significance. This tumour was not discussed further in the previous EU review of glyphosate.

Some evidence for carcinogenicity (SE)
Kidney tumours in the male Swiss albino mouse (Kumar study):
In the re-analysis of the tumour data of the Kumar study by K. Weber (submitted in 2017) no statistically significant trend was found for systemic neoplasms in the Peto test. When analyzed using the Fischer's exact test no statistically significant increase in incidence was found in pair-wise comparisons of all dose groups with the control group. It is important to emphasize that this study was compromised by non-identified ecto-and endoparasites in a large number of animals. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Mammary tumour in the female Wistar rat (Wood study):

At interim and terminal sacrifice combined mammary neoplasia was seen in 6 female mice. There were no mammary neoplasms in the control group but carcinomas were seen with incidences of 2/51, 3/51, and 1/51 at 153.2, 786.8, and 4116 mg/kg bw/day, respectively. All neoplasms were adenocarcinomas with the exception of one adenosquamous carcinoma seen in a low dose group animal. No increase in the incidence of these tumours was reported in the females of other rat studies. The authors concluded that there was no effect of treatment upon the incidence of mammary neoplasia in this study. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Malignant lymphoma in the male Swiss albino mouse (Kumar study):
In the re-analysis of the tumour data of the Kumar study by K. Weber (2017) no statistically significant trend was found for systemic neoplasms in the Peto test. When analyzed using the Fischer's exact test no statistically significant increase in incidence was found in pair-wise comparisons of all dose groups with the control group. It is important to emphasize that this study was compromised by non-identified ecto-and endoparasites in a large number of animals. Therefore, this tumour was not considered as relevant for the assessment of glyphosate.

Pituitary adenomas in the male and the female Wistar rat (Wood study):
Pituitary adenomas were only seen in female mice with incidences of 0/51, 1/51, 0/51, 2/52 at 0, 104.5, 348.6, and 1381.9 mg/kg bw/day. The group distribution was unrelated to treatment. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Testicular interstitial cell tumour in the male SD rat (Lankas study):
The incidences of this tumour were 0/50, 3/50, 1/50, 6/50 at 0, 3.05, 10.30, and 31.49 mg/kg bw/day, respectively. The positive trend is statistically significant and the incidence at the high dose level (12 %) is statistically significantly different from the control and greater that the historical control rate of the test laboratory (3.4-6.6 %). However, there was no dose-response relationship for interstitial cell hyperplasia (1/50, 1/50, 1/50, 0/50). Since the dose range considered in this study (0-31.5 mg/kg bw/day) is approximately at least 30-fold lower than that of all the other studies in rats where no increase of such tumours was found this finding should be considered as spontaneous in nature. Therefore, this tumour was not considered relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because some of the statistical methods employed were not described in sufficient detail. Besides, the results of this study are not in agreement with the findings of Crump *et al*. 2020 in relation to the estimation of false positives and the overall evaluation of the significance of the tumours by the EU regulatory authorities. All the tumours that were identified by the author as providing clear evidence for the carcinogenicity of glyphosate have been previously dismissed in the EU regulatory process.

Reliability criteria for *in vivo* toxicology studies

Publication: Portier, 2020.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N.A.	
Study performed according to GLP	N. A.	Most of the study reports analysed were GLP compliant.
Study completely described and conducted following scientifically acceptable standards	Y	Re-analysis of the tumour data of 13 selected glyphosate cancer bioassays.
Test substance		
Test material (Glyphosate) is sufficiently documented and	Y	Purity of glyphosate used in

reported (i.e. purity, source, content, storage conditions)		every cancer bioassay mentioned.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	N.A.	Described in the test reports of 12 of the selected studies. The data from one study were derived from a JMPR review.
Route and mode of administration described	Y	Oral *via* the diet.
Dose levels reported	Y	Dose range from 71.4 to 8690 mg/kg bw in the mouse and from 3.05 to 4348 mg/kg bw in the rat.
Number of animals used per dose level reported	Y	About 50 per dose group.
Method of analysis described for analysis test media	N.A.	Described in the original test reports.
Validation of the analytical method	N.A.	
Analytical verifications of test media	N.A.	
Complete reporting of effects observed	Y	
Statistical methods described	Y	All statistical methods used in the re-analysis of the tumour data were reported, however sometimes not in sufficient detail.
Historical control data of the laboratory reported	N.A.	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because some of the statistical methods employed were not described in sufficient detail. Besides, the results of this study are not in agreement with the findings of Crump *et al*. 2020 in relation to the estimation of false positives and the overall evaluation of the significance of the tumours by the EU regulatory authorities. All the tumours that were identified by the author as providing clear evidence for the carcinogenicity of glyphosate have been dismissed in the EU regulatory process.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Xia Y. *et al*.
Report year	2020
Report title	The endoplasmic reticulum stress and related signal pathway mediated the glyphosate-induced testosterone synthesis inhibition in TM3 cells
Document No	Environmental Pollution, (2020) 260, 113949 DOI: 10.1016/j.envpol.2020.113949
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The authors investigated the effects of glyphosate on testosterone secretion and the role of endoplasmic reticulum (ER) stress in the process in TM3 cells. The effects of glyphosate at different concentrations on the viability of TM3 cells were detected by the CCK8 method. The effect of glyphosate exposure on testosterone secretion was determined by enzyme-linked immunosorbent assay (ELISA). The expression levels of testosterone synthases and ER stress-related proteins were detected by Western blot and Immunofluorescence stain. Results showed that exposure to glyphosate at concentrations below 200 mg/L had no effect on cell viability, while the glyphosate above 0.5 mg/L could inhibit the testosterone secretion in TM3 cells. Treatment TM3 cells with glyphosate at 5 mg/L not only reduced the protein levels of testosterone synthase StAR and CYP17A1, inhibited testosterone secretion, but also increased the protein level of ER stress molecule Bip and promoted the phosphorylation of PERK and eIF2α. Pre-treatment cells with PBA, an inhibitor of ER stress, alleviated glyphosate-induced increase in Bip, p-PERK, and p-eIF2α protein levels, meanwhile rescuing glyphosate-induced testosterone synthesis disorders. When pre-treatment with GSK2606414, a PERK inhibitor, the glyphosate-induced phosphorylation of PERK and eIF2α was blocked, and the glyphosate-inhibited testosterone synthesis and secretion were also restored. Overall, the authors suggest that glyphosate can interfere with the expression of StAR and CYP17A1 and inhibit testosterone synthesis and secretion via ER stress-mediated the activation of PERK/eIF2α signalling pathway in Leydig cells.

Materials and methods

Chemicals and antibodies

The following chemicals and antibodies were used in this study: Cell Counting Kit (CCK)-8 (Dojindo Molecular Technologies, Shanghai, China), Testosterone ELISA Kit (Mlbio, Shanghai, China), GSK2606414 (Absin, Shanghai, China), 4-phenylbutyric acid (PBA), goat anti-rabbit IgG-TRITC antibody and mouse anti-3β-HSD antibody (Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-Bip antibody, rabbit anti-PERK antibody, rabbit anti-PERK (phosphor T980) antibody, rabbit anti-eIF2α antibody, rabbit anti-eIF2α (phosphor S51) antibody, rabbit anti-StAR antibody, rabbit anti-CYP11A1 antibody, rabbit anti-CYP17A1 antibody, rabbit antiGAPDH antibody, rabbit anti-β-actin antibody, goat anti-mouse IgG, HRP-linked antibody and goat anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Beverly, MA, USA), glyphosate, poly-L-lysine solution (sigma-aldrich, Santa Louis, MO, USA), goat serum blocking solution (ZSJB-BIO, Beijing, China), 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China). PBA was dissolved as a stock solution (0.1 M) in PBS and the final concentration was 10 μM. GSK2606414 was dissolved as a stock solution (2 mM) in DMSO and the final concentration was 0.5 μM.

Cell cultures and treatment

TM3 cells (purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Shanghai, China), a mouse Leydig cell line, were cultured in DMEM/F12 complete medium containing 5 % horse serum and 2.5 % FBS (Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Shanghai, China) at 37 °C in a humidified atmosphere with 5 % CO_2. For cell viability test, cells were seeded at a density of 5 x 10^3 cells/well in 96-well plates. For ELISA, cells were seeded in 24-well plates (4 x 10^4 cells/well) and the concentration of testosterone in cell culture medium was detected by ELISA. For western blotting analysis, cells were seeded at a density of 4 x 10^5 cells/dish in Ø 60 mm Petri dishes. Cells were cultured for 24 h before experimental treatment.

Cell viability test

Cell viability was tested using CCK-8 Kit according to the manufacturer's protocol. Briefly, TM3 cells were seeded in 96-well plates for 24 h before the treatment. After exposed to glyphosate at different concentrations from 0.01 to 2000 mg/L for 24 h, cells in each well were added with 10 μL reagent from the Cell Counting Kit and then incubated at 37 °C for 80 min. Finally, OD value of each well at the wavelength of 450 nm was read by a multimode plate reader (Thermo Scientific, NYC, NY, USA). The experiment was repeated independently four times, and six duplicate wells were set in each group.

Testosterone determination by ELISA

TM3 cells were cultured in a 24-well plate and exposed to glyphosate at different concentrations for 24 h with or without PBA or GSK2606414 pre-treatment. Then, the medium was collected and centrifuged at 12,000 rpm for 5 min at 4 °C. The testosterone in supernatant was assayed using ELISA kit according to the manufacturer's protocol. Briefly, each well of ELISA plate was added with 40 μL sample dilution, 10 μL sample and 100 μL enzyme labelling reagent respectively, then the plate was covered and incubated at 37 °C for 60 min. After thoroughly aspirating solution from wells, the plate was washed with washing solution for five times. Then each well was added with 50 μL developer A and 50 μL developer B, and incubated at 37 °C for 15 min in the dark. Finally, 50 μL stop solution was added to terminate the reaction. The absorbance of each well was measured at a wavelength of 450 nm. The experiment was repeated independently four times, and three duplicate wells were set in each group.

Western blotting analysis

Cells were seeded in Ø 60 mm Petri dishes. After exposed to glyphosate for 24 h with or without PBA or GSK2606414 pre-treatment, cells were extracted using the RIPA lysis buffer (Beyotime, Shanghai, China) containing 1 % phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail. Protein concentrations were detected by BCA protein assay kit (Beyotime, Shanghai, China). Equal amount of protein was prepared and separated by 10 % SDS-PAGE. Then, protein was transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 5 % bovine serum albumin in Tris buffered saline (TBS) for 2 h, the membranes were incubated with primary antibodies overnight at 4 °C. Next day, the membranes were washed 3 times with TBS containing 0.1 % tween-20 (TBST) for 10 min/time and incubated with secondary antibody for 1 h at room temperature. After washed with TBST, the bolts were detected with BeyoECL Star (Beyotime, Shanghai, China) and the grey scales of protein bands were measured by a Chemiluminescence Imager (BIO-RAD, Hercules, CA, USA). Experiments were repeated independently for eight times.

Immunofluorescence analysis

For immunofluorescence analysis, glass slides were coated with 0.01 % (w/v) poly-L-lysine solution for 5 min. The excess solution was removed and the slides were dried at room temperature. Then the slides were put into Ø 35 mm Petri dishes and cells were seeded in them. After exposed to glyphosate for 24 h with or without PBA or GSK2606414 pre-treatment, cells were fixed with 4 % paraformaldehyde for 20 min and permeabilized with 0.5 % Triton X-100 for 15 min at 4 °C. Then, cells were treated with goat serum blocking solution for 2 h at room temperature and incubated with primary antibodies overnight at 4 °C. Subsequently, cells were incubated with TRITC-conjugated secondary antibody for 1 h and DAPI for 15 min at room temperature. Finally, the stained slides were photographed with laser scanning confocal microscope (Olympus, Tokyo, Japan) and the fluorescence intensity was evaluated using ImageJ software. Each experiment was repeated four times and two background groups (without primary antibody or secondary antibody) were set.

Statistical analysis
Data were presented as mean ± SD and analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test using SPSS 22 statistical software. A difference at $p < 0.05$ was considered statistically significant.

Results

Glyphosate at low concentration inhibited testosterone secretion but not to affect cell viability in TM3 cells
After treated with glyphosate at different concentrations from 0.01 to 2000 mg/L for 24 h, cell viability was measured using CCK8 and the concentration of testosterone in the culture medium was detected using ELISA kit. The results showed that glyphosate above 500 mg/L could decrease TM3 cell viability ($p < 0.05$), whereas glyphosate at concentrations below 200 mg/L had no significant effect on TM3 cell viability (Figure 1). In contrast to cell viability, as shown in Figure 2, exposure to glyphosate at a concentration above 0.5 mg/L could significantly decreased the concentrations of testosterone in culture medium ($p < 0.05$). These findings suggested that glyphosate at low concentrations which had no effect on cell viability could inhibit testosterone secretion in TM3 cells.

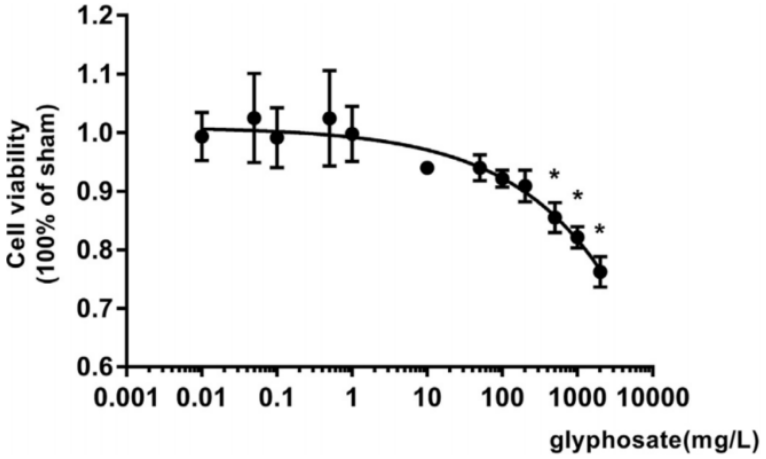
Glyphosate inhibited the expression of testosterone synthases StAR and CYP17A1
In the experiment, the effect of glyphosate on expression of testosterone key synthases was investigated. TM3 cells were treated with 5 mg/L glyphosate for different durations (1, 3, 6, 12 or 24 h), and the protein level of testosterone synthases was detected by western blotting. The results showed that glyphosate exposure significantly reduced the protein levels of StAR and CYP17A1 ($p < 0.05$) (Figure 3A and B). However, the protein levels of CYP11A1 and 3β-HSD were not affected by glyphosate (Figure 3C and D). It suggested that glyphosate decreased testosterone secretion through selectively inhibiting expression levels of key synthesis enzymes of StAR and CYP17A1 in TM3 cells.

Glyphosate induced ER stress and activated the PERK/eIF2α signaling pathway in TM3 cells
To investigate whether glyphosate exposure causes ER stress and activates the PERK/eIF2α signaling pathway in TM3 cells, the expression levels of related proteins were detected by western blotting. Results found that exposure to glyphosate at 5 mg/L for 1 h significantly increased all of the protein levels of Bip, p-PERK, PERK and p-eIF2α, which peaked at around 12 h ($p < 0.05$) (Figure 4A-D), but the protein level of eIF2α was not affected by glyphosate (Figure 4E). When TM3 cells were pretreated with 10 mM PBA, an inhibitor of ER stress for 2 h, all of the glyphosate-induced increases in protein levels of Bip, p-PERK, PERK and p-eIF2α were completely inhibited (Figure 5A-D) ($p < 0.05$). These results indicated that glyphosate could induce ER stress and the activation of PERK/eIF2α signaling pathway in TM3 cells.

Glyphosate-induced testosterone synthesis disorders depended on ER stress
In the present experiment, the role of ER stress in glyphosate-induced testosterone synthesis disorders was investigated. The results showed that PBA, the inhibitor of ER stress not only significantly rescued glyphosate-inhibited expressions both of StAR and CYP17A1 ($p < 0.05$), but also effectively restored the testosterone secretion inhibited by glyphosate ($p < 0.05$). It indicated that glyphosate-induced testosterone synthesis disorders were mediated by the ER stress.

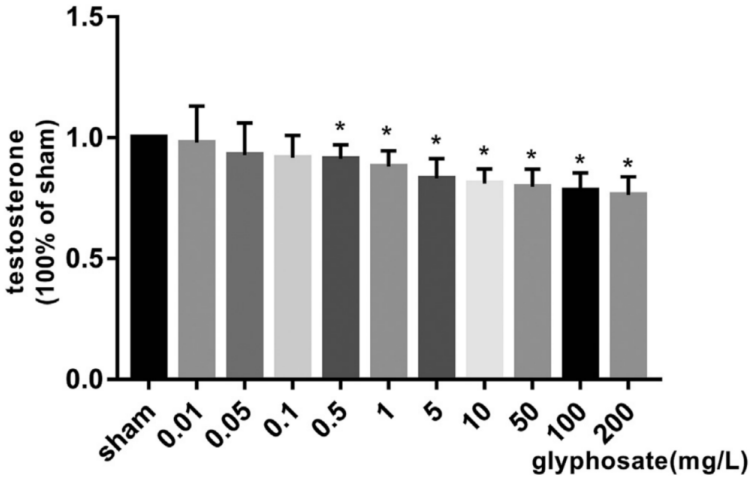
PERK/eIF2α signaling pathway mediated glyphosate-induced testosterone synthesis disorders
To investigate the role of PERK/eIF2α pathway in the glyphosate-induced testosterone synthesis disorders, TM3 cells were pretreated with 0.5 μM GSK2606414, an inhibitor of PERK activation for 2 h, and then incubated with 5 mg/L glyphosate for 24 h. The results showed that GSK2606414 pre-treatment significantly reduced glyphosate-induced phosphorylation of PERK and eIF2α ($p < 0.05$). In addition, the decrease both of StAR and CYP17A1 protein expression induced by glyphosate was completely blocked ($p < 0.05$), and the glyphosate-inhibited testosterone secretion also was effectively rescued ($p < 0.05$). These results suggested glyphosate-induced testosterone synthesis disorders depended on StAR and CYP17A1 which mediated by PERK/eIF2α signaling pathway.

Figure 1: **Effects of glyphosate on TM3 cell viability**



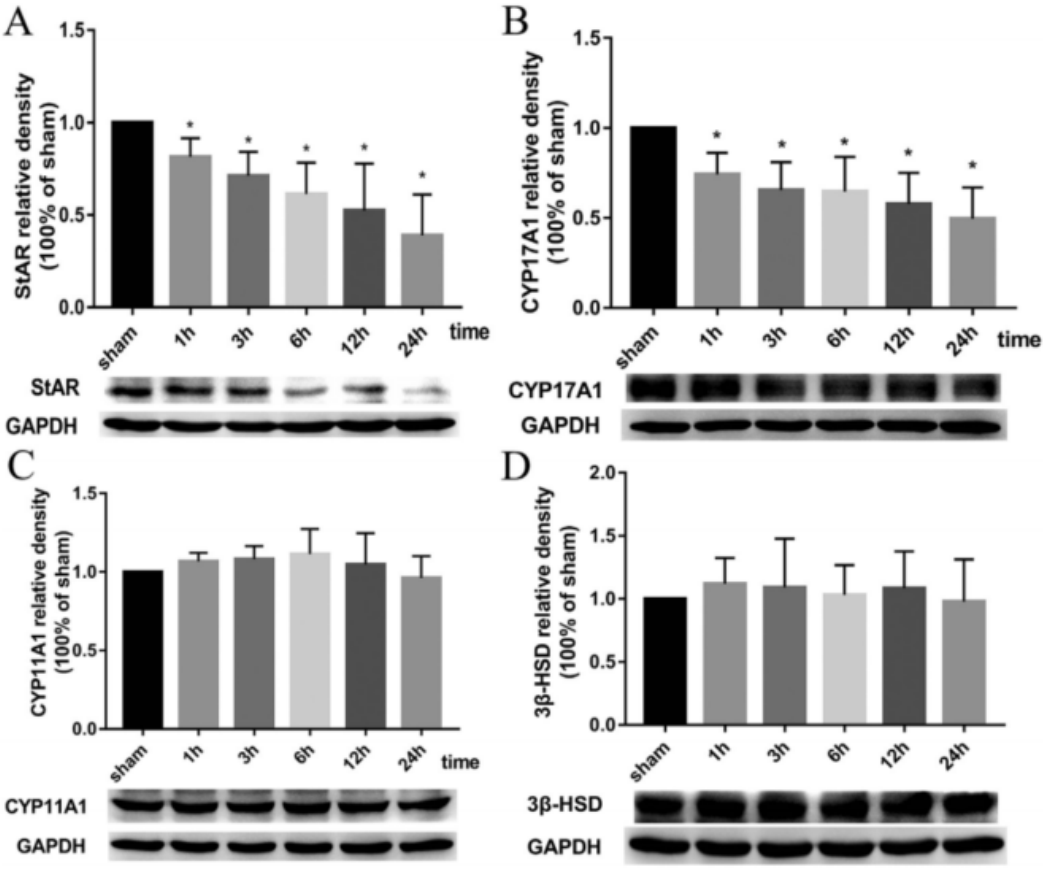
TM3 cells were exposed to different doses of glyphosate in 96-wells plate for 24 h and cell viability was measured using CCK8. Data were presented as the mean ± SD for n = 4 independent experiments.
*p <0.05, compared with the sham group.

Figure 2: **Glyphosate inhibited testosterone secretion of TM3 cells**



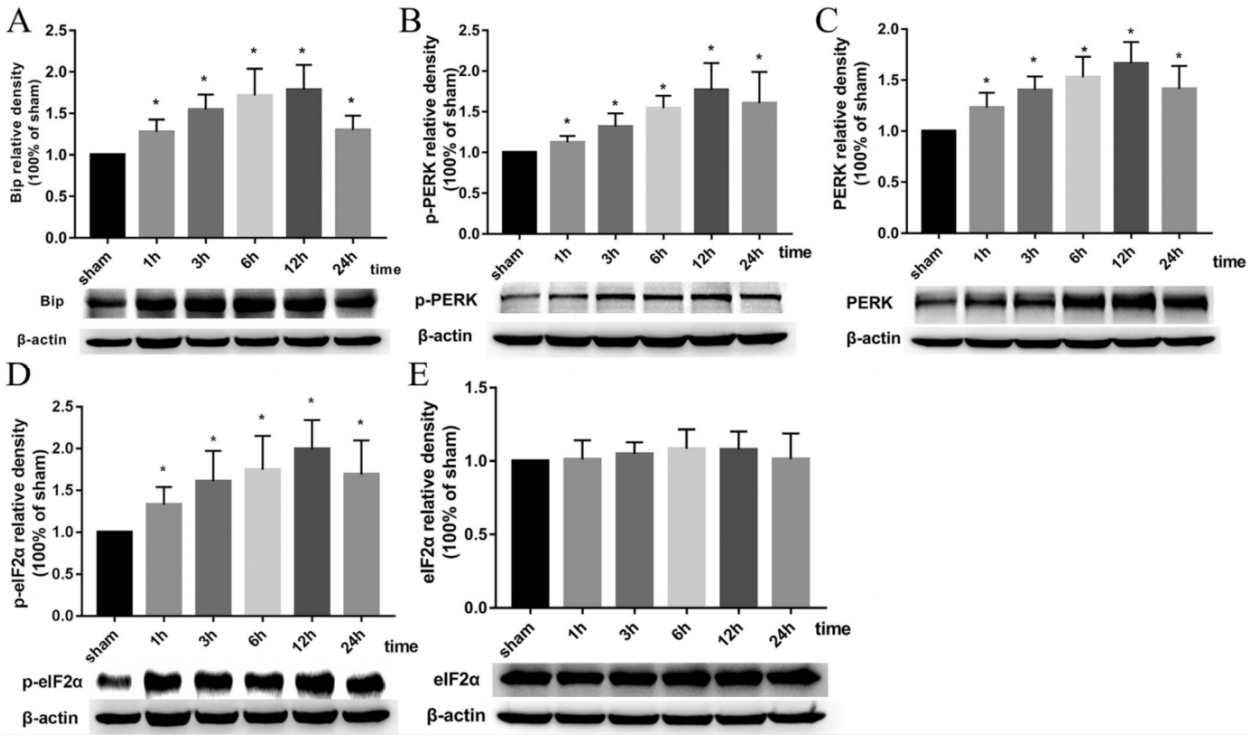
TM3 cells were exposed to different doses of glyphosate for 24 h. The cell supernatants were collected and the testosterone was assayed using ELISA kit. Data were presented as the mean ± SD for n = 4 independent experiments.
*p <0.05, compared with the sham group.

Figure 3: **Glyphosate inhibited the protein expression levels of testosterone synthesis enzymes in TM3 cells**



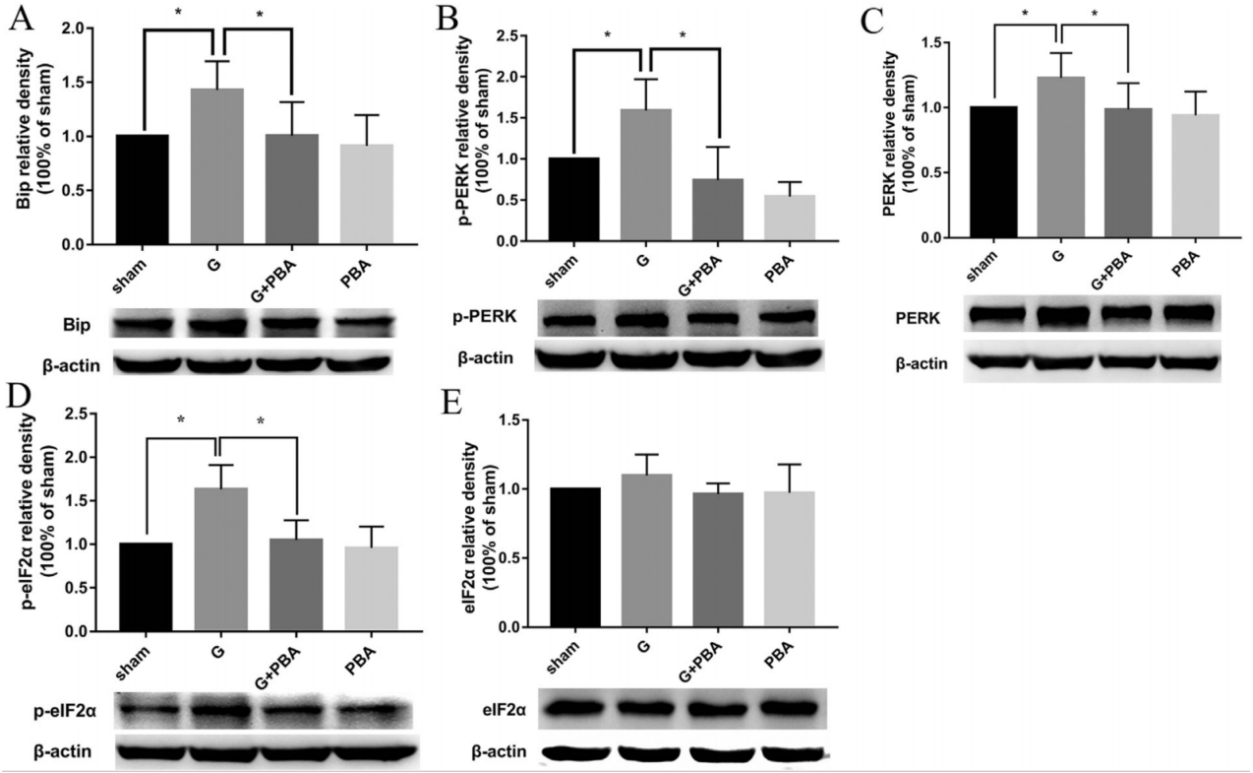
TM3 cells were exposed to 5 mg/L glyphosate for 1-24 h. The level of StAR, CYP17A1, CYP11A1 and 3β-HSD were detected with western blotting. Data were presented as the mean ± SD for n = 8 independent experiments.
*p <0.05, compared with the sham group.

Figure 4: **Glyphosate induced ER stress and activated the PERK/eIF2α signalling pathway in TM3 cells**



TM3 cells were exposed to 5 mg/L glyphosate for 1-24 h. The level of Bip, p-PERK, PERK, p-eIF2α and eIF2α were detected with western blotting. Data were presented as the mean ± SD for n = 8 independent experiments.
*p <0.05, compared with the sham group.

Figure 5: **PBA alleviated ER stress induced by glyphosate in TM3 cells**



TM3 cells were pre-treated with 10 μM PBA for 2 h, and then incubated with 5 mg/L glyphosate for 24 h. The level of Bip, p-PERK, PERK, p-eIF2α and eIF2α were detected with western blotting. Data were presented as the mean ± SD for n = 8 independent experiments.

*p <0.05. Panel labels are Sham for negative control, G for glyphosate exposure only, G + PBA for PBA treatment before glyphosate exposure, and PBA for PBA treatment only. G: glyphosate; PBA: phenylbutyric acid.

Conclusions

The authors conclude that glyphosate could inhibit testosterone synthesis via decreasing the expression of testosterone synthase StAR and CYP17A1, which depends on the ER stress and activation of PERK/eIF2α signalling pathway in Leydig cells.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In vitro study on the effects of glyphosate on testosterone secretion and the role of endoplasmic reticulum stress in the process were investigated in TM3 cells. Results showed that exposure to glyphosate at concentrations below 200 mg/L had no effect on cell viability, while glyphosate at concentrations above 0.5 mg/L could inhibit the testosterone secretion in TM3 cells. Treatment of TM3 cells with glyphosate at 5 mg/L not only reduced the protein levels of testosterone synthase StAR and CYP17A1 but also inhibited testosterone secretion.

The article is classified as reliable with restrictions for the following reason: not enough information on the tested material (purity) was provided, no positive controls were used, and no statistical methods were described. Furthermore, no OECD guidelines were followed, no GLP status was stated, and no historical control data (HCD) were provided to compare the relevance of data. In addition, key literature in disagreement with the authors' findings appear to have been disregarded, suggesting bias within the research and the following publications: Hecker (2011), OECD validation of the H295R steroidogenesis assay with glyphosate; Levine (2007), demonstrating a lack of effect of glyphosate on the StAR protein; US EPA (2015), glyphosate EDSP weight of evidence evaluation; and EFSA (2017), peer review of glyphosate potential endocrine-disrupting properties.

Hecker M et al (2011), The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study, Environmental Science and Pollution Research 18(3):503-15

Levine S. L. Et al. (2007), Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis, Cell Biol Toxicol (2007) 23:385-400

US EPA (2015), EDSP Weight of Evidence Conclusions on the Tier 1 Screening Assays for the List 1 Chemicals - EDSP: WEIGHT OF EVIDENCE ANALYSIS OF POTENTIAL INTERACTION WITH THE ESTROGEN, ANDROGEN OR THYROID PATHWAYS - CHEMICAL: GLYPHOSATE

EFSA (2017): Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate, Question number: EFSA-Q-2016-00663

Assessment and conclusion by RMS:

Reliability Criteria: *In Vitro* Toxicology Studies		
Publication: Xia Y. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	Not stated
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate Purity not stated

Reliability Criteria: *In Vitro* Toxicology Studies		
Publication: Xia Y. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	N	TM3 cells (no purity stated)
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	Not necessary
Test concentrations in physiologically acceptable range (<1 mM)	N	0.01 to 200 mg/L
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive controls
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	Not enough information on the tested material (purity), no positive controls were used, no statistical methods were described. Furthermore, no OECD guideline followed, no GLP status was stated. In addtion, no HCD were available in order to compare the relevance of the data. In addition, key literature in disagreement with the authors' findings appear to have been disregarded, suggesting bias within the research and publication: Hecker (2011) OECD validation of the H295R steroidogenesis assay with glyphosate; Levine (2007) demonstrating a lack of effect of glyphosate on the StAR protein; US EPA (2015) glyphosate EDSP weight of evidence evaluation; EFSA (2017) peer review of glyphosate potential endocrine-disrupting properties.
Not reliable	N	

1. Information on the study

Data point:	CA 5.8.2
Report author	Yahfoufi Z. A. *et al.*
Report year	2020
Report title	Glyphosate Induces Metaphase II Oocyte Deterioration and Embryo Damageby Zinc Depletion and Overproduction of Reactive Oxygen Species
Document No	Toxicology, (2020) Vol. 439, Art. No. 152466
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study effects of glyphosate (0 – 300 μM) on metaphase II mouse oocyte quality and embryo damage was investigated to obtain insight on its mechanisms of cellular action and the tolerance of oocytes and embryos towards this chemical. The work shows that glyphosate exposure impairs metaphase II mouse oocyte quality via two mechanisms: 1) disruption of the microtubule organizing center and chromosomes such as anomalous pericentrin formation, spindle fiber destruction and disappearance, and defective chromosomal alignment and 2) substantial depletion of intracellular zinc bioavailability and enhancement of reactive oxygen species accumulation. Similar effects were found in embryos.

Materials and methods

Materials

Metaphase II noncumulus oocytes and embryos from a B6C3F1 mouse crossed with a B6D2F1 mouse were obtained commercially from Embryotech Inc. in cryopreserved straws using the ethylene glycol-based slow freeze cryopreservation protocol. Human tubular fluid (HTF) medium with gentamicin was obtained from Irvine Scientific Inc. Anti-α tubulin antibody, fluorescein isothiocyanate (FITC) conjugate anti-goat antibody, 4′,6′-diamino-2-phenylindole (DAPI), and 0.1% Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The anti-fade agent, formaldehyde, SYTO 64 Red Fluorescent Nucleic Acid Stain, Alexa Fluor® 633 goat anti-mouse IgG, and dimethylsulfoxide (DMSO) were obtained from Thermo Fisher Scientific Inc. The Purified Mouse Anti-Mouse Pericentrin was purchased from BD Biosciences and Fab Fragment Affinity Purified Antibody was purchased from Jackson ImmunoResearch Inc (West Grove, PA, USA). The cellular ROS detection assay kit and zinc detection kit (Zinquin ethyl ester) were obtained from Abcam (Cambridge, UK). Glyphosate ($C_3H_8NO_5P$, powder form) was purchased from Research Products International (Mt. Prospect, IL, USA; catalog G36060, CAS 1071-83-6).

Methods

Frozen mouse metaphase II oocytes and embryos were obtained from Embryotech (MA, USA). The concentrations of glyphosate treatment used here ranged from 0 to 300 μM.

Study the effect of glyphosate on MT and CH alignment of metaphase II noncumulus oocytes (total number of oocytes ≈ 180)

Oocytes were thawed and transferred from straws to phosphate buffer saline (Dulbecco PBS) and washed for 3 min to remove cryo-protectant. Oocytes were then transferred to HTF media and incubated at 37 °C and 5% carbon dioxide (CO_2) for 60 min to allow spindle re-polymerization and attainment of normal oocyte architecture. The oocytes were then screened for the presence of the polar body confirming their Metaphase II stage. Immature oocytes or those that displayed disrupted zona pellucida were discarded.

In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average; total ∼180 oocytes used across replicates) were exposed to 0, 25, 50, 75, 100, 200, and 300 μM glyphosate for a 2 h incubation period (37 °C, 5% CO_2) to observe the effects on oocyte MT and CH alignment. Only some concentrations were selected for presentation to improve focus. Untreated oocytes served as controls.

Immunostaining of the oocytes and confocal microscopy examination

Oocytes were subsequently fixed in a solution prepared from 2% formaldehyde and 0.2% Triton X-100 for 30 min at 25 °C, then washed with PBS for 3 min. Subsequently, the oocytes were subjected to indirect immunostaining in mouse primary anti-α-tubulin antibody (1:300) against the MT overnight at 25 °C, then washed and placed in secondary FITC conjugated anti-goat antibody (1:50) for 45 min at 25 °C. After washing in PBS, the chromosomes were stained using DAPI (1:100) for 15 min at 25 °C. For immunostaining of pericentrin, oocytes were treated with primary antibody, Purified Mouse Anti-Mouse Pericentrin (1:100), overnight at 4 °C prior to staining with secondary antibody, Alexa Fluor® 633 goat anti-mouse IgG (1:200) for 2 h at 25 °C. Oocytes were then washed 3 times before immunoglobulin blocking with Fab Fragment Affinity Purified Antibody (20 μg/ml) for 30 min at 25 °C, prior to washing and staining for MT and chromosomes.
Stained oocytes were loaded into anti-fade agent on slides with two etched rings and cover slips placed using transparent nail varnish. Slides were stored at −20 °C and protected from light until they were evaluated for more details by confocal microscopy. To obtain confocal images, slides were examined using a Zeiss LSM 510 META NLO microscope using FITC (green), DAPI (blue), and Alexa Fluor® 633 (bright red) fluorescent filters with excitation and emission wavelengths of 495 and 519 nm, 358 and 461 nm, and 632 and 647 nm, respectively. Oocytes were localized using a 10x magnification lens and spindle alterations were assessed using 40x oil immersion lens. The MT was stained fluorescent green, which was distinct from the fluorescent blue staining of chromosomes.

Assessment of MT AND CH Alignment (Scoring System)

Each treated and control oocyte from every experiment set was closely examined for spindle and chromosome configurations. Three independent observers who were blinded to treatment group assignment and used comprehensive evaluation of the individual optical sections performed the categorization of oocytes based on MT and CH configurations.
Three observers scored the oocytes and compared the alterations in the MT and CH alignment to controls. Scores of 1-4 were assigned for both MT and CH alterations, with good outcomes associated with scores 1 or 2 and poor outcomes associated with scores 3 or 4. Good spindle configurations (score 1 or 2) generally exhibit barrel-shaped microtubules, whereas poor or abnormal configurations (score 3 or 4) display shortened spindle length, disorganization, or completely missing spindles. Good chromosomal configurations (score 1 or 2) generally exhibit chromosomes arranged at the equator of the spindle, while poor chromosomes (score 3 or 4) appear dispersed, irregular, or less condensed.

Detection of intracellular zinc in oocytes and embryos after glyphosate exposure (total number of oocytes ≈ 180; total number of embryos ≈ 180)

In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average) were thawed, prepared, and pretreated with increasing concentration of glyphosate for 2 h (0, 25, 50, 75, 100, 200, and 300 μM). Subsequently, oocytes were stained with 25 μM Zinquin ethyl ester in combination with 1 μM SYTO64 and incubated for 1 h at 37 °C, 5% CO_2. A single dilution of Zinquin ethyl ester was used in each dish to ensure that all dishes received the same concentration of dye. This zinc-selective fluorescent probe permeates the cell and labels the intracellular zinc ions to emit a quantifiable blue fluorescent signal. Cells were then washed in PBS for 3 min to remove any excess dye before fixation with 2% formaldehyde and 0.2 % Triton X-100 for 15 min at 25 °C. After washing, cells were loaded into anti-fade agent on slides for subsequent evaluation by confocal microscopy. A similar procedure was used for embryos (n = 7 embryos per group on average).

Detection of ROS generation in oocytes after glyphosate exposure (total number of oocytes ≈ 180; total number of embryos ≈ 180)

In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average) were thawed, prepared,

and exposed to 0, 25, 50, 75, 100, 200, and 300 μM glyphosate as outlined above in detail (under "oocyte preparation"). Oocytes were incubated at 37 °C, 5% CO_2 in glyphosate treatment for 2 h and in 100 μM pyocyanin for 45 min. Oocytes treated with pyocyanin served as positive control, while untreated oocytes served as negative control.
After incubating with the treatment, oocytes were stained with 500 μL volume of ROS deep red working solution (a mixture of 250 μL HTF media, 249 μL ROS buffer, and 1 μL ROS Deep Red dye) and incubated for 1 h at 37 °C, 5% CO_2 before subsequent washing in PBS. After fixation by 2% formaldehyde and permeabilization with 0.2% Triton X- 100 for 30 min, cells were washed and nuclei were lastly stained with DAPI (1:100) for 15 min at 25 °C. Cells were loaded into anti-fade agent on slides for subsequent evaluation by confocal microscopy. A similar procedure was used for embryos (n = 7 embryos per group on average).

Results

Glyphosate altered MT and CH alignment of metaphase II mouse oocytes

The untreated oocytes displayed the same normal spindle morphology throughout the 180 min incubation time, however, the treated oocytes displayed maximum MT and CH alterations (80-85%) at 120 min and remained unchanged beyond this point.
Untreated oocytes had a well-organized barrel shaped spindle structure (green) with chromosomes tightly aligned at the equatorial plate of the spindles (blue). Oocytes exposed to low glyphosate concentration had altered spindle configuration indicated by an enlarged "balloon" shape and some disturbance in the chromosome configuration, while oocytes treated with high glyphosate concentration (> 100 μM) exhibited a scattered pattern of chromosomes and missing microtubules. The damaging effects of glyphosate on both MT and CH quality were exhibited by the significant increase in the quantity of oocytes with poor scores when exposed to higher concentrations of glyphosate compared to control ($p < 0.001$). The control group had approximately 8.69% and 21.7% poor scores for both MT and CH scoring, respectively. The remaining percentage of oocytes with poor MT scores were 22.2% ($p < 0.05$), 72.2%, 77.7%, and 80% ($p < 0.001$) and poor CH scores were 50% ($p < 0.05$), 77.7%, 72.2%, and 80% ($p < 0.001$) for 50, 100, 200, and 300 μM glyphosate, respectively.
Overall, the results demonstrate that increasing glyphosate concentration induces damage to MT and CH alignment as well as substantial oocyte deterioration exhibited beyond 100 μM.

Glyphosate mediates pericentrin disappearance

Control oocytes had normal pericentrin (bright red) localized at each spindle pole, maintaining the spindle force balance with a well-organized MT structure (green) and chromosomes (blue) tightly aligned at the equatorial plate. Low treatment groups (50 μM) displayed pericentrin proteins moving closer together from each spindle pole, while remaining within the polar axis. However, at higher glyphosate concentrations (> 75 μM), pericentrin disappears disrupting the spindle force balance and causing chromosomal dispersion and spindle loss. Pericentrin was diminished significantly at 50 μM ($p < 0.01$), and the effect increases dramatically at 100 μM and higher ($p < 0.001$).
Overall, these findings demonstrate that increasing glyphosate concentration can cause substantial pericentrin damage, thus influencing MT and CH alignment and morphology.

Glyphosate reduced intracellular zinc content

Increasing glyphosate concentration correlated with zinc depletion as indicated by a decrease in blue fluorescent intensity compared to untreated control, which is likely attributed to glyphosate's chelating ability. Normalized CTCF was calculated for the groups tested. The difference between the CTCF of each treatment group compared to the untreated control was found to be statistically significant ($p < 0.001$). Oocytes treated with 300 μM had the lowest zinc-mediated fluorescence, while oocytes treated with 50 μM had the highest fluorescence with an average normalized CTCF of 3.8 ± 1.6 and 19.4 ± 3.4, respectively (mean ± SEM).

Effect of glyphosate exposure on intracellular zinc content in embryos: Similar to oocytes, intracellular zinc declined with glyphosate treatment. 50 μM glyphosate treatments reduced in tracellular zinc by 37%, and increasing the concentration of glyphosate maintained this effect ($p < 0.01$ for comparisons of treatment groups with the control).

Glyphosate increased ROS generation

Increasing glyphosate concentration correlated with an increase in ROS formation as signified by an increase in deep red fluorescence compared to negative control. Normalized CTCF was calculated for the groups tested. The difference between the CTCF of each treatment group compared to the negative control was found to be statistically significant ($p < 0.001$). Oocytes treated with 50 μM had the lowest ROS fluorescence, while oocytes treated with 300 μM had the highest ROS fluorescence with an average normalized CTCF of 28.3 ± 1.1 and 73.8 ± 2.6, respectively (mean ± SEM).
Similarly, changes in ROS production resulting from glyphosate exposure were also examined in embryos Glyphosate exerted a comparable effect in embryos, which showed increase in ROS production as the embryos were exposed to glyphosate. The experiment showed a significant increase in ROS production in the treatment groups when compared to control ($p < 0.01$ for comparisons of all treatment groups with the control).

Conclusion

The work demonstrates for the first time that glyphosate exposure causes metaphase II oocyte quality deterioration in dual mechanisms involving i) pericentrin abnormalities, spindle fiber destruction and disappearance, and disturbance in chromosomal alignment and ii) significant interference with intracellular zinc bioavailability and ROS accumulation. The work also links ROS enhancement and zinc deficiency mediated by glyphosate exposure to possible embryo damage.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The quality of metaphase II noncumulus oocytes and embryos from mice were investigated following glyphosate exposure at different concentrations (max 300 μM). The concentrations were in the range of those found in human blood following accidental acute exposure or suicidal attempts.

Results indicate that glyphosate provokes disruption of the microtubule organizing center and chromosomal disorganization at the mid-position of the spindle due to spindle disappearance, and defective chromosomal alignment as well as depletion of intracellular zinc bioavailability and enhancement of reactive oxygen species (ROS) accumulation in the mouse oocytes. In the embryos (not specified the source and the embryonal stage) zinc depletion and accumulation of ROS was also observed in a dose-related manner.

The article is classified as *reliable with restrictions* for the following reason: Not performed according to GLP or an OECD test guideline. No purity of the test substance stated. No information of the source and the embryonal stage of the embryos were provided. There were no concurrent positive control or substances known to deteriorates oocyte quality through disassembly of microtubule organizing centers (like peroxynitrite) or ROS accumulation (like hydrogen peroxide, and hypochlorous acid) or dimercapto-1-propanesulfonic acid (DMPS) for zinc depletion.

Assessment and conclusion by RMS:

Reliability criteria for *in vitro* toxicology studies		
Publication: Yahfoufi Z. A. et al. 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	No GLP or OECD test guideline followed
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	No purity, content, storage conditions
Only glyphosate acid or one of its salts is the tested substance	Unknown	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	No metabolic activation system present
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Max. concentration 300 µM
Cytotoxicity tests reported	N	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	Not performed according to GLP or an OECD test guideline. No purity of the test substance stated. No appropriate information on the tested embryos was provided. Tested dose correspond to level of glyphosate that may be found in human blood following human accidental acute exposure intoxication. No reference compound were tested in parallel to confirm the appropriateness of the testing procedures and the relevance of the results.
Not reliable	N	

グリホサートカリウム塩

要旨及び評価結果

（環境動態）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 7.5
Report author	De Polo, A. *et al.*
Report year	2019
Report title	From the traces in the wells of the urban aqueduct network to the subsequent prohibition of the use of glyphosate: the case of an area of high-intensity wine production in the province of Treviso, Veneto. Original Title: Dai residui nei pozzi della rete acquedottistica urbana al successivo divieto di utilizzo del glifosate: il caso di un'area ad alta intensità vitivinicola in provincia di Treviso, Veneto.
Document No	Igiene e sanità pubblica, (2019) Vol. 75, No. 6, pp. 451-460
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

In 2016, extraordinary samplings of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were carried out in 12 wells of the water network intended for domestic consumption in the territory of the Ulss2 (*Unità Locale Socio-Sanitaria*, Local Health Unit) 2 - District of Pieve di Soligo, Province of Treviso, Veneto region, Italy. The area includes 13 municipalities at high-intensity "Prosecco d.o.c.g." wine production. Traces of glyphosate (maximum reached 0.08 µg/L) and AMPA (maximum reached 0.25 µg/L) were detected in 2 wells supplying an urban area. Following these findings, an inter-municipal order to suspend the use of glyphosate was introduced and then entered definitively in the rural police regulation concerning all the municipalities in the Prosecco d.o.c.g. area, which led to the elimination of glyphosate and AMPA also in the initially contaminated wells. The case shows that high-consumption herbicides can reach the drinking water network of a city surrounded by territories with high agricultural activity. Moreover, the combined intervention of the institutions was fundamental to eliminate a "probable carcinogen" from the urban drinking water and to promote the abandonment of potentially harmful agricultural practices in favour of solutions with reduced environmental and health impact.

Materials and methods

The monitoring plan was developed in accordance with the 2014-2018 Regional Prevention Plan of Veneto "Plant protection products and health protection: raising awareness of compliance with correct sales conditions and the adoption of good usage practices". In 2016, the Prevention Department of the Ulss2 - *Marca Trevigiana* of Veneto prepared a monitoring plan for glyphosate and AMPA. This involved twelve drinking water sampling works in the territory (ex Ulss7) of the district of *Pieve di Soligo* (Treviso), a territory (of about 215,000 inhabitants) including 13 of the 15 municipalities forming the area of high wine business intensity for the production of Prosecco d.o.c.g. (Figure 1).

It should be pointed out that the two molecules, sampled by USGS Techniques and Methods 5-A10:2009, are subject to the same limits:

- The lower detection rate, intrinsic to the precision of the instrument, which at the beginning of the monitoring period was 0.05 µg/L, then lowered to 0.02 µg/L from 01/05/2017 thanks to technical improvements.
- The higher limit, defined by law as 0.1 µg/L (as per Legislative Decree 31/01).

Figure 1: **Location of the geographical area of interest. Province of Treviso, Ulss2 *Marca Trevigiana*, District of *Pieve di Soligo*.**

Results

As reported in the Table 1, ten of the twelve sampled sites showed no traceable glyphosate or AMPA residues. The other two, belonging to the water network of the city of *Conegliano* (Treviso), reported significant traces of glyphosate while the AMPA level reached the limit value imposed by law.

Table 1: **List of 12 drinking water collection works sampled in the *Pieve di Soligo* district by the Ulss2 Prevention Department - *Marca Trevigiana del Veneto* (Food Hygiene and Nutrition Service), in the period March-July 2016**

ID	Aqueduct	Sample date	Glyphosate [µg/L]	AMPA [µg/L]
1	*Conegliano* (*Scomigo*)	30/03/2016	0.05	0.10
2	*Vittorio Veneto*	27/04/2016	<0.05	<0.05
3	*Moriago della Battaglia*	11/05/2016	<0.05	<0.05
4	*Conegliano* (*Colnù*)	25/05/2016	0.08	<0.05
5	*Susegana*	11/07/2016	<0.05	<0.05
6	*San Pietro di Feletto*	15/06/2016	<0.05	<0.05
7	*Santa Lucia di Piave*	22/06/2016	<0.05	<0.05
8	*Tarzo*	29/09/2016	<0.05	<0.05
9	*Conegliano*	06/07/2016	<0.05	<0.05
10	*Farra di Soligo*	13/07/2016	<0.05	<0.05
11	*Farra di Soligo*	20/07/2016	<0.05	<0.05
12	*Conegliano*	27/07/2016	<0.05	<0.05

The two wells that tested positive at the first sampling have a depth of 22 m (*Scomigo*) and 30 m (*Colnù*) respectively. Both were then monitored approximately every month from March 2016 to February 2017. Traces of glyphosate and AMPA were detected in numerous subsequent samplings, exceeding the legal limit in three of them (Figures 2 and 3).

Following these findings, the municipality of *Conegliano* and four of its neighbouring municipalities (*Colle Umberto*, *San Pietro di Feletto*, *Tarzo* and *Vittorio Veneto*) have adopted, starting from 2 March 2017, a mayoral decree to suspend the use of glyphosate-based herbicides on the respective municipal soils, in compliance with the measures proposed by the Ulss2 Public Health and Hygiene Service. Following this ordinance, the monthly checks on the two wells in the city of *Conegliano*, which were positive at the first sampling, gave a stable negative result for all the following 12 months (Figures 4 and 5). It should be noted that, also as a result of other problems, the *Scomigo - Cal dell'Ebreo* well was excluded from the *Conegliano* drinking water network following the first evidence of contamination. However, a further check carried out on a public fountain in the urban centre of *Conegliano* (sampled monthly starting from 2017) reported traces of glyphosate (0.04 µg/L) on 14/06/2017 (Figure 6). Although this value is well below the legal limit and even lower than the traceability limit of the previously used instrument (until 01/05/2017), it confirmed the persistence of sporadic residual uses of glyphosate, as well as the ease with which the groundwater is subject to contamination.

Based on these considerations, the mayoral decree prohibiting the use of glyphosate was initially reiterated in all five municipalities also for the year 2018; it was then included in the inter-municipal regulation of the rural police, effective from the 1 January 2019 and concerning all 15 municipalities of the Prosecco d.o.c.g wine production area. This legislation banned the use of herbicides containing glyphosate, as well as all other herbicides, with the exception of those of natural and/or organic origin, on all crops, herbaceous, arboreal, arable land and orchards. An exception is made for orchards and vineyards that are young within three years of life or located on slopes where mechanical weeding is not feasible, for which the use of herbicides is allowed as long as they do not contain glyphosate, hazard warnings or risk phrases for human health.

Figure 2: **Levels of glyphosate and AMPA in sequential sampling at the well located in the hamlet of *Scomigo* in the municipality of *Conegliano* in the period March 2016 to February 2017**

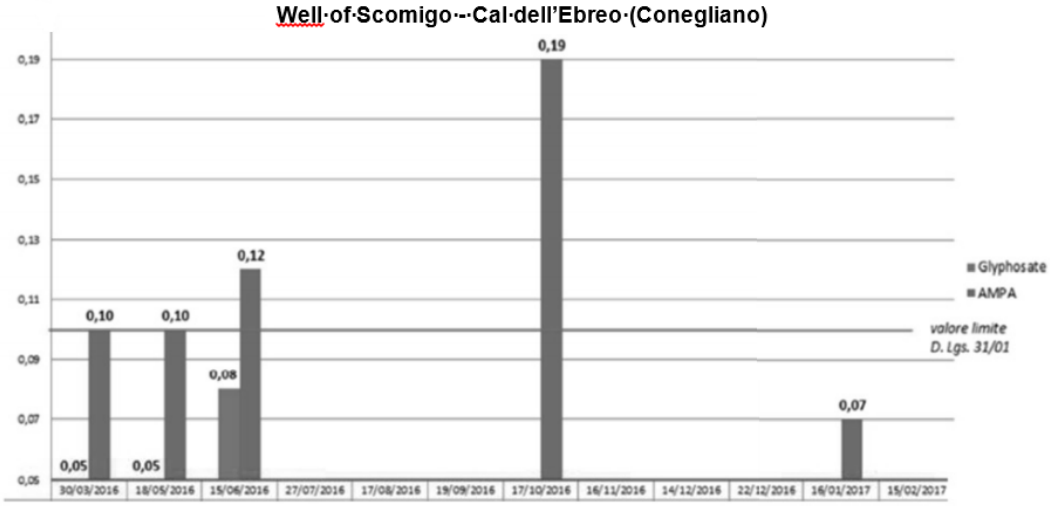


Figure 3: **Levels of glyphosate and AMPA in sequential sampling at the well located in *Colnù* in the municipality of *Conegliano* in the period May 2016 to February 2017**

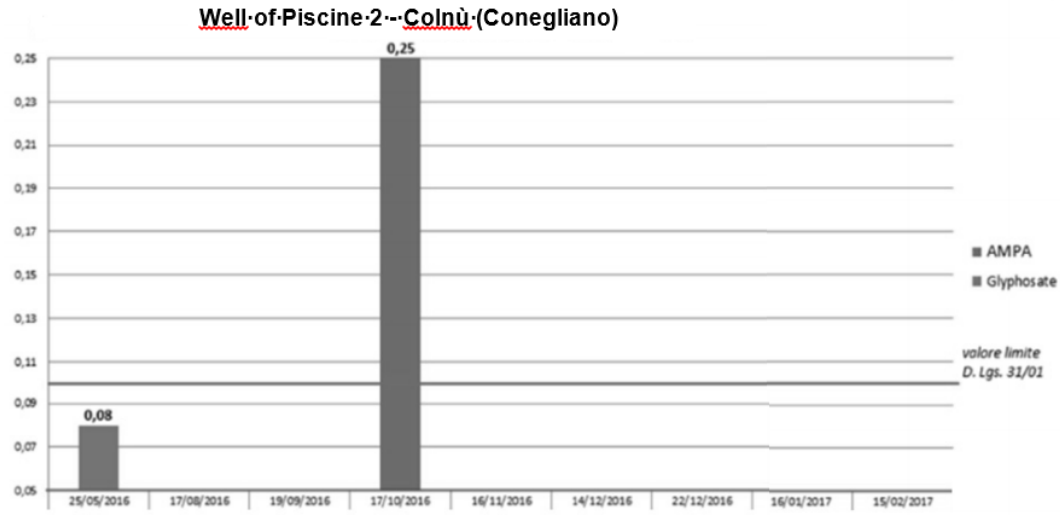


Figure 4: **Levels of glyphosate and AMPA in sequential sampling at the well located in the hamlet of *Scomigo* in the municipality of *Conegliano* in the period March 2017 to February 2018**

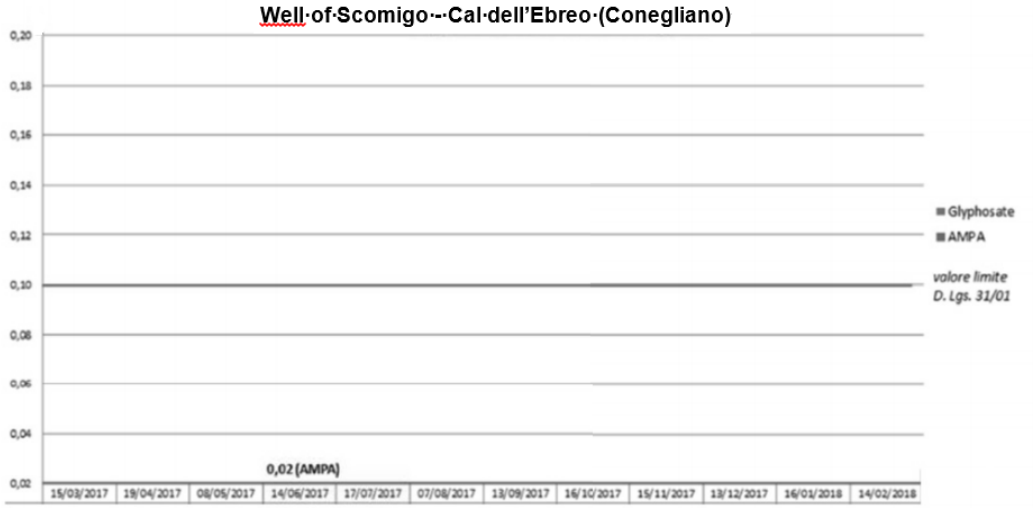


Figure 5: **Levels of glyphosate and AMPA in sequential sampling at the well located in the *Colnù* locality of the municipality of *Conegliano* in the period March 2017 to February 2018**

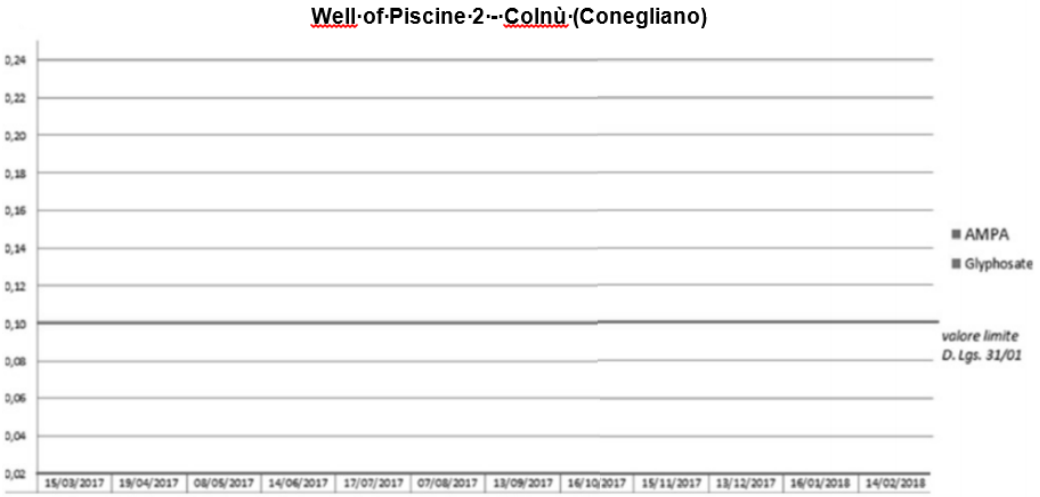
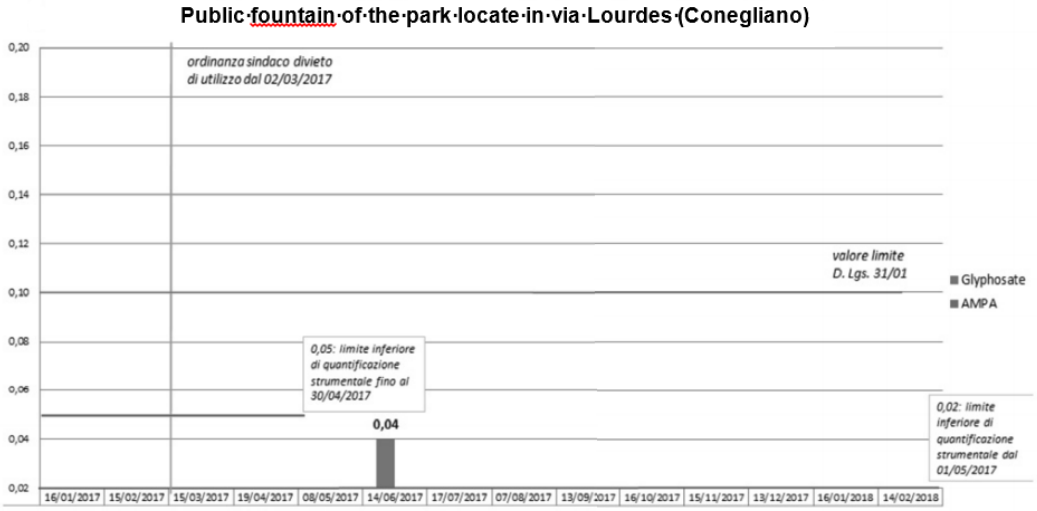


Figure 6: **Levels of glyphosate and AMPA in sequential sampling at the fountain of the public garden in *via Lourdes, Conegliano* in the period January 2017 to February 2018**



Conclusions

High-consumption herbicidal products such as glyphosate, and consequently its metabolite AMPA, can reach the drinking water network of an urban settlement surrounded by areas with high agricultural and wine-growing activity. Although the issue about the toxicity of glyphosate for humans is still widely debated, the experience of the Venetian Ulss2 demonstrates how the joint intervention of the institutions (in this case, the local Hygiene and Public Health Service and the municipal administrations) can contribute to eliminating the presence of a "probable carcinogen" in the city's drinking water. Furthermore, such interventions can make it mandatory to abandon potentially harmful agricultural practices, in favour of alternative solutions characterized by reduced impact on the environment and the health of local populations.

3. Assessment and conclusion

Assessmentand conclusion by applicant:
The article reports concentrations of glyphosate and AMPA found in 12 wells used for drinking water consumption in Northern Italy, situated within a wine growing area. Traces of glyphosate (maximum reached 0.08 µg/L) and AMPA (maximum reached 0.25 µg/L) were detected in 2 wells supplying an urban area. The article is therefore considered as reliable with restrictions. Sampling and analytical methods are not described. Nature of groundwater wells not described, point sources could be possible. No information on precipitation is reported. No description of monitoring sites other than very rough map of area and aqueduct names.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.1.4.2
Report author	Gros, P. *et al.*
Report year	2020
Report title	Leaching and degradation of $^{13}C_2$-^{15}N-glyphosate in field lysimeters
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Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate (GLYP) may have effects in various compartments of the environment such as soil and water. Although laboratory studies showed fast microbial degradation and a low leaching potential, it is often detected in various environmental compartments, but pathways are unknown. Therefore, the objective was to study GLYP leaching and transformations in a lysimeter field experiment over a study period of one hydrological year using non-radioactive $^{13}C_2$-^{5}N-GLYP labelling and maize cultivation. ^{15}N and ^{13}C were selectively measured using isotopic ratio mass spectrometry (IR-MS) in leachates, soil, and plant material. Additionally, HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) was used for quantitation of GLYP and its main degradation product aminomethylphosphonic acid (AMPA) in different environmental compartments (leachates and soil). Results show low recoveries for GLYP (<3 %) and AMPA (<level of detection) in soil after the study period, whereas recoveries of ^{15}N (11–19 %) and ^{13}C (23–54 %) were higher. Time independent enrichment of ^{15}N and ^{13}C and the absence of GLYP and AMPA in leachates indicated further degradation. ^{15}N was enriched in all compartments of maize plants (roots, shoots, and cobs). ^{13}C was only enriched in roots. Results confirmed rapid degradation to further degradation products, e.g., $^{15}NH_4^+$, which plausibly was taken up as nutrient by plants. Due to the discrepancy of low GLYP and AMPA concentrations in soil, but higher values for ^{15}N and ^{13}C after the study period, it cannot be excluded that non-extractable residues of GLYP remained and accumulated in soil.

Materials and methods

The leaching experiment was set up in two field lysimeters (non-weighing zero tension), which were installed in the Lysimeter Station at the Helmholtz Centre for Environmental Research-UFZ (Falkenberg, Germany; 52°51′ N, 11°48′ E). These lysimeters were constructed in 1981 in sheet steel vessels with cuboid shape of 1 × 1 m surface area and 1.25 m depth. The lysimeters were filled with sandy loam (0–30 cm topsoil: 74 % sand, 14 % silt, 12 % clay, pH 4.8, organic C = 1.1 %; 30-100 cm subsoil: 75 % sand, 17 % silt, 8 % clay, pH 5.6, organic C = 0.2 %) and an additional 25 cm-drainage layer composed of three sublayers (sand, gravel, and coarse gravel) at the bottom. The soil texture is representative for the river Elbe valley in the Federal State Saxony-Anhalt. Conventional agricultural management was oriented according to best management practice. In 2017, maize was planted which was embedded in a regionally typical crop rotation of sugar beets-winter wheat-potatoes-winter barley-maize. The present study investigated a period of one hydrological year starting in the hydrological summer semester in May 2017. Any weeds were removed mechanically, followed by $^{13}C_2$-^{15}N-GLYP (GLYP*i*) application (2017/24/04) via spraying as a worst-case scenario. Application rate was equivalent to maximum allowed annual for Germany (3.6 kg/ha/a) with practical concentration of GLYP formulations (480 g/kg) (360 mL GLYP*i*, dissolved in 750 mL H_2O). Drift by air flow was prevented by temporally fencing the application area with a ring of steel (1 m in height). Three days after GLYP*i* application, 5 L of the conservative KBr tracer solution was

applied at a rate corresponding to 40 kg/KBr/ha to each of the lysimeters to provide information on the movement of water through the soil column. Lysimeters were cultivated with maize (9 plants per lysimeter, equally spaced). No fertilizers or treatments for weeding were executed during the study period.

Sampling of leachates, soil, and plant material

Lysimeter soils were sampled from 0 to 5 cm depth (5 spots equally spaced in each lysimeter) at 4 dates over the study period (before and directly after application, 165 and 360 days after application). Soil sampling before application characterizes the basic level of GLYP*i* concentration, whereas the sample directly after application represents 100 % of initial GLYP*i*. To keep the soil column intact, samples from the whole topsoil (0-30 cm) and the subsoil (30-60 cm) were taken only at the end (day 360 after application) of the study period. Soil samples were air dried and sieved (2 mm). Subsamples of the sieved soils were finely ground for further measurement with IR-MS. Residues of GLYP*i* and AMPA*i* were extracted from 5 g of the sieved soil in 40 mL of a 1 M KOH solution (shaking overnight and centrifugation for 10 min at 1558 g) and stored at -20 °C until quantitation via HPLC coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS/MS).

Leachates were collected weekly in polyethylene canisters and volumes were recorded. Subsamples of 150 mL were taken and stored in a freezer at -20 °C in 3 × 50 mL centrifuge tubes for further measurements with ion chromatography (IC) and HPLC-ESI-MS/MS. A total of 50 mL of each sample were lyophilized to dryness (-50 °C, 0.025 mbar; Christ Alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, D-37250 Osterode, Germany) and solid residue amounts were weighed back and stored for measurements with isotopic ratio mass spectrometry (IR-MS).

Mature maize plants (roots, shoots, and cobs) were harvested in September 2017 from the two treated lysimeters and one untreated neighbouring plot as reference. Subsamples of 3 plants per lysimeter were harvested for further measurements of plant biomass. Moist weight was determined followed by drying at 60 °C and measuring of dry matter weight. Plant compartment samples (root, shoot, and cobs) were shredded and subsequently finely ground separately and stored until further measurements with IR-MS.

Sample analyses

Conservative tracer and isotope ratio analyses

Br^- tracer analysis in the leachate was performed using ion chromatography (column: Metrosep A SUPP 5150 × 4.0 mm, pre-column: Metrosep A SUPP 4/5 Guard, eluent: 0.3 mM Na_2CO_3 and 1.0 mM $NaHCO_3$, flow: 0.7 mL/min, separation mode: isocratic; Metrohm, D-70794 Filderstadt, Germany).

Isotopic ratios for $^{15}N/^{14}N$ and $^{13}C/^{12}C$ in soil, plant compartments, and lyophilized leachate samples were measured through the elemental analyser (Eurovector EA, Via F.lli Cuzio 42, 27100 PAVIA, Italy; IR-MS GVIsoprome, Elementar Analysensysteme GmbH, Elementar-Straße 1, 63505 Langenselbold, Germany) in the Institute for Nutritional Sciences, University of Gießen, Germany. For this purpose, finely ground soil and plant samples from the two treated sites and one untreated site (reference) were measured in triplicates. Lyophilized leachate samples from lysimeter leachates were measured in duplicates. Equations 1 and 2 show the calculation of $\delta^{15}N$ and $\delta^{13}C$ derived from isotopic ratios of the sample in relation to defined standard isotopic ratios from air for N and Pee Dee Belemnite (PDB) for C; values are generally given in ‰.

$$\delta^{13}C = \left(\frac{\left(^{13}C/^{12}C\right)_{\text{sample}}}{\left(^{13}C/^{12}C\right)_{\text{PDB}}} - 1 \right) \qquad (1)$$

$$\delta^{15}N = \left(\frac{\left(^{15}N/^{14}N\right)_{\text{sample}}}{\left(^{15}N/^{14}N\right)_{\text{air}}} - 1 \right). \qquad (2)$$

GLYP*i* and AMPA*i* analyses

Soil extracts and leachate samples were analysed for GLYP*i* and AMPA*i* with HPLC-ESI-MS/MS after derivatization with fluorenylmethyloxycarbonyl chloride (FMOC-Cl), as described in Wirth *et al.* (2019). The utilized system was composed of an LC-2040C Nexerai and a triple quadrupole mass spectrometer LCMS8060 (Shimadzu, Duisburg, Germany) equipped with a heated ESI-source. The FMOC derivatives were separated on a Gemini 3 µm NX-C_{18} column (Column 1: 150 × 2 mm, Aschaffenburg, Phenomenex, Germany).

Non-isotope-labelled GLYP (LGC Standards, Wesel, Germany) was used as internal standard for GLYP*i* (Sigma Aldrich, Taufkirchen, Germany) quantitation. Since AMPA*i* is not commercially available as a standard substance, no HPLC-ESI-MS/MS-optimization and, thus, no calibration could be carried out for this compound. Therefore, AMPA*i* was determined only qualitatively. Analytes were detected in the multiple reaction monitoring (MRM) mode. The MRM transitions were determined and optimized utilizing standard compounds (Table 1). However, as AMPA*i* is not commercially available, instrumental MRM optimization for AMPA*i*-FMOC could not be performed. Therefore, the settings for the MRM transitions for this compound were chosen as follows: optimization was carried out for ^{13}C-^{15}N-AMPA-FMOC and AMPA-FMOC (LGC Standards, Wesel, Germany) and their fragmentation patterns were utilized to derive the expected masses of the precursor and product ions for ^{15}N-AMPA-FMOC (AMPA*i*-FMOC). Further parameters of the MRM transitions were set by averaging values for ^{13}C-^{15}N-AMPA-FMOC and AMPA-FMOC (Table 1).

To further verify that the targeted and detected compound was the ^{15}N-AMPA-FMOC, a selection of samples was additionally separated on a different LC-column (Column 2: Kinetex 2.6 µm EVO C18 100 Å, 150 × 2.1 mm, Phenomenex, Aschaffenburg, Germany). The proposed AMPA*i*-FMOC was eluted from both columns at similar retention times as AMPA-FMOC (Table 1) which confirms its presence. Due to the lack of an AMPA*i*-FMOC calibration, these data could be evaluated only semi-quantitatively. Quantitation of GLYP*i* was carried out through weighting with the glyphosate internal standard signal.

Table 1 **Measurement modes for identification and quantitation of $^{13}C_2$-^{15}N-glyphosate and ^{15}N-aminomethylphosphonic acid using high performance liquid chromatography tandem mass spectrometry (HPLC-ESI-MS/MS)**

Component	Measurement mode	Precursor m/z	Product m/z	Collision energy	Retention time column 1(min)	Retention time column 2 (min)
$^{13}C_2$-^{15}N-Glyphosate-FMOC (GLP*i*)	-	392.10	170.15	14	9.10	8.77
			152.20	24		
			63.10	48		
Glyphosate-FMOC	-	390.00	168.15	14	9.10	8.76
			150.20	23		
			63.05	49		
^{15}N-AMPA-FMOC* (AMPA*i*)	+	335.20	179.05	−23	9.47	9.18
			178.15	−48		
			157.05	−10		
			113.05	−15		
AMPA-FMOC	+	334.20	179.05	−23	9.47	9.19
			178.15	−46		
			156.00	−10		
			112.05	−15		
^{13}C-^{15}N-AMPA-FMOC	+	336.20	179.05	−22	9.46	n.a.
			178.10	−50		
			158.15	−10		
			114.10	−15		

*derived from the optimized MRM transitions of ^{13}C-^{15}N-AMPA-FMOC and AMPA-FMOC

Results

Precipitation and leachate analysis

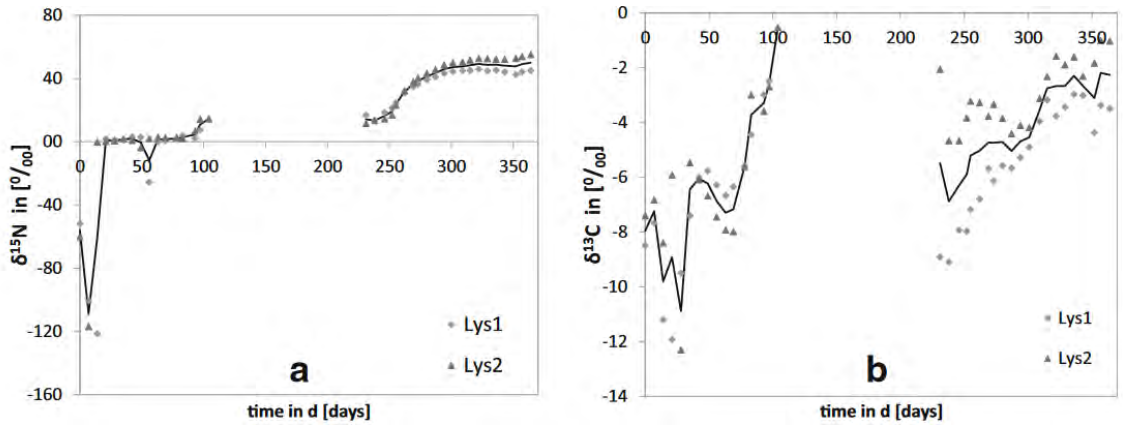
The study period from May 2017 to April 2018 was characterized by overall high amounts of precipitation that exceeded the monthly 30-year mean values (1981-2010) for this region, except for the months May, September, and February. Especially, June and July were characterized by heavy rainfall events that summed up to 123 and 125 mm per month precipitation, greatly exceeding the mean values of 57 ±22 mm (June) and 61 ±32 mm (July). These events resulted in large amounts of leachate in July 2017 (60.4 and 66.3 L). Weekly leachate amounts, collected from May 2017 until July 2017 to December 2017 until April 2018, had a mean volume of 5.1 L per week. For the period from August 2017 to November 2017, no leachates were received although precipitation occurred, most likely because of transpiration and water uptake by plants. Total volumes of leachates for the two lysimeters were 203 and 215 L over the study period. The Br^--breakthrough started in week 10 after application, where 35 and 37 L of leachate were received in the two tested lysimeters. Residues from the conservative tracer KBr were detected later on in all leachates. Due to the occurrence of Br^- in the leachates after 10 weeks and its slowly increasing concentrations over the following weeks along with continually received leachates, the main transport mechanism through the soil column can be assumed as matrix flow for the studied period.

For the natural ^{15}N background representing the ratio of $^{15}N/^{14}N$ of the air nitrogen, the $\delta^{15}N$ has been set to 0 (Fig. 1a). Discrepancies towards higher values indicate an enrichment of ^{15}N. In the first 2 weeks after application, a strong decrease of leachate $\delta^{15}N$ to negative values was detected, indicating an enrichment of ^{14}N. In the following weeks 3 to 10, the $\delta^{15}N$ in the leachate was constant between 0 and 1.7 ‰, and it increased over time from week 11 after GLYP*i* application. After the period with no leachates, the trend of $\delta^{15}N$ had a sigmoidal shape with an assumed maximum limit of about 50 ‰ for the last 10 weeks of the experimental period. This maximum level corresponds to a mass rate of about 10 µg ^{15}N/week of leached GLYP*i* active ingredient equivalent or its N-containing degradation products.

Values for $\delta^{13}C$ started at about -8 ‰ and fluctuated between -12 and -6 ‰ for the first 10 weeks before they strongly increased and reached values of -2.6 and -0.5 ‰ in the two lysimeters (Fig. 1b). After the period with no leachates, the $\delta^{13}C$ started at lower levels of -8.9 and -2.1 in lysimeters 1 and 2, respectively. The trend of increasing $\delta^{13}C$ values starting at -5.5 ‰ went on and ended at -2.3 ‰ for the remaining 20 weeks of the study, although with a less steep slope than in the first experimental phase.

Trends of ^{13}C and ^{15}N originating from GLYP*i* in the leachate (Fig. 1) did not correlate, which may be an indication for an independent movement of these isotopes through the soil column. Also, IR-MS cannot distinguish between GLYP*i* and its degradation products, but simultaneous occurrence and parallel trend would be an indication for a displacement of intact GLYP*i*, which appears unlikely from these data. The analyses for GLYP*i* and AMPA*i* using HPLC-ESI-MS/MS in the leachates showed no occurrence of residues of these compounds above detection limits (0.1 µg/L). Therefore, the leached ^{15}N and ^{13}C residues are most likely no constituents of intact GLYP*i* or AMPA*i* but originated from further degradation products.

Fig. 1 **^{15}N (a) and $\delta^{13}C$ (b) values for lyophilized leachates over the one-year study period in lysimeter 1 (Lys1) and lysimeter 2 (Lys2) and mean values (continuous line)**

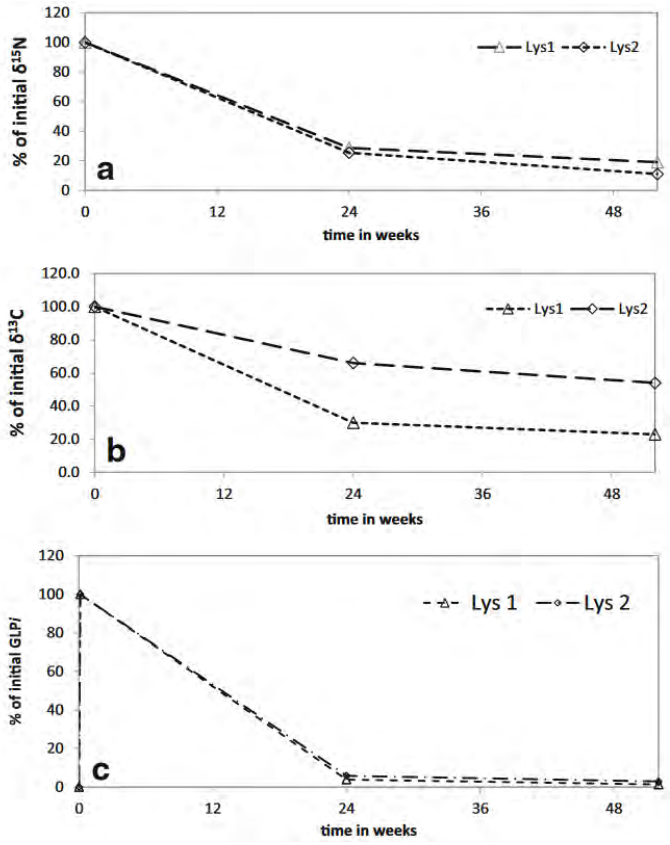


Soil analyses

The concentrations of GLYP*i*, ^{15}N and ^{13}C in the lysimeter soils derived from HPLC-ESI-MS/MS and IR-MS, respectively, were normalized and set to 100 % since GLYP*i* was not detectable in soil extracts sampled before GLYP*i* application (data not shown) (Fig. 2a). The $\delta^{15}N$ decreased within 165 days after application to 24 and 29 % of the initial value and decreased further to 11 and 19 % until the end of the study period. This indicates that amounts of the added artificial ^{15}N isotopes in soil decreased over time. The same was true for $\delta^{13}C$ which decreased down to 30 and 66 % compared with the initial value and ended at 23 and 54 % in the two lysimeters (Fig. 2b).

Measurement of the GLYP*i* residues through HPLC-ESI-MS/MS showed that about 4 and 6 % of the initial GLYP*i* concentration remained in the soil after 165 days and the recovery decreased further down to 1 and 3 % in the two lysimeters until the end of the study (Fig. 2c). AMPA*i* was detected in the topsoil extracts of all samples after application, and GLYP*i* and AMPA*i* were not detected in the subsoil (results not shown). This indicates that AMPA*i* had not been formed, and GLYP*i* was already decomposed by microorganisms or scarcely displaced from surface into subsoil. Therefore, leaching of GLYP*i* or AMPA*i* can be considered as insignificant in this experiment and rapid degradation to further products most likely happened. Nevertheless, it is still possible that strongly bound non-extractable, and therefore non-detected residues of GLYP*i* or AMPA*i* could have remained in soil too, partly explaining the higher amounts of ^{13}C and ^{15}N after 165 and 360 days (Fig. 2a and b).

Fig. 2 **Development of $\delta^{15}N$ (a), $\delta^{13}C$ (b), and $^{13}C_2$-^{15}N-glyphosate (c) in topsoil samples compared with initial values (set to 100 %) over the studied period in lysimeter 1 (Lys1) and lysimeter2 (Lys2)**



Plant material analyses

^{15}N was enriched highly significantly ($p < 0.01$) in all sampled plant compartments (root, 39 ±10 ‰ and 54 ±16; shoot, 28 ±13 ‰ and 51 ±16 ‰; cob, 34 ±12 ‰ and 51 ±14 ‰) compared with reference plant parts from a lysimeter that was not treated with GLYP*i* (root, 2.5 ±1.6 ‰; shoot, 2.0 ±0.9 ‰; cob, 4.0 ±1.9 ‰) (Fig. 3). By comparison, ^{13}C was highly significantly enriched only in the plant roots from the two lysimeters treated with GLYP*i* (-12.75 ±0.07 ‰ and -12.84 ±0.06 ‰) compared with maize roots from the lysimeter with no herbicide treatment (-13.03 ±0.08 ‰). In contrast, ^{13}C was significantly depleted ($p < 0.01$) in the cob material of plants from lysimeters with GLYP*i* treatment (-23.24 ±0.18 ‰ and -24.06 ±0.96 ‰) compared with those with no treatment (-22.84 ±0.25 ‰). There was not a significant difference in the shoots between the treated (-13.69 ±0.06 ‰ and -13.58 ±0.05 ‰) and non-treated lysimeters (-13.56 ±0.45 ‰).

The enrichment of ^{15}N in roots, shoots, and cobs can result only from uptake from the soil and distribution through the plant. Since ^{15}N is bound in GLYP*i* or its ^{15}N containing degradation products (Fig. 4), those degradation products must have acted as plant nutrients. Furthermore, as plants do not take up organic substances like GLYP*i* or AMPA*i* over the root system, the occurrence of ^{15}N can be plausibly explained only by an uptake of mineral ^{15}N ($^{15}NH_4^+$ and/ or $^{15}NO_3^-$) as mineralized degradation products from GLYP*i*, which are formed by microbial degradation in the rhizosphere (Duke *et al.* 2012).

The enrichment of ^{13}C in the roots compared with plants from the non-treated lysimeter may be explained by attachment, possibly due to mycorrhizal fungi associated with the maize roots (Bott *et al.* 2011) that utilize organic substances as nutrients for growth.

In summary, since (i) ^{15}N has been taken up by the maize roots and distributed into all plant compartments and (ii) ^{13}C is only associated with the plant roots, the interaction of these labelled atoms with the plants most plausibly resulted from the independent interaction of the inorganic degradation products of GLYP*i*.

$^{13}CO_2$ and $^{15}NH_3$ as the inorganic end-products of the degradation process can be emitted via the air path. This was shown for ^{14}C labelled GLYP (Grundmann *et al.* 2008). But since (i) $^{15}NH_3$ is water-soluble and forms $^{15}NH_4^+$ in soil solution and (ii) ^{15}N was taken up by plants, it is rather unlikely for inorganic N to be emitted into the air.

Fig. 3 **$\delta^{15}N$ and $\delta^{13}C$ mean values in plant material of roots, shoots, and cobs of maize plants of the tested lysimeter 1 (Lys1), lysimeter 2 (Lys2), and a reference lysimeter (LysRef)**

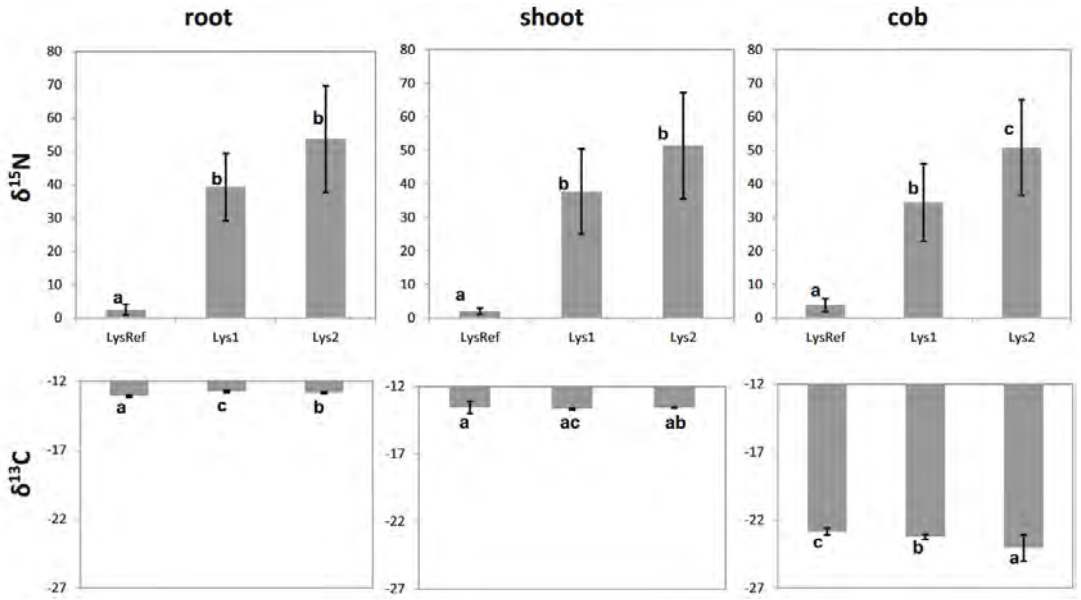
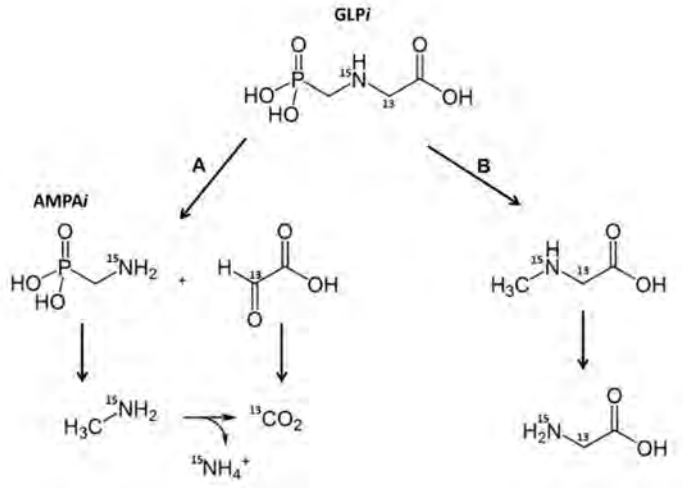


Fig. 4 **Degradation pathways of isotopic labelled $^{13}C_2$-^{15}N-glyphosate (GLP*i*) and its main degradation product ^{15}N-aminomethylphosphonic acid (AMPA*i*) with indicated positions of labelling (modified from Giesy *et al.* 2000)**



Discussion

The present study was designed in detail so that field conditions are reflected and also all relevant compartments are considered.

In the present study, concentrations of extracted GLYP*i*-residues were low in soil at the end of the study period compared with the initial concentrations at the beginning. But fractions of ^{15}N and ^{13}C above extracted GLYP*i*-residues indicate that either non-extractable GLYP*i* is still left and/ or further degradation products accumulated in soil.

The ^{13}C and ^{15}N are signals of leachates (Fig. 1), but absence or low concentrated (<LOD) residues of GLYP*i* and AMPA*i* indicate that further degradation products have been leached through the soil column. The noncorrelated appearance of ^{13}C and ^{15}N signals in leachates (Fig. 1) makes the degradation pathway B in Fig. 4 rather unlikely. Instead, pathway A in Fig. 4 is supported by the noncorrelated appearance of ^{13}C and ^{15}N signals in leachates, among which ^{13}C can originate from glyoxylic acid and ^{15}N from detected AMPA*i* or further degradation products, such as methylamine and ammonium ions (Fig. 4). Along with small concentrations of extracted GLYP*i* (Fig. 2c) under practically optimal leaching conditions in the very wet hydrological year 2017/ 2018, the present findings indicate that rapid degradation most likely is the best explanation for the absence of concentrations above LOD of GLYP*i* and AMPA*i* in leachates.

Conclusions

Isotopic ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ and resulting changes of $\delta^{13}C$ and $\delta^{15}N$ from isotopically labelled glyphosate (GLYP*i*) and its degradation products were successfully quantified using isotopic ratio mass spectrometry (IR-MS) in different compartments (leachates, soil, and plant material) of a field lysimeter. Therefore, this experimental approach was well suited to trace GLYP*i* under practice-near experimental conditions.

Since (i) the great decline of GLYP*i* content down to <3 % of initial amounts in soil during the one-year study period and (ii) a lower decline of ^{13}C (<60 %) and ^{15}N (<20 %), we conclude that either further degradation products had been formed and/ or non-extractable and, therefore, strongly bound GLYP*i* remained in soil and accumulated. The disparate increase of $\delta^{13}C$ and $\delta^{15}N$ values in leachates and plant material is explained plausibly by (i) rapid degradation of GLYP*i* within one vegetation period and, also (ii) the selective uptake of mineralized ^{15}N species from degraded GLYP*i* as plant nutrient, most likely NH_4^+ or NO_3^-. These findings from a wet hydrological year support the assumption that the risk of leaching of applied GLYP to other waterbodies can be considered to be low under central European climatic conditions. Accumulation in soil may enhance the risk of further distribution in the environment by soil erosion.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article reports results of a lysimeter experiment with $^{13}C_2$-^{5}N-glyphosate in Germany. Besides analysis of lysimeter leachate, also soil and plant (maize) samples were analyzed. Although, the methods and results are well described, no endpoint can be derived due to some deviations from the relevant guideline (OECD 22). For example, it is not clear whether an undisturbed soil monolith has been used, and the origin and storage of soil is not reported. Amounts of precipitation are not reported in sufficient detail (only for 2 months) and amounts of leachate are only given as overall sum and weekly averages. glyphosate, the sensitivity of the analytical method is not reported (AMPA was analyzed only qualitatively), and the stability of analytes in leachates and extracts during frozen storage was not shown. Results of leachate analysis were not reported in µg/L (only as $\delta^{13}C$ and $\delta^{15}N$), and results of soil analysis were only given in % of initial concentration.
The article is therefore considered as reliable with restrictions.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 8.6.2
Report author	Rogacz D. *et al.*
Report year	2020
Report title	Ecotoxicological effects of new C-substituted derivatives of *N*-phosphonomethylglycine (glyphosate) and their preliminary evaluation towards herbicidal application in agriculture.
Document No	Ecotoxicology and environmental safety, (2020) Vol. 194, Art. No. 110331.
Guidelines followed in study	OECD 208 (for NTTPs)
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable

2. Full summary of the study according to OECD format

In this paper, ecotoxicological and herbicidal effect of glyphosate was studied on monocotyledonous oat (*A. sativa*) and dicotyledonous radish (*R. sativus*) following the OECD/OCDE methodology (OECD 2006). The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure of glyphosate are presented. The EC_{50} values of shoots was 373.7 mg a.s./kg s.d.w. for oat, and 357.8 mg a.s./kg s.d.w. for radish. The EC_{50} values of roots was 269.3 mg a.s./kg s.d.w. for radish and 556.9 mg a.s./kg s.d.w. for oat. The EC_{50} values of fresh weight was 333.2 mg a.s./kg s.d.w. for radish and 418 mg a.s./kg s.d.w. for oat.

Materials and methods

Glyphosate (≥ 98.0 %) was purchased from Aldrich, Poznań, Poland.

Plant growth test

The plant growth test of glyphosate was performed in laboratory conditions following the Organisation for Economic Co-operation and Development standard Guideline Terrestrial Plants Growth Test (OECD 2006). According to the OECD 208 standard, the plant growth test was carried out in sandy soil of the following parameters: granulometric composition of soil: 77% sand, 16% dust and loam, organic carbon content of approx. 1.7%, pH (KCl) = 6.5.
Tests were carried out in polypropylene pots (diameter of 90 mm and capacity of 300 cm^3), which were filled with the control soil or with the soil mixed with the tested compounds added at following concentrations: 100, 200, 400, 800 and 1000 mg/kg of soil dry weight (s.d.w.). Each concentration was done in triplicate (3 pots for oat, 3 pots for common radish).
Twenty seeds of each of the selected plant species were sown into the soil. Oat (*Avena sativa*) and radish (*Raphanus sativus*) were selected as the representing species of monocotyledonous and dicotyledonous plants respectively. Seeds of each species originated from the same source. Plants were grown for 14 days under controlled conditions: a constant humidity content at the level required for the plants (70% field water capacity), temperature (22 ± 2 °C), constant light intensity (7000 lux), were maintained in the system of 16h/day and 8 h/night.
The evaluation of phytotoxicity at applied concentrations was made by comparing the germination, dry weight of control plants sprouts (seedlings) with germination and of dry and fresh plants sprouts grown in the soil with an admixture of given amounts of the tested compounds. Inhibition of fresh mass, root and shoot of plants was measured as described in the work by Lewkowski et al. 2016a, 2016b; Rogacz et al. 2018, Lewkowski et al. 2017a, 2017b, Matusiak et al. 2013.

Pigment assay

Photosynthetic pigments content was determined according to a method reported by Oren et al. 1995. Fresh leaves (200 mg) were thoroughly homogenized in 20 mL of 80% acetone in a cooled mortar and centrifuged afterward. The content of chlorophyll A, chlorophyll B and carotenoids was calculated based on the absorbance at wavelength 470, 647 and 664 nm. The content of photosynthetic pigments was expressed in mg/g of dry weight.

Statistics

One-way analysis of variance (ANOVA) has been used for comparison of obtained results related to phytotoxicity. Tukey's test with $p < 0.05$ (STATISTICA 13.3) was used to determine the significance of reported differences. The data presented are expressed as the mean with standard deviation obtained from 3 measurement replicates.

Results

Seedling emergence and growth test on oat and radish

The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure to glyphosate are summarized in the table below.

Table 1: Effects of glyphosate on the shoot height, the root length and the fresh matter of oat and radish seedlings plant compared to control (mean ± SD, n = 15).

Compound concentration [mg a.s./kg s.d.w.]	**Mean shoot height inhibition ± SD [%]**	**Mean root length inhibition ± SD [%]**	**Mean inhibition to fresh matter ± SD [%]**
	Oat		
100	7.2 ± 0.1	18.6 ± 1.1	10.7 ± 0.1
200	18.2 ± 0.4	25.1 ± 1.0	19.6 ± 0.4
400	53.4 ± 1.2	33.1 ± 0.6	43.8 ± 0.5
800	84.2 ± 2.0	41.2 ± 0.3	85.4 ± 0.7
1000	94.0 ± 0.9	90.4 ± 0.3	88.2 ± 0.1
	Radish		
100	7.3 ± 0.5	19.2 ± 0.3	11.1 ± 0.1
200	18.3 ± 0.7	26.7 ± 1.1	21.4 ± 0.1
400	57.1 ± 0.6	88.0 ± 0.8	60.7 ± 0.1
800	85.4 ± 0.3	91.8 ± 0.1	87.1 ± 0.1
1000	95.6 ± 0.1	94.2 ± 0.1	94.1 ± 0.1

Inhibition of shoots, roots and fresh matter in both plants (oat/radish) have been observed at all glyphosate concentrations tested (100, 200, 400, 800 and 1000 mg a.s./kg of soil dry weight).

Germination

The percentage germination of the tested plants treated with glyphosate is shown in the table below.

Table 2: Average changes (mean of three replicates) of germination of oat and radish treated with glyphosate.

Compound concentration [mg a.s./kg s.d.w.]	Number of Emerged Seedlings		Germination [%]	
	Oat	Radish	Oat	Radish
Control	20	19	100	100
100	19	17	98	88
200	19	16	98	86
400	18	15	93	77
800	18	13	92	70
1000	17	12	86	65
% Germination refers to number of emerged plants expressed as a percent of control plants.				

The higher the concentration of glyphosate in the soil, the stronger adverse effect was observed. At the highest concentration (1000 mg/kg), percentage germination was lower for radish (65%) when compared to oat (86%).

Dry matter

Dry matter of both treated plants oat/radish increased with growing concentration of glyphosate in the soil.

NOEC, LOEC and EC_{50} values

Changes of dry matter level caused by glyphosate are in accordance LOEC and NOEC values for oat and radish seedlings.

Calculated values of NOEC and LOEC of glyphosate for oat/radish were **100 and 200 mg a.s./kg of s.d.w.**, respectively.

The EC_{50} values of shoots was **373.7 mg a.s./kg s.d.w.** for oat, and **357.8 mg a.s./kg s.d.w.** for radish.

The EC_{50} values of roots was **269.3 mg a.s./kg s.d.w.** for radish and **556.9 mg a.s./kg s.d.w.** for oat.

The EC_{50} values of fresh weight was **333.2 mg a.s./kg s.d.w.** for radish and **418 mg a.s./kg s.d.w.** for oat.

Changes of pigment levels

The chlorophyll content measured in the radish/oat leaves decreased upon exposure to glyphosate. Glyphosate caused accumulation of carotenoids in leaves of both tested plants.

Conclusion

In this paper, ecotoxicological and herbicidal effect of glyphosate was studied on monocotyledonous oat (*A. sativa*) and dicotyledonous radish (*R. sativus*) following the OECD/OCDE methodology (OECD 2006). The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure of glyphosate are presented. The EC_{50} values of shoots was 373.7 mg a.s./kg s.d.w. for oat, and 357.8 mg a.s./kg s.d.w. for radish. The EC_{50} values of roots was 269.3 mg a.s./kg s.d.w. for radish and 556.9 mg a.s./kg s.d.w. for oat. The EC_{50} values of fresh weight was 333.2 mg a.s./kg s.d.w. for radish and 418 mg a.s./kg s.d.w. for oat.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study investigated the effects of glyphosate on the seedling emergence and growth of non-target terrestrial plants (oat and radish) based on OECD 208 guideline. Plants were exposed to glyphosate mixed into sandy soil at 5 concentrations between 100 and 100 mg a.s./kg of soil dry weight with 3 replicates each. There were 20 seeds of each plant species per test concentration sown into the soil. Evaluations were based on fresh mass, root length and shoot height of plants after 14 days of exposure.

The test design was adequately described, but the application of the test item into the soil is not specified. The seedling emergence was acceptable as recommended in the guideline (100 and 95 % in the control for oat and radish, respectively). However, the phytotoxic effects and the survival of the control plants during the study is not reported.
Reliable endpoints for the risk assessment of NTTPs can be obtained for glyphosate: EC_{50} value of 373.7 mg a.s./kg s.d.w. for oat based on shoot height and an EC_{50} value of 357.8 mg a.s./kg s.d.w. for radish based on shoot height.

The article is classified as reliable for NTTPs.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 8.1.4
Report author	Turhan D. Ö *et al.*
Report year	2020
Report title	Developmental and lethal effects of glyphosate and a glyphosate-based product on *Xenopus laevis* embryos and tadpoles
Document No	Bulletin of Environmental Contamination and Toxicology, (2020) Vol. 104, No. 2, pp. 173-179
Guidelines followed in study	ASTM (2003) American Society for Testing and Materials, Standard guide for conducting the Frog Embryo Teragonesis Assay-*Xenopus* (FETAX), E1439-98
Deviations from current test guideline	Jelly coats should be removed from developing embryos mid blastula stage embryos – ca. stage 8, prior to 96 hour exposure well plates. The removal is achieved using 2% cysteine solution followed by rinsing. No reference is provided in the article to see whether this step was completed (it is a relevant as it informs on ion-exchange and impacts from concentrated solution / osmotic and diffuse pressures on the embryos in the increasing concentrations of test media.) Test guideline followed for the late development stage 46 (96 hr test) exposure test was not stated. The FETAX assay runs until 80-90% of the control tadpoles reach developmental stage 47. This cannot be confirmed. Water quality at test start appears to be within specification, although after 96 hrs now water quality data appeared to have been determined.
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

Effects of pure glyphosate were evaluated using two embryonic development stages of *Xenopus laevis* as model system. No lethal or developmental effects were observed at all concentrations tested (up to 500 mg glyphosate/L). Measured biological parameters included growth (length) and measuring enzyme levels. Growth of tadpoles was measured after exposure of developing embryos in cell well plates for 96 hrs, exposed from developmental stage 8 for 96 hours. In a late stage tadpole exposure assay (from Stage 46) tadpoles were exposed to a range of concentrations prior to homogenising whole tadpoles for enzyme level analysis.

[Information in the publication relating to a potassium salt based glyphosate formulation has been excluded from this summary as it cannot be related to the representative formulation MON 52276].

Materials and methods

Glyphosate was purchased from Sigma-Aldrich (PESTANAL®, analytic grade, 45521).

The embryos and tadpoles used in the tests were obtained from male and female frog pairs from an adult *X. laevis* colony in the Author's laboratory. *X. laevis* breeding and acquisition of embryos were performed according to ASTM-E1439-98 (ASTM 2003). Embryos and tadpoles were maintained in a standard Frog Embryo Teratogenesis Assay Xenopus (FETAX) test medium (ASTM 2003) with the following composition: 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 \times 2H_2O$, 30 mg KCl, and 15 mg $CaCl_2$ per liter of distilled water.

The exposure solutions of glyphosate were prepared fresh daily in the standard FETAX test medium. The pH of glyphosate solutions was adjusted to 7.9, as recommended for FETAX tests, using NaOH. Embryos and tadpoles were exposed to test solutions under semi-static test conditions with a 12:12 h light:dark photo-period at 23 °C (± 1 °C).
Before starting the FETAX test, glyphosate levels in the test medium were measured using high-performance liquid chromatography (HPLC) (1100 system, Agilent Technologies, Santa Clara, CA, USA). The measured glyphosate concentrations in exposure media were determined to be at least 92% of the nominal concentrations.

For the FETAX test, stage 8 embryos were exposed to different glyphosate concentrations for 96 h. Four embryos were randomly selected, and placed with 2 mL of test medium into each well of 24-well plates, serving as one replicate per treatment. All concentrations were tested with eight replicates and thus a total of 32 embryos. In the FETAX test, seven concentrations of glyphosate (282–500 mg/L) were tested plus controls. The test medium was changed every 24 h. Dead tadpoles were removed and incidences were recorded. The median lethal concentrations (LC_{50}) were determined after 24, 48, 72, and 96 h of exposure. At termination of the bioassays, surviving embryos were euthanized and observed for developmental abnormalities.

For the tadpole-toxicity bioassays, stage 46 tadpoles were exposed to different glyphosate concentrations for 96 h. Five randomly selected tadpoles were placed in each well of 12-well plates containing 3 mL of test solution. All concentrations were tested with 6 replicates resulting in a total of 30 tadpoles per concentration. In the tadpole-toxicity tests, seven concentrations of glyphosate (250–403 mg/L) were tested excluding control groups.

For biochemical assays, stage 46 tadpoles were exposed to three glyphosate concentrations (50-250 mg/L) for an exposure period of 96 h. Fifteen randomly selected tadpoles and 10 mL of test solution were placed into 25 mL polycarbonate containers. All concentrations were tested with five replicates resulting in a total of 75 tadpoles per treatment. At the end of the exposure period, surviving tadpoles were euthanized and their enzyme activities were measured (glutathione S-transferase (GST), glutathione reductase (GR), carboxylesterase (CaE), acetylcholinesterase (AChE), superoxide dis-mutase (SOD)).

Graphpad Prism software (Version 5, USA) was used to calculate the average lethal concentration (LC_{50}) and 95% confidence intervals (CI) and for other statistical analyses. A log(dose)-normalized response curve ($Y = 100/ (1 + 10^{(logEC50-x)} \times hillslope$) to fit mortality data. For statistical analysis of biomarkers, data were tested initially for homogeneity of variances and normality distributions by the Bartlett and Kolmogorov-Smirnov tests, respectively. Nonparametric data were analyzed using Kruskal-Wallis test followed by pairwise comparisons of groups using Mann-Whitney U tests. Parametric data were analyzed using the One-way Analysis of Variance (ANOVA) followed by the unpaired t test. A Bonferroni correction was applied (0.05/3 = 0.016). In order to determine growth inhibition, the head-to-tail lengths were measured and the lengths were compared using ANOVA (Dunnett's post hoc test, $p < 0.05$).

Results

Even the highest glyphosate concentrations did not cause a lethality higher than 17% for both *X. laevis* embryos and tadpoles in this study.
Moreover, the highest glyphosate concentrations (403 and 500 mg/L) caused no growth inhibition in embryos or tadpoles (see table below).

Table 1: Length of stage 8 embryos and stage 46 tadpoles of *Xenopus laevis* after 96-h exposure to different concentrations of glyphosate.

Glyphosate					
Conc. (mg/L)	***n***	**Length (mm)[a)]**	**Conc. (mg/L)**	***n***	**Length (mm)[a)]**
8th stage			**46th stage**		
Control	32	7.14 ± 0.07	Control[b]	30	10.13 ± 0.10
282	31	7.15 ± 0.08	250	25	10.52 ± 0.07
310	31	7.14 ± 0.07	275	29	10.36 ± 0.08
342	26	7.04 ± 0.08	303	26	10.44 ± 0.09
376	32	7.22 ± 0.06	333	29	10.28 ± 0.04
413	32	7.21 ± 0.08	367	26	10.23 ± 0.08
455	30	7.38 ± 0.08	403	25	10.48 ± 0.07
500	29	7.04 ± 0.10	---	---	---

[a)] Lengths are expressed as mean± standard errors. These values were obtained from the lengths of the surviving individuals (n).
[b)] The average length of tadpoles at the beginning of this test is 7.30 ±0.10 mm (*n* = 32).

In addition, the selected biochemical markers in tadpoles exposed to glyphosate did not show any statistically significant changes (see table below).

Table 2: The enzyme activities of stage 46 tadpoles after 96-h exposure to different concentrations of glyphosate.

Glyphosate						
Concentration (mg/L)	***n***	**GST[a)]**	**GR[a)]**	**CaE[a)]**	**AChE[a)]**	**SOD[b)]**
Control	5	130 ± 3.9	11.2 ± 0.43	167 ± 4.4	142 ± 3.2	0.65 ± 0.05
50	5	134 ± 4.9	11.3 ± 0.38	155 ± 3.9	128 ± 6.9	0.60 ± 0.02
100	5	131 ± 1.0	9.7 ± 0.47	154 ± 2.5	126 ± 5.4	0.60 ± 0.05
250	5	141 ± 3.5	10.7 ± 0.28	170 ± 3.1	132 ± 6.5	0.64 ± 0.03

[a)] The enzyme activity was expressed as nmol/min × mg protein ± standard error.
[b)] The enzyme activity was expressed as U/mg protein ± standard error.

Conclusion

Effects of pure glyphosate were evaluated using two embryonic development stages of *Xenopus laevis* as model system. No lethal or developmental effects were observed at all concentrations tested (up to 500 mg glyphosate/L). Measured biological parameters included growth (length) and measuring enzyme levels. Growth of tadpoles was measured after exposure of developing embryos in cell well plates for 96 hrs, exposed from developmental stage 8 for 96 hours. In a late stage tadpole exposure assay (from Stage 46) tadpoles were exposed to a range of concentrations prior to homogenising whole tadpoles for enzyme level analysis.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Effects of pure glyphosate and a glyphosate-based product Roundup® Star (containing glyphosate in a form of a potassium salt and including 6% surfactant as ethoxylated alkylamine based, were evaluated comparatively using two embryonic development stages of the amphibian *Xenopus laevis* as model system. As the glyphosate-based product Roundup® Star is not the representative formulation for the European renewal of glyphosate, the summary only provides information for pure glyphosate.

However, this publication confirms a general trend that toxic effects caused by glyphosate-based products, compared to pure glyphosate, are increased mainly due to additives present in glyphosate formulations and that it may be a result of synergistic effects between glyphosate and adjuvant in the formulations.

In this study, no lethality >17 % or developmental effects (growth inhibition) were observed in embryos or tadpoles with pure glyphosate at any glyphosate concentration tested (282-500 mg/L for stage 8 embryos) and (250-403 mg/L stage 46 tadpoles)
In addition, no effect was observed with regards to enzymatic activity of stage 46 tadpoles at any glyphosate concentration tested (50-250 mg/L).

The article is classified as reliable with restrictions for the following reason : The specific purity of the test item was not reported. No OECD guidance has been followed. The American Society for Testing and Materials, Standard guide for conducting the Frog Embryo Teragonesis Assay-Xenopus (FETAX test), E1439-98 has been followed with some deviations to the recognised approach (see deviations above). The FETAX assay is a developmental toxicity screening test, which for the most part has been superseded by amphibian metamorphosis and developmental toxicity assays using *Xenopus laevis* (OECD 231 and OECD 241). Studies performed according to both of these recognised test guidelines were submitted with the Annex I dossier (M-CA Section 8.2.3/002 and M-CA Section 8.2.3/003). Whilst the FETAX assay is not directly recognised at the EU level, elements of the FETAX assay are considered in the conduct of the OECD 231 test guideline.
Control mortalities were not reported (only LC_{50} final results). Analytical verifications of the concentrations in the test medium were reported only before starting the test, but exposure medium was changed every 24 h to maintain the desired concentrations.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（農作物及び畜産物への残留）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 6.9
Report author	Panseri, S. *et al.*
Report year	2020
Report title	Occurrence of perchlorate, chlorate and polar herbicides in different baby food commodities
Document No	Food chemistry, (2020) Vol. 330, Art. No. 127205 DOI 10.1016/j.foodchem.2020.127205
Guidelines followed in study	SANTE/11813/2017: Method validation & quality control procedures for pesticide residues analysis in food & feed US FDA FVM (2015): FDA Foods and Veterinary Medicine Science and Research Steering Committee: Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data within the Office of Foods and Veterinary Medicine. US FDA FVM (2019): FDA Foods and Veterinary Medicine Science and Research Steering Committee: Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition. (Analytical methods)
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable

2. Full summary of the study according to OECD format

The incidence of glyphosate, its metabolite aminomethylphosphonic acid (AMPA) and other substances was estimated in baby food commodities (meat, fish, cheese, vegetable and fruit). Ion chromatography coupled to high resolution mass spectrometry analysis of the 105 samples did not show traces of glyphosate or its metabolite AMPA.

Materials and methods

Chemicals and reagents
Glyphosate and aminomethylphosphonic acid (AMPA) (certificated standards) and the internal standard (IS) *N*-Acetyl-D3-glufosinate were purchased from Merck (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Formic acid (98-100 %) was from Riedel-de Haën (Sigma-Aldrich). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

Standard solutions
The principal standard solutions of each compound (1 mg/mL) were prepared in water and stored as recommended by the EU Reference Laboratory for pesticides (Anastassiades *et al.*, 2015). The working solution was kept in 4 °C plastic flasks to avoid pesticide interaction with glass-surfaces.

Sample collection
A total of 105 baby food samples were collected; all details are specified in Table 1. They were from different commercial brands, present in international markets, and bought in different Italian supermarkets (94 samples) and in some Serbian supermarkets (11 samples). In particular, all products are commercialized as homogenized food, packaged in sterile conditions (jar of 80 g) made from vegetable, fruits, meats, fish, cheese or combining different of these matrices, directly ready-for-eat. No sample processing (rehydration/mixing) is necessary before consumption.

Table 1 **Sample collection details according to the different matrix typology**

Meat / meat+vegetables n=43[#]	**Fish / fish+vegetable n=13**	**Fruit / vegetables n=42**	**Cheese / cheese+ham n=7**
veal	plaice	apple	cheese (bovine milk)
swine	hake	plum	cheese and ham**
horse	plaice and potatoes*	pear	
lamb	trout and vegetables*	pear and blueberry	
rabbit	bream and vegetables*	apple and blueberry	
chicken	bream and potatoes*	apple and banana	
turkey	bass and vegetables*	apple and peach	
veal and ham	cod and potatoes*	apple and apricot	
chicken and carrots*	cod and vegetables*	banana and kiwi	
chicken with green beans and zucchini*	salmon and vegetables*	zucchini mix fruit	
turkey, corn and potatoes*		carrot and apple	
veal and carrots*		legumes	
veal and potatoes*		sweet potato and carrots	
veal, potatoes and mushrooms*		broccoli	
veal, broccoli and carrots*		carrots, potatoes and zucchini	
veal and vegetables*		mixed vegetables	
		tomato and vegetables	
		peas and spinach	

[#]- number of samples per category
*for mixed categories, meat and fish represented the major component as declared in the label
**for mixed categories, cheese is the dominant component as declared in the label

Sample extraction

The samples (1 g) were extracted as designated by Chiesa, Nobile, Panseri, and Arioli (2019). The only changes were the internal standard (IS), *N*-Acetyl-D3-glufosinate, and its concentration (0.1 µg/g) for each matrix category. Briefly, 1 g, representative of each single purchased sample, was spiked with the IS, extracted with a mixture of 3 mL of methanol and 7 mL of 1 % formic acidified water, vortexed and sonicated for 15 min, after centrifugation (4 °C, 10 min, 2500 ×g), 1 mL of the supernatant was filtered in a vial, ready for the analysis.

IC-HRMS Orbitrap parameters

Ion chromatography high resolution mass spectrometry (IC-HRMS) instrumentations, parameters and software are described in Chiesa *et al.* (2019). Briefly, the instrumental analysis was performed by an Ion Chromatography Dionex ICS-5000 + system (Sunnyvale, CA, USA) made up of Dual Pump, a Conductivity Detector and an Autosampler. The column was a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a guard column Dionex IonPac AG19-4 µm (2 × 50 mm) kept at 30 °C. The column was chosen on the basis of preliminary trials. In fact, the Thermo Scientific Dionex IonPac AS11 column provided similar results, as evaluated also by Rajski, Díaz Galiano, Cutillas, and Fernández-Alba (2018).

The KOH eluent was converted to water by a Dionex AERS 144 500, 2 mm suppressor (Thermo Scientific). The eluent flow rate (0.3 mL/min), chromatographic run duration (30 min) gradient and injection volume were the same as described by Chiesa *et al.* (2019). Chromatographical separation started with an isocratic 15 mM $KOH_{(aq)}$ elution for the first 8 min, than increased linearly (from 8 to 20 min) up to 55 mM $KOH_{(aq)}$, and was held in these conditions for next 4 min. The initial KOH concentration was brought back at 24.1 min, which was followed by 6 min equilibrium time. The injection volume was 50 µL.

Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), high resolution mass spectrometer equipped with heated electrospray ionization (HESI) source operating in negative mode was used for characterisation of compounds / anions of interest with the same operative conditions described in our original method (Chiesa *et al.*, 2019). Briefly, capillary temperature and vaporizer temperature were set at 330 °C and 280 °C, while the HESI voltage was 3.50 kV. Sheath and auxiliary gas were adjusted at 35 and 15 arbitrary units, with S lens RF level of 60. Instrument calibration was done every analytical session with a direct infusion of a LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The Full Scan (FS, resolution – 70,000 FWHM) was accompanied by a Data-Independent Acquisition (DIA resolution – 35,000 FWHM). DIA method recorded the MS/MS fragmentation events for all compounds/ anions enrolled in this study. On the basis of our compound list, a scan range of *m*/z 50–250 was chosen in FS with the automatic gain control (AGC) of 1×10^{-6}, while maximum injection time was 100 ms. For the DIA segment the AGC target was set to 5×10^{-4}, with an auto regulated maximum injection time. The precursor ions were filtered by the quadrupole which operated at an isolation window of 1 *m*/z. Fragmentation of precursors was optimised as three-stepped normalized collision energy (NCE) (10, 25 and 50 eV). Detection of analytes was achieved by comparing the retention time at which negative molecular ions with exact *m*/z value appeared, accompanied with two specific characteristic fragments. The chemical formulas, retention times, the theoretical mass of the precursors and the corresponding diagnostic fragments / isotopic ratios applied for confirmation purposes are reported in Table 2.

Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system while XcaliburTM 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) to control the HRMS system, the exact mass of the compounds, record and elaborate data.

Method validation

Validation was assessed according to the European Commission (2017) SANTE/11813/2017 Guidance document on method validation & quality control procedures for pesticide residue analysis in food & feed, as described in our recently published papers (Chiesa *et al.*, 2019). Moreover, the method follows the US Food and Drug Administration Foods Veterinary Medicine Research Steering Committee (US FDA FVM) recommendations that regard the validation of chemical methods (US FDA FVM, 2019) and criteria for confirmation of identity of chemical residues using Exact Mass Data (US FDA FVM, 2015). The method was validated for four different blank samples (baby food based on meat, fish, vegetables and cheese) to assess selectivity / specificity, linearity, recovery, precision (as coefficient of variation, CV %) and limit of quantification (LOQ). Besides quantitative validation aspects, also the identification parameters were assessed e.g. ion ratio and retention time. A number of 6 replicates were analysed to check the recovery and precision at two concentration levels (LOQ and 50 ng/g). The LOQ was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120 % and an RSD ≤20 %. The matrix effect was also evaluated by comparing the response produced from the analyte in a solvent solution with that obtained from the same quantity of analyte in the sample extract, expressed as percentage. The matrix-matched calibration curves were made of 5 calibration points in triplicate at 5, 10, 20, 50 and 100 ng/g for all analytes to cover the high concentrations found in the samples.

Table 2 **IC-HRMS data of the studied compounds acquisitioned in negative ionisation mode: formulas, retention time (RT) exact mass, of parent pseudo-molecular anion confirmation fragments and isotopic pattern**

Compound	Formula	R_t (min)	Precursor (*m/z*)	Main products (*m/z*)		
AMPA	CH_6NO_3P	14.15	110.00125	62.96417	78.95904	80.97468
Glyphosate	$C_3H_8NO_5P$	23.66	168.00673	62.96417	124.01687	149.99612
N-Acetyl-glufosinate-D3 (IS)	$C_7D_3H_{11}NO_5P$	13.98	225.07251	62.96413	137.05953	181.08277

Results

The method, already described in a previous study (Chiesa *et al.*, 2019), confirmed the satisfactory validation parameters for all compounds analysed, in Table 3. Briefly, the validated method for different baby food typology showed high specificity, without any interference close to the retention time of selected analytes; good selectivity with a S/N ≥3 and ions with mass accuracy ≤5 ppm since the lowest concentration. Our LOQs were 5 ng/g, recoveries were from 90 to 102 %, linearities demonstrated a good fit with a R^2 >0.99 and precisions expressed as CVs % were lower than 11 %, for all the matrices investigated. The ion ratio was always compliant with the validation guidelines within ±30 % and the matrix effect was always within ±20 % signal suppression or enhancement, as recommended. The internal standard glyphosate-2-^{13}C, ^{15}N used in the previous work (Chiesa *et al.*, 2019), was substituted by *N*-acetyl-glufosinate-D3, more stable and consistent with an absolute recovery ranging from 98 to 102 % in the four different investigated matrices. The proposed method, in comparison to the original QuPPE one (Anastassiades *et al.*, 2015) shows better analytical limits and the protocol is the identical for matrices, not only for vegetables but also for those of animal origin.

The European Union has always paid attention to baby food components and contaminants, glyphosate and its metabolites, as shown by the directives and the regulations on the subject. Regulation (EU) No. 609/2013 (European Regulation, 2013) defines a 'baby food' as a "food intended to fulfil the particular requirements of infants in good health while they are being weaned, and of young children in good health as a supplement to their diet and/ or for their progressive adaptation to ordinary food". The same document specifies that follow-up on the use of pesticides in baby food shall be updated regularly and suggests limiting the use of pesticides as much as possible. The 105 baby food samples analysed for this research, divided on the bases of the matrix origin, did not show traces of glyphosate or its metabolite AMPA. The absence of these analytes is therefore an important result, given the worldwide alert and debates on the glyphosate issue, as well as decisions that will be taken in coming years. The lack of data in literature on screening that covers all types of baby food matrices and the encouraging results of this study, also considering the different origin context of our samples, show that the products of the most common brands present on the Italian and international market are safe for infants. Maybe the attention or the presence of glyphosate is mostly linked to soy-based products or cereals, in particular from American products, differently regulated, as can also be seen from the results of Rodrigues and de Souza (2018).

Table 3 **Validation parameters about all selected compounds in the four different baby food analysed by IC-HRMS**

Vegetable baby food	LOQ (ng g^{-1})	Matrix effects % at LOQ	CV % (at 2 Levels*)	Recovery % (at 2 Levels*)	Linearity R^2
AMPA	5	92	7, 5	95, 97	0.9906
Glyphosate	5	95	5, 4	97, 100	0.9915
Cheese baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	90	9, 8	90, 93	0.9996
Glyphosate	5	94	5, 3	94, 97	0.9984
Fish baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	95	9, 8	96, 98	0.9931
Glyphosate	5	93	5, 3	93, 95	0.9965
Meat baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	99	10, 9	97, 99	0.9936
Glyphosate	5	107	6, 4	102, 100	0.9973

*The 2 concentration levels were LOQ and 50 ng/g.

Conclusions

The 105 baby food samples analysed for this research, divided on the bases of the matrix origin, did not show traces of glyphosate or its metabolite AMPA. Generally summarising, the levels reported for glyphosate in exanimated baby food commodities indicate compliance with existing / forthcoming legislation.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a monitoring of residues of glyphosate and AMPA in 105 commercially available baby food samples. The article is well described, the samples were analysed using validated analytical methods and the methodology is considered as reliable. The publications shows clearly absence of residues of glyphosate and AMPA in all tested commercially available baby food. This finding indicates that infants and toddlers that are mainly fed with ready-to-eat baby food are not exposed to significant levels of glyphosate or AMPA residues. However the finding cannot be directly related to the supported representative uses of glyphosate for renewal.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（ヒトに対する毒性）

検索期間：2020 年 7 月 1 日～2020 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 5.8.3
Report author	Ferramosca A. *et al.*
Report year	2021
Report title	Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency.
Document No	Reproductive Toxicology (2021), Vol. 99, pp. 48-55
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under not conducted under GLP/Officially recognised testing facilities. However, all experiments were performed according to principles of good laboratory practice.
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of the study was to evaluate sperm mitochondria as a target of potential endocrine disrupting chemicals. In an ex vivo experimental model with human sperm cells, the oxygen consumption rate was measured under exposure to glyphosate and glufosinate. **The present summary focusses on the experiments and results obtained with glyphosate (batch # 45521; purity not reported) only.**
Using sperm cells pooled from 11 human donors, quadruplicate cell suspensions were exposed to glyphosate, solvent, negative (carbonyl cyanide 4-chlorophenyl hydrazine, CCCP) and positive controls (nicotinamide adenine dinucleotide, NADH) for one hour at 37 °C. Each substance was tested in a concentration range of 0.1 – 1000 nM. Following treatment, mitochondrial oxygen consumption was measured, based on the active (V_3, rate of oxygen uptake measured in the presence of respiratory substrates and ADP) and in the passive state (V_4, rate of oxygen uptake measured with respiratory substrates pyruvate and malate alone) of mitochondrial respiration, as well as the respiratory control ratio (RCR), an index of mitochondrial respiration efficiency.
In order to evaluate the role of physiologically relevant hormone concentrations in endocrine-disrupting chemical mitochondrial targeting, human sperm cells were co-incubated with the respective test chemical and the steroid hormones progesterone (P4), testosterone (T), di-hydroxytestosterone (DHT) and 17β-estradiol (E2). In addition, the effect of quercetin, a mitochondria-targeting flavonoid, on mitochondria oxygen consumption was assessed. Finally, sperm cells were co-incubated with glyphosate and quercetin.
In human sperm cells, glyphosate was able to reduce mitochondrial functionality, by decreasing the oxygen consumption rate in the active (V_3) and in the passive state (V_4) of mitochondrial respiration. Although the effect on V_3 values was more evident than V_4 and was already observed at 1nM, the mitochondrial respiration efficiency was negatively affected only at concentrations of ≥ 100 nM.
The positive control NADH and the negative control CCCP demonstrated the validity of the test system. NADH exposure caused a statistically significant, dose-dependent increase in the active state of mitochondrial respiration (V_3), resulting in a dose-dependent increase of the respiration control ratio. Conversely, CCCP treatment caused a dose-dependent increase in the resting state of respiration (V_4), hence resulting in a dose-dependent decrease of the respiration control ratio.
The mitochondria-targeted flavonoid quercetin had a direct effect on the passive state of mitochondrial respiration and statistically significantly induced V_3 and V_4 in a dose dependent manner. The RCR value was decreased by -43% when compared to the solvent control at the highest test concentration of 1000 nM. Upon treatment with steroid hormones, DHT was identified as the only hormone significantly reducing mitochondrial functionality at physiologically relevant concentrations. Concentrations of 10 and 100 nM caused a statistically significant decrease in the active state (V_3), as well as a dose-dependent, statistically significant increase in the resting state (V_4), thus significantly decreasing the respiration control ratio (RCR). For the remaining steroid hormones P4, T and E2, no adverse effect on

mitochondria functionality was observed.
In the presence of DHT, the negative effect of glyphosate was evident at concentrations at ≥ 0.1 nM already. V_4 values were increased, suggesting a stimulus of mitochondrial respiration independent of ADP phosphorylation. After co-stimulation with glyphosate and the mitochondria-targeting flavonoid quercetin, an increase in oxygen consumption rate was observed at concentrations in the range of 0.1 – 10 nM, reaching the highest levels at glyphosate and quercetin concentrations at 10 nM. Hence, it was concluded that glyphosate targets mitochondria by using a mechanism that is different from that of DHT and quercetin.

Materials and methods

Test material

Test material:	Glyphosate*
Source:	Sigma Aldrich
Batch no.:	# 45521
Purity:	Not reported

*: Besides Glyphosate, also glufosinate was investigated in this study. The present summary focusses on the experiments and results obtained with glyphosate only.

Control materials

Solvent controls*:	Dimethylsulfoxide (DMSO), final concentration 1%
Negative control:	Carbonyl cyanide 4-chlorophenyl hydrazone (CCCP), 0.1 – 1000 nM
Positive control:	Nicotinamide adenine dinucleotide reduced (NADH), 0.1 – 1000 nM

*: Besides DMSO, also water and ethanol were specified as solvents. It was not further specified which solvent was used for which chemical. However, solvent controls for all solvents were included in the respective experiments.

Male recruitment and semen collection
Semen samples were obtained from 11 healthy human volunteers. The donors were 19 – 38 years old and not on medication with sex steroids. The research on human sperm cells was approved by the Institutional Review Board of Department of biological and environmental sciences and technologies at the University of Salento.
Semen samples were collected by masturbation after 3-5 days of sexual abstinence and examined directly after liquefaction within 30 minutes. All samples underwent computer-assisted sperm analysis prior to use (CASA – SCA® 5.3.0.1: Sperm Class Analyser, LabIVF Asia Pte Ltd, Singapore).

Sperm cell suspension establishment
Sperms from all donors were pooled, collected by centrifugation and re-suspended in isotonic salt solution (2 g/L BSA, 113 mM KCl, 12.5 mM KH2PO4, 2.5 mM K2HPO4, 3 mM MgCl2, 0.4 mM EDTA, 20 mM Tris, adjusted to pH 7.4 with HCl).

Sperm mitochondria respiration assay
A total of 10 x 10^6 sperm cells/mL were incubated with the test item, vehicle, negative or positive control at concentrations in the range of 0.1 – 1000 nM for 1 hour at 37 °C. Each experiment was performed in quadruplicates.
In order to evaluate the role of physiologically relevant hormone concentrations in endocrine-disrupting chemical mitochondrial targeting, human sperm cells were co-incubated with the test item or respective controls and steroid hormones. In the co-incubation experiments, the sperm cells were pre-incubated with the test item, solvent, negative and positive controls in the same concentration range for 20 minutes before adding 10 nM of the respective hormones (progesterone (P4), testosterone (T), di-hydroxytestosterone (DHT) and 17β-estradiol (E2) or the flavonoid quercetin (QRC).
After treatment, spermatozoa were subjected to hypotonic treatment and used for polarographic assays

of oxygen consumption as described by Ferramosca et al. (2008)[1].
Oxygen consumption was measured by using a Clark-type oxygen probe, in the presence of a solution of 10 mM pyruvate and 10 mM malate (respiratory substrates) and 0.76 μM of adenosine diphosphate (ADP).

The following parameters were evaluated:

- V_3, active state of mitochondrial respiration: Rate of oxygen uptake measured in the presence of respiratory substrates and ADP, reported as nmol O2 x ml- 1x min- $1/(10 \times 10^6$ cells).
- V_4, passive state of mitochondrial activation: Rate of oxygen uptake measured with respiratory substrates pyruvate and malate alone, reported as nmol O2 x ml- 1x min- $1/(10 \times 10^6$ cells).
- Respiratory control ratio (RCR): Index of mitochondrial respiration efficiency, calculated by dividing V_3 by V_4.

Statistical analysis:
Statistical analysis was carried out by the software GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as the mean of three samples with standard deviation. Student's *t*-test was performed to detect significant differences between the blank and chemicals treated spermatozoa. Differences were considered statistically significant at $p < 0.05$.

Results

Effects of glyphosate and the flavonoid quercetin on sperm oxygen consumption

Upon treatment with glyphosate, there was a reduction in mitochondrial respiration efficiency. Both parameters V_3 and V_4 were statistically significantly decreased starting at 1 nM and 10 nM, respectively, reaching a maximum decrease of -31% when compared to the solvent control at the highest concentration of 1000 nM. The respiration control ratio (RCR) was statistically significantly decreased at 1000 nM.
The positive control NADH and the negative control CCCP demonstrated the validity of the test system. NADH exposure caused a statistically significant, dose-dependent increase in the active state of mitochondrial respiration (V_3), resulting in a dose-dependent increase of the respiration control ratio. Conversely, CCCP treatment caused a dose-dependent increase in the resting state of respiration (V_4), hence resulting in a dose-dependent decrease of the respiration control ratio.

The mitochondria-targeted flavonoid quercetin had a direct effect on the passive state of mitochondrial respiration. Quercetin induced a statistically significant increase of V_3 values from 0.1 nM constantly kept up to 100 nM, followed by a drop down to control values at the highest concentration, a statistically significant, dose-dependent increase of V_4 and a dose-dependent RCR decrease up to -43% when compared to control levels at the highest concentration.

[1] A. Ferramosca, R. Focarelli, P. Piomboni, L. Coppola, V. Zara, Oxygen uptake by mitochondria in demembranated human spermatozoa: a reliable tool for the evaluation of sperm respiratory efficiency, Int. J. Androl. 31 (2008) 337–345, https://doi.org/10.1111/j.1365-2605.2007.00775.x

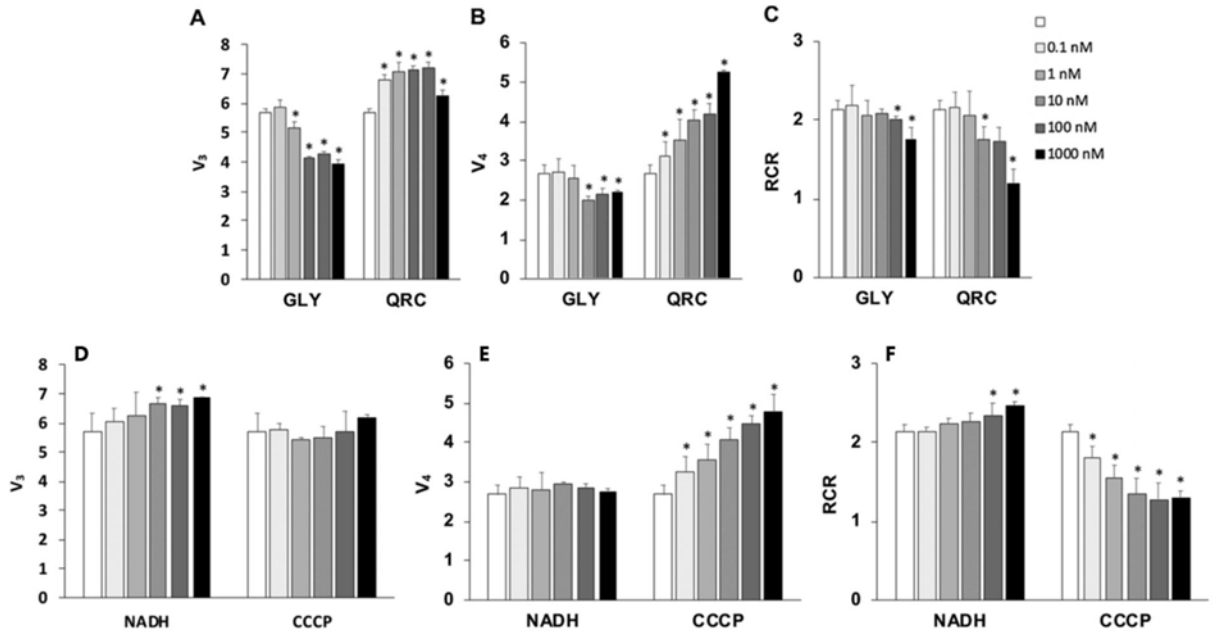


Figure 1 (modified from the publication Ferramosca *et al.*, 2021 to report data for glyphosate only): V_3, V_4 and RCR values upon dose-dependent treatment with glyphosate (GLY), the flavonoid quercetin (QRC), the positive control nicotinamide adenine dinucleotide (NADH) and the negative control carbonyl cyanide 4-chlorophenyl hydrazone (CCCP). **Fig. 1A**: V_3 of GLY and QRC; **Fig 1B**: V_4 of GLY and QRC; **Fig 1C**: RCR of GLY and QRC; **Fig. 1D**: V_3 of NADH and CCCP; **Fig 1E**: V_4 of NADH and CCCP; **Fig 1F**: RCR of NADH and CCCP. All data were subjected to Student's t-test ($p< 0.05$).

Effect of steroid hormones on sperm mitochrondrial oxygen consumption

Upon treatment with steroid hormones, di-hydroxytestosterone (DHT) was identified as the only hormone significantly reducing mitochondrial functionality at physiologically relevant concentrations. DHT at concentrations of 10 and 100 nM caused a statistically significant decrease in the active state (V_3), as well as a dose-dependent, statistically significant increase in the resting state (V_4), thus significantly decreasing the respiration control ratio (RCR).
For the remaining steroid hormones progesterone (P4), testosterone (T) and 17β-estradiol (E2) no adverse effect on mitochondria functionality was observed. Although P4 concentrations at 0.1 and 1 nM had a statistically significant increasing effect on V_3, mitochondrial respiration efficiency was not affected.

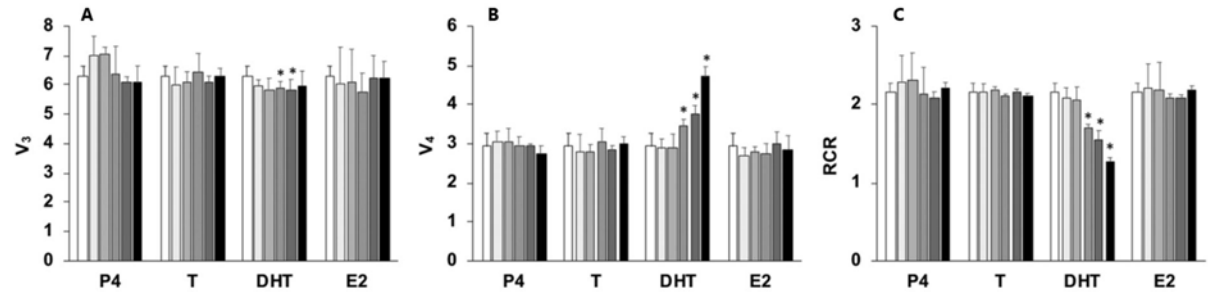


Figure 2 (modified from the publication Ferramosca *et al.*, 2021 to report data for glyphosate only): Sex steroid treatment of human sperms to set up the experimental model to measure oxygen consumption (V_3 and V_4) and the V_3:V_4 ratio (RCR). Human sperms were dose-dependently treated with the steroid hormones progesterone (P4), testosterone (T), di-hydroxy-testosterone (DHT) and 17β-estradiol (E2). **Fig. 2A**, V_3 of P4, T, DHT and E2; **Fig. 2B**, V_4 of P4, T, DHT and E2; **Fig. 2C**, RCR of P4, T, DHT and E2. All data were subjected to Student's *t* test (* $P < 0.05$).

Effects of glyphosate and quercetin on sperm oxygen consumption upon co-treatment with sex steroids

After co-incubation of sperm cells with glyphosate and steroid hormones, only treatment with di-hydroxytestosterone (DHT) was able to change the effects observed when glyphosate has been used alone. At the lowest tested concentration of 0.1 nM glyphosate, the respiration control ratio (RCR) was -31% decreased when compared to the solvent control. The decrease was attributed to an increase in the oxygen consumption rate, however, the increase in V_4 values much higher (55% vs. 5% at 0.1 nM; 58% vs. 114% at 1000 nM).
Also for Quercetin, a slight decrease in the RCR value was observed in the presence of DHT only.

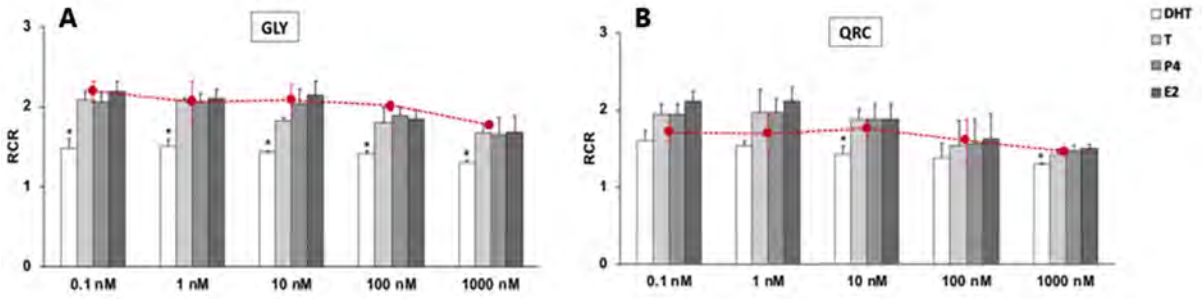


Figure 3 (modified from the publication Ferramosca *et al.*, 2021): RCR values upon dose-dependent treatments with GLY and QRC in the presence of 10 nM DHT, or 10 nM testosterone (T), or 10 nM progesterone (P4), or 10 nM 17β-estradiol (E2). **Fig. 3A**, RCR of GLY (0.1- 1000 nM) in presence of 10 nM DHT, T, P4 or E2; **Fig. 3B**, RCR of QRC (0.1- 1000 nM) in presence of 10 nM DHT, T, P4 or E2. The red dotted line shows RCR values obtained when GLY or QRC were tested individually. All data were subjected to Student's *t*-test (* $P <$ 0.05).

Table 1: Mitochondrial oxygen uptake in the active (V_3) and in the resting (V_4) state after treatment with glyphosate alone (GLY) and glyphosate in combination with di-hydroxytestosterone (DHT) published by Ferramosca *et al*., 2021

Concentrations (nM)	V_3 (nmol O_2 x ml^{-1}x min^{-1}/(10 x 10^6 cells)		V_4 (nmol O_2 x ml^{-1}x min^{-1}/(10 x 10^6 cells)	
	GLY	GLY + DHT	GLY	GLY + DHT
0.1	5.85 ± 0.15	6.17 ± 0.76	2.70 ± 0.50	4.20 ± 0.20
1	5.17 ± 0.29	6.07 ± 0.40	2.53 ± 0.35	4.03 ± 0.25
10	4.12 ± 0.12	6.30 ± 0.66	1.98 ± 0.12	4.40 ± 0.40
100	4.30 ± 0.20	6.67 ± 0.21	2.15 ± 0.15	4.70 ± 0.10
1000	3.93 ± 0.28	6.23 + 0.25	2.23 ± 0.02	4.77 ± 0.23

Effects of glyphosate on sperm oxygen consumption upon co-treatment with quercetin

In the presence of 10 nM quercetin, glyphosate slightly reduced V_3 and V_4 in a dose-dependent manner (statistically not significant). RCR values were dose-dependently decreased, reaching statistical significance at the highest concentrations tested. When compared to individual glyphosate treatments, the co-incubation with quercetin caused an increased consumption rate mainly at concentrations in the range of 0.1 – 10 nM, reaching the highest values when glyphosate and quercetin concentrations were in the same range. The findings suggest a stimulus of mitochondrial respiration independent of ADP phosphorylation.

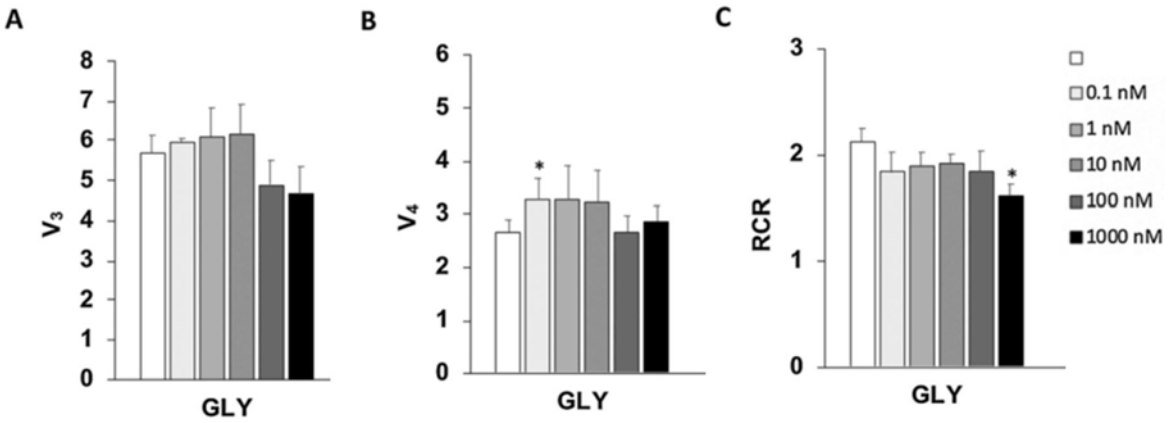


Figure 4 (modified from the publication Ferramosca *et al.*, 2021): V_3, V_4 and RCR values upon dose-dependent treatments with glyphosate (GLY) in the presence of the flavonoid quercetin (QRC). **Fig. 4A,** V_3 of GLY in presence of 10 nM QRC; **Fig. 4B**, V_4 of GLY in presence of 10 nM QRC; **Fig. 4C**, RCR of GLY in presence of 10 nM QRC. All data were subjected to Student's *t*-test (* $P < 0.05$).

The effects of di-hydroxytestosterone, glyphosate and quercetin on sperm mitochondria are summarised in the figure below.

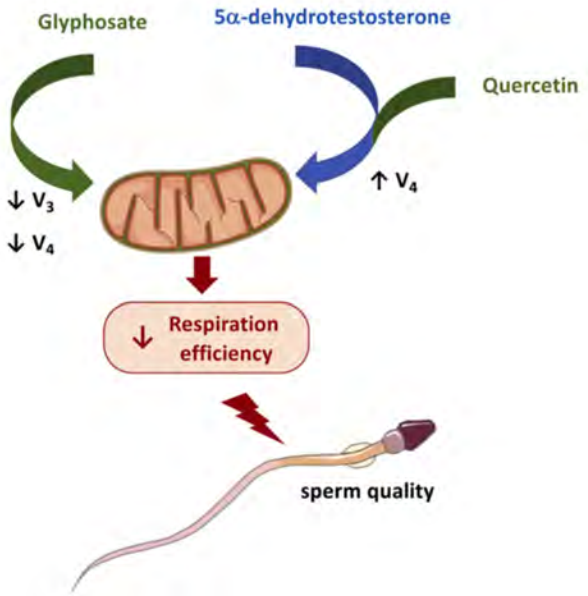


Figure 5: Effects overview of di-hydroxytestosterone, glyphosate and quercetin on sperm mitochondria published by Ferramosca *et al*., 2021

Conclusion

In human sperm cells, glyphosate was able decrease oxygen consumption rate in the active (V_3) and in the passive state (V_4) of mitochondrial respiration, which is assumed to reflect a reduction in mitochondrial function. The mitochondrial respiration efficiency (respiratory concentration ratio, RCR) was negatively affected only at concentrations of ≥ 100 nM.

In the presence of the steroid hormone di-hydroxytestosterone (DHT), the negative effect of glyphosate was evident at concentrations at ≥ 0.1 nM already. V_4 values were increased, suggesting a stimulus of mitochondrial respiration independent of ADP phosphorylation.

After co-stimulation with glyphosate and the mitochondria-targeting flavonoid quercetin, an increase in oxygen consumption rate was observed at concentrations in the range of 0.1 – 10 nM, reaching the highest levels at glyphosate and quercetin concentrations at 10 nM. The authors concluded that glyphosate targets mitochondria by using a mechanism that is different from that of DHT and quercetin.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Glyphosate was reported to reduce mitochondrial functionality, by decreasing the oxygen consumption rate in the active and in the passive state of mitochondrial respiration. The mitochondrial respiration efficiency was negatively affected only at concentrations of ≥ 100 nM. In the presence of the sex steroid hormone di-hydroxytestosterone (DHT), the negative effect on mitochondria functionality caused by glyphosate was observed from ≥ 0.1 nM. The passive state of mitochondrial respiration was found to be increased, suggesting a stimulus of mitochondrial respiration independent of ADP phosphorylation. In the presence of the mitochondria-targeting flavonoid quercetin, an increase in oxygen consumption rate was observed at concentrations in the range of 0.1 – 10 nM, reaching the highest levels at glyphosate and quercetin concentrations at 10 nM. Glyphosate was concluded to target mitochondria by using a mechanism that is different from that of DHT and quercetin but not described.
The study did not follow any OECD guideline and was not performed under GLP. No information on the test item with regard to purity was given, however, the supplier and batch number were reported. Cytotoxicity tests were not included, but a broad concentration range from 0.1 - 1000 nM was tested to cover the sexual hormones physiologically relevant concentrations (10 nM), triggering endogenously hormone-dependent signalling pathways, and the estimated (nM range) QRC dietary intake. However, it is not clear how these concentrations may be relevant in term of exposure to glyphosate. The authors stated that these concentration are below the NOAEL and acceptable daily intake (ADI) for the glyphosate (50 and 0.5 mg/kg bw per day, respectively). But no calculations have been presented to show whether spermatozoa could be exposed under the normal condition of glyphosate use. Given the novel study type and underlaying assumptions, evaluation of other comparator molecules to which humans are regularly systemically exposed (e.g. in the diet) would provide context to the relevance of these results and credibility to the assay's predictive capacity for effects in humans.
It was not clear from the publication which solvent has been used for which chemical. As solvent controls were included for all solvents used, the weakness was considered to be of minor degree. The criteria for a biological response were not provided.
Overall, the study is sufficiently documented to generally accepted scientific principles. It is considered to be reliable with restriction, but the information provided are not robust enough to impact the risk assessment.

Assessment and conclusion by RMS:

Reliability criteria for *in vitro* toxicology studies

Publication: Ferramosca, 2021: Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency.	**Criteria met? Y/N/Uncertain**	**Comments**
Guideline-specific		
Study is in accordance to valid internationally accepted testing guidelines.	N	
Study is performed according to GLP.	N	
Study is completely described and conducted following scientifically acceptable standards.	Y	
Test substance		
Test material (glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity for glyphosate not reported but batch No. given (#45521, Sigma Aldrich)
Only glyphosate acid or one of its salts is the tested substance.	N	Glyphosate alone and in combination with steroid hormones.

Publication: Ferramosca, 2021: Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency.	**Criteria met? Y/N/Uncertain**	**Comments**
AMPA or other glyphosate metabolites is the tested substance.	N	
Study		
Test system is clearly and completely described.	Y	
Test conditions are clearly and completely described.	Y	
Metabolic activation system is clearly and completely described.	N	
Test concentrations is in physiologically acceptable range (< 1 mM).	Y	0.1-1000 nM
Cytotoxicity tests are reported.	N	Concentration range covers both the sexual hormones physiologically relevant concentrations (10 nM), triggering endogenously hormone-dependent signaling pathways, and the estimated (nM range) QRC dietary intake.
Positive and negative controls.	Y	
Complete reporting of effects observed.	Y	
Statistical methods described.	Y	
Historical negative and positive control data reported.	N	Criteria for a biological relevant response not provided.
Dose-effect relationship reported.	Y	
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	No information on the test item with regard to purity was given, however, the supplier and batch number were reported. Cytotoxicity tests were not included, but a broad concentration range from 0.1 - 1000 nM was tested. Historical control data were not reported. Results contradict higher tier in vivo multigenerational studies dosed at several orders of magnitude higher, which do not report any adverse outcomes in fecundity or reproductive outcome. No information on whether the tested concentration may reflect physiological exposure to human spermatozoa in vivo following exposure to the accepted regulatory dose levels following glyphosate use as herbicide.
Not reliable	N	

1. Information on the study

Data point:	CA 5.9.4
Report author	Shrestha S. *et al.*
Report year	2020
Report title	Pesticide use and incident Parkinson's disease in a cohort of farmers and their spouses.
Document No	Environmental Research (2020), Vol. 191, Article No. 110186 https://doi.org/10.1016/j.envres.2020.110186
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes (Relevance Category A)/Reliable without restrictions

2. Full summary of the study according to OECD format

The authors studied the use of pesticides and possible associations with incident Parkinson's disease (PD) for 38,274 pesticide applicators and 27,836 of their spouses in the Agricultural Health Study (AHS) cohort with follow-up of more than 20 years. **Methods:** Self-reported AHS enrollment questionnaire information regarding use of 50 specific pesticides and follow-up questionnaire information from applicators and spouses was employed in the analysis. There were two exposure metrics: ever use of specific pesticides and intensity-weighted lifetime days of use (IWLD). The statistical analysis involved Cox regression, adjusted for covariates, to estimate hazard ratios (HRs) and 95% confidence intervals (95% CIs) for incident PD for each of the 50 pesticides. PD risk was also evaluated based on head injury and the use or non-use of chemical resistant gloves. **Results:** A total of 373 applicators and 118 spouses self-reported incident doctor-diagnosed PD after enrollment. Ever-use of the insecticide terbufos (HR 1.31, 95% CI 1.02, 1.68) and the herbicides trifluralin (HR 1.29, 95% CI 0.99, 1.70) and 2,4,5-T (HR 1.57, 95% CI 1.21, 2.04) was associated with elevated PD risk. On the other hand, diazinon (HR 0.73, 95% CI 0.58, 0.94) and 2,4,5-TP (HR 0.39, 95% CI 0.25, 0.62) were associated with reduced risk. The authors observed higher risk for certain pesticides among those who reported a history of head injury or who did not use chemical resistant gloves. PD risk was also elevated for applicators in the highest category of IWLD for dichlorvos, permethrin (animal use), and benomyl. **Conclusions:** The authors found evidence of increased PD risk for some pesticides and concluded that there was higher susceptibility for pesticide-associated PD among individuals with a prior head injury as well as protection from PD with use of chemical resistant gloves.

Materials and methods

The AHS cohort enrolled 52,394 applicators and 32,345 spouses. Excluded for the purposes of this study were those who did not participate in any AHS follow-up: 13,596 applicators and 4,107 spouses. Also excluded were those who: reported PD at enrollment (96 applicators and 34 spouses), did not respond to follow-up PD questions (249 applicators and 306 spouses), had PD reported on a death certificate but no other information available (84 applicators and 25 spouses), reported PD that could not be confirmed (88 applicators and 36 spouses), or gave inconsistent responses across surveys (7 applicators, 1 spouse). Thus, the analysis cohort for this study included 38,274 applicators and 27,836 spouses.

Two pesticide exposure metrics were used in the analysis. First, the authors evaluated PD risk according to ever use of 50 pesticides. Second, PD risk was evaluated according to exposure intensity-weighted lifetime days of use (IWLD) calculated based on information on days of use for specific pesticides, mixing practices, application methods, equipment repair, and personal protective equipment use. Cox proportional hazards regression was used to estimate HRs and 95% CIs for specific pesticides and PD. In the IWLD analyses, a test for trend was conducted using the median IWLD value as an ordinal variable in regression models. Multiple imputation was used to fill in missing covariate data.

Results

In a combined analysis of applicators and spouses, the authors found positive associations for the organophosphate insecticide terbufos (HR 1.30, 95% CI 1.02, 1.68) and the herbicides trifluralin (HR 1.29, 95% CI 0.99, 1.70) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) (HR 1.57, 95% CI 1.21, 2.04), and inverse (viz., decreased risk) associations for ever-use of the organophosphate insecticide diazinon (HR 0.73, 95% CI 0.58, 0.94), the fumigant ethylene dibromide (HR 0.35, 95% CI 0.14, 0.84), and the herbicide 2,4,5-TP [2,4,5-T,P, 2-(2,4,5-trichlorophenoxy) propionic acid] (HR 0.39, 95% CI 0.25, 0.62). The corresponding analysis for glyphosate showed no association with PD (HR 1.10, 95% CI 0.87, 1.39).

In analyses stratified by presence or absence of head injury, the authors found increased risk for those with a history of head injury, versus no increased risk for those without head injury, for chlordane, dichlorodiphenyltrichloroethane (DDT), toxaphene, diazinon, phorate, permethrin, methyl bromide, paraquat and pendimethalin. Results for glyphosate were not presented for those with/without a history of head injury.

In analyses stratified by use or non-use of chemical resistant gloves, the authors found 5 herbicides (dicamba, imazethapyr, metolachlor, trifluralin, and metribuzin) that were associated with increased PD risk among those who did not wear chemical resistant gloves when there was no increased risk for these herbicides among users of chemical resistant gloves. Results for glyphosate were not presented stratified by use of non-use of chemical resistant gloves.

In IWLD analyses, none of the 50 pesticides were found to have a significant trend of increasing PD HRs with increasing IWLDs, though there were elevated HRs for individuals in the highest category of IWLD for the insecticides dichlorvos (HR 1.46, 95% CI: 0.98, 2.19, p-trend 0.06) and permethrin (animal use) (HR 1.44, 95% CI: 0.85, 2.44, p-trend 0.21), and the fungicides benomyl (HR 1.34, 95% CI 0.64, 2.80, p-trend 0.31), captan (HR 1.27, 95% CI 0.74, 2.20, p-trend 0.36), and chlorothalonil (HR 1.29, 95% CI 0.66, 2.56, p-trend 0.41) as compared to those who never used those pesticides. Glyphosate did not show a positive trend with increasing IWLDs and the HR in the highest IWLD category was 0.85 (95% CI 0.62, 1.17).

Conclusions

The authors concluded that they found evidence of increased PD risk for terbufos, trifluralin, and 2,4,5-T and decreased PD risk for diazinon and 2,4,5-TP. They also concluded that some pesticide-PD associations were more apparent in those with a past head injury and that using chemical resistant gloves reduced some pesticide-PD associations. The authors did not reach a conclusion specific for glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Glyphosate was not associated with PD in analyses based on ever use or in analyses based on IWLDs of use. Given that there is no plausible mechanism for glyphosate causing PD and that glyphosate systemic dose has been found to be minimal for applicators and spouses (Acquavella et al. 2004), those results are considered to be a valid.

References

Acquavella JF, Alexander BH, Mandel JS, et al. Glyphosate biomonitoring for farmers and their families: Results from the farm family exposure study. Environ. Health Perspect. 2004; 112:321-326.

Assessment and conclusion by RMS:

Reliability criteria for epidemiology studies

Publication: Shrestha S. et al., 2020, Pesticide use and incident Parkinson's disease in a cohort of farmers and their spouses.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study is in accordance to valid internationally accepted testing guidelines/practices.	n/a	Not applicable
Study is completely described and conducted following scientifically acceptable standards.	Yes	
Test substance		
Exposure to formulations with only glyphosate as a.i.	Yes	
Exposure to formulations with glyphosate combined with other a.i.	No	
Exposure to various formulations of pesticides.	Yes	50 pesticides total
Study		
Study design – epidemiological method followed.	Yes	
Description of population is investigated.	Yes	
Description of exposure circumstances.	Yes	
Description of results.	Yes	
Have confounding factors been considered.	Yes	
Statistical analysis.	Yes	
Overall assessment		
Reliable without restrictions	Yes	The finding of no association between glyphosate and Parkinson's disease risk in this study is considered to be valid. The results fit with what is known about glyphosate toxicology and exposure potential.
Reliable with restrictions	No	
Reliability not assignable	No	
Not reliable	No	

1. Information on the study

Data point:	CA 5.9.4
Report author	Werder E. J. *et al.*
Report year	2020
Report title	Herbicide, fumigant, and fungicide use and breast cancer risk among farmers' wives.
Document No	Environmental Epidemiology (2020), Vol. 4, No. 3, Art. No. e097
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes (Relevance Category A)/Reliable without restrictions

2. Full summary of the study according to OECD format

Methods: The authors examined exposure to herbicides, fumigants, and fungicides in relation to breast cancer risk among farmers' wives with no prior history of breast cancer in the Agricultural Health Study (AHS). Women provided information on pesticide use, demographics, and reproductive history at enrollment (1993–1997) and at a 5-year follow-up interview. Cox proportional hazards regression was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) between the women's and their husbands' self-reported use of individual pesticides and incident breast cancer risk. **Results:** Out of 30,594 women, 38% reported using herbicides, fumigants, or fungicides and 1,081 were diagnosed with breast cancer during a median 15.3 years of follow-up. There were elevated HRs in relation to women's ever use of the fungicide benomyl (HR = 1.6; 95% CI = 0.9, 2.7) and the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (HR = 1.6; 95% CI = 0.8, 3.1) and for their husbands' use of the herbicide 2-(2,4,5-trichlorophenoxy) propionic acid (2,4,5-TP) (HR = 1.5; 95% CI = 0.9, 2.7). Few other chemical associations were observed and there was little evidence of differential risk by tumor estrogen receptor status or linear exposure-response relationships. **Conclusions:** The authors did not find a general pattern of increased breast cancer risk with use of specific pesticides, though they did find some evidence of increased risk with women's use of benomyl and 2,4,5-T and husbands' use of 2,4,5-TP.

Materials and methods

The underlying biological hypothesis for this study was that some pesticides are endocrine disruptors or exhibit estrogenic activity, so research is warranted into whether direct pesticide use by spouses or possible indirect pesticide exposure through the applicator's use of specific pesticides may relate to higher breast cancer risk.

The study population was restricted to female wives of AHS pesticide applicators. Of the 32,126 AHS wives who enrolled in the AHS cohort, 19,578 (61%) completed the enrollment spouse questionnaire about farm exposures and the female health questionnaire providing a reproductive health history. In addition, 23,676 of the wives (74%) completed a 5-year follow-up telephone interview. Of the 32,126 AHS wives, 478 were excluded for having prevalent breast cancer at enrollment, 113 were excluded for living outside of Iowa or North Carolina at enrollment (residence was necessary for detection of breast cancers in the statewide cancer registries), and 948 were excluded for having provided no data on pesticide use. This left a study population of 30,594 women [Note, the numbers actually add up to 30, 587.]

Cox proportional hazards models were used to estimate HRs and 95% CIs adjusted for time-varying menopausal status, race, state, parity, age at first birth, and for all other pesticides found to be associated with breast cancer. Missing covariate data were imputed. Results were similar with/without the imputed covariate data. Estrogen and progesterone receptor status, known for 87% of cases, was considered via joint proportional hazards models.

Results

Risk of breast cancer was not associated with spouses ever using any herbicide (HR 0.9; 95% CI 0.8, 1.0), any fungicide (HR 0.9; 95% CI 0.6, 1.2), or any fumigant (HR 0.9; 95% CI 0.5, 1.4). For specific pesticides, breast cancer HRs were elevated non-significantly for any use of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (HR 1.6; 95% CI 0.8, 3.1) and the fungicide benomyl (HR 1.6; 95% CI 0.9, 2.7) and the HR was reduced for any use of butylate (HR 0.4; 95% CI 0.2, 1.0). For glyphosate, the HR of 0.9 (95% CI 0.8, 1.0) indicated no association with breast cancer risk with any personal use.

The authors considered possible indirect exposure based on the husbands reported use of pesticides for women who did not apply pesticides themselves (n = 13,500). Ever use of 2-(2,4,5-trichlorophenoxy) propionic acid (2,4,5-TP) was associated with a non-significantly elevated breast cancer risk (HR 1.5; 95%CI 0.9, 2.7) as was husbands' use of 2,4,5-T below the cumulative days of use median (HR 1.5; 95% CI 0.7, 2.9) and above the median (HR = 1.3; 95% CI = 0.8, 2.0), with limited evidence of a monotonic exposure-response trend (p trend = 0.08). Husbands' ever use of trifluralin was associated with a significant reduction in breast cancer risk (HR 0.7; 95% CI 0.5, 0.9) with a significant, but nonmonotonic, exposure-response trend across quartiles of exposure (p trend 0.01). There was no association between husband's extent of use of glyphosate and breast cancer risk. The HR was 1.0 (95% CI 0.7, 1.3) for ever use and HRs varied from 0.8 to 1.1 over cumulative days of use quartiles.

Conclusions

The authors concluded that they did not observe any clear associations between use of pesticides by female spouses and breast cancer or from possible indirect exposure based on pesticides that were used by the husbands. However, they did note elevated HRs associated with women's use of benomyl and 2,4,5-T and husbands' use of 2,4,5-TP.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study was undertaken based on the assumption that the properties of pesticides – on the endocrine disruption and estrogenic activity scales – are such that an increase in breast cancer risk is possible from direct use of specific pesticides by female AHS spouses or from presumed indirect exposure related to their husbands' use of specific pesticides. The presumed biologic properties of pesticides underlying the study's hypotheses do not apply to glyphosate, at systemic doses from direct or indirect exposure (10^{-4} mg/kg direct, 10^{-5} mg/kg indirect – see Acquavella et al. 2004). The results of the study did not find clear associations between pesticide use and breast cancer risk and results for glyphosate were consistent across the various analyses in indicating no association with breast cancer.

We conclude that this study provides evidence that glyphosate is not related to breast cancer risk.

References

Acquavella JF, Alexander BH, Mandel JS, et al. Glyphosate biomonitoring for farmers and their families: Results from the farm family exposure study. Environ. Health Perspect. 2004; 112:321-326.

Assessment and conclusion by RMS:

Reliability criteria for epidemiology studies

Publication: Werder E. J. et al., 2020, Herbicide, fumigant, and fungicide use and breast cancer risk among farmers' wives.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study is in accordance to valid internationally accepted testing guidelines/practices.	n/a	Not applicable
Study is completely described and conducted following scientifically acceptable standards.	Yes	
Test substance		
Exposure to formulations with only glyphosate as a.i.	Yes	
Exposure to formulations with glyphosate combined with other a.i.	Uncertain	
Exposure to various formulations of pesticides.	Yes	26 pesticides
Study		
Study design – epidemiological method followed.	Yes	
Description of population is investigated.	Yes	
Description of exposure circumstances.	Uncertain	No description of how farm spouses applied pesticides.
Description of results.	Yes	
Have confounding factors been considered.	Yes	
Statistical analysis.	Yes	Good.
Overall assessment		
Reliable without restrictions	Yes	This study did not show a relationship between glyphosate and breast cancer. That result is consistent with glyphosate's exposure and toxicological properties.
Reliable with restrictions	No	
Reliability not assignable	No	
Not reliable	No	

グリホサートカリウム塩

要旨及び評価結果

（環境動態）

検索期間：2020 年 7 月 1 日～2020 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 7.1.4.2
Report author	Albers C. N. *et al.*
Report year	2020
Report title	Leaching of herbicidal residues from gravel surfaces - A lysimeter-based study comparing gravels with agricultural topsoil.
Document No	Environmental pollution (2020), Vol. 266, Art. No. 115225
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The approval of herbicides such as glyphosate for use on paved and unpaved gravel surfaces in the European Union is based on their behaviour and fate in agricultural soils. However, this might be very different from their fate in gravel surfaces. Therefore, an outdoor study with 21 small lysimeters containing different gravel types and a sandy arable topsoil as control was conducted. The lysimeters were sprayed with a commercial product for gardening, containing diflufenican and glyphosate. The concentrations of glyphosate and its relevant degradation product AMPA in the outlet was followed for 19 months. Glyphosate and AMPA did not leach from any of the mini-lysimeters. It can be concluded, that the different properties of gravel compared to those of agricultural soils may lead to different herbicide leaching patterns but also that the leaching depends on the type of gravel and highly on the type of herbicide.

Materials and methods

Gravels and arable soil

The first gravel type included in the study is sold under the tradename Slotsgrus® and is a mixture of different size classes. This gravel is used as the only gravel course or as a surface course on gravel paths, driveways etc. but also as a carrying layer under top layers of tiles and rock chippings. It contains particles up to 11 mm with ~10 % fine particles (<0.063 mm), 50 % sand-sized and ~40 % gravel-sized particles (>2 mm). For this gravel type, the term “pathway gravel” is used. The organic matter content is ~0.3 %, pH (H_2O) is 8.4, pH (0.01 M $CaCl_2$) is 7.7, amorphous Fe-oxides is 814 mg/kg and amorphous Al-oxides is 364 mg/kg. Amorphous metal oxides were extracted with ammonium oxalate solution and quantified on an ICP-MS.

The second gravel type of the study is typically used below tiles, asphalt and rock chippings in gardens and roads either as a single layer or as the lowest layer with a surface course on top, which could be pathway gravel or another type of finer gravel. It contains particles from clay size up to 63 mm, the particles being a mixture of silicates, granites, flint and calcite with ~4 % fine particles (<0.063 mm) and ~60 % gravel sized particles (>2 mm). For this gravel type, the term “basecourse gravel” is used. The organic matter content is ~0.6%, pH (H_2O) is 9.0, pH (0.01 M $CaCl_2$) is 8.4, amorphous Fe-oxides is 202 mg/kg and amorphous Al-oxides is 49 mg/kg.

The rock chippings used on top of the basecourse gravel were Norwegian diabas with a nominal size distribution from 11 to 16 mm. The chippings were washed in tap water before being packed into the

lysimeters to remove fine particles. For this material, the term “rock chippings” is used. The organic matter content is ~0.1%.

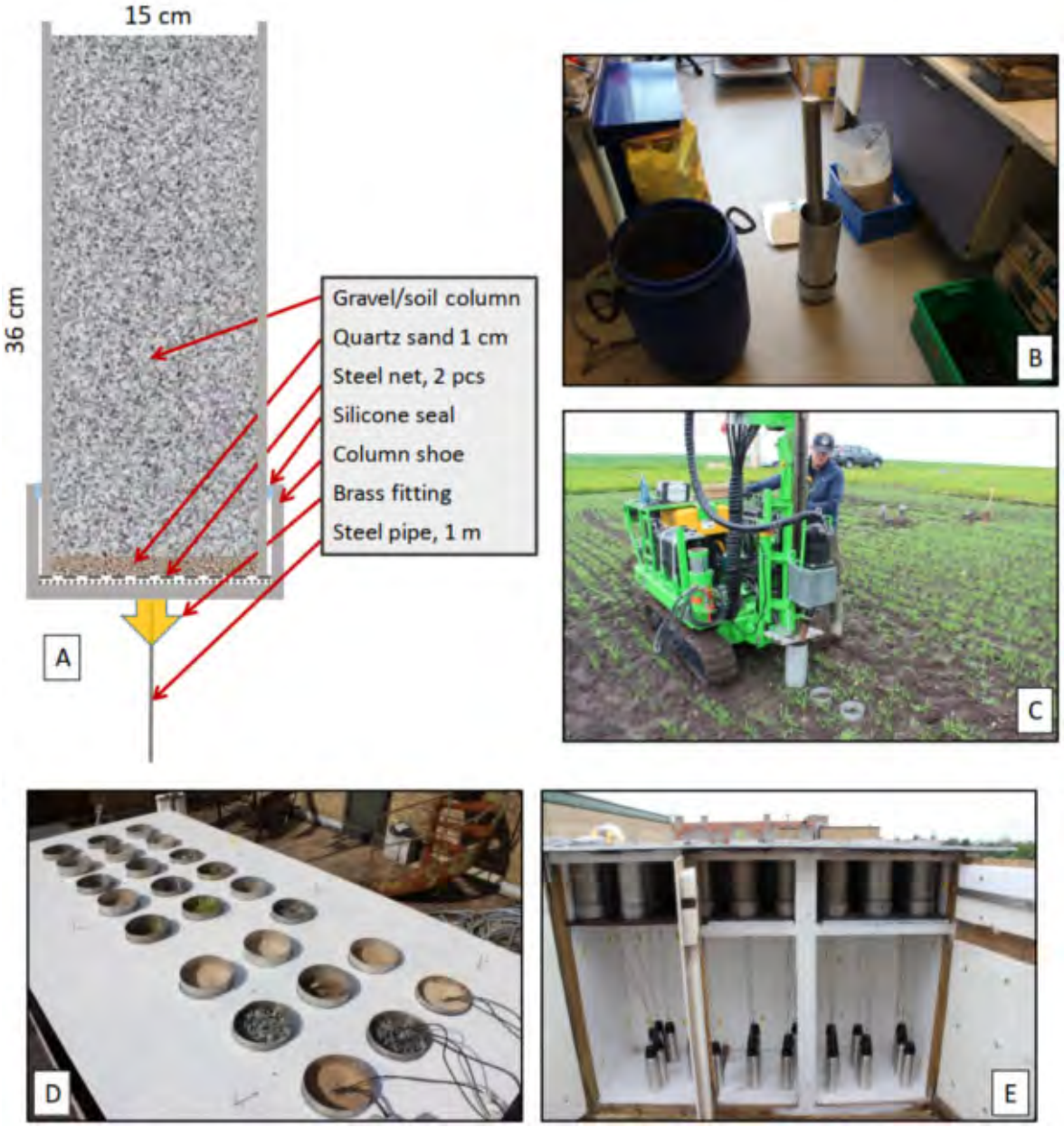
The model agricultural soil is an arable soil from a Late Weichselian outwash plain in south-western Denmark. It is very sandy (mainly coarse sand) and contains ~10 % fine particles (<0.063 mm). The organic matter content is ~3 %, pH (H_2O) is 6.0, pH (0.01 M $CaCl_2$) is 5.4, amorphous Fe-oxides is 1598 mg/kg and amorphous Al-oxides is 961 mg/kg.

Lysimeter construction and installation

The lysimeters were constructed in a way, that the leachate is in contact with no other material than austenitic stainless steel to exclude adsorption of the test substance. The lysimeters consisted of a 40 cm long stainless steel tube (inner diameter 15 cm) inserted into a “column shoe” with a 1 m stainless steel tube (inner diameter 2 mm) hanging in the middle (Figure 1A). To avoid clogging of the steel tube, two steel nets with different mesh sizes (1 mm and 80 mm) plus 12 mm fine quartz sand (0.2-0.3 mm) were placed in the column shoe. Leachate was collected in 1 L stainless steel bottles placed in an insulated cabinet heated to 2°C during winter and cooled to 10°C during summer. The lysimeters containing gravel were packed in the laboratory by compacting gently with a heavy steel rod for each 5 cm of gravel (Figure 1B). The gravel used for packing had a moisture of 6 weight-% (basecourse gravel) or 7 weight-% (pathway gravel) suitable for packing to the prescribed optimal packing density of 2.0 g/cm^3, which was ensured by weighing.

Each lysimeter was packed with either 36 cm basecourse gravel, 36 cm pathway gravel or 26 cm basecourse gravel plus10 cm rock chippings on top. Four replicates were prepared for each gravel type/combination. In addition, one more lysimeter of each gravel type/combination was prepared with temperature and soil moisture-sensors installed, where measurements were taken every hour. Four lysimeters containing arable soil were made by pushing the lysimeter tube 34-36 cm into the soil using the weight of a drilling rig (Figure 1C) and then excavated out of the soil to ensure as little disturbance as possible. The lower 12 mm of each soil core was replaced with fine quartz sand before fitting it into the column shoe. The soil was sampled into the lysimeter tubes in May 2017 and kept outside with no direct sunlight for ten weeks before being installed at the same date as the packed gravel lysimeters. In addition, one empty lysimeter was installed as a negative control, so in total 20 lysimeters of which 16 would receive herbicides.

Figure 1 **Principal sketch of a lysimeter (a), packing gravel lysimeters in the lab (b), sampling soil lysimeters (c), overview of lysimeter setup (d) and a look into the lysimeter housing (e). The insulated housing was heated to 2°C during winter and cooled to 10°C during summer**



After a month of equilibration, a bromide tracer study was conducted before applying the herbicides. Each lysimeter received 88 mL of a 500 mg/L Br-solution (dissolved as KBr in tap water diluted three times with Milli-Q water) corresponding to 5 mm of precipitation. Bromide in the leachate was analysed by ion chromatography after every larger rain event. After 8 weeks, when bromide concentrations were very low in all leachates, leachate was collected for analysis of background herbicide concentrations and the lysimeters were then sprayed with a commercial herbicide product for use in private gardens (Keeper L®). The Keeper L diluted solution was prepared in Milli-Q water added 25 % tap water and dosed according to the manufacturer's instructions (75 mg/m^2 of glyphosate) so that each lysimeter received 1.8 mL Keeper L diluted solution. The solution was distributed evenly across the gravel/soil surface using a pipette with a 2 mL glass tip. The Keeper L spraying solution should contain 750 mg/L glyphosate, which was confirmed by LC-MS/MS analysis. None of the degradation products were detected in the spraying solution.

Precipitation and air temperature were measured directly by the lysimeters to get the most relevant input for the leaching experiment. Precipitation was measured with a rain gauge with a 0.2 mm resolution at accuracy: ±2 %. Data were measured on an hourly basis. Further, shielded air temperature and temperature at the base of the lysimeters were measured hourly.

Sampling and analysis of pesticide residues

Leachate for pesticide analysis was collected after spraying in October 2017 until May 2019. Leachate was sampled in dry periods whenever leachate was present, and in wet periods before the bottles were full. The amount of leachate was quantified. The leachates equal the net precipitation, which in case of no vegetation equals precipitation minus evaporation. Replicate samples were collected at30 time points within the monitoring period. Sub-samples of 3 mL were stored at 5 °C until analysis for a maximum of two weeks. Prior to spray application, leachate from all lysimeters was collected and analysed, but none of the pesticides or their degradation products were detected. Glyphosate and AMPA were directly analysed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLCMS/MS). Before analysis, samples were vortex shaken for 30 s for homogenization and further centrifuged at 2500 RFC for 10 min to avoid particles being injected in the HPLC. One mL of the supernatant was transferred to a HPLC vial to which internal standard solution of glyphosate was added. Finally, 10 mL of the water samples were injected into the HPLC system. Compound concentration was calculated by linear regression of standard calibration (1/x weighting). The peak areas were corrected by isotope-labelled internal standards. For quality control, procedural blanks were analysed within every experimental sequence to determine instrumental noise and possible contaminations during the sample treatment. Good linearity for the different compounds was obtained ($n \geq 12$; $R^2 > 0.98$). Method limit of detection was 0.04 µg/L for glyphosate and 0.14 µg/L for AMPA. Detailed results of the methods performance are provided in Table 1.

Table 1 **Retention time as well as optimized MS-conditions for the analysis of glyphosate and AMPA. For each compound, two MRM transitions were used as quantifiers**

Substance / Acronym	Internal standard	Recovery rate [%] ± standard deviation [%] (Internal calibration)[a]	Recovery rate [%] ± standard deviation [%] (Internal calibration)[b]	Instrument LOD (µg/L)[c]	Instrument LOQ (µg/L)[c]
Glyphosate	Glyphosate-^{13}C	98 ± 9	102 ± 3	0.04	0.13
AMPA	AMPA-(^{13}C, ^{15}N, D_2)	98 ± 4	97 ± 2	0.14	0.44

[a] recovery function tested for a spiking level of 0.1 µg/L in three different water samples: Roskilde Fjord, leachate from basecourse gravel + rock chippings lysimeter and leachate from basecourse gravel lysimeter (duplicate injections; 3 water samples; n=6)

[b] recovery function tested for a range of concentrations (0.4, 4 and 40 µg/L) using leachate from the basecourse gravel, pathway gravel and sandy soil lysimeters (duplicate injections; 3 water samples; 3 concentrations; n=9)

[c] calculated as MeOH blanks average concentration + 3x or 10x MeOH concentration standard deviation (n=3) and cross-validated against S/N.

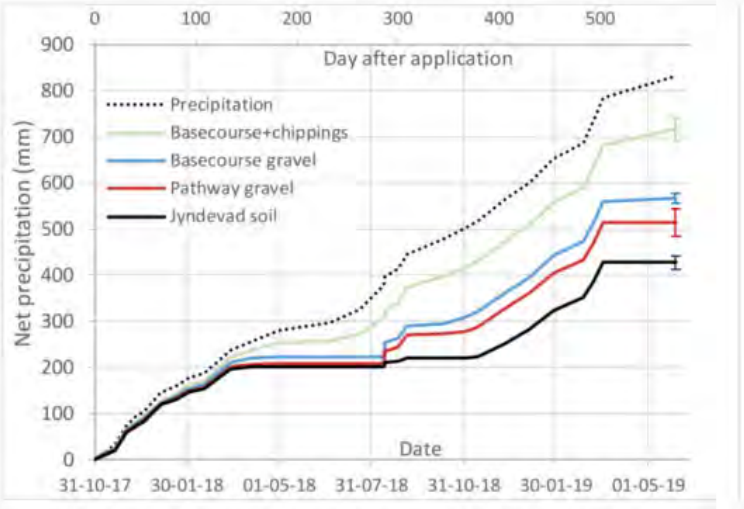
Results

Tracer study and leachate data

The bromide tracer study showed no differences between replicate gravel lysimeters indicating that these were identically packed. Some differences in active pore volumes were observed between different gravel packings. The peak bromide concentrations were seen after ~30 mm leachate for basecourse gravel plus chippings, ~40 mm leachate for basecourse gravel and ~50 mm leachate for pathway gravel. The intact arable soil showed peak concentrations at ~50 mm leachate indicating a similar active pore volume as that in pathway gravel, but with a slightly higher variation between replicate lysimeters. This variation probably reflects the natural heterogeneity that exists even in a sandy arable soil that is tilled regularly. The net precipitation (leachate) over the complete experimental period was very similar

among replicate lysimeters, but differed markedly between the different types of gravel/soil (Figure 2).

Figure 2 **Net precipitation (cumulated mean) in the different lysimeters (n = 4) compared to precipitation (n = 1) from the time of pesticide application until end of experiment. Error bars on the total net precipitation are standard deviation**



Also the periods in which leachate was formed was dependent on the type of gravel/soil. The highest evaporation was observed from the arable soil, probably due to capillary forces drawing moisture to the surface, which would not occur in the gravel due to coarser structure and low organic matter. The arable soil therefore had a net precipitation of only half the gross precipitation and with no leachate during the late spring and summer months. Pathway gravel and basecourse gravel had quite similar leachate patterns concerning both timing and amount ending up with 516-567 mm net precipitation over the whole period compared to 834 mm gross precipitation. The lysimeters with chippings on top had very little evaporation ending up with an average total net precipitation of 717 mm. The lysimeters with chippings on top were the only ones showing significant leachate during late spring and summer. It should be noted that in 2018 this period was very warm and dry (Figure 3). Temperature was more or less the same in all types of gravel lysimeters and fluctuated in the same ways (Figure 4). Daily average temperatures in the lysimeters reached up to ~25 °C during summer and down to ~0 °C during winter and the average temperature from October 2017 to May 2019 was ~9.5 °C at both levels in all lysimeters, very close to the average air temperature during that period (9.6 °C). Fluctuations in soil moisture were also synchronal between different gravel types but with larger fluctuations in the pathway gravel (Figure 4).

Figure 3 **Precipitation and air temperature by the lysimeter**

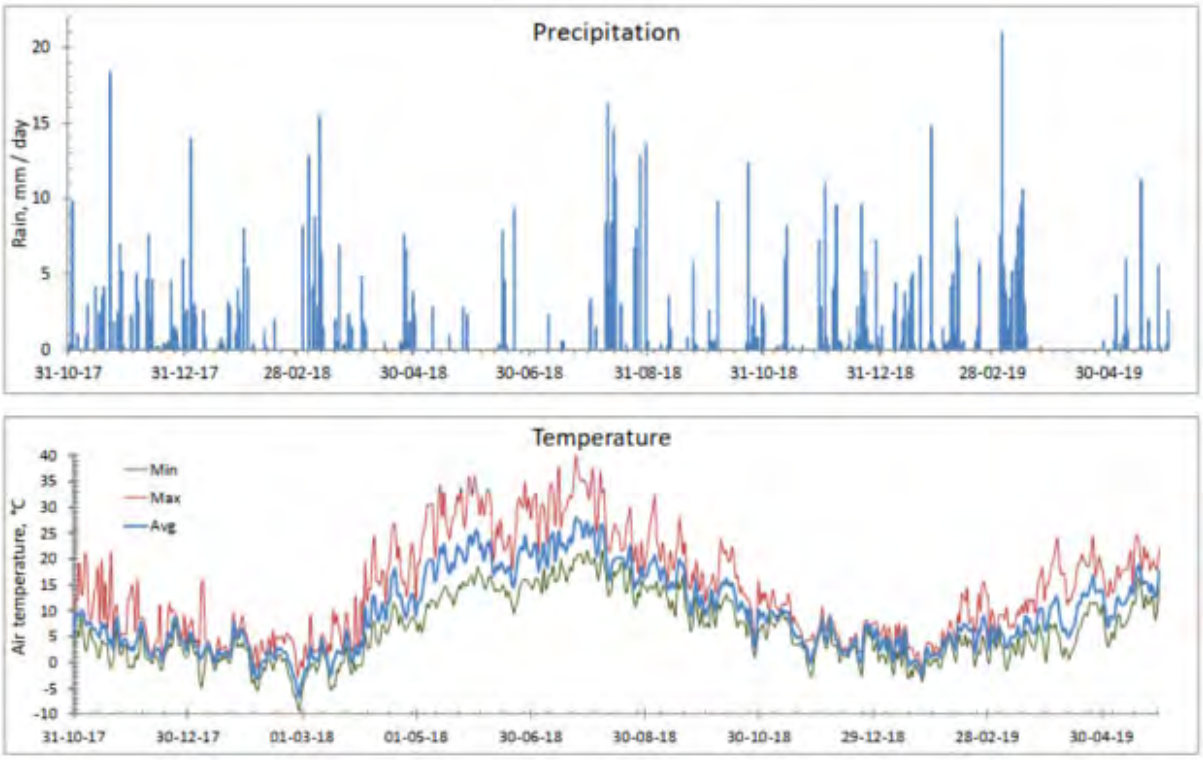
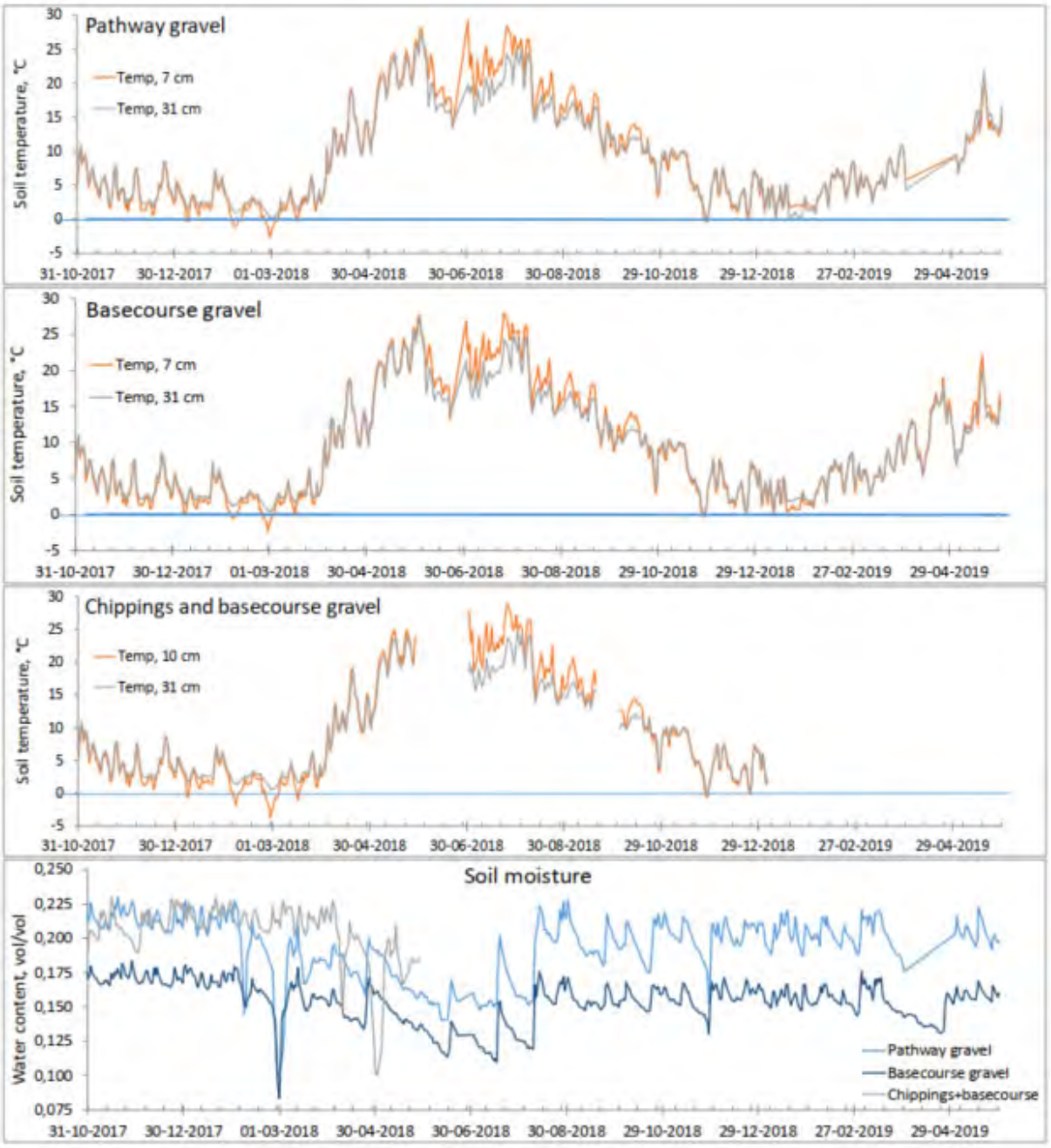


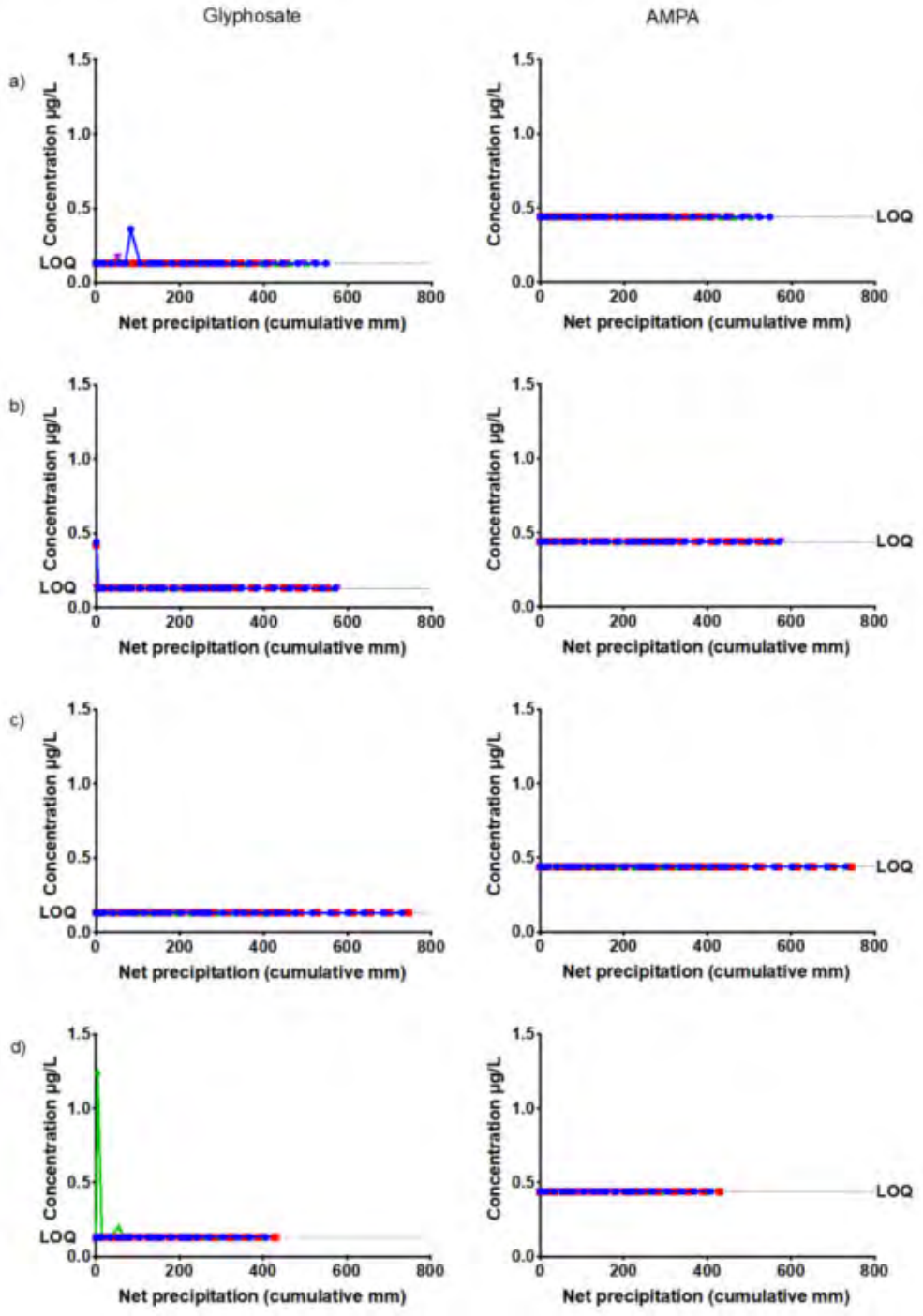
Figure 4 **Daily averages of soil temperature (at 7 or 10 and 31 cm depth) and soil moisture (15-20 cm depth) for gravel lysimeters from the day of pesticide application until end of experiment. Sudden decreases in moisture during winter is not due to desiccation but is caused by freezing of the pore water. The TDR-probe broke down in the Chippings+bascourse-lysimeter, so the measurements stops in May 2018 and for this lysimeter, temperature readings stopped in January 2019**



Leaching of glyphosate

After the pesticide application in October 2017, the concentration of glyphosate as well as of its known degradation product AMPA were followed for 19 months (Figure 5). Except for a few non-systematic glyphosate detections, glyphosate and AMPA were never measured in concentrations above the limit of quantification (0.13 and 0.44 µg/L, respectively).

Figure 5 **Glyphosate and AMPA leaching as a function of cumulated net precipitation in individual lysimeters a) 45 pathway gravel, b) basecourse gravel, c) basecourse gravel + chippings, d) arable soil. Different colours represent each 46 of the replicates (n=4) for each lysimeter type**



Conclusion

Agricultural soils are all in all poor proxies for the behaviour of pesticides in gravel surfaces and potentially other hard and paved surfaces. If pesticides are planned to be used in non-agricultural context new guidelines for their assessment should be developed to protect groundwater. For relatively strong adsorbing pesticides such as glyphosate, the degradation products can be more relevant than the parent compounds with respect to leaching to lower soil layers, also in gravel where generally a low adsorption

is expected, although in case of AMPA not indicated due to high adsorption of AMPA itself. Different properties of gravel compared to those of agricultural soils may lead to very different herbicide leaching patterns, but also the leaching depends highly on the type of gravel and type of herbicide.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes mini-lysimeter experiments with glyphosate in agricultural soil as well as in gravel used for construction of gravel paths or driveways. The experiments and results are well described. The LOQ value of 0.13 µg/L for glyphosate is above the current regulatory limit concentration. Glyphosate was not applied as active substance only, but as a product with another strong sorbing active (diflufenican). Therefore, the article is considered reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.5
Report author	Papagiannaki D. *et al.*
Report year	2020
Report title	Effect of UV-A, UV-B and UV-C irradiation of glyphosate on photolysis and mitigation of aquatic toxicity.
Document No	Scientific Reports (2020), Vol. 10, No. 1, Article No. 20247
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with regard to photolysis experiments

This summary includes only information related to photolysis. With regards to aquatic toxicity, the publication was considered not relevant.

2. Full summary of the study according to OECD format

The active herbicide ingredient glyphosate [N-(phosphonomethyl)glycine] is frequently detected as a contaminant in groundwater and surface waters. This study investigated effects of UV-A (365 nm), UV-B (302 nm) and UV-C (254 nm) irradiation of glyphosate in water on photolysis. High-resolution mass spectrometry analyses of samples after UV-C irradiation showed > 90 % decreases in glyphosate concentrations and the presence of multiple transformation products. The study suggests that UV mediated indirect photolysis can decrease concentrations of glyphosate.

Materials and methods

Chemicals
Stock solutions of glyphosate were prepared in autoclaved distilled deionized water from a 40 % wt/vol *N*-(phosphonomethyl)glycine, monoisopropylamine salt solution (CAS 38641-94-0), and from *N*-(phosphonomethyl)glycine (CAS 1071-83-6). Stock solutions of commercial brands of glyphosate-based herbicides were prepared in autoclaved distilled deionized water from Roundup Ready to Use containing 7.2 % wt/vol glyphosate, Roundup Garden containing 12 % wt/vol glyphosate, Gallup Super 360 containing 36 % wt/vol glyphosate, and Glyfonova 450 Plus containing 45 % wt/vol glyphosate. According to the product datasheets, these formulations also contain water and various surfactants and formulating agents. All stock solutions of glyphosate and glyphosate-based herbicides were stored in the dark at 5 °C.

Drinking water samples
Drinking water samples were collected at Aalborg, Aarhus, Skagen and Sønderborg municipalities (Denmark). Water samples were also collected at the influent (raw water) and effluent (treated water) of Elsted drinking water treatment plant (Denmark). All water samples originated from groundwater but with regional differences in organic and inorganic constituents according to the national Danish well database on water quality.

UV irradiation of glyphosate in aqueous samples.
Glyphosate and glyphosate-based herbicides dissolved in distilled deionized water or drinking water were exposed to different doses of UV-A, UV-B or UV-C irradiation. Stock solutions with 50 and 100 mg/L glyphosate were exposed to UV irradiation at 22 °C in 10 mm 3.5 mL quartz cuvettes. Dilute glyphosate concentrations were prepared in UV bottom-transparent 96 well microplates to examine the

effects of glyphosate concentrations between 0.18 and 100 mg/L on the outcome of UV irradiation. The UV microplates with different glyphosate concentrations were exposed from the top or bottom using similar UV doses as the quartz cuvettes with uniform glyphosate concentrations. The 96-well UV microplates were placed on a cooling plate to avoid heating and maintain the temperature at around 22 °C to minimize evaporation from the small sample volumes used in these plates (100 µL). In all UV experiments, parallel control samples without UV exposure were covered with aluminium foil and stored in the dark for later analysis together with UV exposed samples.

UV irradiation experiments were carried out using a 4 W UVP UVGL-25 lamp equipped with separate UV-A (365 nm) and UV-C (254 nm) tubes, and an 8 W UVP 3UV lamp equipped with separate tubes for UV-A (365 nm), UV-B (302 nm) and UV-C (254 nm). The two UV lamps gave comparable results in experiments for comparable UV doses, and the 8 W lamp with greater intensity was selected to shorten exposure times. Irradiation intensity was measured using an Extech SDL470 Light meter equipped with UV-AB and UV-C sensors. The irradiation intensity at 15 cm distance from the UVP 3UV lamp was 970 µW/cm^2/s for UV-A, 1900 µW/cm^2/s for UV-B, and 327 µW/cm^2/s for UV-C. UV doses (J/cm^2) were calculated from the measured UV irradiation intensity (µW/cm^2/s) and the exposure time (s). No detectable UV-C irradiation was measured from the UV-A and UV-B lamps and vice versa. UV exposure doses (J/cm^2) were controlled by changing exposure times and distances to the UV lamps. For example, an exposure UV dose of 20 J/cm^2 was obtained using the same exposure time but different distances to the UV lamp (20 cm for UV-A, 35 cm for UV-B, and for 5 cm for UV-C). The presence of active oxygen species in aqueous samples after UV irradiation was confirmed by the addition of different oxygen radical probes. Superoxide radical ($\cdot O_2^-$), was detected by measuring chemiluminescence after post-treatment addition of 1 mM luminol. Hydroxyl radical ($\cdot$OH) was detected by measuring fluorescence after pre-treatment addition of 1 mM coumarin, terephthalic acid or benzoic acid. Chemiluminescence and fluorescence originating from the oxygen radical probes after reaction with active oxygen species were quantified using a Victor X2 Multilabel Plate Reader.

Analysis of glyphosate phototransformation products

Liquid chromatography-high resolution mass spectrometry analysis (LC-HRMS) of water samples with glyphosate was performed before and after UV exposure to identify transformation products. Analyses were carried out using an Ultimate 3000 High-Pressure Liquid Chromatography coupled through an ESI source to an LTQ-Orbitrap mass spectrometer. Chromatographic separation was achieved using a reversed-phase C18 column by injecting a 10 µL sample volume at a mobile phase consisted of a mixture of 0.1 mM Formic Acid (eluent A) and Acetonitrile (eluent B). The gradient profile started with 5 % B, increased up to 100 % B in 40 min and to 100 % A in 10 min. Samples were ionized in both positive and negative ionization modes. The LC effluent was delivered to the ESI ion source using nitrogen as sheath and auxiliary gas with the following parameters: sheath gas 34 arbitrary unit (arb), auxiliary gas 15 arb, capillary voltage 4.48 kV, and capillary temperature of 270 °C. Full mass spectra were acquired in positive ion mode with a resolution of 30.000. Data analysis was performed using the MZmine 2.53 for peak alignment, peak grouping, background noise and retention time correction, and the METLIN database was used to identify the transformation products.

Results

Identification of glyphosate phototransformation products

Liquid chromatography high-resolution mass spectrometry analysis (LC-HRMS) of water samples with glyphosate was performed before and after UV-C irradiation to identify transformation products after UV doses of 20 J/cm^2 and 70 J/cm^2 (Table 1). The analysis was carried out as a semi-targeted analysis focusing on likely glyphosate transformation products using the METLIN database. The LC-HRMS analysis confirmed that aqueous concentrations of glyphosate decreased by 96 % after UV-C irradiation and that degradation had occurred. More than 20 glyphosate ($C_3H_8NO_5P$) transformation products were observed after UV-C irradiation (Table 1) including main products such as sarcosine ($C_3H_7NO_2$), glycine ($C_2H_5NO_2$), glyoxylic acid ($C_2H_2O_3$), aminomethylphosphonic acid (CH_6NO_3P; AMPA), acetic acid (CH_4O_2) and phosphoric acid (H_3PO_4). CO_2 was also a potential transformation product but was not targeted in the analysis due to a high background concentration of CO_2/HCO_3^- in the water samples. Increases in AMPA and glycine concentrations were observed after UV-C irradiation at 70 J/cm^2,

whereas sarcosine concentrations decreased. Phosphoric acid was detected at both high and low UV-C doses whereas glyoxylic and acetic acid were only observed after UV-C irradiation at 70 J/cm^2. Glyphosate concentration decreased in samples irradiated with 20 J/cm^2 and was no longer detectable in samples treated with 70 J/cm^2.

Table 1 **Identified transformation products by LC-HRMS analysis in negative ESI (-) and positive ESI(+) modes after UV-C irradiation of aqueous glyphosate**

Compound	*m/z*	RT (min)	ESI	Compound	m/z	RT (min)	ESI
H_3O_4P	96.968	25.94	–	$C_5H_9NO_2$	116.069	15.66	+
CH_6NO_3P	110.001	1.75	–	$C_3H_3NO_2$	84,009	27.82	–
$C_2H_5NO_2$	74.020	1.86	–	$C_3H_4O_4$	103.003	1.69	–
$C_2H_2O_3$	72.908	16.64	–	$C_3H_6O_4$	105.017	26.14	–
$C_3H_7NO_2$	90.054	29.62	+	C_3H_6O	58.080	11.8	+
$C_2H_7NO_2$	77.084	1.77	–	$C_3H_9NO_2$	92.069	1.66	+
C_2H_5NO	59.070	35.32	–	$C_4H_{10}O_2$	91.074	28.37	+
$C_2H_6N_2O_4$	91.016	28.99	–	C_4H_8O	73.028	4.43	+
$C2H_4O_2$	59.015	8.52	–	$C_4H_8O_4$	119.033	6.85	–
$C_3H_8N_2O$	89.070	30.13	+	$C_4H_{10}O_3$	106.120	29.42	–
$C_6H_{11}NO_2$	130.085	28.58	+	C_5H_5N	80.048	32.84	+

Conclusion

This study investigated effects of UV-A, UV-B and UV-C irradiation of glyphosate on photolysis. The results suggested that UV-C and to some extend UV-B mediated indirect photolysis of glyphosate in water could attenuate concentrations of this pesticide. UV-C mediated formation of less bioactive glyphosate transformation products may subsequently facilitate environmental biodegradation. The results of our study can be relevant for further developments of UV mediated treatment processes for aquatic contaminants.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes experiments on the effects of UV-A, UV-B and UV-C irradiation on glyphosate photolysis. Experiments on aquatic toxicity were neglected in this evaluation as not found relevant. The photolysis experiments and results are well described. Therefore, the article is considered reliable with regard to the photolysis experiments.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.1.1.2, CA 7.1.3, CA 7.5
Report author	Tauchnitz N. *et al.*
Report year	2020
Report title	Assessment of pesticide inputs into surface waters by agricultural and urban sources - A case study in the Querne/Weida catchment, central Germany.
Document No	Environmental pollution (2020), Vol. 267, Art No. 115186
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Degradation experiments: not reliable Adsorption experiments: not reliable Monitoring data: reliable

2. Full summary of the study according to OECD format

Relevant pesticide substances were measured in surface waters in agricultural and urban surroundings and in soil samples within the catchment area of the Querne/Weida catchment. Pesticides application data from farmers were analysed. Additionally, batch tests were performed to determine sorption and degradation of relevant pesticides for site-specific soil properties. Frequency of detection, number of pesticides and maximum concentrations were much higher in the surface water samples in mainly urban surroundings compared to those in agricultural surrounding. Glyphosate and AMPA contributed to the highest concentrations in surface water samples (max. 58 mg L^{-1}) and soil samples (max. 0.19 mg kg^{-1}). In most cases, pesticide detections in surface water and soil were not consistent with application data from farmers, indicating that urban sources may affect water quality in the catchment area substantially. However, it was observed that pesticide substances remain in the soil over a long time supported by sorption on the soil matrix. Therefore, delayed inputs into surface waters could be suspected. For the implementation of reduction measures, both urban and agricultural sources should be considered.

Materials and methods

Test site

The study was conducted in the Querne/Weida catchment located in Saxony-Anhalt (central Germany). The entire catchment area covers 247 km^2. The selected sub-catchment comprises 150 km^2 with the largest portion (97%) used as arable land. The main agricultural crops during the study period (2015-2016) were winter wheat (36 %), silage corn (16 %), winter barley and winter oilseed rape (13 %), sugar beets (6 %) and grain corn (4 %). Several towns are located within the catchment, with Querfurt being the largest (11,562 inhabitants). In the catchment, 92 % of inhabitants were connected to two central water run-off wastewater treatment plants (WWTPs) and 8 % to small decentralized WWTPs, which release their water to groundwater, to a septic tank or directly to surface waters.

The climate of the study site is subcontinental and characterized by low precipitation amounts with a long-term (1981-2010) precipitation level of 550 mm and maximum precipitation falling during the summer months. The long-term average annual air temperature was 9.0 °C. The geology consists of Mesozoic rocks (shell limestone, mottled sandstone) which are largely covered by a loess layer. Main soil types at the study site are Chernozems and Luvisols.

Precipitation and discharge
Precipitation amounts were obtained from the German Weather Service station Lodersleben (elev. 204 m, 51°23N, 11°37E). Discharge amounts were registered at two gauge stations in the Kriebuschbach and Weida surface waters by continuously measurement of water levels and periodic measurement of flow velocities. Discharge amounts were calculated by the rating curve method (stage-discharge relation). Discharge data from the two gauge stations were stored at 15 min intervals by the data logger of an automatic water sampler.

Water and soil sampling
Grab water samples were collected monthly from July 2015 to June 2016 at 9 sampling sites in different surface waters. At two sampling sites, one with a mainly agricultural surrounding (SP_{agr}) and one with a mainly urban surrounding (SP_{urb}), water samples were taken with daily resolution and pooled to one composite weekly sample from June 2015 to July 2016. For this purpose, automatic water samplers equipped with polytetrafluoroethylene (PTFE)-tubes and polypropylene bottles were installed in the surface waters. Composite samples were taken at weekly frequencies (7 aliquots every 24 h). Water samples were transferred to glass bottles and stored in the dark at 4 °C until analyses. Topsoil samples (0-30 cm) were taken at 25 different sampling sites in the vicinity of surface waters, in areas which were assessed to be vulnerable for erosion. In addition, soil samples were taken at five sampling sites at permanent soil observation plots up to a depth of 5 m by the core drilling method (drill diameters of 105 mm).

Analysis of water and soil samples
Water samples were measured unfiltered after suspended particles had deposited on the bottom of the glass bottles. The samples were analysed for 53 pesticide substances, amongst them glyphosate and its degradation product aminomethylphosphonic acid (AMPA). Glyphosate and AMPA were analysed by HPLC-MS according to DIN 38407-22 (2001), modified with pre-column derivatization. The limit of determination (LOD) 10 ng L^{-1} for glyphosate and AMPA).

The soil was air-dried and passed through a 2 mm sieve. For analyses of glyphosate and AMPA, 5 g soil was extracted with 10 mL of 0.6 M potassium hydroxide. The sample was shaken for 30 min and centrifuged for 30 min at 5000 rpm. A portion of 600 mL of 32 % hydrochloric acid was added to precipitate humic acids for 15 min. Four mL of the sample were diluted with 6 mL water and filtered through a 500 mg Chromabond®C18 ec filter. The filter was previously conditioned with 5 mL methanol and 1 mL water. Samples were analysed with LC coupled to a triple quadrupole MS (LC-MS/MS). The limit of detection was 0.025 and LOQ was 0.05 mg kg^{-1} soil dry matter (DM).

Pesticide application data
For the analyses of pesticide application in the catchment area, farmers were contacted and asked to provide their pesticide application data for a retroactive period of 6 years (2011-2016). Farmers managing arable land adjacent to surface waters were primarily considered. The following information was requested: date of pesticide application, treated crop, indication of treatment, name of applied pesticide and amount (l ha^1, g ha^1). The amount of active ingredients of each pesticide was searched in the BVL (2020) database. Data from 7 farms encompassing a total of 9573 ha arable land (547 fields), corresponding to 66 % of the total farmland in the catchment area was obtained. Application data were tested for improper use by random checks with respect to application amounts, application date and indications. Pesticide detections in surface waters were compared to the application data of farmland directly adjacent to the considered surface waters at a radius of approx. 3 km around the sampling point. Pesticide detections in the topsoil samples were compared directly to the application data from the respective field (if available). For the depth profiles at permanent soil observation plots, application data from farmers for a retroactive period of 10 years (2007-2016) were available for analysis. For all pesticide detections in water and soil samples, the days between last application and detection were calculated, if application data were available.

Sorption and degradation laboratory study
For the laboratory study, homogenized soil samples from depth profiles (sorption study: 0-1 m, degradation study: 2-4 m) with the following soil properties were used: sandy silt, pH 7.8, 0.12 % of OC DM for degradation studies and clayey silt, pH 7.3, 1.29 % of OC DM for the sorption studies.

Sorption studies were performed by carrying out two classic batch test series (A & B). For each batch series, a solution consisting of synthetic rainwater (distilled water with 1.64 mg L^{-1} $KHCO^3$, 6.86 mg L^{-1} $NaNO_3$ and 40.04 mg L^{-1} $CaSO_4$ * 2 H_2O) spiked with the pesticide substances (glyphosate A: 170 & B: 400) were prepared. For this purpose, pesticide substances were added to the synthetic rainwater, stirred at 40 °C for 12 h and then filtered. For the different approaches, soil dry matters of 226 g, 452 g, 903 g, 1355 g and 1806 g along with corresponding process water amounts to achieve liquid-solid ratios of 8.5, 4.0, 1.7, 1.0 and 0.6 were filled in 2 L glass bottles. As reference for blank subtraction, process water without added soil was used. The batches were shaken overhead for 22 h at 10 °C in the dark and left for 2 h for sedimentation. Then samples were centrifuged for 20 min at 4000 rpm. Supernatants were decanted and filtered and analysed as described above. The difference between the initial pesticide concentration in solution and the concentration after equilibration was assumed to equal the amount of pesticides sorbed to the soil. The isotherm models Henry and Freundlich have been applied to determine the sorption coefficients (K_d, K_{Fr}).

For examination of the microbial degradation, static batch tests (soil saturation extracts, method embedded in LfULG, 2004) were prepared under anaerobic conditions. Analogous to the sorption study, the homogenous soil samples were mixed with synthetic rainwater, which was spiked with the pesticide substances at a concentration of 200 mg L^{-1} (solution stirred at 40 °C for 12 h and then pressure filtered). The produced suspensions with a liquid-solid ratio of 0.3:1 were incubated in the dark in airtight 1 L glass jars at 10 °C. For the analyses, pore water was extracted by a stainless-steel pressure-filtration-cell equipped with a 0.45 mm filter up to 2 bar with N_2. The first sampling of pore water was conducted 1 day after preparation of soil saturation extracts, sufficient time for equilibration between liquid and solid phase. Pore water samples were analysed for glyphosate and AMPA as described above every 1-3 months (time wise increasing sampling intervals) in a time span of up to 333 days. Pesticide degradation was described using first-order kinetics. Correlations between discharge amounts and pesticide concentrations and regression coefficients for the sorption studies were calculated.

Results

Precipitation and discharge
Yearly precipitation during the study period amounted to 479 mm (2015) and 441 mm (2016) whereas the most precipitation fell during the summer months (May to September) with amounts of 287 and 246 mm in 2015 and 2016, respectively. One heavy rain event with 30 mm d^{-1} occurred on 5 July 2015. Average registered discharge in 2015 at the Kriebuschbach gauge station was 0.053 m^3 s^{-1} and in 2016, it was 0.054 m^3 s^{-1}. The high discharge was associated with several heavy rain events in February 2016. Recorded mean discharge at the gauge station Weida in 2015 and 2016 was 0.28 and 0.22 m^3 s^{-1}, respectively. Accumulated for the weekly intervals highest discharge was registered in August 2015. Compared to the long-term mean (2006-2016) of 0.091 m^3 s^{-1} (Kriebuschbach) and 0.308 m^3 s^{-1} (1966-2016, Weida) measured discharge in the study period was markedly lower.

Pesticide application and detection in water and soil
Analyses of pesticide use data in the catchment area showed an application of 112 different pesticide substances during the period of 2011-2016, whereas the number of applied pesticide substances ranged between 29 and 70 per farm and 1 to 58 per field. Types of applied pesticides were mainly herbicides (53 %) and fungicides (26 %) e.g. glyphosate. The proportion of applied insecticides, growth regulators and rodenticides was smaller with 10 %, 10 % and 1 %, respectively. Indications of incorrect uses of pesticides by farmers (e.g. exceedances of allowed pesticide amounts) were not noticeable.

<u>Grab water samples</u>
In the monthly grab water samples, numerous pesticide substances were detected, ranging from 6 to maximum 35 different substances. At five sampling sites, pesticide substances were found in all

analysed water samples. The lowest number of detections was observed at sampling site Kriebuschbach, which is located next to a forest. The highest number of pesticides detected, was observed at sampling sites in the vicinity of urban areas. One of the most frequently detected substances was glyphosate and its metabolite AMPA. The pesticide glyphosate and AMPA contributed to the highest concentrations in the water samples with maximum values of 58.0 µg L^{-1}, and 5.4 µg L^{-1}, respectively. (see Figure 3).

Weekly composite water samples

In the composite water samples, in mainly agricultural surroundings (SP_{agr}), nine different pesticide substances were detected during the study period overall (Table 1). The most frequently detected substance was glyphosate (9 % of water samples), followed by AMPA with 7 % frequency of detection, respectively. Glyphosate and AMPA also contributed to the highest concentrations of maximum 0.20 and 0.21 µg L^{-1} and on average of 0.021 and 0.025 µg L^{-1}, respectively (Table 1). At SP_{agr}, positive detections were found in 26 % of analysed water samples. In contrast to SP_{agr}, at the sampling point in a mainly urban surrounding (SP_{urb}), higher concentrations of pesticide substances and a higher frequency of detection was observed (Table 2, see Figure 2). Here, 24 different substances were present and 90 % of analysed water samples showed positive detections. One of the most frequently determined substance was glyphosate and its metabolite AMPA with 63 % and 39 % frequency of detection, respectively. A total of 11 of the detected substances are known to be used for dual (agricultural and urban) or solely urban purposes. The highest mean concentration was measured for glyphosate (0.15 mg L^{-1}) and AMPA was measured at 0.04 mg L^{-1}. The highest concentration of glyphosate was 0.9 mg L^{-1}. Pesticide substances were found throughout the whole course of the year (Figure 2). Total pesticide concentrations in the water samples were positive correlated with discharge amounts ($r = 0.57$, $p < 0.05$, $n = 59$).

Figure 1 **Total pesticide concentration, number of positive findings and discharge at the gauge station Kriebuschbach in mainly agricultural surroundings (SP_{urb})**

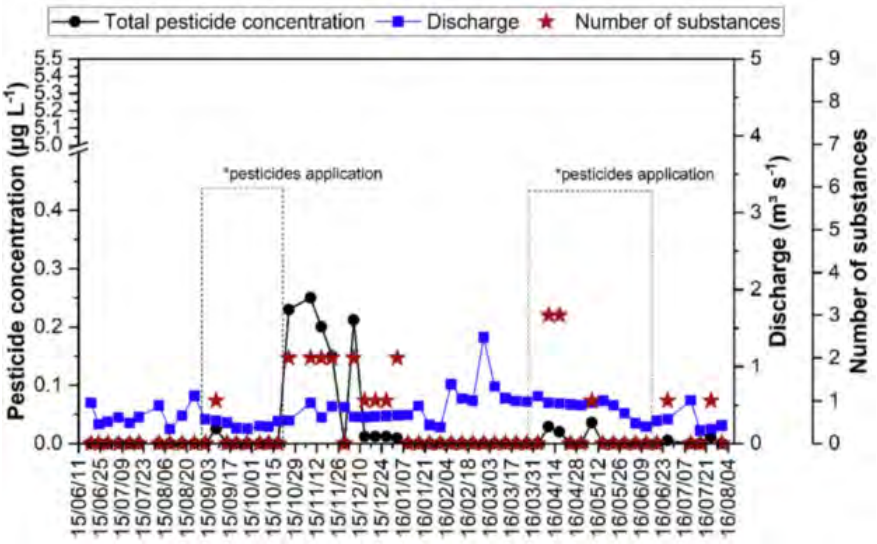


Figure 2 **Total pesticide concentration, number of positive findings and discharge at the gauge station Weida in mainly urban surroundings (SP_{urb})**

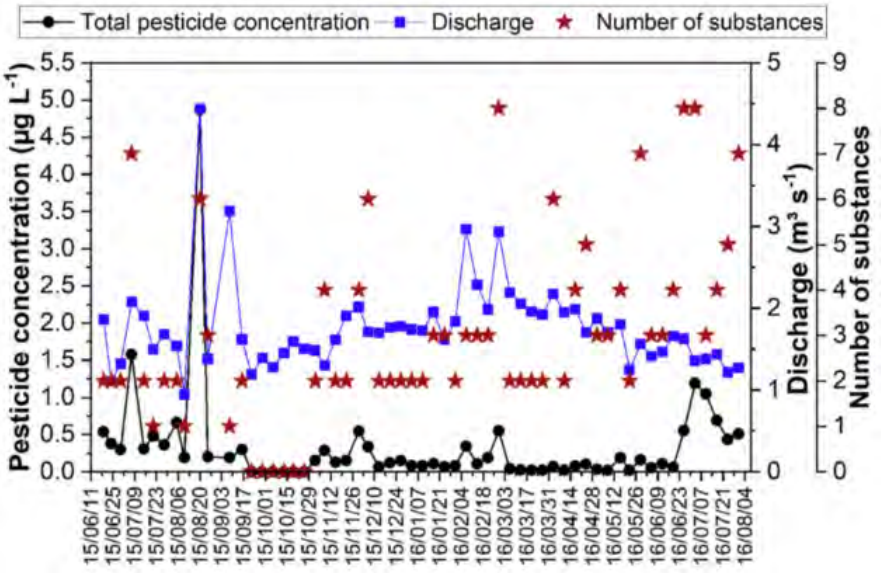


Figure 3 **Number of positive detections and maximal concentrations of pesticides in surface water samples (grab water) in the catchment area**

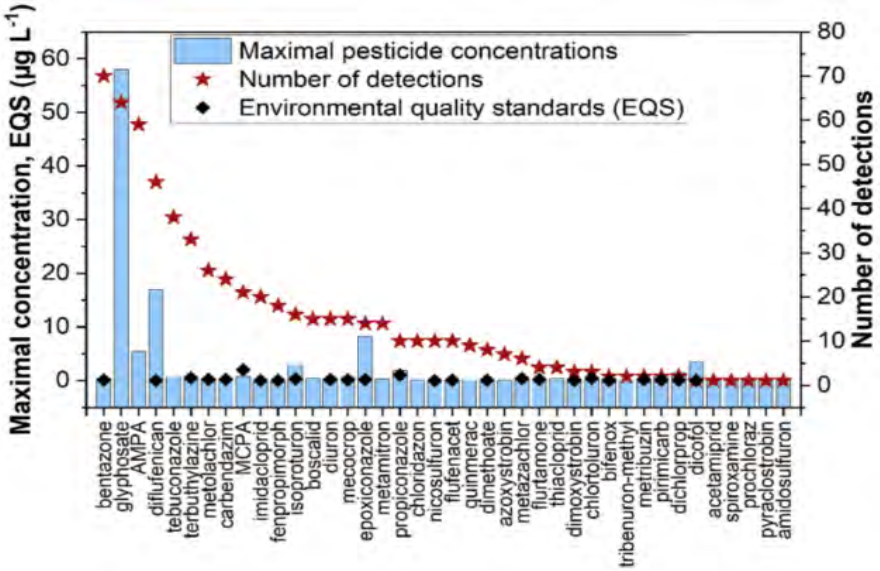


Table 1 **Minimum, maximum, mean concentrations and frequency of detection of pesticide substances in the weekly composite water samples with mainly agricultural surrounding (SP_{agr}) in the study period of 15/06/19 to 16/07/31 (n = 59), pesticide types and their use for agricultural (agric) or dual (urban and agricultural) purposes**

Substances	Frequency of detection (%)	Concentration (µg L^{-1})			Use (type)
		Min	Max	Mean	
AMPA[a]	7.0	0.12	0.21	0.025	–
boscalid	1.8	0.01	0.01	0.005	agric (h)
diflufenican	3.5	0.005	0.006	0.002	dual (h)
fenpropimorph	7.0	0.008	0.036	0.003	dual (f)
glyphosate	8.8	0.03	0.20	0.021	dual (h)
imidacloprid	3.5	0.0007	0.0008	0.0004	dual (i)
metazachlor	1.8	0.03	0.03	0.006	agric (h)
quinmerac	7.0	0.01	0.01	0.006	agric (h)
tebuconazole	3.5	0.011	0.014	0.005	dual (f)

h: herbicide, f: fungicide, i: insecticide
[a] Aminomethylphosphonic acid: metabolite of glyphosate

Table 2 **Minimum, maximum, mean concentrations and frequency of detection of pesticide substances in the weekly composite water samples with mainly urban surrounding (SP_{urb}) in the study period of 15/06/19 to 16/07/31 (n = 59), pesticide types and their use for agricultural (agric), urban or dual (urban and agricultural) purposes**

Substances	Frequency of detection (%)	Concentration (µg L^{-1})			Use (type)
		Min	Max	Mean	
amidosulfuron	1.7	0.018	0.018	0.005	agric (h)
AMPA[a]	38.9	0.03	0.18	0.04	–
bentazone	50.8	0.01	0.04	0.009	agric (h)
chlorotoluron	3.4	0.01	0.08	0.006	agric (h)
dichlorprop	18.6	0.02	0.33	0.015	agric (h)
diflufenican	3.4	0.003	0.004	0.002	dual (h)
dimethoate	3.4	0.02	0.07	0.006	dual (i)
diuron	1.7	0.05	0.05	0.006	urban (h)
fenpropimorph	16.9	0.008	0.03	0.004	dual (f)
flufenacet	1.7	0.08	0.08	0.006	agric (h)
flurtamone	1.7	0.09	0.09	0.006	agric (h)
glyphosate	62.7	0.04	0.90	0.15	dual (h)
imidacloprid	3.4	0.0007	0.0007	0.0004	dual (i)
isoproturon	3.4	0.017	0.15	0.007	dual (h)
MCPA	16.9	0.01	0.42	0.015	dual (h)
mecocrop	16.9	0.01	0.14	0.01	dual (h)
metamitron	3.4	0.02	0.11	0.007	agric (h)
metazachlor	1.7	0.02	0.02	0.005	agric (h)
metolachlor	5.1	0.01	0.05	0.006	agric (h)
prochloraz	18.6	0.01	4.00	0.08	agric (f)
quinmerac	3.4	0.01	0.06	0.006	agric (h)
tebuconazole	6.8	0.01	0.08	0.007	dual (f)
terbuthylazine	11.9	0.01	0.04	0.007	agric (h)
tribenuron-methyl	3.4	0.01	0.02	0.005	agric (h)

h: herbicide, f: fungicide, i: insecticide
[a] Aminomethylphosphonic acid: metabolite of glyphosate

Soil samples

The soil of the catchment area was characterised by pH values in the range of 7.3-7.9 and organic carbon (OC) contents of 0.07-0.8 % depending on the soil depth. In the analysed soil profiles, 22 different substances were found (Table 3). Concentrations ranged from 0.003 to maximum 0.19 mg kg^{-1} dry matter (DM). Glyphosate and its metabolite AMPA contributed to the highest concentrations of 0.19 and 0.17 mg kg^{-1} DM, respectively but were only detected in 13 % (glyphosate) and 17 % (AMPA) of the considered topsoil samples. Most detected substances were only present in the topsoil, however some amounts of glyphosate were also measured in the subsoil (>30 cm depth) at concentrations between 0.01 and 0.19 mg kg^{-1} DM.

Table 3 **Detected pesticide substances in topsoil (n = 30) and subsoil (n = 5), sampling date: 15/11/01, number of detections (Detect.) and minimal (Min) and maximal (Max) concentrations (Concentr.)**

	Topsoil (0–30 cm)		Subsoil (30–90 cm)		Subsoil (>100 cm)	
	Detect.	Concentr. (mg kg^{-1} DM) (Min-Max)	Detect.	Concentr. (mg kg^{-1} DM) (Min-Max)	Detect.	Concentr. (mg kg^{-1} DM) (Min-Max)
AMPA[a]	5	0.03–0.17	–	–	–	–
azoxystrobin	4	0.003–0.005	–	–	–	–
beta-cyfluthrin	1	0.004	–	–	–	–
boscalid	20	0.003–0.06	–	–	–	–
diflufenican	26	0.003–0.06	2	0.003–0.01	–	–
dimoxystrobin	3	0.003–0.003	–	–	–	–
epoxiconazole	17	0.003–0.03	1	0.003	–	–
glyphosate	4	0.03–0.09	–	–	3	0.01–0.19
imidacloprid	1	0.003	–	–	–	–
lambda-cyhalothrin	1	0.003	–	–	–	–
MCPA	4	0.003–0.014	2	0.008–0.01	1	0.01
metamitron	4	0.003–0.01	–	–	–	–
metazachlor	1	0.01	–	–	–	–
S-metolachlor	5	0.003–0.01	1	0.01	1	0.01
nicosulfuron	1	0.005	–	–	–	–
pendimethalin	14	0.003–0.04	2	0.003–0.01	1	0.007
prochloraz	5	0.003–0.02	–	–	–	–
propiconazole	1	0.03	–	–	–	–
pyraclostrobin	1	0.005	–	–	–	–
quinmerac	1	0.003	–	–	–	–
tebuconazole	18	0.003–0.09	2	0.003–0.004	–	–
terbuthylazin	10	0.003–0.01	1	0.004	–	–

[a] Aminomethylphosphonic acid: metabolite of glyphosate; DM: dry matter

Pesticide sorption and degradation

Calculated non-linear Freundlich isotherm coefficients (K_{FR}) provided the best fit of the sorption data with coefficients of determination (R^2) in the range of 0.393-0.977 (on average: 0.855, Table 4). The K_{FR} isotherm coefficients covered a wide range, from 0.39 to 50.1 mg^{1-n} L^n kg^{-1} (Table 4), whereby the highest K^{FR} value was observed for glyphosate. The calculated $T_{1/2}$ values ranged from 26 to 1420 days (Table 4) with a half-life of 33 days for glyphosate.

Table 4 **Henry (K_d) and Freundlich (K_{FR}) sorption coefficients and half-lives ($T_{1/2}$) of selected pesticide substances (anaerobic degradation)**

Substances	K_d L kg^{-1}	R^2	K_{Fr} mg $^{1-n}$ L^n kg^{-1}	N	R^2	$T_{1/2}$ days
bentazone	0.7	0.075	n. d.	n. d.	n. d.	839
diflufenican	n. d.	n. d.	n. d.	n. d.	n. d.	76
epoxiconazole	23.6	0.834	18.4	0.86	0.941	1420
glyphosate	455.9	0.899	50.1	0.63	0.952	33
imidacloprid	4.8	0.320	0.67	0.41	0.942	n. d.
MCPA	1.6	0.441	0.39	0.66	0.393	34
metazachlor	2.5	0.828	0.49	0.41	0.977	398
terbuthylazin	3.1	0.694	1.11	0.61	0.926	26

n.d.: not determinable due to no significant decrease in concentration (degradation studies), too low solution concentrations or concentration changes (sorption studies).

Detected pesticide substances related to the application time

Numerous positive detections of up to 35 different substances were found in the studied surface waters. Considering the pesticide application data from farmers, it can be shown that pesticide detections in water and soil samples were not consistent with their application. It was observed that pesticide substances remain in the soil over a long time supported by sorption on the soil matrix. Therefore, delayed inputs into surface waters could be suspected.

Table 5 **Detected pesticide substances in water and soil samples (listed after frequency of detection) related to the application time (for soil samples only analyses of 5 soil depth profiles)**

Water samples	Days between detection and application		Soil samples	Days between detection and application	
Detected substances	Min	Max	Detected substances	Min	Max
bentazone	1556	1893	diflufenican	30	3650
glyphosate	11	298	boscalid	730	730
AMPA[a]	11	298	tebuconazole	150	1095
diflufenican	1	193	epoxiconazole	150	150
tebuconazole	6	139	pendimethalin	n.a.[1]	n.a.[1]
terbuthylazin	15	370	terbuthylazin	365	3285
S-metolachlor	23	370	S-metolachlor	365	3285
carbendazim	n.a.	n.a.	AMPA	60	n.a.[1]
MCPA	n.a.	n.a.	prochloraz	365	n.a.[1]
imidacloprid	n.a.	n.a.	MCPA	365	n.a.[1]
fenpropimorph	65	372	glyphosate	60	n.a.[1]
isoproturon	197	227	metamitron	1460	1460
mecocrop	42	103	azoxystrobin	730	n.a.[1]
diuron	n.a.	n.a.	dimoxystrobin	730	730
metamitron	55	362	metazachlor	2920	2920
epoxiconazole	20	204	imidacloprid	n.a.[1]	n.a.[1]
propiconazole	44	44	lambda-cyhalothrin	2920	2920
chloridazon	820	820	propiconazole	180	180
nicosulfuron	92	372	pyraclostrobin	180	180
quinmerac	362	362	quinmerac	n.a.	n.a.
acetamiprid	785	785			

n.a.: no application in the last 6 years, n.a.[1]: no application in the last 10 years
[a] Aminomethylphosphonic acid: metabolite of glyphosate

Conclusion

Numerous pesticide substances were detected in the water and soil samples within the considered catchment area, where they might substantially contribute to environmental pollution. Analyses of pesticide application data from farmers showed in most cases that pesticide detections in water and soil samples were not consistent with their application. Analyses of soil samples and the accompanying batch tests showed that primarily strong sorbing substances might accumulate in the soil over long periods, exhibiting strong temporal delay compared to their application. This persistence presumably resulted from a decreased microbial availability and thus delayed degradation of the pesticides. It can be assumed by the present study that both agricultural and urban sources affect the water quality in the catchment area substantially. Urban inputs, however, led to the highest pesticide concentrations and the highest number of detected pesticide substances over the entire course of the year.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes pesticide analyses, amongst them glyphosate, in surface waters and in soil samples within a German catchment area. Additionally, batch adsorption and anaerobic soil degradation experiments were conducted.

For the analyses in surface waters and soil samples, methods and results are well described and conclusive. The respective results are therefore considered reliable.

Maximum concentrations found in composite weekly water samples were 0.90 µg/L for glyphosate and 0.21 µg/L for AMPA. In soil, maximum concentrations comprised of 0.09 mg/kg for glyphosate and 0.17 mg/kg for AMPA.

For the batch adsorption experiment, the methods are well described, however the experimental design is not in agreement with the relevant guideline (OECD 106), e.g. due to use of synthetic rainwater instead of $CaCl_2$ solution, insufficient information on the test concentrations and a temperature of 10 °C. From the information provided, it cannot be concluded on the representativeness of the soils used. Further, besides the final adsorption parameters, no detailed results are reported. Thus, no conclusion can be made on the quality of the results. The adsorption results are therefore considered not reliable.

For the anaerobic degradation experiment, the methods are well described, however the experimental design is not in agreement with the relevant guideline (OECD 307), e.g. due to use of synthetic rainwater, insufficient information on the test concentrations, use of slurry of a liquid-solid ratio of 0.3:1, air-tight incubation and analysis of pore water, only. From the information provided, it cannot be concluded on the representativeness of the soils used. Further, besides the final half-live, no detailed results are reported. Thus, no conclusion can be made on the quality of the results. The results from the anaerobic degradation experiment are therefore considered not reliable.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2020 年 7 月 1 日～2020 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 8.2.2 and CA 8.2.2.1 and CP 10.2.2
Report author	Forner-Piquer I. *et al.*
Report year	2021
Report title	Differential impact of dose-range glyphosate on locomotor behavior, neuronal activity, glio-cerebrovascular structures, and transcript regulations in zebrafish larvae.
Document No	Chemosphere (2021), Vol. 267, Art. No. 128986
Guidelines followed in study	OECD TG 236 partially
Deviations from current test guideline	Deviations from OECD TG 236: • Indicators of lethality as coagulation of fertilised eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat not reported as recommended in the OECD TG, but growth, cardiac and brain edema, general and brain necrosis, curled tail, pigmentation, tail fin, notochord, eyes, optic capsule and muscles indicators are reported instead. • Validity criteria of overall fertilisation rate, water temperature in test chambers, exposure to positive control and dissolved oxygen at the end of the 96 hrs exposure are not reported. • Apical observations of embryos 24 - 96 hrs post fertilisation are not reported • Water quality (pH, total hardness and conductivity in the control) is not reported • Glyphosate concentration in the lowest test concentration is not reported • Tested concentrations are not spaced by a factor not exceeding 2.2
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

Zebrafish (*Danio rerio*) larvae were exposed to glyphosate concentrations between 0.05 and 10.000 µg/L from 1.5 to 120 h post fertilization (hpf). Mortality was assessed at 24 hpf and cumulative hatching at 72 and 96 hpf. Gross morphology parameters were recorded until 120 hpf. The exposure of zebrafish to glyphosate concentrations had no increasing effect on mortality after 24 hpf. No significant effects were observed at the hatching rates at 72 hpf and 96 hpf and for morphological differences at 120 hpf. Zebrafish larvae showed behavioural changes in locomotor activity at concentrations equal and higher 1000 µg a.s./L. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

Materials and methods

Test item: Glyphosate (N-(Phosphonomethyl) glycine); Sigma–Aldrich; CAS Number 1071-83-6; content of active substance (AS) 98.5%.

Test species: Zebrafish (*Danio rerio*); wild type AB strain.

Test design: Glyphosate was diluted in water-based E3 medium to obtain the requested concentrations. Zebrafish larvae were exposed to 8 concentrations of glyphosate: 0.05, 0.1, 0.5, 1, 10, 100, 1000, 10.000 µg/L from 1.5 to 120 h post fertilization (hpf). Solutions were renewed every day.

Mortality was assessed at 24 hpf and cumulative hatching at 72 and 96 hpf. Gross morphology was observed daily (e.g. growth, necrosis, eyes) until 120 hpf, using a stereomicroscope. Standard length was measured daily. Swimming bladder area, eye diameter and head-trunk angle were assessed at 120 hpf.

Locomotor behavioural activity like total distance travelled and velocity were assessed and recorded in a dark environment in an observation chamber coupled with video tracking. Therefore, all larvae at 120 hpf were transferred to a multi-well plate and acclimatized, for one hour and additionally for further 3 minutes after re-positioning in an incubator at 28°C and darkness, prior testing. Each treatment consists of 2 replicate plates with 24 larvae each plate. Locomotor behavioural activity data were smoothed with a Minimal Distance Moved threshold of 0.2 mm and with a Maximum Distance Moved filter of 8 mm to exclude small movements.

Glyphosate concentrations were analysed using liquid chromatography-mass spectrometry (LC-MS/MS).

Statistical analysis of morphology data and behavioural changes in locomotor activity was carried out with one-way ANOVA followed by Dunnett's multiple comparisons test. Cumulative hatching rate was analysed using two-way ANOVA followed by Dunnett's multiple comparisons test ($p < 0.05$).

Results

Analytical results (from supplementary data)

Analysis of glyphosate concentration was limited to high concentrations because of the limit of quantification of the technique used (10 µg/L). In particular, nominal levels of 1000 and 10.000 µg/L corresponded to actual values of 980 ± 310 µg/L and 6800 ± 1600 µg/L respectively.

Biological results

Table 1: Hatching rates and gross morphological parameters of zebrafish exposed to glyphosate

Treatment (µg a.s./L)	Mean hatching rate ± SD [a]		Mean total length ± SD (µm)	Mean swim bladder area ± SD (μm^2)	Mean eye diameter ± SD (µm)	Mean trunk - head angle ± SD (deg)
	72 hpf	96 hpf				
Control	73.20 ± 16.70	98.50 ± 2.60	3717 ± 89.3	61955 ± 4388	340.2 ± 18.0	157.3 ± 4.6
0.05	46.1 ± 7.70	99.30 ± 1.20	3696 ± 94.2	63331 ± 3172	325.1 ± 11.5	155.4 ± 2.9
0.1	63.80 ± 15.50	100 ± 0.00	3730 ± 67.2	63023 ± 6547	324.9 ± 18.5	156.0 ± 3.3
0.5	51.50 ± 20.30	100 ± 0.00	3730 ± 67.0	62653 ± 4355	324.8 ± 18.5	155.7 ± 2.10
1	63.80 ± 15.50	97.80 ± 3.90	3710 ± 40.0	61320 ± 5652	325.2 ± 15.6	156.8 ± 2.6
10	51.50 ± 20.30	100 ± 0.00	3676 ± 112.9	61930 ± 3973	323.5 ± 12.0	155.4 ± 2.3
100	39.30 ± 14.80	94.80 ± 5.10	3738 ± 109.3	62309 ± 2714	315.6 ± 12.1	155.5 ± 1.9
1,000	50.40 ± 23.90	98.70 ± 1.20	3736 ± 59.1	62089 ± 5004	325.0 ± 28.4	154.5 ± 2.7
10,000	62.10 ± 26.40	97.60 ± 2.50	3762 ± 62.9	64713 ± 4389	332.7 ± 8.42	157.0 ± 3.3

hpf Hours post fertilization
SD Standard deviation
[a] Expressed as hatched eggs/total x 100
deg Degree

No increase in mortality was observed following exposure to glyphosate (data not shown).

A trend decrease of hatching rates was observed at 72 hpf, although by 96 hpf no difference occurred.

No significant morphological differences were observed at any of the glyphosate concentrations tested.

All locomotor behavioural activities, observed at 120 hpf, showed significant effects at glyphosate concentrations equal or higher than 1000 µg/L compared to the control. Especially distance, mean velocity, number of rotations, and body mobility were reduced. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

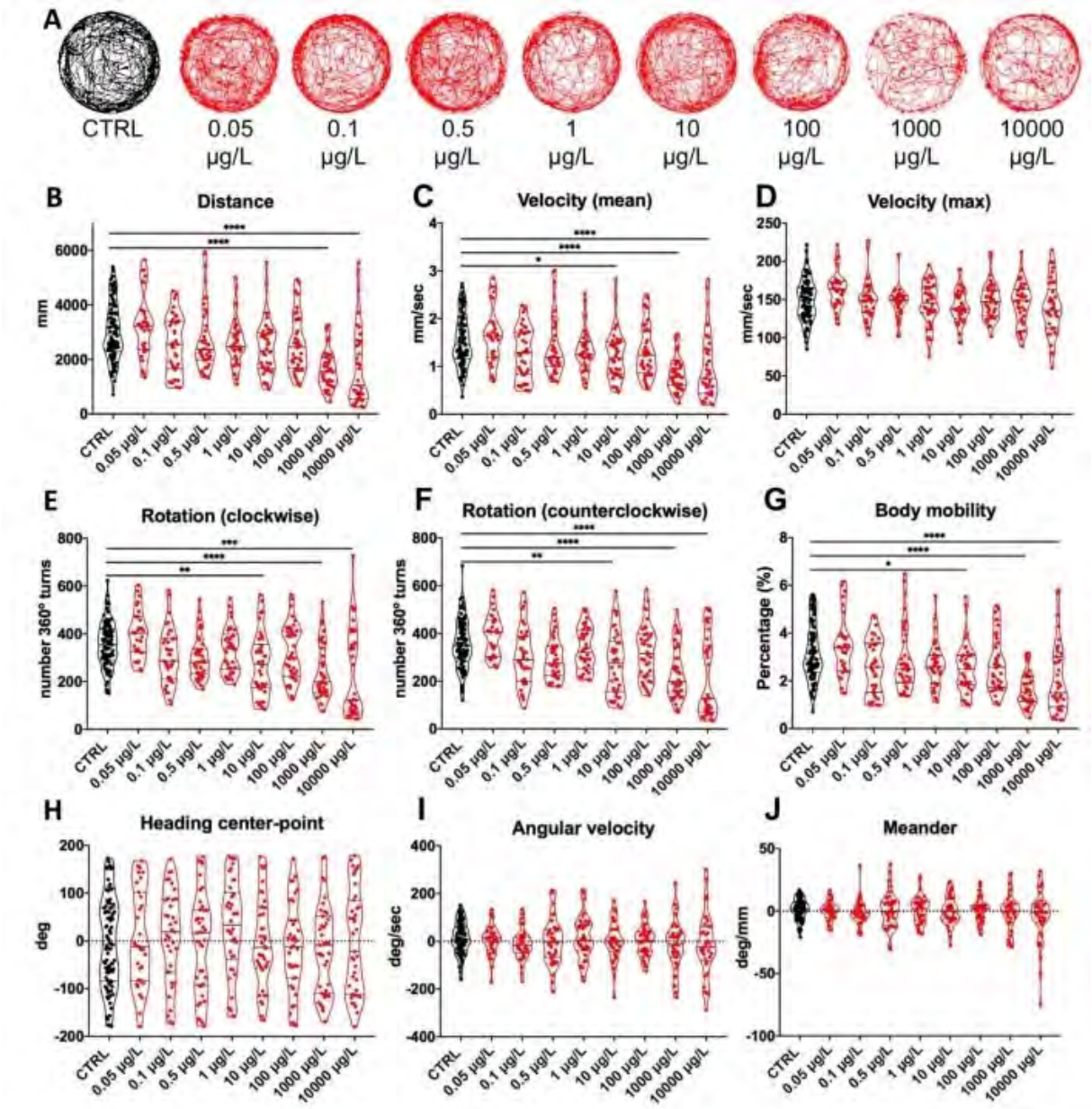


Figure 1: Behavioural defects in zebrafish larvae elicit with increasing glyphosate concentrations. A) Examples of 30-min swimming paths for each experimental group. B) Distance in mm. C) Mean velocity in mm/s. D) Maximum velocity in mm/s. E) Clockwise rotation in number of 360 turns. F) Counter-clockwise rotation in number of 360 turns. G) Percentage of body mobility. H) Direction of the body in degrees I) Angular velocity in degrees/s. J) Convolution of the movement in degrees/mm. Data are reported as mean ± SD, (1-way ANOVA, Dunnett's multiple comparison test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Experiments were performed in duplicate (CTRL n = 90, glyphosate groups n = 40).

Conclusion

The exposure of zebrafish to glyphosate concentrations had no increasing effect on mortality after 24 hpf. No significant effects were observed at the hatching rates at 72 hpf and 96 hpf and for morphological differences at 120 hpf.

The effect of glyphosate on zebrafish larvae showed behavioral changes in locomotor activity at concentrations equal and higher 1000 µg a.s./L. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Zebrafish (*Danio rerio*) larvae were exposed to glyphosate concentrations between 0.05 and 10.000 µg/L from 1.5 to 120 h post fertilization (hpf). Mortality, cumulative hatching rate and morphological changes showed no significant effects at any glyphosate concentration. Behavioural changes in locomotor were observed at concentrations equal and higher 1000 µg a.s./L. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

A wide range of concentrations (8 between 0.05 and 10.000 µg/L) were tested, which does not allow the estimation of EC_xs. Not all validity criteria according to OECD TG 236 (Fish Embryo Acute Toxicity Test) can be evaluated. Analytical verifications of the test concentrations and some methodological data are not fully reported. In addition, water quality parameters were not reported. The study is partially compliant with the OECD TG 236: Fish Embryo Acute Toxicity Test. Several LOEC and/or NOEC (hatching rate, morphological and behavioural parameters, etc.) can be obtained from the study.

This study has been classified as relevant (Category A acc. EFSA GD 2092, Point 5.4.1) and reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 8.2.2 and CP 10.2.2
Report author	Du-Carree J.L. *et al.*
Report year	2021
Report title	Impact of chronic exposure of rainbow trout, *Oncorhynchus mykiss*, to low doses of glyphosate or glyphosate-based herbicides.
Document No	Aquatic toxicology (2021), Vol. 230, Art No. 105687
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The effects of direct and chronic exposure of glyphosate on the health status of the rainbow trout, *Oncorhynchus mykiss* was assessed for ten months. In a continuous flow-through test, 12 fishes were exposed daily to a dose of around 1 µg a.s./L for one hour with the water flow stopped and then set up at 135 L/hour for the rest of the day, allowing for the gradual dilution of glyphosate. Additionally, an unexposed control group was included. The effect on the reproductive capacities was analysed over a period of 4 months covering spawning.
No mortality was recorded before the spawning period. Spawning induced mortality rates between 15% and 30%, without significant differences between the treatment groups. Growth and reproduction showed no statistical differences between the glyphosate treatment and the control group. These results suggest that a 10 months exposure of rainbow trout to a daily mean theoretical concentration of 0.123 µg/L of glyphosate administered using the pure active substance did not significantly modify their global health including during the spawning period.

Materials and methods

Test item: Glyphosate; Sigma-Aldrich; ref. 45521; CAS Number 1071-83-6; content of active substance (AS) 98%.

Test species: Rainbow trout (*Oncorhynchus mykiss*); three-year-old specific pathogen free (SPF) males and females from the protected and monitored fish facilities of the ANSES Plouzané Laboratory site (France).

Test conditions: In 400 L water tanks, fish were maintained under continuous flow-through conditions (300 L/h) and oxygen levels above 60% of saturation with aeration. Fish were fed daily at 1.5% of the biomass and submitted to a natural photoperiod and a temperature increasing from 9 to 21ºC between April and July and decreasing from 21 to 8ºC between July and November. Eggs during embryonic development were maintained at 8 ± 2°C.

Test design: 12 fish (male/female proportion of 1:2) per test item and control treatment were exposed every working day to 100 mL of a prepared concentrated glyphosate solution of 4 mg a.s./L, resulting in around 1 µg a.s./L of exposure. During exposure, the continuous flow-through (300 L/h) was stopped for one hour, so that the fish get in contact with the test substance. Afterwards, the flow-through was set up at 135 L/h for the rest of the day for a gradual dilution of glyphosate. Taking theoretical kinetics of glyphosate concentrations in contaminated tanks into account, the integrated mean theoretical concentration of glyphosate for each contamination day was approx. 123 ng/L.

The spawning period occurred in November 2018 and the maturation of fish was tested every week by exerting manual abdominal stripping to ensure spawning on a day when most of the fish were mature. Stripping events were done twice over the spawning period to collect eggs and sperm from the most mature fish, with approximately two weeks between them. In each treatment, sperm of all mature males were used to fertilize eggs of mature females. Females were weighed before and after stripping to measure the total weight of eggs. A fraction of eggs for each mature female was sampled for egg numbering and weighing. Relative fecundity was considered as the number of eggs divided by the weight of each respective female after stripping.

The embryonic development was conducted in tank containing approximately 300 L continuously renewed with a river water flow (approximately 300 L/h), after fertilising the eggs of each female with pooled sperm from each treatment. For the control four females and for glyphosate seven females were considered. Fertility was considered as the proportion of eggs surviving at five days post fertilization. Survival of eggs was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes.

Water tank samples were analysed for the actual concentration of glyphosate at the beginning (after 1 month of contamination) and at the end (after 10 month of contamination) of the exposure period using ELISA method for the first and HPLC for the second sampling. The metabolite AMPA was also quantified by HPLC.

For statistical analysis, data were tested for normality and homoscedasticity. In the case of normal and homoscedastic data, one-way Anova tests were used to compare means, followed by a post-hoc test of Dunnett. In the case of normal and heteroscedastic data, modified one-way Anova tests were used to compare means, followed by a post-hoc test of Tamhane-Dunnett. In the case of non-normal data, a Kruskal-Wallis test was used to compare means, followed by a post-hoc test of Dunn. Differences between survival and fecundity percentages were compared with a Chi-squared test. A p-value of 0.05 was used as the threshold for statistical significance.

Results

Analytical results

Glyphosate was not detected in control tank throughout the experiment. The glyphosate metabolite AMPA was never detected in any of the exposure tanks. One hour after the addition of glyphosate, concentrations ranged between 1.05 and 1.45 µg a.s./L in all exposure tanks. The glyphosate concentration measured by HPLC was approx. 30-40% lower (0.77 µg a.s./L) at the same point of the kinetic. Two hours after restarting the water flow, glyphosate concentrations were similar (between 0.24 and 0.55 instead of the modelled concentration of 0.51 µg/L predicted by the theoretical dilution curve) regardless of the method used.

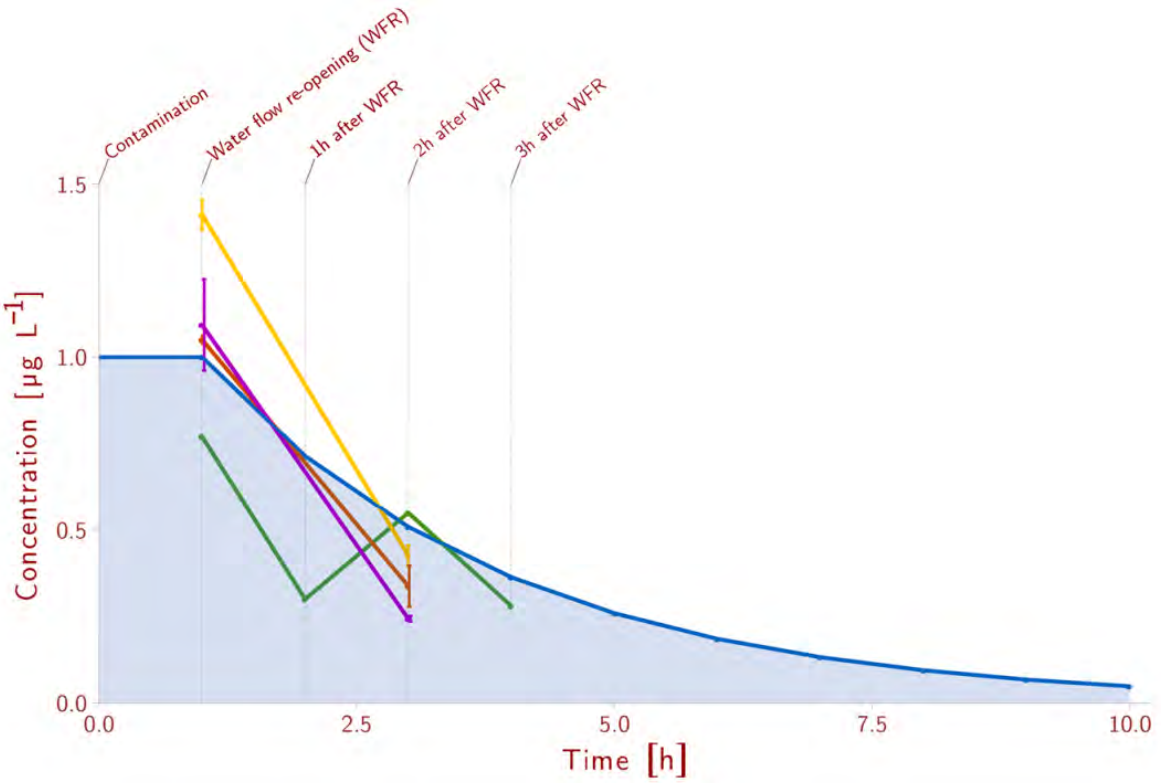


Figure 1: Mean concentration of glyphosate as a function of time (µg/L). Theoretical concentrations (in blue) are compared to concentrations measured at different times of the kinetic using ELISA or HPLC methods. ELISA analysis was done after 1 month of contamination for each condition (Glyphosate – G in yellow) and two points of the kinetics. HPLC analysis was done after 10 months of contamination in the tank contaminated with G for all points of the kinetics (in black). Error bars represent standard-errors.

Biological results

No mortality was recorded before the spawning period. Spawning induced mortality rates between 15% and 30% (data not shown), without significant differences between the treatment groups.

Outside of the spawning period, mortality events occurred in control and contaminated groups but no abnormal behaviour or clinical signs were observed.

Mean ratios of weights before and after the spawning period were 1.14 and 1.11 for the control and glyphosate, respectively. No statistical differences were found among the means of the treatment groups.

Table 1: Mean weights of mature rainbow trouts before and after spawning period

Treatment	**Mean weight ± SD (g)**		
	Before spawning [a]	**After spawning [b]**	**Ratios before/after**
Control	1208 ± 117	1387 ± 213	1.14 ± 0.13
Glyphosate 1 µg a.s./L	1131 ± 193	1249 ± 222	1.11 ± 0.11

SD Standard deviation
[a] measured in September 2018
[b] measured in November 2018

High fertility was observed for female rainbow trout in both treatments. Relative fecundity and fertility between control and contaminated fish were not significantly different.

Table 2: Mean reproductive parameters of female rainbow trout

Treatment	Reproduction	
	Relative fecundity ± SE (eggs/g fish)	**Fertility (%)**
Control	2.63 ± 0.72	99.6 ± 0.4
Glyphosate 1 µg a.s./L	2.71 ± 0.30	95.8 ± 3.9

SE Standard error

Conclusion

The effect of low and environmentally relevant concentrations of glyphosate on rainbow trout was tested in a long-term exposure. No mortality was recorded before the spawning period. Spawning induced mortality rates between 15% and 30%, without significant differences between the treatment groups. Growth and reproduction showed no statistical differences between the glyphosate treatment and the control group.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Rainbow trouts were exposed daily to glyphosate (1 µg a.s./L) for 10 month under flow-through conditions. Mortality, reproduction and growth showed no statistical differences between the glyphosate treatment and the control group.

In this study only one concentration was tested (1 µg a.s./L; low-dose). The characteristics and composition of the media used (water) were not fully described. The data on the technical material supports low chronic exposure risk. The test design is such that the fish were too big when used in the study and there is some uncertainty over the influence of the body size on the outcome of the study.

This study has been classified as relevant (Category A acc. EFSA GD 2092, Point 5.4.1) and reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 8.3.1.4, CP 10.3.1.5, CP 10.3.1.6
Report author	Odemer R. *et al.*
Report year	2020
Report title	Chronic High Glyphosate Exposure Delays Individual Worker Bee (*Apis mellifera* L.) Development under Field Conditions.
Document No	Insects (2020), Vol. 11, No. 10, Art. No. 664
Guidelines followed in study	OECD TG 75 modified and partially followed in Experiment 1.
Deviations from current test guideline	Deviations to OECD TG 75: • The temperature during the field experiments is only reported as an average of 18.9 °C without knowing if the recommended range of min. 15°C and max. 30°C was kept, to enable a sufficient flight activity of the bees. • Relative humidity and rainfall is not reported for the field experiments. • An assessment of control performance and acceptability of the study has not been stated despite the study appearing to have been conducted according to recognized approaches e.g. OECD 75 semi-field and tunnel test and the Oomen (1992) brood feeding approaches.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable without restrictions

2. Full summary of the study according to OECD format

In this study three field and one semi-field tests were conducted in Germany to assess the effect of glyphosate based herbicides (Glyfos Unkraut-Frei® 360 g a.i./L and Roundup® Power Flex 480 g a.i./L) to honey bees (*A. mellifera*). The field studies assessed the effect on the brood and colony development, adult survival, and overwintering success of honey bees, while residues were measured, whereas the semi-field study determined residues of glyphosate based herbicides in different bee relevant matrices.
For brood effects and survival, mini-hives housed in the "Kieler mating-nuc" system were orally exposed to two concentrations of Glyfos Unkraut-Frei® 360 g a.i./L (T1: 4.8 mg a.i./kg and T2: 137.6 mg a.i./kg feeding syrup) over a period of one brood cycle (21 days). Brood development and colony conditions were assessed after a modified OECD method (No. 75). On day 21, pooled samples of the stored syrup were collected from in-hive storage cells for residue analyses. For adult survival, weighed and labelled freshly emerged workers were introduced into non-contaminated mini-hives to monitor their life span for 25 consecutive days.
For colony conditions under field conditions, honey bee colonies were exposed during summer to flowering *Phacelia* sprayed with Roundup® Power Flex (480 g a.i./L) at a rate of 3.75 L/ha. The number of bees, brood cells, and stores (honey and pollen) were estimated on DAT-6 (20 July) before the exposure and DAT+15 (10 August) and DAT+57 (21 September) after the exposure. Beebread (stored pollen), food (stored syrup), and plant samples were taken at different time intervals for residue analyses.
For the overwintering success, honey bee colonies were fed with one concentration of Roundup® Power Flex 480 g a.i./L (5.439 mg a.i./kg feeding syrup) and were assessed on DAT-1 (1 October) before the exposure and DAT+43 (14 November) and DAT+170 (21 March) after the exposure, using the "Liebefeld method". Samples of stored food were collected from in-hive storage cells for residue analyses at different time intervals.

For the determination of GBH residues, honey bee colonies placed in tunnel tents were exposed to flowering *Phacelia* sprayed with Roundup® Power Flex (480 g a.i./L) at a rate of 3.75 L/ha. Stored food, pooled honey sac, pooled corbicular pollen, and pooled plant samples were taken at different time intervals after the treatment from all tents for residue analyses.
Feeding exposure revealed no significant differences between groups in colony condition and development as well as in brood development. Significantly increased brood termination (BFD+21) and significantly lower hatching weight of adult workers in the test item treatment T2, was assessed when compared to the control.With a hazard ratio (HR) of 0.93 for T1 and 1.43 for T2, the treated bees were not expressing higher mortality when compared to the control.
At the foliar application experiments glyphosate based herbicides did not significantly affect colony development but a matrix-dependent exposure gradient could be identified, which could be presented from high to low glyphosate residues, as follows: corbicular pollen > plants > honey sac > stored food/nectar.

Materials and methods

Experiment 1: Brood Development and Survival
Test item: Glyfos Unkraut-Frei® (Dr. Stähler, Köln, Germany); content of active ingredient (a.i.) 360 g glyphosate/L.
Tested concentration: Glyfos Unkraut-Frei® was directly mixed into 5 L of feeding syrup (Apiinvert, Südzucker GmbH, Mannheim, Germany) to achieve the desired concentrations of 0.090 g product/5 L for T1 and 2.610 g product/5 L for T2, corresponding to measured concentrations of 4.8 mg a.i./kg (T1) and 137.6 mg a.i./kg (T2) feeding syrup. The control was fed with untreated syrup, which was provided in a weekly interval like in all other groups. Chronic treatment in T1 and T2 was maintained for 21 consecutive days to cover a full worker-brood cycle with a total amount of 0.81 kg feeding syrup per hive, corresponded to a calculated amount of 3.859 mg (T1) and 111.428 mg (T2) glyphosate per hive, respectively.
Test species: Honey bee (*Apis mellifera L.)*; mini-hives originating from brood frames of two healthy donor colonies with no clinical symptoms of adult bee or brood diseases visible during inspection.
Location of the field site: Near Wurmberg, Germany (LAT 48.867414°, LON 8.808538°) between June and September (field phase). Within proximity of 250 m, no other hives were set up, whereas in an extended radius (>250 m) a sufficient number of unrelated colonies were located to provide enough drones for mating. At the present time, bees could forage on *Tilia* spp., *Cyanus segetum,* and other floral sources in the surroundings.
Test conditions: Natural field conditions with an average outdoor temperature of 18.9 °C and a precipitation of 53.97 L/m2. The incubator for brood development was set at 33 °C, 70% RH and total darkness.

Experiment 2: Field Exposure
Test item: Roundup® Power Flex (Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany); content of active ingredient (a.i.) 480 g glyphosate/L.
Tested concentration: Roundup® Power Flex (480 g glyphosate/L) was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering *Phacelia*. The control remained unsprayed.
Test species: Honey bee (*Apis mellifera L.)*; healthy and queen-right colonies with one hive body including ten combs and no clinical symptoms of adult bee or brood disease were visible; colonies were as homogenous as possible with approx. 15,000 bees per colony; queens originated from one breeding line.
Location of the field site: Near Braunschweig, Germany (Control plot: LAT 52.296415°, LON 10.437062°; treatment plot: LAT 52.202098°, LON 10.623331°) during July and August. Colonies were placed at the edge of two flowering *Phacelia tanacetifolia* plots.

Experiment 3: Overwintering
Test item: Roundup® Power Flex (Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany); content of active ingredient (a.i.) 480 g glyphosate/L.
Tested concentration: Roundup® Power Flex was directly mixed into 5 L of feeding syrup (1:1 water sugar, w/w, density: 1.2296) for each colony to achieve the desired nominal concentration of 8.13 mg

a.i./kg. The control was fed with untreated syrup. Chronic exposure in the Roundup® Power Flex treatment was maintained until the feeder was emptied. The average measured concentration of 5.439 mg a.i./kg in the feeding solution corresponded to a calculated total amount of 33.436 mg glyphosate per hive.
Test species: Honey bee (*Apis mellifera L.)*; healthy and queen-right colonies with one hive body including ten combs and no clinical symptoms of adult bee or brood disease were visible; colonies were as homogenous as possible with approx. 10,000 bees per colony; queens originated from one breeding line.
Location of the field site: Near Braunschweig, Germany (LAT 52.202098°, LON 10.623331°) between October and March.

Experiment 4: Determination of Glyphosate Based Herbicides Residues
Test item: Roundup® Power Flex (Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany); content of active ingredient (a.i.) 480 g glyphosate/L.
Tested concentration: Roundup® Power Flex (480 g glyphosate/L) was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering phacelia. The control was sprayed with water.
Test species: Honey bee (*Apis mellifera L.)*; healthy and queen-right colonies with one hive body including ten combs and no clinical symptoms of adult bee or brood disease were visible; colonies were as homogenous as possible with approx. 15,000 bees per colony; queens originated from one breeding line.
Location of the field site: Near Braunschweig, Germany (LAT 52.296415°, LON 10.437062°) during July and August.

Test design:

Experiment 1: Brood Development and Survival
In this field study, 19 mini-hives were established with about 800 worker bees. Subsequently, unmated sister queens were introduced, and the mini-hives were placed at a remote apiary for mating. Mini-hives were each housed in a Styrofoam box with four top-bars, a strip of a beeswax foundation attached to it and a feeder attached to it. After five weeks, the established hives showed all the relevant brood stages (eggs, larvae, and patches of sealed brood) and newly built combs.
Mini-hives of one treatment group, consisted of five replicate colonies, were orally exposed to sublethal concentrations of 4.8 mg glyphosate/kg (T1, low) and 137.6 mg glyphosate/kg (T2, high) over a period of one brood cycle (21 days). Additionally, an untreated control was included. One T2 mini-hive was discovered to be queenless shortly after the start of the experiment and was therefore removed (n: C = 5, T1 = 5, T2 = 4).
After the 21 days chronic exposure, one sealed brood comb containing brood cells ready to hatch was removed from the mini-hives of all groups and put together treatment-wise (five combs from control and T1, and four from T2) in an incubator. After 24 h, all hatched bees were pooled treatment-wise, and 30–40 young workers were collected randomly. After documenting the hatching weight bees were introduced into the remaining four mini-hives from a total of 19 and were divided equally according to their treatment to total numbers of 152 (control), 149 (T1), 141 (T2) introduced individuals per treatment. Survival was then monitored for 25 days.
For adult survival, freshly emerged workers from control and exposed colonies were marked with a coloured and numbered opalith plate on the thorax for identification and then introduced into non-contaminated mini-hives for 25 consecutive days of monitoring. Every two to three days all combs, including the inside of the hive, were photographed for the subsequent counting of the marked bees on a computer screen.
Hatching weight was documented by weighting the bees according to their treatment and respective pool, resulting in a total number (n) of control (50), T1 (38), and T2 (36).
Colony conditions and brood development were assessed after a modified OECD method No. 75.
Colony conditions: The total number of bees was estimated for each colony. In addition, the absolute hive weight was recorded at the time of the colony assessments. The assessments were performed shortly before the first application, DAT0, DAT+30 and DAT+56.
Brood development: For the colony condition assessment on DAT0, one or two brood combs were taken out of each replicate from the control, T1, and T2 (Brood area Fixing Day = BFD) groups. The

development of the bee brood was continuously assessed and followed. Approximately 100 cells per replicate containing eggs were selected, uniquely identified and pictures were taken from the comb-area containing eggs. From this evaluation, the brood termination rate was respectively calculated.

Experiment 2: Field Exposure

In this field study, twelve honey bee colonies were split into two groups with six replicates each control and treatment group. Eight days before application (Day after treatment = DAT-8) the colonies were placed to the field site.

Roundup® Power Flex was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering *Phacelia* (BBCH 64–65) with a field sprayer. The control remained unsprayed. Colonies from the treatment plot were migrated after nine days of exposure (DAT+8) to the control plot.

On DAT-6 (20 July) before the exposure and DAT+15 (10 August) and DAT+57 (21 September) after the exposure, respectively, the number of bees, brood cells, and stores (honey and pollen) were estimated.

Experiment 3: Overwintering

In this field study, bees were exposed to Roundup® Power Flex orally with treated feeding syrup provided in feeder.

On DAT-1 (1 October) before the exposure and DAT+43 (14 November) and DAT+170 (21 March) after the exposure, respectively, the number of bees, number of brood cells, and the number of stores were estimated.

Experiment 4: Determination of Glyphosate Based Herbicides Residues

In this semi-filed study, twelve honey bee colonies were split into two groups with three replicates each (control and treatment group). Queens were removed on DAT-14 to stimulate foraging in the tunnel tents. On the day of application (DAT0, 2 August), each colony was placed in a tunnel tent with an area of approximately 33.5 m2 on a flowering *P. tanacetifolia* plot. On the application day (DAT0) Roundup® Power Flex was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering *Phacelia* (BBCH 65) using a portable boom sprayer. The control was sprayed with water. Due to the loss of forage in the GBH-treated tents, all colonies were migrated after 13 days of exposure (DAT+13, 15 August) to a remote apiary.

A total of four stored food, two pooled honey sac, two pooled corbicular pollen, and four pooled plant samples were taken at different time intervals after the treatment from all tents for residue analyses. On DAT+5, plants could no longer be sampled due to the action of the herbicide.

Residue analyses

Experiment 1: Brood Development and Survival

For residue analyses of glyphosate, a sample of each feeding regime and the untreated feeding syrup was collected before the experiment and on day 21 pooled samples of the stored syrup from all groups were collected from in-hive storage cells.

Experiment 2: Field Exposure

For residue analyses, 12 beebread (stored pollen), 60 food (stored syrup), and 10 plant samples (whole plant except the roots) were taken at different time intervals before and after the treatment from both plot sites.

Experiment 3: Overwintering

For residue analyses, 66 samples of stored food were collected from in-hive storage cells from all groups at different time intervals.

Experiment 4: Determination of Glyphosate Based Herbicides Residues

For residue determination of glyphosate (GLY) and its metabolite aminomethylphosphonic acid (AMPA) in the samples, LC-MS/MS was used. The samples of all the experiments (1-4) were analysed together. A total of 176 samples were measured, including controls (experiment 1: six samples, experiment 2: 92 samples, experiment 3: 66 samples, experiment 4: 12 samples).

Statistic (for all experiments)

Statistical analysis of the Mortality in the mini-hives was evaluated with a Kaplan–Meier survival analysis (KM) and with log-rank tests (Cox–Mantel) using the "pairwise_survdiff" function.

Statistical analysis of the estimated number of bees and brood cells from all the colony condition assessments, the hatching weight and weight gain of the mini-hives as well as the brood termination rate, and the number of selected eggs from the BFD was carried out with a Shapiro–Wilk test for normal distribution. Normally distributed date were analysed with a one-way ANOVA and non-normally distributed data with a Kruskal-Wallis test to compare multiple experimental groups, respectively. Statistically significant results were further tested pairwise with a Student's t-test or Wilcoxon rank-sum test. All analyses were performed in R version 3.6.2 with a significance level of $\alpha = 0.05$ for all tests.

Results

Experiment 1: Brood Development and Survival

Survival of the bees showed no significant differences between the Glyfos Unkraut-Frei® treatments T1 and T2 and the control.

The hatching weight of adult workers in T2 was significantly lower (16.7% reduction) when compared to the control with median values of control: 0.14, T1: 0.13, and T2: 0.12 g.

In addition, a Cox proportional hazards model was applied to determine the hazard ratio (HR), displayed as a forest plot (Supplementary data). With an HR of 0.93 for T1 and 1.43 for T2, the treated bees were not expressing higher mortality when compared to the control (T1: $p = 0.73$, T2: $p = 0.051$, log-rank test).

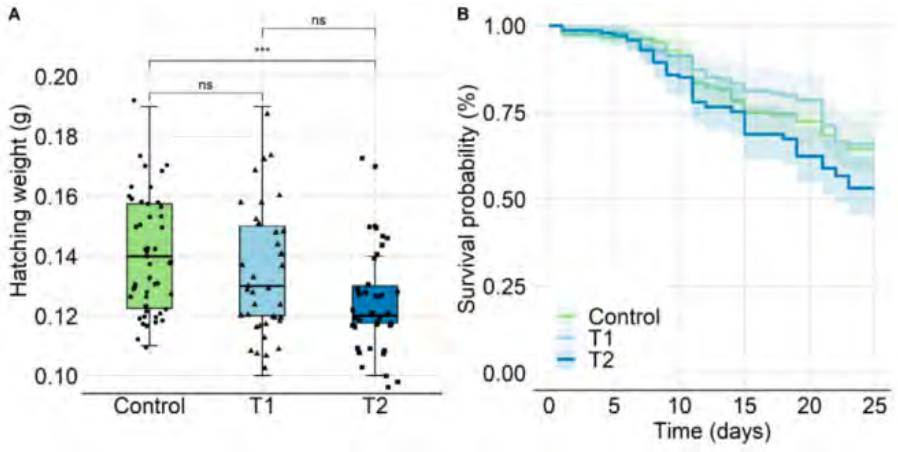


Figure 1. Effect of chronic Glyfos Unkraut-Frei® feeding exposure (T1: 4.8, T2: 137.6 mg a.i./kg) on hatching weight and survival of adult worker bees. (A) Hatching weight was assessed on the day of emergence after 24 h in the incubator and displayed as boxplot. Weight in T2 was significantly lower (16.7%) when compared to the control ($p < 0.01$, Wilcoxon rank-sum test, pairwise). (B) Survival of individual workers is illustrated with a Kaplan–Meier survival curve and 95% confidence intervals. A pairwise comparison with corrections for multiple testing did not confirm differences between the respective treatments and the control ($p > 0.05$, log-rank test, pairwise).

No significant effects in colony conditions between treatments and the control were observed.

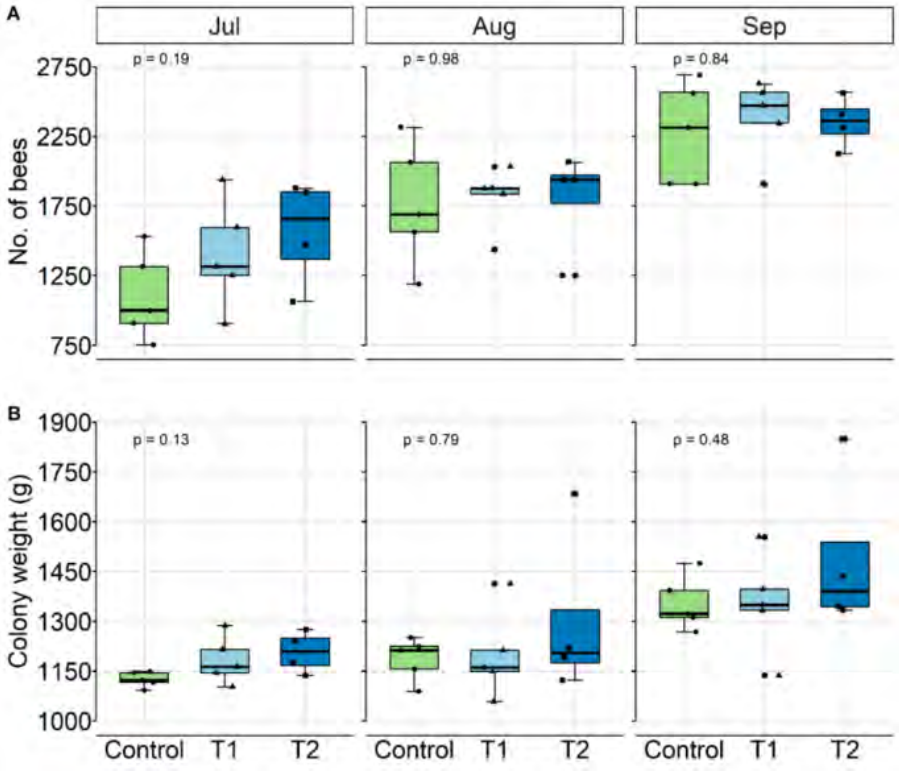


Figure 2. Effect of chronic Glyfos Unkraut-Frei® feeding exposure (T1: 4.8, T2: 137.6 mg a.i./kg) on colony conditions, displayed as a boxplot. (A) Number of worker bees increased similarly and was not significantly affected by the test item ($p > 0.05$, ANOVA). (B) Absolute colony weight and its increase during the study. There were no significant differences between groups ($p > 0.05$, Kruskal–Wallis test).

Successful brood development was observed in the control, T1, and T2 groups, in the majority of the marked brood cells. On BFD+13, the median termination rate was 13.04% in the control, 10.53% in T1 and 24.05% in T2. On BFD+21, the median termination rate was 22.11% in the control, 20.69% in T1 and 49.84% in T2. The T2, brood termination was significantly increased when compared to the control.

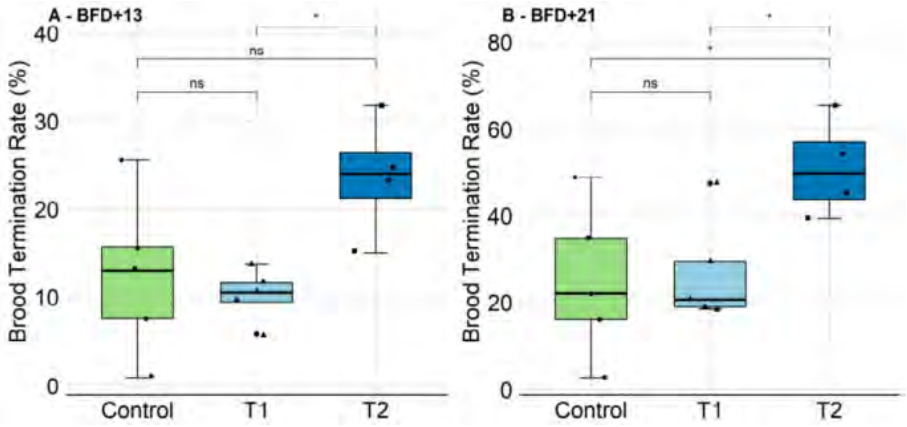


Figure 3. Effect of chronic Glyfos Unkraut-Frei® feeding exposure (T1: 4.8, T2: 137.6 mg a.i./kg) on the Brood Termination Rate (BTR) displayed as a boxplot. (A) The BTR of treatments T1 and T2 did not show significant differences when compared to the control ($p > 0.05$, t-test, pairwise) on BFD+13 (Brood area Fixing Day). (B) Eight days later, on BFD+21, T1 did not show differences when compared to the control ($p > 0.05$, t-test, pairwise). T2, however, revealed a significantly higher BTR ($p < 0.05$, t-test, pairwise).

Experiment 2: Field Exposure

Roundup® Power Flex did not significantly affect colony development. Bees and brood showed a pattern of increases and decreases in an alternate sequence. Brood cells were decreasing faster in the control on DAT+57 due to a greater winter food intake.

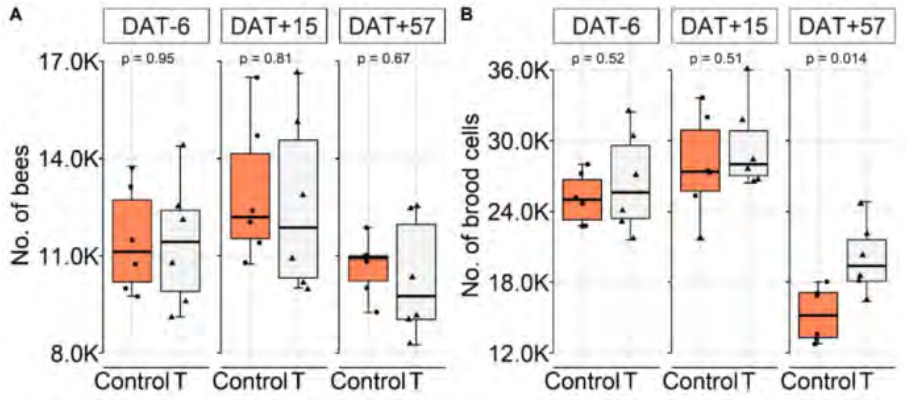


Figure 4. Effect of GBH field exposure (foliar spray) on colony conditions, displayed as boxplot. (A) Number of worker bees. (B) Number of brood cells during the study. On DAT+57, the treatment T had significantly more brood cells when compared to the control (Wilcoxon rank-sum test). For all other assessments, conditions were not significantly different. Dates correspond to the following months: DAT−6 (=20 July), DAT+15 (=10 August), and DAT+57 (=21 September).

Experiment 3: Overwintering

Shortly before hibernation (DAT+43), brood activity was reduced in all colonies independent of their treatment, followed by a continuous decrease of bees. At the end of overwintering, a decrease of stored food was observed (Supplementary data) as a result of the returning brood activity. Roundup® Power Flex did not significantly affect colony development. On DAT+170, we found that two colonies in the control and one in the treatment group had not survived winter.

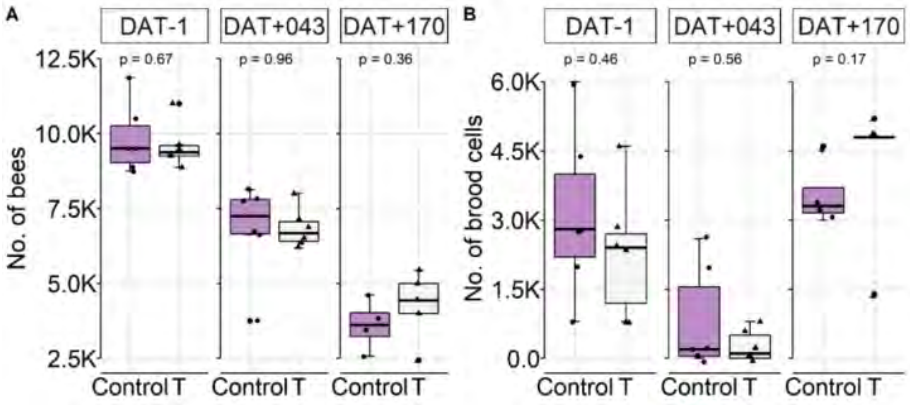


Figure 5. Effect of Roundup® Power Flex exposure (feeding) on colony conditions over winter, displayed as boxplot. (A) Number of worker bees. (B) Number of brood cells during the course of the study. For all assessments, conditions were not significantly different. Dates correspond to the following months: DAT−1 (1 October), DAT+43 (14 November), and DAT+170 (21 March).

Experiment 4: Determination of Glyphosate Based Herbicides Residues

In experiments 1, 2, and 3, irrespective of the time interval after the application (short term: three days, or long term: 170 days), glyphosate residues remained constant in all the sugar matrices.

In experiment 2, a 15.9-fold average increase, and a 24-fold peak increase was measured in stored pollen (beebread) when compared to stored nectar. In plants, a range similar to experiment 4 (tunnel tents) was measured shortly after the application on DAT0. Degradation of glyphosate in the tunnel tents, however, was faster than in the field.

In experiment 4, a matrix-dependent exposure gradient could be identified, which could be presented from high to low glyphosate residues, as follows: corbicular pollen > plants > honey sac > stored

food/nectar.
Honey sac residues were measured with a 3.3-fold reduction when compared to their floral source (plants). In turn, a 27.4-fold increase was measured in pollen when compared to honey sac contents, similarly to what was reported in experiment 2 for the stored products.
In experiment 1, trace residues of glyphosate were found in the pooled control food on DAT+21 (0.18 mg a.i./kg, AMPA was not detectable).
In experiment 3, trace residues of glyphosate were found in one control and one treatment colony before the application on DAT−1 (C2: 0.05 and T2: 0.04 mg a.i./kg, AMPA was not detectable).
In honey and plants, the LOD of GLY was 5.0 µg/kg and in pollen, it was 12.5 µg/kg, respectively. The LOQ was 12.5 µg/kg and 25 µg/kg, respectively. In honey, the LOD of AMPA was 2.5 µg/kg, and in plants and pollen, it was 12.5 µg/kg, respectively. The LOQ was 5.0 µg/kg and 25 µg/kg, respectively.

			Stock Solution		Stored Food/Nectar		Honey Sac	
	Time	Samples (n)	GLY	AMPA	GLY	AMPA	GLY	AMPA
Experiment 1: Brood development & survival	DAT0 (T1)	1	4.764 ± 0.424 +	0.020 ± 0.003 +	-	-	-	-
	DAT0 (T2)	1	137.566 ± 4.318 +	0.428 ± 0.001 +	-	-	-	-
	DAT+21 (T1)	5 *	-	-	5.103	0.013	-	-
	DAT+21 (T2)	4 *	-	-	99.861	0.319	-	-
Experiment 2: Field exposure	DAT0 (+0.5 h)	6 *	-	-	n.d.	n.d.	-	-
	DAT+1	6	-	-	0.327 ± 0.246	0.087 ± 0.194	-	-
	DAT+3	6	-	-	0.490 ± 0.455	n.d.	-	-
	DAT+6	6	-	-	0.347 ± 0.362	n.d.	-	-
	DAT+7	6	-	-	-	-	-	-
Experiment 3: Overwintering	DAT −1	12	-	-	0.004 ± 0.012	n.d.	-	-
	DAT+6	6	-	-	4.462 ± 1.651	0.006 ± 0.005	-	-
	DAT+13	6	-	-	5.159 ± 1.011	0.012 ± 0.004	-	-
	DAT+43	6	-	-	7.148 ± 3.234	0.033 ± 0.015	-	-
	DAT+170	6	-	-	4.985 ± 0.613	0.019 ± 0.004	-	-
Experiment 4: Residues under semi-field conditions	DAT0 (+1 h)	3 *	-	-	-	-	24.918	0.092
	DAT+1	3 *	-	-	-	-	-	-
	DAT+2	3 *	-	-	-	-	-	-
	DAT+3	3 *	-	-	-	-	-	-
	DAT+5	4	-	-	0.182 ± 0.225	n.d.	-	-

Figure 6. Glyphosate (GLY) and aminomethylphosphonic acid (AMPA) residues in stock solution, stored food/nectar and honey sack corresponding to the assessment date (DAT = day after treatment) and matrix from all four experiments (Treatment groups only). All values are rounded and presented as means (±SD) in mg a.i./kg where appropriate. *: pooled sample, +: analyzed twice, n.d.: not detectable, -: not measured).

			Beebread		Corbicular Pollen		Plants	
	Time	Samples (n)	GLY	AMPA	GLY	AMPA	GLY	AMPA
Experiment 1: Brood development & survival	DAT0 (T1)	1	-	-	-	-	-	-
	DAT0 (T2)	1	-	-	-	-	-	-
	DAT+21 (T1)	5 *	-	-	-	-	-	-
	DAT+21 (T2)	4 *	-	-	-	-	-	-
Experiment 2: Field exposure	DAT0 (+0.5 h)	6 *	-	-	-	-	96.429 ± 13.540 *	0.360 ± 0.005 *
	DAT+1	6	-	-	-	-	44.715 ± 1.429 *	0.255 ± 0.007 *
	DAT+3	6	-	-	-	-	33.634 ± 0.016 *	0.218 ± 0.017 *
	DAT+6	6	5.512 ± 6.922	0.007 ± 0.017	-	-	-	-
	DAT+7	6	-	-	-	-	31.051 ± 1.930 *	0.224 ± 0.007 *
Experiment 3: Overwintering	DAT −1	12	-	-	-	-	-	-
	DAT+6	6	-	-	-	-	-	-
	DAT+13	6	-	-	-	-	-	-
	DAT+43	6	-	-	-	-	-	-
	DAT+170	6	-	-	-	-	-	-
Experiment 4: Residues under semi-field conditions	DAT0 (+1 h)	3 *	-	-	614.841	2.122	82.407	0.217
	DAT+1	3 *	-	-	-	-	103.892	0.362
	DAT+2	3 *	-	-	-	-	62.845	0.229
	DAT+3	3 *	-	-	-	-	92.886	0.395
	DAT+5	4	-	-	-	-	-	-

Figure 7. Glyphosate (GLY) and aminomethylphosphonic acid (AMPA) residues in beebread, corbicular pollen and plants corresponding to the assessment date (DAT = day after treatment)

and matrix from all four experiments (Treatment groups only). All values are rounded and presented as means (±SD) in mg a.i./kg where appropriate. *: pooled sample, +: analyzed twice, n.d.: not detectable, -: not measured).

Conclusion

Glyfos Unkraut-Frei® showed no significant differences in bee survival between the treatments of 4.8 (T1) and 137.6 mg a.i./kg diet (T2) and the control. The hatching weight of adult worker bees was significantly lower (16.7% reduction) and the brood termination (BFD+21) significantly increased when treated with 137.6 mg a.i./kg diet, compared to the control.
Roundup® Power Flex did not significantly affect colony development either in summer or during overwintering.
The tested glyphosate based herbicides did not affect the lifespan of individuals, colony conditions, and overwintering, but delayed worker brood development when applied at a chronic high concentration.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study three field and one semi-field tests were conducted in Germany to assess the effect of glyphosate based herbicides (Glyfos Unkraut-Frei® 360 g a.i./L and Roundup® Power Flex 480 g a.i./L) to honey bees (*A. mellifera*). The field studies assessed the effect on the brood and colony development, adult survival, and overwintering success of honey bees, while residues were measured, whereas the semi-field study determined residues of glyphosate based herbicides in different bee relevant matrices.
Glyfos Unkraut-Frei® showed no significant differences in bee survival between the treatments of 4.8 (T1) and 137.6 mg a.i./kg diet (T2) and the control, but hatching weight of adult worker bees was significantly lower (16.7% reduction) and the brood termination (BFD+21) significantly increased when treated with 137.6 mg a.i./kg diet, compared to the control. Roundup® Power Flex did not significantly affect colony development either in summer or during overwintering.
Therefore, the study shows that the tested glyphosate based herbicides did not affect the lifespan of individuals, colony conditions, and overwintering, but delayed worker brood development when applied at a chronic high concentration (137.6 mg a.i./kg diet).

This study has been classified as relevant (Category A acc. EFSA GD 2092, Point 5.4.1) and reliable without restrictions.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（ヒトに対する毒性）

検索期間：2021 年 1 月 1 日～2021 年 5 月 14 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 5.6
Report author	Gorga A. *et al.*
Report year	2021
Report title	Low doses of glyphosate/Roundup alter blood-testis barrier integrity in juvenile rats
Document No	Frontiers in Endocrinology, 2021, 12, 615678
Guidelines followed in study	None
Deviations from current test guideline	No guideline followed.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, the possible effects of glyphosate (G) or Roundup (R) treatment of juvenile male rats on blood-testis barrier function and on adult male sperm production was investigated. Pups were randomly assigned to the following groups: control group (C), receiving water; G2 and G50 groups, receiving 2 and 50 mg/kg/day G respectively; and R2 and R50 groups receiving 2 and 50 mg/kg/day R respectively. Treatments were performed orally from postnatal day (PND) 14 to 30, period of life that is essential to complete a functional blood-testis barrier. Evaluation was done on PND 31. No differences in body and testis weight were observed between groups. Testis histological analysis showed disorganized seminiferous epithelium, with apparent low cellular adhesion in treated animals. Blood-testis barrier permeability to a biotin tracer was examined. A significant increase in permeable tubules was observed in treated groups. To evaluate possible mechanisms that could explain the effects on blood-testis barrier permeability, intratesticular testosterone levels, androgen receptor expression, thiobarbituric acid reactive substances (TBARS) and the expression of intercellular junction proteins (claudin11, occludin, ZO-1, connexin43, 46, and 50 which are components of the blood-testis barrier) were examined. No modifications in the abovementioned parameters were detected. To evaluate whether juvenile exposure to G and R could have consequences during adulthood, a set of animals of the R50 group was allowed to grow up until PND 90. Histological analysis showed that control and R50 groups had normal cellular associations and complete spermatogenesis. Also, blood-testis barrier function was recovered and testicular weight, daily sperm production, and epididymal sperm motility and morphology did not seem to be modified by juvenile treatment. The authors concluded their results show that continuous exposure to low doses of G or R alters blood-testis barrier permeability in juvenile rats. However, considering that adult animals treated during the juvenile stage showed no differences in daily sperm production compared with control animals, the authors proposed it is feasible to think that blood-testis barrier impairment is a reversible phenomenon and that ore studies are needed to determine possible damage in the reproductive function of human juvenile populations exposed to low doses of G or R.

Materials and methods

Animals

3-month old pregnant Sprague Dawley rats (250-300 g) were purchased from the University of Buenos Aires. In that facility, nulliparous female rats were mated with a stud male. The animals were separated after confirmed copulation either by detection of vaginal plug or spermatozoa in vaginal smear samples. Five days prior to pup delivery, rats were transported to the facility in transportation crates and were housed singly in free exchange cages (30 cm x 40 cm x 23 cm) with stainless steel tray containing pine wood shavings as bedding. Animals were maintained under controlled conditions of temperature (20±2°C), relative humidity and lighting (12 h light-2 h dark cycle) with free access to water and pellet laboratory chow (Rat-Mouse Diet).

Experimental design

At delivery, pups were sexed according to the anogenital distance. Litters were adjusted to 8 pups, prioritizing a maximum of 8 male pups per litter when possible. Male pups were randomly assigned to one of the following treatment groups: control group (C), receiving water; G2 and G50 groups, receiving 2 and 50 mg/kg/day G, respectively; and R2 and R50 groups receiving 2 and 50 mg/kg/day R, respectively. G was provided by Sigma-Aldrich (St Louis, USA). R formulation was a liquid water-soluble formulation containing 66.2% of G potassium salt. Treatments were given orally from PND 14 to 30. Pups were weaned on PND 21 and euthanized on PND 31. A set of animals were treated from PND 14 to 30 with water or 50 mg/kg/day R and kept without further treatment until PND 90. Tissues and blood were collected from euthanized animals in PND 31 or PND 90.

Rats were treated from PND 14 to 30 to comprise the full period of maturation of the BTB. The dose of 2 mg/kg/day was selected because it is in order of magnitude of the reference dose (RfD) of 1 mg/kg/day recently reassigned for glyphosate by the US EPA. The dose of 50 mg/kg/day was selected based on the no observed adverse effect level (NOAEL) for G and on a previous report that proposed this dose of R as appropriate for future toxicological analyses. No alterations in maternal care and nursing among the experimental groups were detected. Treatments caused no overt signs of toxicity.

Collection of blood and tissues

Blood was obtained by intracardiac puncture. The samples were allowed to clot at room temperature for 15 min, and then they were centrifuged at 950 g for 5 min in order to obtain the serum. Supernatants were immediately frozen at -20°C for subsequent analysis. Biochemical parameters were determined with an automated Cobas c 501 analyser.

At PND 31, animals were euthanized by CO_2 asphyxiation, and testes were dissected, weighed, and used for histological analysis and BTB assay. Also, testes were dissected and snap frozen for reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and Thiobarbituric Acid Reactive Substances (TBARS) determination. Tissue samples (liver, kidney, stomach, and intestine) were also collected at that time.

At PND 90, animals were anesthetized with a mixture of ketamine-xylazine and tissues were sampled and weighed. Testes were used for histological analysis and BTB assay and to determine daily sperm production (DSP). Epididymides were used to evaluate sperm parameters.

Histological analysis

For histological analysis, testes were removed and fixed in Bouin solution, and the other tissue samples were fixed in 10% formalin. Dehydration was carried out at room temperature using ascending concentrations of ethanol and shifting to xylene. After clearing, tissues were embedded in paraffin wax and 3- to 5-mm-thick sections were cut using a microtome. Sections were transferred to albumenized slides that were preheated to 37°C. Tissues were rehydrated in descending concentrations of ethanol, stained with haematoxylin/eosin and covered with a coverslip. The prepared slides were observed under an Eclipse 50i microscope equipped with a digital camer). For TUNEL assay, testicular sections were revealed with In Situ Cell Death Kit.

Intratesticular and serum testosterone determination

Testosterone was extracted from PND 31 testis homogenates with diethyl ether followed by evaporation of the organic phase and reconstitution of extracted testosterone in 0.1% PBS. Serum testosterone of PND 90 animals were also evaluated. Testosterone concentration was measured with an electrochemiluminescence assay using a Cobas e 411 analyser. Testosterone assay sensitivity was 10 ng/dl, and intra and interassay CV were 2.4% and 2.6%, respectively.

Thiobarbituric acid reactive substances assay

Lipid oxidation was determined by the colorimetric assay of TBARS. Testis homogenates were performed in PBS containing 0.4% w/v butylated hydroxyl-toluene on ice and then disrupted by ultrasonic irradiation. An aliquot (25 µL, corresponding to 450 µg protein) was added to 175 µL mixed reaction solution (0.15% w/v SDS, 0.5 N HCl, 0.75% w/v phosphotungstic acid, and 0.175% w/v 2-thiobarbituric acid). The mixture was heated in a boiling water bath for 45 min. TBARS were extracted with 200 µL of n-butanol. After a centrifugation at 10,000 g for 5 min at 4°C, the absorbance at 532 nm of the butanolic phase was measured. A calibration curve was performed using malondialdehyde (MDA), generated from 1,1,3,3-tetramethoxypropane (0.4-8 µM), as standard to express the absorbance changes as nmol MDA/µg protein.

Blood-testis barrier integrity assay

The permeability of the BTB was assessed with a biotin tracer. Immediately after testes isolation, a solution of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin dissolved in PBS containing 1 mM $CaCl_2$ was injected into the testis. The administered volume represented 10% of testis weight. Testes were then incubated at 34°C for 30 min, immersed in 4% paraformaldehyde and embedded in paraffin. For localization of the biotin tracer, testis sections (5 µm thick) obtained from different levels were deparaffinised and hydrated. To avoid nonspecific staining, sections were blocked with 5% non-fat dry milk in PBS containing 0.01% Triton X-100 for 15 min prior to incubation with streptavidin-rhodamine (1:300) for 45 min at room temperature. After nuclear staining with DAPI, sections were mounted in buffered glycerine and observed by Ahiophot fluorescent microscope with epi-illumination. At least 50 seminiferous tubules from 3 non-consecutive testis sections from each rat were examined. Results are expressed as % of permeable tubules.

RT-qPCR analysis

Total RNA was isolated from testis homogenates with TRI Reagent. The amount of RNA was estimated by spectrophotometry at 260 nm. RT was performed on 2 µg RNA at 42°C for 50 min with a mixture containing 200 U MMLV reverse transcriptase enzyme, 125 ng random primers and 0.5 mM dNTP Mix.

qPCR was performed by a Step One Real Time PCR System. Amplification was carried out as recommended by the manufacturer: 25 µL reaction mixture containing 12.5 µL of SYBR Green PCR Master mix, the appropriate primer concentration and 1 µL of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The relative standard curve method was used to calculate relative gene expression. Relative mRNA levels were normalized to the reference genes HPRT1 and β-actin.

Determination of daily sperm production

At PND 90, testes were collected and processed. The tunica albuginea was removed from one testis and the parenchyma was homogenized in 0.9% w/v NaCl containing 0.5% w/v Triton X-100. Then, the homogenates were ultrasonic disrupted for 30 s. Samples were diluted at 1:10 and transferred to a Neubauer chamber and counted in quadruplicate. Elongated spermatid nuclei with a shape characteristic of step 19 spermatids and resistant to homogenization were counted to determine the number of elongated spermatid nuclei. To calculate the DSP, the number of spermatids was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium.

Assessment of sperm parameters

At PND 90, sperm were recovered from cauda epididymis. The epididymides were placed in a conical tube, covered with 750 µL of fresh medium and sperm were allowed to swim up at 37°C. After 10 min, aliquots of the upper sperm layer were recovered for motility and morphology evaluation. To evaluate total motility (progressive + nonprogressive), sperm suspensions were placed on pre-warmed slides and analysed subjectively under a light microscope (400× magnification). To assess sperm morphology, a 10 µL aliquot of the sperm suspension was smeared over a microscope slide. After drying in air, the smear was fixed with methanol for 5 min, washed with distilled water, stained with Harris haematoxylin for 15 min, and finally washed with tap water. Sperm morphology was evaluated using a Nikon microscope at 1000× magnification. In all cases, at least 200 spermatozoa from each sample were assessed.

Statistical analysis

At least five animals per treatment group were used, and data were presented as mean ± SD. The variables under study were first submitted to tests of normal distribution (Shapiro-Wilk's Test) and for homogeneity of variances (Levene's Test). Then, one-way ANOVA followed by Tukey-Kramer test for the comparison of multiple groups was performed. To assume normal distribution, percentages were expressed as ratios and subjected to the arcsine square root transformation. Results were compared using the unpaired Student t test. Probabilities <0.05 were considered statistically significant. InfoStat 2016 was used.

Results

Glyphosate and Roundup effects on serum biomarkers and testis and body weight

Initially, rats were exposed to low doses (2 and 50 mg/kg/day) of G or R from PND 14 to 30. Serum biomarkers for renal (urea, creatinine) and liver (aspartate and alanine aminotransferases: AST and ALT) function were analysed. These biomarkers did not change in treated groups compared with the control (see table below). In addition, treatments did not alter liver, kidney, stomach, and intestine histology (data not shown). To explore the impact of G or R exposure on male reproductive system the authors assessed body and testicular weights after treatments. No differences in body and testis weight and in the ratio testis/body weight at any G or R dose tested were observed (see table below).

Table 1. Effect of G and R treatment on serum biochemical markers and on body weight, testis weight, and relative testis weight.

	Control	G 2 mg/kg/day	G 5O mg/kg/day	R 2 mg/kg/day	R 50 mg/kg/day
Urea (mg/dl)	31.5 ± 3.3	34.9 ± 4.2	33.0 ± 7.1	32.0 ± 5.7	35.9 ± 6.2
Creatinine (mg/dl)	0.13 ± 0.09	0.12 ± 0.05	0.18 ± 0.11	0.17 ± 0.04	0.21 ± 0.03
AST (UI/l)	134 ± 18	128 ± 13	136 ± 15	142 ± 16	111 ± 12
ALT (UI/l)	37.8 ± 7.0	37,9 ± 6,4	35.0 ± 5.3	37.2 ± 7.8	33.0 ± 8.2
Body Weight(g)	112.4 ± 11.2	101.1 ± 12.7	111.3 ± 17.0	105.3 ± 12.5	112.8 ± 13.0
Testis Weight (mg)	375.7 ± 90.6	327,7 ± 53,4	366.9 ± 80.2	332.3 ± 83.8	348.9 ± 77.4
TW/BW Ratio (%)	0.33 ± 0.05	0.32 ± 0.03	0.33 ± 0.03	0.31 ± 0.06	0.31 ± 0.04

Animals (n = 5/group) were treated with 2 and 50 mg/kg/day of G or R from PND 14 to 30. At PND 31, body and testicular weights were assessed, and blood was sampled by intracardiac puncture to determine serum parameters. Results are presented as mean ± SD.

Glyphosate and Roundup effects on testicular histology and blood-testis barrier function

Histological examination of the testes revealed differences in the seminiferous tubules among control and treated groups. Some seminiferous tubules of G2, G50, and R2 groups showed a disorganized epithelium, with an apparent low cellular adhesion. In R50 treated males, tubules presented severe disorganization and epithelial desquamation of the most differentiated cells (spermatocytes and round spermatids). In this group, the percentage of affected tubules is higher than in the other groups tested. Although epithelium disorganization was observed in treated groups, the histological characteristics of Sertoli cells-nucleus located parallel to basement membrane next to spermatogonia and premeiotic spermatocytes and triangular in shape remained unaltered. Additionally, TUNEL analysis of rat testis section from control and R50 treated animals were performed. Only a few seminiferous tubules with some TUNEL positive cells located in areas not corresponding to positions occupied by Sertoli cells were observed in both groups.

To analyse the effects of G or R treatment on BTB integrity, the permeability of the BTB was evaluated using a biotin tracer which is excluded from the ad luminal compartment of intact seminiferous tubules. The tracer entered the ad luminal compartment in animals treated with G or R at both doses tested. A significant increase in the percentage of seminiferous tubules with a permeable barrier in G and R groups was observed.

The next set of experiments was performed to evaluate possible mechanisms that would explain the deleterious effects of G or R treatments on BTB permeability. Firstly, G and R treatments did not modify either intratesticular testosterone (ITT) levels or androgen receptor (AR) expression. Secondly, TBARS levels were not modified by G or R exposure. Thirdly, G or R treatments did not modify claudin11, occludin, ZO-1, connexin43, 46, and 50 mRNA levels.

Juvenile Roundup treatment effects on adult animals

In order to evaluate possible consequences of juvenile herbicide treatment in adulthood, a set of animals was treated with 50 mg/ kg/day of R from PND 14 to 30 and then allowed to grow until PND 90. Several parameters were analysed at this age. As it was observed in PND 31, no differences in urea,creatinine, AST, and ALT levels between groups were observed. The histological examination of testis at PND 90 in control and R50 groups showed that tubules had normal cellularassociations and complete spermatogenesis. The analysis of the frequency of the stages of the seminiferous epithelium showed that no alteration of the presence of the different stages of the cycle of the seminiferous epithelium was found between groups. The study with the biotin tracer and the data obtained after determining the percentage of permeable tubules show a small increase in the percentage of permeable tubule in R50 group. Despite this small increase in BTB permeability, testicular weight, and DSP were not modified by juvenile treatment with R.

Cauda epididymal sperm were used to analyse sperm motility and morphology. No differences in motility and morphology were observed in sperm of both groups. In addition, no changes were observed in epididymal weight and in epididymal/body weight ratio between groups.

Conclusion

The authors concluded their results show that continuous exposure to low doses of G or R alters blood-testis barrier permeability in juvenile rats. Considering that DSP in adult animals, which have been unexposed to the herbicide for a prolonged period, is indistinguishable from that in control animals, the authors suggested it is feasible that BTB impairment is a reversible phenomenon. These results warrant further investigation of glyphosate-mediated reproductive damage of human juvenile populations exposed to low doses of G or R. Such analysis may include the determination of follicle stimulating hormone (FSH), luteinizing hormone (LH), anti-Müllerian hormone (AMH), inhibin B, and testosterone serum levels to get insight into Sertoli cell maturation in juvenile people as well as semen analysis in adult population.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The aim of the study was to investigate the possible effects of glyphosate (G) or the commercial formulation Roundup (R) treatment of juvenile male rats on blood-testis barrier function and on adult male sperm production. Pups were randomly assigned to the following groups: control group (C), receiving water; G2 and G50 groups, receiving 2 and 50 mg/kg/day G respectively; and R2 and R50 groups receiving 2 and 50 mg/kg/day R respectively. Treatments were performed orally from postnatal day (PND) 14 to 30, period of life that is essential to complete a functional blood-testis barrier. Evaluation was done on PND 31.
In conclusion, the results presented show that continuous exposure to low doses of G or R alters blood-testis barrier permeability in juvenile rats, but have no effect on clinical chemistry parameters, intertesticular testosterone levels, androgen receptor expression, or testis weight. Furthermore, adult animals treated during the juvenile stage showed no differences in daily sperm production compared with control animals, demonstrating that blood-testis barrier effects are a transient, reversible phenomenon.

The article is considered relevant (Category A) and reliable with restrictions for the following reason: Purity missing for glyphosate technical, exact number of animal/group difficult to find, no dose-response relationship, no HCD, and no positive control to confirm assay validity.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies

Publication: Gorga A., 2021, Low Doses of Glyphosate/Roundup Alter Blood-Testis Barrier Integrity in Juvenile Rats	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	Study performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the National Institute of Health of USA
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Glyphosate was provided by Sigma-Aldrich (St Louis, USA). No information about purity. Roundup formulation was a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt.
Only glyphosate acid or one of its salts is the tested substance	Y	Yes, glyphosate and glyphosate potassium salt as GBH
AMPA or other glyphosate metabolites is the tested substance	N	
Study		
Test species clearly and completely described	N	3-month-old pregnant Sprague Dawley rats (250-300 g), no number available of male and females. No details on the litters.

Publication: Gorga A., 2021, Low Doses of Glyphosate/Roundup Alter Blood-Testis Barrier Integrity in Juvenile Rats	**Criteria met? Y/N/?**	**Comments**
Test conditions clearly and completely described	Y	Housing, diet and environmental conditions described
Route and mode of administration described	N	Treatment given orally from PND 14 to 30, assumed via gavage but not described. Euthanasia on PND 31 for a group and on PND 90 for another group. Mode of administration not properly described.
Dose levels reported	Y	0, 2 and 50 mg/kg/day
Number of animals used per dose level reported	Y	At least 5 animals per treatment group were used, sometimes 7 animals/group. Difficult to find the exact number
Method of analysis described for analysis test media	N	
Validation of the analytical method	N/A	
Analytical verifications of test media	N/A	
Complete reporting of effects observed	N	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	Purity missing for glyphosate technical, exact number of animal/group difficult to find, no dose-response relationship, no HCD and no concurrent positive control to verify assay.
Not reliable	N	

1. Information on the study

Data point:	CA 5.6
Report author	Refaie A. A. *et al.*
Report year	2020
Report title	Hematological, biochemical, antioxidant and histopathological alterations in kidneys of Wistar rat pups exposed to glyphosate herbicide during lactation period
Document No	Current Topics in Pharmacology, 2021, 24, 69-76
Guidelines followed in study	None
Deviations from current test guideline	No guideline followed.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In the present study, the authors evaluated the effects of glyphosate administered during lactation period of pup rats at a daily dose of 0.3 mg/kg body weight [the acceptable daily intake (ADI)], 31 mg/kg body weight [no-observed-adverse-effect level (NOAEL)] and 56 mg/kg body weight [1/100 LD50] for 21 days. At the end of each treatment, the suckling pups were separated into male and female, and the relative kidney weights were measured. The levels of creatinine and urea were determined in serum. Oxidative stress was measured using lipid peroxidation and the activities of glutathione peroxidase (GPx) and catalase (CAT) in kidney tissue were also measured. Red blood corpuscle (RBC) and white blood cell (WBC) counts and haemoglobin content (Hb) were measured. Histopathological examination was also performed in kidney tissues. The pups showed increased relative kidney weight with the highest increment in 1/100 LD50 dose followed by NOAEL and ADI doses. Also, marked increment in malondialdehyde (MDA) level along with significant inhibition in catalase (CAT) and glutathione peroxidase (GPx) activities were detected in pup's kidney tissues. Counts of WBCs and RBCs and Hb content exhibited significant reduction in the blood ofpups that received 1/100 LD 50 dose which is more than the NOAEL and ADI doses. Histopathological examination of kidney tissue demonstrated focal fibrosis with inflammatory cells between glomeruli and tubules of male and female pup kidney tissue (lower scoring severity (+) in ADI and NOAEL doses than 1/100 LD50 (+++)). Additionally, at 1/100 LD50 dose focal haemorrhages, degradation in tubular lining epithelial cells (+) and renal arteries congestion (++) were observed.

Materials and methods

Chemicals

Analytical grade glyphosate (96%) purchased from Sigma-Aldrich (St. Louis, MO, USA). The kits used for biochemical studies of creatinine, urea, catalase, glutathion peroxidase, malondialdehyde (MDA) and total protein were obtained from Biodiagnostic company, 29 Tahrir Street, Dokki, Giza, Egypt. All other chemicals were of reagent grades and obtained from local scientific distributors in Egypt.

Animals

The pregnant albino rats of the Wistar strain weighing 220-245 g were obtained from the animal house, National Research Centre of Egypt. The rats were acclimatized to the laboratory conditions for one week prior to the experiment. After acclimation period, the pregnant female rats (n = 8) were housed in stainless steel cages (one pregnant female in each cage) in breeding animals' room at 25±2°C with 45% relative humidity, dark/light cycle (12-h), and were fed standard pellet diet and tap water ad libitum. The number of male and female pups in each litter was recorded and weighed. To maximize the lactation performance, male and female pups in each litter were randomly divided into five male and female pups.

Experimental design

Eight adult pregnant female Wistar rats were randomly divided into four groups of two rats each. Glyphosate was dissolved in corn oil and administrated via the oral route to dams at a fixed volume of 0.5 mL/dam from the first day after parturition for 21 days (lactation period). Dams were weighed weekly to adjust the dose of glyphosate. The suckling pups were grouped as follows: G1: male and female pups (five each) of the two mother female rats who were administered 0.5 mL corn oil/dam daily served as control, G2: male and female pups (five each) of the two mother female rats who were administered glyphosate at 0.3 mg/kg body weight for acceptable daily intake (ADI), G3: five male and female pups of the two mother female rats who were administered glyphosate at no-observed-adverse-effect level (NOAEL) for 21 days (31 mg/kg body weight), G4: five male and five female pups suckling from the two mother rats who were administered glyphosate at 1/100 of acute oral toxicity (1/100 LD50) (56 mg/kg body weight).

Blood sample collection and tissue preparation

Post lactation cycle of pups (21 days), rats were fasted overnight and blood samples were obtained by puncturing the retero-orbital venous plexus of animals with a fine sterilized glass capillary. The blood sample was divided into two portions; the first portion was collected in an anticoagulant (EDTA) tube and used for blood cell count and Hb content measuring. The second portion of blood was collected in centrifugal tubes without anticoagulant, and left to clot in these dry tubes and centrifuged at 3000 rpm (600xg) for 10 min at 4°C using Heraeus Labofuge 400R to get the sera. Sera were stored at -20°C for further biochemical analysis including blood urea, and creatinine determination. Then, the rats were sacrificed by decapitation. Kidney was removed immediately after sacrification, weighed, washed in saline and relative kidney weight was calculated. A small part of the kidney was homogenized in 10% (w/v) ice cold 100 mM phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm for 15 min at 4°C, then the supernatant was obtained and used for oxidative stress (lipid peroxidation) and antioxidant enzyme measurements (CAT and GPx). Then, small pieces of kidney samples were cut and kept in 10% natural formalin and used for histopathological investigations.

Biochemical parameter determination

Oxidative stress biomarkers in kidney tissues

Kidney glutathione peroxidase and catalase enzyme activities were determined according to the method of Paglia and Valentin and Abei. Lipid peroxidation (LPO) was estimated by determining thio-barbituric acid reactive substances (TBARS) and was expressed in relation to malondialdehyde (MDA) content using a colorimetric technique. The MDA values were expressed as nanomoles of MDA per gram tissue. Total protein content in homogenate was measured.

Kidney function in serum

Urea and creatinine were measured according to the method of Tietz.

Measurements of blood constituents

Red blood corpuscles (RBC) and white blood cells (WBC) were counted according to Britton and Seivered; Heamoglobin (Hb) measurement was carried out as per Wintrobe.

Histopathological examination

Kidney specimens of male and female pups were cut and dehydrated in graded series of alcohol, cleaned in xylene and fixed in paraffin wax. Five micrometer thick pieces were cut and stained by haematoxylin and eosin (H&E). Two slides were prepared for this organ; each slide contained ten field areas and two sections were examined for histopathological changes. The examination was done using a light microscope with a digital camera. The histopathological alterations in kidney tissues were scored as follows: normal appearance ( ), mild (+), moderate (++) and severe (+++).

Statistical analysis

Data were reported as mean ± SE. Data were analysed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test as a post-hoc test. The level of significance between mean values was set at $p \leq 0.05$. All statistical analyses were performed using SPSS software.

Results

The results indicated a significant increase in relative kidney weight (%) post-exposure of male and female pups to glyphosate during lactation period with G4>G3>G2, compared to the control group (G1) (Table 1).

Table 1. Relative kidney weight and kidney dysfunction biomarkers in serum of female and male pups exposed to glyphosate during lactation period.

Treatments	Creatinine (mg/dl)		Urea (mg/dl)		Relative kidney weight (%)	
	Female	Male	Female	Male	Female	Male
G1	1.25 ± 0.04^{a}	1.54 ± 0.011^{a}	34.34 ± 0.32^{a}	36.14 ± 0.42^{a}	0.52 ± 0.005^{a}	0.63 ± 0.006^{a}
G2	$1.35 \pm 0.01^{a\,b}$	1.63 ± 0.005^{b}	41.71 ± 0.39^{b}	44.91 ± 0.33^{b}	0.63 ± 0.006^{b}	0.73 ± 0.007^{b}
G3	1.43 ± 0.03^{b}	1.73 ± 0.012^{c}	45.95 ± 0.38^{c}	53.95 ± 0.58^{c}	0.74 ± 0.005^{c}	0.77 ± 0.008^{c}
G4	1.78 ± 0.02^{c}	1.77 ± 0.008^{d}	55.90 ± 0.67^{d}	66.10 ± 0.43^{d}	0.78 ± 0.002^{d}	0.83 ± 0.005^{d}

G1: control group, G2: glyphosate (ADI), G3: glyphosate (NOAEL), G4: glyphosate (1/100 LD50). Values expressed as a mean of 5 animals ± S.E.; $^{a, b, c, d}$ values having different letters significantly different at $p \leq 0.05$.

The effects of glyphosate on renal function revealed a significant increase in creatinine and urea levels in both male and female pups in all treated groups compared with the control group, where G4 exhibited a more noticeable significant increase in both blood urea and creatinine levels than G3 and G2 (G4>G3>G2) at $p \leq 0.05$ (Table 1). The significant increase observed in our work suggested the nephrotoxic effect of the herbicide.

The results indicated that the sub-chronic exposure to glyphosate (21 days) caused a high level of serum urea in all selected doses of treatedpups compared to control.

The authors pointed out that the significant reduction in the RBC count and haemoglobin level post 21 days of lactation is indicative of anaemic condition.

Pups exposed to glyphosate herbicide showed significant inhibition in antioxidant enzyme activities CAT and GPx with G4>G3>G2, as compared to control levels ($p \leq 0.05$), while a significant increase in MDA level in all treated groups was recorded (G>G3>G2) (Table 2).

Table 2. Effect of glyphosate on kidney antioxidant enzyme activities and lipid peroxidation level in the kidney tissue of female and male pups.

Treatments	Catalase (u/mg protein)		Glutathion–peroxidase (u/mg protein)		Lipid peroxidation (nmol MDA/g tissue)	
	Female	Male	Female	Male	Female	Male
G1	38.46 ± 0.36^{d}	39.86 ± 0.35^{b}	4.54 ± 0.01^{c}	6.24 ± 0.05^{d}	0.48 ± 0.004^{a}	0.68 ± 0.004^{a}
G2	36.41 ± 0.07^{c}	37.86 ± 0.54^{d}	4.51 ± 0.02^{c}	5.41 ± 0.09^{c}	0.53 ± 0.006^{b}	0.69 ± 0.004^{a}
G3	34.66 ± 0.70^{b}	34.06 ± 0.43^{c}	4.25 ± 0.03^{b}	5.10 ± 0.02^{b}	0.57 ± 0.006^{c}	0.71 ± 0.006^{b}
G4	31.98 ± 0.28^{a}	25.19 ± 0.71^{a}	3.78 ± 0.01^{a}	4.19 ± 0.03^{a}	0.72 ± 0.003^{d}	0.91 ± 0.004^{c}

Control (G1), Glyphosate (ADI) (G2), Glyphosate (NOAEL) (G3), and 1/100 LD50 (G4). Values are means ± SE, n = 5; values having different letters are significantly different from each other.

The histopathological examination indicated focal fibrosis with inflammatory cells between glomeruli and tubules of renal tissue with severity score (+) in G2 and G3 and (+++) in G4. Also, focal haemorrhages and degradation in tubular lining epithelial cells were noticed in G4 (+). Besides these, renal artery congestion (++) was noticed.

Overall, the authors indicated that elevation in creatinine and total urea together with the histopathological results proved that glyphosate induced renal injury in pups.

Conclusion

The authors concluded that exposure of pups to glyphosate herbicide via breastfeeding showed increase in relative kidney weight and kidney function, while, it lowered haematological values and induced perturbations in antioxidant status in male and female pups. Severe histopathological alterations were also examined in renal tissue of pups. Glyphosate showed a dose-dependent relationship, where 1/100 LD50 showed more drastic effects than the other selected two doses.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The aim of this study was to evaluate the effects of glyphosate administered orally to the dams during lactation period of pup rats at a daily dose of 0.3 mg/kg body weight (acceptable daily intake ADI), 31 mg/kg body weight (no observed adverse effect level NOAEL) and 56 mg/kg body weight (1/100 LD50) for 21 days. However, the underlying assumption of glyphosate transfer to pups via milk is not supported by the data – this is well explained in mammals including humans (Bus, 2015*; McGuire et al, 2016**). At the end of the treatment, the suckling pups were separated into male and female, and the relative kidney weights were measured. The levels of creatinine and urea were determined in serum. Oxidative stress was measured using lipid peroxidation and the activities of glutathione peroxidase (GPx) and catalase (CAT) in kidney tissue were also measured. Test substance administration in this study was to two pregnant rats per group, and thus the experimental unit is the litter, not all the individual pups across two litters with the same dose. Therefore, in contrast to the statistical analyses presented, there are only two experimental units (litters) per dose group (2-1 = 1 degree of freedom; **not** 10-1 = 9 d.f.), which lacks adequate statistical power for meaningful analysis in both the ANOVA and LSD test.

The pups showed increased relative kidney weight with the highest increment in 1/100 LD50 dose followed by NOAEL and ADI doses. Also, marked increment in malondialdehyde (MDA) level along with significant inhibition in catalase (CAT) and glutathione peroxidase (GPx) activities were detected in pup's kidney tissues. Counts of WBCs and RBCs and Hb content exhibited significant reduction in the blood of pups that received 1/100 LD50 dose which is more than the NOAEL and ADI doses. Histopathological examinations of kidney tissue demonstrated focal fibrosis with inflammatory cells between glomeruli and tubules of male and female pup kidney tissue (lower scoring severity (+) in ADI and NOAEL doses than 1/100 LD50 (+++)). Additionally, at 1/100 LD50 dose focal haemorrhages, degradation in tubular lining epithelial cells (+) and renal arteries congestion (++) were observed. These results for F1 kidney weights and histopathology data are not consistent with the weight of evidence across in numerous multigenerational studies up to the limit dose of 1000 mg/kg bw/day.

The article is considered relevant (Category A) and reliable with restrictions for the following reasons: No method of analysis, no HCD, and inappropriate statistical assumptions on the experimental unit.

References:

*Bus, J. S. (2015). Analysis of Moms Across America report suggesting bioaccumulation of glyphosate in U.S. mother's breast milk: Implausibility based on inconsistency with available body of glyphosate animal toxicokinetic, human biomonitoring, and physico-chemical data. *Regulatory Toxicology and Pharmacology* (2015), Vol. 73, Issue, 3, pp. 758-64. doi: 10.1016/j.yrtph.2015.10.022.

**McGuire M. K. at al. (2016). Glyphosate and aminomethylphosphonic acid are not detectable in human milk. *The American Journal of Clinical Nutrition* (2016), Vol. 103, Issue 5, pp 1285-1290. https://doi.org/10.3945/ajcn.115.126854

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies

Publication: Refaie 2020, Hematological, biochemical, antioxidant and histopathological alterations in kidneys of Wistar rat pups exposed to glyphosate herbicide during lactation period	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	Protocol corresponds to the guidelines of the National Research Center (NRC, 2011)
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Analytical grade glyphosate (96%) was purchased from Sigma Aldrich (St. Louis, MO, USA).
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA or other glyphosate metabolites is the tested substance	N	
Study		
Test species clearly and completely described	Y	8 pregnant albino rats of the Wistar strain weighting 220-245 g
Test conditions clearly and completely described	Y	Housing, diet and environmental conditions described.
Route and mode of administration described	Y	Glyphosate was dissolved in corn oil and administrated via the oral route to dams at a fixed volume of 0.5 ml/dam from the first day of parturition for 21 days (lactation period).
Dose levels reported	Y	0.3, 31 and 56 mg/kg body weight
Number of animals used per dose level reported	Y	8 adults divided in 4 groups of 2 rats that receive the vehicle or one of the 3 dose of glyphosate. Male and female pups in each litter were randomly divided into 5 male pups and 5 female pups.
Method of analysis described for analysis test media	N	No analysis performed
Validation of the analytical method	N/A	No analysis performed
Analytical verifications of test media	N/A	No analysis performed
Complete reporting of effects observed	Y	
Statistical methods described	Y	ANOVA with LSD post-hoc test, however, incorrect application of experimental unit to offspring rather than lactating mother. Therefore all statistical analyses are incorrect.
Historical control data of the laboratory reported	N	No HCD
Dose-effect relationship reported	Y	Yes
Overall assessment		
Reliable without restrictions	N	

Publication: Refaie 2020, Hematological, biochemical, antioxidant and histopathological alterations in kidneys of Wistar rat pups exposed to glyphosate herbicide during lactation period	**Criteria met? Y/N/?**	**Comments**
Reliable with restrictions	Y	No method of analysis, no HCD, and inappropriate statistical assumptions on the experimental unit.
Not reliable	N	

1. Information on the study

Data point:	CA 5.6
Report author	Zhao L. *et al.*
Report year	2021
Report title	Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of *StAR* expression in mouse Leydig cells
Document No	Science of the Total Environment, 2021, 785, 147323
Guidelines followed in study	None
Deviations from current test guideline	No guideline followed.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The involvement of NR1D1 in glyphosate-mediated inhibition of testosterone synthesis was investigated both in vitro and in vivo. Glyphosate exposure of TM3 cells significantly increased *Nr1d1* mRNA levels, but decreased *Bmal1*, *Per2*, *StAR*, *Cyp11a1*, and *Cyp17a1* mRNA levels. Western blotting confirmed elevated NR1D1 and reduced StAR protein levels following glyphosate exposure. Glyphosate exposure also reduced testosterone production in TM3 cells. In primary LCs, glyphosate exposure also upregulated *Nr1d1* mRNA levels and downregulated the mRNA levels of other clock genes (*Bmal1* and *Per2*) and steroidogenic genes (*StAR*, *Cyp17a1*, *Cyp11a1*, and *Hsd3b2*), and inhibited testosterone synthesis. Moreover, glyphosate exposure significantly reduced the amplitude and shortened the period of *PER2::LUCIFERASE* oscillations in primary LCs isolated from *mPer2*^{Luciferase} knock-in mice. Four weeks of oral glyphosate upregulated NR1D1 at both the mRNA and protein levels in mouse testes, and this was accompanied by a reduction in StAR expression. Notably, serum testosterone levels were also drastically reduced in mice treated with glyphosate. Moreover, dual-luciferase reporter and EMSA assays revealed that in TM3 cells NR1D1 inhibits the expression of *StAR* by binding to a canonical RORE element present within its promoter. Together, these data demonstrate that glyphosate perturbs testosterone synthesis via NR1D1 mediated inhibition of *StAR* expression in mouse LCs.

Materials and methods

Animals

BALB/c wild-type (WT) mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.. *mPer2*^{Luc} knock-in reporter mice carrying the firefly *luciferase* (*Luc*) gene in exon 23 of the circadian clock gene *Per2* were obtained from the Jackson Laboratory (Sacramento, CA, USA). Mice (10-12 weeks old) were housed under a 12 h light/dark cycle (Zeitgeber time, ZT0: 0800 light on; ZT12: 2000 light off) for at least 2 weeks to synchronize their circadian clock system. All mice were provided with food and water ad libitum. The mouse house was maintained at a temperature of 23±2°C and a relative humidity of 50±10%.

Animal treatments

Pure glyphosate powder was purchased from Shanghai Yuanye Bio-Technology Co., Ltd.. Glyphosate was dissolved in distilled water to obtain a 0.5% solution, as previously described (5 g glyphosate/L water). Twenty WT mice were randomly assigned to two equally sized groups. Mice in one group were orally administered 0.5% glyphosate via daily drinking water (Glyphosate group). The control group received distilled water alone (CTRL group). Four weeks later, the mice from the Glyphosate and CTRL groups were euthanized at two representative time points (ZT0 and ZT12; n = 5 per group for each time point). Testes, epididymis, seminal vesicles, liver, and blood samples were collected. Body, testes, epididymis, seminal vesicles, and liver weights were recorded. The blood samples were stored at 4°C for 6-8 h to enable clotting. The serum was separated from the blood clot by centrifugation at 1000×g at 4°C for 15 min, collected, and stored at -80 °C.

Cell preparation and culture

TM3 cells were seeded on 35 mm collagen coated dishes and cultured in DMEM supplemented with 2.5% foetal bovine serum (FBS), 5% horse serum, and 1× antibiotic-antimycotic (AA, containing penicillin, streptomycin, and amphotericin B) in a humidified atmosphere of 5% CO_2 at 37°C. Primary LCs were isolated from 10 to 12-weeks-old adult *mPer2*^{Luc} knock-in mice or WT mice and cultured. Briefly, testes were de-capsulated and digested using 0.05% collagenase I in DMEM/F12 for 15 min at 34°C. The suspended LCs were collected by filtration using a 0.074 mm nylon cell strainer and purified over a discontinuous Percoll gradient (5%, 30%, 58%, and 70%). After centrifugation at 800×g at 4°C for 30 min, the majority of the purified LCs were found to be concentrated in the third layer. The LCs were seeded onto 35 mm collagen-coated dishes in 2 mL of DMEM/F12 containing 10% FBS supplemented with 1× AA and cultured in a humidified atmosphere of 5% CO_2 at 37°C for 2 days until they reached 70%-80% confluence. All cells were incubated with various concentrations of glyphosate at the indicated time points for further experiments.

TM3 cell viability assay

The effects of glyphosate on TM3 cell viability were assessed using the CCK-8 assay. TM3 cells were seeded in 96 well plates (1×10^4 cells per well) and cultured for 24 h. TM3 cells were then exposed to different concentrations of glyphosate (0, 0.0001, 0.001, 0.01, 0.1, 1, 5, 10, 50, 100, or 200 mM) for 24 h or 48 h, after which CCK-8 (10 μL) was added to each well, and the cells were incubated for 1 h at 37°C. The absorbance of each well at 450 nm was measured using a microplate reader. The data were expressed as a percentage of the untreated cells, which were assigned 100% viability.

TM3 cell apoptosis assay

TM3 cells were plated into 35 mm collagen-coated dishes at a density of 3×10^5 cells and incubated for 24 h prior to exposure with or without 0.1 mM glyphosate for 24 h or 48 h. TM3 cells were collected and washed twice with PBS. Apoptosis was evaluated using the Annexin V-FITC/PI Apoptosis Detection Kit. The cells were then stained with 5 μL Annexin V-FITC (FITC) and 5 μL propidium iodide (PI) in 100 μL binding buffer for 15 min at room temperature in the dark. Apoptotic cells were quantified by measuring the fluorescent signals using a flow cytometer.

Real-time monitoring of *PER2::LUC* oscillations

Primary LCs isolated from 10 to 12 weeks old adult *mPer2*^{Luc} knock-in mice were synchronized with 100 nM dexamethasone (DXM) for 2 h in serum-free DMEM/F12 medium containing 1× AA. The culture medium was changed to DMEM/F12 medium supplemented with 10% FBS, 1× AA, and 100 μM luciferin with or without 0.1 mM glyphosate. Luciferase activity was chronologically monitored at 37°C with a Kronos Dio AB-2550 luminometer, which was interfaced with a computer to continuously acquire data. The data are shown as photon counts per minute. The correction of the baseline was carried out using a 24 h moving average, while the first 12 h of data were excluded. The phase time was determined from the peak that appeared between 24 and 48 h after DXM synchronization. The amplitude and period of the *PER2::LUC* oscillations were determined using Cosinor Periodogram software.

RNA extraction and real-time quantitative PCR

Cultured cells or testes tissue samples were harvested at the indicated time points. Total RNA was extracted from cells or tissues using TRIzol reagent and subsequently treated with RNase-free DNase. cDNA was generated using the PrimeScript RT Reagent Kit. All qPCR reactions were performed in a final volume of 20 μL containing 10 ng of cDNA, the SYBR Premix Ex Taq II kit, and 200 nM specific primers. Amplification was performed using the CFX96™ qPCR system. All reactions were performed in triplicate. The relative expression levels of each sample were normalized to the average level of the constitutively expressed housekeeping gene *36b4*, which is a reliable and consistent standard for use in gene expression analysis.

Protein extraction and western blotting (WB)

Proteins were extracted using a protein extraction kit. Cultured cells were harvested 24 h or 48 h after exposure with 0.1 mM glyphosate. For in vivo analysis, 80 mg of testes samples were collected at the indicated time points (ZT0 and ZT12) after oral administration of Glyphosate or CTRL for 4 weeks. Protein concentrations were measured using a BCA Protein Assay Kit. Equal amounts of protein were resolved by 12% SDS-PAGE and then electrophoretically transferred onto PVDF membranes. The membranes were then incubated with primary antibodies diluted in Tris-buffered saline Tween (TBST) overnight at 4°C after blocking with 10% non-fat milk for 1 h at room temperature, and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) for 1 h. The primary antibodies used were anti-NR1D1 (1:2000), anti-StAR (1:2000), and anti-β-actin (1:5000). After washing five times in TBST for 5 min each time, peroxidase activity was measured using a Western Bright ECL HRP substrate kit. Immunoreactive bands were visualized using a gel imaging system (GBox-chemi-XRQ). β-actin was used as an internal control for normalization and quantitative analysis to compare the relative protein levels in a group of samples.

Haematoxylin and eosin (HE)

Testes samples collected at ZT0 and ZT12 were fixed with Blouin's fluid and then embedded in paraffin wax using standard protocols and sectioned at a thickness of 5 μm, which were deparaffinised with xylene and ethanol, stained with HE, dehydrated, and sealed with neutral gum for microscopic examination.

Measurement of secreted testosterone levels

Culture supernatants from LCs and TM3 cells were collected after incubation of the cells with or without 0.1 mM glyphosate for 24 h or 48 h. Serum was collected at ZT0 and ZT12 from mice orally administered with Glyphosate or CTRL for 4 weeks. The secreted testosterone levels were measured using an ELISA kit. The fluorescence intensity at 450 nm was determined using a microplate reader. The intra- and inter-assay coefficients of variability were <10%.

Dual-luciferase reporter

Genomic inspection revealed that there is a canonical sequence for the RORE cis-element (-859 to -864, +1 indicates the transcription start site) within the *StAR* promoter. A dual-luciferase reporter was first used to determine whether NR1D1 inhibits *StAR* transcription by binding to the *StAR* promoter. A 1773 bp fragment of the *StAR* gene (−1377 to +396, +1 indicates the transcription start site) was amplified from mouse liver genomic DNA using forward and reverse primers containing the KpnI restriction enzyme site and a 15 bp homologous arm (F, 5′-CTGGCCTAACTGGCCGGTACCTTCAAGGACAGACCAGGTTAGA-3′; R, 5′-AGGCTAGCGAGCTCAGGTACCGCGAGGAACATGCTGAGTG-3′). The 1773 bp fragment of the *StAR* gene was cloned into the *KpnI* site of the pGL4.10 vector using the seamless connection method (ClonExpress II One Step Cloning Kit), which was then named pGL4.10-*StAR*-Luc reporter plasmid (*StAR-Luc*).The target coding sequence (CDS) fragment of NR1D1 was amplified using PrimerSTAR HS DNA Polymerase using cDNA generated from mouse liver tissue as the template (F, 5′-CGATATCGGATCCGGTACCCTCGAGATGACGACCCTGGACTCCAATA-3′; R, 5′-GCGGTTTAAACTTACCGGTCTCGAGTCACTGGGCGTCCACCCGGAAG-3′). The CDS fragment of NR1D1 was cloned into the *XhoI* site of the pcDNA 3.1 plasmid, which was named pcDNA 3.1-*Nr1d1*. TM3 cells were plated in 24-well plates and transfected with *StAR*-Luc, pRL-CMV (an internal control plasmid), pcDNA 3.1-*Nr1d1*, and pcDNA 3.1 empty vector using Turbofect transfection reagent. Thirty-six hours after transfection, the cells were washed and lysed with 200 μL of reporter lysis buffer. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and the SPARK® Multimode Microplate Reader at 25°C. The light output, as a result of transcriptional activity, was divided by the light output from Renilla luciferase activity to normalize the samples.

Electrophoretic mobility shift assay (EMSA)

TM3 cells were transfected with the NR1D1 overexpression vector, pcDNA 3.1-*Nr1d1*. Nuclear proteins were isolated using a nuclear protein extraction kit. A 72 bp biotinylated probe was designed from the mouse *StAR* promoter, including the RORE cis-element (-859 to -864). The unlabelled and mutated probes were not biotinylated. All probes were synthesized by Sangon Biotech. Biotinylated and unlabelled probe sequences were: F, 5′-TTTCTCACCTTTGTGCACAGGTCAGGACCTGTAGCAGGGCAGGCCAGCCTTAGCTGC ATGAGGAAAGGGTGA-3′. Mutated probe: F, 5′-TTTCTCACCTTTGTGCACCCTAGCGGACCTGTAGCAGGGCAGGCCAGCCTTAGCTGC ATGAGGAAAGGGTGA-3′. EMSA was conducted using a chemiluminescent EMSA kit. A total of 0.04 pmol of biotinylated probe was added to each reaction. Unlabelled probes and mutated probes were added in a 15× excess in the competition assays. The probes were mixed with EMSA reagent and 3 μg of nuclear proteins, according to the manufacturer's instructions. The complexes obtained were separated by electrophoresis on 6% polyacrylamide gels and then electrophoretically transferred onto nylon membranes (Beyotime). The bands were visualized using a gel imaging system (GBox-chemi-XRQ).

Statistical analyses

All data are expressed as the mean ± standard error (SE) of at least three separate experiments, each performed in triplicate. Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparisons post-test or a two-way ANOVA with a Sidak's multiple comparisons post-test or a Student's t-test using GraphPad Prism Version 7.0. ANOVA assumptions were corroborated by the Kolmogorov-Smirnov test for normality and Bartlett's test for homogeneity of variances. The Durbin-Watson test was also used to evaluate the independence of observations. Differences were considered significant at $p < 0.05$.

Results

Determination of the optimal concentration of glyphosate exposure of TM3 cells

To investigate the effect of glyphosate on the cellular circadian clock and testosterone synthesis in LCs, the TM3 cell line was used as a representative LC model. A CCK-8 assay was used to determine the appropriate concentration of glyphosate that did not markedly affect cell viability. Exposure of TM3 cells to different concentrations of glyphosate for 24 h significantly reduced cell viability at concentrations of 5 mM or more. However, after incubation for 48 h, 1 mM glyphosate exposure markedly inhibited the viability of TM3 cells. Thus, 0.1 mM was the highest concentration of glyphosate, which did not significantly affect the viability of TM3 cells relative to the CTRL (0 mM) group at 24 h and 48 h. In addition, the cell apoptosis assay results confirmed that exposure with 0.1 mM glyphosate for 24 h and 48 h did not significantly affect the apoptotic rate of TM3 cells compared to the CTRL group. Therefore, 0.1 mM was selected as the optimal glyphosate exposure concentration for further analysis in this study.

Effect of glyphosate exposure on the expression of circadian clock genes and steroidogenic genes in TM3 cells

To investigate the effect of glyphosate on the circadian clock system and testosterone synthesis, the expression levels of several circadian clock genes and steroidogenic genes were examined in DXM-synchronized TM3 cells cultured in the presence or absence of 0.1 mM glyphosate for 24, 28, 32, 36, 40, and 44 h. Glyphosate exposure significantly downregulated the mRNA expression levels of circadian clock genes (*Bmal1* and *Per2*) (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0123$ and $p = 0.0002$, respectively). However, the mRNA expression levels of *Nr1d1* were markedly increased after glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Additionally, WB analysis verified the elevated expression levels of NR1D1 in TM3 cells after glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0021$) (Figure 2B-C). For steroidogenic genes, 0.1 mM glyphosate exposure significantly decreased the mRNA expression levels of *StAR*, *Cyp17a1*, and *Cyp11a1*, respectively, except for *Hsd3b2* (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$, $p = 0.0057$, and $p < 0.0001$, respectively). Glyphosate exposure also consistently inhibited *StAR* expression at the protein level (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0066$). In addition, the ELISA results revealed a decline in secreted testosterone levels at both 24 h and 48 h in TM3 cells after 0.1 mM glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). These results demonstrate that 0.1 mM glyphosate exposure alters the circadian clock system and attenuates testosterone production in TM3 cells.

Effects of glyphosate exposure on *PER2::LUC* oscillations in primary LCs

To further investigate the influence of glyphosate on the circadian clock system in LCs, primary LCs isolated from *mPer2*^{Luc} knock-in mice were treated with or without 0.1 mM glyphosate and changes in *PER2::LUC* oscillations were examined. Several *PER2::LUC* oscillations were clearly observed in DXM-synchronized primary LCs isolated from *mPer2*^{Luc} knock-in mice in the presence or absence of glyphosate. However, the amplitude of the *PER2::LUC* oscillations was significantly attenuated by exposure to 0.1 mM glyphosate. In addition, exposure to glyphosate significantly advanced the peak time and shortened the period of the *PER2::LUC* oscillations relative to the CTRL group. These data provide additional evidence that 0.1 mM glyphosate not only alters the circadian clock system in TM3 cells, but also does so in primary LCs.

Effect of glyphosate exposure on the expression of circadian clock genes and steroidogenic genes in primary LCs

To further determine the effect of glyphosate on the expression of circadian clock genes and steroidogenic genes in primary LCs, their mRNA expression levels were examined in DXM-synchronized primary LCs for 24, 28, 32, 36, 40, and 44 h in the presence or absence of glyphosate (0.1 mM). Glyphosate exposure upregulated the mRNA expression levels of *Nr1d1* in primary LCs (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Additionally, WB analysis confirmed that NR1D1 protein levels were consistent with elevated mRNA expression levels after glyphosate exposure ($p < 0.01$). In contrast, other circadian clock genes (*Bmal1* and *Per2*) were significantly decreased (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Notably, glyphosate exposure also decreased the mRNA expression levels of *StAR*, *Cyp11a1*, and *Cyp17a1* in primary LCs (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Moreover, the expression levels of *Hsd3b2* were markedly decreased in primary LCs treated with glyphosate, which was different from that observed in TM3 cells (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0001$). A significant reduction in testosterone production was also observed at both 24 h and 48 h following glyphosate administration (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). These results further confirmed that glyphosate exposure perturbs the transcription of the circadian clock and steroidogenic genes and alleviates testosterone synthesis in LCs.

The effect of glyphosate on the expression of circadian clock genes, steroidogenic genes, and testosterone production in vivo

The above results revealed that the expression levels of circadian clock genes at the mRNA and protein levels were affected in both TM3 cells and primary LCs after glyphosate exposure. Notably, the mRNA and protein expression levels of *StAR* were significantly reduced in TM3 cells and primary LCs after glyphosate addition. To further investigate whether the influence of glyphosate on the testicular circadian clock and steroidogenic genes in vitro is consistent with that in vivo, glyphosate was orally administered to mice through drinking water for 4 weeks. No significant changes in body weight, relative testis weight, relative epididymal weight, relative seminal vesicle weight, or relative liver weight in glyphosate-treated mice compared to mice in the CTRL group were found. In addition, HE analysis of the testis sections revealed that exposure to glyphosate did not affect testis morphology. Consistent with the results in TM3 cells and primary LCs, the mRNA expression levels of *Nr1d1* were significantly increased in the testes of both ZT0 and ZT12 after glyphosate exposure for 4 weeks (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). WB analysis also confirmed the elevated NR1D1 expression at the protein level (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Compared with CTRL, the mRNA expression levels of *Bmal1* and *Per2* were significantly reduced in the testes after 4 weeks of glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Declines in *StAR*, *Cyp17a1*, and *Hsd3b2* mRNA expression levels were observed in testes at ZT0 and ZT12 following glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). The mRNA expression levels of *Dbp* were increased at ZT0 and decreased at ZT12 following glyphosate administration. The protein levels of *StAR* were reduced in testes at ZT0 and ZT12 after 4 weeks of glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). An ELISA verified that serum testosterone levels were significantly reduced at ZT0 and ZT12 after 4 weeks of glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$).

NR1D1 inhibits *StAR* transcription by binding to a RORE cis-element within its promoter

Genomic inspection revealed that the canonical sequence for the RORE cis-element (-859 to -864) was located within the -2000 bp region from the transcriptional start site of the *StAR* gene in mice (NC_000074.7). To assess whether *StAR* expression is regulated by NR1D1 through its direct binding to the *StAR* promoter, the authors constructed a *StAR*-Luc reporter vector containing a 1773 bp fragment (-1377 to +396, +1 indicates the transcription start site) of the *StAR* gene. As indicated above, *StAR*-Luc, pRL-CMV (Promega, an internal control plasmid), pcDNA 3.1-*Nr1d1*, and pcDNA3.1 empty vector were transfected into TM3 cells for 36 h. As expected, the transcriptional activity of the *StAR* fragment was significantly inhibited following transfection with pcDNA 3.1-*Nr1d1* compared with the pcDNA 3.1-empty group ($p < 0.05$). To assess whether NR1D1 inhibits *StAR* transcription primarily through the canonical RORE cis-element located within its promoter, the authors performed an EMSA assay. A clear band representing a DNA-protein complex was detected when NR1D1 nuclear proteins from TM3 cells were incubated with a biotinylated probe encompassing RORE (-859 to -864). The band was absent after the addition of a competitor (unlabelled probe). However, the band reappeared in the presence of a mutated competitor. In summary, these results revealed that NR1D1 inhibits *StAR* expression by binding to a RORE cis-element within its promoter.

Conclusion

The authors concluded that this study is the first to examine the detrimental effects of glyphosate on the transcription of the circadian clock and steroidogenic genes, as well as testosterone production in TM3 cells, primary LCs, and in vivo. The authors also indicated they proved that *StAR* transcription can be directly repressed by elevated NR1D1 expression levels induced by glyphosate exposure in LCs, which is a reason for the dysregulation of testosterone synthesis in LCs. Finally, the authors stated that this study not only provides a basis for explaining the detrimental effects of glyphosate on the circadian clock system in LCs and mouse testes, but also expands the understanding of the underlying mechanisms of the reproductive toxicity of glyphosate in mammals.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article hypothesize a mode of action to perturb testosterone synthesis via NR1D1 mediated inhibition of StAR expression in mouse Leydig cells.

This study examined the effects of glyphosate on the expression of the circadian clock and steroidogenic genes, as well as testosterone production in TM3 cells and primary LCs. The authors also investigated the effects of glyphosate on PER2::LUCIFERASE (PER2::LUC) oscillations in mouse primary LCs isolated from mPer2Luciferase (mPer2Luc) reporter gene knock-in mice. Furthermore, the effects of glyphosate exposure on the expression of core clock and steroidogenic genes in mouse testes as well as serum testosterone levels were determined in vivo. Finally, dual-luciferase reporter and EMSA assays were used to uncover the underlying molecular mechanism of glyphosate impairment of testosterone production involving NR1D1 regulation of StAR expression in LCs. The lack of a concurrent and historical positive control data substantially diminishes confidence in the predictability of the methodology employed to probe the proposed mechanism.

The results indicate that glyphosate perturbs testosterone synthesis via NR1D1 mediated inhibition of StAR expression in mouse LCs. These results contradict a large body of high-quality data assessing endocrine disruption and specifically steroidogenesis. Furthermore, the results and conclusions of this publication are not consistent with Levine et al. (2007), which specifically investigated glyphosate & formulation in mouse Leydig cells and StAR protein expression.

The article is considered relevant (Category A) and reliable with restrictions for the following reason: No purity of the test material provided, no HCD/positive control reported. Only one dose evaluated/no dose-effect relationship for the in vivo experiment. The lack of a concurrent and historical positive control data substantially diminishes confidence in the predictability of the methodology employed to probe the proposed mechanism.

References:
Levine s. et al; Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis, Cell Biol Toxicol (2007) 23:385–400.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies

Publication: Zhao L. 2021, Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	All animal usage and experimental procedures were approved in accordance with the Guidelines for Animal Experiments by the Committee for the Ethics on Animal Care and Experiments of Northwest A&F University and performed under the control of the "Guidelines on Ethical Treatment of Experimental

Publication: Zhao L. 2021, Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells	Criteria met? Y/N/?	Comments
		Animals" (2006) No.398 set by the Ministry of Science and Technology, China
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Pure glyphosate powder, purchased from Shanghai Yuanye BioTechnology Co., Ltd. (Shanghai, China). No information on purity, content etc.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA or other glyphosate metabolites is the tested substance	N	
Study		
Test species clearly and completely described	Y	BALB/c wild-type (WT) mice (10–12 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China); No BW information was included
Test conditions clearly and completely described	Y	Standard laboratory conditions of temperature, humidity, and lighting described
Route and mode of administration described	Y	Mice in one group were orally administered 0.5% glyphosate via daily drinking water (Glyphosate group). No distinction if animals were group-caged or not. The control group received distilled water alone (CTRL group). Four weeks later, the mice from the Glyphosate and CTRL groups were euthanized. Cell preparation described, cytotoxicity assay performed.
Dose levels reported	Y	Glyphosate was dissolved in distilled water to obtain a 0.5% solution, as previously described (5 g glyphosate/L water). The pH levels of dose & control groups were not adjusted.
Number of animals used per dose level reported	Y	Twenty WT mice were randomly assigned to two equally sized groups.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N/A	
Analytical verifications of test media	N	pH of glyphosate acid test groups were not reported, which would be quite acidic compared to the control dose.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	No HCD, no positive control for the *in vitro* experiment to verify this novel method.
Dose-effect relationship reported	N	Only one dose
Overall assessment		
Reliable without restrictions	N	

Publication: Zhao L. 2021, Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells	**Criteria met? Y/N/?**	**Comments**
Reliable with restrictions	Y	No purity of the test material provided, no HCD/positive control reported. Only one dose evaluated/no dose-effect relationship for the *in vivo* experiment. The lack of a concurrent and historical positive control data substantially diminishes confidence in the predictability of the methodology employed to probe the proposed mechanism.
Not reliable	N	

1. Information on the study

Data point:	CA 5.9.4
Report author	Ferreira C. *et al.*
Report year	2021
Report title	Urine biomonitoring of glyphosate in children: Exposure and risk assessment
Document No	Environ Res 2021; 198: 111294 https://doi.org/10.1016/j.envres.2021.111294
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes (Relevance Category A)/Reliable with restrictions

2. Full summary of the study according to OECD format

Purpose: To determine the concentrations of glyphosate in the urines of 41 Portuguese children (2–13 years old) and to identify potential determinants of urine concentration. **Methods:** First morning spot urine samples were collected from study participants and glyphosate concentrations were estimated using the glyphosate ELISA Plate Kit® with a 0.6 μg/L limit of detection. **Results:** Glyphosate was detected in 95.1% of the samples with a mean concentration of 1.77 ± 0.86 μg/L and a maximum value of 4.35 μg/L. Glyphosate concentrations were higher in the urine of children aged 7–9 years, those living within 1 kilometer of agricultural areas, those with families that have a higher consumption of home produced foods, and those whose parents applied herbicides in the backyard. The authors' risk assessment estimated oral intake of glyphosate to range from 1% to 5.6% of the established Acceptable Daily Intake (ADI) (0.5 mg/kg bw/day). **Conclusions:** The results should be further analyzed considering the age of the participants, for whom no adjusted ADI exists.

Materials and methods

Study subjects and data collection: 41 healthy children, ages 2 to 13 years, were recruited from different regions of Portugal. Participants were 46% male (age 2 to 12 years) and 54% female (age 2 to 13 years). The participants were concentrated in the Coimbra region (82%, n = 32). The other 9 participants were distributed across 3 municipalities: Porto (n =1), Lisbon (n = 2), and Aveiro (n = 6). All of the children participating in the study lived in municipalities that subscribed to the "Municipalities without Glyphosate" campaign.

Parents completed a questionnaire about their child's anthropometric data and health including sex, age, weight, height, medication, and exposure to tobacco smoke. Parents also provided data concerning the family's sociodemographic status, parents' educational levels, distance from the family residence to an industrial zone or agricultural field or nearest highway, and residential use of agrochemicals.

First morning spot urine samples were collected between July 2018 and February 2019. Samples were collected in polystyrene containers and returned to the laboratory on the collection day, divided into aliquots, and stored at - 20 C until extraction for analysis.

Urine analysis: Urine samples were analyzed using the glyphosate ELISA Plate Kit®. The first step was to filter 500 uL of the urine samples through Millipore Amicon Ultra filters into 2 ml tubes. Samples were then centrifuged at 8000 g for 15 minutes. Then 300 uL of the filtrate was placed in a new tube and 200 uL of ethyl acetate was added. Then the sample was centrifuged again at 8000 g for 3 minutes. The initial preparation was completed by adding 350 uL of the sample diligent to 50 uL of the previous sample.

The next step was derivatization. To dilute the derivatization agent, 3.5 mL of the diluent were added to the derivatization reagent and centrifuged at 5444 g at 4 ◦C for 15 minutes. Then, 250 μL of each standard, control and sample was added to the respective labeled tubes having 1 ml of buffer. The tubes were centrifuged for approximately 2 seconds.

The previously prepared diluted derivatization reagent (100 µL) was added to each labeled tube (standard, control, and sample). Each tube was centrifuged for 15–30 seconds. Then, the tubes were incubated at room temperature for 10 minutes. To start the ELISA analysis, 50 µL of each standard concentration (0, 0.075, 0.2, 0.5, 1 and 4 ng/L), control (0.75 ± 0.2 ng/mL) and derivatized samples were added to the respective wells of the plate. Then 50 µL of the anti-glyphosate antibody was added to all wells.

The tubes were covered with aluminum foil and homogenized through rapid circular movements for 30 seconds. Then, they were incubated at room temperature for 30 minutes, protected from direct sunlight. Subsequently, 50 µL of enzyme conjugate were distributed in each individual well. The microplate was again covered, shaken, and incubated for 60 min at room temperature, protected from light.

Then 150 µL of substrate was added. The microplate was covered again, shaken, and incubated for 30 minutes at room temperature, protected from light. Finally, 100 µL of stop solution was added and the absorbance was read at 450 nm, using an ELISA microplate reader. According to the manufacturer, the detection limit of the kit corresponded to 0.6 µg/L.

Statistical analysis: Data were analyzed descriptively in Microsoft Excel. Other statistical analyses were performed using the GraphPad Prism 8 (CR) program. Where the data were found to be skewed, non-parametric tests were implemented. The Kruskal-Wallis test was used to compare variables with more than 2 categories and the Mann-Whitney test was used to compare variables with only two categories. A p value less than 0.05 was considered to be statistically significant.

The authors estimated the internal dose of glyphosate as the product of each child's urine concentration (in µg/L) times the average daily urinary volume for children of 1.5 L. The resulting internal dose was then divided by the average body weight (in kg) for the 4 age categories (14,4 kg for those < 3 years old, 20.5 kg for those 4 to 6 years old, 29.3 kg for those 7 to 9 years old, and 38.3 kg for those 10 to 13 years old) to yield dose per kg body weight (equation 1) or the probable daily intake (PDI).

$$\text{PDI (µg / kg bw)} = \frac{\text{concentration}_{urine} \text{ X volume}_{urine}}{\text{body weight (kg)}} \qquad \text{equation (1)}$$

Assuming that diet was the most likely route of exposure and assuming 1% absorption based on a human feeding study by Zoller et al. (2020), the amount ingested (or external dose) was estimated to be (equation 2 as in Niemann et al. 2015)

$$\text{External dose (µg / kg bw)} = \frac{\text{100 X internal dose}}{\text{1 \% oral absorption}} \qquad \text{equation (2)}$$

Results

Glyphosate was detected in 95% of samples (39 out of 41). The average urine concentration was 1.77 +- 0.86 ug/L. The maximum urine concentration was 4.35 ug/L. Urinary values were evaluated with respect to possible determinants of exposure. Average values were slightly higher for girls (2.00 +- 0.94 ug/L) than for boys (1.56 +- 0.57 ug/L). Average values were slightly lower for those less than 3 years of age (1.20 ug/L) compared with older children (4 to 6 years old 1.99 ug/L, 7 to 9 years old 2.12 ug/L, and 10 to 13 years old 1.74 ug/L) ($p = 0.063$). Glyphosate concentrations were slightly higher in the urine of those living within 1 kilometer of agricultural areas, those from families with a higher consumption of home-produced foods, and those whose parents applied herbicides in the backyard.

The authors conducted a risk assessment by taking the minimum, average, and maximum glyphosate urinary concentrations for each of 4 age groups, back calculating the amount of glyphosate ingested assuming 1% absorption, and then calculating estimated glyphosate intake as a percent of the Acceptable Daily Intake (ADI) of glyphosate (0.5 mg/kg bw) The following table summarizes those % ADI calculations. Values ranged from 1% to 5.6% of the ADI.

Glyphosate concentration	% ADI ≤ 3 years old	% ADI 4 to 6 years old	% ADI 7 to 9 years old	% ADI 10 to 13 years old
Minimum	1.8%	1.3%	1.0%	1.0%
Average	2.5%	3.0%	2.2%	1.3%
Maximum	3.6%	5.6%	4.5%	1.5%

Conclusion

The authors concluded that glyphosate was detected in 95% of the urine samples with a mean concentration of 1.77 µg/L and a maximum concentration of 4.35 µg/L. They also concluded that higher concentrations of glyphosate in urine were found among those children aged 7–9 years old, those living within 1 kilometer of agricultural areas, those who consumed higher amounts of home-produced foods, and those that had herbicides applied in the yard of their residences. Based on their risk assessment, the authors concluded that oral intake of glyphosate ranged from 1% to 5.6% of the established ADI for glyphosate (0.5 mg/kg bw). They considered the frequency of detection and the estimated oral intake to be of concern and, considering the vulnerability of children, encouraged that further biomonitoring studies should be conducted.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This is a small biomonitoring study with limited urine sample collection for each participant. The sampling strategy was not described, but the focus on recruiting participants from municipalities that subscribed to the "Municipalities without Glyphosate" campaign means that the results cannot be taken to be representative of the population of Portuguese children ages 2 to 13. Also, since 82% of the participants came from one region (Coimbra), and other regions had as few as 1 or 2 participants, it seems almost certain that the sampling strategy was a convenience sample. The results should be interpreted accordingly.

The authors concluded that age, living near agricultural areas, consumption of home-produced foods, and having herbicides applied in the backyard were predictors of urinary glyphosate concentrations. The actual differences in urinary concentrations across the categories of these factors were slight and none of the differences were statistically significant. Further, the analysis for each potential predictive factor was univariate and did not control for the other potential predictors. Thus, the authors' evaluation of potential predictors of urinary concentration is relatively uninformative.

The authors estimated that the study participants had glyphosate oral ingestion ranging from 1% to 5.6% of the glyphosate ADI (0.5 mg/kg bw). These estimates were based on assuming 1% oral glyphosate absorption as estimated by Zoller et al. (2020). One percent absorption is 20-fold lower than the absorption factor used previously in the literature (e.g., Niemann et al. 2015). However, the Zoller et al. (2020) study was a well-controlled human study and the 1% absorption is potentially representative for low oral exposures to glyphosate. Zoller et al. (2020) noted, with respect to the 1% absorption factor, that human glyphosate intake estimated from urinary concentrations could be approximately 20 times greater than previously estimated and that would be counterbalanced by

human systemic availability being 20 times lower than previously assumed. Assuming 1% absorption is valid, the estimated intakes in this study are 18-fold to 100-fold less than the ADI.

To conclude, the study by Ferreira and colleagues (2021) has several important limitations. The study participants are unlikely to be representative of children in Portugal. Urine collection per participant was limited to one spot sample, which requires assumptions about the daily volume of urine for each participant and about whether the single sample is representative of that participant's steady state throughout the year. The statistical analysis was superficial with respect to factors that might predict oral intake. Setting aside these limitations and taking the % ADI results at face value, the highest value seen in this study is a small percentage of the ADI.

References

Niemann L, Sieke C, Pfeil R, Solecki R. A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers. Journal für Verbraucherschutz und Lebensmittelsicherheit 2015;10(1):3-12.

Zoller O, Rhyn P, Zarn JA, Dudler V. Urine glyphosate level as a quantitative biomarker of oral exposure. International Journal of Hygiene and Environmental Health 228 (2020) 113526.

Publication: Ferreira C. et al. Urine biomonitoring of glyphosate in children: Exposure and risk assessment.	**Criteria met? Y/N/Uncertain**	**Comments**
Guideline-specific		
Study is in accordance to valid internationally accepted testing guidelines/practices.	n/a	
Study is completely described and conducted following scientifically acceptable standards.	Yes	
Test substance		
Exposure to formulations with only glyphosate as active substance.	Uncertain	Source of glyphosate exposure is not specified.
Exposure to formulations with glyphosate combined with other active substance(s).	Uncertain	Same comment as above
Exposure to various formulations of pesticides.	Uncertain	Same comment as above
Study		
Study design – epidemiological method is followed.	n/a	
Description of population investigated.	No	Portuguese children 2 to 13 years of age from municipalities that were participating in the "Municipalities without Glyphosate" campaign. The methodology within that sampling frame (e.g., random, convenience, etc.) was not specified
Description of exposure circumstances.	No	Also, it seems that the investigators did not ask pesticide users about which specific pesticides were used – especially

Publication: Ferreira C. et al. Urine biomonitoring of glyphosate in children: Exposure and risk assessment.	**Criteria met? Y/N/Uncertain**	**Comments**
		relating to glyphosate use among the herbicide users.
Description of results.	Yes	Superficial analyses
Have confounding factors been considered?	n/a	
Statistical analysis reported / described.	Yes	Analyses of exposure determinants were univariate and very limited. Most differences were small. Conclusions about factors that determine urinary levels were not based on multivariate evaluation and statistical conventions.
Overall assessment		
Reliable without restrictions	No	
Reliable with restrictions	Yes	This is a small study with only a single spot urine sample per participant. The sampling methodology and participation rate were not specified.
Reliability not assignable	No	
Not reliable	No	

グリホサートカリウム塩

要旨及び評価結果

（環境動態）

検索期間：2021 年 1 月 1 日～2021 年 5 月 14 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 7.3.1/009
Report author	Holtomo O. *et al.*
Report year	2021
Report title	Insight of UV-vis spectra and atmospheric implication for the reaction of •OH radical towards glyphosate herbicide and its hydrates
Document No	RSC Advances (2021), 11(27), 16404-16418
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Relevant (Category A acc. to EFSA GD, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The rate constant of the reactions of •OH radicals with glyphosate glyphosate and its hydrates (glyphosate$(H_2O)_{n=1-3}$) were evaluated using the dual method M06-2X/6-311++G(df,p)//6-31+G(df,p) over the temperature range of 200–400 K. The results served to estimate the atmospheric lifetime along with the photochemical ozone creation potential (POCP). The calculations yielded an atmospheric lifetime of 2.34 hours and a POCP of 24.7 for glyphosate. Upon addition of water molecules, there is an increase of lifetime and decrease of POCP for water monomer and water dimer. The POCP for water trimer is slightly above the gaseous glyphosate. However, the POCPs of glyphosate and its hydrates are comparable to that of alkanes. The glyphosate and its hydrates were found to be a potential reservoir of CO_2. The acidification potential (AP) of glyphosate was found to be 0.189 and decreases upon addition of water molecules. This shows negligible contribution to rain acidification as the AP is less than that of SO_2. The UV-vis spectra were attained using the M06-L/6-311++G(3df,3pd) method and cover the range 160–260 nm.

Materials and methods

The rate constant of the oxidative degradation of glyphosate were determined through H-abstraction mechanism in the presence of •OH radical. This mechanism is suspected to happen by five plausible channels as presented in reactions (R1a)–(R1f).

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + {}^{\bullet}OH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COO^{\bullet} + H_2O \quad (R1a)$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + {}^{\bullet}OH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH^{\bullet}\text{–}COOH + H_2O \quad (R1b)$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + {}^{\bullet}OH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}N^{\bullet}\text{–}CH_2\text{–}COOH + H_2O \quad (R1c)$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + {}^{\bullet}OH \rightarrow (OH)_2\text{–}PO\text{–}CH^{\bullet}\text{–}NH\text{–}CH_2\text{–}COOH + H_2O \quad (R1d)$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + {}^{\bullet}OH \rightarrow (HO)O^{\bullet}\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + H_2O \quad (R1e)$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + {}^{\bullet}OH \rightarrow {}^{\bullet}O(HO)\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + H_2O \quad (R1f)$$

The thermochemistry of these five pathways was assessed. The enthalpy and free energy of these reactions were calculated in order to predict the feasibility and spontaneity of the reaction channels. The

bond dissociation enthalpy (BDE) of X–H (X = C, N, or O atom) was used to compare the heat required to break the X–H bonds in glyphosate and its hydrates. The BDE was calculated at five sites as presented in the reactions (R2a)–(R2f).

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COO^{\bullet} + H^{\bullet} \quad \text{(R2a)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH^{\bullet}\text{–}COOH + H^{\bullet} \quad \text{(R2b)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}N^{\bullet}\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2c)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH^{\bullet}\text{–}NH\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2d)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (HO)O^{\bullet}\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2e)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow {}^{\bullet}O(HO)\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2f)}$$

The present work was completed by the means of the density functional theory (DFT) and the time dependent variant (TD-DFT). The exchange–correlation functionals M06-2X and M06-L combined with the appropriate split valence basis sets were employed, respectively. This is in order to yield accurate results of structures, electronic energies, UV-vis spectra, vibrational frequencies, enthalpies and free energies of the species in the reaction processes.

Computational details

Computations were carried out using the density functional theory (DFT) implemented in the Gaussian 09 suite package.

The time dependent DFT (TD-DFT) and DFT schemes through the meta-hybrid exchange correlation functional M06-2X were employed. The potential energy surfaces (PESs) of glyphosate were attained through the rotation of dihedral angles using the M06-2X/6-31G(df) method. The equilibrium geometries and frequencies of glyphosate and its hydrates were carried out using the dual method M06-2X/6-311++G(df,p)//6-31+G(df). However, for the kinetics of the reactions of •OH radical towards glyphosate and its hydrates, the M06-2X/6-311++G(df,p)//6-31+G(df,p) method was assigned. This composite basis set produced excellent results regarding the rate constant of •OH radical towards greenhouse gases. The single point energy calculations were done without orbital symmetry constraints. The direct inversion in the iterative subspace (DIIS) procedure was used for geometry optimizations. The equilibrium geometries of the different species of this study were validated based on the real frequencies obtained, except for the transition state equilibrium where only one imaginary frequency should be observed. The TD-DFT was performed based on the Runge–Gross scheme at the M06-L/6-311++G(3df,3pd) level of theory, in order to have the UV-vis spectra of glyphosate and its hydrates.

UV-vis spectral generation

The UV-vis spectrum of chemical species is turned out by the list of wavelengths and oscillator strengths employing the Gaussian broadening function. This function is the molar extinction coefficient ε(ω) that quantifies the ability of chemical species to absorb light at a given wavelength λ. This is expressed as follows:

$$\varepsilon(\lambda) = \sum_{k=1}^{N} \frac{A_k}{\sigma_k \sqrt{2\pi}} \exp\left(- \frac{(\lambda_k - \lambda)^2}{2{\sigma_k}^2} \right) \qquad (1)$$

where N indicates the number of excited states. The subscript k refers to the k^{th} absorption peak in the spectrum, λ_k is the wavelength at the origin, λ is any given wavelength, and σ_k is the dispersion that is related to the full width at half-maximum (Γ) by $\Gamma = 2\sigma\sqrt{2ln2}$. A_k indicates the absorption intensity of each band including the oscillator strength $f_k = 2m\omega_k|\mu_k|^2/3\hbar$ by the relation:

$$A_k = \frac{2\pi^2 e^2 N_A}{mc \ln(10)} f_k \qquad (2)$$

where m and e are the mass and electric charge of electron, respectively. N_A is the Avogadro's number, c is the speed of light, and μ_k is the transition dipole moment from ground state to k^{th} excited state.

Thermodynamics and kinetics of reactions

The H-abstraction mechanism presented in reactions (R2a)–(R2e) is characterized by the bond dissociation enthalpy (BDE) of all the X–H bonds of glyphosate molecule (C–H, O–H, and N–H). This is assessed using eqn (3).

$$\mathrm{BDE}_{298} = \Delta_f H^\circ(\mathrm{GPS–X^\bullet}) + \Delta_f H^\circ(\mathrm{H^\bullet}) - \Delta_f H^\circ(\mathrm{GPS–XH}) \qquad (3)$$

However, the H-atom transfer (HAT) from gaseous glyphosate to $^\bullet$OH radical is governed by the enthalpies and Gibbs free energies of reactions (R1a)–(R1e). These thermal parameters were calculated using the expression of eqn (4) and (5).

$$\Delta H_{r,298} = \Delta_f H^\circ(\mathrm{GPS–X^\bullet}) + \Delta_f H^\circ(\mathrm{H_2O}) - \Delta_f H^\circ(\mathrm{GPS–XH}) - \Delta_f H^\circ(^\bullet\mathrm{OH}) \qquad (4)$$

$$\Delta G_{r,298} = \Delta_f G^\circ(\mathrm{GPS–X^\bullet}) + \Delta_f G^\circ(\mathrm{H_2O}) - \Delta_f G^\circ(\mathrm{GPS–X–H}) - \Delta_f G^\circ(^\bullet\mathrm{OH}) \qquad (5)$$

where $\Delta_f H^\circ(Y)$ and $\Delta_f G^\circ(Y)$ are the enthalpy and Gibbs free energy of formation of species Y. The physical quantity $\Delta_f H^\circ(Y)$ is the sum of electronic energy, zero-point vibrational energy (ZPVE), and the thermal correction to enthalpy, while $\Delta_f G^\circ(Y) = \Delta_f H^\circ(Y) + RT$, where R represents the gas constant. The rate constants of the reactions (R1a)–(R1e) were calculated based on the transition state theory (TST). This theory is the most popular issue for assessing rate constants of chemical processes. Over the past two decades, significant progress has been made in developing methods for quantitative predictions of rate constants based upon the dynamical formulation of TST. The conventional TST rate constant at temperature T for a bimolecular reaction reads as:

$$k_{TST}(T) = \frac{\sigma}{2\pi\beta\hbar} \frac{Q_{TS}(T)}{\Phi_{React}(T)} \exp(-\beta V_0) \qquad (6)$$

$\beta = 1/k_B T$, k_B is the Boltzmann constant, $\hbar$ is the reduced Planck constant, and σ denotes the number of indistinguishable ways the reactants may approach the activated complex regions. $Q_{TS}(T)$ is the partition function at transition state, and $\phi_{React}(T) = Q_A Q_B$ is the total partition function of reactants A and B per unit volume at temperature T. V_0 is the barrier height of the reaction. Another variant of TST is the vibrational transition state theory (VTST) in which, $Q_{TS}(T)$ and V_0 varies with the reaction coordinate as $Q_{TS}(T,s)$ and $V_0(s)$. In VTST variant, the rate constant is expressed as the minimum of $k_{VTST}(T,s)$. This method was performed in the present work for the reaction of $^\bullet$OH radical towards glyphosate and its hydrates. In the total partition function of the reactants ($\phi_{React}(T) = Q_{glyphosate} Q_{OH}$), the contribution of electronic partition function of $^\bullet$OH radical takes into account the splitting of the ground state π^2 into $\pi_{1/2}^2$ and $\pi_{3/2}^2$. The separation 139.7 cm^{-1} of these sub-states was used in the calculations. The electronic partition of $^\bullet$OH radical is then written as follows $Q_{elec}(OH) = 2 + 2\exp((139.7\ \mathrm{cm}^{-1})\hbar c/k_B T)$, where $\hbar$ and c are the Planck's constant and speed of light, respectively. It is worth mentioning that, the internal rotation modes of glyphosate and its hydrates were treated using the hindered rotor approximation, whereas the other vibrational frequencies were treated harmonically.

Considering the quantum tunnelling effects, the rate constant is multiplied by the quantum tunnelling factor $\eta(T)$ as per eqn (7).

$$k(T) = \eta(T) k_{VTST}(T) \qquad (7)$$

The $\eta(T)$ factor is defined as the ground state transmission coefficient at temperature T. This factor is quantitatively the ratio of the thermally averaged multidimensional semi-classical transmission probability to the thermally averaged classical transmission probability for scattering by the effective

potential.

Results

Structural study of molecular systems

To come out with the atmospheric implication of this study, the geometrical structures of glyphosate (glyphosate) and its hydrates was paramount challenges to deal with. A computational protocol was used to yield the low-energy conformers of glyphosate and its hydrates.

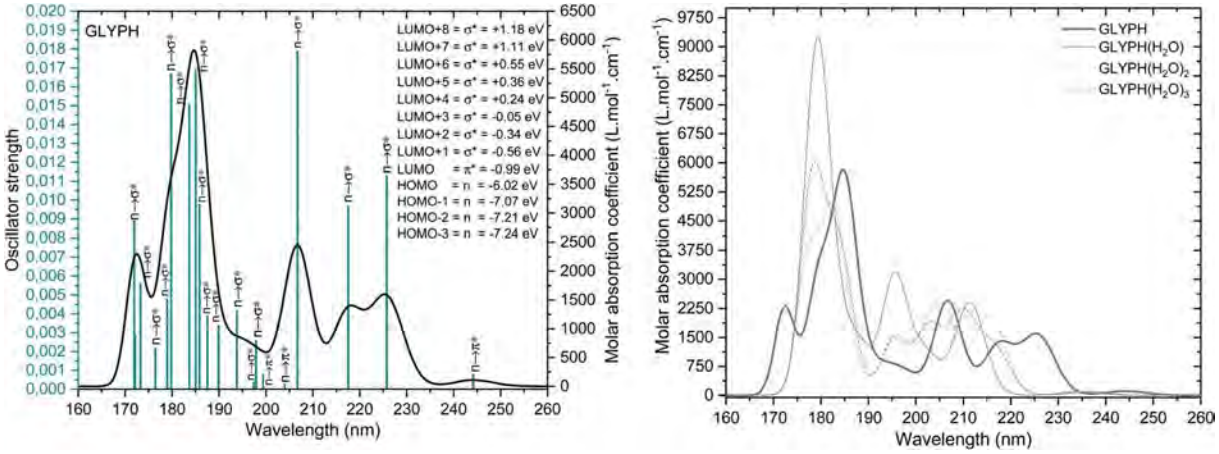
The order of degradation of glyphosate and its hydrates from the dipole moment analysis was stated as follows: glyphosate(H_2O) > glyphosate > glyphosate$(H_2O)_2$ > glyphosate$(H_2O)_3$. Thus, in humid air, glyphosate reduces its dipole moment and resist to degradation. This is in discordance with the average polarizability analysis where, the order of degradation follows as glyphosate$(H_2O)_3$ > glyphosate$(H_2O)_2$ > glyphosate(H_2O) > glyphosate. In this case the humid air favours the degradation of glyphosate.

UV-vis spectroscopies of glyphosate and its hydrates

In order to identify glyphosate in the atmosphere, the TD-DFT calculations were first performed at M06-2X/6-311++G(3df,3pd) level of theory. The results failed while comparing with experiment. To improve this result, a diagnostic based on the fact that HF/6-311++G(d,p) yielded good results, thus glyphosate molecule is a single reference system. Therefore, a pure DFT can be a good deal for the spectroscopy of glyphosate and its hydrates. Then the pure functional M06-L of the same family of Menesota University was selected to perform TD-DFT calculations. The obtained results gave satisfaction (206.74 nm).

The UV-vis spectra of the glyphosate and its hydrates are presented in Fig. 5. The main peak at 206.74 nm has a weak absorption coefficient value. This is due to the nearby peaks in which the overall Gaussian function accumulates the individual ones. The effects of water molecules impact seriously the spectrum of glyphosate. The blue shifts of peaks are noticeable along with the enhancement of the absorption peaks (hyperchromic effects). However, it is seen that, the spectra are localized in the UV window range 160–260 nm. This advises that, glyphosate and its hydrates are colourless.

Figure 5: **UV-vis spectra of glyphosate its hydrates. The full width at half maximum was taken at 0.1 eV**



Thermodynamics of reactions

In the presence of •OH radical, the reaction process of glyphosate can advent from six different issues as presented in the reactions (R1a)–(R1e). The bond dissociation mechanisms from the five channels are stated at equations (R2a)–(R2e). The enthalpies of these reactions (BDE) at 298.15 K advises on the amount of heat required to dissociate the C–H, N–H, and O–H bonds of glyphosate. The results are compared to the computational BDEs of other molecules as quercetin (282.8 kJ/mol at B3LYP/6-31++G*), caffeic acid phenethyl ester (312.0 kJ/mol at B3LYP/6-31+G**), 3,4-dihydroxyphenylpyruvic acid (304 kJ/mol at B3LYP/6-311++G**), *p*-phenylenediamine (340 kJ/mol at B3LYP/6-31++G**) and tetracyano-*p*-phenylenediamine (381 kJ/mol at B3LYP/6-31++G**), and the experimental BDE of 3,4-dihydroxycinnamic acid (285 kJ/mol is in excellent agreement with theory at B3LYP/6-31++G** fit). In these molecules, the BDE were obtained from the O–H bonds except for *p*-phenylenediamine and tetracyano-*p*-phenylenediamine where the BDE was calculated from the N–H

bonds. The computational results were assessed using the B3LYP functional combined with the Pople's basis sets 6-31++G*, 6-31+G**, 6-31++G**. It is seen, that the computational BDEs from the N–H bond are greater than those obtained from O–H bonds. This is consistent with what was observed in glyphosate and its hydrates. However, the BDEs of glyphosate increase in the following order: BDE (site C_6) < BDE (site O_{16}) <BDE (site C_3) < BDE (site N_1) < BDE (site O_{10}) = BDE (site O_{12}). For wetted glyphosate, it follows the order: BDE (site C_3) < BDE (site O_{16}) < BDE (site C_6) < BDE (site N_1) < BDE (site O_{12}) = BDE (site O_{10}). Therefore, the most favourable site of radical attacks is C_6 for gaseous glyphosate and C_3 for wetted glyphosate. In addition, the H-atom at site O_{10} and O_{12} are strongly attached; they require a great amount of heat for dissociation. The BDEs from the exothermic paths ((R1a)–(R1d)) are reliable with the molecules used for comparison, while the BDE from the endothermic path (R1e) is out of order. The glyphosate radical obtained after the dissociation of O_{16}–H bond involved in the carboxylic group, is thermodynamically unstable. It dissociates in turns into carbon dioxide CO_2 and $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$ radical. This mechanism is written as per equation (R3).

$$(OH)_2 - PO - CH_2 - NH - CH_2 - COOH \rightarrow (OH)_2 - PO - CH_2 - NH - CH_2^{\cdot} + CO_2 + H^{\bullet} \quad (R3)$$

In the calculation of the BDE of O_{16}–H, the enthalpy of the overall system $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$-CO_2 was considered as it takes into account the interaction between individual subsystems.

On the other side, the enthalpies $\Delta H_{r,298}$ and Gibbs free energies $\Delta G_{r,298}$ of reactions involving glyphosate (and its hydrates) and $^{\bullet}$OH radical were estimated at 298.15 K in order to predict the feasibility and spontaneity of the reaction channels.

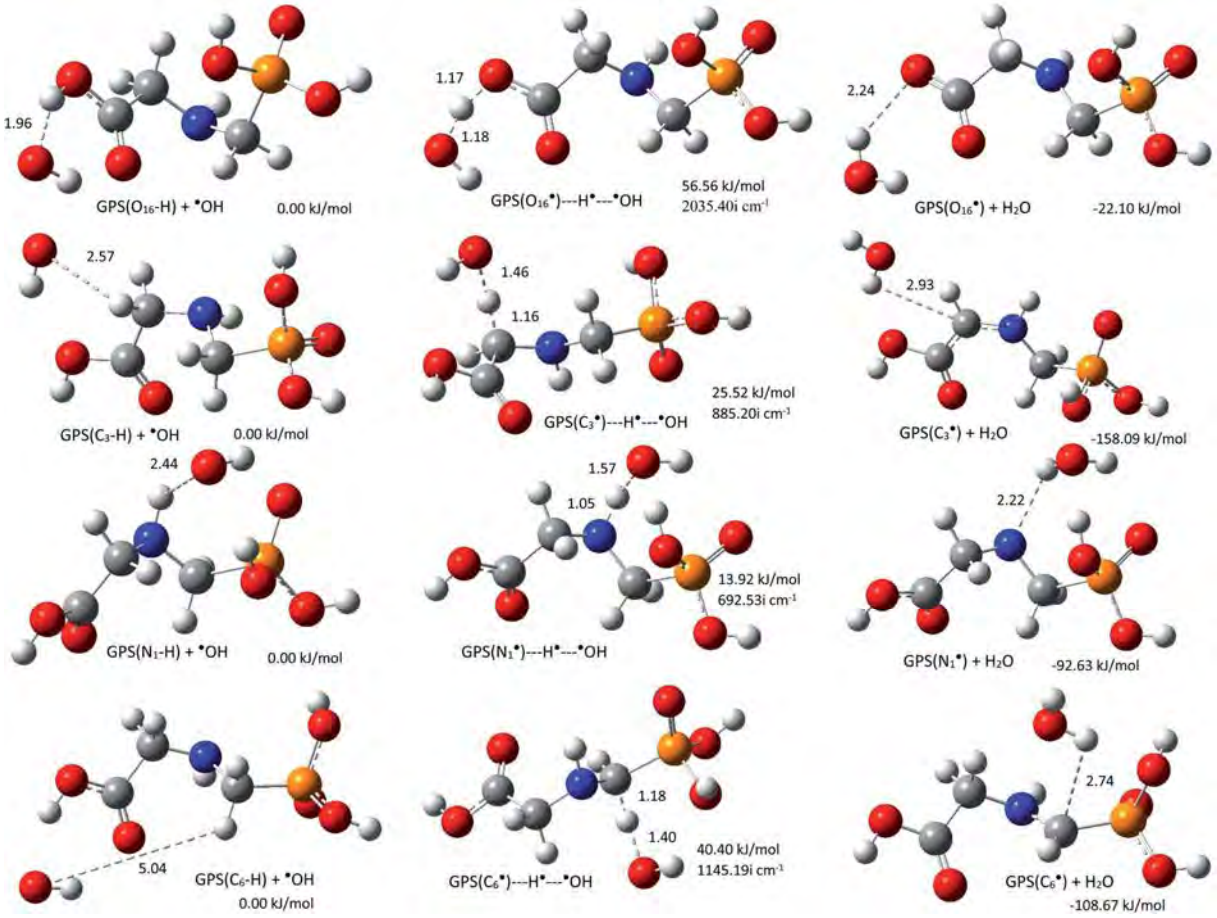
It turns out that, the reaction channels (R1a)–(R1d) are exothermic, while the channel (R1e) is endothermic. This is due to the nucleophilic character of the molecular systems at the level of P-atom. In fact, in the glyphosate molecule, the charge hole is made on P-atom and the electron lone pairs take place on the O-atoms and reinforce the enthalpy of the O–H bonds. It is noticeable that, this fact is correlated with the BDE and explains why channel (R2e) has the greatest value of BDE. Dealing with $\Delta G_{r,298}$, it comes out that, the reaction channels (R1a)–(R1d) are spontaneous, while the channel (R1e) is non-spontaneous. Thus, we can conclude that, the reaction channel (R1e) is unfeasible; therefore, an attention was paid only on the reaction channels (R1a)–(R1d) in the section devoted to kinetics of chemical reactions.

Moreover, in the presence of $^{\bullet}$OH radical, the reaction process (R3) yields three different products such as H_2O, CO_2 and $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$. As in the case of BDE calculation, the enthalpy and Gibbs free energy of formation of the overall system $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$-CO_2 was considered in the calculations of $\Delta H_{r,298}$ and $\Delta G_{r,298}$. In view of the production of CO_2 by the channel (R1a) and the feasibility of the reaction, one can assert that glyphosate is a reservoir of CO_2 in the lower atmosphere.

Kinetics of reactions

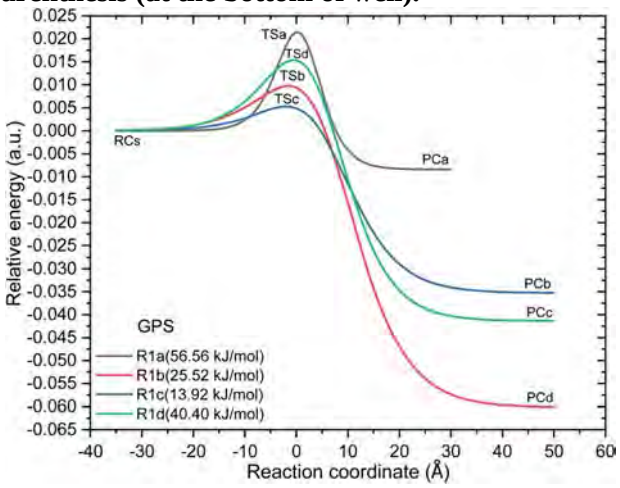
The rate constants k_{OH} of the reactions of $^{\bullet}$OH radical towards glyphosate and its hydrates were evaluated at all the thermodynamically feasible hydrogen sites. This includes the first four channels ((R1a)–(R1d)). The equilibrium geometries at reaction complex (RC), transition state (TS), and product complex (PC) for each pathway in gas phase are presented in Fig. 6 along with their relative energy (relative to RC). The stabilization of RCs and TSs for glyphosate and glyphosate(H_2O) were examined by the basis set superposition error (BSSE) corrected binding energies using the M06-2X/6-311++G(df,p) method. They were calculated from two fragments; $^{\bullet}$glyphosate/ $^{\bullet}$glyphosate(H_2O) and $^{\bullet}$OH radicals for RCs and three fragments; $^{\bullet}$glyphosate/ $^{\bullet}$glyphosate(H_2O), $H^{\bullet}$, and $^{\bullet}$OH radicals for TSs. As shown, the difference between binding energies of RCs and TSs are within 10^{-3} au, which can be ignored in the rate constant calculation.

Figure 6: **Reaction complex (RC), transition state (TS), and product complex (PC) for each reaction pathway of •OH radical towards glyphosate. The relative electronic energy of each step of the process are presented in kJ mol^{-1}. The internuclear lengths along the reaction coordinates and the imaginary frequency at TS are given in Å and cm^{-1}, respectively.**



As shown along the reaction coordinate, RC is first formed and as the reaction progresses, the intermediate (TS) is appeared and finally PC takes place. The three states along the minimum energy path (MEP) are stabilized by the interactions between glyphosate and •OH. All the species involved in the reaction paths (R1a)–(R1d), are verified by real vibrational positive frequencies, while TSs are confirmed by one imaginary frequency (IF) in the list of frequencies. The magnitude of these imaginary frequencies follows the order IF(R1a) > IF(R1d) > IF(R1b) > IF(R1c) for glyphosate. The larger the IF, the shorter is the width of the MEP. This is noticeable on the MEP presented in Fig. 7, where the narrowest and the largest potential barriers are along the (R1a) and (R1d), respectively. This behaviour is also observed for glyphosate hydrates. The relative energies of TSs are the energy barrier height (EBH) of the reaction path, which follows the order (R1a) > (R1d) > (R1b) > (R1c) for glyphosate. For glyphosate hydrates, one can read (R1a) > (R1b) > (R1d) > (R1c) for glyphosate (H_2O), (R1a) > (R1d) > (R1b) > (R1c) for glyphosate $(H_2O)_2$ and glyphosate $(H_2O)_3$. Thus, this order for glyphosate with water dimer and water trimer agrees with the one established for glyphosate in gas phase. Upon addition of water molecule, the EBH decreases in the reaction path (R1a), while the other paths show a disharmony. The EBHs of all the paths are further lowered or broken by water continuum. The continuum was assessed using the solvation model based on the quantum mechanical charge density of a solute molecule interacting with a continuum (SMD). In fact, the calculations show that the mechanism of H-abstractions from glyphosate in water continuum are spontaneous since the reaction complexes (RCs) are unstable. Once a RC is formed, the reaction moves to the product complex (PC).

Figure 7: **Minimum energy potentials of the reaction of •OH towards gas phase glyphosate. With the values of electronic energies at reaction complex (RC), transition state (TS), and product complex (PC), these potentials were interpolated by the Eckart unsymmetrical function. The barrier height from the reaction complex of each reaction path are presented in parenthesis (at the bottom of well).**



The calculated values of rate constant (298 K) are reported in Table 5 along with the branching ratio of each pathway. The internal rotation modes of glyphosate and its hydrates were used in place of vibrational modes using the hindered rotor approximation.

Table 5: **Rate constant (cm^3 per molecule per s) along with branching ratio (%) for each reaction path of glyphosate and its hydrates at 298 K from the processes of H-atom abstraction by •OH radicals. The total rate constant is given by $k_{OH} = k^a_{OH} + 2k^b_{OH} + k^c_{OH} + 2k^d_{OH}$**

Reaction	(R1a)	(R1b)	(R1c)	(R1d)	Total
Rate constant	k^a_{OH}	k^b_{OH}	k^c_{OH}	k^d_{OH}	k_{OH}
GPS	1.14×10^{-10}	1.74×10^{-12}	9.07×10^{-13}	8.83×10^{-14}	1.19×10^{-10}
GPS(H_2O)	7.56×10^{-12}	1.85×10^{-14}	2.96×10^{-11}	9.22×10^{-12}	5.56×10^{-11}
GPS(H_2O)$_2$	2.27×10^{-10}	4.09×10^{-15}	7.79×10^{-14}	6.76×10^{-11}	3.62×10^{-10}
GPS(H_2O)$_3$	5.20×10^{-13}	8.91×10^{-15}	5.10×10^{-14}	1.33×10^{-13}	8.56×10^{-13}
GPS(H_2O)$_\infty$					
Branching ratio	k^a_{OH}/k_{OH}	k^b_{OH}/k_{OH}	k^c_{OH}/k_{OH}	k^d_{OH}/k_{OH}	
GPS	96.15	1.47	0.76	0.08	—
GPS(H_2O)	13.60	0.033	53.20	16.60	—
GPS(H_2O)$_2$	62.60	0.001	0.022	18.70	—
GPS(H_2O)$_3$	60.80	1.04	5.96	15.60	—

The results show that, the dominant reaction is (R1a) with the highest value of branching ratio. The exception is seen for the hydrate glyphosate (H_2O), where the highest branching ratio appeared at channel (R1c). The discrepancy with the barrier height comes from the narrow and large widths. (R1a) is kinetically more favourable for its narrow barrier (small width). The available experimental result is $k_{OH} = (3.1 \pm 0.08) \times 10^{-13}$ cm^3 per molecules per s^{-1} obtained in water continuum. This result is affected by the complexation with iron. Nevertheless, the findings with the maximum size of water cluster (n = 3) fit the experimental results at 78.71%. The remaining percentage can be corrected by supplementing explicit water molecules to the hydrate glyphosate $(H_2O)_3$.

The fit of the total rate constant over the temperature range 200–400 K was completed for glyphosate and its hydrates (Fig. 8). The fit yielded the non-Arrhenius equation of the form $k^{env}_{OH}(T) = \exp(c + bT^{-1} + aT^{-2})$, where a, b and c are real constants of the fit, and T the temperature in K. The superscript 'env' indicates the environment of glyphosate, which can be gaseous state or explicit water. The equations of the rate constant as a function of temperature are given as per eqn (8)–(11).

$$k_{OH}^{gas}(T) = \exp\left(-2.263 - \frac{7268.6}{T} + \frac{338.63 \times 10^3}{T^2} \right) \quad (8)$$

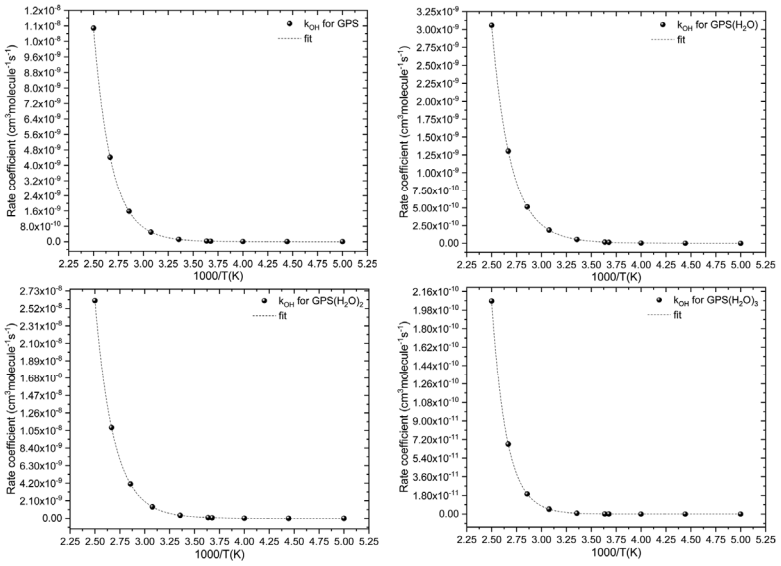
$$k_{OH}^{H_2O}(T) = \exp\left(-2.542 - \frac{8428.99}{T} + \frac{641.46 \times 10^3}{T^2} \right) \quad (9)$$

$$k_{OH}^{(H_2O)_2}(T) = \exp\left(-1.625 - \frac{7334.33}{T} + \frac{400.07 \times 10^3}{T^2} \right) \quad (10)$$

$$k_{OH}^{(H_2O)_3}(T) = \exp\left(-2.563 - \frac{9003.8}{T} + \frac{443.53 \times 10^3}{T^2} \right) \quad (11)$$

These equations were obtained with a coefficient of determination equal to unity.

Figure 8: **Temperature-dependent rate constant of the reaction between glyphosate (and its hydrates) with the •OH radical over the temperature range 200–400 K.**



Atmospheric lifetime

With the rate constants of the reactions of •OH radical towards glyphosate and its hydrates, the atmospheric lifetime τ_{OH} of glyphosate and its hydrates were estimated as the inverse of the product of rate constant and the concentration of •OH radical in the troposphere. This is expressed as per eqn (12).

$$\tau_{OH} = \frac{1}{k_{OH}[\bullet OH]} \quad (12)$$

where k_{OH} represents the rate constant of the reaction of •OH radical with glyphosate and its hydrates. The quantity [•OH] = 1.0×10^6 molecule cm^{-3} is the global average atmospheric concentration of the •OH radicals. In fact, other oxidants as O_3, $\bullet NO_{x=1-3}$ radicals, and Cl atom also contribute to the degradation of the gas in the troposphere. However, they are less reactive compared to •OH radical, which is known as the 'atmospheric detergent'. This advises the use of •OH radical in the determination of atmospheric lifetime.

The results of atmospheric lifetime are reported in Table 6. They are of the order of hours and follow

the order glyphosate $(H_2O)_2$ < glyphosate < glyphosate (H_2O) < glyphosate $(H_2O)_3$. Thus, glyphosate and its hydrates are washed out from the troposphere once its release. However, the growth of the size of these hydrates may form aerosols and increase the atmospheric lifetime. However, glyphosate and its hydrates are not atmospherically well-mixed as they are short-live compounds. Therefore, they cannot be assigned to a unique atmospheric lifetime. The reported results represent the global atmospheric lifetime average. The local lifetime could significantly vary with the seasons, the physical and chemical conditions of atmosphere, and the location of emission.

Table 6: **Atmospheric lifetime (hours), photochemical ozone creation potential (POCP), and acidification potential**

	Atmospheric lifetime (hours)	POCP	Acidification potential
GPS	2.34	24.7	0.189
GPS(H_2O)	4.99	23.0	0.171
GPS(H_2O)$_2$	0.77	20.0	0.156
GPS(H_2O)$_3$	324.49(13.52 days)	32.7	0.144

GPS = glyphosate

Photochemical ozone creation potential

Photochemical ozone creation potential (POCP) has been suggested to quantify the ability of a gas to create ozone in the troposphere. In fact, the emitted gas reacts with the oxidants ($^{\bullet}OH$, $^{\bullet}NO_3$, Cl, etc.) in the troposphere, which yields the corresponding peroxy/alkoxy radicals. Further, these peroxy/alkoxy radicals react with $^{\bullet}NO$ to form $^{\bullet}NO_2$, which in turn regenerates $^{\bullet}NO$ along with the ozone molecule in the presence of light and oxygen. The procedure to estimate the POCP was recently improved and the new estimation procedure is written as per eqn (13).

$$POCP_X = (A \times \gamma_s \times R \times S \times F) + P + R_{O_3} - Q) \qquad (13)$$

where the subscript X stands for the molecular system glyphosate$(H_2O)_{n=0-3}$. The quantities A, γ_s, R, and S are core parameters used for any compound, while F, P, R_{O_3} and Q are the parameters used for specific compound. These can take the default values of one (1) for F and zero (0) for P, R_{O_3} and Q. In North West European conditions, the parameter A = 100 is a multiplier. γ_s is the ozone formation index obtained from the structure of molecule X. it is expressed as per eqn (14).

$$\gamma_s = \frac{n_B}{6} \times \frac{28.05}{M_X} \qquad (14)$$

where n_B indicates the total number of reactive bonds (C–C, N–C, P–C, C–H, N–H, and O–H) in the molecule, M_X denotes the molecular weight of molecule X. R is a reactivity element that is related to the $^{\bullet}OH$ reactivity of X. It takes the following from $R = 1 - (B\gamma_R + 1)^{-1}$. The parameters $B = 4.0$ under North West European conditions and γ_R is the ozone formation index obtained from the reactivity of molecule X. It is expressed as follows.

$$\gamma_R = \frac{6}{n_B} \times \frac{k_{OH}^X}{k_{OH}^{ethene}} \qquad (15)$$

k^X_{OH} is the rate constant for the reaction of molecule X with $^{\bullet}OH$ radical at 298 K and 1 atm of air, and k^{ethene}_{OH} is the rate constant for the reaction of ethene with $^{\bullet}OH$ radical at 298 K and 1 atm of air. k^{ethene}_{OH} was found to be 8.64×10^{-12} cm^3 per molecule per s. The parameter S is related to the size of the molecule X and takes the form $S = (1-\alpha) \times \exp(-C^* n_c^{\beta}) + \alpha$, where n_c is the number of carbon of the gas molecule X. under North West European conditions, the values of α, C, and β are 0.56, 0.0038, and 2.7, respectively. The default values were assigned to the other parameters. As such, for glyphosate and its

hydrates, the difference between their POCP is seen from their structure and reactivity indexes.
The results of the estimated POCPs are reported in Table 6. It comes out that, the gaseous glyphosate and its hydrates are not efficient ozone producer, as their POCPs values are low. It is 24.7 for glyphosate and decreases with the water monomer (23.0) and water dimer (20.0). The POCP of water trimer is 32.7 larger than the other ones including gaseous glyphosate. It lies on the range of the ozone formation potential of alkane.

Acidification potential
Acid rain is one of the environmental concerns today. It impacts negatively on plants and animals on the earth's surface. When the substances containing the atoms N, F, Cl and S, get emitted into the atmosphere, they form the acid species such as HNO_3, HF, HCl, and H_2SO_4 which contributes naturally to the acid rain. In accordance with the above, the acidification potential (AP) is the number of acid equivalent potentials (H^+) per unit mass of a given compound X with respect to the number of H^+ per unit mass of the reference compound SO_2. It is thus a parameter to measure the ability of an emitted compound to contribute towards acid rain with respect to SO_2 in the local environment. It is expressed as per eqn (16).

$$\mathrm{AP_X} = \frac{M_{\mathrm{SO_2}}}{M_{\mathrm{X}}} \times \frac{1}{2}(n_{\mathrm{Cl}} + n_{\mathrm{F}} + n_{\mathrm{N}} + 2n_{\mathrm{S}}) \qquad (16)$$

where M_{SO_2} and M_X represent the molecular weights of SO_2 and molecule X = (glyphosate$(H_2O)_{n=0-3}$), respectively. The quantities n_{Cl}, n_F, n_N, and n_s are the number of Cl, F, N, and S atoms, respectively, in the molecular system X. The estimated results are compiled in Table 6. It turns out that, the AP of glyphosate is 0.189 and decreases upon addition of water molecules. This shows negligible contribution to rain acidification as the AP is less than that of SO_2.

Conclusion
The rate constant was fitted and expressed in non-Arrhenius model equation $k_{OH}^{env}(T) = \exp(c + bT^{-1} + aT^{-2})$ over the temperature range 200–400 K, where a, b, and c are real constants. The atmospheric lifetime was estimated from the rate constant along with the photochemical ozone creation potential (POCP). It turns out that, glyphosate and its hydrates are short-live compounds as their atmospheric lifetime are in the order of hours. glyphosate and its hydrates are inefficient ozone producers. The acidification potentials of glyphosate and its hydrates show negligible impact in the acidification of rain.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The aim of the study was the estimation of the atmospheric half-life of glyphosate considering reactions with OH-radical or Cl-atoms. The calculations yielded an atmospheric lifetime of glyphosate of 2.34 hours. The EU agreed method to determine the half-life of an active substance is the Atkinson approach. The calculation in the publication cannot be considered a common method and the endpoint should not supersede the endpoint calculated using the Atkinson method.
The study is considered reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.5
Report author	Geissen, V. *et al.*
Report year	2021
Report title	Cocktails of pesticide residues in conventional and organic farming systems in Europe - Legacy of the past and turning point for the future
Document No	Environmental Pollution (2021):278, 116827
Guidelines followed in study	SANTE/11813/2017
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Relevant (Category A acc. to EFSA GD, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, 340 EU agricultural topsoil samples were analysed for multiple pesticide residues (glyphosate and AMPA were among them). These samples originated from 4 representative EU case study sites, which covered 3 countries and four of the main EU crops: vegetable and orange production in Spain (abbreviated as S-V and S-O, respectively), grape production in Portugal (abbreviated as P-G), and potato production in the Netherlands (abbreviated as N-P). Soil samples were collected between 2015 and 2018 after harvest or before the start of the growing season, depending on the site.

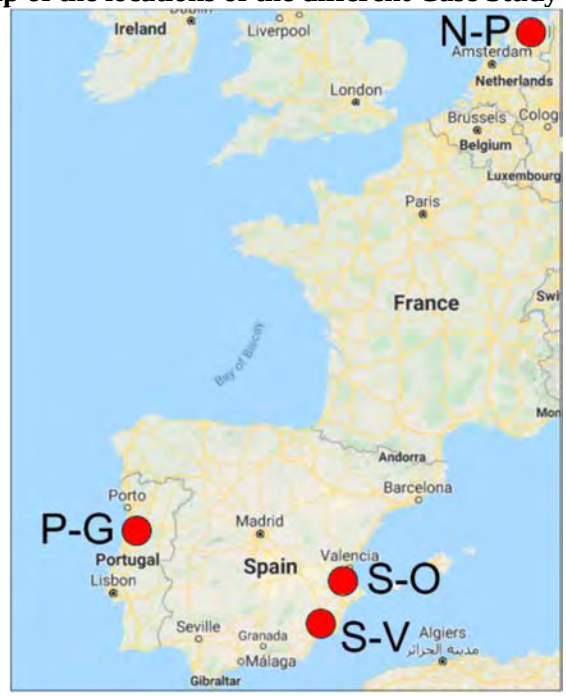
For glyphosate, the maximum amounts detected in conventional fields were 7.843, 1.3 and 0.18 mg/kg in grapes in Portugal, potatoes in the Netherlands and oranges in Spain, respectively. For AMPA, the maximum amounts detected in conventional fields were 4.294, 0.528 and 1.626 mg/kg in grapes in Portugal, potatoes in the Netherlands and oranges in Spain, respectively. In organic fields, glyphosate was detected only at S-O-O with a maximum amount of 0.105 mg/kg while AMPA was detected with maximum 0.014 and 0.593 mg/kg in N-P-O and S-O-O, respectively.

Materials and methods

Case study sites overview

For this study, data was compiled from 4 Case Study Sites (Figure 1) from 3 EC funded projects addressing soil quality (RECARE, iSQAPER and DIVERFARMING). In all three projects, pesticide application patterns and distribution of pesticide residues in agricultural soils were studied at a study site level. These case study sites represented typical cropping systems and covered different climate zones: vegetable production under plastic mulch in Southeast-Spain (S-V; Glyphosate was not determined in this site, therefore, no information or results of this site is summarised), orange production in Eastern Spain (S-O), grape production in Northern Portugal (P-G), and potato production in Northern Netherlands (N-P). The study site included both organic and conventional production systems, except for P-G (organic grape farms were not common in the sampled area of Portugal). The organic fields were converted more than 5 years ago (S-O) or more than 10 years ago (N-P). The conventional farms were managed as such for at least the last 10 years. Overall, 340 topsoil samples were collected and analysed (0-10/15 cm depth). Soil samples were collected between 2015 and 2018 at the end of the growing season (S-O, P-G) or before the growing season (N-P). Sampling at the end of the growing season aimed to assess the accumulative soil contamination scenario after the application period (expected to be the worst-case scenario) while sampling before the growing season tested the background situation. The characteristics of the study sites and the sampling pattern for each site are presented in Table 1. The soil samples were air-dried (at ambient temperatures, under dark conditions, and for a maximum of 1 week), sieved to 2 mm and frozen (-20°C) until the extraction and determination of pesticide residues could be carried out.

Figure 1: **Map of the locations of the different Case Study Sites**



S-V = vegetables production in Southeast-Spain (no glyphosate determination), S-O = orange production in Eastern Spain, P-G = grape production in Northern Portugal, and N-P = potato production in Northern Netherlands.

Table 1: **Characteristics of the case study sites and respective sampling details**

CSS code	Location	Crop	Climate	No. harvests (year period)	Soil type; Texture
P–G (P–G–C)	Bairrada, N-Portugal	Grapes	Temperate Mediterranean	1 (October)	Cambisols and Luvisols; clay, sandy loam, sandy, clay loam
N–P (N–P–C, N–P–O)	Groningen, N-Netherlands	Potatoes (in rotation with cereals)	Atlantic	1 (September)	Cambisols; sandy loam, clay
S–O (S–O–C, S–O–O)	Valencia, E-Spain	Oranges	Hot Mediterranean	1 (December/ January)	Cambisols; Sandy loam

CSS code	Location	Organic matter (mean ± SDev)	pH in H_2O (mean ± SDev)	Timing of soil sampling	No. of fields sampled	No. of samples per field	No. of samples per CSS
P–G (P–G–C)	Bairrada, N-Portugal	6.2 ± 1.8%	7.1 ± 1.3	October 2016	C: 9; O: 0	12	C: 108; O: 0
N–P (N–P–C, N–P–O)	Groningen, N-Netherlands	3.6 ± 0.9%	7.7 ± 0.4	April 2018	C: 9; O: 1	C: 3/4; O: 6	C: 28; O: 6
S–O (S–O–C, S–O–O)	Valencia, E-Spain	3.5 ± 1.5%	8.1 ± 0.2	February 2015	C:6; O: 6	C:9; O: 6	C: 54; O: 36

C = conventional, O = organic, SDev = standard deviation, No. = Number.

Analysis of pesticide residues in soil samples

The list of the pesticide residues tested in soil samples included 47 residues in P-G, 36 in N-P and 75 in S-O.

All soil samples were thawed and homogenised (hand mixed until a visual homogeneous sample was obtained) and split into two aliquots: one for the determination of basic soil properties (pH, organic matter and texture) and one (2 g) for determination of glyphosate and its main metabolite AMPA (in S-O, P-G and N-P). Since none of the parties interviewed for this study reported that glyphosate was applied in S-V, it was not analysed in those samples. Glyphosate and AMPA were determined using LC-MS/MS (liquid chromatography-tandem mass spectrometry; Instrument: Quattro Ultima from Micromass coupled to an Acquity UPLC system).

Analyses were performed according to the analytical quality control and method validation procedures for pesticides residues analysis in food and feed. The guidelines for the current version, at the time of analysis, of the SANTE document were applied. Analyses involved the use of calibration standards, reference standards and isotope labelled internal standards. The calibration standards were prepared from a mix solution that combined the reference standards of all the compounds that were going to be analysed. Isotope labelled internal standards were used for normalisation of response of glyphosate and AMPA. The reference standards were purchased from LGC Standards (Germany), HPC Standards (Germany) or Sigma-Aldrich (USA). The isotope labelled internal standards of glyphosate and AMPA were obtained from LGC Standards (Germany). Limits of quantification (LoQ) were used as reporting limits. The LoQ of glyphosate and AMPA was 0.050 mg/kg.

Data analysis - Residues in soil

The frequency of detection, the median and the range of concentrations for each compound from each organic and conventional farming system per study site was calculated. Furthermore, the range and the median number of pesticide residues found in organic and conventional soils for each site is presented. The content of the different pesticide residues found in each sample to obtain the total residues content per sample was added. Nonparametric Mann Whitney U tests were used to test significant differences in the number of residues and in the total residues content in soils between conventional and organic farms within the same site, and between sites within the same farming strategy. Statistical analyses were performed using STATISTICA, version 12. The significance level was set at 0.05.

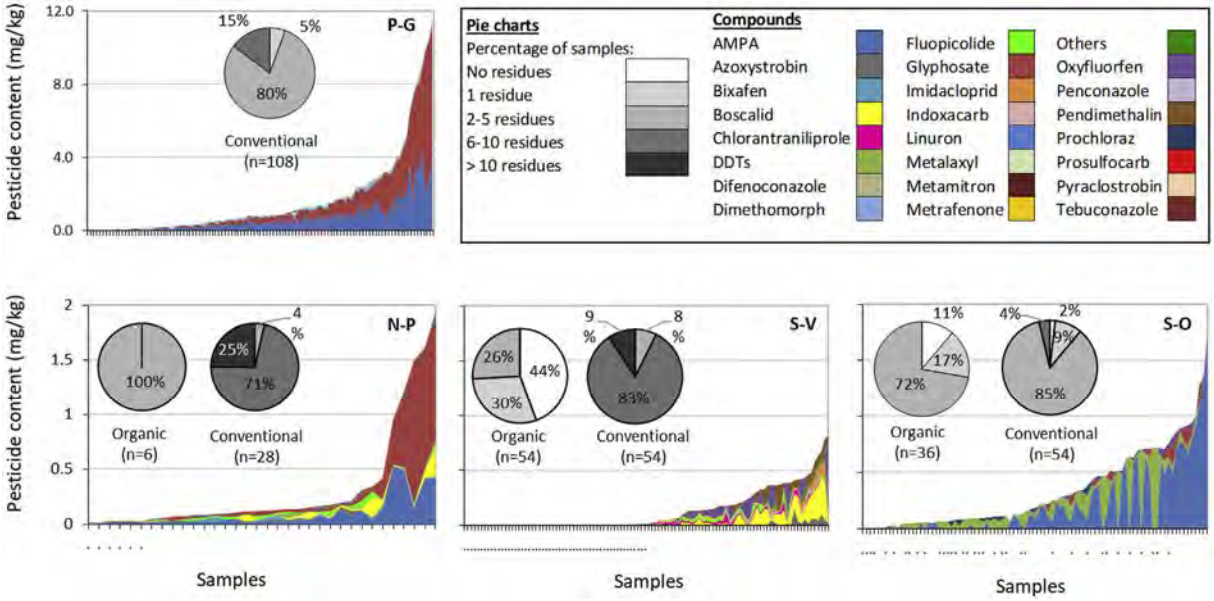
Results

Pesticide residues identified in the study sites - Frequency of detection

The only pesticide residue-free soils under conventional farming were identified in conventional orange farming in Spain (2% of all conventional soils; all other soils under conventional farming contained one or more pesticide residues. In conventional farming of grapes and oranges, more than 80% of the soils contained 2 to 5 residues, while most potatoe samples contained 6 to 10 different residues (71%). A substantial part of potato (25%) soil samples contained even more complex mixtures, with more than 10 residues. Soils from organic farms contained significantly fewer residues, with 11% of the soils in orange farming being free of tested pesticide residues. However, 100% of organic potato soils and 72% of organic orange soils contained mixtures of two to five residues. In total, 15 residues were detected with a frequency above 50% in one or more of the study sites and 7 residues with a frequency between 20 and 50% in one or more of the sites (Figure 2).

The detection frequency of glyphosate was 78% in P-G-C, 93% in N-P-C, 0% in N-P-O, 22% in S-O-C and 6% in S-O-O (Table 2).

Figure 2: **Number of pesticide residues and total pesticide content found in the organic and conventionally managed soils from P-G, N-P, S-V, S-O**



The number of residues in soils were aggregated per classes, being the distribution of each class is presented in the pie-charts. The total pesticide content is presented as a cumulative curve, being the topsoil samples from each site organized by increasing total pesticide content. The. below the x axis indicates soils under organic management, the remaining values are related to conventionally managed fields. Each colour under the curves represent a different pesticide residue.

Table 2: **Frequency, maximum value and median value of glyphosate and AMPA in the 3 tested study sites (in mg/kg)**

		P-G (conv)				N-P (conv/org)				S-O (conv/org)			
Compound	Persistence	% freq	Q1	Max	Median	% freq	Q1	Max	Median	% freq	Q1	Max	Median
AMPA (M-H)	VP	83	0.260	4.294	0.505	96/ 83	0.026/ 0.012	0.528/ 0.014	0.037/ 0.013	87/ 17	0.102/ 0.069	1.626/ 0.593	0.261/ 0.070
Glyphosate (H)	P	78	0.179	7.843	0.452	93/ 0	0.018/ <LoQ	1.300/ <LoQ	0.029/ <LoQ	22/ 6	0.070/0.076	0.180/ 0.105	0.090/ 0.090

known to be applied and detected

H = herbicide, M = metabolite; VP = very persistent, P = persistent. LoQ - Limit of quantification. Q1 - The first quartile concentration, i.e. 25% of the values above LoQ. orange production in Eastern Spain (S-O), grape production in Northern Portugal (P-G), and potato production in NorthernNetherlands (N-P), conv = conventional, org = organic. No reporting on glyphosate content in vegetable production in Spain (S-V).

Pesticide residues identified in the study sites - Pesticide residues content in the study sites

The highest pesticide content was found in P-G-C, with a total residue content of nearly 12 mg/kg, a value approximately 6 times higher than the maximum content in N-P-C and S-O-C fields (with a maximum content of 2 and 1.7 mg/kg, respectively; Figure 2). The residue content under organic farming in N-P did not exceed 0.2 mg/kg, i.e. 10% of the maximum content in the respective conventional fields. In S-O-O, the maximum residue content was 0.6 mg/kg, which was about 30% of the maximum residue content of the conventional fields. AMPA and glyphosate contributed among other the most to the total residue content of P-G, N-P and S-O (in S-O only AMPA contributed to the total residue content). In organically managed fields, AMPA and DDT metabolites had the highest contributions, and specially for S-O; in the other organic farming systems, the total content was low.

For glyphosate, the maximum amounts detected in conventional fields were 7.843, 1.3 and 0.18 mg/kg in P-G-C, N-P-C and S-O-C, respectively. For AMPA, the maximum amounts detected in conventional fields were 4.294, 0.528 and 1.626 mg/kg in P-G-C, N-P-C and S-O-C, respectively. In organic fields, glyphosate was detected only at S-O-O with a maximum amount of 0.105 mg/kg while AMPA was detected with maximum 0.014 and 0.593 mg/kg in N-P-O and S-O-O, respectively.

Conclusion

Mixtures of pesticide residues were present in all case study sites under conventional farming, both in samples taken at the start of the crops season, and samples taken post-harvest.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study several compounds, including glyphosate and AMPA, were analysed in 340 agricultural topsoil samples from 4 representative EU study sites in Spain, Portugal and the Netherlands. Soil samples were collected between 2015 and 2018 after harvest or before the start of the growing season.

No information on the sampling procedure and storage time of the topsoil samples is provided. This does not allow to assess the representativeness of the soil samples. Furthermore, only maximum and median values are reported and the results cannot be assigned to a respective sampling period. Therefore, the article is considered reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.5/
Report author	Piel S. *et al.*
Report year	2021
Report title	Understanding the origins of herbicides metabolites in an agricultural watershed through their spatial and seasonal variations
Document No	Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes, Part B (2021), Vol. 56(4), pp. 313-332
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Relevant (Category A acc. to EFSA GD, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of this study is to understand the spatial and seasonal variations of persistent herbicides metabolites and to determine their origins in the Vilaine River watershed, Britany-France. Data were collected at 13 sampling stations during five sampling campaigns in 2016 and 2017. The influence of land use and wastewater treatment plants (WWTP) on streams water quality has been identified. Aminomethylphosphonic acid (AMPA) is associated to anthropogenic urban contamination and nutrient loads. AMPA has its major sources in both glyphosate and phosphonate detergents issued from WWTP.

Among the studied compounds, glyphosate was observed at a detection frequency of 8 to 23% with concentration levels between 0.09 and 0.56 µg/L. AMPA was found with a detection frequency of 100% and reached its maximum concentration (2.48 µg/L) during summer downstream of the WWTP of the major urban area.

Materials and methods

River water sampling locations

The main River Vilaine crosses one major urban area (Rennes, 300,000 inhabitants) on the upstream part of the watershed. Redon city (10,000 inhabitants) in the south of the watershed is the only other major city crossed by the Vilaine River. At the mouth of this river is located a major drinking water treatment plant (DWTP), producing about 100k m^3/day of drinking water for approximately 1,000,000 inhabitants. The water treatment process is composed of a first step of sedimentation with ferric salts followed by sand filtration, a second step of interozonation followed by granular activated carbon filtration and a last step of disinfection with chlorine.

Vilaine River has four main tributaries: Oust, Meu, Seiche, and Isac (Figure 1). Meu and Seiche Rivers are located downstream of Rennes urban area, in the upper part of the watershed. Oust River joins the Vilaine River at Redon, south of the watershed. Oust river is known to be agriculturally influenced. Finally, Isac River is the last tributary to join the main river 25 km before the DWTP.

Thirteen sampling stations were chosen to describe influences based on previous work (mostly agricultural), land use and tributaries (Figure 1). Sampling stations were located on each major tributary before their confluence with the main river Vilaine (Meu (NW5), Seiche (NW7), Oust (NW10) and Isac (NW12)). Then, on the main river, stations upstream and downstream of river confluences were sampled (NW4, NW6, NW8, NW9, NW11 and NW13). NW0 is the “reference

station" corresponding to the source of Vilaine River. NW1 and NW4 are respectively located upstream and downstream of Rennes in order to measure urban impact on water quality.

NW4, corresponding to Rennes, shows 116.7 km^2 of urban land cover and 577.2 km^2 of dedicated agricultural land. NW10 corresponds to a bigger agricultural sub-watershed than NW4, with 185.6 km^2 of urban facilities and 3867.7 km^2 of dedicated agricultural land. NW1 station located upstream of Rennes shows a similar agricultural land use to NW4. The two main tributaries of the upper watershed, the Meu (NW5) and the Seiche (NW7) Rivers, show similar land use with small urban areas and agricultural area similar to NW4. NW6 and NW8 are the stations measuring the influence of Meu and Seiche tributaries on the River Vilaine water quality. NW9 station follows a long river stretch without major tributaries. Inhabitant-equivalent (IE) density is smaller than in the upper part of the watershed (Table 1). Consequently, this station shows a mix of agricultural (NW9) and urban (waters from upstream, NW4 to NW8) influences, depending on seasons and rainfalls.

DWTPi is located at the extreme downstream of the watershed; close to the inlet of the DWTP (Figure 1). An additional sampling point, the treated water of the DWTP, has been chosen to assess the treatment process efficiency for the removal of herbicides and metabolites of interest. Two major WWTP are located in this watershed. The largest - Beaurade (360,000 IE) is located just downstream of Rennes and upstream of sampling station NW 4. Beaurade WWTP is composed of low charge activated sludge and clarification processes. The second main WWTP is in Loudeac (175,000 IE) – in the upstream part of the Oust watershed and upstream of sampling station NW10. This WWTP receives only industrial effluents from slaughterhouses and laundry (effluents are more concentrated, for instance, in organic matter, grease, phosphorus) and consequently its influence on the Vilaine water quality is different from Beaurade WWTP which receives urban wastewater only. Loudeac WWTP is composed of low charge activated sludge, clarification and physicochemical dephosphatation processes.

Railways are concentrated in the upstream of Vilaine watershed and especially in NW4 drainage area (440.4 km). Moreover, railways are closely following Vilaine River course on the major part of the watershed. Railway lines in urban areas can be one of the major sources of glyphosate, used as weed controller.

Figure 1: Simplified Vilaine watershed map, with the main rivers, sampling stations, weather stations, and flow measurement stations.

Table 1: Land use characteristics of the sampling points sub-watersheds (*Inhabitant-Equivalent)

		Facilities			Urban	Agricultural					
Sampling station	Total area (km^2)	Total IE* (thousands of IE)	IE* density (IE/km^2)	Railways (km)	Area (km^2)	Cereals (km^2)	Including maize (km^2)	Oleaginous (km^2)	Proteaginous (km^2)	Pasture/ Meadow (km^2)	Other vegetables (km^2)
NW0 - Vilaine source	**93**	1	11	0,0	2,0	39,7	29,9	0,8	–	40,2	–
NW1 - Vilaine upstream Rennes	**698**	109	156	234	73,8	287,3	154,1	11,0	1,1	239,2	0,2
NW4 – Vilaine downstream Rennes	**784**	540	689	440	185,6	236,2	120,2	12,3	1,2	170,9	1,5
NW5 - Meu	**744**	122	164	123	41,2	311,2	149,1	14,3	1,7	163,4	1,3
NW6 - Vilaine downstream confluence Meu	**22**	11	500	0	2,3	5,4	2,0	0,6	–	3,9	–
NW7 - Seiche	**801**	110	137	217	53,9	356,1	156,4	28,8	2,7	215,2	0,8
NW8 - Vilaine donwstream confluence Seiche	**168**	16	95	23	12,4	70,1	26,1	6,8	0,4	51,3	0,3
NW9 - Vilaine upstream confluence Oust	**2173**	97	45	260	88,4	767,9	297,4	63,7	11,1	677,7	4,4
NW10 - Oust	**3554**	473	183	196	116,7	1309,8	453,2	75,4	10,3	785,8	43,2
NW11 - Vilaine downstream confluence Oust	**81**	1	12	27	26,2	19,9	8,7	1,4	0,3	31,4	0,0
NW12 - Isac	**632**	61	96	17	38,2	158,7	73,9	10,4	2,9	282,9	0,3
NW13 - Vilaine downstream Isac confluence	-	–	–	–	–	–	–	–	–	–	–
DWTPi - Férel	**388**	57	147	17	26,4	105,4	55,8	2,8	0,4	118,1	1,9

Hydro climatic conditions of sampling campaigns

In order to characterise hydroclimatic conditions of each sampling campaign, flows and meteorological data have been collected. River daily average flow measures at a limnimetric station are provided by the database Hydro (French Ministry for Ecology, Sustainable Development and Energy). The height flow measurement stations location is provided in Figure 1. Where limnimetric station does not exist, river flows have been calculated by the difference of measured flows at the upstream station and downstream station. The sampling stations with a calculated flow are NW4 (difference between NW1 and NW5 flows), NW6 (difference between NW4 and NW5 flows) and NW9 (difference between NW11 and NW10 flows).

The four weather stations are Ernee, close to the Vilaine source (NW0); Rennes-Saint Jacques, located in Rennes agglomeration (NW4); Saint Jacut-les-pins, located at the downstream of the Oust sub-watershed (NW10); and finally, Arzal, which is located few kilometres downstream of the DWTP (DWTPi).

Data for each sampling station are taken from the closest weather station (air temperature, rainfall, insolation duration and solar radiation). Those data are imported from the database “Publitèque”, powered by the French national service of meteorology (Meteo France). Acquisition frequency is one measure per hour.

Five sampling campaigns were conducted between 2016 and 2017 on the Vilaine river and its tributaries at 13 sampling stations (Figure 1), three campaigns during rain periods (C3 January 17, C4 May 17, C5 November 17) and two during warm and dry periods (C1 July 16 and C2 September 16). A sampling campaign is considered as rainy when conducted for a minimum rainfall of 10 mm/24h before sampling (according to the weather forecast).

Sampling campaigns C1 and C2 were characterised by low flow ($Q < 5$ m^3/s) and by low rainfalls (respectively < 1.8 and < 6.2 mm the week before sampling). Sampling campaigns C3 and C5 were characterised by high flow (respectively 16 and 11 m^3/s) and high rainfalls (respectively between 8.9 and 30.7 and between 12.9 and 32.9 mm the week before sampling). C3 and C5 corresponded to minimal period of pesticides application, and minimal degradation of pesticides in soils. Sampling campaign C4 was characterised by high flow (13 m^3/s) and significant rainfall (between 16.5 and 43.8 mm the week before sampling), C4 was conducted following a significant rainfall (22.8 mm 24 h before sampling) during the principal period of application of glyphosate. Details of the hydroclimatic parameters and the physiochemical characteristics are shown in Table 2 and Table 3.

Table 2: **Hydroclimatic parameters (minimum, maximum, median) for the 5 sampling campaigns (C1 to C5).**

Sampling campaigns	C1 (n = 13) July 16			C2 (n = 13) September 16			C3 (n = 13) January 17			C4 (n = 12) May 17			C5 (n = 13) November 17		
Parameters	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median
Rainfall (mm)															
Maximum daily, the week before the sampling day - RR (Vmax-1w)	0.2	1.6	0.4	0.4	2.2	1.3	7.1	12.3	10.7	7.9	22.8	16.7	5.7	13.9	7.9
Cumulative 1 week before sampling day - ∑RR (-1w)	0.0	1.8	0.3	1.0	6.2	2.9	8.9	30.7	25.7	16.5	43.8	32.8	12.9	32.9	20.2
Cumulative 2 week before sampling day - ∑RR (-2w)	0.3	7.9	4.2	15.5	44.0	27.8	12.7	37.2	29.5	17.9	45.0	34.2	14.1	34.3	21.2
Cumulative 4 week before sampling day - ∑RR (-4w)	6.9	14.2	9.6	28.1	47.4	35.9	36.2	53.7	45.9	18.7	51.2	39.8	34.1	82.5	49.1
Air temperature (°C)															
Average of the sampling day - TM(d)	16.0	18.0	16.9	15.6	17.7	17.5	0.0	1.3	1.1	11.4	12.4	12.0	3.1	6.4	4.9
Insolation duration															
The day of sampling - INST(d) (hour)	1.7	3.9	3.8	*NA*	*NA*	*NA*	7.8	8.3	8.3	4.9	9.1	5.2	4.3	5.0	4.6
Cumulative 1 week before sampling day - ∑INST(-1w) (days)	2.5	2.7	2.6	1.6	1.8	1.7	0.8	0.8	0.8	1.3	1.3	1.3	0.8	1.0	0.9
Cumulative 2 weeks before sampling day - ∑INST(-2w) (days)	5.7	5.8	5.8	2.3	2.4	2.4	1.0	1.4	1.1	4.3	4.8	4.8	1.6	1.6	1.6
Solar radiation (kilo joules/cm^2)															
Cumulative 1 week before sampling day - ∑GLOT(-1w)	17.2	17.3	17.2	9.2	9.9	9.8	2.5	2.7	2.5	9.8	10.1	9.8	3.1	3.3	3.2
Cumulative 2 weeks before sampling day - ∑GLOT(-2w)	34.5	34.7	34.7	15.7	16.9	16.8	4.3	4.8	4.4	26.1	26.1	26.1	6.4	6.4	6.4

Table 3: **Physiochemical characteristics of the Vilaine River for 5 strategic sampling stations.**

Sampling stations	NW1				NW4			NW9			NW10			DWTPi		Quality limits*
Parameters	Min.	Median	Max	Min.	Median	Max	Min.	Median	Max	Min.	Median	Max	Min.	Median	Max	
pH	7.6	**7.9**	8.3	7.6	**7.8**	8.4	7.5	**8.2**	8.8	7.3	**8.2**	8.6	7.8	**7.8**	8.4	**5.5 – 9**
Temperature of water - Tw (°C)	5.7	**11.2**	21.0	6.2	**11.8**	22.4	4.7	**13.6**	24.5	5.0	**13.3**	23.6	6.1	**15.1**	24.7	**25**
Dissolved oxygen - O_2 (%)	78	**90**	100	83	**89**	95	77	**86**	104	74	**90**	93	69	**90**	101	**> 30**
Conductivity - Cond (µS/cm) (25 °C)	361	**411**	448	322	**382**	517	394	**404**	478	250	**328**	393	298	**333**	498	**1 100**
Turbidity - Turb (NFU)	4.4	**5.8**	21.2	6.1	**7.8**	45.6	3.0	**6.2**	10.0	2.6	**3.1**	3.7	0.2	**2.8**	9.4	-
Fluorescence of dissolved organic matter - fDOM (RFU)	28.1	**29.3**	31.6	27.3	**41.0**	57.3	29.3	**34.0**	44.1	15.8	**24.9**	26.8	0.0	**27.2**	32.0	-
Dissolved organic carbon - DOC (C mg/L)	6.0	**6.5**	7.2	5.2	**6.2**	6.6	5.3	**5.9**	6.3	3.3	**5.0**	5.3	4.3	**5.7**	7.1	-
Total organic carbon - TOC (C mg/L)	6.4	**7.3**	8.7	6.2	**7.0**	7.2	5.8	**6.1**	6.5	3.6	**5.2**	5.6	4.4	**5.8**	7.6	**10**
Suspended matter - SM (mg/L)	8	**12**	34	8	**9**	36	6	**9**	12	5	**8**	16	2	**4**	9	-
Nitrate - NO_3 (NO_3 mg/L)	3.4	**10.0**	15.1	6.3	**8.1**	12.3	4.2	**9.3**	13.6	6.7	**16.8**	27.7	3.9	**12.0**	21.8	**50**
Ammonium - NH_4 (NH_4 mg/L)	0.02	**0.05**	0.32	0.15	**0.18**	0.37	0.03	**0.06**	0.09	0.01	**0.05**	0.10	0.04	**0.06**	0.08	**4.0**
Kjeldahl nitrogen KN (N mg/L)	0.8	**1.0**	1.6	0.9	**1.1**	1.3	0.7	**0.8**	1.1	0.5	**0.7**	1.4	0.5	**0.6**	1.0	-
Orthohosphate - PO_4 (PO_4 mg/L)	0.0	**0.1**	0.3	0.1	**0.3**	0.5	0.1	**0.3**	0.6	0.0	**0.1**	0.1	0.0	**0.1**	0.2	-
Total phosphorus - TP (P_2O_5 mg/L)	0.3	**0.3**	0.4	0.3	**0.4**	0.5	0.2	**0.3**	0.5	0.1	**0.2**	0.5	0.1	**0.1**	0.2	**0.7**

Sample collection

In each sampling station (NW), the samples were collected from a bridge using a stainless-steel bucket, according to the standardised method AFNOR FD T90-523-1. Before sampling, the bucket was rinsed 3 times with sampling water. The samples were carried out at a depth between 0.5 and 1 m in a homogeneous zone in the middle and upstream of the bridge. At the station DWTPi, raw water was sampled at the inlet of the drinking water treatment plant (DWTP). Temperature, pH, turbidity, conductivity (at 25°C), dissolved oxygen (% O_2), fluorescence of dissolved organic matter (fDOM) (excitation 365 nm – emission 480 nm) were analysed in situ using an EXO2 multi-parameters probe. Specific flasks were used according to laboratory analysis procedures. Samples were maintained at 5°C ± 3°C during transports, in coolers filled with ice blocks.

Analysis of herbicide compounds

Polypropylene flasks with sodium thiosulfate were used to store samples for glyphosate and AMPA analyses. For glyphosate and AMPA quantification, analyses were performed by Liquid Chromatography (LC)-Fluorescence after derivatisation with 9-fluorenylmethoxycarbonyl-chloride (FMOC-Cl) according to an internal method. This method has been developed in accordance with the

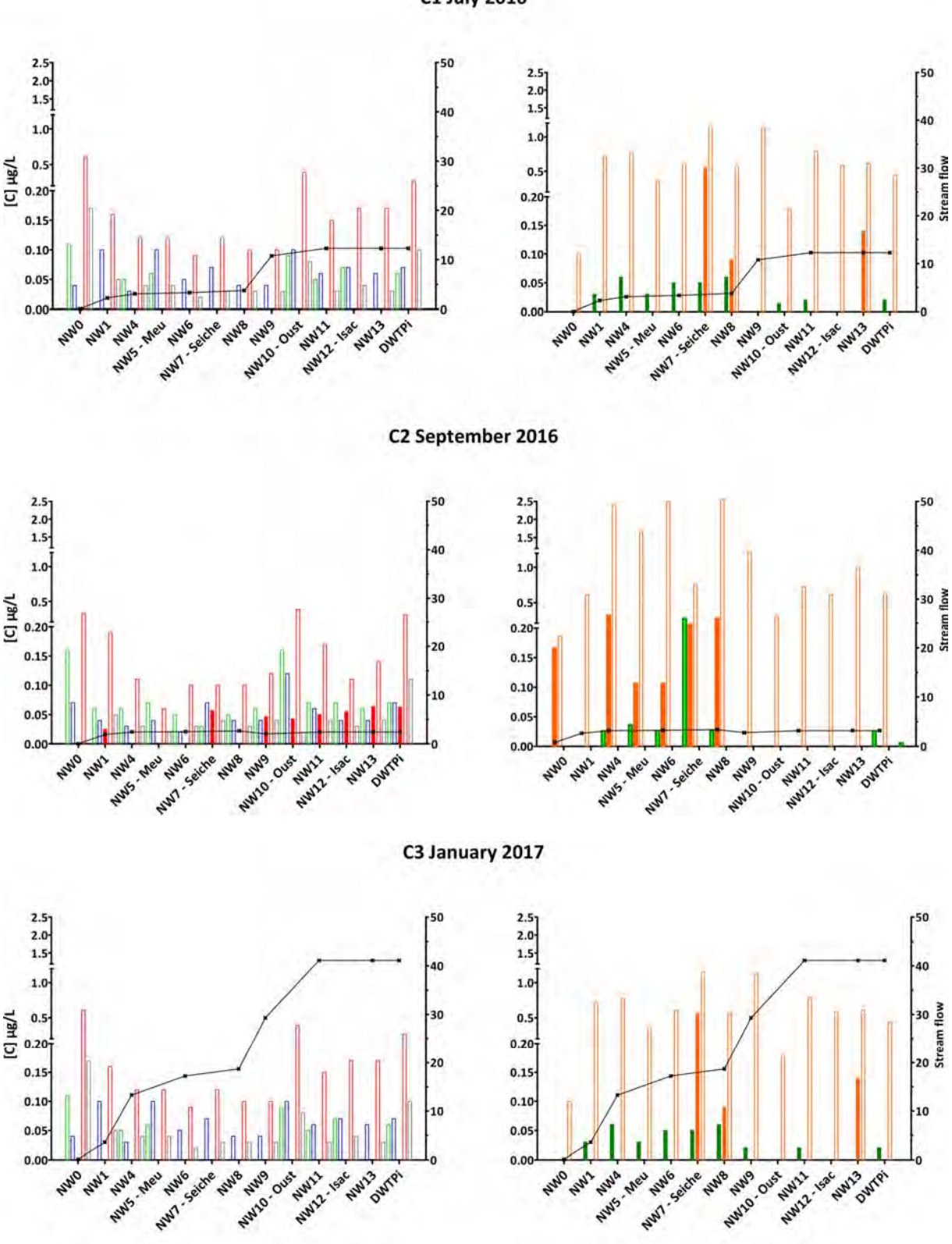
international standard ISO/IEC 17025:2005 and has been accredited by the French national accreditation committee (Cofrac). Analyses were performed using a LC system (Agilent 1200 Infinity) coupled to fluorescence detector (1200 G1321A). The limit of quantification (noted LoQ) is 0.05 µg/L for glyphosate and AMPA.
Chromatographic separation was performed at a flow rate of 0.4 mL/min on a X Bridge TM BEH C18 column (100 mm length × 2.1 mm I.D., 2.5 µm particle size) maintained at a constant temperature of 35°C. The binary mobile phase comprised ultra-pure water with 0.01% formic acid (solvent A) and CH_3CN with 0.01% formic acid (solvent B). The sample injection volume was 5 µL. Samples were dissolved in mobile phase at initial conditions (60% A) prior to injection. The gradient elution program was as follows: 0-2 min, 60% A; 2-13 min, 60-30% A; 13-15 min, 30-0% A; 15-27 min, 0-60% A (return to initial conditions); 27-28 min, 60% A (equilibration).

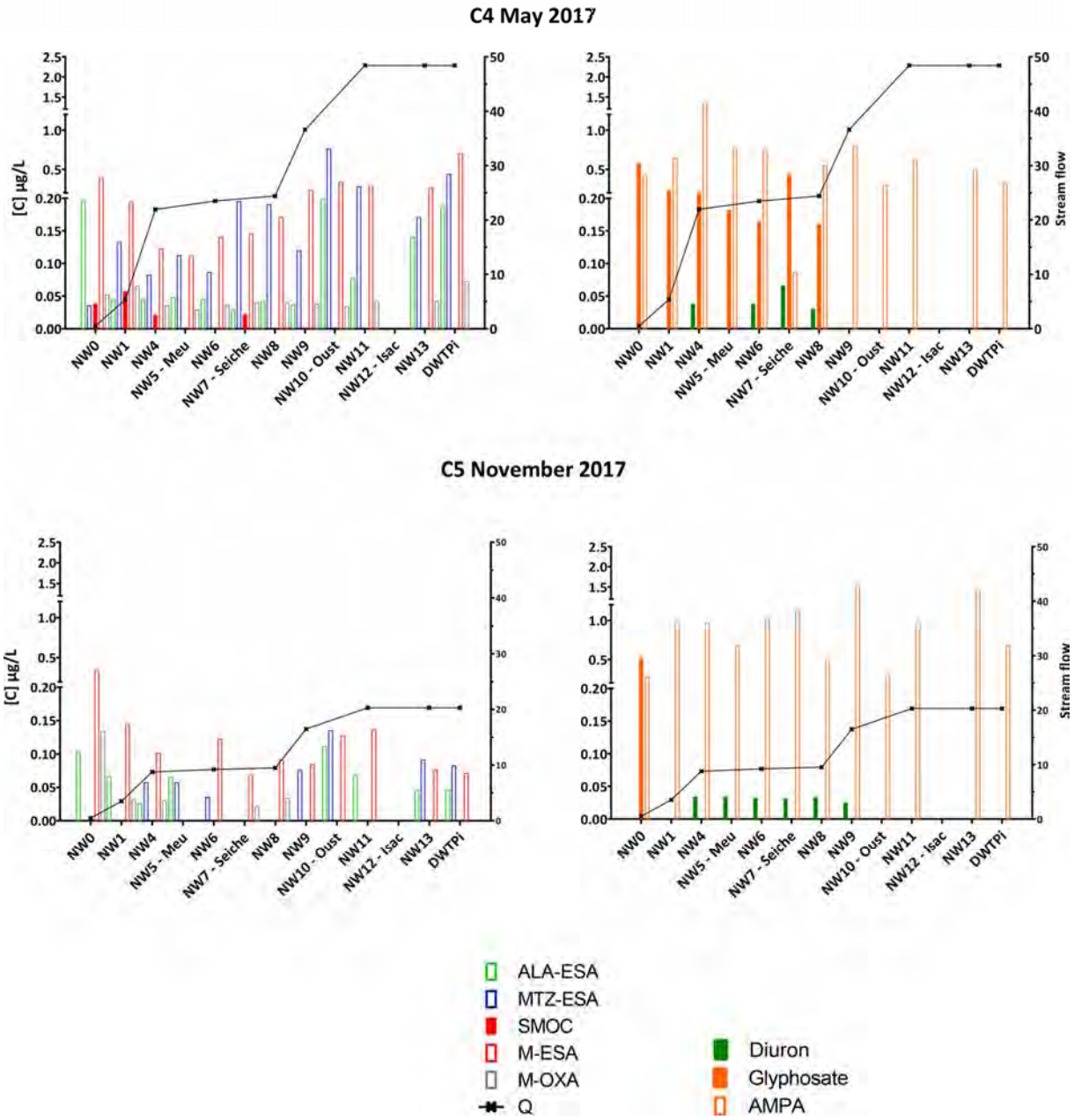
Risk assessment
A risk assessment was performed by first comparing the concentration levels with the environmental quality standards (EQS) in surface water for priority substances in the Directive 2013/39/EU. In France, AA-EQS and MAC-EQS have been established for AMPA (45 and 45,000 µg/L) and glyphosate (28 and 70 µg/L).
A second comparison could be performed with parametric values on the quality of water intended for human consumption. In Europe, the 98/83/EC European Council directive monitored the anthropogenic compounds and potential other pollutants in water. The directive fixed concentration limits in raw waters used to produce drinking water: 2 µg/L for any individual pesticide (including metabolites) and 5 µg/L for total pesticides concentration (including metabolites). In accordance with this directive, pesticides concentration in drinking water is limited to 0.1 µg/L for individual pesticides (included relevant metabolites) and to 0.5 µg/L for the sum of all detected and quantified individual pesticides and metabolites.

Results
Occurrence of herbicide compounds
Among the 15 compounds selected, glyphosate and AMPA have been frequently quantified (Frequency of Quantification (FoQ) > 30% of the samples). Figure 2 presents herbicides and their metabolites (glyphosate and AMPA) repartition in the watershed during the 5 campaigns.
Glyphosate was rarely observed in waters during cold months with 23% and 8% of FoQs during C3 and C5 campaigns, respectively. Concentration levels ranged from 0.09 to 0.56 µg/L.

Glyphosate was mainly found during spring and summer (C1, C2 and C4). During those seasons, it frequently occurred the same way that its metabolite AMPA in the upstream watershed (NW0 to NW8). Conversely, in the downstream watershed (NW9 to DWTPi), AMPA was found alone, indicating either that glyphosate had been already degraded in water (7 weeks < half-life < 10 weeks) or stored in the sediments. AMPA may also have a different source than glyphosate in this part of the watershed. AMPA can be derived from treated water of WWTPs representing 689,000 IE situated between NW9 and DWTPi.
AMPA was the compound with the highest concentrations (mean concentration 0.77 µg/L) and FoQ (100%). During C2 campaign (late summer - low flow), AMPA reached its maximum concentration of 2.48 µg/L (NW4). During this period WWTPs were the main contributors to the river flow, explaining the high AMPA concentrations in rivers.

Figure 2: **Herbicides and metabolites concentrations for each sampling campaign.**





Environmental and sanitary quality standards compliance in the Vilaine River watershed
Glyphosate and AMPA concentrations, up to 0.58 and 2.48 respectively, were systematically lower than their EQS (AA-EQS 28 and 45 µg/ L; MAC-EQS 70 and 45,000 µg/L).

The second comparison was performed with parametric values for raw waters intended for human consumption (2 µg/L quality limit for individual substance). Among the 15 studied herbicides and metabolites, only AMPA exceeded 2 µg/L during C2 campaign at NW4, NW6 and NW8 sampling stations (respectively 2.30, 2.38 and 2.48 µg/L). C2 campaign carried out during warm and dry period were characterized by low flow (Q < 5 m3/s) and low rainfalls (< 6.2 mm the week before sampling). The exceedance of threshold values during this campaign could be explained by the impact of WWTP whose discharges mainly contribute to the river flow and quality.

Concerning the drinking water sampled at the DWTP, AMPA was quantified with a concentration of 0.16 µg/L. The literature suggests that AMPA can be removed by clarification and sand filtration. AMPA is adsorbed on the flocs created by iron or aluminum salts and thus will settle down and be filtered with suspended particles. Ozonation and chlorination can also participate to the removal of AMPA depending on operation conditions, including dose, contact time and pH.

Spearman correlation
Two correlation matrices were built with Spearman correlation rank method (alpha = 0.05) on 64 samples (surface waters) of the 5 described campaigns. The first one was applied to herbicides and metabolites concentrations dataset and the second, on herbicides and metabolites concentrations and physicochemical water quality parameters datasets.

Diuron and AMPA were positively correlated together. The herbicide glyphosate and its metabolite (AMPA) showed no significant relationship. Glyphosate, diuron and AMPA were correlated with phosphorus forms (PO4 and TP) and nitrogen forms (KN and NH4). AMPA was also positively correlated with conductivity and negatively correlated with NO3. These physicochemical parameters are related to urban land use. This first group is primarily of urban origin.

Principal component analysis
First dimension (PC1) accounted for 26.28% of total variance, showed high positive loadings of turbidity, KN, DOC and TOC, SM, TP and glyphosate. There was no high negative loading for this factor. PC1 can be related to anthropogenic (wastewater) pollution sources.

AMPA showed a different behaviour: it is opposed to the agricultural factor PC2 and is orthogonally projected to glyphosate. Consequently, other activities, like food processing, laundry and milk industries, may be considered as potential sources of AMPA in the watershed. Indeed, AMPA is known to be a by-product and a major impurity of phosphonate detergents used in these industries.

Conclusion
Among the studied compounds, glyphosate was rarely observed (detection frequency of 8 to 23%) with concentration levels between 0.09 and 0.56 µg/L. AMPA reached its maximum concentration (2.48 µg/L) during summer downstream of the WWTP of the major urban area.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The aim of this study was to understand the spatial and seasonal variations of glyphosate and AMPA and to determine their origins in the Vilaine River watershed, Britany-France.
The sample storage time prior to analysis is not reported. Individual concentrations for glyphosate and AMPA assigned to sampling stations and sampling campaigns is presented graphically. Thus, no exact but narrative concentrations can be given for respective sampling locations and periods.
Therefore, the study is considered reliable with restrictions.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2021 年 1 月 1 日～2021 年 5 月 14 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 8.2.6.1 and CA 8.2.7
Report author	Tajnaiová L. *et al.*
Report year	2020
Report title	Determination of the Ecotoxicity of Herbicides Roundup® Classic Pro and Garlon New in Aquatic and Terrestrial Environments
Document No	Plants, 2020, 9, 1203
Guidelines followed in study	No OECD guideline mentioned. Modified EN ISO 8692, EN ISO 20079 and EN ISO 23753-1
Deviations from current test guideline	• No OECD guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities (no indication)
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The authors compared the effects of the glyphosate-containing herbicide Roundup® Classic Pro (Monsanto Canada, 28.85% w/v of glyphosate + surfactant ether alkylamine ethoxylate) to that of a metabolite of its active substance, aminomethylphosphonic acid (AMPA). Changes in the growth rates of two tested aquatic organisms, duckweed *Lemna minor* and green algae *Desmodesmus subspicatus*, the total chlorophyll content of duckweed fronds, and the levels of dehydrogenase activity (DHA) in soil were all analysed. The results of an algal growth test showed that AMPA has a 1.5× weaker inhibitory effect on the growth of *D. subspicatus* than the Roundup formula (IC_{50}AMPA = 117.8 mg/L). The authors concluded that AMPA was ecotoxic to the tested organisms.

Applicant's comment: *Glyphosate-based formulations that contain polyethoxylated tallow amine (POEA) surfactants or surfactants of similar chemical structure (e.g. ethoxylated ether alkylamine), are generally more toxic than the active substance itself. In addition, the composition of formulations is an important consideration when comparing the endpoints achieved in public literature with those achieved in regulatory studies conducted with either the technical material or studies conducted with the representative formulation MON 52276. Co-formulants may ameliorate or enhance potential effects on test organisms. For example, POEA-based surfactants (not permitted for use in Europe) or surfactants with similar structures, may lead to enhanced sensitivity.*

For this reason and the fact that the formulation tested contains ethoxylated ether alkylamine (similar chemical structure to POEA), only the results for the metabolite AMPA should be considered as relevant for the AIR5 glyphosate renewal dossier.

Materials and methods

Chemicals
The chemical substance aminomethylphosphonic acid (AMPA) was tested. AMPA was supplied by Tokio Chemical Industry co. Ltd., and was used to prepare a stock solution.

For aquatic tests, Bold's basal medium (BBM), pH 6.5 and Steinberg growth medium, pH 5.5 were prepared. Methanol was used to determine chlorophyll content. For the determination of DHA in soils, analytical grade ethanol (96%), hydrochloric acid (HCl, 1 mol/L), tris buffer solution (100 nmol/L, pH 7.6), substrate solution (TTC, 300 nmol/L), and triphenylformazan solutions (TPF) were used. All solutions for the determination of DHA were prepared according to ISO 23753-1.

Organisms

Two aquatic organisms were chosen for this experiment. The first one was green alga *Desmodesmus subspicatus* (R. Chodat), E. Hegewald et A. Schmidt, Brinkmann 1953/SAG 8681 BU AV Czech Republic, Třeboň. The second one was duckweed *Lemna minor* L., sterile culture, Steinberg: Federal Environmental Agency (FDA), Berlin, Germany.

Experimental design

The experimental design was carried out according to the following standards: EN ISO 8692, EN ISO 20079, EN ISO 23753-1, with modifications.

Algal growth inhibition test

The algal growth inhibition test was performed in 25 mL Erlenmeyer flasks. Pre-cultivation of green algae *D. subspicatus* in a 500 mL flat bottom flask was started three days prior to the inhibition test. Throughout the pre-cultivation, the flask was aerated by a tube with a microbiological filter. The cells were inoculated into the growth medium and subsequently cultured at 23±2ºC under continuous illumination provided by daylight lamps with an intensity of 8000 lx under sterile conditions. Test substances in different concentrations, BBM, and inoculum of a total volume of 15 mL were pipetted into the Erlenmeyer flasks under sterile conditions in a laminar flow box. Subsequently, the flasks were stoppered with pulp plugs and cultured under the same heat and light conditions as during pre-cultivation. The initial algal density of *D. subspicatus* depended on the concentration of the algal inoculum. During the test, the flasks were continuously shaken at 160 rpm by an orbital shaker. After 72 h, the cell concentration of *D. subspicatus* was determined with a light microscope. Before the application of testing organisms and the sterilization, the pH was changed using NaOH (0.1 mol/L) to pH 6.6, which is the standardized pH of an algae cultivation medium. For AMPA, the concentrations corresponded to the molar equivalent of glyphosate present in the respective dilutions of Roundup® Classic Pro. The highest concentration used was 2851 mg/L and other values used were as follows: 2138.6; 1425.7; 712.8; 356.4; 178.2; 128.3; 85.5; 64.1; 42.7; 21.3; 10.6; 5.3; 2.6; 1.3 mg/L and a control. In the pH-adjusted assay, decimal dilution was used and five concentrations plus a control were tested 2851.5; 285.1; 28.5; 2.8; and 0.2 mg/L. To determine the acute toxicity of the tested substance after 72 h, its IC_{50} value was calculated using the endpoint of growth inhibition. The specific growth rate was calculated as the logarithmic increase. The inhibition of growth rate in percent was calculated using the specific growth rate.

Duckweed growth inhibition test

The duckweed growth inhibition test was initiated by pre-cultivating *L. minor* in the Steinberg medium, whose pH was adjusted to pH 5.5±0.2, for 7 days. After the adaptation period, several healthy colonies with 2-3 fronds (12 fronds per beaker total) were transferred from the inoculum culture into 150 mL glass beakers containing 100 mL of modified Steinberg medium used for dilution of the prepared ready-to-use herbicide solution. All beakers were covered and placed in a cultivator under continuous cool fluorescent light at 24±1ºC. All fronds of *L. minor* were equidistant from the light (7000 lux). The conditions throughout the entire test were sterile. The test with the AMPA solution was complemented with a parallel test using a pH-adjusted AMPA solution of pH 5.5, the same pH as the modified Steinberg medium. Duckweed growth was determined by tallying the frond numbers (FN) and the area of the fronds. The frond numbers were taken at the first, fourth, and seventh day of the experiment. All visible fronds were counted. A photo of the surface of the fronds was taken simultaneously for area evaluation using the NIS-Elements 4.2 software. Fronds of *L. minor* were observed at the first, fourth, and seventh day of the experiment for toxicity symptoms, such as chlorosis, necrosis, and frond disconnection. For AMPA, 10 concentrations plus a control were used, each of which corresponded to the molar equivalent of glyphosate present in its respective concentration of Roundup® Classic Pro. The AMPA concentrations tested were as follows: 2851.6; 2138.7; 1425.8; 712.9; 356.4; 178.2; 85.5; 42.7; 21.3 and 10.6 mg/L. In the pH-adjusted assay, decimal dilution was used and 5 concentrations plus a control were tested: 2851.6; 285.1; 28.5; 2.8; and 0.2 mg/L. The specific growth rate for was calculated as the logarithmic increase. The inhibition of growth rate in percent was calculated using the specific growth rate. To verify the validity of the test, the doubling time of the frond area was calculated. Chlorophyll *a* (chl *a*), *b* (chl *b*) concentrations were determined by extraction in methanol. After 7 days of incubation, duckweed fronds were gathered and placed in a plastic vial. The roots of the fronds were removed. Next,

the plastic vials were filled up with 8 mL of methanol. The extraction was carried out in a cold environment (4ºC) and darkness. After 24 h of extraction, the samples were centrifuged at 5000 rpm for 15 min and then a spectrophotometric analysis of the supernatant was performed. To measure the concentration of chlorophyll *a* and *b*, the absorbance of the samples was measured at different wave lengths.

The determination of dehydrogenase activity in soil

A sample of soil was taken according to ČSN ISO 10381-6 from a depth of 0-15 cm in Dejvice, Prague. It is a loess aluminosilicate soil with an admixture of silicates on slate subsoil. After sieving through a 2 mm sieve, the soil was placed in a resealable plastic bag and stored in a refrigerator in the dark at 4ºC for 7 days. Prior to the DHA test, the pH was determined according to ČSN ISO 10390. The moisture content of the soil was gravimetrically measured according to ČSN ISO 11465. After an initial oven drying at 105 ◦C for water loss, the organic matter content of the soil was determined by the loss on ignition (LOI) method. The content of elements C, H, N, and S in the soil was quantified by burning the sample at 1200ºC. The gaseous combustion products (N_2, CO_2, H_2O, and SO_2) were purified, separated into individual components and analyzed on a TCD detector. The results of the analysis include all of the combustible sulfur, both organic and inorganic, as well as all of the combustible carbon, both organically bound and inorganically bound. The content of the elements was determined in two replication measurements. In this study, the effect of AMPA on DHA was compared to the effect of the herbicide Roundup on DHA. The highest AMPA concentration corresponded to the molar equivalent of glyphosate salt present in the Roundup formula, which is 2852 mg/L. The dehydrogenase activity, when AMPA was applied, was higher at all dilutions than when Roundup was applied. The soil was homogenized in a beaker and put in U-bottom tubes with 5 different concentrations of the herbicide, three per each concentration. The method used for measuring DHA in soil required that a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) as an artificial electron acceptor be added to the soil samples. Tubes with stoppers filled with 2 g of soil were covered with aluminum foil and placed in an incubator to be incubated in the dark at 25ºC for 24 h. Subsequently, methanol was added, and the tubes were vortexed to better homogenize the sample. The samples were then centrifuged for 10 min and the supernatant was pipetted off. Finally, the absorbance was measured by a UV-VIS spectrophotometer at 485 nm and the concentration of triphenylformazane was established.

Statistical analysis

All samples and controls were prepared in triplicate. The results are represented as the mean values ± their respective standard deviations. All statistical analyses were performed with the Data Analysis Tools in Microsoft Excel 2016. The standard deviations of the experimental data were calculated by means of the Microsoft Excel software. The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 8.4.3.

Results

After adding the test substances into the BBM medium, the pH of the medium decreased. AMPA caused the most significant change in the pH (see table below), so the authors adjusted the pH of a set of AMPA solutions.

Table 1. pH values

AMPA – pH value										
Concentration of AMPA(mg/L)	2852	2139	1426	713	356	178	86	43	21	11
pH of AMPA solution	3.67	3.75	3.80	3.90	4.08	4.16	4.30	4.43	4.64	4.81
Algae inhibition test, pH after 72 h	4.37	4.56	4.63	5.40	5.67	6.50	6.93	7.39	7.63	7.71
Duckweed inhibition test, pH after 7 d	4.49	4.56	4.75	4.82	5.07	5.62	5.75	5.85	5.91	5.85

Green algae acute toxicity test

In the case of AMPA, a direct metabolite of glyphosate, the half maximal inhibitory concentration (IC_{50}) was higher than that of the herbicide Roundup® Classic Pro, namely IC_{50} = 117.8 mg/L. In summary, AMPA alone is less toxic to green algae than Roundup® Classic Pro.

In the pH-adjusted solutions, the value of IC_{50} of AMPA was determined to be 192.1 mg/L, indicating a mild mitigation of its toxic effects. A pH-adjusted solution better represents in situ conditions where a pH buffering system, which would prevent the pH from decreasing in such an extreme way, is naturally present. The results of the toxicity tests are presented in the table below, showing the inhibition values corresponding to all of the tested concentrations:

Table 2. Algae toxicity test

Algae (*D. subspicatus*) toxicity test							
AMPA							
%	c (mg/L)	log c	I (%)	**%**	c (mg/L)	log c	I (%)
100.00	2,851.6	6.4	89.6	**0.78**	64.1	4.8	4.7
75.00	2,138.7	6.3	88.4	**0.39**	42.7	4.6	0.9
50.00	1,425.8	6.1	88.6	**0.19**	21.3	4.3	-5.7
25.00	712.9	5.8	87.0	**0.09**	10.6	4.0	-3.1
12.50	356.4	5.5	89.6	**0.04**	5.3	3.7	-3.7
6.25	178.2	5.2	81.7	**0.02**	2.6	3.4	-4.5
3.12	128.3	5.1	67.2	**0.01**	1.3	3.1	-5.6
1.56	85.5	4.9	6.2				

For *D. subspicatus*, the inhibition caused by AMPA in the pH-adjusted medium at a dose corresponding to the ready-to-use dose was higher than that caused by AMPA without pH adjustment. At the highest dose tested (2851.6 mg/L), the inhibition caused by AMPA with and without pH adjustment was 92.8% and 89.6%, respectively. Inhibition values corresponding to AMPA without pH adjustment were in the range of 87.0%-89.6% for the first five most concentrated doses tested. The first major difference in the inhibition effect was observed at the dose of 128.3 mg/L where inhibition dropped to 67.2%, and at the next dose of 85.5 mg/L, where inhibition was measured at 6.2%. Stimulation of growth occurred in AMPA without pH adjustments at a concentration of 21.3 mg/L. On the contrary, in AMPA with pH adjustment, inhibition effects persisted even at the concentration of 0.2 mg/L, which is over 10,000 times more diluted than the highest tested dose.

The criteria for the validity of the test according to the OECD guidelines for freshwater algae were met. The coefficient of variation of the daily growth rates in the control cultures during the test did not exceed 35% and the coefficient of variation of the average growth in the replicated control cultures did not exceed 15%. The pH of the control samples increased from the original 6.6 value in all three replicates, but it never exceeded the value of 8.0.

Duckweed acute toxicity test

The observed doubling time in the control was 2.4 days on average. The pH of the control samples increased from an initial value of 5.5 to an average value of 5.9. The table below shows the inhibition values corresponding to the AMPA concentrations used.

Table 3. Duckweed toxicity test

Duckweed (*L. minor*) toxicity test							
AMPA							
%	c (mg/L)	log c	I (%)	I (%)	c (mg/L)	log c	I (%)
100	2,851.6	6.4	100.0	6.2	178.2	5.2	86.1
75	2,138.7	6.3	100.0	3.1	85.5	4.9	85.2
50	1,425.8	6.1	100.0	1.5	42.7	4.6	84.7
25	712.9	5.8	100.0	0.7	21.3	4.3	70.7
12.5	356.4	5.5	99.2	0.3	10.6	4.0	36.8

At a concentration of 10.69 mg/L, duckweed growth inhibition for AMPA was 36.83%. When added to Steinberg's growth medium, the test substances lowered its pH, with the most significant decrease brought about by AMPA. For this reason, in addition to comparing pure AMPA, pH-adjusted AMPA was prepared as well. After adjusting the pH for the AMPA metabolite, a 10-fold dilution was used. The highest concentration corresponded with molar conversion to the glyphosate content in Roundup® Classic Pro and as such was determined to be 2851 mg/L. At this concentration, duckweed growth inhibition for AMPA with pH-adjustment was 95.3%. The measured data shows that after a pH adjustment in the medium with the tested AMPA, the inhibitory effect was lower than when using AMPA with no pH adjustment, but the differences were not significant ($I_{(c=285.1\ mg/L)}$ = 85.5%; $I_{(c=28.5\ mg/L)}$ = 80.4%). There was an increase in the total area of the duckweed fronds after four and seven days in the medium with the pH-adjusted AMPA. Furthermore, when the concentration of AMPA was lowered, no necrosis occurred; although partial chlorosis was present in all samples, even at the lowest concentration of 10.7 mg/L. Root growth, with the exception of controls, was not observed or only very stunted roots grew at the lowest concentrations. After seven days, all L. minor fronds turned white. The values of total chlorophyll in the fronds of the duckweed corresponded to the loss of biomass. None of the measured values exceeded the control samples. In contrast, at the four highest concentrations, no chlorophyll was detected after applying any of the three test substances. When diluted to 12.5%, a small amount of chlorophyll remained in the AMPA samples, and even at the lowest tested concentration, the decrease caused by AMPA was the least severe.

The determination of dehydrogenase activity in soil

A preliminary analysis of the soil sample showed that it was slightly acidic, with pH~6.34. The moisture of the soil was measured to be 72.27%. Ignition losses were determined at 15.84%. The total average sulfur content was set at 0.14% of the soil weight, hydrogen at 1.04%, nitrogen at 0.78%, and the total average carbon content was at 6.45%. The highest DHA was observed at the lowest dose tested, which corresponded to a dilution of 6.2%. The DHA value of the control was not exceeded at any tested dose of AMPA. At the highest tested AMPA concentration of 2852 mg/L, which corresponds to 100% of the measured concentration, the determined DHA was 1.6 higher than in the samples treated with Roundup® Classic Pro. This trend was maintained at all concentrations tested (in the range of 1.2-1.6). All values for both substances were lower than that of the control sample.

Conclusion

AMPA, being a metabolite of glyphosate, cause morphological changes in non-target organism *L. minor*. Furthermore, this substance exhibited toxicity to both duckweed *L. minor* and freshwater green algae *D. subspicatus* after short-term exposure, not only in application doses but also in much lower concentrations. When evaluating the effect of substances on the DHA of soil, the level of activity after the application of the test substances was shown as follows: AMPA > Roundup® Classic Pro.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study reports on endpoints with possible relevance to the EU-level risk assessment for aquatic organisms, specifically freshwater algae (*Desmodesmus subspicatus*) and aquatic plants (*Lemna minor*) for the glyphosate metabolite AMPA. For *D. subspicatus*, a regulatory relevant endpoint is reported (IC_{50} for inhibition of growth rate, usually described as ErC_{50}). For the *Lemna* test, no regulatory relevant endpoint was reported but the assessed parameters (growth rate, front area and chlorophyll content) could potentially serve as supporting information in a weight of evidence approach.
As the tests differed both in design as in the reported endpoints, assessments of reliability should be looked at separately.

Test with *D. subspicatus*: The report states that the test was conducted according to ISO 8692 with not further specified modifications. Furthermore, it is reported, that the test fulfilled the validity criteria laid down by OECD test guideline for freshwater algae. There are however important information missing from the report. Specifically, no data of the negative control are presented not even in the supplementary material. No raw data are provided so, the validity criteria could not be checked. Furthermore, the IC_{50} value was calculated without confidence intervals and no parameters to judge the fit of the curve used for calculating the IC_{50} are presented. The certainty of the level of protection can therefore not be fully assessed.

Test with *L. minor*: While relevant parameters were assessed (such as growth rate and front area), no regulatory relevant endpoints, such as ECx values, were calculated. No justifications are provided. Furthermore, control data are only presented for the parameters front area and chlorophyll content, and only in a graphical format. Due to the lack of raw data, no regulatory relevant endpoints can be calculated retroactively.

For both tests: The purity of the test material was not reported. Furthermore, exposure concentrations were not analytically verified. Especially for the *Lemna* test, which lasted 7 days without renewal of the test substance, actual exposure concentrations could vary significantly from the nominal concentrations.

Based on the above mentioned issues for both tests, the study can only be deemed reliable with restrictions.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		

1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	The study states that tests were conducted according to standardized test guidelines with modifications (not specified); EN ISO 8692 (growth inhibition of green algae) and EN ISO 20079 (duckweed growth inhibition test) with modifications. For algae: validity criteria according to OECD guidelines for freshwater algae were met For *Lemna*: no validity criteria mentioned
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Test cultures were obtained from research institutes in Germany and Czech Republic
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Uncertain	Not reported whether a solvent was used to prepare the test solutions
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Uncertain	Source of the test material was reported but no information on purity
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	Non-vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	Standardized test guidelines were inferred (see point 1.); validity criteria were met for the freshwater algae assay, for the *Lemna* assay validity was not discussed
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Uncertain	Glyphosate metabolite (AMPA) was tested but the preparation of test solutions was not described
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	No	Missing information on preparation of test solutions (no info on use of solvent)

11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes and No	Algae: Endpoints reported: IC_{50} *Lemna*: no regulatory relevant endpoint reported
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	*Lemna*: 10 levels and a control; 5 levels pH adjusted assay Algae: 15 levels and a control; 5 levels pH adjusted assay 3 replicates for all treatment and control levels
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Uncertain	Sufficient number of organisms and treatments was included but control results were not sufficiently reported for all endpoints (only for front area and chlorophyll content for the *Lemna* test)
17. Assessment of the statistical power of the assay is possible with reported data.	Uncertain	No raw data reported, however statistical test results are reported
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical methods were reported
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study

20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Uncertain	Medium type mentioned: Bolds Basel Medium (BBM) and Steinberg medium; composition not mentioned
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Algae: 23 ± 2 ◦C *Lemna*: 24 ± 1 ◦C.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	-
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	No residue measurements
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	IC_{50} value reported without C.I.

Overall assessment		
Reliable without restrictions	No	

Reliable with restrictions	Yes	The study reports on endpoints with possible relevance to the EU-level risk assessment for aquatic organisms, specifically freshwater algae (*Desmodesmus subspicatus*) and aquatic plants (*Lemna minor*) and the glyphosate metabolite AMPA. For *D. subspicatus,* a regulatory relevant endpoint is reported (IC_{50} for inhibition of growth rate, usually described as ErC_{50}). For the lemna test, no regulatory relevant endpoint was reported but the assessed parameters (growth rate, front area and chlorophyll content) could potentially serve as supporting information in a weight of evidence approach. As the tests differed both in design as in the reported endpoints, assessments of reliability should be looked at separately. Test with *D. subspicatus:* The report states that the test was conducted according to ISO 8692 with not further specified modifications. Furthermore, it is reported, that the test fulfilled the validity criteria laid down by OECD test guideline for freshwater algae. There are however important information missing from the report. Specifically, no data of the negative control are presented not even in the supplementary material. Furthermore, the IC_{50} value was calculated without confidence intervals and no parameters to judge the fit of the curve used for calculating the IC_{50} are presented. The certainty of the level of protection can therefore not be fully assessed. Test with *L. minor:* While relevant parameters were assessed (such as growth rate and front area), no regulatory relevant endpoints, such as ECx values, were calculated. No justifications is provided. Furthermore, control data are only presented for the parameters front area and chlorophyll content, and only in a graphical format. Due to the lack of raw data, no regulatory relevant endpoints can be calculated retroactively. For both tests: The purity of the test material was not reported. Furthermore, exposure concentrations were not analytically verified. Especially for the lemna test, which lasted 7 days without renewal of the test substance, actual exposure concentrations could vary significantly from the nominal concentrations. Based on the above listed limitations, the study can only be deemed reliable with restrictions.
Not reliable	No	

1. Information on the study

Data point:	CP 10.2.1
Report author	Gustinasari K. *et al.*
Report year	2021
Report title	Acute toxicity and morphology alterations of glyphosate-based herbicides to *Daphnia magna* and *Cyclops vicinus*
Document No	Toxicological Research, 2021, 37, 197-207
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities (no indication)
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, acute toxicity test of Glyphosate-Based Herbicides (GBHs) was conducted to *Daphnia magna* and *Cyclops vicinus*. Acute toxicity test was performed to both organisms at the Glyphosate concentrations of 20, 80, 160, 320, and 640 mg/L in exposure time of 12 h, 24 h, and 48 h, under static conditions. The mortality and morphology were observed to determine the LC_{50} and the effect of its morphology. The test showed that *D. magna* was more susceptible than *C. vicinus*. The LC_{50} of GBHs to *D. magna* and *C. vicinus* for its different exposure time were respectively show as follows: 76.67 mg/L and 207.89 mg/L (12 h); 36.2 mg/L and 159.8 mg/L (24 h); and 21.34 mg/L and 92.93 mg/L (48 h). There were no significant differences of the alteration of spin length, body length, and head length of *D. magna* to exposure of GBHs, except the head width. While body length alteration of *C. vicinus* was significantly different towards the increase in concentration.

Materials and methods

Test organism management

For this toxicity test, *D. magna* Straus, 1820 was obtained from cultures by the Institute of Biology, University of Szczecin, Poland. During the cultivation, *D. magna* was fed green algae, powder was weighed as much as 0.3 g, gently stir in order to dissolve with water in the container. Aeration was also supplied to provide oxygen requirements for *D. magna*. The culture was run for 5 days prior to the experiment. *C. vicinus* Uljanin, 1875 were obtained from Odra river, Poland and were sufficient in the amount and the size needed. A total of 180 specimens *D. magna* and *C. vicinus* were randomly assigned to give a loading of ten specimens per tank.

Experimental design:

The acute 12 h, 24 h, and 48 h static bioassay was performed in the Hydrobiology Laboratorium, Biology Institute of Szczecin University based on internal methods. The glyphosate-based herbicides used was from a brand named SUMIN ATUT 360 SL. It contained glyphosate in the form of isopropylamine salt at 360 g/L and 30.85% of detergent. Six different concentrations of glyphosate were made: 20 mg/L, 80 mg/L, 160 mg/L, 320 mg/L, 640 mg/L, and a control with no toxicant (0 mg/L). These concentrations were made by diluting the isopropylamine salt in distilled water. Each concentration was prepared in 100 mL volume and replicated three times on both *D. magna* and *C. vicinus*.

The ten individuals of *D. magna* and *C. vicinus* were introduced into each concentration in the container. There were three replicates for each group.

Observations:
The survival and mortality were counted for 12 h, 24 h, and 48 h. The experiments were kept at the same temperature without food. At the end of the test, the parameters of pH, temperature, EC (conductivity), TDS (Total Dissolved Solid), and DO (Dissolved Oxygen) were measured. Mortality was determined when the specimen did not respond to a repeated prodding.
Observations of the morphological response of *D. magna* and *C. vicinus* exposed to glyphosate were conducted after 48 h. The morphological indicators of *D. magna* were the spin length, body length, head width, and long head. While the morphological indicator of *C. vicinus* was body length. These indicators were observed using Zeiss Stereo microscope Discovery V12 (Germany). Each variable was measured from digital photographs, using the software Axio Vision. Responses were recorded if they differ from the controls.

Statistical analysis
As partial mortality in any replicate was observed, probit method was suitable to calculate the LC_{50}. The percentage of mortality was carried out for probit values against the logarithm of concentration using Microsoft excel. The LC_{50} was then calculated by substituting the probit value of 50% in the equation $y = b + ax$ in which variable x is known and a = unknown and b = intercept. The anti-logarithm value of "a" was taken as the LC_{50}. To determine the highest concentration value that does not cause an impact (NOEC), for this acute toxicity test the LC_{50} min/1000 was used.
The morphology indicators were analysed using one-way analysis of variance (ANOVA). The significant difference was level at $P = 0.05$. Further, the linear regression was also done. Regression correlation is divided into six level classifications, namely: $R^2 = 0$ (no correlation); R^2 >0-0.25 (very low correlation); R^2 >0.25-0.5 (moderate correlation); R^2 >0.5-0.75 (strong correlation); and R^2 >0.75-0.99 (very strong correlation); $R^2 = 1$ (perfect correlation).

Results

Acute toxicity
In the present work, acute toxicity test with GBHs (in the form of 360 g/L isopylamine salt) was performed using *D. magna* and *C. vicinus*. The table below shows the mortality percentage of *D. magna* and *C. vicinus* based on the influence of exposure time and concentration variation. All *D. magna* died at a concentration of 160 mg/L with an exposure time of 48 h. Exposure time had a significant effect on the death of *D. magna* at a concentration of 160 mg/L. All *D. magna* had died at 320 mg/L and 640 mg/L. Exposure time had a significant effect on death of *C. vicinus* at a concentration of 320 mg/L. At the highest concentration (640 mg/L), exposure time had no significant effect on death because almost all *C. vicinus* died. Concentration significantly affected the death of both *D. magna* and *C. vicinus*, at the time of exposure 12, 24, or 48 h.

Table 1. Percentage mortality of *Daphnia magna* and *Cyclops vicinus* exposed to glyphosate-based herbicides

Concentration (mg/L)	Species	Hours		
		12	24	48
0	*D. magna*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	C. vicinus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20	*D. magna*	10.00 ± 17.32	46.67 ± 25.17	53.33 ± 25.17
	C. vicinus	10.00 ± 17.32	10.00 ± 17.32	23.33 ± 15.28
80	*D. magna*	23.33 ± 40.41	53.33 ± 25.17	70.00 ± 26.46
	C. vicinus	16.67 ± 28.87	16.67 ± 28.87	26.67 ± 20.82
160	*D. magna*	63.33 ± 11.55	83.33 ± 15.28	100.00 ± 0.00
	C. vicinus	30.00 ± 0.00	36.67 ± 11.55	43.33 ± 11.55
320	*D. magna*	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	C. vicinus	46.67 ± 5.77	50.00 ± 10.00	73.33 ± 5.77
640	*D. magna*	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	C. vicinus	90.00 ± 10.00	93.33 ± 11.55	100.00 ± 0.00

The table below presents the value of LC_{50} for both *D. magna* and *C. vicinus* of the exposure of GBHs at the period bioassay of 12 h, 24 h, and 48 h. The LC_{50} of *D. magna* for each exposure time were 76.67 (12 h), 36.20 (24 h) and 21.34 (48 h), so the NOEC estimated 0.021 mg/L. While the LC_{50} of *C. vicinus* for each exposure time were 207.89 (12 h), 159.81 (24 h) and 92.93 (48 h), so the NOEC estimated 0.09 mg/L.

Table 2. The LC_{50} values of *Daphnia magna* and *Cyclops vicinus* exposed to glyphosate-based herbicides

Hours	Species	Equation for the regression analysis	LC_{50}
12	*D. magna*	y = 3.3246x − 1.2657	76.67
	C. vicinus	y = 1.564x + 1.3749	207.89
24	*D. magna*	y = 2.4367x + 1.202	36.20
	C. vicinus	y = 1.8428x + 0.9393	159.81
48	*D. magna*	y = 2.4035x + 1.8052	21.34
	C. vicinus	y = 2.276x + 0.5205	92.93

The table below showed the environmental parameters of water as media exposure after 48 h period bioassay. The pH and DO decrease with increasing glyphosate concentration, from 6.84 to 4.83 and from 3.95 to 2.49 mg/L, respectively. While the conductivity and TDS increase with increasing concentration, from 26.87 to 424.8 S/m and from 0.02 to 0.27 mg/L, respectively. This proved that concentration influences pH, conductivity, TDS, and DO. However, temperature is not affected by glyphosate concentration.

Morphology alterations

The boxplot charts of concentration correlation to changes in morphological indicators comprise spin length, body length, dead width, and head length for *D. magna* and body length for *C. vicinus.* There was no significant differences on concentration of glyphosate to the spin length of *D. magna*. At a concentration of 80 mg/L, it appeared that the spin length was longer than the concentration of 20 mg/L. However, at a concentration of 160 mg/L the effects of shortening of the spin length began to be seen and continued to decline to 640 mg/L.

The difference in body length of *D. magna* after being exposed to glyphosate was not significant difference. The concentration of 20 mg/L until 160 mg/L almost had the same body length. Decrease in body length was only seen at a concentration of 320 mg/L and 640 mg/L. The head width alteration of *D. magna* had a significant difference.

The concentration of glyphosate to head length had no significant difference. At the concentration of 80mg/L, the head was longer than 20 mg/L, although there was a slight decrease to a concentration of 320 mg/L. At a concentration of 640 mg/L the head length was much shorter than at other concentrations. The morphological indicators of *C. vicinus* was the body length of *C. vicinus.* There was the significant difference for the body length alteration. There was a decrease in body length with each concentration.

The alteration of morphological with the measuring of body length, head length, head width, and spin length of *D. magna* were quantitative data. Visually, there were different appearance of both *D. magna* and *C. vicinus* at each concentration. *D. magna* changed colour to slightly white with increasing concentration. In addition, the internal organs of *D. magna* with increasing concentration loose from the outer layer of the body. *C. vicinus* changed colour as well. However, the internal organs of the *C. vicinus* could not be separated from their outer layers.

Conclusion

The findings showed that the toxicant effects on the *D. magna* and *C. vicinus* increased as the GBHs concentration of exposure and the longer period of exposure. The NOEC of GBHs of *D. magna* and *C. vicinus* were 0.021 mg/L and 0.092 mg/L, respectively. The alteration morphology occurred in both *D. magna* and *C. vicinus* as the concentration of GBHs increases. The test showed that *D. magna* was more susceptible than *C. vicinus*.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study provides regulatory relevant endpoints, specifically the LC_{50} for *D. magna* and *C. vicinus* after 48-hours exposure. However, the study cannot be deemed fully reliable due to the following aspects: First, the exposure concentrations were not analytically verified. Furthermore, previous exposure of *C. vicinus* to contaminants cannot be excluded as they were collected from a river with unknown contamination history. In addition, the exposure medium was not specified (possibly distilled water, but the phrasing is ambiguous) and important info on the culturing conditions is missing (i.e. temperature, etc.). Life-stage and size of the organisms at test start were not documented. Finally, for the morphological alterations, the control values were not reported.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes (*D. magna*) and No (*C. vicinus*)	*D. magna* were obtained from a culture, *C. vicinus* were sampled from a river

3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in distilled water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source and content reported
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Invertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	No	Reported: Source, feeding, aeration Not reported: Culturing conditions (incl. type of medium, O_2-content, temperature, pH, conductivity), life-stage or size of test organisms
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	Test with glyphosate formulation SUMIN ATUT 360 SL
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Uncertain	Doses are reported but constitution of exposure medium not clear, no dilution scheme provided
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoints reported: LC_{50} (12, 24 and 48-h) and NOEC (but not correctly determined)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	5 treatment levels and a negative control
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	3 replicates of 10 organisms per treatment group and control; control data reported
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data

18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	No	No information on test medium
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature roughly between 21-22 °C at the end of the test, not reported for acclimation period
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	No residue measurements
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported

27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Uncertain	Underlying equation of the probit curve was reported but no confidence intervals for the LC_{50} or raw data to calculate confidence intervals retroactively
Overall assessment		
Reliable without restrictions	No	
Reliable with restrictions	Yes	The study provides regulatory relevant endpoints, specifically the LC_{50} for *D. magna* and *C. vicinus* after 48-hours exposure. However, the study cannot be deemed fully reliable due to the following aspects: First of, the exposure concentrations were not analytically verified. Furthermore, previous exposure of *C. vicinus* to contaminants cannot be excluded as they were collected from a river with unknown contamination history. In addition, the exposure medium was not specified (possibly distilled water, but the phrasing is ambiguous) and important info on the culturing conditions is missing. Life-stage and size of the organisms at test start were not documented. For the morphological alterations, the control values were not reported
Not reliable	No	

1. Information on the study

Data point:	CP 10.3.1
Report author	Luo Q.H. *et al.*
Report year	2021
Report title	Effects of a commercially formulated glyphosate solutions at recommended concentrations on honeybee (*Apis mellifera* L.) behaviours
Document No	Scientific Reports, 2021, 11, 2115
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities (no indication)
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, a series of behavioural experiments were conducted to investigate the effects of Commercially Formulated Glyphosate (CFG) on honeybees after a chronic exposure. The results showed that there was a significant decline in water responsiveness at 1/2×, 1× and 2× the Recommended Concentration (RC) after 3 h of exposure to CFG for 11 days. The CFG significantly reduced sucrose responsiveness at 1/2× and 1× the RC. In addition, CFG significantly affected olfactory learning ability at 1/2×, 1×, and 2× the RC and negatively affected memory ability at 1/2× and 1× the RC. The climbing ability of honeybees also significantly decreased at 1/2×, 1×, and 2× the RC. Our findings indicated that, after they were chronically exposed to CFG at the RC, honeybees exhibited behavioural changes. These results provide a theoretical basis for regulating field applications of CFG, which is necessary for establishing an early warning and notification system and for protecting honeybees.

Materials and methods

Test organisms

Sampling sites were located at the Zhuifengshan Forest Apiary, Dachengzi town, Miyun district, Beijing. In order to avoid taking too many emerging bees at one time and affecting bee colonies, each experiment randomly selected six capped combs in six colonies from forty colonies and maintained in an incubator to take the emerging honeybees the next day (these combs were put back into the original colonies after collection). Each cage (15×15×10 cm, with mesh on two sides) contained 70 honeybees that were captured within 24 h. A total of 4 cages were randomly selected for four treatments in the LD_{50} experiment. The entire experiment was repeated for 6 times in total and thirty-six colonies involved. A total of 16 cages (70 bees/cage), 4 of which were randomly selected for water responsiveness assay (four treatment: 0, 1/2, 1, and 2× RC), and the rest for sucrose responsiveness, learning and memory and climbing assay (4 cages for four treatments in each behavioural experiment) respectively. Six combs were randomly selected again after such an experiment completed, and the entire experiment was repeated for a total of 3 times and 18 colonies involved (5 times for climbing assay).

LD_{50} experiment

LD_{50} was tested to assess the dose level used in this study. The sucrose solution contained 41% glyphosate isopropylamine salt (Monsanto Roundup Original; 356 g of glyphosate acid equivalent per liter) at concentrations of 0, 0.72, 3.6, 7.2 g(s) of glyphosate acid equivalent per liter respectively (abbreviated as g/L). Plastic feeder were inserted vertically into the cages and changed daily. On the

basis of the number of honeybees that survived daily, each honeybee was fed with an average of 33 μL of sucrose solution and then fed with sucrose solution 50% (w/w) and water ad libitum. The honeybees were fed in an incubator whose temperature was 30±1°C and whose relative humidity was 65%~70%. The insects in the control groups were fed 50% (w/w) sucrose solution and water. The total mortality rate was calculated at 48 h after treatment.

Test organisms preparation and treatment for experiments

At the beginning of the experiment, to keep the honeybees at the same age and adapt to the laboratory feeding, the honeybees were fed 50% sucrose for one week (during which each caged honeybee was fed with sufficient amounts of pollen, which was replaced daily). The recommended concentration of Monsanto Roundup Original was 50 mL-500 mL formulation solution in 30-40 L water according to different application. The formulation in our study was applied at a rate equivalent to 50 mL formulation solution in 40 L water, which was the lowest recommended concentration and equal to 445 mg a.i./L (1× recommended concentration). Beginning when they were 8 days old, the honeybees were continuously fed three concentrations of glyphosate (the 1/2× , 1× and 2× RC used in this study), which were equal to doses of 7, 14 and 28 μg/bee/day in 33 μL of 50% sucrose solution/day, on average.Tested honeybee were 18 days old. The control groups were fed with a 50% sucrose solution. All tested honeybees were starved for 4 h before the test. The honeybees were fixed in a tube such that the antennae and head could move freely.

Water responsiveness experiment

Water was placed on the antennae of the honeybees to detect the proboscis extension response (PER). The honeybees were tested twice: at both 1 h and 3 h after treatment. Thirty honeybees were tested per replicate, and three replicates were used per treatment (0, 1/2×, 1× and 2× RC).

Sucrose responsiveness experiment

Water was first put on the antennae of the honeybees to determine whether the insects sucked water, eliminating the proboscis extension response phenomenon due to thirst. The proboscis extension response was then used to test honeybee sucrose responsiveness to increasing concentrations of sucrose solution (0.3%, 1%, 3%, 10%, 30%; w/v). The sucrose solution was absorbed and placed on the antennae of the honeybees to detect the proboscis extension response. For each concentration, the percentage of proboscis extension response released by the honeybees was recorded.

Olfactory learning and memory ability experiment

Water was first placed on the antennae of the honeybees as described above. A 30% sucrose solution was used for the proboscis extension response assay, and the percentage of proboscis extension response was recorded. Linalool (Sigma, 95% purity) was used as the conditioned stimulus and was presented for 6 s first. During odour presentation, the proboscis extension response was elicited after 3 s by contacting the antennae with the sucrose solution (30%), and the same solution was immediately given as a reward. Conditioning trials were conducted three times (C1, C2, C3) at 20-min intervals. Five test trials (T1, T2, T3, T4, T5) were then conducted with linalool stimulation for 6 s without sucrose feedback. Proboscis extension responses were also recorded at 20-min intervals.

Climbing ability experiment

After 11 days of continuous feeding, the honeybees were starved for 1 h before testing. A box (65 cm in length, 35 cm in width and 4 cm in height) was divided into 10 lanes (50 cm×3.5 cm×4 cm) with a fluorescent lamp on the top and covered with glass. The tests were conducted in the dark and with the box tilted at 45°. One honeybee per lane was placed in the box, and the lamp was on. The time it took for a honeybee to climb 50 cm was recorded. Ten honeybees were tested in each trial (which was performed in five replicates) in the control and treatment groups.

Statistical analysis

All the data were processed by SPSS 19 (IBM Corp., Armonk, NY, USA). The ingestion LD_{50} was determined on the basis of the mortality of honeybees for each dose via Probit analysis. The effects on water responsiveness, sucrose responsiveness, learning and memory and climbing were analysed by ANOVA for both the Roundup-treated and control groups. And LSD (homogeneity of variance) or Tamhane's T2 test (heterogeneity of variance) was performed. A difference was considered significant when the P-value was <0.05.

Results

LD50

The mortalities were respectively 3.67±1.13%, 7.77±3.04%, 35.25±7.94%, and 75.00±2.85% at the concentrations of 0 g a.i./L, 0.72 g a.i./L, 3.6 g a.i./L, and 7.2 g a.i./L. The ingestion LD_{50} was 309 μg/bee ($y = 5.454x - 1.684$).

Roundup decreased water responsiveness

No significant effect of Roundup on water responsiveness was detected at 1 h after treatment ($P = 0.49$). Compared with the control solution, Roundup significantly decreased the proboscis extension response to water at 3 h after treatment at all tested concentrations. The highest percentage of proboscis extension response was 28.33% in the control groups, and the lowest was 6.67% in the Roundup treatment of 2× the recommended concentration.

Roundup affected sucrose responsiveness

The proboscis extension response percentage increased with increasing sucrose concentration in the control and treatment groups. A significant decrease in the proboscis extension response percentage was detected in response to the 0.03% and 0.3% sucrose solutions after treatment with Roundup at 1/2× the RC for 11 days. In honeybees treated with Roundup at 1× the RC, the proboscis extension response percentage in response to the 0.03%, 0.1% and 0.3% sucrose solutions was significantly lower than that of the control groups. Moreover, there was no significant effect of the proboscis extension response percentage for the ≥1% sucrose solution between the control and treatment groups. The groups of honeybees treated with 2× the RC of Roundup did not exhibit any significant effects on the proboscis extension response percentage in any of the treatments.

Roundup affected olfactory learning and memory ability

The olfactory learning and memory ability of honeybees was expressed as the proboscis extension response percentage. Fixed honeybees were evaluated in the training phases C1, C2, and C3 and then in the test phases T1, T2, T3, T4 and T5. The proboscis extension response percentage of honeybees treated with Roundup at 1/2× the RC indicated that the learning ability (the relationship between odour and sucrose feedback) during the second and third presentation of odour (C2 and C3) was significantly lower than that in the control groups. And compared with that in the control groups, the proboscis extension response percentage in the treatment group significantly decreased during all test phases. Compared with those in the control groups, the honeybees treated with Roundup at 1× the RC presented a significantly increased proboscis extension response percentage at C1 but a significantly decreased proboscis extension response at T1, T4 and T5. A significant reduction in olfactory learning performance was also noted during C2 and C3 in honeybees treated with the highest dose of Roundup (2× the RC). The T1-T5 tests revealed no significant effect of the highest concentration of Roundup.

Roundup significantly reduced climbing ability

The climbing ability of the honeybees was significantly affected by Roundup. Honeybees in the control groups required the shortest time to climb 50 cm, whereas those in the Roundup treatment groups at 2× the RC required the longest time. During the climbing activity tests, honeybees treated with Roundup at all tested concentrations took longer to walk through the 50-cm track than did those in the control groups after 11 days of exposure.

Conclusion

In this study, the authors provided new information on the influence of commercially formulated glyphosate at the recommended concentration on the behaviours of honeybees. The authors concluded that their findings showed that the water responsiveness, sucrose responsiveness, learning and memory ability and climbing ability of honeybees were affected by commercially formulated glyphosate at or below the recommended concentration. Also, the authors stated that additional research needs to be conducted to determine the effects of actual applications of commercially formulated glyphosate at the recommended or lower concentration rather than the pure glyphosate on honeybees.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study reports on possibly relevant endpoints for the acute bee risk assessment, specifically a 48-hour oral LD_{50} value, as well as behavioural assessments on bees after glyphosate exposure. However, the study has to be deemed reliable with restrictions based on the following aspects: The colonies used for this study were obtained from an apiary about which no further information was provided. This pertains especially to possible pre-existent contaminant exposure of the colonies as well as health aspects regarding bee specific illnesses and treatments. As stated in OECD Guideline 213 (Honeybees, Acute Oral Toxicity Test), "… bees treated with chemical substances, such as antibiotics, anti-varroa, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment". This aspect cannot be adequately assessed as information on the bee colonies health or possible contaminant contact points is missing from the study report. The reported temperature during the test was 30 ± 1°C, which is outside the temperature range of 25 ± 2 °C recommended in OECD 213. The study report states an observation timeframe of 48 hours, however exact time-points of observation are not reported. Test concentrations were not analytically verified. Lastly, the LD_{50} value was reported without confidence intervals. The certainty on the level of protection offered by the median LD_{50} value can hence not be assessed. Based on the above mentioned aspects, the reliability of the study is restricted.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	No	Bees were acquired from a forest apiary, previous contact of the colony with contaminants is unknown
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	-	No carrier was used, test substance was directly dissolved in sucrose solution
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Uncertain	Content reported, Source and purity not reported

5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	Non-vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	Source, life-stage and haltering conditions reported
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	Roundup formulation; 356 g/L
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	An oral LD_{50} (48-hours) is reported
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	3 treatment levels and a negative control were tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	Increased mortality with increased test item concentration was observed
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	70 individuals per treatment group per experiment, experiment was repeated 6 times in total
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error as well as curve equation are reported but no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical methods were described incl. level of power
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Uncertain	Mortality was observed over 48-hours but exact time-points of observations not mentioned

20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, Bee study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, Bee study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, Bee study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, Bee study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, Bee study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, Bee study
20.6. Data on precipitation is recorded.	-	Not applicable, Bee study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	No	According to OECD 213 (Honeybees, Acute Oral Toxicity Test) the temperature should be 25 ± 2 °C; the reported temperature was 30 ± 1 °C
22. For bee studies, temperature of the study should be appropriate to species.	No	See above
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	-	Not applicable, Bee study
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	-	Not applicable, Bee study
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	No residue measurements
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical measurements conducted
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	LC_{50} reported but not the corresponding confidence interval; no raw data to re-calculate retroactively

Overall assessment		
Reliable without restrictions	No	

Reliable with restrictions	Yes	The study reports on possibly relevant endpoints for the acute bee risk assessment, specifically a 48-hour oral LD_{50} value, as well as behavioural assessments on bees after glyphosate exposure. However, the study has to be deemed reliable with restrictions based on the following aspects: The colonies used for this study were obtained from an apiary about which no further information was provided. This pertains especially to possible pre-existent contaminant exposure of the colonies as well as health aspects regarding bee specific illnesses and treatments. As stated in OECD Guideline 213 (Honeybees, Acute Oral Toxicity Test), "… bees treated with chemical substances, such as antibiotics, anti-varroa, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment". This aspect cannot be adequately assessed as information on the bee colonies health or possible contaminant contact points is missing from the study report. The reported temperature during the test was 30 ± 1 °C, which is outside the temperature range of 25 ± 2 °C recommended in OECD 213. The study reports states an observation timeframe of 48 hours, however exact time-points of observation are not reported. Test concentrations were not analytically verified. Lastly, the LD_{50} value was reported without confidence intervals. The certainty on the level of protection offered by the median LD_{50} value can hence not be assessed. Based on the above mentioned aspects, the reliability of the study is restricted.
Not reliable	No	

グリホサートカリウム塩

要旨及び評価結果

検索期間：2021 年 5 月 14 日～2021 年 8 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 8.1.5
Report author	Diaz-Martín R. D. *et al.*
Report year	2021
Report title	Short exposure to glyphosate induces locomotor, craniofacial, and bone disorders in zebrafish (*Danio rerio*) embryos
Document No	Environmental toxicology and pharmacology (2021), Vol. 87, Article No. 103700
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors used the zebrafish model to assess the effects of early life glyphosate exposure on the development of cartilage and bone tissues and organismal responses. The authors found functional alterations, including a reduction in the cardiac rate, significant changes in the spontaneous tail movement pattern, and defects in craniofacial development. These effects were concomitant with alterations in the level of the oestrogen receptor alpha osteopontin and bone sialoprotein. The authors also found that embryos exposed to glyphosate presented spine deformities as adults. These developmental alterations are likely induced by changes in protein levels related to bone and cartilage formation.

Materials and methods

Fish maintenance and embryo production

The authors followed all procedures and policies stated in the NOM-062-ZOO-1999 Mexican guideline (these guidelines apply mostly to mammalian species but the authors applied the same animal welfare principles to the fish in this study). The authors also followed the British guidelines for fish welfare. Zebrafish handling and maintenance complied with the guidelines of the Zebrafish Book. Wild-type adult zebrafish (*Danio rerio*) were obtained from commercial distributors and maintained in 6-L tanks (6 fish per tank) with dechlorinated water in a closed flow-through system at 27ºC, a 13:11 h light:dark cycle, and two feedings each day. Zebrafish embryos were generated by massive mating using 4 females and 2 males. Embryos were examined 2 h post-fertilization (hpf) under a Stemi 305 stereoscopic microscope to select normally developed embryos at the 16-cell stage. These embryos were then placed in Petri dishes containing EM buffer (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, and 0.7 mM $NaHCO_3$ at pH 7±0.2) for the exposure trials.

Glyphosate exposure

Fifty millilitres of stock solution (50 µg/mL) were prepared using 2.5 mg of glyphosate (99% purity, CAS Number 1071-83-6; Sigma-Aldrich, St. Louis, USA) dissolved in EM buffer. Glyphosate test solutions were then prepared at 0 (control), 1, 5, 10, and 50 µg/mL. The concentration of glyphosate in stock solution was determined by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS). The experiment was carried out with five completely independent replicates. Three replicates were used for craniofacial morphometrics. For each trial, 60 randomly distributed embryos were placed into 6-well plates (10 embryos per well) and exposed to glyphosate for up to 96 h. In all cases, after 48 h of exposure, 50% of the medium was replaced by a recently prepared solution. Plates

were incubated at 28.5°C, and the biological parameters of survival and hatching were evaluated every 24 h. No differences were observed in either survival or hatching rates among concentrations. After exposure, five embryos per group were selected and raised for 10 months until adulthood for skeletal assessment. Glyphosate exposure was performed according to the guidelines of the Institutional Animal Care and Use Committee of CIAD (Centro de Investigación en Alimentación y Desarrollo, A. C.).

Cardiac rate

The cardiac rate was measured at 48 and 72 hpf. Embryos were placed under the Stemi 305 stereoscopic dissection microscope coupled to a D3200 digital camera in a lateral position to visualize the heart, and heartbeats were recorded over 30 s and expressed in beats per minute (bpm). Three randomly selected embryos from each of the five repetitions were recorded for each glyphosate concentration.

Locomotor activity

Locomotor activity was measured by counting spontaneous tail movements at 24 hpf, with some modifications. Individual 1-min recordings were collected for 15 embryos per group. Each embryo was categorized depending on its motile capacity (i.e., number of tail movements within one minute) as follows: a) the non-motile embryos (NME) were those without spontaneous movements; b) moderately mobile embryos (MME) were those with a maximum of three spontaneous movements; and c) highly motile embryos (HME) were those that displayed more than four tail movements.

Craniofacial morphometrics

The morphological measurements of craniofacial cartilage were conducted following the method of Carvan et al. (2004). In addition, the authors incorporated Meckel's-Palatoquadrate (M-PQ) angle due to its relevance in analysing toxicity in zebrafish. Embryos at 96 hpf were anesthetized using tricaine and fixed with 4% formaldehyde overnight at 25ºC. Embryos were dehydrated stepwise in ethanol, stained with Alcian Blue solution (70% ethanol, 30% acetic acid, and 2 mg/mL Alcian Blue), and stored in glycerol-KOH. Fifteen stained embryos per group were photographed with the Stemi 305 Stereo Microscope coupled to the D3200 digital camera. The otic capsule length (OT), lower jaw length (LJL), ceratohyal cartilage length (CCL), hyosymplectic cartilage length (HS), intracranial distance (ID), the distance between Meckel's cartilage and the palatoquadrate cartilage (MPQ), and the distance between Meckel's cartilage and basihyal cartilage (MBH) were measured in mm and analysed with Fiji software.

Protein isolation and western blot analysis

Twenty embryos from the control and exposed groups were homogenized using a cellular homogenizer in lysis buffer (2% β-mercaptoethanol, 1% SDS, 20 mM EGTA, 2 mM Tris-HCl at pH 7.5, and protease inhibitors cocktail). The lysate was centrifuged at 6000×g for 10 min at 4ºC, and the proteins were precipitated with acetone at -20ºC overnight. The precipitated protein samples were dissolved in a lysis buffer. The total protein concentration was estimated using Bradford reagent. Protein samples were resuspended in sample buffer (0.1 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.2% bromophenol blue) and boiled for 5 min. The proteins (20 µg per lane) were separated by electrophoresis under reducing conditions with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly (vinylidene fluoride) (PVDF) membranes. For western blot analysis, the PVDF membranes were blocked with 6% milk in TBST (in mM: 10 Tris HCl and 75 NaCl, pH 8.0, 0.1% Tween 20) for 2 h and then incubated with the corresponding primary antibody. The anti-ERα antibody (SC-543) and anti-BSP antibody (SC-73634) were obtained from Santa Cruz Biotechnology. The anti-alpha tubulin antibody (T6199) and the anti-OPN antibody (ab166709) were obtained by Sigma-Aldrich and Abcam, respectively. After incubation with the primary antibody, the membranes were incubated with the corresponding secondary antibody conjugated to horseradish peroxidase (HRP). Detection was performed by chemiluminescence in a ChemiDoc XRS system using Image Lab Software.

Adult skeletal assessment

Adult fish (10 months) from the glyphosate exposed groups (n = 5 per group) were euthanized with tricaine and fixed with 4% formaldehyde overnight at 25ºC. Alizarin Red staining was performed. Samples were rinsed several times with phosphate buffered saline with 0.1% Tween 20 (PBST) and then dehydrated with ethanol overnight at 50%, 70%, and 100%. Subsequently, samples were incubated in

2 mg/mL of Alizarin Red in 1% potassium hydroxide (KOH) for 48 h. Pigmentation was bleached with H_2O_2 (3%) and KOH (0.5%) for 40 min. After which, fish were incubated with 25% glycerol/0.1% KOH for 48 h and rinsed in a 50% glycerol and 0.1% KOH solution to remove excess stain. Then, samples were kept at 4ºC in the same solution. Stained fish were observed and photographed to detect morphological alterations in the spine and skull using the Stemi 305 Stereo Microscope coupled to the D3200 digital camera. Craniofacial measurements of the length and height of the orbital cavity (LOC and HOC, respectively), skull length (SL), ID, LJL, width where the ceratohyal and palatoquadrate cartilage join (WCP), and the distance between the mandibular symphysis and the ceratohyal (DMSC), were measured in mm. The analysis of the data was performed using Fiji software.

Data analysis

Comparisons among groups with regard to the cardiac rate, craniofacial morphometrics, and protein level of embryos and adult skeletal measurements were evaluated by one-way analysis of variance (ANOVA) and post-hoc Tukey tests. Results were considered significant at $p < 0.05$. Shapiro-Wilk and Levene tests were used to evaluate normality and homoscedasticity. If the normality assumption was not fulfilled, a non-parametric Kruskal-Wallis test was used. Spontaneous tail movement comparisons at 24 hpf were conducted using Chi-square category tests. All analyses were performed in the R environment (R Core development team, 2018).

Results

Changes in heart rate and tail movements

At 96 hpf, no differences were observed in the survival rate (1 µg/mL = 98% and 100% in the other concentrations) or hatching rate (0 and 10 µg/mL = 98% and 100% of the embryos in the other groups hatched at 72 hpf). The heart rate showed a differential response related to the developmental stage. Compared to that of the control, lower heart rates were consistently observed in all exposed groups. Significant differences in heart rate were observed for the 10 µg/mL test concentration at 48 hpf, whereas at 72 hpf, significant reductions of ~10 and 12 bpm were observed with the 10 and 50 µg/mL test concentrations, respectively. A negative correlation between glyphosate concentration and the number of beats per minute was only observed at 72 hpf ($\rho = -0.36$, $p < 0.01$). Glyphosate appeared to alter the locomotor activity of zebrafish embryos. In general, most of the organisms were classified within the MME category, with significant differences observed with the 50 µg/mL test concentration (chi-square = 6, df = 2, $p = 0.04$).

Craniofacial alterations

Some craniofacial structures were responsive to glyphosate exposure. In particular, the CCL showed shortened lengths at all concentrations, with significant differences observed with the 50 µg/mL test concentration ($p < 0.05$). At this concentration, similar length reductions were observed for the MPQ, HS, and ID, although with higher variability and no significant differences. The M-PQ also shows significant differences and high variability.

Modification of proteins related to the ossification process

Levels of OPN were upregulated 4-fold in embryos exposed to glyphosate concentrations of 5 µg/mL or higher, with a significant difference observed at 50 µg/mL. A similar response was observed for BSP but with significant differences observed at concentrations of 5 µg/mL or higher. The level of ERα was also significantly higher at 10 and 50 µg/mL and was more than 2-fold higher in the 50 µg/mL exposed group compared to that of the control.

Early exposure to glyphosate generated bone defects in adulthood

Adult fish embryonically exposed to glyphosate showed distinctive bone deformities. Fifteen and 9.8% of the adults exposed to 50 and 10 µg/mL concentrations, respectively, presented marked curvatures in the spine as adults as well as deformities towards the caudal region of the column, which resembled the patterns of scoliosis. In contrast, the morphometric analysis of craniofacial measurements in adult fish exposed to glyphosate as embryos indicated that differences in the cranium were not present between the exposed groups and the control group.

Conclusion

The authors concluded that the changes induced by glyphosate regarding estrogenic activity and proteins related to the formation of bones and cartilage, such as OPN and BSP, could be related to the presence of skeletal deformities in both embryos and adult zebrafish. Evidence indicates that glyphosate is able to interfere with a variety of endocrine signalling systems, including steroid hormone signalling, while oestrogens could induce major disruptions in cartilage and bone formation, indicating that precise control of oestrogen signalling is required for the appropriate development of skeletal structures. Whereas further studies are necessary to corroborate the mechanisms linking the regulation of bone development proteins through ERα modifications, this study provides evidence that early and short exposure to glyphosate can produce skeletal alterations in zebrafish, which are likely to have ecotoxicological consequences on growth, reproduction, and survival.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This article used the zebrafish model to assess the effects of early life glyphosate exposure on the development of cartilage and bone tissues and organismal responses. The evidence suggests functional alterations, including a reduction in the cardiac rate, significant changes in the spontaneous tail movement pattern, and defects in craniofacial development. These effects were concomitant with alterations in the level of the oestrogen receptor alpha osteopontin and bone sialoprotein. Embryos exposed to glyphosate presented spine deformities as adults. These developmental alterations are likely induced by changes in protein levels related to bone and cartilage formation.

This article is of importance for the assessment of the endocrine disrupting properties of glyphosate. It reports the acute and chronic effects on zebra fish. The study seems to have been well conducted and reports a regulatory relevant and reliable endpoint: 96 h NOEC = 1 mg/L (based on the effects on bone sialoprotein (BSPII) relative expression).The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase. Temperature during the test is a bit high for the species tested.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Zebrafish embryos used for the present study were generated from individuals obtained from commercial distributors and cultured in the lab.

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in the buffer solution, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, purity and CAS number reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Batch specifications are not provided and the assessment of the ecotoxicological equivalence cannot be conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were analytically verified only in the stock solutions
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: NOEC (96-h)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	No positive control tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Concentrations were analytically verified only in the stock solutions
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Uncertain	Temperature set at 28.5°C for the tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	Concentrations were analytically verified only in the stock solutions, but not during the test
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	Concentrations were analytically verified only in the stock solutions, but not during the test
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical	Yes	The concentration of glyphosate

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
methods is included.		in-stock solution was determined by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS)
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No assessment of the ECX median values was conducted.

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This article is of importance for the assessment of the endocrine disrupting properties of glyphosate. It reports the acute and chronic effects on zebra fish. The study seems to have been well conducted and reports a regulatory relevant and reliable endpoint: 96-h NOEC = 1 mg/L (based on the effects on bone sialoprotein (BSPII) relative expression). The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase. Temperature during the test is a bit high for the species tested.
Not reliable	No	-

1. Information on the study

Data point:	CA 8.2.2, CP 10.2.2
Report author	Le Du -Carrée J. *et al.*
Report year	2021
Report title	Developmental effect of parental or direct chronic exposure to environmental concentration of glyphosate on the larvae of rainbow trout, *Oncorhynchus mykiss*
Document No	Aquatic Toxicology, 2021, 237, 105894
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

This study investigated the impact of parental and direct exposure to 1 µg/L of glyphosate using the active substance (AS) alone or one of two Glyphosate-Based Herbicide (GBH) formulations (i.e. Roundup Innovert® and Viaglif Jardin®) in the early developmental stages of rainbow trout. Three different modes of exposure on the F1 generation were studied: (1) intergenerational; (2) direct and (3) multigenerational. The impact of chemical treatments on embryo-larval development (survival, biometry and malformations), swimming behaviour, biochemical markers of oxidative stress equilibrium (TBARS and catalase), acetylcholine esterase (AChE) and energy metabolism (citrate synthase, CS; cytochrome-c oxidase, CCO; lactate dehydrogenase, LDH; glucose-6-phosphate dehydrogenase, G6PDH) was explored. Chemical exposure did not affect the survival of F1 embryos or malformation rates. Direct exposure to the AS induced some biometric changes, such as reduction in head size (with a 10% decrease in head length), independently of co-formulants. Intergenerational exposure to the AS or the Roundup GBH increased swimming activity of the larvae, with increase of between 78 and 102% in travel speeds. Viaglif co-formulants appear to have counteracted this behavioural change. The minor changes detected in the assayed biochemical markers suggested that observed effects were not due to oxidative damage, AChE inhibition or alterations to energy metabolism. Nonetheless, multi- and intergenerational exposure to Roundup increased CS:CCO and LDH:CS ratios by 46% and 9%, respectively, with a potential modification of the aerobic-to-anaerobic energy production balance. These biochemical effects were not correlated with those observed on individual level of biological organization. Therefore, further studies on generational toxicity of glyphosate and its co-formulants are needed to identify the other mechanisms of glyphosate toxicity at the cellular level.

Materials and methods

Ethics statement

Assays on fish were done in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). Experimental procedures were validated by the animal ethics committee ANSES/ENVA/UPC No. 16 and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS#2019010812403065). A lethal dose of 100 ppm of eugenol into tank water was used to euthanize fish.

Chemical compounds

We used the AS glyphosate (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6) and two GBHs, Roundup Innovert® (R) and Viaglif Jardin® (V). The purity of G was 98%, and the glyphosate concentration of Roundup and Viaglif were 360 and 420 g/L, respectively. For each product, aqueous concentrated solutions (4 mg/L) were prepared and stored under appropriate conditions (darkness, 4ºC). Pure glyphosate was diluted first in 10 mL of pure methanol (solvent concentration of concentrated solution was 10 mL/L so the final dose of methanol exposure was kept under 4 µL/L).

Fish

Specific pathogen free mature rainbow trout (3 years old; F0 generation) were exposed daily for 8 months to control (C) or to a mean glyphosate concentration of 123 ng/L using pure glyphosate, Roundup or Viaglif, before producing the F1 generation. Embryonic development was conducted in a shallow bottom tank containing approximately 300 L. All fish were maintained in filtered river water, with a water flow rate to ensure complete renewal once an hour and maintain appropriate physicochemical conditions and oxygen saturation greater than 60%. A photoperiod of 12 h of daylight was maintained throughout the experiments. Fish feed (Le Gouessant), adapted for fish size, was given ad libitum.

Reproduction and embryonic development

Reproduction of fish from F0 generation is described in the article of Le Du-Carrée *et al*. Embryonic development of the F1 generation until the eyed stage was conducted in tanks, containing approximately 300 L, continuously renewed with river water (at a flow rate of approximately 300 L/h). After the eyed stage, rainbow trout embryos were placed in 10 tanks (40 L) in a confined room. Temperature was maintained at 8º±2ºC throughout embryonic development.

Chemical exposure

Once the embryos reached the eyed stage, they were exposed to 10 conditions of chemical exposure. The name of each condition is composed of two letters separated by a slash, the first letter represents the chemical exposure of the F0 generation and the second letter represents the chemical exposure of the F1 generation. As an example, fish from non-contaminated parents, but directly contaminated make up the C/G, C/R, and C/V conditions. Chemical exposure of the F1 generation was conducted using the same methodology as that used for the F0: every working day (generally 5 days a week), 10 mL of the condition respective concentrated chemical solution was added to the experimental tanks in which water input was stopped for 1 h. After 1 h of contact, water flow was set to 13.5 L/h for the rest of the day, resulting in the gradual dilution of glyphosate. The theoretical kinetics of glyphosate concentrations was modelled and the integrated mean daily expected concentration was approximately 123 ng/L.

Samples and sampling dates

To measure hatching frequencies, egg survival was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes until all eggs were either dead 34 or hatched. The hatched eggs were placed in plastic breeding boxes 35 days after hatching.

For each condition, 25 larvae were sampled at 320 degree-day (DD; sampling date S1) after 14 days of direct exposure and at 328DD (S2) for intergenerational exposure. They were placed in a 3% glutaraldehyde solution, at 4ºC until biometric and malformation analyses. At 488DD (S3), 25 larvae were sampled in each tested condition to measure oxidative stress and metabolic markers. Whole larvae were flash-frozen in liquid nitrogen and stored at -80 ºC for future analyses. Proteins were extracted by homogenizing whole larvae in phosphate buffer (0.1 M, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor using the tissue homogenizer Precellys 24 (Bertin Technologies, France).

Determination of glyphosate concentrations in exposure tank

Glyphosate was quantified in water after two months of chemical contamination using a direct competitive ELISA assay (Novakits, ref. 1500086) at two time points in the daily experimental procedure (i.e. just before restarting the water flow [wfr] and 2 h after wfr). A total of 250 µL of filtered water was used to conduct the ELISA assay and final absorbance was read at 450 nm on a TECAN's Spark 10M microplate spectrophotometer. The four-parameter log-logistic function, LL.4, of the "drc" R package, was used to generate the standard curve. The OD value obtained for the sample was plotted on the quantification standard curve to determine the glyphosate concentration in each sample.

Biometric index measurements and malformation analyses

Biometric index measurements and malformations were analysed on images of individual larvae taken on a stereo microscope (Leica MZ75) combined with a ToupCam camera (U3CMOS05100KPA). Body and head length, eye diameter and yolk-sac surface were measured using the software ToupView 3.7

Swimming behaviour analysis

The protocol of photomotor assay was adapted from the study of Weeks Santos et al. (2019) and applied to swimming larvae (i.e. 855DD; S4) maintained at 11ºC throughout the experiment. To avoid perturbation due to the circadian rhythm, analyses were done during a maximum duration of 4 h per day for three days. An infrared camera coupled to the DanioVision (Noldus, version 12.1) system was used to record the behaviour of larvae placed in six-well cell culture plates (Nunc, ref. 140685) with each flat well containing 2 mL of water. For each assay, larvae were acclimated in the plate for 10 min in the dark before the measurements began. The 30 min recording was divided into three phases at different light intensities: 10 min of darkness (Dark 1), followed by 10 min of light (light 1), finally followed by 10 min of darkness (Dark 2). The distance (in meters) travelled by each larva during these three light-darks phases was then used to compare the effect of the different chemical treatments.

Assays for oxidative stress and metabolic markers and choline esterases

Colorimetric analysis was carried out on a TECAN Spark 10M microplate spectrophotometer. Choline esterases (ChE) were measured at 412 nm. Each assay was performed in duplicate or triplicate. Oxidative stress markers were assayed in whole larvae homogenates at 532 nm and 240 nm, respectively. Metabolic markers were measured in whole larvae samples at 412 nm, 550 nm, 340 nm and 340 nm. The slope of the optical density f time curve plotted on a calibration curve allowed the calculation of enzymatic activity. Calibration curves were generated using pure enzymes purchased at Sigma-Aldrich. Protein concentrations were measured using the Pierce BCA protein assay kit (ThermoFisher Scientific), and results were expressed as specific activity (IU/mg of protein).

Data processing and statistical analyses

Statistical analyses and data processing were done using R software (R Core Team, 2018). Data sets were tested for normality (Shapiro-Wilk) and homoscedasticity (test of Levene for parametric data and Fligner-Killeen for non-parametric data). When normal and homoscedastic data were confirmed, one-way ANOVA tests were used to compare means, followed by Dunnett's post-hoc test. For normal and heteroscedastic data, modified one-way ANOVA were employed to compare means, followed by Tamhane-Dunnett post-hoc test. For non-normal data, a Kruskal-Wallis test was used to compare means, followed by Dunn's post-hoc test. Differences between hatching and malformation rates were compared using a chi-squared test. Survival rates for the different chemical treatments were compared using the log-rank test in the "survival" R package. A p -value of 0.05 was used as the threshold for statistical significance.

Results

Glyphosate concentration in water

Glyphosate was never detected in the control tank during the experiment. Glyphosate concentrations between 1.18±0.036 and 1.95±0.086 µg/L were detected in all contaminated tanks 1 h after adding the chemical solutions. Two hours after restarting the water flow, measured concentrations were slightly below those predicted by the theoretical kinetics (from 0.30±0.015 to 0.34±0.021 instead of expected value of 0.51 µg/L).

Hatching and larvae survival, malformations and biometric indices

Neither the hatching percentage nor the larval survival rate during the 35 days after hatching was significantly affected by chemical exposure.

No yolk-sac oedemas were observed. Jaw appeared in larvae at frequencies varying from 0 to 4%, depending on the exposure condition. Spinal curvature was the most frequent malformation observed (12 to 24%). Chemical treatments had no impact on malformation rates.

No statistical significant differences between control and directly contaminated larvae were observed for body length, yolk-sac surface or eye:head length ratio. However, there were significant reductions in head ($p < 0.0001$, $df = 3$ and $f = 9.46$), eye diameter ($p < 0.0001$, $df = 3$ and $\chi^2 = 28.06$) and head:body length ratio ($p < 0.008$, $df = 3$ and $f = 6.51$). For head length, post-hoc tests revealed significant differences for the C/G (-11%), C/R (-11%) and C/V (-8%) conditions compared with the control ($p < 0.05$). For eye diameter, significant differences were observed for the C/G (-11%), C/R (-10%) and C/V (-7%) conditions ($p < 0.05$). For head:body length ratio, significant differences were observed for the C/G (-7%), C/R (-6%) and C/V (-6%) conditions compared with the control ($p < 0.05$). Intergenerational exposure induced no statistically significant differences between control and chemically contaminated larvae for most of the biometric indices considered. However, a significant effect of chemical concentration was observed in body length ($p < 0.0001$, $df = 3$ and $\chi^2 = 45.05$) and in the ratio between eye and head length ($p = 0.0004$, $df = 3$ and $f = 6.56$). Post-hoc tests revealed significant increases compared with the control, with +7% in body length for the R/C condition and +6% in eye:head length ratio for the V/C condition ($p < 0.05$).

Metabolic activity in whole larvae

No major significant change in mean activity was observed at 488DD for the oxidative stress markers (i.e. CAT and TBARS), or for AChE and the metabolic markers (i.e. CS, CCO, G6PDH) except for LDH ($p = 0.004$, $df = 9$ and $\chi^2 = 24.02$). A post-hoc test revealed a significant increase in LDH activity for the R/R condition compared with the control (+16%, $p < 0.05$).

Means of CS:CCO ratio values were $8.8 \times 10^{-3} \pm 5.2 \times 10^{-4}$ for the control condition and comprised between $8.0 \times 10^{-3} \pm 4.5 \times 10^{-4}$ and $9.7 \times 10^{-3} \pm 4.2 \times 10^{-4}$ for the other conditions except for the R/R condition which showed a ratio of $13 \times 10^{-3} \pm 1.7 \times 10^{-3}$. This latter ratio was significantly different to the control condition (global mean difference: $p = 0.004$, $df = 9$ and $\chi^2 = 24.27$; +46%, $p < 0.05$).

For the LDH:CS ratio, values were 458.98 ± 7.18 for the control condition, comprised between 458.56 ± 8.42 and 482.19 ± 13.62 for the other conditions, except for R/C and R/R, which showed values of 512.21 ± 10.57 and 501.93 ± 9.09, respectively. For the LDH:CS ratio, a statistical difference was observed among all the conditions ($p = 0.002$, $df = 9$ and $\chi^2 = 25.86$) and a post-hoc test revealed significant differences to the control for the R/C (+12%, $p < 0.05$) and R/R (+9%, $p < 0.05$) conditions.

Swimming behaviour of intergenerationally exposed trout

During the photomotor assay, larvae travelled approximately 4.95 ± 0.49 m and 6.86 ± 0.51 m in the first and the second period of darkness. Speed dramatically decreased in the presence of light with a mean travelled distance of 0.81 ± 0.12 m. No effect of chemical exposure was found in the presence of light, but global mean differences were observed between distance travelled by larvae in the first ($p < 0.0001$, $df = 3$ and $\chi^2 = 30.46$) and in the second period of darkness ($p < 0.0001$, $df = 3$ and $\chi^2 = 41.78$). Post-hoc tests revealed significant differences in means obtained for the G/C and R/C conditions compared with the control during the first period of darkness (+83 and 102%, respectively, $p < 0.05$) and during the second period of darkness (+78 and 83%, respectively, $p < 0.05$).

Conclusion

The authors studied the effect of parental and/or direct exposure to an environmental concentration of glyphosate, focusing on the embryo-larval development of the F1 generation. Although no effect was shown on embryo or larval survival rates regardless of the mode of exposure or the contaminants considered, some effects were observed at different levels of biological organization. Glyphosate, apparently independently of co-formulants and only in the case of direct exposure, appeared to induce developmental changes, such as reductions in head size and that of associated organs, but did not cause developmental malformations. Intergenerational exposure to glyphosate increased the swimming activity in larvae, without any correlation with AChE inhibition. The authors indicated that this behavioural change may potentially have an important impact on larvae survival in a stressful natural environment. Viaglif co-formulants seemed to counteract this behavioural change. No oxidative stress response or damage was detected in rainbow trout larvae. Nonetheless, parental exposure to one of the two tested GBHs may modify energy production by increasing the amount of energy produced via anaerobic metabolism compared with that produced by aerobic metabolism. Also, multigenerational exposure to the same GBH may potentially cause a reduction in the capacity of mitochondria for energy production. These effects, not detected with the AS alone, were probably not responsible for the other effects observed. Thus, there are likely other mechanisms of glyphosate toxicity at the cellular level and further investigations are needed to fully understand how they are responsible for both direct and generational toxicity of glyphosate and its co-formulants.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study investigates the impact of parental and direct exposure to 1 µg/L of glyphosate using the active subtance alone or one of two Glyphosaet-Based Herbicide formulations (i.e. Roundup Innovert® and Viaglif Jardin®) in the early developmental stages of rainbow trout. Three different modes of exposure on the F1 generation were studied: (1) intergenerational (i.e. fish only exposed through their parents); (2) direct (i.e. fish exposed only directly) and (3) multigenerational (i.e. fish both exposed intergenerationally and directly). The impact of chemical treatments on embryo -larval development (survival, biometry and malformations), swimming behaviour, biochemical markers. Chemical exposure did not affect the survival of F1 embryos or malformation rates. Direct exposure to the a.s. induced some biometric changes, such as reduction in head size (with a 10% decrease in head length), independently of co -formulants. Intergenerational exposure to the a.s. or the Roundup GBH increased swimming activity of the larvae, with increase of between 78 and 102% in travel speeds. Therefore, 1 generation LOEC = 1 µg/L.
The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline -compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non -guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals were cultured in the lab.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non -toxic) and a carrier control / positive control is considered in the test design.	Uncertain	Pre -dilution of pure glyphosate was done in pure methanol in a concentration so the final dose of methanol exposure was kept under 4 µL/L
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, purity, CAS number and content reported
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	The assessment of the ecotoxicological equivalence was not conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non -guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Concentrations were analytically verified just before and 2-h after restarting the water flow

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: LOEC (1 generation)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Just 1 concentration was tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	No	Only 1 concentration was tested
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time -points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5 -8.0), cation exchange capacity, organic carbon (0.5 -2 -5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A -horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/ - 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20 -25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	River water filtered
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15 -25ºC.	Uncertain	Temperature set at 8°C during the embryonic development and at 11°C for the swimming behaviour analysis. Main test temperature not reported
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	-
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		time was generated.
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	The concentration of glyphosate in water was determined by HPLC and fluorometric methods
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No assessment of the ECX median values was conducted.

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study investigates the impact of parental and direct exposure to 1 µg/L of glyphosate using the AS alone or one of two GBH formulations (i.e. Roundup Innovert® and Viaglif Jardin®) in the early developmental stages of rainbow trout. Three different modes of exposure on the F1 generation were studied: (1) intergenerational (i.e. fish only exposed through their parents); (2) direct (i.e. fish exposed only directly) and (3) multigenerational (i.e. fish both exposed intergenerationally and directly). The impact of chemical treatments on embryo -larval development (survival, biometry and malformations), swimming behaviour, biochemical markers. Chemical exposure did not affect the survival of F1 embryos or malformation rates. Direct exposure to the AS induced some biometric changes, such as reduction in head size (with a 10% decrease in head length), independently of co -formulants. Intergenerational exposure to the AS or the Roundup GBH increased swimming activity of the larvae, with increase of between 78 and 102% in travel speeds. Therefore, 1 generation LOEC = 1 µg/L. The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.
Not reliable	No	-

1. Information on the study

Data point:	CA 8.2.2, CP 10.2.2
Report author	Le Du-Carrée J. *et al.*
Report year	2021
Report title	Generational effects of a chronic exposure to a low environmentally relevant concentration of glyphosate on rainbow trout, *Oncorhynchus mykiss*
Document No	Science of the Total Environment, 2021, 801, 149462
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors investigated the impact of an environmentally relevant concentration of glyphosate and its co -formulants on an F2 generation issued from exposed generations F0 and F1. Trans, inter and multigenerational toxicity of 1 µg/L of the active substance was evaluated on early stages of development and juvenile rainbow trout (*Oncorhynchus mykiss*) using different molecular, biochemical, immuno-hematologic, and biometric parameters, behaviour analysis, and a viral challenge. Reproductive parameters of generation F1 were not affected. However, developmental toxicity in generation F2 due to glyphosate alone or co -formulated was observed with head size changes (e.g. head surface up to +10%), and metabolic disruptions (e.g. 35% reduction in cytochrome -c -oxidase). Moreover, larvae exposed transgenerationally to Viaglif and intergenerationally to glyphosate and Roundup presented a reduced response to light, potentially indicating altered escape behaviour. Overall methylation was, however, not altered and further experiments using gene -specific DNA methylation analyses are required. After several months, biochemical parameters measured in juvenile fish were no longer impacted, only intergenerational exposure to glyphosate drastically increased the susceptibility of rainbow trout to hematopoietic necrosis virus. This result might be due to a lower antibody response in exposed fish. The authors concluded that their results show that generational exposure to glyphosate induces developmental toxicity and increases viral susceptibility. Co -formulants present in glyphosate -based herbicides can modulate the toxicity of the active substance. The authors indicated that further investigations are required to study the specific mechanisms of transmission but their results suggest that both non -genetic mechanisms and exposure during the germinal stage could be involved.

Materials and methods

Ethics statement

Fish experimentation was carried out in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). Experimental procedures were validated by the animal ethics committee ANSES/ENVA/ UPC No. 16 and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS#2019010812403065). Euthanasia involved the addition of a lethal dose of 100 ppm of Eugenol into the tank water.

Chemicals

Three chemical compounds were tested: glyphosate active substance (G; Sigma -Aldrich, ref. 45,521, CAS Number 1071 -83 -6) and two GBHs: Roundup Innovert® (R; Agrilisa - for professional use) and

Viaglif Jardin® (V; Agrilisa - for home gardens). The purity of G was 98%, and the concentrations of R and V were 360 and 420 g/L of glyphosate, respectively.

Fish

Experiments were conducted using specific pathogen -free (SPF) rainbow trout reared in the protected and monitored fish facilities of the ANSES Plouzané Laboratory site (France).

Fish were maintained in tanks of 40 L (juveniles) and 400 L (adult) containing river water filtered with sand filter (approximately 20 µm), with a water flow rate to ensure complete renewal once an hour and maintain appropriate physicochemical conditions and oxygen saturation greater than 60%. The experimental facilities were supplied with river water with a mean pH of 7.8, a conductivity of 400 µS/m and a hardness of 12°TH. Concentrations of ammoniac, nitrate and nitrite are close to 0. Physicochemical analyses of the water were regularly carried out to guarantee excellent maintenance conditions for all the fish. A photoperiod of 12 h of daylight was maintained throughout the experiments. After the eyed stage period, embryos were placed in 8 tanks (40 L) positioned in a confined room. Temperature during embryonic development was maintained at 8±2°C. After this stage water temperature varied from 6 to 15°C. A trout -specific feed (Le Gouessant®), adapted to the fish size, and was given ad libitum.

Chemical exposure

For each product, concentrated aqueous solutions (4 mg/L in distilled water) were prepared and stored under appropriate conditions (darkness, 4ºC±2). A pre -dilution of pure glyphosate was done in 10 mL of pure methanol (concentration of solvent in concentrated solution was 10 mL/L so the final dose of methanol exposure was kept under 4 µL/L). The F0 and F1 generations were exposed daily for 10 days and 24 months, respectively, to a mean concentration of 123 ng/L of glyphosate using Glyphosate (G); Roundup (R); Viaglif (V). Unexposed control conditions (C) were included. F0 engendered the F1 generation. The F2 generation was produced by 2 to 5 females and 2 to 8 males of the F1 generation. Conditions C/V and V/V were lost during the experiment due to material dysfunction, so 8 conditions only could give birth to the F1 generation.

Reproduction

Procedure of trout reproduction is described in the article of Le Du-Carrée *et al* and embryonic development until the eyed stage was conducted in 300 L tanks containing two breeding boxes by tank and continuously renewed with river water (filtered with sand and cotton wool filters) with a flow rate of approximately 300 L/h.

Experimental design

Chemical exposure

Once the F2 larvae reached eyed stage, rainbow trout embryos were exposed to the 8 conditions. Fish produced from non-contaminated F0 but directly contaminated in F1 were C/G/C, and C/R/C and are considered intergenerationally exposed fish. Fish only contaminated through F0 are considered transgenerationally contaminated, and were G/C/C, R/C/C, and V/C/C. Finally, multigenerational exposure designated fish that were contaminated continuously for three generations. They are represented by the conditions G/G/G and R/R/R. F1 and F2 chemical exposure was conducted with the same methodology as for F0. Theoretical glyphosate concentration kinetics were modelled and the integrated mean daily theoretical concentration was approximately 123 ng/L.

Water sampling was performed in after approximately three months of chemical contamination to measure concentrations of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) in water tanks. Quantification of glyphosate and AMPA was done within 72 h after sampling using HPLC and fluorometric methods (Method ref. ANA -I10.MOA.69.B) by an external provider (Labocea, France).

Viral challenge

The virus used for viral challenge was the N61 strain (genotype E) of the infectious hematopoietic necrosis *IHN* virus (i.e. *IHNv*) isolated from diseased rainbow trout fry displaying typical signs of the disease. The infectious titre of the viral production, was $4x10^7$ $TCID_{50}$/mL. After approximately

6 months of chemical exposure, 280 F2 fish for each chemical treatment were randomly distributed to four 10 L tanks with constant water renewal (i.e. 70 fish per replicate of a condition). Three of these tanks were infected with *IHNv*, and one was used as the uninfected control. Non -infected EPC cell supernatant was used for uninfected control tanks. For 6 weeks after *IHNv* infection, general behaviour, the appearance of clinical signs (lethargy, darkening of the skin, exophthalmia), and mortality were recorded twice a day. Dead individuals were stored at -20°C for viral examination.

Sampling date

Fertility was considered to be the proportion of eggs surviving at 5 days post -fertilization. To perform this measurement, egg survival was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes. Larvae for biometric indices and malformation measurements were sampled at 350 DD (S1) and stored in a 3% glutaraldehyde solution at 4 DD until the analysis. Condition C/R/C was lost during storage and could not be analysed.

At 541 DD (S2), invasive sampling was done on 20 larvae exposed to the different conditions. They were flash -frozen in liquid nitrogen and stored at -80ºC for future analyses.

Eleven days before infection (S4), 96 h post -infection (96 hpi; S5), and 42 days post -infection (42 dpi; S6) to *IHNv*, invasive sampling was done on 20 juvenile rainbow trout aged approximately 6 months, with size and length (mean ± se) of 5.69±5.69 and 83.83±0.75, respectively. A blood sample was taken by withdrawing 10 µL of blood from the caudal vein with a lithium heparin haematocrit tube (Greiner ref. KG454244), and fish were euthanized. Then, at S4, gills were sampled, flash -frozen in liquid nitrogen and stored at -80ºC for future analyses. Between S3 and S4, condition G/G/G was lost due to an incident during fish maintenance and could not be sampled.

Biomarkers analysed

Biometric index measurements and malformation analysis

Biometric index measurements and malformation analysis were done on a picture of larvae taken with a binocular magnifier (Stemi 508) coupled to a camera (Canon DS126431) permitting 40x magnification. Body length (without tail fin), head surface, eye diameter, and yolk sac surface were analysed using ToupView software, version 3.7 on a total of 30 larvae per condition. Frequencies of malformation were determined on larvae considering jaw malformations, yolk sac oedema (not observed), and spinal curvature.

Swimming behaviour analysis

The analysis was done during a time window of seven days with free -swimming larvae (i.e. from 615 to 681 DD, S3 sampling date) maintained at 11 throughout the experiment.

Viral examination and immune parameters during the viral challenge

The presence and concentration of *IHNv* were checked individually from 3 dead fish collected at peak mortality, by replicate chemical condition. Extracted organs (kidneys, spleen, heart, and brain) were pooled and crushed using a mortar and pestle, diluted to 10^{-1} with Eagle medium, and centrifuged for 15 min at 2,000x g at 4°C. The supernatant was then diluted to 10^{-8}, and the virus concentration was determined for each fish. At S5, red blood cell (RBC) and white blood cell (WBC) counts were performed on a Thoma cell haemocytometer using whole blood diluted to 1/200 in Giemsa solution. At S6, detection and semi -quantification of anti -IHNv antibodies in the plasma of surviving fish were performed using a modified procedure.

Methylation

Extraction of total DNA was performed using a DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. Global DNA methylation was then measured on 10 ng of total DNA extracted from whole fish larvae sampled at S2, using a MethylFlash Global DNA Methylation (5 -methylCytosine or 5 -mC) ELISA Easy Colorimetric Kit (Epigentek), following the manufacturer's instructions. Absorbance of the products were measured on a TECAN Spark 10 M microplate spectrophotometer at 450 nm. Calculation of the percent methylated DNA for each sample was carried out by reporting the optical density values on the standard curve.

Oxidative stress and metabolic parameters

Choline esterases (ChE), oxidative parameters, namely thiobarbituric acid reactive substances (TBARS), catalase (CAT), and glutathione peroxidase (GPx), and metabolic parameters, i.e. citrate synthase (CS); cytochrome c oxidase (CCO); lactate dehydrogenase (LDH); and glucose -6 -phosphate dehydrogenase (G6PDH), were assayed in larvae (sampled at S2) and gills of juvenile (sampled at S4) following the same procedures described in a previous article (Le Du-Carrée et al., 2021).

Data processing and statistical analyses

Statistical analyses and data processing were performed with R software (R Core Team, 2018). Figures were generated using the ggplot2 package. Quantitative data sets were tested for normality (Shapiro-Wilk) and homoscedasticity (test of Levene for parametric data and Fligner-Killeen for non-parametric data). When normal and homoscedastic data were confirmed, one -way ANOVA tests were used to compare means, followed by a post -hoc test of Dunnett. In the case of normal and heteroscedastic data, modified one -way ANOVA tests were used to compare means, followed by a post -hoc test of Tamhane-Dunnett. In the case of non-normal data, a Kruskal-Wallis test was used to compare means, followed by a post -hoc test of Dunn. Differences between malformation rates were compared using a chi -squared test. Survival rates for the different chemical treatments were compared using the "survival" package. A p -value of 0.05 was used as the threshold for statistical significance. A test of correlations between variables was carried out using the lmrob R function (R package robustbase).

Results

Glyphosate concentrations in exposure tank water

Validation of the experimental chemical contamination procedure for the exposure of F0 and F1 generation trout in 400 L tanks was performed in a previous experiment. During this experiment, the river water supplying the tanks did not present any detectable concentration of glyphosate. After 1 h of exposure and just before water flow reopening, concentrations of glyphosate of 0.54 and 0.57 µg/L were quantified in tanks contaminated with glyphosate and Roundup. These concentrations were 46 and 43% below the theoretically expected value of 1 µg/L. One hour after water flow reopening, the concentration of glyphosate in the tanks contaminated with the active substance was close (2% variation) to the expected concentration of 0.71 µg/L, whereas it was 47% lower in the Roundup contaminated tanks. Two hours after restarting water flow, glyphosate concentrations were 45% below expected values for both tanks (0.28 instead of 0.51 µg/L theoretically). AMPA, the main metabolite of glyphosate, was not detected in any of the samples including those artificially contaminated with the active substance or Roundup.

Fertility and fecundity of the F1 generation

Relative fecundity of the F1 generation aged of two years varied between 1.79±0.61 and 3.05±0.40 eggs/g, regardless of the condition, with no detectable impact of the chemical contamination (Table 1). Fertility was calculated for control and intergenerationally contaminated fish of the F2 generation. It was greater than 97% regardless of the condition considered. No statistically significant difference was measured.

Table 1. Relative fecundity of the F1 generation (expressed in eggs/g; mean ± standard error, se) as a function of the chemical exposure conditions ($2 \leq n \leq 5$).

Mode of exposure	Condition	Relative fecundity	
		Mean	se
Control	C/C	2.17	0.44
Trans.	G/C	2.85	0.28
	R/C	2.10	0.33
	V/C	3.05	0.40
Direct	C/G	2.27	0.38
	C/R	1.79	0.61
Multi.	G/G	2.14	0.86
	R/R	2.53	0.44

Biometric indices and malformations observed on the F2 generation

No differences between control and chemically contaminated F2 larvae sampled at S1 were observed for body length and yolk sac surface (Table 2). Different chemical conditions induced significant changes in other biometric indices, such as an increase in head surface, df = 6 and $\chi^2 = 30.50$), eye surface ($p < 0.0001$, df =6 and $\chi^2 = 43.56$), and eye:head surface ratio ($p = 0.001$, df = 6 and $\chi^2 = 26.97$). For head surface, a post -hoc test revealed significant differences for the G/C/C (+10%) and G/G/G (+9%) conditions, compared to the control ($p < 0.05$). For eye surface, a post -hoc test revealed a significant increase (+9%) for the multigenerationnally exposed G/G/G condition ($p < 0.05$). For eye:head surface ratio, a post -hoc test revealed a significant reduction for C/G/C (-5%) and R/R/R (-9%) compared to the control ($p < 0.05$).

Table 2. Mean biometric indices measured at 350 (S1) in F2 larvae exposed directly or through their parents to glyphosate or GBHs. Standard errors are given in parentheses under each respective mean (n = 30). Lengths are expressed in, surfaces in and ratios in %. Numbers in bold with an asterisk indicate conditions significantly different ($p < 0.05$) from the control.

Parameter	Mode of exposure						
	Control	Transgenerational			Inter.	Multigenerational	
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	G/G/G	R/R/R
Body length	14.9 (0.121)	15.35 (0.148)	15.01 (0.113)	15.15 (0.117)	15.12 (0.091)	15.42 (0.09)	14.36 (0.186)
Head surface	6.25 (0.089)	**6.89*** (0.196)	6.32 (0.138)	6.39 (0.129)	6.47 (0.109)	**6.82*** (0.114)	5.85 (0.179)
Eye surface	1.17 (0.023)	1.34 (0.097)	1.21 (0.022)	1.2 (0.025)	1.15 (0.018)	**1.28*** (0.03)	1.01 (0.051)
Eye:head surface ratio	18.78 (0.29)	19.11 (0.71)	19.18 (0.27)	18.8 (0.25)	**17.89*** (0.2)	18.71 (0.4)	17.04 (0.67)

Spinal curvatures and jaw malformations were detected in F2 larvae (the analyses were done on a total of 89 113 larvae per condition). Low frequencies of jaw malformations were observed for all conditions, with proportions ranging from 0 to 7%. Spinal curvature was the most frequent malformation detected, with frequencies ranging from 0 to 11%. No yolk sac oedema was observed. Chemical treatments did not induce statistically significant induction of malformations compared to the non-exposed condition (i.e. the control).

Metabolic activity

Chemical contamination induced changes in certain enzymatic levels in F2 larvae sampled at S2 (Table 3). While no changes were observed for AChE, LDH, or TBARS regardless of the chemical condition considered, statistically significant reductions in enzymatic activities were detected for CAT, CCO, and CS. CAT activity was affected by chemical exposures ($p = 0.003$, df = 7 and f = 3.25), with a significant reduction of 13, 18, 15, and 23% found by a post -hoc test for the G/C/C, G/G/G, R/C/C and V/C/C conditions, respectively, compared to the control ($p < 0.05$). CCO and CS activities were also affected by chemical exposure ($p < 0.0001$, df = 7 and f = 12.30; $p = 0.04$, df = 7 and f = 2.14, respectively), with reductions for G/C/C and V/C/C comprised between 12 and 33%, compared to the control ($p < 0.05$). A reduction of 35% in CCO activity was also observed between the control and G/G/G ($p < 0.05$). The ratio between CS and CCO activities (CS:CCO ratio) was affected by chemical contamination ($p < 0.0001$, df = 7 and $\chi^2 = 53.60$). Increases of 33 and 31% were observed for G/C/C and G/G/G compared to the control, respectively ($p < 0.05$). The LDH:CS ratio was also altered by the chemical treatments ($p < 0.0001$, df = 7 and f=11.39). Conditions G/C/C, C/G/C, and V/V/V showed increased values of 19, 24, and 14% compared to the control, respectively ($p < 0.05$).

At S4, chemical contamination did not induce changes in LDH and GPx activities nor in CS:CCO and LDH:CS ratios in rainbow trout gills (see Table 4). However, changes were observed in CCO and CS activities ($p = 0.002$, df = 6 and f = 3.87 and $p = 0.003$, df = 6 and $\chi^2 = 19.94$), enzymes involved in aerobic metabolism.

Mean CCO activity ranged between 0.16±0.0089 and 0.26±0.011 IU/mg, while CS activities ranged between 0.32±0.0098 and 0.36±0.0082 IU/mg according to the considered treatment. A post -hoc test revealed that CCO and CS activities were 24% higher in the C/G/C condition compared to the control ($p < 0.05$) (see Table 4).

Table 3. Mean specific activities and TBARS levels measured in whole F2 larvae at 541 DD (S2) for the different chemical conditions. Specific activities are expressed in IU/mg of protein and MDA concentrations in nmol/mg of protein. Standard errors are given in parentheses under each respective mean ($11 \leq n \leq 20$). Values in bold with an asterisk are significantly different ($p < 0.05$) from the control condition.

Parameter	Mode of exposure							
	Control	Transgenerational			Intergenerational		Multigenerational	
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	C/R/C	G/G/G	R/R/R
AChE	0.31	0.29	0.31	0.28	0.29	0.31	0.3	0.35
	(0.0094)	(0.007)	(0.0079)	(0.0099)	(0.0117)	(0.0105)	(0.011)	(0.0145)
CAT	7.46	**6.3***	**6.32***	**5.78***	6.46	6.31	**6.14***	6.96
	(0.21)	(0.27)	(0.29)	(0.25)	(0.27)	(0.26)	(0.33)	(0.38)
CCO	0.25	**0.16***	0.23	**0.2***	0.23	0.22	**0.16***	0.26
	(0.0173)	(0.0089)	(0.0076)	(0.0084)	(0.0113)	(0.0089)	(0.007)	(0.0109)
CS	0.36	**0.32***	0.34	**0.32***	0.32	0.34	0.32	0.35
	(0.0082)	(0.0098)	(0.0092)	(0.0117)	(0.0128)	(0.0123)	(0.0116)	(0.013)
CS:CCO	1.56	**2.07***	1.54	1.59	1.47	1.6	**2.03***	1.38
	(0.111)	(0.154)	(0.047)	(0.056)	(0.066)	(0.066)	(0.051)	(0.044)
LDH	13.4	14.05	14.41	13.5	14.89	12.54	12.07	13.03
	(0.5)	(0.4)	(0.52)	(0.58)	(0.59)	(0.47)	(0.53)	(0.65)
LDH:CS	37.44	**44.65***	42.7	**42.68***	**46.56***	36.84	37.53	37.33
	(1.53)	(1.24)	(1.34)	(1)	(1.28)	(0.84)	(0.95)	(0.87)
TBARS	0.92	0.57	1.18	0.54	1.07	0.97	0.65	0.82
	(0.284)	(0.114)	(0.198)	(0.064)	(0.104)	(0.095)	(0.077)	(0.095)

Table 4. Mean specific activities measured in gills of F2 juveniles (S4) for the different chemical conditions. Specific activities are expressed in of protein. Standard errors are given in parentheses under each respective mean ($11 \leq n \leq 20$). The numbers in bold with an asterisk are significantly different ($p < 0.05$) from the values obtained for the control condition.

Parameter	Mode of exposure						
	Control	Transgenerational			Intergenerational		Multigenerational
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	C/R/C	R/R/R
CCO	1.7	1.8	1.62	1.66	**2.11***	1.95	1.71
	(0.085)	(0.096)	(0.083)	(0.077)	(0.14)	(0.062)	(0.067)
CS	0.42	0.43	0.42	0.43	**0.52***	0.46	0.44
	(0.018)	(0.014)	(0.015)	(0.021)	(0.026)	(0.011)	(0.016)
CS:CCO	0.25	0.24	0.26	0.26	0.26	0.24	0.26
	(0.0084)	(0.0073)	(0.0085)	(0.0108)	(0.0119)	(0.0073)	(0.0139)
GPx	0.04	0.046	0.038	0.045	0.052	0.044	0.042
	(0.004)	(0.0029)	(0.004)	(0.0035)	(0.006)	(0.0044)	(0.0041)
LDH	27.94	27.49	26.05	27.58	31.21	30.79	27.93
	(1.08)	(1.57)	(1.07)	(1.1)	(1.31)	(1.09)	(0.87)
LDH:CS	64.9	66.24	61.67	65.44	61.14	67.36	64
	(2.65)	(3.05)	(2.42)	(2.61)	(2.11)	(2.35)	(3.22)

Global methylation in whole F2 larvae

The proportion of 5 -methyl Cytosine (5 -mC) measured in total DNA of whole larvae, comprised between 2.68±0.26 and 3.42±0.21 5mc/total DNA, was similar among the different chemical exposure conditions.

Swimming behaviour

Distances travelled by larvae in darkness were comprised between 4.18±0.64 m and 6.89±1.11 m and between 7.97±0.69 m and 10.16±0.89 m (mean ± se) during the first and second periods. A drastic speed reduction was observed under light exposure, with distances travelled comprised between 1.87±0.24 m and 2.65±0.40 m. Chemical exposure did not induce statistically significant changes in the travelled distance for the different light intensity periods considered. However, a peak of swimming activity was observed during the minute following opening of the light, where larvae from the control condition presented the highest speed. A comparison of the speed of larvae during the minute following the opening of the light, for the different chemical exposure conditions, revealed a statistically significant effect of chemical exposure on the response to light ($p = 0.004$, $df = 7$ and $\chi^2 = 20.35$). A post -hoc test showed that control speed was 47, 40 and 42% higher than C/G/C, C/R/C, and V/C/C, respectively ($p < 0.05$).

Mortality induced by the viral challenge

Mortality was observed for all conditions following the viral challenge with *IHNv*. The lowest cumulative mortality rate was obtained for the non-chemically exposed larvae (i.e. 39.1±5.6%, mean ± se, n = 3), while values ranging from 46.0±1.6% to 83.0±5.7% were obtained for chemically exposed fish, with a significant difference between groups ($p < 0.0001$, $df = 6$ and $\chi^2 = 234$). A post -hoc test revealed a significant difference ($p < 0.05$) in mortality between the C/G/C, R/R/R, and C/R/C conditions, which presented restricted mean survival times (RMSTs) of 14.6±2.6, 23.0±3.9, and 25.3±2.5 dpi (mean ± se, n = 3), compared to 32.9±1.1, 33.8±0.5, 33.8±1.1, and 36.6±2.1 dpi for the control, V/C/C, R/C/C and G/ C/C conditions, respectively.

Maximum daily mortality (i.e. the mortality peak) was observed 6 to 10 dpi for all conditions. On the three pools of dead fish analysed at the mortality peak, *IHNv* was detected in 33.3 to 100% of the samples, depending on the exposure condition (Table 5). The mean viral titre of positive pooled fish was between $2.38x10^9 \pm 1.97x10^9$ and $4.88x10^3 \pm 1.76x10^3$ for the different conditions of chemical contamination, and was not correlated with the cumulative mortality observed.

Table 5. Proportion of *IHNv* -positive pools of fish per condition and mean viral titres at the mortality peak. Viral titres are expressed in 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) as means ± standard error (n = 3).

Condition	Parameters		
	Proportion of *IHNv* -positive fish	Mean titer ($TCID_{50}mL^{-1}$)	se
C/C/C	3/3	2.38×10^9	1.14×10^9
G/C/C	1/3	6.32×10^3	–
R/C/C	2/3	1.03×10^6	6.85×10^5
V/C/C	2/3	4.88×10^3	8.31×10^2
C/G/C	2/3	3.26×10^5	2.16×10^5
C/R/C	3/3	7.40×10^7	3.65×10^7
R/R/R	3/3	8.84×10^4	3.38×10^4

Immuno-hematologic parameters

After 96 h of viral infection (S4), no effects of chemical contamination were detected in the RBCCs or WBCCs of the F2 juvenile rainbow trout. RBCCs ranged between 0.32±0.031 and 0.56±0.11 TL, while WBCCs ranged between 15.69±1.55 and 25.5±3.97 GL.

At 42 dpi (S6), similar proportions of *IHNv* seropositive fish, ranging between 10/15 and 14/15 fish, were detected in the serum of the survivor fish for all exposure conditions (Table 6). However, the control and transgenerationally exposed fish presented higher proportions of highly seropositive fish (comprised between 60 and 85%), compared with fish intergenerationally exposed to glyphosate and Roundup and multigenerationally exposed to Roundup (comprised between 36 and 55%). No statistically significant differences in proportions of highly seropositive fish were found, but the p -value was just above the significance threshold ($p = 0.10$, $df = 6$ and $\chi^2 = 10.51$). Nevertheless, the highly positive individuals presented mean anti -*IHNv* antibody titres that were not significantly affected by chemical contamination.

Table 6. Proportions of *IHNv* -seropositive and highly seropositive survivors per condition and mean anti -*IHNv* antibody titres (mean ± standard error, n = 15) at 60 dpi (S5). High seropositivity is defined as specific antibody titres greater than ≥640. The proportion of highly seropositive fish per condition corresponds to the ratio between the number of fish with a titre greater than ≥640 and the total number of seropositive fish. Mean antibody titres were calculated for highly seropositive fish only.

Condition	Parameters			
	Seropositivity	High seropositivity	Mean anti-*IHNv*	se
		Frequency (%)	antibody titer	
C/C/C	11/15	73	1360	282
G/C/C	10/15	60	1280	286
R/C/C	12/15	83	1280	165
V/C/C	13/15	85	2153	369
C/G/C	13/15	54	3474	813
C/R/C	11/15	55	2560	859
R/R/R	14/15	36	1280	351

Conclusion

The authors concluded that their complex experimental design was an efficient approach to investigate generational effects on rainbow trout exposed to an environmentally relevant concentration of both pure glyphosate and two GBHs. Although no impact was observed on certain reproductive parameters of the F1 generation, the early development of the F2 generation was affected by chemical exposure of previous generations, with effects observed on metabolism, biometrics, and swimming behaviour. The intergenerational effects may be due to direct contact of the F2 organism with contaminants at the stage of germinal cells, while transgenerational effects could reflect epigenetic modifications inherited from the F0 generation. Biochemical parameters appeared to be restored as the fish develop. Intergenerational exposure to pure glyphosate drastically reduced the ability of rainbow trout to face a viral infection, potentially due to the inability of fish to elicit an efficient antibody response. The authors stated that their results demonstrated that glyphosate exposure induced both inter and transgenerational toxicity, sometimes with different effects depending on the physiologic functions considered. GBHs, particularly during early development, seemed to occasionally modulate the effects of the active substance. Re -exposure to glyphosate (i.e. multigenerational exposure) did not increase the toxicity compared to inter or transgenerational exposures. The authors indicated these results need to be strengthened by integrating more specific parameters allowing for an in -depth investigation of the mechanisms of glyphosate toxicity, the relationship between the active substance and the co -formulants, and also toxicity inheritance through generations, which will be helpful to adopt future regulations for the use of glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study investigates the impact of an environmentally relevant concentration of glyphosate on a F2 generation issued from exposed generations F0 and F1. Trans, inter and multigenerational toxicity of 1 µg/L of the active substance was evaluated on early stages of development and juvenile rainbow trout (*Oncorhynchus mykiss*) using different molecular, biochemical, immuno-hematologic, and biometric parameters, behaviour analysis, and a viral challenge. Reproductive parameters of generation F1 were not affected. However, developmental toxicity in generation F2 due to glyphosate alone or co -formulated was observed with head size changes (e.g. head surface up to +10%), and metabolic disruptions (e.g. 35% reduction in cytochrome -c -oxidase). Therefore, LOEC = 1 µg/L.
The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline -compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non -guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals were cultured in the lab.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Uncertain	Pre -dilution of pure glyphosate was done in pure methanol in a concentration so the final dose of methanol exposure was kept under 4 µL/L
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, purity, CAS number and content reported
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	The assessment of the ecotoxicological equivalence was not conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non -guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: LOEC (2 generations)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Just 1 concentration was tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	No	Only 1 concentration was tested

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time -points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5 -8.0), cation exchange capacity, organic carbon (0.5 -2 -5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A -horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/ - 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20 -25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	River water filtered
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15 -25ºC.	Uncertain	Temperature varied from 6 to 15°C (it should have been 12 to 15.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	-
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate,	Yes	Concentrations were analytically

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full -text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
and where relevant its metabolites.		verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	The concentration of glyphosate in water was determined by HPLC and fluorometric methods
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No assessment of the ECX median values was conducted.

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study investigates the impact of an environmentally relevant concentration of glyphosate on a F2 generation issued from exposed generations F0 and F1. Trans, inter and multigenerational toxicity of 1 µg/L of the active substance was evaluated on early stages of development and juvenile rainbow trout (*Oncorhynchus mykiss*) using different molecular, biochemical, immuno-hematologic, and biometric parameters, behaviour analysis, and a viral challenge. Reproductive parameters of generation F1 were not affected. However, developmental toxicity in generation F2 due to glyphosate alone or co -formulated was observed with head size changes (e.g. head surface up to +10%), and metabolic disruptions (e.g. 35% reduction in cytochrome -c -oxidase). Therefore, 2 generations LOEC = 1 µg/L. The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.
Not reliable	No	-

1. Information on the study

Data point:	CA 8.2.6.1
Report author	Kaeoboon S. *et al*.
Report year	2021
Report title	Toxicity response of *Chlorella* microalgae to glyphosate herbicide exposure based on biomass, pigment contents and photosynthetic efficiency
Document No	Plant Science Today, 2021, 8(2), 293-300
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

In this study, the effects of glyphosate at different concentrations (50-500 µg/mL) on three *Chlorella* species including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* especially in relation to the biomass, pigment contents and photosynthetic efficiency were assessed. After treatment for 24 h, the acute toxicity results showed that *C. vulgaris* (IC_{50} = 449.34±6.20 µg/mL) was more tolerant to glyphosate than *C. ellipsoidea* (IC_{50} = 288.23±23.53 µg/mL) and *C. sorokiniana* (IC_{50} = 174.28±0.50 µg/mL). After a 72-h chronic toxicity treatment with glyphosate, glyphosate concentrations decreased to 400-500 µg/mL in *C. ellipsoidea*, 200-300 µg/mL in *C. sorokiniana* and 200-500 µg/mL in *C. vulgaris* respectively. During 24-h acute toxicity exposure to glyphosate, the pigment contents and maximum quantum efficiency of photosystem II (Fv/Fm) decreased as the concentration of glyphosate increased. Overall, the biomass, pigment contents and photosynthetic efficiency presented a high positive correlation. The authors indicated that their study provides detailed information on the toxicity and sensitivity of these *Chlorella* species to glyphosate.

Materials and methods

General chemicals and materials

The reagents and chemicals were purchased as follows: tris base ($H_2NC(CH_2OH)_3$ Tris (hydroxymethyl)-aminomethan; Carlo Eraba, France), NH_4Cl (FLUKA, Switzerland), $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, UK), $CaCl_2 \cdot 2H_2O$ (Ajax Finechem, Australia), K_2HPO_4 (Ajax Finechem, Australia), KH_2PO_4 (Merck, Germany), $Na_2EDTA \cdot 2H_2O$ (Fisher Scientific, UK), $ZnSO_4 \cdot 7H_2O$ (Ajax Finechem, Australia), H_3BO_3 (Merck, Germany), $MnCl_2 \cdot 4H_2O$ (Carlo Eraba, France), $FeSO_4 \cdot 7H_2O$ (Merck, Germany), $CoCl_2 \cdot 6H_2O$ (Ajax Finechem, Australia), $CuSO_4 \cdot 5H_2O$ (Merck, Germany), $(NH_4)6MoO_3$ (Mallinckrodt Chemical, USA), Acetic acid glacial (Carlo Eraba, France), Dimethyl sulfoxide (DMSO; Fisher Scientific, UK) and glyphosate (*N*-(phosphonomethyl)glycine; HPLC grade, Sigma-Aldrich, Germany). Spectrophotometric determinations were performed using a UV-1800 UV-visible spectrophotometer (Shimadzu, Japan). The effective quantum yield was determined by pulse amplitude modulation (PAM 2500, Walz, Germany).

Herbicide stock preparation

The glyphosate used in this study was analytical grade and prepared in sterile distilled water. Serial dilution was performed to achieve the required range of concentrations from 100-500 µg/mL for *C. ellipsoidea* and *C. vulgaris* and 50-300 µg/mL for *C. sorokiniana*. The culture medium was used as diluent.

Strains and culture conditions

C. ellipsoidea (TISTR 8260) and *C. vulgaris* (TISTR 8580) were purchased from the Thailand Institute of Science and Technology. *C. sorokiniana* strain KU.B2, which was isolated from an agricultural drainage in Nonthaburi Province, Thailand (July 2018), was obtained from the culture collection of the Department of Botany, Faculty of Science, Kasetsart University. *Chlorella* species was cultured in liquid TAP medium containing the following micronutrients: 2.42 gm tris base, 25 mL TAP-salt (15 gm/L NH_4Cl, 4 gm/L $MgSO_4 \cdot 7H_2O$ and 2 gm/L $CaCl_2 \cdot 2H_2O$), 1 mL phosphate solution (288 gm/L K_2HPO_4 and 144 gm/L KH_2PO_4), 1 mL trace elements solution (Hutner's trace elements; 50 gm/L $Na_2EDTA \cdot 2H_2O$, 22 gm/L $ZnSO_4 \cdot 7H_2O$, 11.4 gm/L H_3BO_3, 5 gm/L $MnCl_2 \cdot 4H_2O$, 5 gm/L $FeSO_4 \cdot 7H_2O$, 1.6 gm/L $CoCl_2 \cdot 6H_2O$, 1.6 gm/L $CuSO_4 \cdot 5H_2O$ and 1.1 gm/L $(NH_4)6MoO_3$) and 1 mL acetic acid. Then, the medium was adjusted to pH 7.0. *Chlorella* species were cultivated at 30±1°C, under controlled conditions using white cool fluorescent light lamps (330 µmol photons m^{-2} s^{-1}). The cultures were shaken five times per day during incubation. The concentration of each *Chlorella* mixture was quantified by cell counting with a Neubauer chamber. The regression equation for the relationship between cell density (y; $1.0x10^6$ cells/mL) and absorption (x; wavelength at 750 nm) was calculated as follows: $y = 33.944x - 0.8972$ ($R^2 = 0.9998$) for *C. ellipsoidea*, $y = 44.001x - 0.7281$ ($R^2 = 0.9999$) for *C. sorokiniana* and $y = 46.72x - 0.3799$ ($R^2 = 1$) for *C. vulgaris*. The same incubation conditions were used for the determination of IC_{50} and in the glyphosate toxicity treatments.

Toxicity experiments

The biomass was measured as the optical density (OD) at 750 nm. The exponential growth phase of samples was cultured in 150 mL culture medium in an Erlenmeyer flask with initial cell density of $1.0x10^6$ cells/mL for 24, 48 and 96 h. The biomass yield was calculated at 24, 48 and 72 h. Inhibition of growth was monitored as an index to determine the glyphosate toxicity and the IC_{50} value for biomass was calculated based on OD_{750}. In order to enable direct comparison of dose responses for IC_{50}, all responses were converted to % of control.

Photosynthetic pigment determination

The pigment contents were determined by spectrophotometry. 5 mL of each treatment was harvested then centrifuged at 3000 gm for 15 min. Subsequently, the supernatant was discarded and 5 mL DMSO was added to the extract of photosynthetic pigments. The samples were sonicated for 1 h and stored in the dark. After 24 h, each sample was centrifuged at 3000 gm for 15 min. The pigments of the *Chlorella* microalgae samples in the supernatant were analysed by using a spectrophotometer at appropriate wavelengths (470, 649 and 665 nm). The resulting absorbance measurements were obtained from Wellburn A.R. The equations used to calculate the pigment concentrations are as follows:

Chlorophyll *a* (Chl *a*) = 12.19 A665 - 3.45 A649
Chlorophyll *b* (Chl *b*) = 21.99 A649 - 5.32 A665
Total Chlorophyll = Chlorophyll *a* + Chlorophyll *b*
Total Carotenoids = (1000 A470 - 2.86 Chl *a* - 129.2 Chl *b*)/221

The pigment contents are represented as the concentrations in µg/mL.

Chlorophyll fluorescence

The effect of glyphosate on chlorophyll fluorescence was measured as the effective quantum yield using a pulse amplitude modulation (PAM) fluorometer with a suspension cuvette (KS2500; diameter of 7.5 mm, depth of 9.0 mm). Monitoring of the photoinhibition in microalgae was performed using standard method (Magnusson M. *et al*.). The effective quantum yield was determined by immersing the probe directly into the culture (measuring light intensity = 9, gain = 4) and one measurement was taken per replicate after 24 h treatment. The toxic response in each treatment was expressed as a percentage of control values.

Statistical analysis

The experimental treatments had three independent replicates. The results were analysed using GraphPad Prism 6 Software (San Diego, USA). P-values lower than 0.05 were considered as statistically significant with two-way analysis of Dunnett's test. Pearson's correlation was obtained for all treatments.

Results

Growth inhibition test to assess the glyphosate toxicity

The study investigated the glyphosate toxicity in *Chlorella* microalgae, including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* for further use as a biological indicator model. To examine the toxicity effects, *C. ellipsoidea*, *C. sorokiniana* and *C. vulgaris* were treated with 100-500, 50-300 and 100-500 µg/mL glyphosate, respectively. The growth inhibition test clearly manifested that glyphosate treatment induced a significant inhibitory effect on the acute toxicity in the three species after 24 h. Compared to the control, the biomass of *C. ellipsoidea* and *C. sorokiniana* was significantly inhibited by 50-500 µg/mL glyphosate and that of *C. vulgaris* was inhibited at 200 µg/mL. *C. vulgaris* (IC_{50} = 449.34±6.20 µg/mL) showed the greatest tolerance to glyphosate compared to *C. ellipsoidea* (IC_{50} = 288.23±23.53 µg/mL) and *C. sorokiniana* (IC_{50} = 174.28±0.50 µg/mL).

The glyphosate caused a decrease in biomass of all species as both concentration and time increased, leading to chronic toxicity. Between 24 and 48 h treatment, the biomass of *C. ellipsoidea* and *C. sorokiniana* did not exceed 300 µg/mL and 150 µg/mL respectively. After 72 h of treatment, the glyphosate concentration inhibit growth in the following concentrations: 400-500 µg/mL for *C. ellipsoidea*, 200-300 µg/mL for *C. sorokiniana* and 200-500 µg/mL for *C. vulgaris*.

Pigment contents after glyphosate exposure

After the 24 h treatment, the concentrations of total chlorophyll and carotenoids were significantly different among *Chlorella* sp. and the control. As indicated in Table 1, increased glyphosate concentration had a negative linear effect on the chlorophyll and carotenoids concentrations. For both *C. ellipsoidea* and *C. sorokiniana* inhibited growth, the value of total carotenoid content was relate similar to biomass result at the same concentration.

Table 1. Pigment contents of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), and carotenoid in (A) *Chlorella ellipsoidea*, (B) *Chlorella sorokiniana* and (C) *Chlorella vulgaris* after treated with different concentrations of glyphosate (50-500 µg/mL) for 24 h. The results are presented as the mean ± standard deviation in triplicate (n = 3).

(A)

Pigment content (µg ml⁻¹)	Glyphosate (µg ml⁻¹)					
	control	100	200	300	400	500
chl *a*	6.70 ± 0.06	4.81 ± 0.01	4.25 ± 0.01	2.06 ± 0.02	1.70 ± 0.01	1.96 ± 0.01
chl *b*	5.50 ± 0.04	3.99 ± 0.06	3.57 ± 0.16	1.72 ± 0.02	1.26 ± 0.02	1.42 ± 0.02
total chlorophyll	12.20 ± 0.04^{a}	8.80 ± 0.08^{b}	7.82 ± 0.15^{c}	3.78 ± 0.04^{d}	2.96 ± 0.03^{e}	3.37 ±.0.21^{f}
total carotenoid	1.84 ± 0.09^{a}	1.50 ± 0.01^{b}	1.24 ± 0.06^{c}	0.53 ± 0.01^{d}	0.37 ± 0.00^{d}	0.43 ± 0.01^{d}

(B)

Pigment content (µg ml⁻¹)	Glyphosate (µg ml⁻¹)					
	control	50	100	150	200	300
chl *a*	3.24 ± 0.01	2.36 ± 0.01	0.68 ± 0.01	0.44 ± 0.01	0.12 ± 0.00	0.09 ± 0.00
chl *b*	2.83 ± 0.01	2.08 ± 0.03	0.73 ± 0.00	0.55 ± 0.01	0.22 ± 0.01	0.20 ± 0.01
total chlorophyll	6.08 ± 0.02^{a}	4.44 ± 0.04^{b}	1.41 ± 0.01^{c}	0.99 ±0.02^{d}	0.34 ± 0.01^{e}	0.30 ± 0.01^{f}
total carotenoid	0.82 ±0.01^{a}	0.64 ± 0.00^{b}	0.26 ± 0.00^{c}	0.17 ± 0.01^{d}	0.10 ± 0.00^{e}	0.090 ± 0.00^{e}

(C)

Pigment content (µg ml⁻¹)	Glyphosate (µg ml⁻¹)					
	control	100	200	300	400	500
chl *a*	5.92 ± 0.19	4.69 ± 0.02	2.80 ± 0.01	1.98 ± 0.00	1.66 ± 0.01	1.54 ± 0.01
chl *b*	2.79 ± 0.11	3.41 ± 0.02	2.20 ± 0.01	1.71 ± 0.01	1.30 ± 0.01	1.16 ± 0.02
total chlorophyll	8.71 ±0.09^{a}	8.10 ± 0.01^{b}	5.00 ± 0.02^{c}	3.69 ± 0.01^{d}	2.96 ± 0.02^{e}	2.70 ± 0.01^{f}
total carotenoid	1.38 ± 0.02^{a}	1.26 ± 0.01^{a}	0.86 ± 0.01^{b}	0.67 ± 0.02^{cd}	0.55 ± 0.00^{d}	0.48 ± 0.00^{de}

Photosynthetic efficiency after exposed to glyphosate

The inhibition of effective quantum yield (Fv/Fm) using pulse amplitude modulation (PAM) fluorometer revealed similar patterns in the biomass and pigment contents of the three *Chlorella* species after 24 h cumulative glyphosate exposure. The results demonstrate that 400, 150 and 500 µg/mL glyphosate reached the level of highest inhibition of quantum yield of *C. ellipsoidea*, *C. sorokiniana* and *C. vulgaris*, respectively.Thus, the findings indicate that *C. vulgaris* was least influenced by glyphosate.

Correlation analysis

In this study, the obtained Pearson's correlation was utilized as a guide to evaluate the correlation coefficient (*r*). The correlation coefficients of the biomass, pigment contents and photosynthetic performance of three *Chlorella* species indicate that all species present high positive correlation ($r > 0.7$) after 24 h exposure.

Conclusion

The authors concluded from their experimental results that glyphosate exposure affects biomass, pigment contents and photosynthetic efficiency of *Chlorella* species. After 24-h acute toxicity glyphosate exposure in terms of biomass, *C. vulgaris* showed the greatest tolerance, while *C. sorokiniana* was the most sensitive. After 72-h chronic toxicity, the biomass yield of all *Chlorella* species was at a relatively low concentration level, which indicated, according to the authors, that the effect of glyphosate is both concentration and time-dependent. Similar to the biomass results, glyphosate exposure leads to reduced pigment contents and photosynthetic efficiency following 24-h exposure. This indicated to the authors that the relationship between the biomass, pigment contents and photosynthetic efficiency is significantly correlated in this study. The authors stated that these results could be beneficial to understanding the impact and potential risk of glyphosate toxicity on microalgae in aquatic environments.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This article reports the effects of glyphosate at different concentrations (50-500 mg/L) on three *Chlorella* species including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* in relation to the biomass, pigment contents and photosynthetic efficiency. The study seems to have been well conducted and reports regulatory relevant endpoints: 24-h acute EC_{50} = 449.34, 288.23 and 174.28 mg/L for *Chlorella vulgaris*, *Chlorella ellipsoidea* and *Chlorella sorokiniana*, respectively. Chronic endpoints at 72-h could also be calculated.
The study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium and the temperature during the tests and culture are higher than recommended for green algae. Purity of the test item is not given.

Assessment and conclusion by RMS:

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	-
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	The culture medium was directly used as diluent

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	No	The producer is provided, but not the purity
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Validity criteria cannot be assessed because no raw data are available
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	No analytical verifications of the concentration of glyphosate in the test media were conducted
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint reported: EC_{50} (24-h)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	5 concentrations tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Uncertain	No analytical verifications of the concentration of glyphosate in the test media were conducted
15. A clear concentration response relationship is reported - in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	3 replicates for each treatment group were tested
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation were provided. No raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented	Yes	Statistical analysis

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
(e.g., checking the plots and confidence intervals).		sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not relevant, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not relevant, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not relevant, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not relevant, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not relevant, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not relevant, aquatic study
20.6. Data on precipitation is recorded.	-	Not relevant, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not relevant, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	No bee study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	No	Temperature was 30ºC (should have been in the range 21-24ºC)
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Uncertain	No analytical verifications of the concentration of glyphosate in the test media were conducted
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Uncertain	No analytical verifications of the concentration of glyphosate in the test media were conducted
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria		Criteria met? Yes / No / Uncertain	Comment / Justification
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.		No	No ECx assessment was conducted
Overall assessment			
Reliable without restrictions	No	-	
Reliable with restrictions	Yes	This article reports the effects of glyphosate at different concentrations (50-500 mg/L) on three *Chlorella* species including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* in relation to the biomass, pigment contents and photosynthetic efficiency. The study seems to have been well conducted and reports regulatory relevant endpoints: 24-h acute EC_{50} = 449.34, 288.23 and 174.28 mg/L for *Chlorella vulgaris*, *Chlorella ellipsoidea* and *Chlorella sorokiniana*, respectively. Chronic endpoints at 72-h could also be calculated. The study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium and the temperature during the tests and culture are higher than recommended for green algae. Purity of the test item is not given.	
Not reliable	No	-	

1. Information on the study

Data point:	CA 8.2.7
Report author	Mendes E. J. *et al.*
Report year	2021
Report title	Isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid on the physiology and water remediation capacity of *Salvinia molesta*
Document No	Journal of Hazardous Materials, 2021, 417, 125694
Guidelines followed in study	None (partially based on OECD TG 221)
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors evaluate the isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid (AMPA) and the potential of the aquatic macrophyte *Salvinia molesta* to remove these chemicals from contaminated water. Plants were exposed to environmentally relevant concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) for seven days. Then, based on the effective concentrations of glyphosate found to reduce photosynthetic rates by 10% (EC_{10}) and 50% (EC_{50}), the plants were exposed to combinations of 0, 16 and 63.5 µg glyphosate/L and 0, 5, 15, 25 µg AMPA/L. The $EC_{(10)}$ and $EC_{(50)}$ were lower for AMPA (6.1 µg/L and 28.4 µg/L respectively) than for glyphosate (16 and 63.5 µg glyphosate/L respectively). When occurring together, the deleterious effects of those chemicals to plants increased. *S. molesta* plants removed up to 74.15% of glyphosate and 71.34% of AMPA from culture water. Due to its high removal efficiency, *S. molesta* can be used in phytoremediation programs. It will be important to evaluate the combined effects of glyphosate and AMPA in any toxicological studies of the herbicide.

Materials and methods

Plant material

Salvinia molesta D. Mich (Salviniaceae) plants were collected at Barigui Park (Curitiba, PR, Brazil). Before initiating the experiments, the macrophytes were acclimated in plastic pots (50 L) containing autoclaved and reconstituted water under controlled conditions (temperature 20±2ºC, and a 12/12 h light cycle, at 100 µmol photons m^{-2} s^{-1}) for 60 days, for depuration. The plants were then washed for 3 min in a 0.5% hypochlorite solution, rinsed thoroughly in distilled water, and transferred to 5 L plastic pots containing autoclaved reconstituted water. The pots were kept in biological oxygen demand (B.O.D) chambers under the controlled condition mentioned above for 15 days until used. Tests were performed in 250 mL Erlenmeyer flasks stoppered with cotton wool containing 100 mL of autoclaved reconstituted water and the desired treatments. A density of 15 g plant/L was used in the bioassays.

Bioassays

Plants were submitted to environmental representative concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) in water for seven days following OECD guidelines. Those concentrations were chosen following worldwide occurrences of glyphosate and AMPA in surface waters, thus being environmentally representative concentrations. To investigate the interactive effects of glyphosate and AMPA, plants were exposed for seven days to glyphosate concentrations causing 10% (EC_{10}; 16 µg glyphosate/L) and 50% (EC_{50}; 63.5 µg glyphosate/L)

reductions in photosynthesis, plus increasing concentrations of AMPA (0, 5, 15, 25 µg/L). The AMPA concentrations used in the glyphosate AMPA assays were chosen based on concentrations known to cause photosynthetic reductions in those plants. The experiments were conducted under the same above-mentioned conditions of temperature and illumination. Analytical grade glyphosate and AMPA were used in all experiments. Parallel bioassays were conducted in flasks without plants under the same conditions to evaluate the degradation of chemicals by light, temperature, and hydrolysis.

Photosynthesis and pigment evaluations

After harvesting, the plants were thoroughly washed in distilled water (50 mL, three times) to remove unabsorbed chemical residues that might be present on plant surfaces. Photosynthetic parameters were evaluated using whole plants in a closed-infrared gas analyser system (CI-340 Hand-held Photosynthesis System; CID Bio-Science Inc., USA). Net photosynthetic rates (P_N) were evaluated three times/plant at -100 µmol photons m^{-2} s^{-1}. After the photosynthetic evaluations, the plants were flash-frozen in liquid nitrogen and subsequently stored at -80 ºC in aluminium foil until used for pigment and biochemical analyses. Chlorophyll and pheophytin *a* and *b* concentrations were measured in plants ground in 80% acetone.

Oxidative stress markers

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations were determined according to using 0.1 g of plants. Ascorbate peroxidase and catalase activities were evaluated after the determination of the total protein concentration in 0.1 g of plants ground in liquid nitrogen and homogenized in 1.0 mL of an extraction solution containing 100 mM phosphate buffer (pH 7.8), 100 mM EDTA, 1 mM *L-ascorbate*, and 2% polyvinylpyrrolidone (PVP) (m/v).

Chemical analyses

The concentrations of glyphosate and AMPA in the water medium and plants (initial and final concentrations) were determined using a LC-MS/MS system coupled to HPLC. Glyphosate and AMPA were extracted from 0.4 g of plants, previously thoroughly washed in ultrapure water, using 50 mL of acidified water. The plant extracts and samples of the growth solutions were filtered through C18 SPR cartridges previously conditioned with 15 mL of acidified water and 5 mL of methanol. The cartridges containing the samples were washed with 3 mL of 50% methanol in water (v/v). The eluate was then dried in a SpeedVac machine (RC1010, Thermo), and the residues resuspended in the mobile phase A. Before injection, the samples were filtered through nylon syringe filters.

Chromatographic separations were performed using an Ascentis® C18 column with a mobile phase consisting of 5 mM of ammonium acetate in water (phase A) and 5 mM of ammonium acetate in methanol (phase B), both pH 7.0. Mass spectrometry analyses were operated in a negative ion mode. In addition to glyphosate and AMPA, shikimate concentrations were also quantified in plants using the same LC-MS/MS technique. Analytical-grade glyphosate, AMPA, and shikimate were used to prepare the calibration curves. The six-point calibration curves showed good linearity for the analytes ($r^2 \geq 0.95$; $p < 0.0001$). Each sample batch included three blanks, three standards, and three fortified samples (for quality control). The recovery rates for all of the compounds were greater than 87%. The natural degradation of glyphosate and AMPA in the water (degradation) as well as the efficiency of plant removal of glyphosate and AMPA (removal efficiency) were calculated. as following:

$$Degradation(\%) = 100 - \left(\frac{C_7 \quad without\ plants}{C_0 \quad without\ plants} x \quad 100\right)$$

$$Removal\ efficiency(\%) = 100 - \left(\frac{C_7 \quad with\ plants}{C_0 \quad with\ plants} x \quad 100\right) - degradation$$

where C_0 and C_7 are the initial and final concentration of glyphosate or AMPA in the water.

Statistical analysis

The effective concentrations causing 10% (EC_{10}) or 50% (EC_{50}) photosynthetic reductions were determined from the nonlinear least square fits, using an inverse regression curve.

Statistical analyses were performed on JMP 7.0 software (SAS Institute Inc.). Data were tested for normality (Shapiro-Wilk) and homogeneity (Bartlett), and then statistically evaluated. Data from plants treated only with glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or only with AMPA (0, 10, 20, 30, 40 and 50 µg/L) were evaluated using one-way analysis of variance (ANOVA), and the means compared using a post hoc Tukey test ($P < 0.05$). Data from plants submitted to the factorial treatments with glyphosate (0, 16 and 63.5 µg/L) and AMPA (0, 5, 15, 25 µg/L) were evaluated using two-way ANOVA. Interactions between glyphosate and AMPA were included in the model. When differences were detected by ANOVA, the means were compared by a post hoc Tukey test ($p < 0.05$).

Results

Effects of glyphosate on plant physiology

Visible symptoms of injury (chloroses and some necrotic spots) were only observed in plants exposed to glyphosate concentrations ≥60 µg/L. Any symptoms of leaf injuries were restricted to the bases of the leaves and did not involve more than 30% of the leaf biomass, even among plants exposed to highest glyphosate concentrations. Plants exposed to glyphosate concentrations ≥40 µg/L showed decreased net photosynthetic rates (P_N; $F = 40.47$) and chlorophyll *b* concentrations ($F = 26.86$; $p < 0.001$), while their pheophytin *b*/chlorophyll *b* ratios increased ($F = 29.02$; $p < 0.0001$). The effective concentration causing 10% (EC_{10}) and 50% (EC_{50}) photosynthetic decreases were 16 and 63.5 µg/L respectively. Reductions of chlorophyll *a* concentrations were only observed in plants exposed to the highest glyphosate concentration (100 µg/L) ($F = 7.46$; $p < 0.01$), although their pheophytin *a*/chlorophyll *a* ratios were not significantly affected ($F = 1.16$; $p > 0.05$) by herbicide exposure.

Shikimate concentrations ($F = 57.39$) and ascorbate peroxidase activities (APX; $F = 33.26$) were greater in glyphosate-treated plants as compared to the control ($p < 0.0001$; Figure 3). Hydrogen peroxide (H_2O_2; $F = 36.85$) and MDA concentrations ($F = 47.66$) increased in plants treated with glyphosate concentrations ≥40 µg/L ($p < 0.0001$), while catalase activity (CAT) increased in plants treated with glyphosate at concentrations ≥60 µg/L ($F = 62.49$, $p < 0.0001$).

Effects of AMPA on plant physiology

Visible symptoms of injury (chlorosis) were only observed in plants exposed to AMPA at concentrations ≥30 µg/L. Similar to glyphosate-exposed plants, AMPA-exposed plants survived seven days of exposure to that compound. P_N ($F = 90.23$) and chlorophyll *a* concentrations ($F = 31.08$) significantly decreased ($p < 0.0001$) in plants exposed to AMPA. Chlorophyll *b* concentrations ($F = 9.67$) decreased in plants exposed to AMPA concentrations ≥20 µg/L in relation to the control ($p < 0.0001$). Pheophytin *a*/chlorophyll *a* ($F = 1.92$) and pheophytin *b*/chlorophyll *b* ($F = 1.00$) ratios were not significantly affected ($p > 0.05$) by AMPA treatments in relation to the control.

Shikimate concentrations were lowest in plants treated with 10 µg glyphosate/L ($F = 12.91$; $p < 0.0001$). H_2O_2 ($F = 130.86$) and MDA concentrations ($F = 46.91$), as well as APX ($F = 62.12$) and CAT ($F = 24.73$) activities significantly increased in AMPA-treated plants in relation to controls ($p < 0.0001$).

Interactive effects of glyphosate and AMPA on plant physiology

Chlorosis and necrosis were observed in plants when exposed to combined concentrations of glyphosate and AMPA, regardless of their concentrations. Although some leaves died, most of the leaf tissue appeared to remain viable. Significant interactions between glyphosate and AMPA addition were observed ($p < 0.01$) for all of the physiological parameters evaluated, except shikimate concentrations ($p > 0.05$). The addition of AMPA to plants exposed to glyphosate decreased their P_N and photosynthetic pigments and increased pheophytin/chlorophyll ratios – except among plants treated with 16 or 63 µg glyphosate/L + 5 AMPA/L, when chlorophyll *b* concentrations and pheophytin *b*/chlorophyll *b* ratios did not significantly differ from plants treated solely with equivalent glyphosate concentrations (16 or 63 µg glyphosate/L + 0 µg AMPA/L).

Shikimate concentrations in plants increased as glyphosate concentrations increased ($p < 0.0001$) but were not affected by AMPA addition ($p > 0.05$). Regardless of the glyphosate concentration used, the

addition of AMPA significantly increased plant H_2O_2 and MDA concentrations, as well as APX and CAT activities.

Glyphosate and AMPA degradation in the water

As can be seen in Table 1, no glyphosate or AMPA was detected in the water of flasks at the initial time (T_0) without their purposeful addition, indicating no inter-contaminations between treatments receiving only glyphosate or only AMPA. In systems without plants, glyphosate and AMPA degradation in water after seven days ranged from 0.93% to 4.58% and 1.55-3.57%, respectively, when the chemicals were applied alone. Residual concentrations of AMPA (0.49-1.30 µg/L) were observed after seven days in the water of flasks without plants and receiving only glyphosate. When combined, glyphosate and AMPA degradations ranged from 0.72% to 6.24% and 0.76 to 4.85% respectively. Once again, AMPA was detected in flasks with only glyphosate added.

Table 1. Glyphosate and AMPA concentrations in water media at the beginning of the experiments (T_0) and after seven days (T_7) and chemical degradation percentages in flasks without *Salvinia molesta* plants.

Treatment	Glyphosate (µg l^{-1})			AMPA (µg l^{-1})		
	T_0	T_7	Degradation (%)	T_0	T_7	Degradation (%)
0 µg l^{-1}	n.d.	n.d.	–	n.d.	n.d.	–
Glyphosate						
20 µg l^{-1}	20.12 ± 2.26	19.18 ± 1.97	4.58 ± 1.16	n.d.	0.77 ± 0.24	–
40 µg l^{-1}	41.67 ± 2.66	41.06 ± 2.64	1.45 ± 1.03	n.d.	0.49 ± 0.32	–
60 µg l^{-1}	61.94 ± 1.69	61.23 ± 1.36	1.12 ± 0.70	n.d.	0.56 ± 0.39	–
80 µg l^{-1}	79.84 ± 1.24	79.09 ± 0.97	0.93 ± 0.60	n.d.	0.54 ± 0.38	–
100 µg l^{-1}	102.96 ± 3.52	101.22 ± 4.04	1.70 ± 1.02	n.d.	1.30 ± 0.61	–
AMPA						
10 µg l^{-1}	n.d.	n.d.	–	10.33 ± 0.54	10.17 ± 0.52	1.95 ± 0.53
20 µg l^{-1}	n.d.	n.d.	–	20.39 ± 0.48	19.66 ± 0.43	3.57 ± 1.20
30 µg l^{-1}	n.d.	n.d.	–	30.83 ± 1.07	30.16 ± 0.43	3.29 ± 2.47
40 µg l^{-1}	n.d.	n.d.	–	41.05 ± 0.84	40.40 ± 0.77	1.55 ± 0.99
50 µg l^{-1}	n.d.	n.d.	–	51.17 ± 1.39	49.84 ± 0.93	2.58 ± 0.95
Glyphosate + AMPA						
0 µg glyphosate l^{-1} + 5 µg AMPA l^{-1}	n.d.	n.d.	–	5.20 ± 0.24	4.97 ± 0.15	4.35 ± 1.54
0 µg glyphosate l^{-1} + 15 µg AMPA l^{-1}	n.d.	n.d.	–	15.73 ± 0.58	15.15 ± 0.62	3.66 ± 2.26
0 µg glyphosate l^{-1} + 25 µg AMPA l^{-1}	n.d.	n.d.	–	26.20 ± 1.44	25.03 ± 1.22	4.40 ± 2.00
16 µg glyphosate l^{-1} + 0 µg AMPA l^{-1}	16.87 ± 1.16	16.12 ± 1.28	4.46 ± 1.73	n.d.	0.52 ± 0.15	–
16 µg glyphosate l^{-1} + 5 µg AMPA l^{-1}	16.96 ± 1.29	15.92 ± 1.53	6.24 ± 2.20	5.65 ± 0.28	5.42 ± 0.32	3.93 ± 5.52
16 µg glyphosate l^{-1} + 15 µg AMPA l^{-1}	15.47 ± 0.91	15.09 ± 1.05	2.51 ± 2.02	15.81 ± 0.92	15.22 ± 0.79	4.26 ± 6.73
16 µg glyphosate l^{-1} + 25 µg AMPA l^{-1}	16.74 ± 1.12	16.12 ± 1.28	3.70 ± 2.62	26.06 ± 1.34	25.09 ± 1.59	4.85 ± 6.88
63.5 µg glyphosate l^{-1} + 0 µg AMPA l^{-1}	63.93 ± 0.99	63.47 ± 1.13	0.72 ± 5.52	n.d.	0.58 ± 0.03	–
63.5 µg glyphosate l^{-1} + 5 µg AMPA l^{-1}	65.31 ± 1.71	64.62 ± 1.59	1.04 ± 0.26	5.85 ± 0.68	5.85 ± 0.61	0.76 ± 0.65
63.5 µg glyphosate l^{-1} + 15 µg AMPA l^{-1}	63.80 ± 1.06	62.65 ± 1.41	1.81 ± 0.61	13.00 ± 5.28	12.68 ± 4.58	3.19 ± 2.39
63.5 µg glyphosate l^{-1} + 25 µg AMPA l^{-1}	63.95 ± 1.24	62.90 ± 1.25	1.64 ± 0.17	25.53 ± 0.37	24.09 ± 0.91	1.82 ± 2.21

Values are means ± SD of four replicates. n.d. = not detected

Glyphosate and AMPA uptake by plants

No glyphosate or AMPA was detected in plants at the initial time (T_0). Among plants exposed to only glyphosate, plant glyphosate concentrations increased with the addition of glyphosate up to 80 µg/L, not differing between plants exposed to 80 and 100 µg glyphosate/L ($F = 105.79$; $p < 0.0001$). AMPA was also observed in plants exposed to only glyphosate, and increased as glyphosate addition increased (but did not differ between plants exposed to 60 and 80 µg glyphosate/L) ($F = 134.60$; $p < 0.0001$). In plants treated with glyphosate only, the effective removal of glyphosate ranged from 55% to 78%.

No glyphosate was detected in plants exposed to AMPA only. Among AMPA-treated plants, AMPA concentrations increased with the AMPA addition to the medium up to 30 µg/L, not differing between plants exposed to 30, 40 and 50 µg AMPA/L ($F = 144.33$; $p < 0.0001$). In plants exposed only to AMPA, its effective removal ranged from 62% to 71%.

Significant interactions ($p < 0.001$) between glyphosate and added AMPA were observed in terms of glyphosate and AMPA concentrations in the plants as well as for the removal efficiency of glyphosate ($F = 6.20$) and AMPA ($F = 43.03$). Increased concentrations of glyphosate were observed in plants exposed to 63.5 µg glyphosate/L when AMPA was added. In the absence of glyphosate, AMPA concentration was greatest in plants exposed to 25 µg AMPA/L, and did not differ between plants treated with 5 or 15 µg AMPA/L. In the presence of glyphosate, however, AMPA concentration in plants increased with AMPA addition. AMPA was observed in glyphosate-treated plants without any AMPA addition. The greatest AMPA concentrations were observed in plants treated with 15 and 25 µg AMPA/L and exposed to 16 µg glyphosate/L. The glyphosate removal efficiency of plants ranged

from 58% to 87%, being highest in plants treated with 16 µg glyphosate/L and 15 or 25 µg AMPA/L. AMPA removal efficiency did not differ among glyphosate-treated plants when AMPA was added to water; in the absence of glyphosate, AMPA removal was greatest in plants treated with 5 µg AMPA/L.

Conclusion

The authors concluded that they evidenced the deleterious effects that environmentally representative concentrations of glyphosate and AMPA have on the macrophyte *Salvinia molesta*. Based on photosynthetic rates, glyphosate showed lower EC_{10} and EC_{50} values (16 and 63.5 µg/L respectively) than AMPA (6.1 µg/L and 28.4 µg/L respectively), which was more toxic to the plants. The mechanisms by which glyphosate and AMPA affect photosynthesis were distinct: in addition to disrupting shikimic acid (and, thus, protein biosynthesis), glyphosate appears to induce chlorophyll degradation through ROS accumulation; AMPA, on the other hand, apparently disrupts pigment biosynthesis. Glyphosate and AMPA showed synergistic effects, increasing deleterious effects when occurring together in plant tissues. It will therefore be important to evaluate the combined effects of glyphosate and AMPA in toxicological studies of that herbicide. Despite the negative effects of the contaminants on its physiology, *S. molesta* plants were efficient at reducing glyphosate and AMPA concentrations in the surrounding water. When those compounds were added separately to the medium, the plants removed up to 74.15% of glyphosate and 71.34% of AMPA. Moreover, at a glyphosate concentration of 16 µg/L, the presence of AMPA increased the glyphosate-removal efficiency of *S. molesta*. Therefore, the use of the macrophyte *S. molesta* for the removal of glyphosate and AMPA from water can constitute an environmentally friendly technology, reducing the costs otherwise associated with physicochemical methods of contaminant removal.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study evaluates the isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid (AMPA) on the aquatic macrophyte *Salvinia molesta*. Plants were exposed to environmentally relevant concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) for seven days. Then, based on the effective concentrations of glyphosate found to reduce photosynthetic rates by 10% (EC_{10}) and 50% (EC_{50}), the plants were exposed to combinations of 0, 16 and 63.5 µg glyphosate/L and 0, 5, 15, 25 µg AMPA/L. The EC_{10} and EC_{50} were lower for AMPA (6.1 µg/L and 28.4 µg/L, respectively) than for glyphosate (16 and 63.5 µg glyphosate/L, respectively). When occurring together, the deleterious effects of those chemicals to plants increased.
The study is considered not fully reliable because it is not possible to identify the number of plants tested for each treatment and because plants were collected from the field in Brazil with no record of previous pesticide exposure (although they were maintained in the lab for 60 days for depuration).

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	Study conducted according to OECD TG 221, but validity criteria cannot be verified because the measured variables (mainly photosynthetic rate) are different from those stated in the OECD GD (inhibition of growth).
2. No previous exposure to other chemicals is documented (where relevant).	Uncertain	Plants were collected from the field in Brazil with no record of previous pesticide exposure, but were maintained in the lab for 60 days for depuration
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Analytical grade glyphosate and AMPA (Pestanal®, Sigma-Aldrich, Canada) were used in all experiments
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	Study conducted according to OECD TG 221, but validity criteria cannot be verified
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of	Yes	-

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
glyphosate (or a glyphosate metabolite).		
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	The concentrations of glyphosate and AMPA in the water medium and plants (initial and final concentrations) were determined
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: EC_{50} (7-d) for both glyphosate and AMPA
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	-
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Analytical verifications were conducted at the beginning and the end of the test
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Uncertain	A density of 15 g plant/L was used in the bioassays, but the number of replicates is not known.
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature set at 20°C for the tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Yes	Analytical verifications were conducted at the beginning and the end of the test
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Analytical verifications were conducted at the beginning and the end of the test
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	Analytical methods were described
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	95% CI values were not reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study evaluates the isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid (AMPA) on the aquatic macrophyte *Salvinia molesta*. Plants were exposed to environmentally relevant concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) for seven days. Then, based on the effective concentrations of glyphosate found to reduce

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria			Criteria met? Yes / No / Uncertain	Comment / Justification
		photosynthetic rates by 10% (EC_{10}) and 50% (EC_{50}), the plants were exposed to combinations of 0, 16 and 63.5 µg glyphosate/L and 0, 5, 15, 25 µg AMPA/L. The EC_{10} and EC_{50} were lower for AMPA (6.1 µg/L and 28.4 µg/L, respectively) than for glyphosate (16 and 63.5 µg glyphosate/L, respectively). When occurring together, the deleterious effects of those chemicals to plants increased. The study is considered not fully reliable because it is not possible to identify the number of plants tested for each treatment and because plants were collected from the field in Brazil with no record of previous pesticide exposure (although they were maintained in the lab for 60 days for depuration).		
Not reliable	No	-		

1. Information on the study

Data point:	CA 8.2.8
Report author	Vera M. S. *et al.*
Report year	2021
Report title	First evaluation of the periphyton recovery after glyphosate exposure
Document No	Environmental Pollution, 2021, 290, 117998
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors evaluated the potential of freshwater periphyton to recover from glyphosate exposure using microcosms under laboratory conditions. Periphyton developed on artificial substrates was exposed to 0.4 or 4 mg/L monoisopropylamine salt of glyphosate (IPA) for 7 days, followed by translocation to herbicide-free water. The authors sampled the community 1, 2 and 3 weeks after the transfer. Dry weight, ash-free dry weight, chlorophyll *a*, and periphyton abundances were analysed. The periphyton impacted with the lowest IPA concentration recovered most of the structural parameters within 7 days in clean water, but the taxonomic structure did not entirely recover towards the control structure. Periphyton exposed to 4 mg IPA/L could not recover during 21 days in herbicide-free water, reaching values almost four times higher in % of dead diatoms and four times lower in ash-free dry weight concerning the control at the end of the study. Results suggest a long-lasting effect of the herbicide due to the persistence within the community matrix even after translocating periphyton to decontaminated water. The authors concluded that the exposure concentration modulates the recovery potential of IPA-impacted periphyton. The current research is the first to study the recovery in glyphosate-free water of periphyton exposed to the most commonly used herbicide in the world. Finally, the authors highlighted the need for more studies focused on the recovery potential of freshwater ecosystems and aquatic communities after glyphosate contamination.

Materials and methods

Periphytic community

Ad hoc devices (periphytometers) were set sub-superficially in a 3000 litres artificial outdoor pond ([chlorophyll *a*] = 1.6 μg/L; turbidity = 1.17 NTU) located in an experimental field of the University of Buenos Aires, to support artificial substrates for periphytic colonization. This pond has never been contaminated with glyphosate or any other pesticide. After four weeks, periphytometers with colonized substrates were removed and transferred from the experimental field to the laboratory in dark and cold. When periphytometers were removed, physical parameters were recorded in situ and water samples to determine glyphosate, phytoplanktonic chlorophyll *a* and nutrients concentrations were collected. Also, periphyton samples from colonized substrates were taken to determine periphyton chlorophyll *a* concentration.

Experimental design

Each microcosm (experimental unit, e.u.) consisted of an autoclaved glass beaker with 250 mL of filtered and autoclaved pond water with four colonized substrates vertically immersed. Pond water, where the periphytic colonization carried out, was filtered through a mesh and then autoclaved to remove other communities, which may modify and confound the responses of the periphyton.

Thirty-nine microcosms were placed randomly in a culture chamber with controlled photoperiod (12-h light/dark), light intensity (cool white fluorescent light of 30 µmol photon m^{-2} s^{-1}) and temperature (25±1ºC), and they were acclimatized to laboratory conditions for 48 h. Afterwards, 3 treatments were randomly assigned to the e.u. (by triplicate): two monoisopropylamine salt of glyphosate (IPA) concentrations (40 wt% in H_2O, CAS: 38641-94-0): (i) 0.4 mg/L, low glyphosate (LG), and (ii) 4 mg/L, high glyphosate (HG) treatments, and a (iii) control, without herbicide (C).
After the 48-h stabilization, two periods were considered, the exposure and the recovery. At the exposure period, samples were taken the day the experiment began (T0) and 7 days later (T7). On day 7, the periphyton was transferred by translocating the colonized substrates to beakers with herbicide-free water (filtered and autoclaved). Subsequently, samples were taken at 7, 14, and 21 days (R7, R14, and R21, respectively) after translocation to assess community recovery. At each sampling time, three microcosms per treatment were extracted from the chamber to determine physical, chemical and biological parameters. Also, at the start of the recovery period (R0), before translocating the community, physical and chemical parameters in water were measured to determine the initial recovery conditions of the medium.

Physical, chemical and biological variables

Conductivity, pH, temperature and dissolved oxygen concentration (DO) were measured. The temperature in microcosms was registered to check the correct functioning of the culture chamber. Nephelometric turbidity was assessed. Total phosphorus (TP) and nitrogen (TN), and soluble reactive phosphorus (SRP) were determined. TP and TN were determined from unfiltered water samples after acid digestion and SRP from filtered samples through Whatman GF/F filters.
Glyphosate concentration was measured by ion chromatography using a DIONEX DX-100©. The detection limit achieved was 0.2 mg/L. The standard deviation estimated for this analytical method was 3%. The periphyton was removed by scraping the surface of artificial substrates, brought to a known volume with distilled water, and sub-sampled for different analyses. All periphytic variables were expressed per unit area. Sample for quantitative analysis of the autotrophic fraction was fixed and preserved in cold and dark. Counts were performed using the inverted microscope technique. Periphyton classes' percentages and live and dead diatom abundance were considered. Another sample was used to determine dry weight (DW) and ash-free dry weight (AFDW) of periphyton. The periphytic chlorophyll *a* concentration (Chla) corrected by phaeopigments was estimated from the material retained in Whatman GF/F filters. Pigment extraction was carried out and Chla was quantified. The phytoplankton chlorophyll *a* was only determined in the outdoor pond as described for periphyton.

Statistical analyses

To evaluate the exposure and recovery responses in microcosms, periphytic variables were modelled using a general linear model (GLM) with treatment (three levels: C, LG, HG) and sampling time (five levels: T0, T7, R7, R14 and R21) as fixed effects factors. Instead, for each water variable, the two periods, exposure and recovery, were analysed separately. Water variables were also modelled using GLM with treatment (3 levels) and sampling time (2 levels for the exposure period -T0 and T7-, and 4 levels for the recovery period -R0, R7, R14 and R21-) as fixed factors. Tukey's post hoc tests were used for multiple comparisons of means. A p-value <0.05 was considered to be statistically significant. The recovery of a periphytic variable was considered as the time point with no significant differences between the control and any of the treatments with herbicide addition.
Normality and homoscedasticity were analysed. If homoscedasticity was not achieved, models were fitted using VarIdent with time or treatment as the modelling function. The best model was chosen by comparison of Akaike's Information Criteria (AIC). All analyses were performed using R Statistical Software version 3.6.3 (R Core Team, 2020) with nlme and emmeans packages.

Results

Outdoor pond variables

Physical, chemical and biological parameters were recorded in the outdoor pond, where substrates were colonized. In the pond, glyphosate concentration was under the detection limit.

Microcosms water variables

Mean physical and chemical water characteristics for each treatment and sampling time are shown in Table 1. The minimum mean conductivity value was registered at T0 (0.23±0.09 mS/cm) and the maximums at R21 (0.56±0.11 mS/cm, average between the three treatments). Conductivity only showed differences between times, both in the exposure ($F_{1,12}$ = 15.6615; p = 0.0019) and the recovery periods ($F_{3,24}$ = 69.2929; p <0.0001). Mean conductivity showed an average increase of 50% at T7 with respect to T0. pH was circumneutral to slightly basic throughout the study. The minimum value recorded was 7.60 at R0, and the maximum was 8.26 at R21 on the HG treatment. pH only registered differences in the recovery period due to the time-by-treatment interaction ($F_{6,24}$ = 4.2841; p = 0.0045). There were no differences in pH between C and LG throughout the recovery period (p >0.05). On the other hand, HG showed an increase in pH from 7.60 at R0 to 8.26 at R21, resulting in pH values higher than C at R21 (p <0.05).

Turbidity showed differences between times ($F_{1,12}$ = 15.8897; p = 0.0018) in the exposure period, with an almost two times increase from T0 to T7. On the other hand, there were differences in this variable due to the time-by-treatment interaction ($F_{6,24}$ = 15.7276; p <0.0001) in the recovery period. Mean comparisons revealed no differences in the control over time. Instead, treatments with IPA showed an increment in turbidity throughout the recovery. The three treatments showed different turbidity between them at R21 (p <0.05); LG and HG presented values 2.4 and 5.5 times higher than C (Table 1).

Dissolved oxygen, during the exposure, registered differences due to time-by-treatment interaction ($F_{2,12}$ = 17.697; p = 0.0003). Tukey's post hoc comparisons revealed a ~10% decrease in DO in both treatments with IPA (p <0.05), showing the lowest DO mean values (7.02±0.13 mg/L in LG and 6.83±0.11 mg/L in HG) of the entire experiment at T7 (Table 1). During the recovery, differences in DO were only between sampling times ($F_{3,24}$ = 56.5951; p <0.0001). DO decrease 6% from R0 to R7, and then there was a 20% increase until R21 in all the treatments.

The average SRP during the entire exposure period was 0.13±0.06 mg/L. The SRP only showed differences during the recovery between sampling times ($F_{3,24}$ = 7.7038; p = 0.0009). A marked SRP decrease at R14 revealed the lowest average values of the whole experience (0.07±0.03 mg/L, average between the three treatments) with a subsequent increase on the last sampling date (Table 1). Total phosphorus only displayed differences in the exposure period due to the treatment-by-time interaction ($F_{2,12}$ = 172.8995; p <0.0001). There was an increase in TP from T0, 0.24±0.02 mg/L, to the highest mean value of the experiment in HG at T7 (0.86±0.06 mg/L).

The mean glyphosate concentration at T0 was 0.40±0.02 mg/L and 4.09±0.04 mg/L in LG and HG, respectively. At the end of the exposure period, it was 0.42±0.04 mg/L in LG and 4.13±0.08 mg/L in HG. In controls and during the recovery, glyphosate concentrations were below the detection limit of the method.

Table 1. Physical and chemical water characteristics for each treatment and sampling time of the experiment. Data are presented as mean ± 1 SD. Grey shadow pattern represents the exposure period, white pattern represents the recovery period. When the time-by-treatment interaction was significant (p <0.05): differences between treatments within the same sampling date were indicated by different letters, and differences between consecutive times within a treatment were indicated by asterisks. Instead, when differences between sampling times were significant (p <0.05): differences between consecutive times were indicated by diamonds. n = 3. NTU: nephelometric turbidity.

Table 1.(continued)

	C	LG	HG
Conductivity (mS cm^{-1})			
T0 ♦	0.2±0.1	0.2±0.1	0.2±0.1
T7 ♦	0.4±0.0	0.3±0.0	0.3±0.0
R0	0.3±0.0	0.3±0.0	0.3±0.0
R7	0.4±0.1	0.3±0.0	0.3±0.0
R14 ♦	0.4±0.0	0.5±0.0	0.4±0.0
R21 ♦	0.5±0.0	0.6±0.2	0.6±0.1
pH			
T0	7.7±0.1	7.7±0.1	7.7±0.1
T7	7.7±0.0	7.7±0.0	7.8±0.1
R0	7.7±0.1	7.7±0.1	7.7±0.1
R7	7.9±0.1	7.9±0.1 *	7.8±0.0
R14	7.9±0.1	8.0±0.1	8.0±0.0
R21	7.7±0.1^{a}	7.9±0.1^{ab}	8.1±0.1^{b}

	C	LG	HG
Turbidity (NTU)			
T0 ♦	1.9±0.7	1.9±0.7	1.9±0.7
T7 ♦	3.3±1.0	4.4±1.5	3.1±0.5
R0	1.7±0.4	1.7±0.4	1.7±0.4
R7	1.4±0.5	3.8±2.2 *	6.7±1.1
R14	6.1±1.7	15.3±3.1 *	10.5±3.0
R21	7.9±4.4^{a}	19.1±3.7^{b}	43.2±8.0^{c} *
Dissolved oxygen (mg l^{-1})			
T0	7.7±0.1	7.7±0.1 *	7.7±0.1 *
T7	7.5±0.1^{a}	7.0±0.1^{b}	6.8±0.1^{b}
R0 ♦	7.7±0.0	7.7±0.0	7.7±0.0
R7 ♦	7.3±0.4	7.3±0.2	7.2±0.1
R14 ♦	8.2±0.1	8.2±0.6	7.5±0.1
R21 ♦	8.6±0.2	8.9±0.3	8.6±0.3

	C	LG	HG
Soluble reactive phosphorus (mg l^{-1})			
T0	0.1±0.1	0.1±0.1	0.1±0.1
T7	0.2±0.1	0.1±0.1	0.2±0.0
R0	0.1±0.0	0.1±0.0	0.1±0.0
R7 ♦	0.2±0.1	0.2±0.0	0.1±0.0
R14	0.1±0.0	0.1±0.0	0.1±0.0
R21	0.1±0.0	0.1±0.0	0.1±0.0
Total phosphorus (mg l^{-1})			
T0	0.2±0.0	0.2±0.0	0.2±0.0
T7	0.3±0.0^{a}	0.3±0.0^{a}	0.9±0.1^{b} *
R0	0.2±0.0	0.2±0.0	0.2±0.0
R7	0.2±0.0	0.2±0.1	0.2±0.1
R14	0.2±0.1	0.2±0.0	0.2±0.1
R21	0.2±0.0	0.3±0.1	0.2±0.0

Periphyton: glyphosate impact and recovery in microcosms

The controls, both for DW and AFDW, presented the minimum mean values at T7 (18.343±4.936 μg DW/cm^2; 16.040±2.461 μg AFDW/cm^2) and the maximum at R21 (45.879±3.910 μg DW/cm^2; 43.844±5.188 μg AFDW/cm^2). The mean DW throughout the experiment ranged between 14.351±4.148 μg/cm^2 (HG at R7) and 51.759±11.836 μg/cm^2 (LG at R7); and mean AFDW between 10.796±2.280 μg/cm^2 (HG at R21) and 46.816±11.333 μg/cm^2 (LG at R7). The interaction between treatments and time was significant for these two variables (DW: $F_{8,30} = 9.8840$; $p < 0.0001$; AFDW: $F_{8,30} = 11.0226$; $p < 0.0001$). For both, there was an increase at T7 in periphyton subjected to treatments with IPA, revealing values 2.3 (DW) and 2.6 (AFDW) times higher in HG than C. There were no differences between C and LG treatments, in DW or AFDW, during the recovery ($p > 0.05$). On the other hand, the periphyton exposed to HG presented a decrease at R7 concerning T7 (-66% DW and -73% AFDW). This community recorded the lowest DW and AFDW values during the recovery.

The mean periphytic chlorophyll *a* concentration in C for the whole experiment was 0.022±0.007 μg/cm^2. The Chla displayed differences due to the treatment-by-time interaction ($F_{8,30} = 6.3958$; $p = 0.0001$). There were no differences over time in controls ($p > 0.05$). The Chla increased from 0.015±0.002 μg/cm^2 (T0) to 0.036±0.006 μg/cm^2 in LG and 0.028±0.008 μg/cm^2 in HG (T7), when both treatments presented higher values than C (+147% LG and +93% HG). During the entire recovery period, there were no differences between the community subjected to LG and the control ($p > 0.05$). Instead, during the same period, periphyton exposed to the HG treatment did not differ from C until R14 ($p > 0.05$), but then Chla presented a 62% decrease reaching the lowest mean value of the whole experience (0.011±0.003 μg/cm2) at R21.

The mean periphyton total abundance registered the minimum values at T0 ($2.18x10^4 \pm 1.00x10^3$ ind/cm^2), and the maximums at R21 for all the treatments (C: $4.45x10^4 \pm 5.05x10^3$ ind/cm^2; LG: $5.34x10^4 \pm 1.37x10^4$ ind/cm^2; and HG: $1.08x10^5 \pm 2.71\times10^4$ ind/cm^2). Differences were observed due to the time-by-treatment interaction ($F_{8,30} = 24.2081$; $p < 0.0001$). The only significant difference was in HG at R21, when an increase of more than two times in the total abundance of the periphytic community was recorded concerning R14, resulting in higher values than C and LG at R21 ($p < 0.05$).

At T0, Chlorophyta accounted for 81.9±1.8%, followed by Bacillariophyceae (10.8±2.3%), Cyanobacteria (4.1±0.5%), Dinophyceae (2.9±0.5%) and Euglenophyceae (0.2±0.1%). There were differences due to the interaction between treatment and time for the relative percentage of chlorophytes ($F_{8,30} = 144.0479$; $p < 0.0001$). Chlorophyta proportion decreased ($p < 0.05$) at some point during the experiment in all the treatments. In C and LG, the decrease was recorded at R7 (-6% in C and -28% in LG, with respect to T7). Afterwards, in control, this percentage remained almost invariable ($p > 0.05$) until R21, and periphyton treated with LG underwent a significant increase at R14, reaching ~64% of the total periphyton abundance until R21. On the other hand, the high concentration of IPA generated a decrease in the percentage proportion of chlorophytes throughout almost the entire experience, from 81.9±1.8% at T0 resulting at R21 with only 33.3±0.1% of the total periphyton abundance. There were differences between the three treatments at R14 and R21 ($p < 0.05$).

The control treatment had the lowest values of mean relative abundance of total diatoms at all sampling times, with a minimum of 9.8±1.4% at T7. Differences were due to the treatment-by-time interaction ($F_{8,30} = 151.5796$; $p < 0.0001$), and comparisons revealed that the proportion of diatoms was 66% higher in LG than C at T7. In all the treatments, the transfer of colonized substrates to herbicide-free beakers triggered an increase in the percentage proportion of diatoms ($p < 0.05$), and at R7, the microcosms with IPA presented a percentage almost two times greater than the control ($p < 0.05$). Afterwards, the relative abundance of diatoms decreased (-7%) in LG and increased (+9.5%) in HG at R14 ($p < 0.05$), revealing differences between the three treatments at R14 and R21. The lowest mean relative percentage of Cyanobacteria was at T0 (4.1±0.5%) and the maximum at T7 in HG (24.5±1.6%). This percentage showed differences due to the time-by-treatment interaction ($F_{8,30} = 226.9154$; $p < 0.0001$). The cyanobacteria percentage proportion sharply increased from T0 to T7 in periphyton exposed to HG, reaching values more than five times higher than C and LG at T7 ($p < 0.05$). From R14 to the end of the recovery period, there were no differences in cyanobacteria relative abundance between the periphyton impacted with the LG treatment and the control ($p > 0.05$). On the other hand, during the same period, the community exposed to HG presented higher values than those exposed to the other treatments

($p < 0.05$), reaching at R21 an average proportion (24.4±0.2%) as high as in T7 and more than two times higher than C and LG.
Regarding dead diatoms, differences were registered in the treatment-by-time interaction ($F_{8,30} = 40.0396$; $p < 0.0001$). The control treatment registered the lowest values throughout the experience (15.6±2.0%, average for the entire experiment) without differences over time ($p > 0.05$). The percentage of dead diatoms increased from T0 (14.6±3.3%) to T7, more than two times and almost three times for LG and HG, respectively. At all sampling times during the recovery, there were differences in % of dead diatoms between the three treatments ($p < 0.05$). The HG treatment registered the highest percentages and the maximum value of the experiment at R21 (54.0±1.2%).

Conclusion

The authors concluded this was the first study to provide evidence of the capability of periphyton communities to recover after exposure to the most commonly used herbicide in the world. The authors suggested that the concentration of the herbicide during the exposure period will influence IPA effects on periphyton, and this exposure concentration will impact and play a decisive role in the recovery potential of the community. The authors highlighted the need for more studies focusing on the recovery potential of aquatic communities after glyphosate contamination, considering different scenarios and glyphosate-based commercial formulations, as would occur in natural freshwater ecosystems in agricultural landscapes. Microbial ecotoxicologists have been challenged to understand and predict how community recovery can reflect the recovery of an ecosystem. The authors suggested that the first step to answer this question is to increase the scientific literature on microbial communities' structural and functional recovery studies.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This is a higher tier study for aquatic organisms with two glyphosate concentrations tested (0.4 and 4 mg/L) on aquatic microcosms. The study is structured in two phases: 7 days of exposure to treated water and 21 days of recovery in clean water (both of them under static conditions). It evaluates the potential of freshwater periphyton to recover from glyphosate exposure using microcosms under laboratory conditions. Dry weight, ash-free dry weight, chlorophyll *a*, and periphyton abundances were analysed. The periphyton affected with the lowest concentration recovered most of the structural parameters within 7 days in clean water, but the taxonomic structure did not entirely recover towards the control structure. Periphyton exposed to 4 mg/L could not recover during 21 days in herbicide-free water, reaching values almost four times higher in % of dead diatoms and four times lower in ash-free dry weight concerning the control at the end of the study. Results suggest a long lasting effect of the herbicide due to the persistence within the community matrix even after translocating periphyton to decontaminated water. The study concludes that the exposure concentration modulates the recovery potential of impacted periphyton.
The study is considered reliable with restrictions. Analytical verifications of the tested item in the test medium were conducted at the beginning and the end of the exposure phase.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	The periphytic colonization was conducted in a pond that has never been contaminated with glyphosate or any other pesticide.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Uncertain	It's not clear whether the test item dissolved in distilled water, filtered pond water or a solvent was used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, content and CAS number were reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoints reported: LOEC immediately after the exposure phase

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		(7-d) and after the recovery phase (28-d)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Only 2 concentrations were tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	3 microcosms per treatment to determine physical, chemical and biological parameters
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	Water variables were continuously verified
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature set at 25°C for culture and tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	Analytical methods reported (ion chromatography)
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	LC_{50} 95% confidence interval is reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This is a higher tier study for aquatic organisms with two glyphosate concentrations tested (0.4 and 4 mg/L) on aquatic microcosms. The study is structured in two phases: 7 days of exposure to treated water and 21 days of recovery in clean water (both of them under static conditions). It evaluates the potential of freshwater periphyton (a complex of bacteria, fungi, algae and protozoa) to recover from glyphosate exposure using microcosms under laboratory conditions. Dry weight, ash-free dry weight, chlorophyll *a*, and periphyton abundances were analysed. The periphyton affected with the lowest concentration recovered most of the structural parameters within 7 days in clean water, but the taxonomic structure did not entirely recover towards the control structure. Periphyton exposed to 4 mg/L could not recover during 21 days in herbicide-free water, reaching values almost four times higher in % of dead diatoms and four times lower in

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria			Criteria met? Yes / No / Uncertain	Comment / Justification
		ash-free dry weight concerning the control at the end of the study. Results suggest a long lasting effect of the herbicide due to the persistence within the community matrix even after translocating periphyton to decontaminated water. The study concludes that the exposure concentration modulates the recovery potential of impacted periphyton. The study is considered reliable. Analytical verifications of the tested item in the test medium were conducted at the beginning and the end of the exposure phase.		
Not reliable	No	-		

1. Information on the study

Data point:	CP 10.1.3
Report author	Goodman R. M. *et al.*
Report year	2021
Report title	Influence of herbicide exposure and ranavirus infection on growth and survival of juvenile red-eared slider turtles (*Trachemys scripta elegans*)
Document No	Viruses, 2021, 13(8), 1440
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors exposed hatchling red-eared slider turtles (*Trachemys scripta elegans*) to herbicide and ranavirus treatments to examine direct effects and interactions on growth, morbidity, and mortality. Turtles were assigned to each herbicide treatments or a control group. Turtles were exposed to Roundup ProMax® or Rodeo® via water bath during the first 3 weeks of the experiment. After 1 week, turtles were exposed to either a control (cell culture medium) or ranavirus-infected cell lysate via injection into the pectoral muscles. Necropsies were performed upon death or upon euthanasia after 5 weeks. Tissues were collected for histopathology and detection of ranavirus DNA via quantitative PCR. Only 57.5% of turtles exposed to ranavirus tested positive for ranaviral DNA at the time of death. Exposure to environmentally relevant concentrations of herbicides did not impact infection rate, morbidity, or mortality of hatchling turtles due to ranavirus exposure. The authors also found no direct effects of herbicide or interactions with ranavirus exposure on growth or survival time. Results of this study should be interpreted in the context of the modest ranavirus infection rate achieved, the general lack of growth, and the unplanned presence of an additional pathogen in this study.
The results for combined herbicides (including Atrazine herbicide), reported in the original article, were not presented in this summary.

Materials and methods

The authors obtained 170 juvenile red-eared slider turtles from a commercial supplier (Reptile City; Dallas, TX, USA) in May of 2014. Ten turtles were immediately euthanized and decapitated 1 h after euthanasia. These turtles were necropsied to obtain the following tissues which genomic DNA was then extracted from: approximately 4 cm of mid- and distal intestine, the left kidney, and the left lobe of liver. Testing via quantitative polymerase chain reaction (qPCR; described below) for ranaviral DNA was performed to ensure that supplied animals were not infected with ranavirus. Upon confirmation of negative results for the subsample of 10 turtles, the remaining 160 turtles were placed individually in 5 L plastic containers containing 350 mL of filtered, dechlorinated tap water.

Laboratory temperatures were maintained between 19.5-28ºC with an average temperature of 24ºC. A natural photoperiod (approximately 6:00 a.m.-8:30 p.m. for May-June in Virginia) supplied by large windows in the room was supplemented by overhead fluorescent lights during 9:00 a.m.-6:00 p.m. Throughout the experiment, the authors fed turtles approximately 10% of their weight in Zoo Med natural aquatic turtle feed every other day. Turtle enclosures were placed on shelves in our laboratory and rotated weekly to avoid any bias due to position within the laboratory. Paper dividers were placed between each container and on the outside edge, to reduce possible stress.

After 3 days of acclimation to the laboratory conditions, turtles were assigned to one of eight experimental treatments (n = 20 for each) in a full factorial design with two factors: ranavirus exposure (control, ranavirus-exposed) and herbicide exposure (control, Roundup ProMax®, Rodeo®). At the initiation of the experiment, the authors collected initial weights and measurements of each turtle (mass in g; width and plastron and carapace length in mm using digital calipers) and collected 3-4 mm from each tail tip and sealed the wound. These tissue were collected to sample initial conditions in the event that any turtles not exposed to ranavirus during the experiment turned out to be ranavirus-positive at the conclusion. However, this scenario did not occur; thus, these tail tissues were not tested for ranavirus.

Herbicide exposure consisted of herbicide addition (or lack thereof, for controls) in 200 mL of water in housing containers during the first 3 weeks of the experiment. Herbicide-free dechlorinated water was used for all turtles during the remaining 2 weeks of the 5 week experiment. Levels of herbicide exposure were based on the higher end of but not maximal concentrations reported in the literature: 2000 µg a.e./L glyphosate for Roundup ProMax® and Rodeo® (Monsanto, Creve Coeur, MO, USA).

To constitute treatment water for glyphosate herbicides, 33.4 µL of Rodeo and 29.6 µL of Roundup ProMax® were each added to 500 mL of water and mixed on a stir plate on high for 10 min. The authors then added each 500 mL stock solution to 7500 mL of water in a large bucket and stirred each by hand for 5 min. In two different weeks, the authors took samples from the final herbicide solutions and shipped them overnight on ice to the laboratory to verify target concentrations. Actual concentrations of water samples were 2048-2223 ppb for glyphosate in Roundup Pro Max® and 1952-2292 ppb for Rodeo®.

Ranavirus exposure was executed at the start of the second week by injection of 100 µL of virus-infected cell lysate ($6.3x10^4$ $TCID_{50}$). Control turtles were each injected with 50 µL of Dulbecco's modified Eagle medium (used for fathead minnow cell culture) in each pectoral muscle.

Each week, turtles were weighed, measured, and placed in cleaned, disinfected tubs with reconstitutions of herbicide treatments for the first 3 weeks or herbicide-free dechlorinated water for the last 2 weeks. After virus exposures, turtles were checked visually every 12 h for external signs of ranavirus infection: lethargy, respiratory stress, cutaneous erythema, and ocular/nasal discharge. During weekly measurements and water changes, turtles were examined while in hand for ranaviral disease signs. When turtles expired before the completion of the experiment, they were examined and sampled as described below following euthanasia done at the conclusion of the experiment. Turtles were considered deceased when unresponsive to stimulation of the limbs and corneas. The authors weighed and measured, took note of and photographed any external signs of disease, and then decapitated and necropsied the turtles. The colour and condition of the liver was noted, and tissue samples were taken from the intestine, kidney, and liver, as previously described. Tissues were frozen at 80ºC until use in ranavirus testing. The remainder of the turtle was preserved in 10% buffered formalin for histopathological examination. The experiment was concluded after 5 weeks (4 weeks after virus exposures), when all remaining living turtles were euthanized, measured, and sampled as described above.

The authors extracted DNA from tissue samples with Qiagen DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany). The authors standardized the amount of genomic DNA used in each reaction with an Epoch spectrophotometer (Biotek, Winooski, VT, USA). The authors tested for presence of ranavirus DNA using quantitative polymerase chain reaction (qPCR) following the protocol of Picco *et al*. Each 25 µL PCR reaction contained: 7 µL volume of combined nuclease-free water and genomic DNA; 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems™, Foster City, CA, USA); 1.5 µL each of 10 µM primers: F 5'-ACA CCA CCG CCC AAA AGT AC-3', R 5'-CCG TTC ATG ATG CGG ATA ATG-3'; 2.5 µL of 2.5 µM probe: 5'-/56-FAM/CCT CAT CGT /ZEN/TCT GGC CAT CAA CCA /3IABkFQ/-3' (Integrated DNA Technologies, Coralville, IA, USA). All samples were tested in duplicate using Applied Biosystems™ StepOne Real-time PCR machine with two negative and two positive controls in each run. Samples with Ct values <30 for both runs were considered positive for ranavirus, according to standards established for this machine using known negative and positive controls from water, cultured ranavirus, and ranavirus-infected reptiles. If Ct values from two samples

for an individual were not both <30 or if one approximated 30, the authors ran two additional PCR reactions. Then, the consensus from three out of four total reactions was used.

The authors conducted histopathology on four turtles from each combination of herbicide and virus exposure (eight treatments, 32 turtles total). Four turtles with the lowest Ct values were selected from each of the ranavirus-exposed treatment groups, and the authors performed histological examinations of commonly infected tissues including the liver, spleen, pancreas, and gastrointestinal tract. All turtle tissues were processed at the University of Tennessee Veterinary Medical Center Diagnostic Laboratory where they were embedded in paraffin blocks, cut into 5 µm sections, mounted on glass slides, and stained with haematoxylin and eosin. The authors examined tissues under light microscopy for signs of ranaviral infection, as well as possible tissue lesions caused by herbicide exposure. All work in this study was approved by the Hampden-Sydney College Animal Care and Use Committee, under the protocol number 965 (approved 05 December 2014).

The authors used chi-squared tests of independence to compare ranavirus-positive and -negative PCR results among herbicide treatments and to compare incidence of external white growths (presumed fungus) and liver discoloration among herbicide treatments and ranavirus exposure treatments. To examine potential treatment effects of days survived and change in turtle mass, as well as plastron and carapace length, the authors used a weighted least squares regression model with herbicide and ranavirus exposure and an interaction effect as fixed factors, and time (day of measurement) as a fixed factor. The authors conducted their analyses in SPSS® Statistics version 23 (SPSS Inc., Chicago, IL, USA) and created Figures in R version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

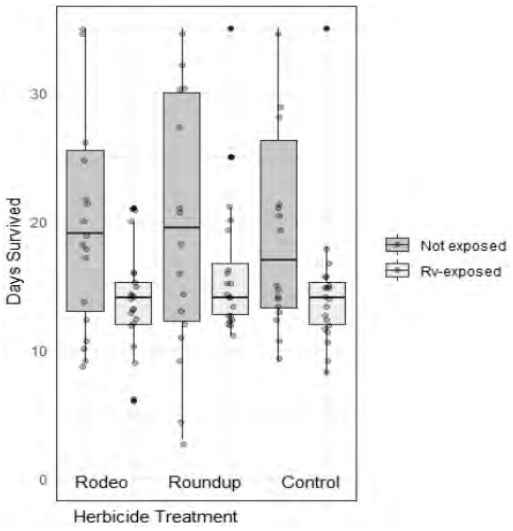
Herbicide treatment did not affect incidence of ranavirus infection among turtles exposed to ranavirus, although there was a non-significant trend for higher infection rates in the herbicide control group relative to other groups infected with ranavirus (Table 1). Neither variable influenced liver colour (tan or red), which was observed in some turtles at the time of necropsy (Table 1). Neither herbicide treatments nor ranavirus exposure treatments affected the incidence of flocculent white growths (typically circular; 1-3 mm in diameter), which were observed in some turtles at the time of necropsy (Table 1). These growths were not observed upon receipt of the shipment of turtles, but they were observed starting on the 10th day of the experiment and culminated in 1-23 growths per turtle, occurring on the skin but not the shells. We attempted to examine the white growths under microscopy but were unable to determine the causal agent. Histopathological changes in ranavirus-exposed turtles with the highest viral loads (lowest Ct values) included necrosis and haemorrhage in the liver, haematopoietic necrosis in the pancreas, and inclusion bodies consistent with ranavirus.

Table 1. Incidence of response variables at time of death among hatchling turtles (*Trachemys scripta elegans*) exposed to herbicide and ranavirus treatments.

Ranavirus Treatment	Herbicide Treatment	*N*	Incidence of Infection	Liver Discol-oration	White Growths
Rv-exposed	Roundup	20	9	6	8
Rv-exposed	Rodeo	20	9	8	11
Rv-exposed	Control	20	16	10	12
Control	Roundup	20	-	7	3
Control	Rodeo	20	-	10	6
Control	Control	20	-	7	7

Ranavirus exposure decreased the number of days survived; however, there was no effect of herbicide treatment (figure 1). The average starting sizes of hatchlings turtles were 7.26 g (SD = 0.97), 33.32 mm in carapace length (SD = 1.54), and 32.07 mm in plastron length (SD = 1.53).

Figure 1. Days survived for hatchling turtles (*Trachemys scripta elegans*) exposed to herbicide and ranavirus treatments.



Conclusion

The authors concluded that ranaviruses are increasingly recognized as significant pathogens worldwide, but their influence on health and survival in reptiles is understudied. The authors stated that their study provides an investigation of interactions between ranaviruses and herbicides, but the importance of many other pollutants and environmental stressors remains to be examined. As expected, ranavirus exposure decreased survival time and reduced mass and plastron length in juvenile turtles. However, contrary to the authors' expectations, they did not detect any direct effects of two formulations of glyphosate on growth or survival or any interaction with ranavirus exposure in terms of susceptibility, morbidity, or mortality. The authors urge caution in the interpretation of their results, because of the low growth rates and infection rates and the unplanned occurrence of another pathogen in our study system. In conclusion, the authors urge replication of ranavirus exposure studies in different study species and with the inclusion of other environmental pollutants and stressors.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study, hatchling red-eared slider turtles (*Trachemys scripta elegans*) were exposed to the formulated glyphosate herbicides Roundup ProMax® and Rodeo® to examine direct effects on growth and mortality. Turtles were exposed to herbicides via water bath during the first 3 weeks of a 5-week experiment. Exposure to a NOEC = 2 mg/L concentration of glyphosate (for both products) did not impact growth or survival time of hatchling turtles.
The study cannot be considered as fully reliable because it is uncertain whether and when the samples from the final herbicide solutions that were analysed to verify target concentrations, were taken from the experimental cages. In addition, the test items were not documented and only one concentration of each product was tested.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals used for the present study were purchased from a commercial supplier and acclimatized for 3 days to laboratory conditions
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	No	Just the name of the supplier was reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Batch specifications are not provided and the assessment of the ecotoxicological equivalence cannot be conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Uncertain	In two different weeks, samples from the final herbicide solutions were analysed to verify target

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		concentrations, but it is not clear whether and when the samples were taken from the experimental cages.
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint reported: chronic NOEC (35-d))
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Only 1 concentration of each product was tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Uncertain	Samples from the final herbicide solutions were analysed to verify target concentrations, but it is not clear whether and when the samples were taken from the exposure cages.
15. A clear concentration response relationship is reported - in studies where the dose response test design is employed.	No	Only 1 concentration of each product was tested
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	20 replicates of 1 individual per treatment
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water	-	Not applicable, aquatic study

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
retention, microbial biomass (~1% of organic carbon).		
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Mean temperature set at 24°C (19.5-28°C).
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Uncertain	Samples from the final herbicide solutions were analysed to verify target concentrations, but it is not clear whether and when the samples were taken from the exposure cages.
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Uncertain	Samples from the final herbicide solutions were analysed to verify target concentrations, but it is not clear whether and when the samples were taken from the exposure cages.
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No ECx assessment was conducted
Overall assessment		

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria		Criteria met? Yes / No / Uncertain	Comment / Justification
Reliable without restrictions	No	-	
Reliable with restrictions	Yes	This article reports the chronic effects of two glyphosate-based pesticides on growth and survival of juvenile Red-Eared Slider Turtles (*Trachemys scripta elegans*). The study seems to have been well conducted and reports a regulatory relevant endpoint: 35-d chronic NOEC = 2 mg/L (1.952 - 2.292). The study cannot be considered as fully reliable because it is uncertain whether and when the samples from the final herbicide solutions that were analysed to verify target concentrations, were taken from the experimental cages. In addition, the test items were not documented and only one concentration of each product was tested.	
Not reliable	No	-	

1. Information on the study

Data point:	CP 10.2.1
Report author	Fernandez C. *et al.*
Report year	2021
Report title	Toxic effects of chlorpyrifos, cypermethrin and glyphosate on the non-target organism *Selenastrum capricornutum* (Chlorophyta)
Document No	An Acad Bras Cienc, 2021, 93(4), e20200233
Guidelines followed in study	OECD TG 201 (2011) partially
Deviations from current test guideline	Deviation from OECD TG 201 (2011): • No analytical verifications of the tested item in the test medium during the exposure phase • The test item is not fully documented.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The toxic effects of the herbicide glyphosate on the growth, biovolume and ultrastructure of the green microalgae *Selenastrum capricornutum* were evaluated. Concentrations between 4.7-60 mg/L of glyphosate were tested along with a control culture. The tested concentrations were prepared using a commercial formulation. After 48 h, all tested concentrations of the pesticide reduced significantly the population growth. The 96 h effective concentration 50 (EC_{50}) was 15.60 mg/L for glyphosate. Cells exposed to the pesticide showed an increase in cellular size related to the increase in pesticide concentration and exposure time. The most significant damages observed on the ultrastructure of cells exposed to the pesticide included thylakoids and mitochondria disruption, formation of electrodense bodies, accumulation of lipids and increase in the size and number of starch granules. The present study demonstrates that the effects of the pesticide also extend to non-target organisms having significant ecological implications.

Materials and methods

Species test and toxicity bioassays

Toxicity tests were performed on the freshwater unicellular green algae *Selenastrum capricornutum* Printz, a standard test organism. Cultures of *S. capricornutum* were obtained from the Universidad Federal de São Carlos (São Paulo) and were kept in Bold´s Basic Medium.

Stock cultures were kept in liquid media during 7 days at a temperature of 24±2ºC under continuous illumination (4800 lux) in order to obtain a culture in an early phase of exponential growth. To avoid the settling of algae, the cultures were re-suspended for a few minutes three times a day on an orbital shaker.

The tested concentrations were prepared using the commercial formulation Round-up® (glyphosate 48% w/v). This formulation was diluted in sterile distilled water and added to sterile Bold´s Basic medium. In order to obtain the desired concentrations of the active substance, microalgae were exposed to one third-dilution in a range of 4.7-60 mg/L. These concentrations were selected through a preliminary assay in which the microalgae were exposed to a wider range of pesticide concentration. A toxicity tests with the reference substance potassium dichromate ($K_2Cr_2O_7$) using a one third-dilution in a range of

0.08-0.52 mg/L was performed to evaluate the physiological conditions of the organisms and hence the validity of the tests.

An initial inoculum of 5 x 10^4 cells m/L *S. capricornutum* was added to each concentration. All assays, i.e., reference toxicant tests, controls and pesticide treatments, were carried out in triplicate without medium replacement and under the same conditions of light, temperature and agitation as the stock cultures.

To evaluate the effects of pesticides on the population growth, the number of microalgae in each replicate and in the control culture was estimated by direct counting in Neubauer chamber every 24 h during the entire assay (96 h). To evaluate the effects of pesticides on cell volume, thirty cells from the control culture and from each replicate were measured every 24 h under a light microscope Olympus CX23 and the biovolume was calculated according to Sun & Liu (2003). Simultaneously, the shape, colour and presence of granules in the microalgae were observed.

Effective concentration 50 values (EC_{50}) and confidence intervals (95%) at each exposure time were calculated by means of a Probit analysis of the percent inhibition of algal growth rate (%I) using the Infostat software package (2017).

One-way analysis of variance (ANOVA) was used to evaluate whether there were significant differences between microalgae population growth (cell concentration and *r*) of the different treatments, when statistical differences among values were detected, Dunnett's test was applied. The non-parametric Kruskal-Wallis test was used to assess differences in microalgae cell volume between treatments since data did not attend criteria for parametric methods.

Electron microscopy techniques

At the end of the bioassay (96 h) the control culture and cultures exposed to the pesticide (60 mg/L of glyphosate) were centrifuged at 4000 rpm during 5 minutes.

Cells were fixed at 4ºC in 2.5% glutaraldehyde and post fixed in 1% OsO_4 using filtered culture medium as fixative vehicle. Fixed cells were subsequently dehydrated in an acetone series from 10% to absolute acetone. It was then embedded drop by drop in Spurr's low-viscosity resin and flat-embedded between glass slides coated with dry Teflon. Sections were cut with a Diatome 2.1 mm diamond knife, mounted on Formvar-coated grids and stained with uranyl acetate and lead citrate. They were examined under a Jeol 100 CX-II electron microscope at CCT-Bahia Blanca.

Results

Effects of the glyphosate on growth

Growth curves of *S. capricornutum* under different concentrations are shown in the figure 1 below, the results of the statistical analysis are listed in the table inside the figure. All tested concentrations inhibited the growth of *S. capricornutum* to some extent.

After 24 h of exposure only treatments with 36 mg/L ($p < 0.05$) and 60 mg/L ($p < 0.01$) significantly inhibited the growth, whereas with longer exposure times all concentrations inhibited the growth.

As shown in the table 1 below, the EC_{50} for the %I decreased with the increase of exposure time for glyphosate, indicating a higher toxicity as the exposure time increases.

Table 2 below shows the growth rates (*r*) of algal cultures exposed to different concentrations of pesticides after 96 h. The growth rate decreased as the concentration of the pesticide increased, and all concentrations assayed of the pesticide reduced significantly the growth rate compared to the corresponding control without pesticide.

Figure 1. 96 h growth curve of *Selenastrum capricornutum* in treatments with different concentrations of glyphosate. The table shows significant differences in cell number of treatments compared to the corresponding controls. One asterisk: $p < 0.05$, two asterisks: $p < 0.01$, ns: no significant differences. Adapted from Fernandez C. *et al*. An Acad Bras Cienc, 2021, 93(4), e20200233.

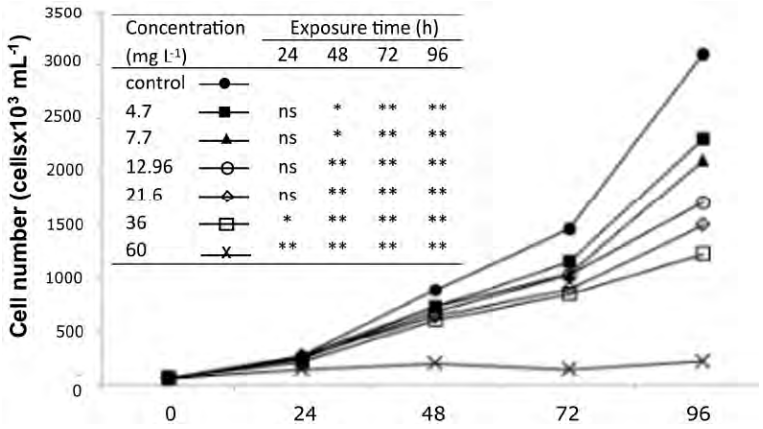


Table 1. Effective concentrations (EC_{50}, mg/L) and the respective 95% confidence limit (95% CI) for percent inhibition of algal growth rate (%I) of the pesticide to *Selenastrum capicornutum* in 24, 48, 72 and 96 h. Adapted from Fernandez C. *et al*. An Acad Bras Cienc, 2021, 93(4), e20200233.

		Glyphosate
24 h	EC_{50}	61.86
	95% CI	49.94-110.55
48 h	EC_{50}	39.02
	95% CI	28.97-62.13
72 h	EC_{50}	24.44
	95% CI	19.13-33.06
96 h	EC_{50}	15.60
	95% CI	12.27-19.67

The algal growth inhibition rate with respect to controls was calculated according to the following equation:

$$\%I = \frac{T - C}{C} \times 100$$

where T is the number of cells in each treatment and C is the number of cells in the control culture.

Table 2. Specific growth rate (r) (/h) of *Selenastrum capicornutum* cultures after 96 h of exposure to different concentrations of the glyphosate. One asterisk ($p < 0.05$) and two asterisks ($p < 0.01$) indicate significant differences compared to the corresponding control without pesticide. Adapted from Fernandez C. *et al*. An Acad Bras Cienc, 2021, 93(4), e20200233.

Concentration (mg/L)	**Growth rate (*r*)**
control	0.0420±0.0003
4.7	0.0388±0.0007**
7.7	0.0380±0.0005**
12.96	0.0358±0.0003**
21.6	0.0344±0.0008**
36	0.0324±0.0005**
60	0.0139±0.0004**

Exponential growth rate (r) was calculated through the following equation:

$$r = \frac{\ln N_1 - \ln N_0}{t}$$

where N_1 is the number of cells at the end of the assay, N_0 is the initial number of cells and t is the exposure time in hours.

Effects of the pesticide on cellular size and morphology

S. capricornutum cells exposed to the glyphosate evidenced an increase in the cellular size; such increase was related to the increase in concentration and exposure time. At the end of the bioassay (96 h), statistically significant differences were found between treatments ($p < 0.01$). Cells exposed to 21.6, 36 and 60 mg/L showed a cell volume significantly higher than control cells ($p < 0.05$).

With regards to morphology, at the end of the bioassay, normal cells were found only in cultures exposed to the lowest concentrations of the pesticide. At higher concentrations (glyphosate 21.6, 36 and 60 mg/L) different degree of anomalies were observed including deformed and chlorotic cells with abundant cytoplasmic granules and destroyed chloroplasts.

Effects of the pesticide on the ultrastructure

Control culture cells showed the characteristics that best typify Chlorococcales. The central nucleus exhibited condensed chromatin next to the nuclear envelope; it also exhibited a pronounced neckline where the large dictyosome was located. The parietal chloroplast occupied most of the cell volume and showed one conspicuous spherical pyrenoid. Thylakoids were arranged in an almost parallel pattern and starch granules were located between them. The cytoplasm was homogeneous, showing fine and regular granulation due to the presence of ribosomes. The cell wall was characterized by the presence of three layers, the outermost of which was involved in forming crests.

S. capricornutum cells exposed to the pesticide showed changes in the cell structure; the level of disturbance on the ultrastructure depended on the pesticide type and increased as the pesticide concentration increases.

In cells exposed to 60 mg/L of glyphosate the chloroplast and mitochondria were the organelles that underwent the greatest changes, with compacted thylakoids and a big development of the condriome. Abundant lipid globules and vacuoles were observed in the cytoplasm and in some cases occupied the entire cell. The nuclear chromatin was dispersed. The cell wall was not affected and the three- layer structure was still evident. At this concentration of glyphosate the formation of autospores could not be evidenced.

Conclusion

The authors concluded that the present study showed the toxic effects of the pesticide glyphosate on population growth and cell morphology and ultrastructure of *Selenastrum capricornutum*. Ultrastructural damage was produced by glyphosate. Besides, the EC_{50} for glyphosate was close to the concentrations found in some superficial waters indicating a higher ecological risk for this compound.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study evaluates the acute toxic effects of glyphosate on the growth, biovolume and ultrastructure of the green microalgae *Selenastrum capricornutum*. After 48 h, all tested concentrations reduced significantly the population growth. The regulatory relevant endpoint 96-h effective concentration 50 (EC_{50}) was 15.60 mg/L. Cells exposed to glyphosate showed an increase in the cellular size related to the increase in pesticide concentration and exposure time. The most significant damages observed on the ultrastructure of cells included thylakoids and mitochondria disruption, formation of electrodense bodies, accumulation of lipids and increase in the size and number of starch granules.
The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase and the test item is not fully documented.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	Study conducted according to OECD TG 201 and Environment Canada Series Report EPS 1/RM/25, but not all validity criteria can be checked.
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Cultures were obtained from a culture collection at the Universidad Federal de São Carlos (São Paulo) and were kept in Bold´s Basic Medium.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in the test medium, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	No	Only the name of the product and glyphosate content were reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	Study conducted according to OECD TG 201 and Environment Canada Series Report EPS 1/RM/25, but not all validity criteria can be checked.
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume	Yes	-

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.		
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Analytical verifications were not conducted
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: EC_{50} (96-h))
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	-
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Analytical verifications were not conducted
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature set at 24°C for the tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	Analytical verifications were not conducted
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	Analytical verifications were not conducted
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	Analytical verifications were not conducted
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Yes	95% CI values were reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study evaluates the acute toxic effects of glyphosate on the growth, bio volume and ultrastructure of the green microalgae *Selenastrum capricornutum*. The 96-h effective concentration 50 (EC_{50}) was 15.60 mg/L. The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase and the test item is not fully documented.
Not reliable	No	-

1. Information on the study

Data point:	CP 10.2.1, CP 10.2.2
Report author	Houssou A. M. *et al.*
Report year	2021
Report title	Acute and chronic effects of a glyphosate and a cypermethrin-based pesticide on a non-target species *Eucypris* sp. Vavra, 1891 (Crustacea, Ostracoda)
Document No	Processes, 2021, 9(4), 701
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The study aims to evaluate the acute and chronic sensitivities of a freshwater ostracod species (*Eucypris* sp.) to a glyphosate herbicide-based formulation. Lethal concentrations (LC_{50}) of the pesticide for the species at 24 and 48 h were determined. The chronic exposure allowed assessing the effects of low concentrations of the pesticide; firstly, on the parthenogenetic reproduction of *Eucypris* sp., and, secondly, on its population growth. Individuals of *Eucypris* sp. were exposed to 4.51 ppm and 9.03 ppm of glyphosate. These concentrations are respectively the 10% and the 20% of the 48-h LC_{50} (median lethal concentration) of the pesticide for the species. For glyphosate, the 24-h LC_{50} was 50.521 ppm, while at 48-h it was 45.149 ppm. After glyphosate chronic exposure, 60%, 50%, and 90% of individuals were able to reproduce at 10% of LC_{50}-48-h, 20% LC_{50}-48-h, and the control treatment, respectively. The population growth was also affected by the tested low concentrations of glyphosate. The low concentrations of glyphosate affected the species at the individual and population levels.

Materials and methods

Test species

A plankton sample (100 mL) was collected from the Oueme River (Benin) at Bétérou, with a plankton net of 50 µm mesh size. Since most ostracod species are benthic, the sampling was conducted during low flow by trailing the net on the substratum of the river. The sample was subdivided into two parts. A subsample was cultured in laboratory conditions, with a natural fertilizer (chicken droppings) at 0.6 g/L. The temperature of the culture medium was 26.9±0.1ºC, the pH was 7.1±0.0, and the dissolved oxygen was 4.2±0.3 ppm. The second subsample was fixed with formalin for ostracod species identification (just one ostracod species was identified in the sample, *Eucypris* sp.). The ostracod was identified at the genus level. After two weeks of culture, 10 specimens of *Eucypris* sp. were isolated and placed in a phytoplankton (*Scenedesmus* sp.) culture to allow the development of a monospecific culture, which was subcultured every two weeks. Individuals used for the present study were of the fifth generation. They may be considered as non-contaminated individuals by environmental pollutants.

Culture monitoring showed that the asexual female of *Eucypris* sp. reached sexual maturity with the deposit of the first eggs between 15 and 17 days of age, under the conditions presented above. Thus, neonates of 24-h of age, obtained from several females maintained individually, were grouped and bred to 14 days of age, when they were used in the experiment.

Chemicals properties

The test solutions were obtained by dissolving known concentrations of the commercial formulation of the pesticide in water. Kumark® 480 g/L (Kumark Company Limited, Kumasi, Ghana) was used. Glyphosate is a non-selective systemic herbicide with the chemical formula $C_3H_8NO_5P$. Its molecular weight is 169.07 g/mol. Glyphosate is not likely to volatilize (vapour pressure = $1.31x10^{-2}$ mPa). Its half-life in water is 9.9 at 20ºC.

Acute test

Twenty-eight glass cups composed the experimental design for the pesticide. Six acute concentrations were tested for the pesticide and one control (water only) was used. Each treatment had four replications. Seven adults of *Eucypris* sp. Vavra, 1891 (14 days old) were placed in each glass cup immediately after the distribution of the test solutions (28 individuals per treatment, and a total of 168 per test). To identify the nominal concentration to be tested for the pesticide, preliminary exposure tests with randomly selected concentrations were carried out (data not presented). For glyphosate, concentrations of 0 (control), 6, 12, 24, 36, 48, and 60 ppm were used in the definitive acute tests. Each test lasted 48 h. The test solutions were not renewed and *Eucypris* sp. individuals were not fed. Considering the chemicals properties, the loss of active ingredient concentrations in the 48-h was considered insignificant. During the experiment, the photoperiod was set at 16/8-h (light/darkness). Tests with glyphosate were performed at temperatures of 27.0±0.3ºC, pH of 7.2±0.1, and dissolved oxygen of 4.6±0.1 ppm. The mobility of individuals and mortality were monitored at 1-h, 24-h, and 48-h of exposure. Individuals were declared dead when a lack of movement was observed after a mild stimulus.

Chronic test

During chronic tests, the parthenogenetic reproduction, the survivorship of neonates, and population growth of *Eucypris* sp. under low concentrations of glyphosate conditions were tested. To assess the reproduction and neonate survival, the treatments (pesticide concentrations and one control) were conducted in glass containers. Each treatment was carried out in ten replications with a total of 30 containers. The tested concentrations were 10% of the estimated 48-h LC_{50} and 20% of the estimated 48-h LC_{50}. These concentrations were 4.51 ppm and 9.03 ppm. Twenty millilitres of the prepared test solutions were immediately placed in each glass container. One adult female of *Eucypris* sp. was placed in each cup. Individuals were fed ad libitum with phytoplankton (*Scenedesmus* sp.). The experiment lasted 10 days and the test solution was renewed every 96-h. The reproduction and survivorship of neonates were controlled daily.

Regarding the population growth test, it was conducted in polyethylene aquaria of 3 L each. The same concentrations were tested as in the reproduction test. Three replications were used with a total of 9 aquaria. 1 L of test solutions enriched with phytoplankton (*Scenedesmus* sp.) was used in each aquarium and 10 *Eucypris* sp. individuals were dropped. The test solution was renewed every 96-h. Once a week, 20 mL of the culture medium of each treatment was collected after homogenization and fixed with formalin in a pillbox. These were used for individual enumeration under a light microscope. The tests lasted 28 days. The photoperiod was 16/8-h (light/darkness), the temperature was 25.0±0.3ºC, the pH was 6.5±0.3, and the dissolved oxygen was 3.6±0.8 ppm.

Data analysis

The mortality values were used to estimate the median lethal concentrations (LC_{50}) at 24-h and 48-h for the pesticide. The LC_{50} were estimated using the probit method in the computing program PoloPlus v.1.0 (LeOra Software LLC, Northampton, UK). Reproduction rates and survival of neonates were calculated for each treatment. The one-way analysis of variance (ANOVA) was used to check differences among treatments (Statistica v.7 Software (StatSoft, Tulsa, OK, USA) was used). Before the ANOVA test, the normality of data was tested with the Kolmogorov–Smirnov (K-S) test, while the homogeneity of variance was assessed with Levene's test.

Results

Swimming ability

Eucypris sp. individuals exposed to acute doses of glyphosate showed a perturbation of their swimming

ability shortly after exposure. They became motionless with an erratic movement following a soft needle stimulus. This steady state preceded individual death in most of the cases, while some remained alive with the imperfection until the end of the study (48 h of acute exposure). Acute exposure, immobilization ranged from 13% to 82% after one hour. In 24 h, it ranged from 45% to 82%, while in 48 h, it was from 63% to 96%. The two highest concentrations were significantly different from control after 24h and 48h. The first four concentrations of glyphosate also showed significant temporal variations of individual immobilization ($p < 0.05$).

Individual mortality

The median 24 h lethal concentration was 50.525 ppm. The interval of confidence was 45.351-58.204 ppm. For exposure of 48 h, the glyphosate LC_{50} was estimated at 45.149 ppm (40.082-51.296 ppm).

Reproduction and neonate survival

After 10 days exposure to glyphosate, 60%, 50%, and 90% of parthenogenetic reproduction were obtained with the 10% LC_{50} (4.51 ppm), 20% LC_{50} (9.03 ppm), and the control (0.0 ppm), respectively. The survival of neonates was 98±0.1%, 83.7±0.5%, and 95±0.3%, respectively.

Population growth

A slowdown of the population growth was observed compared to the control in which an exponential profile was obtained. A maximum density of 1271.7±7.6 ind/L (28^{th} day) and 651.8±8.0 ind/L (21^{st} day) was observed with 4.51 ppm (10% LC_{50}) and 9.03 ppm (20% LC_{50}), respectively, against 2671.7±13.9 ind/L (28^{th} day) in the control.

Conclusion

The authors concluded that freshwater ostracod *Eucypris* sp. showed tolerance to glyphosate with 24-h LC_{50} of 50.525 ppm and 45.149 ppm in 48 h. Sub-lethal concentrations such as 9.03 ppm of glyphosate may affect the parthenogenetic reproduction of the species. These may also affect the survivorship of neonates and, consequently, the population growth over time.

The authors indicated that, in developing countries, where monitoring devices are absent for aquatic ecosystems and the use of pesticides is experiencing a rapid increase, high concentrations of glyphosate can quickly occur in aquatic environments. The risk of ecological disturbances would, therefore, be great with the corollary of a considerable reduction in services.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This article reports the acute and chronic effects of a glyphosate-based pesticide on the fresh-water Ostracoda species *Eucypris* sp. (aquatic invertebrate species other than *Daphnia magna*). The study seems to have been well conducted and reports regulatory relevant and reliable endpoints: 48-h acute LC_{50} = 9.03 mg/L and 28-d chronic LOEC = 0.903 mg/L (10% of the estimated 48-h LC_{50}). However, the study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium. In addition, the culture/test medium is not described.

Assessment and conclusion by RMS:

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals used for the present study are fifth generation of a lab culture from a natural sample. They may be considered as non-contaminated individuals by environmental pollutants.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in distilled water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Uncertain	Source and content reported, but not clear if the tested formulation Kumark® 480 g/L (Kumark Company Limited, Kumasi, Ghana) contains surfactants.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Batch specifications are not provided and the assessment of the ecotoxicological equivalence cannot be conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Uncertain	Culture medium is not described
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when	Yes	-

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.		
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoints reported: acute LC_{50} (24 and 48-h) and chronic LOEC (28-d))
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes (acute test) No (chronic test)	For the chronic test only 2 concentrations were tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	Four replicates with seven adults per tested concentration (acute), ten replicates of 1 adult female per concentration (chronic reproduction) and 3 replicates of 10 individuals per concentration (chronic growth)
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	No	No information on the composition of the culture/test medium
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Uncertain	Temperature set at 27°C for culture and acute tests. No data of the temperature of the chronic tests are reported.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	Exposure concentrations were not analytically verified
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	Exposure concentrations were not analytically verified
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Yes	LC_{50} 95% confidence interval is reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This article reports the acute and chronic effects of a glyphosate-based pesticide on the fresh-water Ostracoda species *Eucypris* sp. The study seems to

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria		Criteria met? Yes / No / Uncertain	Comment / Justification
		have been well conducted and reports regulatory relevant and reliable endpoints: 48-h acute LC_{50} = 9.03 mg/L and 28-d chronic LOEC = 0.903 mg/L (10% of the estimated 48-h LC_{50}). However, the study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium. In addition, the culture/test medium is not described.	
Not reliable	No	-	

1. Information on the study

Data point:	CP 10.4.2.1
Report author	Wee J. *et al.*
Report year	2021
Report title	Temperature and Aging Affect Glyphosate Toxicity and Fatty Acid Composition in *Allonychiurus kimi* (Lee) (Collembola)
Document No	Toxics, 2021, 9, 126
Guidelines followed in study	None (partially based on OECD TG 232)
Deviations from current test guideline	• OECD TG 232 validity criteria cannot be fully checked.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

This study examined the toxicity of glyphosate with the temperature (20ºC and 25ºC) and aging times (0 day and 7 days) in soil using a collembolan species, *Allonychiurus kimi* (Lee). The degradation of glyphosate was investigated. Fatty acid composition of *A. kimi* was also investigated. The half-life of glyphosate was 2.38 days at 20ºC and 1.69 days at 25ºC. At 20ºC with 0 day of aging, the EC_{50} was estimated to be 93.5 mg/kg. However, as the temperature and aging time increased, the glyphosate degradation increased, so no significant toxicity was observed on juvenile production. The proportions of the arachidonic acid and stearic acid decreased and increased with the glyphosate treatment, respectively, even at 37.1 mg/kg, at which no significant effects on juvenile production were observed. The authors concluded that their results showed that the changes in the glyphosate toxicity with temperature and aging time were mostly dependent on the soil residual concentration. Furthermore, the changes in the fatty acid compositions suggest that glyphosate could have a chronic effect on soil organisms.

Materials and methods

Test animals

The test collembolan species, *A. kimi* (formerly known as *Paronychiurus kimi*), was collected from a paddy field in Korea in 1996. The *A. kimi* population was cultured in a plastic petri dish (9.0 cm in diameter and 1.5 cm in height) filled with approximately 0.5 cm depth of moist substrate comprised of plaster of Paris, activated charcoal, and distilled water at a ratio of 4:1:4 by volume in a dark growth chamber at 20±1ºC. Brewer's yeast was provided weekly as food. Age-synchronized collembolans were obtained. After the eggs hatched, the juveniles were reared under the same conditions and used for all subsequent experiments.

Chemical and toxicity test

A commercial formulation of glyphosate (Geunsami®) was obtained from Farm Hannong Ltd. (Seoul, Korea) and used throughout the experiments. The formulation contained glyphosate as an active ingredient (41%, w/w) in the form of isopropylamine salt.

Toxicity tests were conducted according to OECD guideline 232. The effects of glyphosate on the adult survival and juvenile production of *A. kimi* at 20ºC and 25ºC and aging times of 0 day and 7 days were investigated. The test concentrations were 0.0, 0.7, 3.7, 37.1, 74.1, and 370.5 mg/kg soil dry weight. The stock solution was prepared by dissolving the formulation in deionized water. Diluted stock solutions

adjusted to the test concentrations were mixed thoroughly with the soil to obtain the required moisture content of 50% water holding capacity. The spiked soil (30 g) was placed in a polystyrene vessel (55 mm in diameter and 60 mm in height). A total of 120 test vessels (6 concentrations 5 replicates 2 temperatures 2 aging times) were prepared at once, and 60 test vessels were separately stored in a dark growth chamber at 20ºC and 25ºC, respectively. After 0 day and 7 days of aging at each temperature, 30 test vessels (5 vessels per concentration) were randomly selected and used for the toxicity test.
At the beginning of each test, 10 *A. kimi* adults (42-46 day old) were introduced into each test vessel. The test vessels were kept under the condition that the aging proceeded at each temperature. The test vessels were aerated and weighed weekly to replenish the moisture loss by the addition of deionized water, if needed. Brewer's yeast was provided on the soil surface as food at the beginning of the test and biweekly. After 28 d, the surviving adults and juveniles produced in each vessel were counted by flotation with water and transferred to a petri dish filled with a moist substrate.

Fatty acid analysis

The effects of glyphosate treatment on the fatty acid composition of adults were determined at 20ºC and 25ºC. The test concentrations were 0.0 (control), 37.1, and 370.5 mg/kg. Because no significant effect on adult mortality was observed at any of the glyphosate concentrations, the tested concentrations were selected based on the lowest and highest concentrations at which a decrease in juvenile production was observed. After 28 days of exposure to 37.1 mg/kg and 370.5 mg/kg of glyphosate at 20ºC and 25ºC, the adults that survived the glyphosate treatments were transferred to and kept in the plastic petri dish for 1 d without feeding to excrete their gut contents. The adults exposed to the same concentration were pooled and transferred to a 2 mL microtube and then stored at 80ºC until fatty acid analysis.
The total cellular fatty acids in *A. kimi* adults were analysed. Briefly, 20 adults exposed to the same concentration were homogenized in 7.5 mL of a mixture of chloroform, methanol, and 0.05 M of phosphate buffer at a pH of 7.4 (1:2:0.8, v/v/v). After overnight shaking, 0.8 mL of distilled water and 0.8 mL of chloroform were added. The mixture was centrifuged at 1500 rpm for 5 min and then allowed to stand to separate into three layers of methanol, phosphate buffer, and chloroform. The lipids in the chloroform layer were saponified and methylated using the Sherlock Microbial Identification System to produce fatty acid methyl esters (FAMEs). Finally, the FAMEs were analysed using the Sherlock Microbial Identification System. This procedure was repeated three times. In addition, the total cellular lipids (TCLs) in *A. kimi* adults were estimated using an external standard curve of palmitic acid (16:0).

Glyphosate degradation in soil

To investigate the temperature-dependent degradation kinetics of glyphosate, 30 g of soil spiked with 370.5 mg/kg of glyphosate were prepared and stored at 20ºC and 25ºC. The glyphosate concentrations in 5 g of soil were measured at 0, 2, 4, 7, and 14 days after exposure with three replicates each. The control group was treated with distilled water only. In addition, a series of diluted glyphosate solutions (0-20 mg/L) was prepared by dissolving a standard glyphosate solution (1000 μg/mL) in 20 mL of 2 M ammonium hydroxide and used to obtain a standard curve.
The glyphosate concentration in the soil samples was determined after soil samples preparation. A linear relationship between the absorbance (measured at 435 nm) and concentration of glyphosate in standard solutions was obtained ($r^2 = 0.92$). No glyphosate was detected in the control soil, and the recovery rate of glyphosate was 79.59±12.75% in the glyphosate-treated soil. The limit of quantification of glyphosate was 2.08 mg/kg.

Data analysis

The degradation of glyphosate over time was fitted to a single first-order (SFO) kinetic model, as follows:

$$C_t = C_0 e^{-kt}, \qquad (1)$$

where C_t is the concentration of glyphosate at time t, C_0 is the initial concentration of glyphosate ($t = 0$), and k is the degradation rate constant. The degradation half-life time (DT_{50}), the time required for a 90% decline in concentration (DT_{90}), and k were estimated by fitting Equation 1 into a non-linear regression model.
To evaluate the effects of the temperature and aging time on the toxicity, an effective concentration of 50% (EC_{50}) on juvenile production and the corresponding 95% confidence intervals were estimated by

fitting the data to a logistic model. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) on juvenile production were determined by applying one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test ($p < 0.05$).
Prior to statistical analysis of the fatty acid profile, the data were arcsine-root trans- formed to normalize the distribution. The unsaturation index (UI; sum of the percentage of each unsaturated fatty acid multiplied by the number of double bonds) and the ratio of fatty acids with 16 carbons and 18 carbons (C16:C18) were calculated. To test whether the fatty acid profiles were affected by the glyphosate concentration and temperature, each fatty acid profile was compared using a two-way ANOVA with the temperature and concentration as independent variables. The Shapiro-Wilk test was used to confirm the normal distribution of data, and the Bartlett test was used to check for homogeneity of variance. A post hoc test for multiple comparisons was conducted using Tukey's test ($p < 0.05$). All the statistical data analyses were performed using SAS software (version 9.4). In addition, to visualize the differences in the fatty acid profiles across different glyphosate concentrations, principal component analysis (PCA) was conducted to evaluate the effects of glyphosate on the fatty acid composition. A permutational multivariate analysis (PERMANOVA) using the Euclidean distance as a similarity index was performed using the Adonis function in the Vegan package of RStudio version 1.2.5001.

Results

Toxicity test

The mean adult mortality rates in all controls were all less than 10%, and no significant difference was detected between the controls ($p > 0.05$). However, regardless of the aging time, the mean juvenile production per vessel in the control at 25ºC (135.8±31.4 for 0 d and 125.0±25.5 for seven days) was always significantly higher than that observed at 20ºC (82.5±6.5 and 76.0±12.3, respectively) ($p < 0.05$). The coefficient of variation calculated for the number of juveniles was less than 30% in all controls.
In all of the treatments, the adult survival within the entire test concentration range was not significantly different from that of the corresponding control, thereby indicating that glyphosate did not have a negative effect on adult survival. Thus, the median lethal concentration (LC_{50}) for adults could not be determined because all of the mortality values were below 50%. Meanwhile, juvenile production in all of the treatments was reduced in a concentration-dependent manner, but a significant difference in juvenile production from that of the control was observed only at 0 d of aging. At 20ºC with 0 day of aging, the EC_{50} value was 93.5 mg/kg and the NOEC and LOEC values were 3.7 mg/kg and 37.1 mg/kg, respectively. In the test at 25ºC with 0 day of aging, a significant difference from the control was observed only at the highest treatment concentration (370.5 mg/kg); thus, the EC_{50} values could not be estimated. In the seven-day aging test at both temperatures, no significant difference from the control was observed even at the highest concentration, thereby indicating that the toxicity of glyphosate to *A. kimi* was reduced as the aging time and temperature increased.

Fatty acid composition

At both temperatures, the TCL concentration per individual was lower in the glyphosate treatments than in the control, but no significant difference was observed between the treatments (Table 1). The TCL concentration in the control was 6.90±1.10 nmol/ind and 8.20±4.40 nmol/ind at 20ºC and 25ºC, respectively, but that in the treatments ranged from 3.10±0.60 nmol/ind to 3.80±1.90 nmol/ind.
Six fatty acids with a carbon chain length of 16 to 20 were identified as palmitoleic acid (16:1ω7), palmitic acid (16:0), linoleic acid (18:2ω6,9), oleic acid (18:1ω9), stearic acid (18:0), and arachidonic acid (20:4ω6,9,12,15). Each fatty acid composition is presented on a percentile scale in Table 1. Regardless of the temperature and glyphosate concentration, the most dominant fatty acids were oleic acid (39.65% to 44.63%) and palmitic acid (21.37% to 24.83%). The compositions of stearic acid and linoleic acid were very similar, ranging from 12.54% to 15.75% and 12.50% to 14.90%, respectively.
In addition, a significant interaction between the glyphosate concentration and temperature was detected in palmitoleic acid and oleic acid, but no significant differences in the proportion of each fatty acid were observed, except for palmitoleic acid, which was undetected at the highest concentration at 25ºC (Table 1).

Table 1. Proportions of fatty acids (mean ± standard deviation) in *Allonychiurus kimi* (Lee) adults after 28 day of exposure to glyphosate (0.0, 37.1, and 370.5 mg/kg) at 20ºC and 25ºC.

		20 °C			25 °C		
		Glyphosate Concentration (mg kg^{-1})			Glyphosate Concentration (mg kg^{-1})		
Fatty Acids		0.0	37.1	370.5	0.0	37.1	370.5
Palmitoleic acid	16:1ω7	5.77 ± 1.06	5.60 ± 3.68	5.48 ± 0.17	5.16 ± 0.18	6.72 ± 1.34	**0.00 ± 0.00**
Palmitic acid	16:0	21.37 ± 1.15	24.31 ± 0.43	23.79 ± 0.26	22.87 ± 0.71	22.84 ± 0.11	24.83 ± 1.30
Linoleic acid	18:2ω6,9	14.90 ± 0.95	13.41 ± 1.98	13.60 ± 0.93	12.50 ± 1.07	13.03 ± 0.81	13.70 ± 0.57
Oleic acid	18:1ω9	39.65 ± 0.07	44.63 ± 2.96	41.40 ± 1.11	40.18 ± 0.86	39.54 ± 1.68	43.46 ± 1.40
Stearic acid	18:0	12.84 ± 0.39	12.54 ± 1.87	**15.75 ± 0.11**	13.38 ± 0.17	**14.99 ± 0.37**	**15.49 ± 0.30**
Arachidonic acid	20:4ω6,9,12,15	5.49 ± 1.58	2.51 ± 3.55	0.00 ± 0.00	5.93 ± 0.47	**0.00 ± 0.00**	**0.00 ± 0.00**
Total cellular lipids (nmol·ind^{-1})		6.90 ± 1.10	3.80 ± 1.90	3.70 ± 1.20	8.20 ± 4.40	3.70 ± 0.20	3.10 ± 0.60
C16:C18 [a]		0.40 ± 0.04	0.39 ± 0.08	0.41 ± 0.00	0.42 ± 0.01	0.44 ± 0.00	**0.34 ± 0.01**
Unsaturation index [b]		0.97 ± 0.07	0.84 ± 0.11	0.74 ± 0.01	0.94 ± 0.03	**0.72 ± 0.05**	**0.71 ± 0.03**

The bold numbers indicate a significant difference from the control within the same temperature treatment (Dunnett's post hoc test; $p < 0.05$). [a] The ratio of fatty acids with 16 carbons and 18 carbons. [b] The unsaturation index was calculated as described by Haubert et al., 2004, Comp. Biochem. Physiol. B Biochem. Mol. Biol., 138, 41-52.

The two-way ANOVA results indicated that the proportions of palmitic acid, stearic acid, and arachidonic acid were significantly affected by the glyphosate concentration ($p < 0.05$), but no temperature effect on the six fatty acid profiles was observed (Table 2). The proportion of stearic acid, a long-chain saturated fatty acid, increased as the glyphosate concentration increased at 20ºC and 25ºC, but that of arachidonic acid, a polyunsaturated fatty acid (PUFA), decreased as the concentration increased and was not detected at the highest concentration at both temperatures.

Table 2. Two-way analysis of variance results on the effects of glyphosate and temperature on the individual fatty acid contents in *Allonychiurus kimi* (Lee) adults after 28 day of exposure to glyphosate.

Fatty Acids		Glyphosate (G)		Temperature (T)		G × T	
		F	*p*	*F*	*p*	*F*	*p*
Palmitoleic acid	16:1ω7	3.02	0.12	0.76	0.42	8.00	**0.02**
Palmitic acid	16:0	7.91	**0.02**	0.64	0.45	4.05	0.08
Linoleic acid	18:2ω6,9	0.21	0.82	1.78	0.23	1.35	0.33
Oleic acid	18:1ω9c	2.90	0.13	0.81	0.40	5.53	**0.04**
Stearic acid	18:0	9.41	**0.01**	3.72	0.10	2.82	0.14
Arachidonic acid	20:4ω6,9,12,15	14.14	**0.01**	0.78	0.41	1.05	0.41
C16:C18 [a]		1.15	0.38	0.00	0.98	3.03	0.12
Unsaturation index		11.74	**0.01**	2.28	0.18	0.29	0.76

The bold numbers indicate a significant effect of the factor on individual fatty acids ($p < 0.05$). [a] The ratio of fatty acids with 16 carbons and 18 carbons.

Owing to the increase in C18 and saturated fatty acids (stearic acid) and the decrease in C16 and unsaturated fatty acids (arachidonic acid and palmitoleic acid) due to glyphosate treatment, the UI and C16:C18 ratio at 25ºC decreased significantly as the glyphosate concentration increased. This was also evident from the biplot of the PCA, which clearly separated the fatty acid profiles exposed to different glyphosate concentrations according to the changes in the proportions of stearic acid and arachidonic acid. The PERMANOVA results also revealed significant influences of glyphosate application ($F = 3.45$; $p = 0.02$) on the fatty acid profiles.

Glyphosate degradation

The residual glyphosate concentration in the soil decreased exponentially over time at both temperatures. The lag phase was not observed in the initial degradation state and after two days of incubation, and the residual glyphosate concentration at 20ºC (51.8±9.0 mg/kg) was significantly higher than that at 25ºC (27.5±1.2 mg/kg) ($p < 0.05$). At the end of the experiment, the residual glyphosate concentration at 20ºC was 2.2±1.1 mg/kg, but no glyphosate was detected at 25ºC, thereby indicating that glyphosate was

rapidly degraded as the temperature increased.
Because the χ^2 error values for the SFO model were smaller than the validity criteria (15%) and their visual fits and residual plots were acceptable, the SFO model was used to estimate the DT_{50} and DT_{90} values (Table 3). The DT_{50} and DT_{90} values at 20ºC (2.38 day and 7.91 day, respectively) were longer than those at 25ºC (1.69 day and 5.63 day, respectively).

Table 3. Degradation kinetics parameter (k), DT_x values, and χ^2 error for glyphosate fitted with the single first-order model in soil at 20ºC and 25ºC.

Temperature (°C)	k	DT_{50} (d)	DT_{90} (d)	χ^2 Error (%)
20	0.291	2.38 (1.74–3.02)	7.91 (5.79–10.05)	9.55
25	0.409	1.69 (1.27–2.11)	5.63 (4.23–7.01)	14.02

DT_x values indicate the time required for the initial concentration to decrease x%. The degradation kinetics of glyphosate were investigated in soil contaminated with 370.5 mg/kg of glyphosate at both temperatures.

Conclusion

Glyphosate have been known to be toxicologically and environmentally benign. However, the authors concluded that their study showed that glyphosate can have negative effects on the juvenile production of *A. kimi* depending on the temperature and aging time. Glyphosate toxicity is largely determined by the amount of residue in the soil, which is dependent on the soil temperature. Thus, an understanding of the temperature-dependent half-life of glyphosate in various field soils in which the temperature fluctuates considerably is important to assess the ecologically relevant risk under field conditions. No glyphosate-induced adult mortality was observed in any of the treatment conditions, but significant changes in the fatty acid composition of *A. kimi* adults, which play a critical role in energy storage, cell structure, and regulatory physiology, were observed, thereby suggesting that glyphosate could have adverse effects on *A. kimi* at the sub-individual level. Considering the increasing application rate and repeated application of glyphosate in fields, glyphosate concentrations higher than the PEC could be present in the soil. If *A. kimi* is exposed to glyphosate for a long time above the PEC, then the ecological fitness of an individual may also be affected. Therefore, it is necessary to investigate the long-term impacts of glyphosate by conducting higher-tier tests, such as multigenerational tests.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study examined the toxicity of glyphosate with the temperature (20ºC and 25ºC) and aging times (0 day and 7 days) in soil using a collembolan species, *Allonychiurus kimi* (Lee). The degradation of glyphosate in soil was investigated. Fatty acid composition of *A. kimi* was also investigated. The half-life of glyphosate was 2.38 days at 20ºC and 1.69 days at 25ºC. At 20ºC with 0 day of aging, the EC_{50} and NOEC were estimated to be 93.5 and 3.7 mg/kg, respectively. As the temperature and aging time increased, the glyphosate degradation also increased, so no significant toxicity was observed on juvenile production. The proportions of the arachidonic acid and stearic acid decreased and increased with the glyphosate treatment, respectively, even at 37.1 mg/kg, at which no significant effects on juvenile production were observed. The study was conducted according to OECD TG 232 Collembolan Reproduction Test in Soil and is considered reliable with restrictions, the validity criteria cannot be fully checked.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	As no raw data are provided not all validity criteria can be checked
2. No previous exposure to other chemicals is documented (where relevant).	Yes	The species has been cultured for years in the lab and the soil is according to guidance
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	-	No aquatic study
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	-
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	As no raw data are provided not all validity criteria can be checked
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Analytical verifications of the concentration of glyphosate in soil samples were conducted
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint reported: 28-d EC_{50} and NOEC
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	-
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Analytical verifications of the concentration of glyphosate in

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		soil samples were conducted
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	5 replicates of 10 individuals for each treatment group
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation were provided. No raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	Yes	OECD substrate
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Lab study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	Yes	OECD artificial soil used
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	Yes	OECD artificial soil used
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Lab study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Lab study
20.6. Data on precipitation is recorded.	-	Lab study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	Yes	2 temperatures (20 and 25°C) set
22. For bee studies, temperature of the study should be appropriate to species.	-	No bee study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	-	Not aquatic study
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	-	Not aquatic study
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Yes	Analytical verifications of the concentration

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		of glyphosate in soil samples were conducted
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Analytical verifications of the concentration of glyphosate in soil samples were conducted
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	Analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Yes	95% confidence intervals of the EC_{50} were calculated

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study examined the toxicity of glyphosate with the temperature (20ºC and 25ºC) and aging times (0 day and 7 days) in soil using a collembolan species, *Allonychiurus kimi* (Lee). The degradation of glyphosate in soil was investigated. Fatty acid composition of *A. kimi* was also investigated. The half-life of glyphosate was 2.38 days at 20ºC and 1.69 days at 25ºC. At 20ºC with 0 day of aging, the EC_{50} and NOEC were estimated to be 93.5 and 3.7 mg/kg, respectively. As the temperature and aging time increased, the glyphosate degradation also increased, so no significant toxicity was observed on juvenile production. The proportions of the arachidonic acid and stearic acid decreased and increased with the glyphosate treatment, respectively, even at 37.1 mg/kg, at which no significant effects on juvenile production were observed. The study was conducted according to OECD TG 232 Collembolan Reproduction Test in Soil and is considered reliable with restrictions, the validity criteria cannot be fully checked.
Not reliable	No	-