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
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
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.

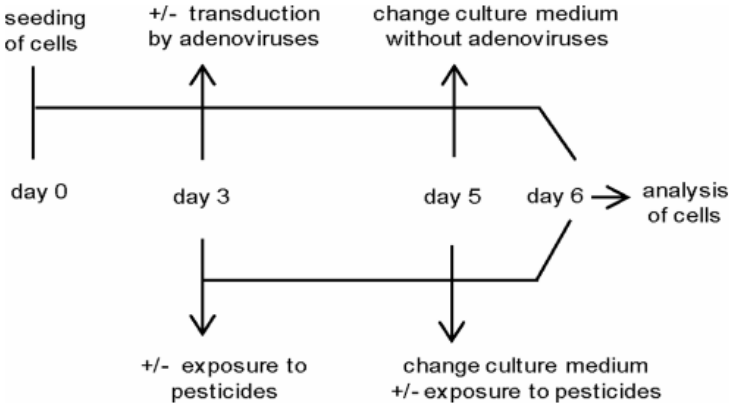


Figure 1. Experimental protocol of exposure to pesticides and recombinant AdV transduction of SH-SY5Y neuroblastoma and SK-MEL-2 melanoma cell lines. At approximately 70% cell confluence (day 3), cells were transduced with recombinant AdVs for protein overexpression (WT α-syn, A53T α-syn, GFP) and/or 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.

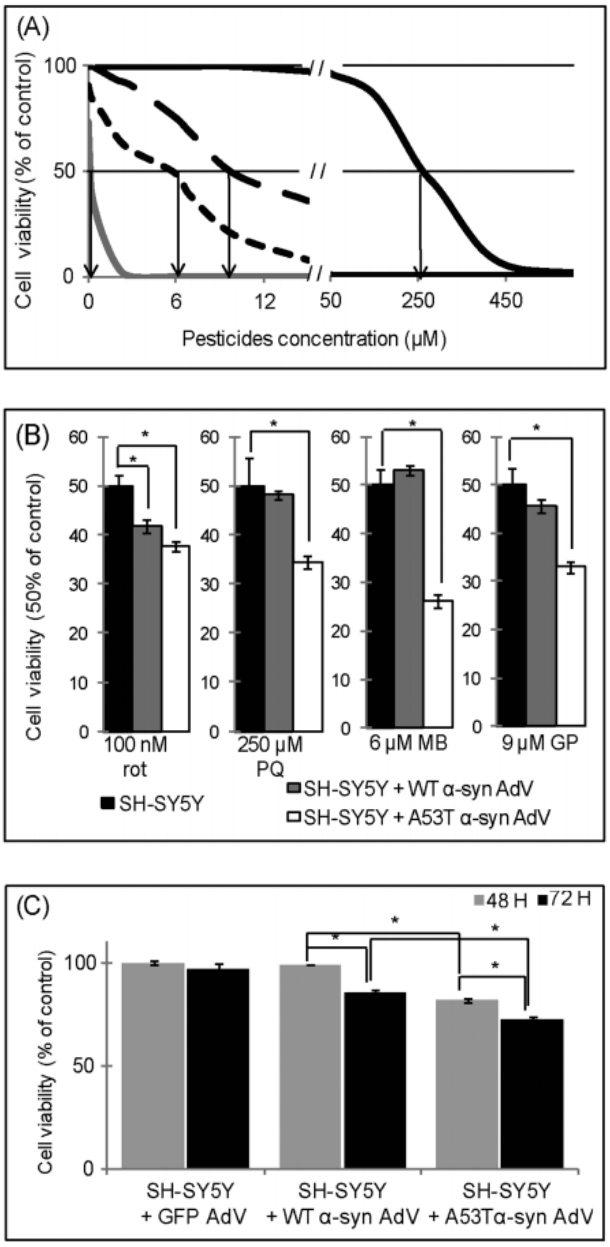


Figure 2. Effects of pesticides and adenoviral transduction on viability of SH-SY5Y neuroblastoma cells. Cell viability was assessed using the MTT assay. Data represent mean ± SEM numbers of viable treated cells/numbers of viable untreated cells from three separate experiments (*$p < 0.05$). The p values 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).

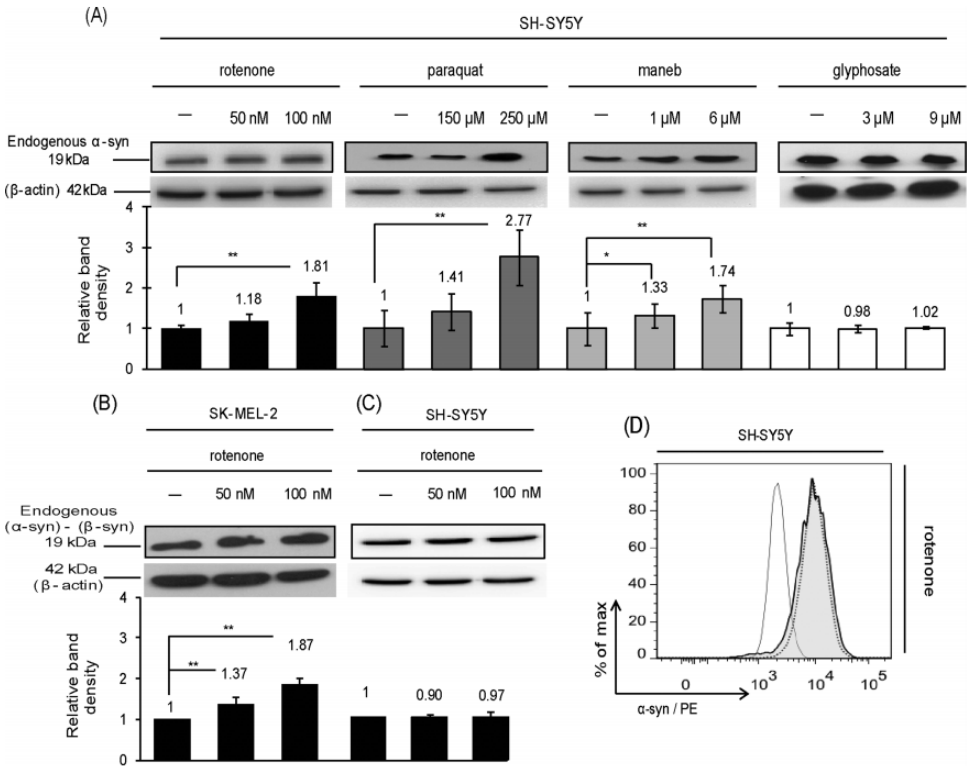


Figure 3. Characterization of the impact of pesticides on endogenous α-syn levels in SH-SY5Y and SK-MEL-2 cells by Western blot and flow cytometry. he levels of endogenous α-syn were estimated by Western blot after exposure of SH-SY5Y cells to different pesticides at concentrations corresponding to 25 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.

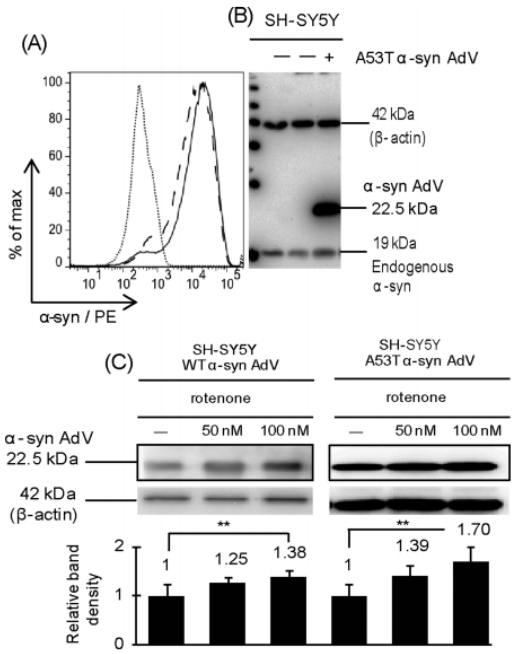


Figure 4. Characterization of the impact of pesticides on recombinant α-syn levels in SH-SY5Y cells by Western blot and flow cytometry. Three days after adenoviral transductions, the cells were permeabilized and immunostained with clone 42 antibody raised against α-syn. The flow cytometry histogram shows 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).

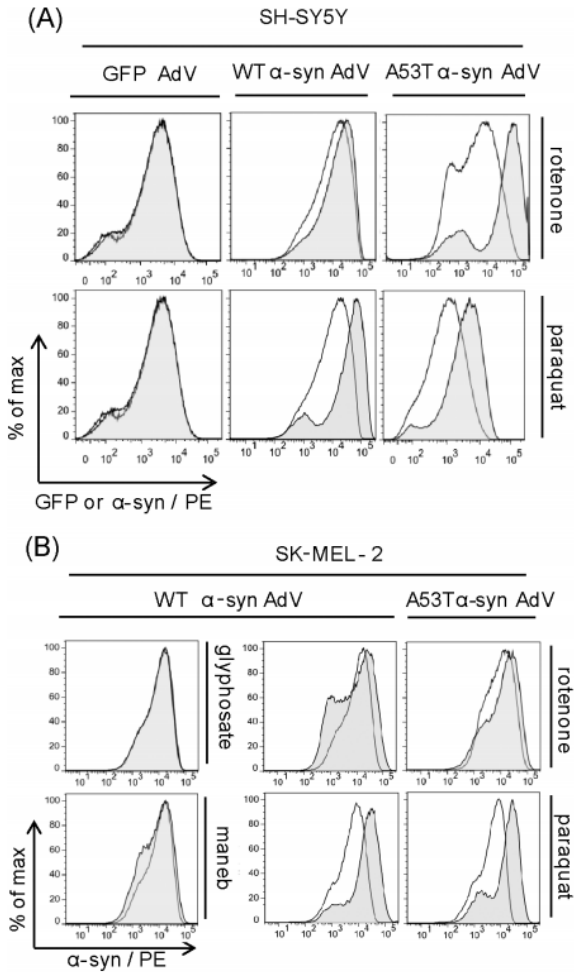


Figure 5. Characterization of the impact of pesticides on α-syn levels in AdV-transduced SH-SY5Y and SK-MEL-2 cells by flow cytometry. Fluorescence curves correspond to the α-syn levels following AdV transduction of SH-SY5Y cells by GFP AdV (left column, solid curve not shaded), WT α-syn AdV (center 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.

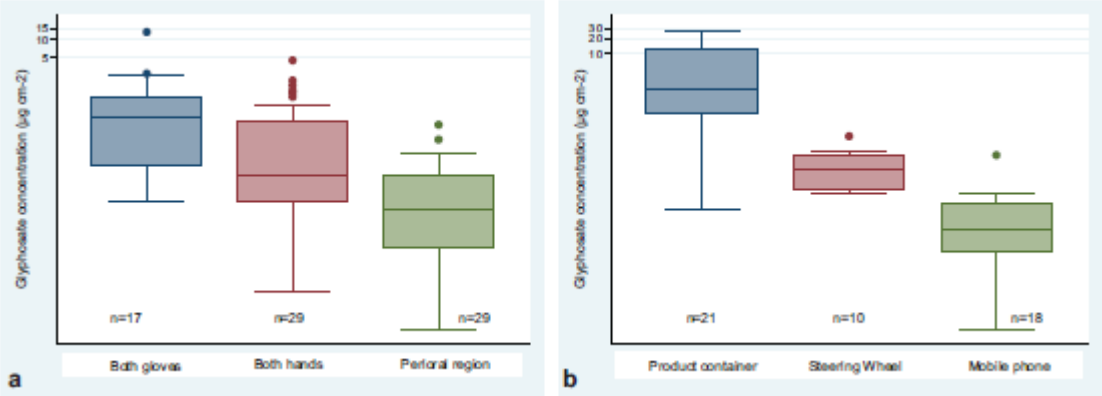


Figure 1. Boxplot showing the post-work task glyphosate concentrations for (a) disposable gloves, the workers hands (under the glove) and the perioral region and (b) for potentially contaminated work surfaces, pesticide product containers, the steering wheel of work vehicles and participants mobile phone (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).

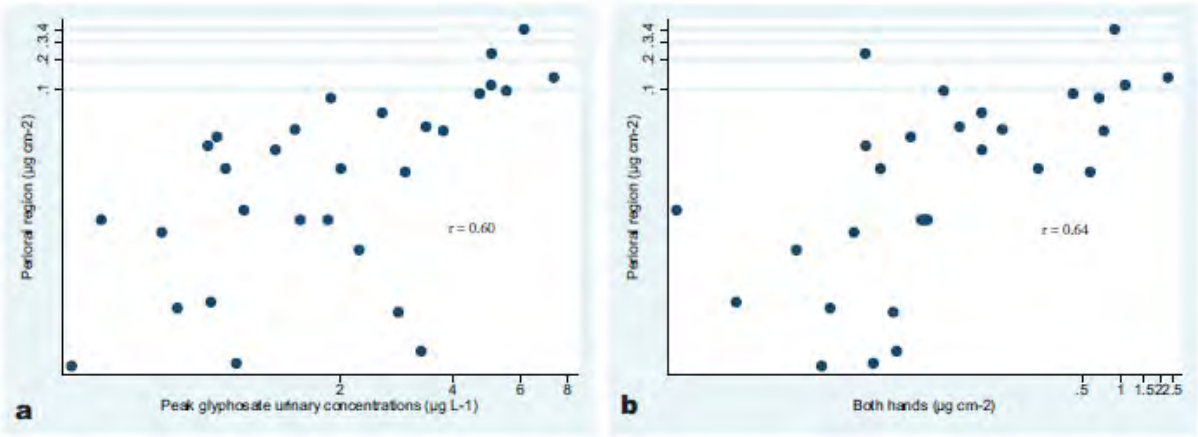


Figure 2. Scatter graph showing moderate relationships between glyphosate perioral glyphosate wipe contamination levels (µg/cm) and (a) peak urinary glyphosate concentrations (µg/L) and (b) both hands glyphosate surface loading contamination levels after a pesticide application task (µg/cm).

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

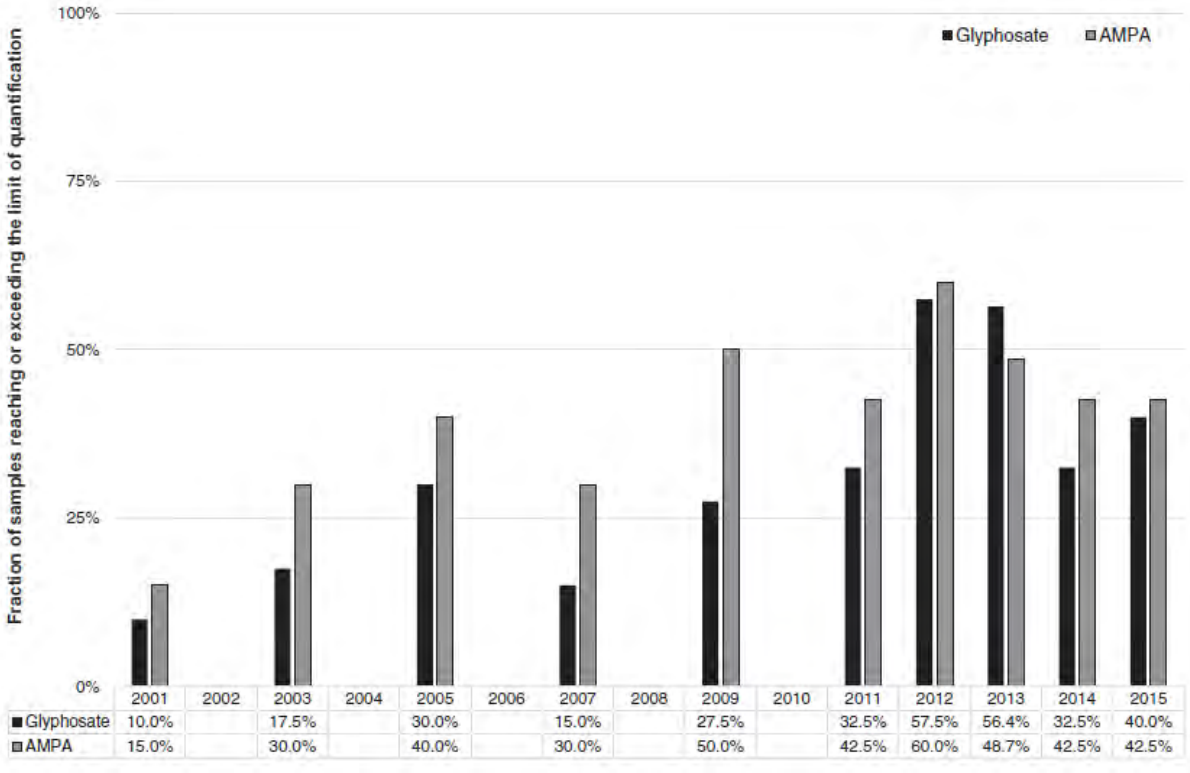
Statistical analysis; Glyphosate and AMPA concentrations below the LOQ were set to LOQ/2 prior to statistical evaluation. All data analyses were carried out in SPSS Statistics Version 20 (IBM Corporation, 2011). Differences between frequencies were tested with Pearson's Chi^2 test of independence after 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.

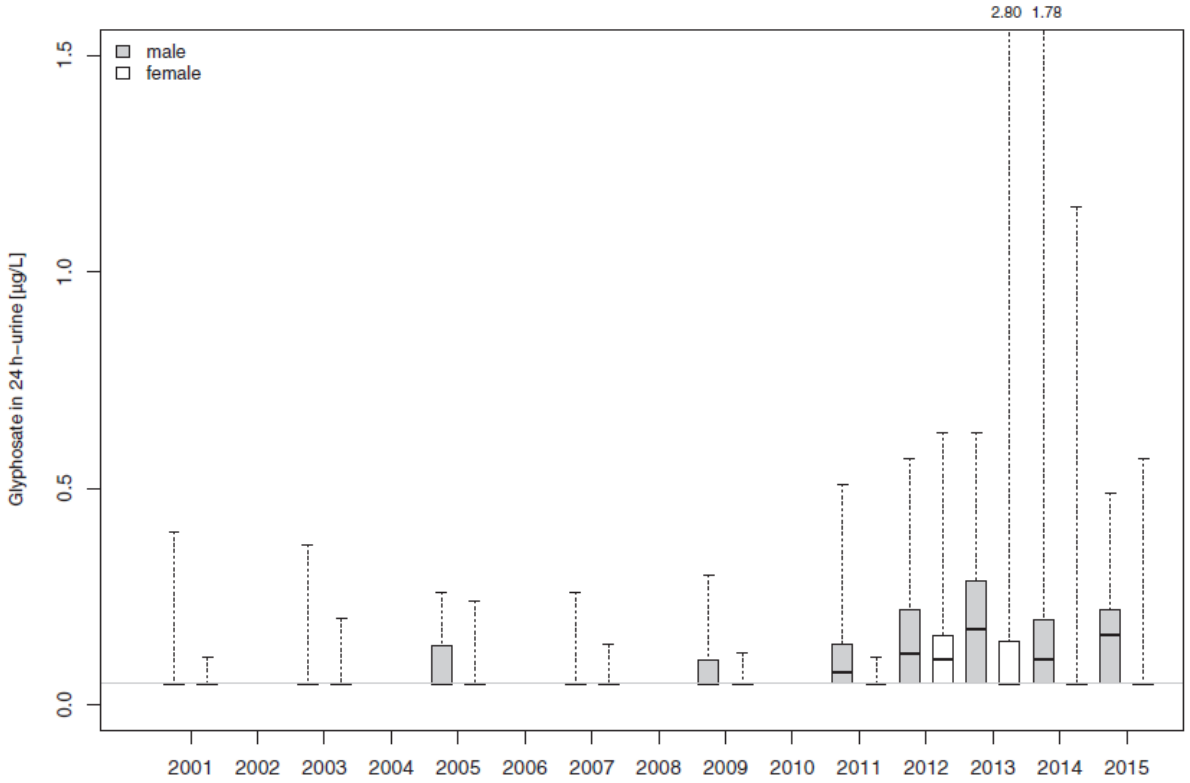


Fig. 2. Box-plots of glyphosate concentrations in 24 h-urine samples by study year and sex (ESB sampling location Greifswald, no self-reported specific dietary restriction, concentrations below LOQ of 0.1 µg/L set to LOQ/2 = horizontal solid line, box displays 25th, median and 75th percentile, whiskers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

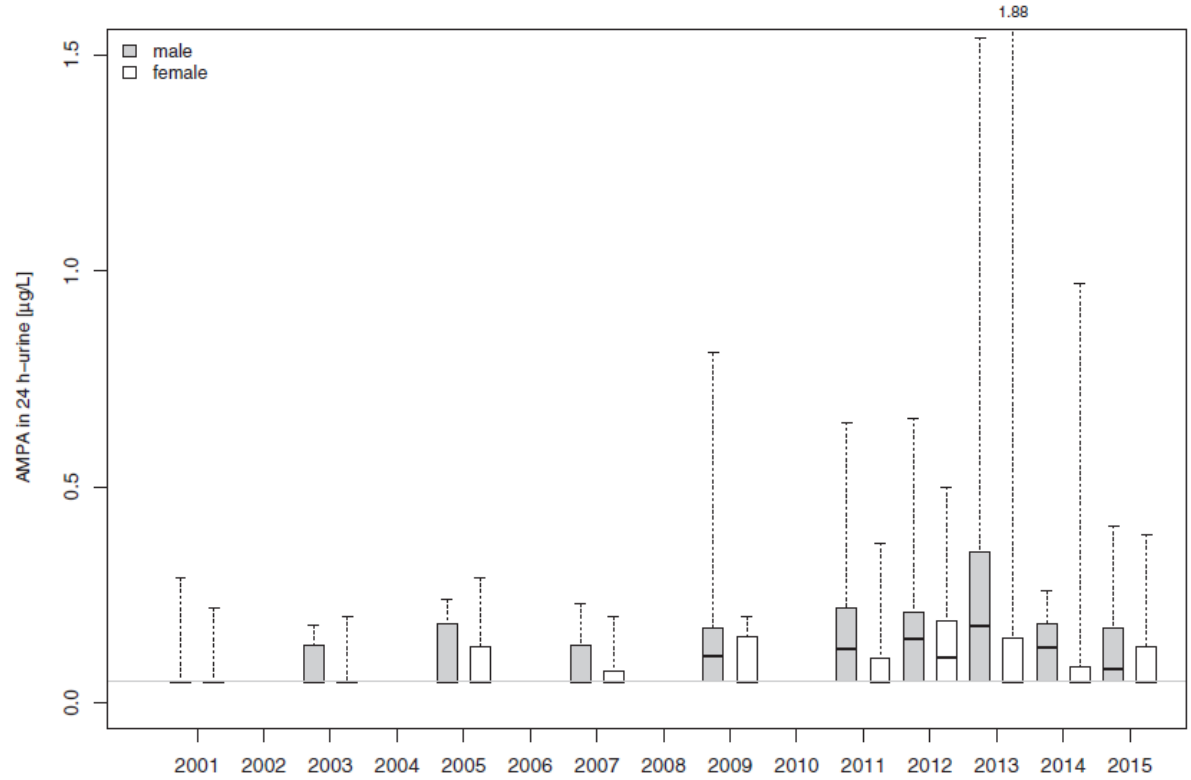


Fig. 3. Box-plots of AMPA concentrations in 24 h-urine samples by study year and sex (ESB sampling location Greifswald, no self-reported specific dietary restriction, concentrations below LOQ of 0.1 µg/L set to LOQ/2 = horizontal solid line, box displays 25th, median and 75th percentile, whiskers extend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

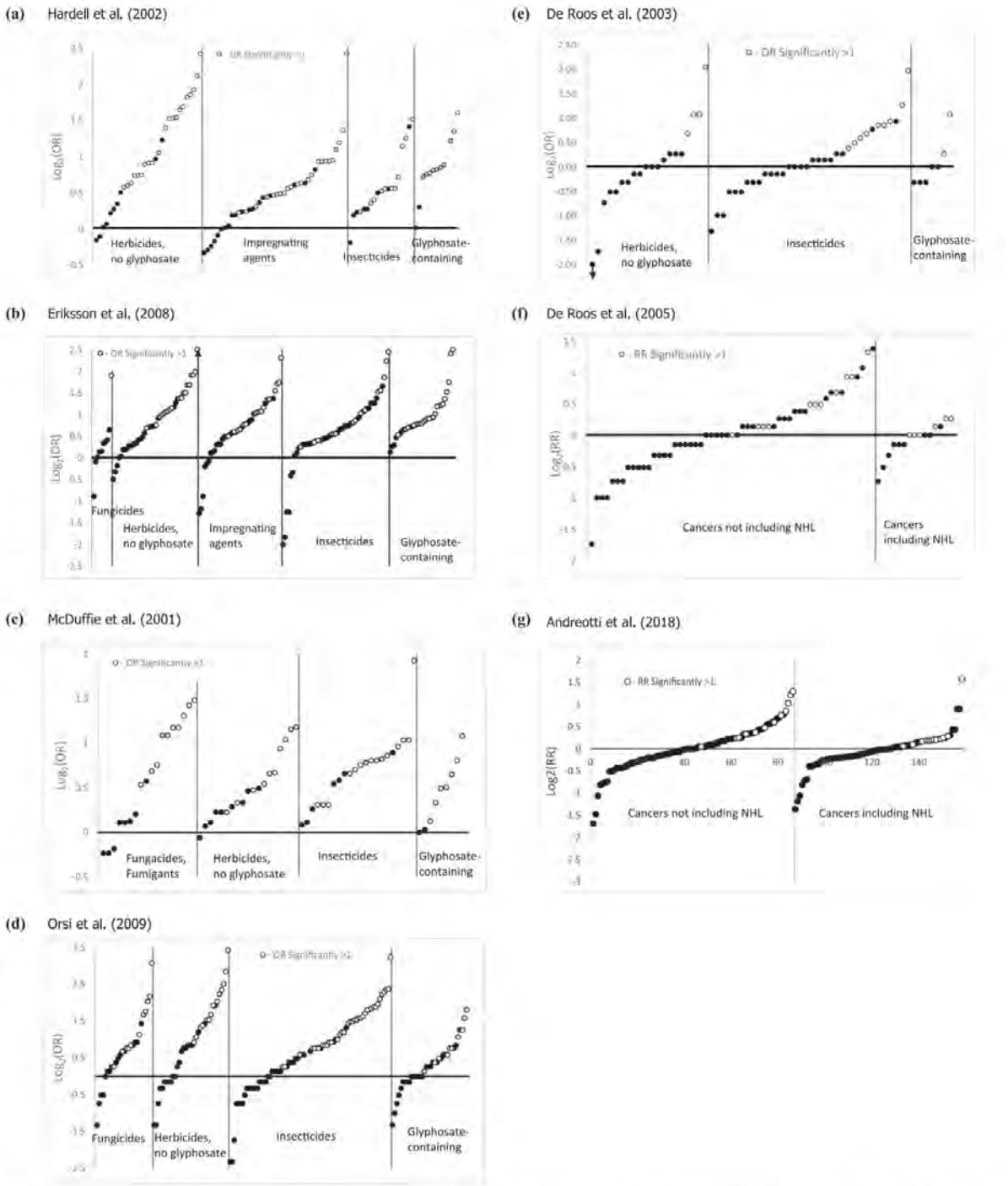


Fig. 1. Graphs of tabulated ORs and RRs. (a) Hardell et al. (2002), (b) Eriksson et al. (2008), (c) McDuffie et al. (2001), (d) Orsi et al. (2009), (e) De Roos et al. (2003), (f) De Roos et al. (2005), (g) Andreotti et al. (2018).

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

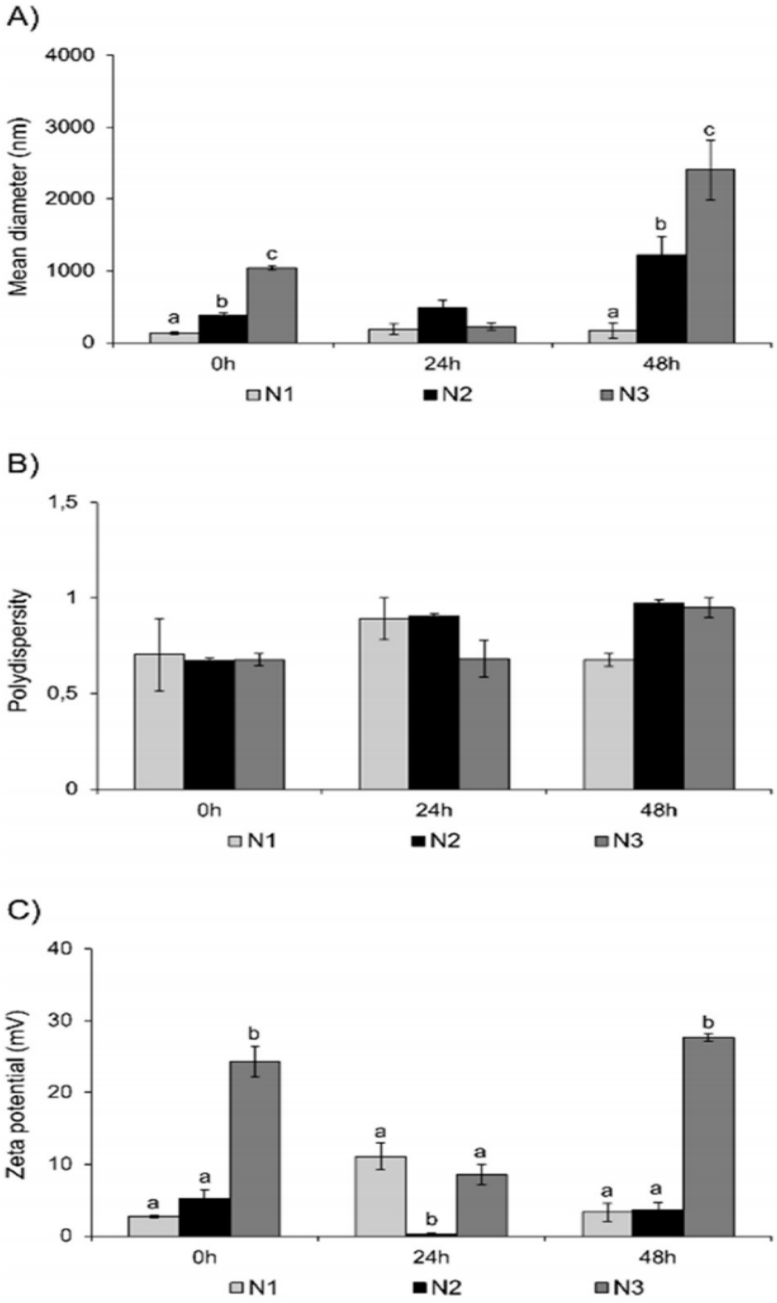


Fig. 1. Mean diameter (A), polydispersity (B), and zeta potential (C) of the goethite NPs present in the exposure solutions of 1, 10, and 100 mg L^{-1} (N1, N2, and N3) for 0, 24, and 48 h. Results are mean ± SE ($n = 3$). Different letters indicate significant differences between treatments for the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

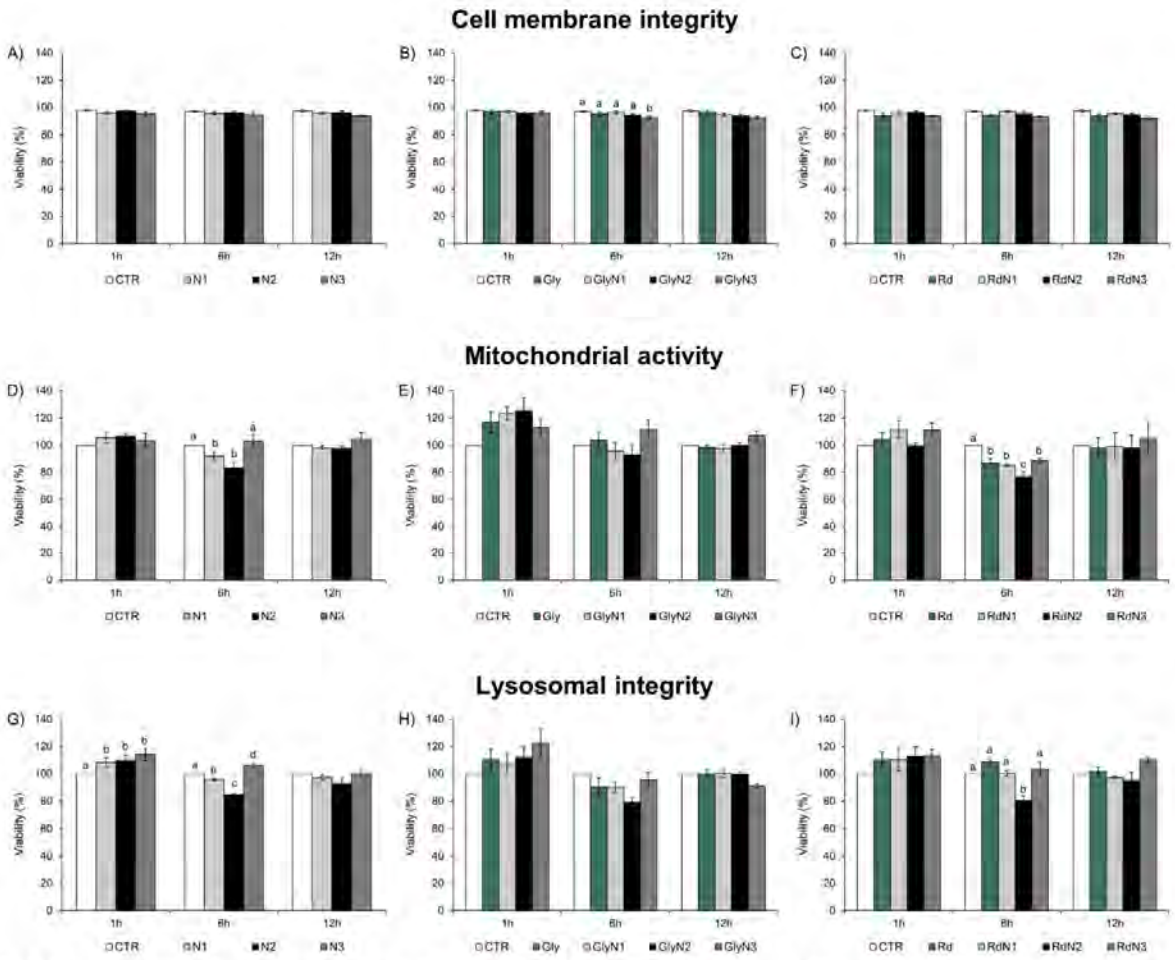


Fig. 2. Cell viability (%) based on plasma membrane integrity (A-C), mitochondrial activity (D-F), and lysosomal integrity (G-I) for ZFL cells exposed to: 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3) 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).

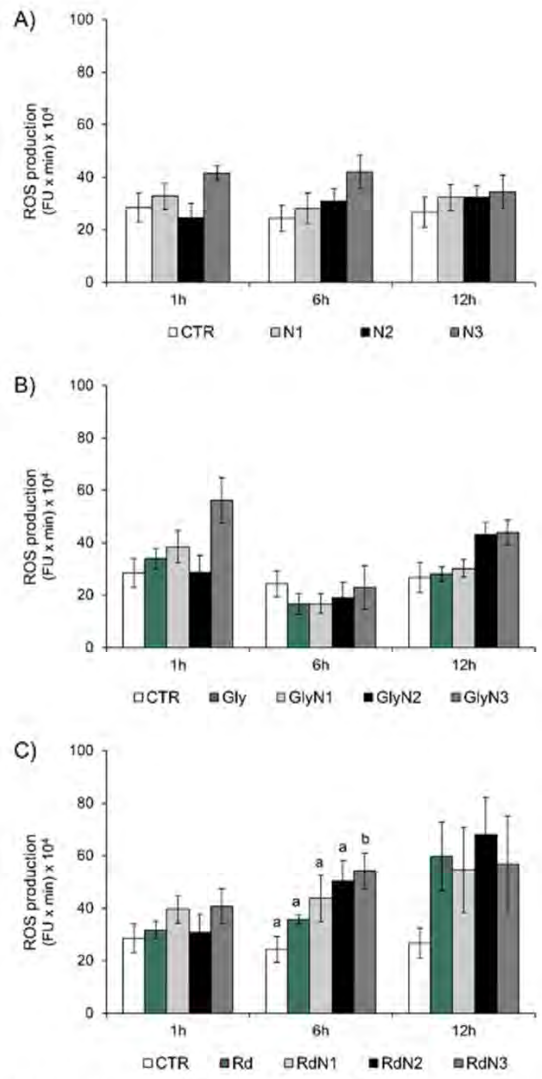


Fig. 3. Production of reactive oxygen species (ROS) in ZFL cells exposed to: A) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); B) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and C) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, 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 ($P < .05$).

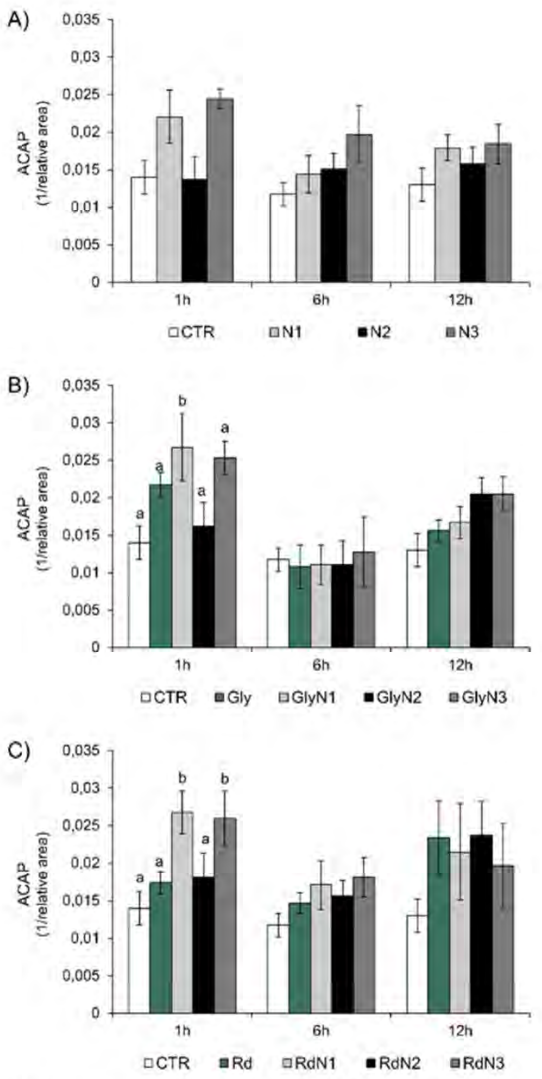


Fig. 4. Antioxidant capacity against peroxyl radicals (ACAP) of ZFL cells exposed to: A) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); B) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and C) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (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.

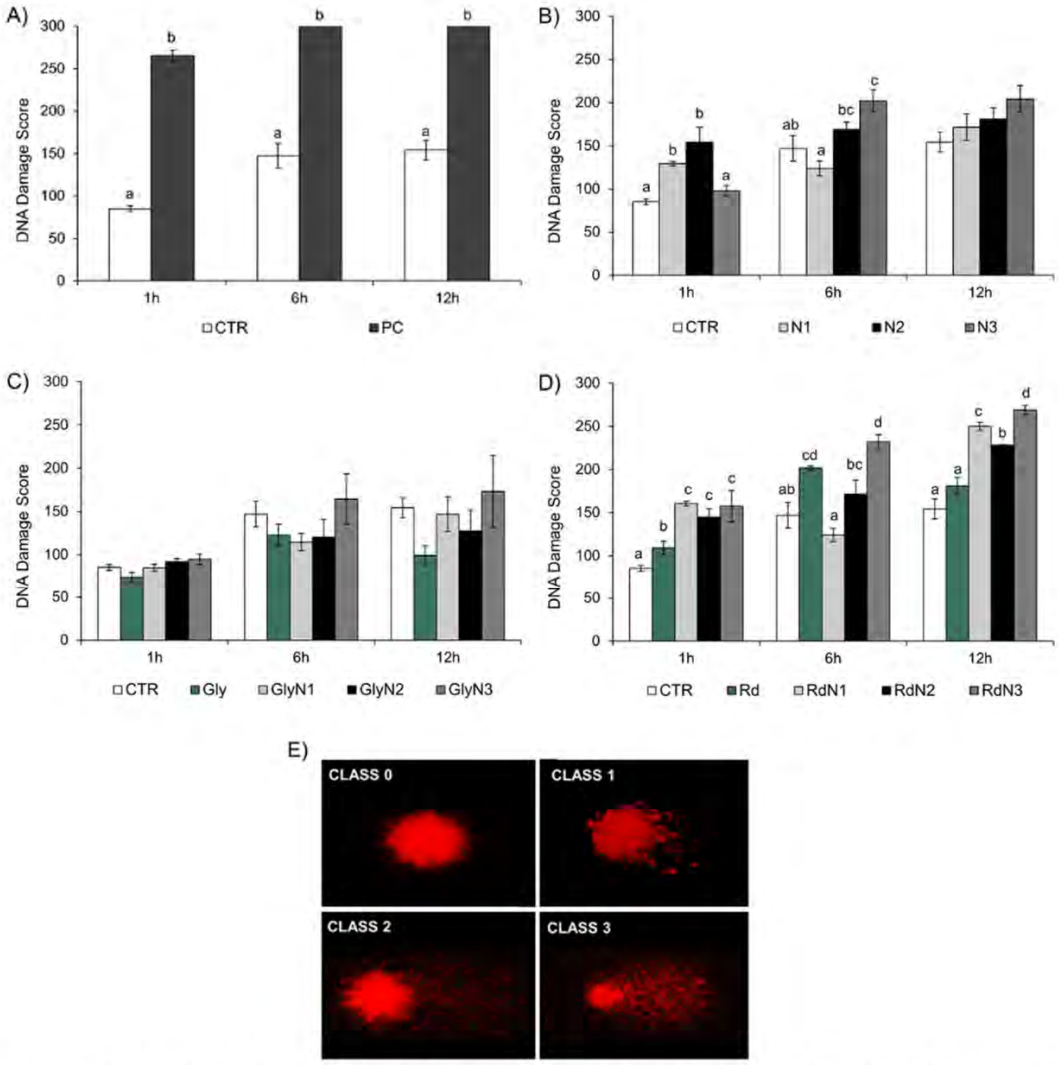


Fig. 5. A) Score of DNA damage in ZFL cells exposed to 0.5 mM of MMS (Positive Control or PC); B) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); C) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and D) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (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).

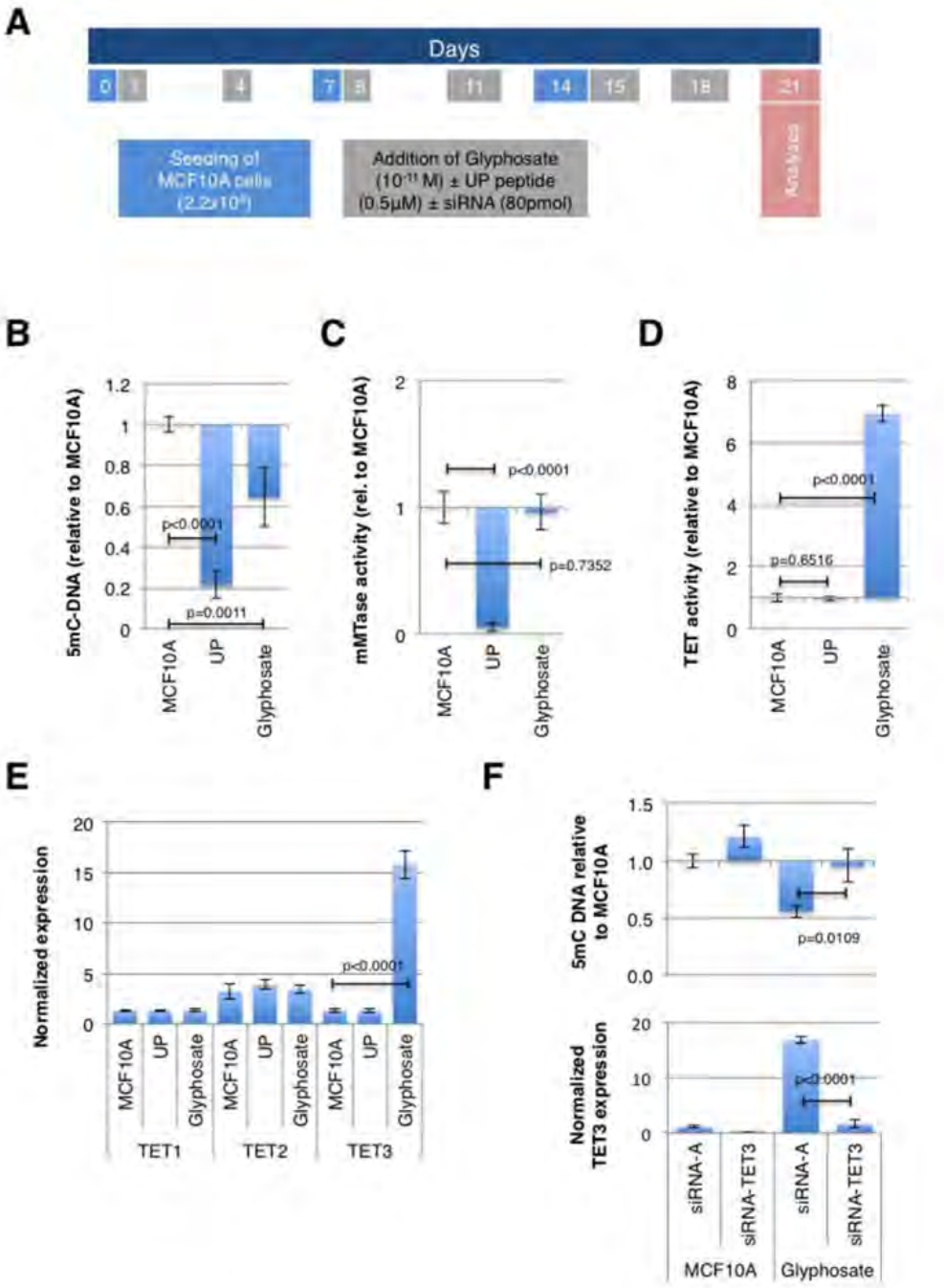


FIGURE 1 | Glyphosate exposure promotes a TET3-mediated global DNA hypomethylation. MCF10A cells were treated according to a timetable shown in **(A)** and analyzed on day 21 of culture. (Explanations for color-coded days are located in corresponding color rectangles underneath the timeline. UP peptide 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.

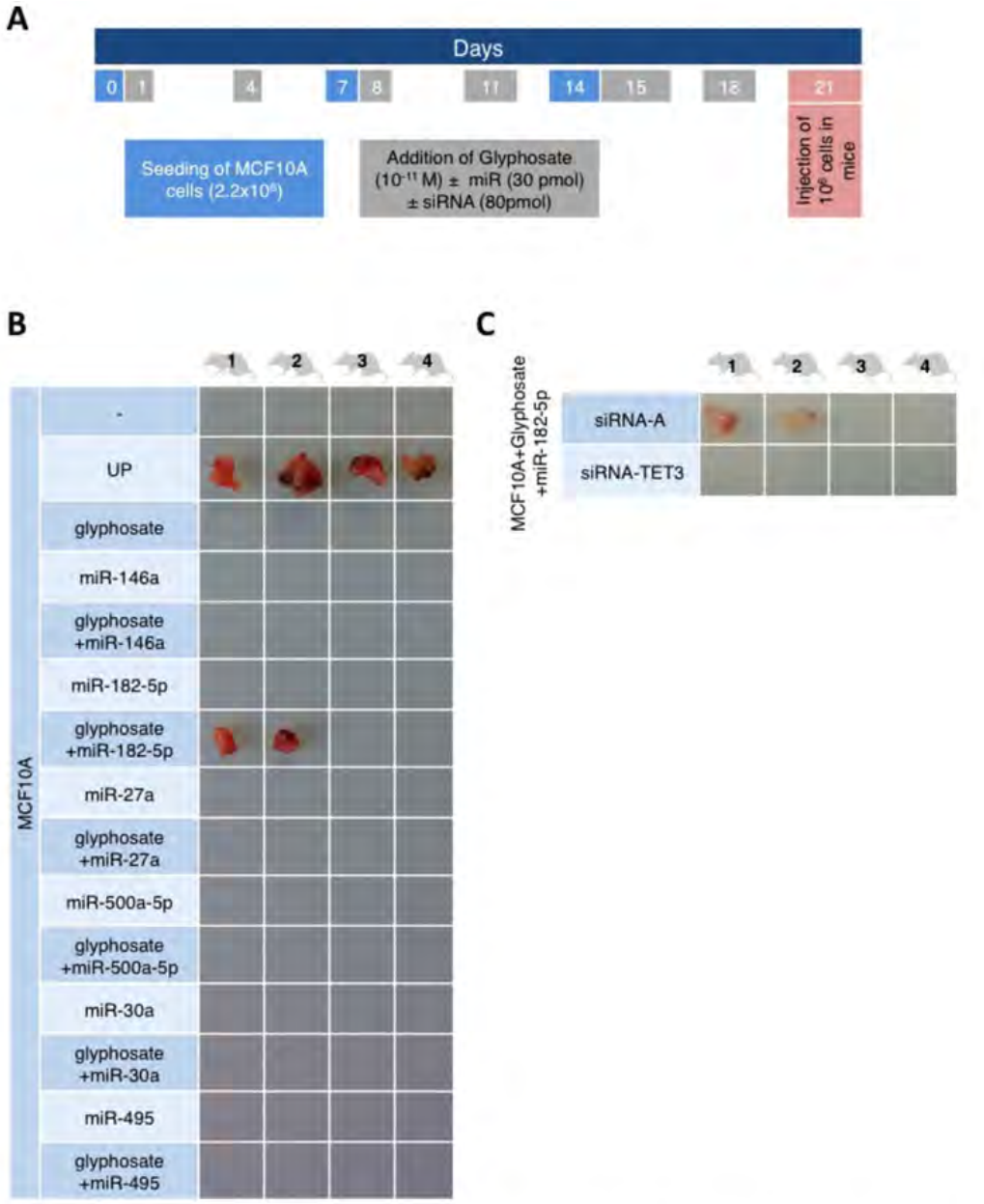


FIGURE 2 | The combination of glyphosate exposure and miR-182 overexpression is tumorigenic for MCF10A cells in a two-factor hit model. **(A)** The timetable illustrates the experiment design. Explanations for color-coded days are located in corresponding color rectangles underneath the timeline. **(B** 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.

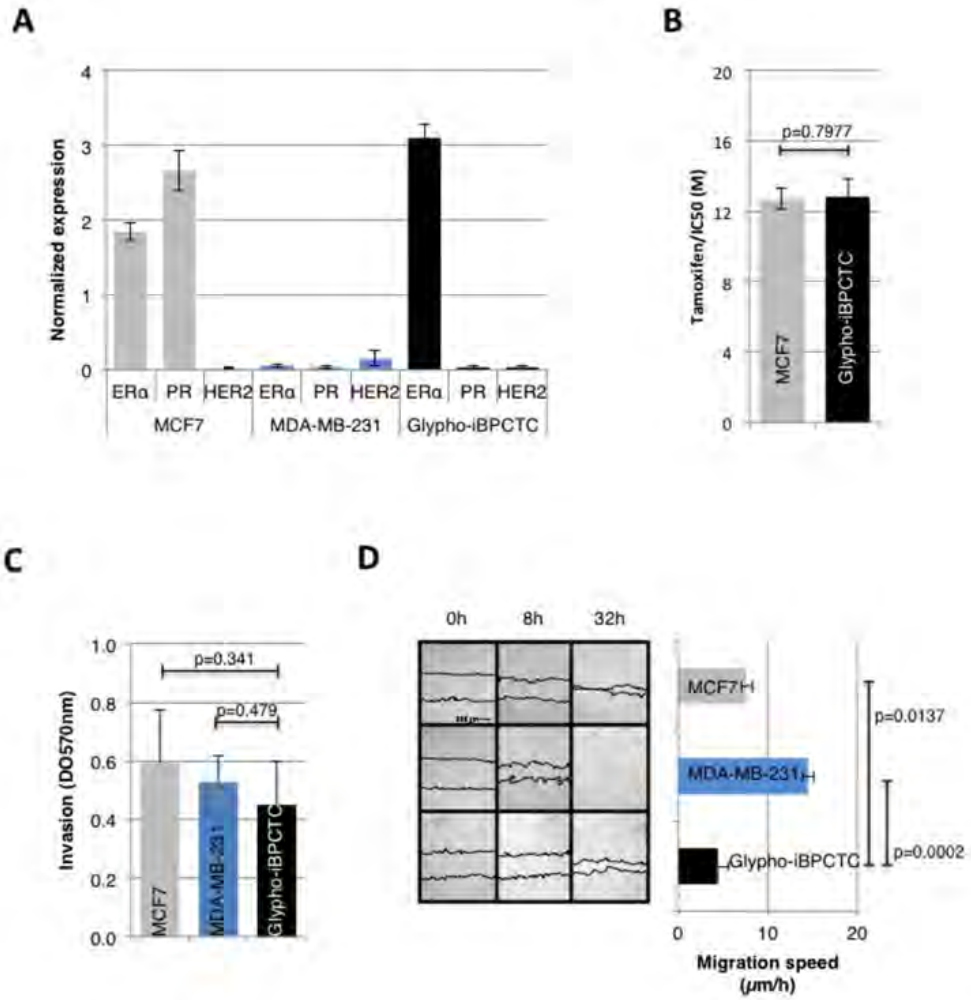


FIGURE 3 | Primary cells from glyphosate-induced breast tumor display characteristics of malignant cells. **(A)** The expression levels of ERα, PR, and HER2 were estimated in MCF7 cells, MDA-MB-231 cells, and Glypho-iBPCTC primary cells using In-Cell ELISA. Normalization to Janus Green staining intensity 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).

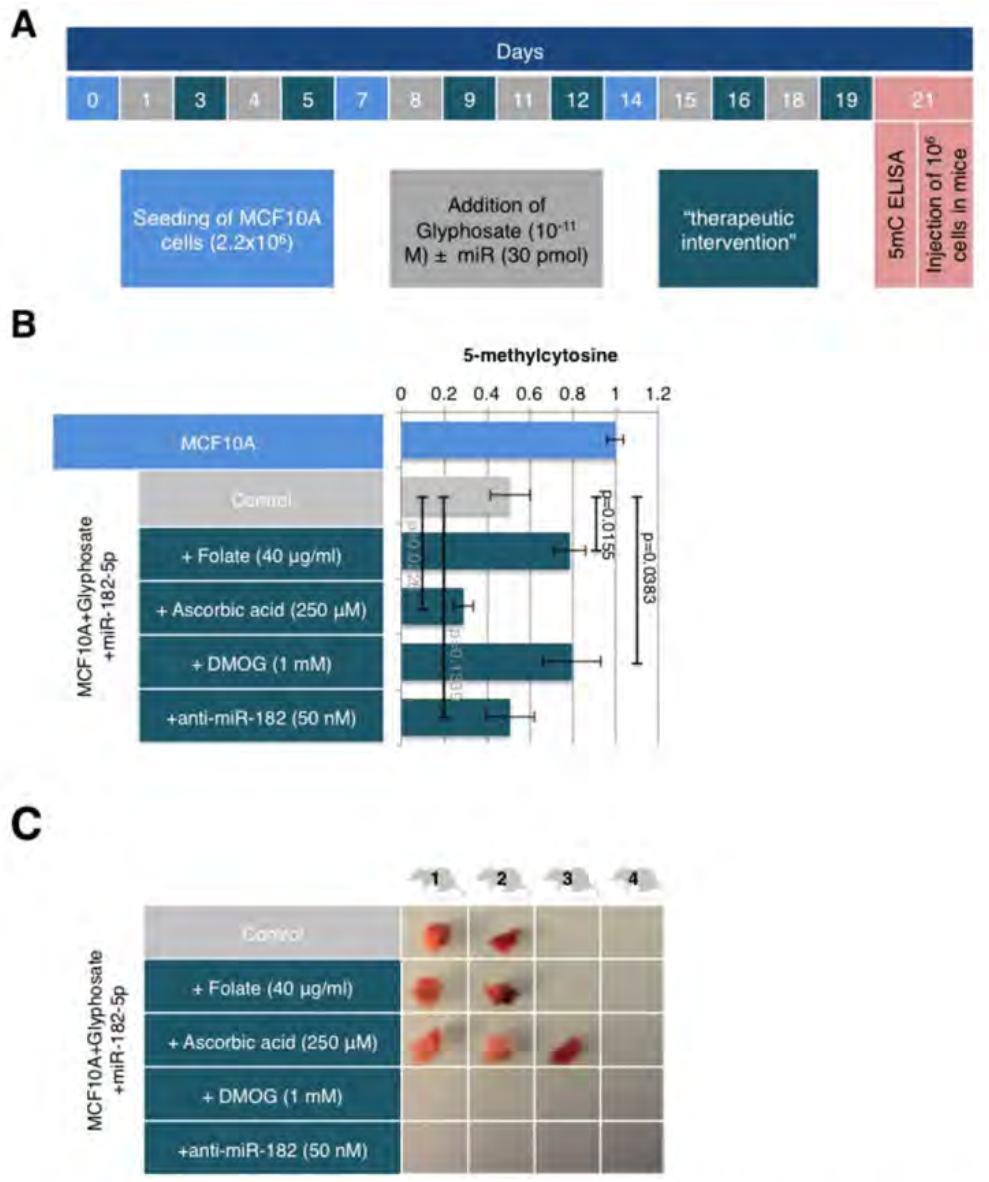


FIGURE 4 | DMOG and anti-miR-182 prevent tumor onset but differentially impact 5-meC level. **(A)** The timetable illustrating the experiment design. Explanations for color-coded days are located in corresponding color rectangles underneath the timeline. Therapeutic interventions on MCF10A cells treated 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.

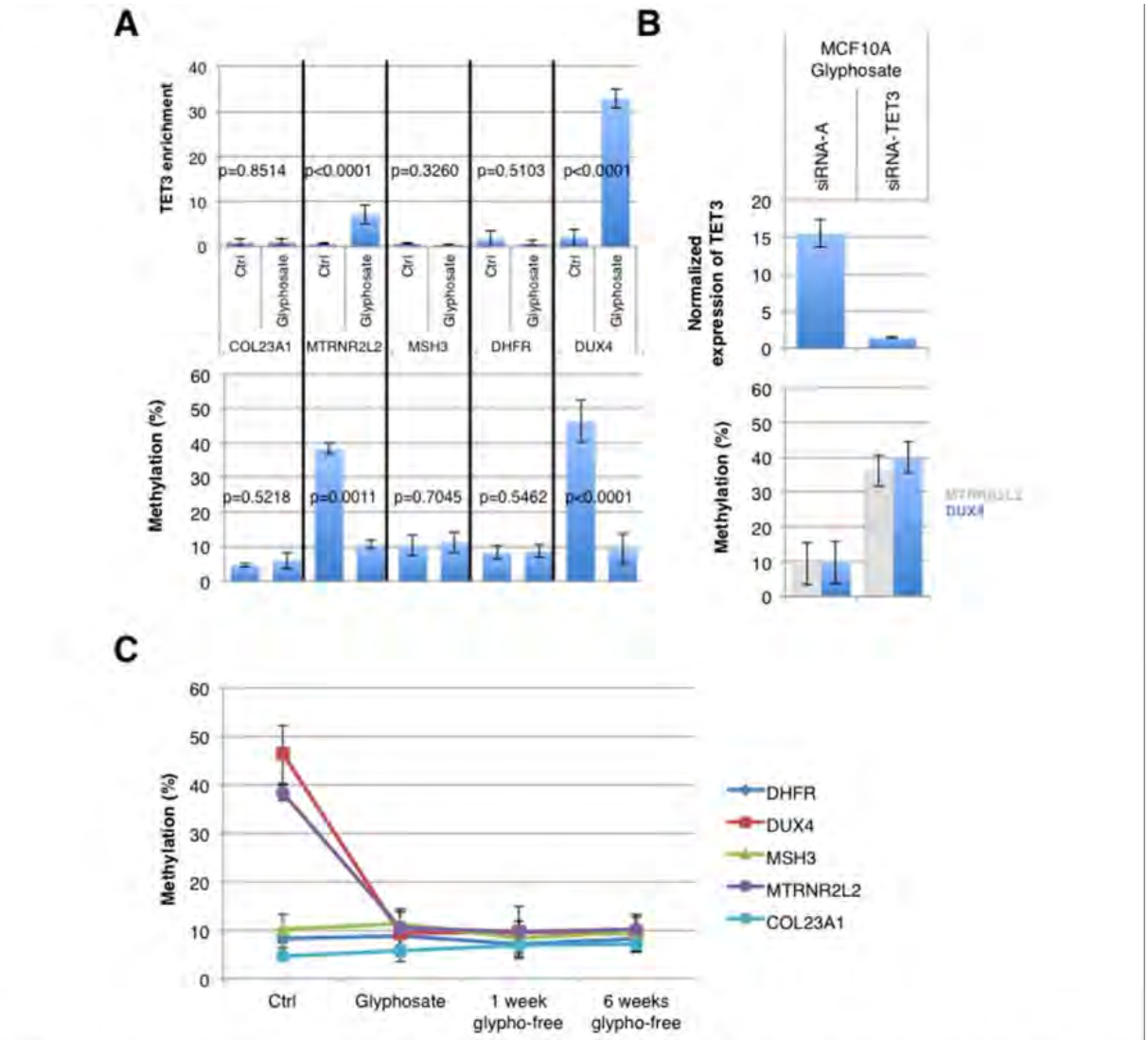


FIGURE 5 | Glyphosate-induced TET3-mediated demethylation affects *MTRNR2L2* and *DUX4* genes. **(A)** MCF10A cells were treated with glyphosate for 21 days as in the schedule shown in **Figure 2**. The graphs illustrate TET3 enrichment (top) following chromatin immunoprecipitation (ChIP) and the methylation 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.		