グリホサートカリウム塩

要旨及び評価結果

（ヒトに対する毒性）

検索期間：2021 年 1 月 1 日～2021 年 5 月 14 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 5.6
Report author	Gorga A. *et al.*
Report year	2021
Report title	Low doses of glyphosate/Roundup alter blood-testis barrier integrity in juvenile rats
Document No	Frontiers in Endocrinology, 2021, 12, 615678
Guidelines followed in study	None
Deviations from current test guideline	No guideline followed.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, the possible effects of glyphosate (G) or Roundup (R) treatment of juvenile male rats on blood-testis barrier function and on adult male sperm production was investigated. Pups were randomly assigned to the following groups: control group (C), receiving water; G2 and G50 groups, receiving 2 and 50 mg/kg/day G respectively; and R2 and R50 groups receiving 2 and 50 mg/kg/day R respectively. Treatments were performed orally from postnatal day (PND) 14 to 30, period of life that is essential to complete a functional blood-testis barrier. Evaluation was done on PND 31. No differences in body and testis weight were observed between groups. Testis histological analysis showed disorganized seminiferous epithelium, with apparent low cellular adhesion in treated animals. Blood-testis barrier permeability to a biotin tracer was examined. A significant increase in permeable tubules was observed in treated groups. To evaluate possible mechanisms that could explain the effects on blood-testis barrier permeability, intratesticular testosterone levels, androgen receptor expression, thiobarbituric acid reactive substances (TBARS) and the expression of intercellular junction proteins (claudin11, occludin, ZO-1, connexin43, 46, and 50 which are components of the blood-testis barrier) were examined. No modifications in the abovementioned parameters were detected. To evaluate whether juvenile exposure to G and R could have consequences during adulthood, a set of animals of the R50 group was allowed to grow up until PND 90. Histological analysis showed that control and R50 groups had normal cellular associations and complete spermatogenesis. Also, blood-testis barrier function was recovered and testicular weight, daily sperm production, and epididymal sperm motility and morphology did not seem to be modified by juvenile treatment. The authors concluded their results show that continuous exposure to low doses of G or R alters blood-testis barrier permeability in juvenile rats. However, considering that adult animals treated during the juvenile stage showed no differences in daily sperm production compared with control animals, the authors proposed it is feasible to think that blood-testis barrier impairment is a reversible phenomenon and that ore studies are needed to determine possible damage in the reproductive function of human juvenile populations exposed to low doses of G or R.

Materials and methods

Animals

3-month old pregnant Sprague Dawley rats (250-300 g) were purchased from the University of Buenos Aires. In that facility, nulliparous female rats were mated with a stud male. The animals were separated after confirmed copulation either by detection of vaginal plug or spermatozoa in vaginal smear samples. Five days prior to pup delivery, rats were transported to the facility in transportation crates and were housed singly in free exchange cages (30 cm x 40 cm x 23 cm) with stainless steel tray containing pine wood shavings as bedding. Animals were maintained under controlled conditions of temperature (20±2°C), relative humidity and lighting (12 h light-2 h dark cycle) with free access to water and pellet laboratory chow (Rat-Mouse Diet).

Experimental design

At delivery, pups were sexed according to the anogenital distance. Litters were adjusted to 8 pups, prioritizing a maximum of 8 male pups per litter when possible. Male pups were randomly assigned to one of the following treatment groups: control group (C), receiving water; G2 and G50 groups, receiving 2 and 50 mg/kg/day G, respectively; and R2 and R50 groups receiving 2 and 50 mg/kg/day R, respectively. G was provided by Sigma-Aldrich (St Louis, USA). R formulation was a liquid water-soluble formulation containing 66.2% of G potassium salt. Treatments were given orally from PND 14 to 30. Pups were weaned on PND 21 and euthanized on PND 31. A set of animals were treated from PND 14 to 30 with water or 50 mg/kg/day R and kept without further treatment until PND 90. Tissues and blood were collected from euthanized animals in PND 31 or PND 90.

Rats were treated from PND 14 to 30 to comprise the full period of maturation of the BTB. The dose of 2 mg/kg/day was selected because it is in order of magnitude of the reference dose (RfD) of 1 mg/kg/day recently reassigned for glyphosate by the US EPA. The dose of 50 mg/kg/day was selected based on the no observed adverse effect level (NOAEL) for G and on a previous report that proposed this dose of R as appropriate for future toxicological analyses. No alterations in maternal care and nursing among the experimental groups were detected. Treatments caused no overt signs of toxicity.

Collection of blood and tissues

Blood was obtained by intracardiac puncture. The samples were allowed to clot at room temperature for 15 min, and then they were centrifuged at 950 g for 5 min in order to obtain the serum. Supernatants were immediately frozen at -20°C for subsequent analysis. Biochemical parameters were determined with an automated Cobas c 501 analyser.

At PND 31, animals were euthanized by CO_2 asphyxiation, and testes were dissected, weighed, and used for histological analysis and BTB assay. Also, testes were dissected and snap frozen for reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and Thiobarbituric Acid Reactive Substances (TBARS) determination. Tissue samples (liver, kidney, stomach, and intestine) were also collected at that time.

At PND 90, animals were anesthetized with a mixture of ketamine-xylazine and tissues were sampled and weighed. Testes were used for histological analysis and BTB assay and to determine daily sperm production (DSP). Epididymides were used to evaluate sperm parameters.

Histological analysis

For histological analysis, testes were removed and fixed in Bouin solution, and the other tissue samples were fixed in 10% formalin. Dehydration was carried out at room temperature using ascending concentrations of ethanol and shifting to xylene. After clearing, tissues were embedded in paraffin wax and 3- to 5-mm-thick sections were cut using a microtome. Sections were transferred to albumenized slides that were preheated to 37°C. Tissues were rehydrated in descending concentrations of ethanol, stained with haematoxylin/eosin and covered with a coverslip. The prepared slides were observed under an Eclipse 50i microscope equipped with a digital camer). For TUNEL assay, testicular sections were revealed with In Situ Cell Death Kit.

Intratesticular and serum testosterone determination

Testosterone was extracted from PND 31 testis homogenates with diethyl ether followed by evaporation of the organic phase and reconstitution of extracted testosterone in 0.1% PBS. Serum testosterone of PND 90 animals were also evaluated. Testosterone concentration was measured with an electrochemiluminescence assay using a Cobas e 411 analyser. Testosterone assay sensitivity was 10 ng/dl, and intra and interassay CV were 2.4% and 2.6%, respectively.

Thiobarbituric acid reactive substances assay

Lipid oxidation was determined by the colorimetric assay of TBARS. Testis homogenates were performed in PBS containing 0.4% w/v butylated hydroxyl-toluene on ice and then disrupted by ultrasonic irradiation. An aliquot (25 µL, corresponding to 450 µg protein) was added to 175 µL mixed reaction solution (0.15% w/v SDS, 0.5 N HCl, 0.75% w/v phosphotungstic acid, and 0.175% w/v 2-thiobarbituric acid). The mixture was heated in a boiling water bath for 45 min. TBARS were extracted with 200 µL of n-butanol. After a centrifugation at 10,000 g for 5 min at 4°C, the absorbance at 532 nm of the butanolic phase was measured. A calibration curve was performed using malondialdehyde (MDA), generated from 1,1,3,3-tetramethoxypropane (0.4-8 µM), as standard to express the absorbance changes as nmol MDA/µg protein.

Blood-testis barrier integrity assay

The permeability of the BTB was assessed with a biotin tracer. Immediately after testes isolation, a solution of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin dissolved in PBS containing 1 mM $CaCl_2$ was injected into the testis. The administered volume represented 10% of testis weight. Testes were then incubated at 34°C for 30 min, immersed in 4% paraformaldehyde and embedded in paraffin. For localization of the biotin tracer, testis sections (5 µm thick) obtained from different levels were deparaffinised and hydrated. To avoid nonspecific staining, sections were blocked with 5% non-fat dry milk in PBS containing 0.01% Triton X-100 for 15 min prior to incubation with streptavidin-rhodamine (1:300) for 45 min at room temperature. After nuclear staining with DAPI, sections were mounted in buffered glycerine and observed by Ahiophot fluorescent microscope with epi-illumination. At least 50 seminiferous tubules from 3 non-consecutive testis sections from each rat were examined. Results are expressed as % of permeable tubules.

RT-qPCR analysis

Total RNA was isolated from testis homogenates with TRI Reagent. The amount of RNA was estimated by spectrophotometry at 260 nm. RT was performed on 2 µg RNA at 42°C for 50 min with a mixture containing 200 U MMLV reverse transcriptase enzyme, 125 ng random primers and 0.5 mM dNTP Mix.

qPCR was performed by a Step One Real Time PCR System. Amplification was carried out as recommended by the manufacturer: 25 µL reaction mixture containing 12.5 µL of SYBR Green PCR Master mix, the appropriate primer concentration and 1 µL of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The relative standard curve method was used to calculate relative gene expression. Relative mRNA levels were normalized to the reference genes HPRT1 and β-actin.

Determination of daily sperm production

At PND 90, testes were collected and processed. The tunica albuginea was removed from one testis and the parenchyma was homogenized in 0.9% w/v NaCl containing 0.5% w/v Triton X-100. Then, the homogenates were ultrasonic disrupted for 30 s. Samples were diluted at 1:10 and transferred to a Neubauer chamber and counted in quadruplicate. Elongated spermatid nuclei with a shape characteristic of step 19 spermatids and resistant to homogenization were counted to determine the number of elongated spermatid nuclei. To calculate the DSP, the number of spermatids was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium.

Assessment of sperm parameters

At PND 90, sperm were recovered from cauda epididymis. The epididymides were placed in a conical tube, covered with 750 µL of fresh medium and sperm were allowed to swim up at 37°C. After 10 min, aliquots of the upper sperm layer were recovered for motility and morphology evaluation. To evaluate total motility (progressive + nonprogressive), sperm suspensions were placed on pre-warmed slides and analysed subjectively under a light microscope (400× magnification). To assess sperm morphology, a 10 µL aliquot of the sperm suspension was smeared over a microscope slide. After drying in air, the smear was fixed with methanol for 5 min, washed with distilled water, stained with Harris haematoxylin for 15 min, and finally washed with tap water. Sperm morphology was evaluated using a Nikon microscope at 1000× magnification. In all cases, at least 200 spermatozoa from each sample were assessed.

Statistical analysis

At least five animals per treatment group were used, and data were presented as mean ± SD. The variables under study were first submitted to tests of normal distribution (Shapiro-Wilk's Test) and for homogeneity of variances (Levene's Test). Then, one-way ANOVA followed by Tukey-Kramer test for the comparison of multiple groups was performed. To assume normal distribution, percentages were expressed as ratios and subjected to the arcsine square root transformation. Results were compared using the unpaired Student t test. Probabilities <0.05 were considered statistically significant. InfoStat 2016 was used.

Results

Glyphosate and Roundup effects on serum biomarkers and testis and body weight

Initially, rats were exposed to low doses (2 and 50 mg/kg/day) of G or R from PND 14 to 30. Serum biomarkers for renal (urea, creatinine) and liver (aspartate and alanine aminotransferases: AST and ALT) function were analysed. These biomarkers did not change in treated groups compared with the control (see table below). In addition, treatments did not alter liver, kidney, stomach, and intestine histology (data not shown). To explore the impact of G or R exposure on male reproductive system the authors assessed body and testicular weights after treatments. No differences in body and testis weight and in the ratio testis/body weight at any G or R dose tested were observed (see table below).

Table 1. Effect of G and R treatment on serum biochemical markers and on body weight, testis weight, and relative testis weight.

	Control	G 2 mg/kg/day	G 5O mg/kg/day	R 2 mg/kg/day	R 50 mg/kg/day
Urea (mg/dl)	31.5 ± 3.3	34.9 ± 4.2	33.0 ± 7.1	32.0 ± 5.7	35.9 ± 6.2
Creatinine (mg/dl)	0.13 ± 0.09	0.12 ± 0.05	0.18 ± 0.11	0.17 ± 0.04	0.21 ± 0.03
AST (UI/l)	134 ± 18	128 ± 13	136 ± 15	142 ± 16	111 ± 12
ALT (UI/l)	37.8 ± 7.0	37,9 ± 6,4	35.0 ± 5.3	37.2 ± 7.8	33.0 ± 8.2
Body Weight(g)	112.4 ± 11.2	101.1 ± 12.7	111.3 ± 17.0	105.3 ± 12.5	112.8 ± 13.0
Testis Weight (mg)	375.7 ± 90.6	327,7 ± 53,4	366.9 ± 80.2	332.3 ± 83.8	348.9 ± 77.4
TW/BW Ratio (%)	0.33 ± 0.05	0.32 ± 0.03	0.33 ± 0.03	0.31 ± 0.06	0.31 ± 0.04

Animals (n = 5/group) were treated with 2 and 50 mg/kg/day of G or R from PND 14 to 30. At PND 31, body and testicular weights were assessed, and blood was sampled by intracardiac puncture to determine serum parameters. Results are presented as mean ± SD.

Glyphosate and Roundup effects on testicular histology and blood-testis barrier function

Histological examination of the testes revealed differences in the seminiferous tubules among control and treated groups. Some seminiferous tubules of G2, G50, and R2 groups showed a disorganized epithelium, with an apparent low cellular adhesion. In R50 treated males, tubules presented severe disorganization and epithelial desquamation of the most differentiated cells (spermatocytes and round spermatids). In this group, the percentage of affected tubules is higher than in the other groups tested. Although epithelium disorganization was observed in treated groups, the histological characteristics of Sertoli cells-nucleus located parallel to basement membrane next to spermatogonia and premeiotic spermatocytes and triangular in shape remained unaltered. Additionally, TUNEL analysis of rat testis section from control and R50 treated animals were performed. Only a few seminiferous tubules with some TUNEL positive cells located in areas not corresponding to positions occupied by Sertoli cells were observed in both groups.

To analyse the effects of G or R treatment on BTB integrity, the permeability of the BTB was evaluated using a biotin tracer which is excluded from the ad luminal compartment of intact seminiferous tubules. The tracer entered the ad luminal compartment in animals treated with G or R at both doses tested. A significant increase in the percentage of seminiferous tubules with a permeable barrier in G and R groups was observed.

The next set of experiments was performed to evaluate possible mechanisms that would explain the deleterious effects of G or R treatments on BTB permeability. Firstly, G and R treatments did not modify either intratesticular testosterone (ITT) levels or androgen receptor (AR) expression. Secondly, TBARS levels were not modified by G or R exposure. Thirdly, G or R treatments did not modify claudin11, occludin, ZO-1, connexin43, 46, and 50 mRNA levels.

Juvenile Roundup treatment effects on adult animals

In order to evaluate possible consequences of juvenile herbicide treatment in adulthood, a set of animals was treated with 50 mg/ kg/day of R from PND 14 to 30 and then allowed to grow until PND 90. Several parameters were analysed at this age. As it was observed in PND 31, no differences in urea,creatinine, AST, and ALT levels between groups were observed. The histological examination of testis at PND 90 in control and R50 groups showed that tubules had normal cellularassociations and complete spermatogenesis. The analysis of the frequency of the stages of the seminiferous epithelium showed that no alteration of the presence of the different stages of the cycle of the seminiferous epithelium was found between groups. The study with the biotin tracer and the data obtained after determining the percentage of permeable tubules show a small increase in the percentage of permeable tubule in R50 group. Despite this small increase in BTB permeability, testicular weight, and DSP were not modified by juvenile treatment with R.

Cauda epididymal sperm were used to analyse sperm motility and morphology. No differences in motility and morphology were observed in sperm of both groups. In addition, no changes were observed in epididymal weight and in epididymal/body weight ratio between groups.

Conclusion

The authors concluded their results show that continuous exposure to low doses of G or R alters blood-testis barrier permeability in juvenile rats. Considering that DSP in adult animals, which have been unexposed to the herbicide for a prolonged period, is indistinguishable from that in control animals, the authors suggested it is feasible that BTB impairment is a reversible phenomenon. These results warrant further investigation of glyphosate-mediated reproductive damage of human juvenile populations exposed to low doses of G or R. Such analysis may include the determination of follicle stimulating hormone (FSH), luteinizing hormone (LH), anti-Müllerian hormone (AMH), inhibin B, and testosterone serum levels to get insight into Sertoli cell maturation in juvenile people as well as semen analysis in adult population.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The aim of the study was to investigate the possible effects of glyphosate (G) or the commercial formulation Roundup (R) treatment of juvenile male rats on blood-testis barrier function and on adult male sperm production. Pups were randomly assigned to the following groups: control group (C), receiving water; G2 and G50 groups, receiving 2 and 50 mg/kg/day G respectively; and R2 and R50 groups receiving 2 and 50 mg/kg/day R respectively. Treatments were performed orally from postnatal day (PND) 14 to 30, period of life that is essential to complete a functional blood-testis barrier. Evaluation was done on PND 31.
In conclusion, the results presented show that continuous exposure to low doses of G or R alters blood-testis barrier permeability in juvenile rats, but have no effect on clinical chemistry parameters, intertesticular testosterone levels, androgen receptor expression, or testis weight. Furthermore, adult animals treated during the juvenile stage showed no differences in daily sperm production compared with control animals, demonstrating that blood-testis barrier effects are a transient, reversible phenomenon.

The article is considered relevant (Category A) and reliable with restrictions for the following reason: Purity missing for glyphosate technical, exact number of animal/group difficult to find, no dose-response relationship, no HCD, and no positive control to confirm assay validity.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies

Publication: Gorga A., 2021, Low Doses of Glyphosate/Roundup Alter Blood-Testis Barrier Integrity in Juvenile Rats	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	Study performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the National Institute of Health of USA
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Glyphosate was provided by Sigma-Aldrich (St Louis, USA). No information about purity. Roundup formulation was a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt.
Only glyphosate acid or one of its salts is the tested substance	Y	Yes, glyphosate and glyphosate potassium salt as GBH
AMPA or other glyphosate metabolites is the tested substance	N	
Study		
Test species clearly and completely described	N	3-month-old pregnant Sprague Dawley rats (250-300 g), no number available of male and females. No details on the litters.

Publication: Gorga A., 2021, Low Doses of Glyphosate/Roundup Alter Blood-Testis Barrier Integrity in Juvenile Rats	**Criteria met? Y/N/?**	**Comments**
Test conditions clearly and completely described	Y	Housing, diet and environmental conditions described
Route and mode of administration described	N	Treatment given orally from PND 14 to 30, assumed via gavage but not described. Euthanasia on PND 31 for a group and on PND 90 for another group. Mode of administration not properly described.
Dose levels reported	Y	0, 2 and 50 mg/kg/day
Number of animals used per dose level reported	Y	At least 5 animals per treatment group were used, sometimes 7 animals/group. Difficult to find the exact number
Method of analysis described for analysis test media	N	
Validation of the analytical method	N/A	
Analytical verifications of test media	N/A	
Complete reporting of effects observed	N	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	Purity missing for glyphosate technical, exact number of animal/group difficult to find, no dose-response relationship, no HCD and no concurrent positive control to verify assay.
Not reliable	N	

1. Information on the study

Data point:	CA 5.6
Report author	Refaie A. A. *et al.*
Report year	2020
Report title	Hematological, biochemical, antioxidant and histopathological alterations in kidneys of Wistar rat pups exposed to glyphosate herbicide during lactation period
Document No	Current Topics in Pharmacology, 2021, 24, 69-76
Guidelines followed in study	None
Deviations from current test guideline	No guideline followed.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In the present study, the authors evaluated the effects of glyphosate administered during lactation period of pup rats at a daily dose of 0.3 mg/kg body weight [the acceptable daily intake (ADI)], 31 mg/kg body weight [no-observed-adverse-effect level (NOAEL)] and 56 mg/kg body weight [1/100 LD50] for 21 days. At the end of each treatment, the suckling pups were separated into male and female, and the relative kidney weights were measured. The levels of creatinine and urea were determined in serum. Oxidative stress was measured using lipid peroxidation and the activities of glutathione peroxidase (GPx) and catalase (CAT) in kidney tissue were also measured. Red blood corpuscle (RBC) and white blood cell (WBC) counts and haemoglobin content (Hb) were measured. Histopathological examination was also performed in kidney tissues. The pups showed increased relative kidney weight with the highest increment in 1/100 LD50 dose followed by NOAEL and ADI doses. Also, marked increment in malondialdehyde (MDA) level along with significant inhibition in catalase (CAT) and glutathione peroxidase (GPx) activities were detected in pup's kidney tissues. Counts of WBCs and RBCs and Hb content exhibited significant reduction in the blood ofpups that received 1/100 LD 50 dose which is more than the NOAEL and ADI doses. Histopathological examination of kidney tissue demonstrated focal fibrosis with inflammatory cells between glomeruli and tubules of male and female pup kidney tissue (lower scoring severity (+) in ADI and NOAEL doses than 1/100 LD50 (+++)). Additionally, at 1/100 LD50 dose focal haemorrhages, degradation in tubular lining epithelial cells (+) and renal arteries congestion (++) were observed.

Materials and methods

Chemicals

Analytical grade glyphosate (96%) purchased from Sigma-Aldrich (St. Louis, MO, USA). The kits used for biochemical studies of creatinine, urea, catalase, glutathion peroxidase, malondialdehyde (MDA) and total protein were obtained from Biodiagnostic company, 29 Tahrir Street, Dokki, Giza, Egypt. All other chemicals were of reagent grades and obtained from local scientific distributors in Egypt.

Animals

The pregnant albino rats of the Wistar strain weighing 220-245 g were obtained from the animal house, National Research Centre of Egypt. The rats were acclimatized to the laboratory conditions for one week prior to the experiment. After acclimation period, the pregnant female rats (n = 8) were housed in stainless steel cages (one pregnant female in each cage) in breeding animals' room at 25±2°C with 45% relative humidity, dark/light cycle (12-h), and were fed standard pellet diet and tap water ad libitum. The number of male and female pups in each litter was recorded and weighed. To maximize the lactation performance, male and female pups in each litter were randomly divided into five male and female pups.

Experimental design

Eight adult pregnant female Wistar rats were randomly divided into four groups of two rats each. Glyphosate was dissolved in corn oil and administrated via the oral route to dams at a fixed volume of 0.5 mL/dam from the first day after parturition for 21 days (lactation period). Dams were weighed weekly to adjust the dose of glyphosate. The suckling pups were grouped as follows: G1: male and female pups (five each) of the two mother female rats who were administered 0.5 mL corn oil/dam daily served as control, G2: male and female pups (five each) of the two mother female rats who were administered glyphosate at 0.3 mg/kg body weight for acceptable daily intake (ADI), G3: five male and female pups of the two mother female rats who were administered glyphosate at no-observed-adverse-effect level (NOAEL) for 21 days (31 mg/kg body weight), G4: five male and five female pups suckling from the two mother rats who were administered glyphosate at 1/100 of acute oral toxicity (1/100 LD50) (56 mg/kg body weight).

Blood sample collection and tissue preparation

Post lactation cycle of pups (21 days), rats were fasted overnight and blood samples were obtained by puncturing the retero-orbital venous plexus of animals with a fine sterilized glass capillary. The blood sample was divided into two portions; the first portion was collected in an anticoagulant (EDTA) tube and used for blood cell count and Hb content measuring. The second portion of blood was collected in centrifugal tubes without anticoagulant, and left to clot in these dry tubes and centrifuged at 3000 rpm (600xg) for 10 min at 4°C using Heraeus Labofuge 400R to get the sera. Sera were stored at -20°C for further biochemical analysis including blood urea, and creatinine determination. Then, the rats were sacrificed by decapitation. Kidney was removed immediately after sacrification, weighed, washed in saline and relative kidney weight was calculated. A small part of the kidney was homogenized in 10% (w/v) ice cold 100 mM phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm for 15 min at 4°C, then the supernatant was obtained and used for oxidative stress (lipid peroxidation) and antioxidant enzyme measurements (CAT and GPx). Then, small pieces of kidney samples were cut and kept in 10% natural formalin and used for histopathological investigations.

Biochemical parameter determination

Oxidative stress biomarkers in kidney tissues

Kidney glutathione peroxidase and catalase enzyme activities were determined according to the method of Paglia and Valentin and Abei. Lipid peroxidation (LPO) was estimated by determining thio-barbituric acid reactive substances (TBARS) and was expressed in relation to malondialdehyde (MDA) content using a colorimetric technique. The MDA values were expressed as nanomoles of MDA per gram tissue. Total protein content in homogenate was measured.

Kidney function in serum

Urea and creatinine were measured according to the method of Tietz.

Measurements of blood constituents

Red blood corpuscles (RBC) and white blood cells (WBC) were counted according to Britton and Seivered; Heamoglobin (Hb) measurement was carried out as per Wintrobe.

Histopathological examination

Kidney specimens of male and female pups were cut and dehydrated in graded series of alcohol, cleaned in xylene and fixed in paraffin wax. Five micrometer thick pieces were cut and stained by haematoxylin and eosin (H&E). Two slides were prepared for this organ; each slide contained ten field areas and two sections were examined for histopathological changes. The examination was done using a light microscope with a digital camera. The histopathological alterations in kidney tissues were scored as follows: normal appearance ( ), mild (+), moderate (++) and severe (+++).

Statistical analysis

Data were reported as mean ± SE. Data were analysed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test as a post-hoc test. The level of significance between mean values was set at $p \leq 0.05$. All statistical analyses were performed using SPSS software.

Results

The results indicated a significant increase in relative kidney weight (%) post-exposure of male and female pups to glyphosate during lactation period with G4>G3>G2, compared to the control group (G1) (Table 1).

Table 1. Relative kidney weight and kidney dysfunction biomarkers in serum of female and male pups exposed to glyphosate during lactation period.

Treatments	Creatinine (mg/dl)		Urea (mg/dl)		Relative kidney weight (%)	
	Female	Male	Female	Male	Female	Male
G1	1.25 ± 0.04^{a}	1.54 ± 0.011^{a}	34.34 ± 0.32^{a}	36.14 ± 0.42^{a}	0.52 ± 0.005^{a}	0.63 ± 0.006^{a}
G2	$1.35 \pm 0.01^{a\,b}$	1.63 ± 0.005^{b}	41.71 ± 0.39^{b}	44.91 ± 0.33^{b}	0.63 ± 0.006^{b}	0.73 ± 0.007^{b}
G3	1.43 ± 0.03^{b}	1.73 ± 0.012^{c}	45.95 ± 0.38^{c}	53.95 ± 0.58^{c}	0.74 ± 0.005^{c}	0.77 ± 0.008^{c}
G4	1.78 ± 0.02^{c}	1.77 ± 0.008^{d}	55.90 ± 0.67^{d}	66.10 ± 0.43^{d}	0.78 ± 0.002^{d}	0.83 ± 0.005^{d}

G1: control group, G2: glyphosate (ADI), G3: glyphosate (NOAEL), G4: glyphosate (1/100 LD50). Values expressed as a mean of 5 animals ± S.E.; $^{a, b, c, d}$ values having different letters significantly different at $p \leq 0.05$.

The effects of glyphosate on renal function revealed a significant increase in creatinine and urea levels in both male and female pups in all treated groups compared with the control group, where G4 exhibited a more noticeable significant increase in both blood urea and creatinine levels than G3 and G2 (G4>G3>G2) at $p \leq 0.05$ (Table 1). The significant increase observed in our work suggested the nephrotoxic effect of the herbicide.

The results indicated that the sub-chronic exposure to glyphosate (21 days) caused a high level of serum urea in all selected doses of treatedpups compared to control.

The authors pointed out that the significant reduction in the RBC count and haemoglobin level post 21 days of lactation is indicative of anaemic condition.

Pups exposed to glyphosate herbicide showed significant inhibition in antioxidant enzyme activities CAT and GPx with G4>G3>G2, as compared to control levels ($p \leq 0.05$), while a significant increase in MDA level in all treated groups was recorded (G>G3>G2) (Table 2).

Table 2. Effect of glyphosate on kidney antioxidant enzyme activities and lipid peroxidation level in the kidney tissue of female and male pups.

Treatments	Catalase (u/mg protein)		Glutathion–peroxidase (u/mg protein)		Lipid peroxidation (nmol MDA/g tissue)	
	Female	Male	Female	Male	Female	Male
G1	38.46 ± 0.36^{d}	39.86 ± 0.35^{b}	4.54 ± 0.01^{c}	6.24 ± 0.05^{d}	0.48 ± 0.004^{a}	0.68 ± 0.004^{a}
G2	36.41 ± 0.07^{c}	37.86 ± 0.54^{d}	4.51 ± 0.02^{c}	5.41 ± 0.09^{c}	0.53 ± 0.006^{b}	0.69 ± 0.004^{a}
G3	34.66 ± 0.70^{b}	34.06 ± 0.43^{c}	4.25 ± 0.03^{b}	5.10 ± 0.02^{b}	0.57 ± 0.006^{c}	0.71 ± 0.006^{b}
G4	31.98 ± 0.28^{a}	25.19 ± 0.71^{a}	3.78 ± 0.01^{a}	4.19 ± 0.03^{a}	0.72 ± 0.003^{d}	0.91 ± 0.004^{c}

Control (G1), Glyphosate (ADI) (G2), Glyphosate (NOAEL) (G3), and 1/100 LD50 (G4). Values are means ± SE, n = 5; values having different letters are significantly different from each other.

The histopathological examination indicated focal fibrosis with inflammatory cells between glomeruli and tubules of renal tissue with severity score (+) in G2 and G3 and (+++) in G4. Also, focal haemorrhages and degradation in tubular lining epithelial cells were noticed in G4 (+). Besides these, renal artery congestion (++) was noticed.

Overall, the authors indicated that elevation in creatinine and total urea together with the histopathological results proved that glyphosate induced renal injury in pups.

Conclusion

The authors concluded that exposure of pups to glyphosate herbicide via breastfeeding showed increase in relative kidney weight and kidney function, while, it lowered haematological values and induced perturbations in antioxidant status in male and female pups. Severe histopathological alterations were also examined in renal tissue of pups. Glyphosate showed a dose-dependent relationship, where 1/100 LD50 showed more drastic effects than the other selected two doses.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The aim of this study was to evaluate the effects of glyphosate administered orally to the dams during lactation period of pup rats at a daily dose of 0.3 mg/kg body weight (acceptable daily intake ADI), 31 mg/kg body weight (no observed adverse effect level NOAEL) and 56 mg/kg body weight (1/100 LD50) for 21 days. However, the underlying assumption of glyphosate transfer to pups via milk is not supported by the data – this is well explained in mammals including humans (Bus, 2015*; McGuire et al, 2016**). At the end of the treatment, the suckling pups were separated into male and female, and the relative kidney weights were measured. The levels of creatinine and urea were determined in serum. Oxidative stress was measured using lipid peroxidation and the activities of glutathione peroxidase (GPx) and catalase (CAT) in kidney tissue were also measured. Test substance administration in this study was to two pregnant rats per group, and thus the experimental unit is the litter, not all the individual pups across two litters with the same dose. Therefore, in contrast to the statistical analyses presented, there are only two experimental units (litters) per dose group (2-1 = 1 degree of freedom; **not** 10-1 = 9 d.f.), which lacks adequate statistical power for meaningful analysis in both the ANOVA and LSD test.

The pups showed increased relative kidney weight with the highest increment in 1/100 LD50 dose followed by NOAEL and ADI doses. Also, marked increment in malondialdehyde (MDA) level along with significant inhibition in catalase (CAT) and glutathione peroxidase (GPx) activities were detected in pup's kidney tissues. Counts of WBCs and RBCs and Hb content exhibited significant reduction in the blood of pups that received 1/100 LD50 dose which is more than the NOAEL and ADI doses. Histopathological examinations of kidney tissue demonstrated focal fibrosis with inflammatory cells between glomeruli and tubules of male and female pup kidney tissue (lower scoring severity (+) in ADI and NOAEL doses than 1/100 LD50 (+++)). Additionally, at 1/100 LD50 dose focal haemorrhages, degradation in tubular lining epithelial cells (+) and renal arteries congestion (++) were observed. These results for F1 kidney weights and histopathology data are not consistent with the weight of evidence across in numerous multigenerational studies up to the limit dose of 1000 mg/kg bw/day.

The article is considered relevant (Category A) and reliable with restrictions for the following reasons: No method of analysis, no HCD, and inappropriate statistical assumptions on the experimental unit.

References:

*Bus, J. S. (2015). Analysis of Moms Across America report suggesting bioaccumulation of glyphosate in U.S. mother's breast milk: Implausibility based on inconsistency with available body of glyphosate animal toxicokinetic, human biomonitoring, and physico-chemical data. *Regulatory Toxicology and Pharmacology* (2015), Vol. 73, Issue, 3, pp. 758-64. doi: 10.1016/j.yrtph.2015.10.022.

**McGuire M. K. at al. (2016). Glyphosate and aminomethylphosphonic acid are not detectable in human milk. *The American Journal of Clinical Nutrition* (2016), Vol. 103, Issue 5, pp 1285-1290. https://doi.org/10.3945/ajcn.115.126854

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies

Publication: Refaie 2020, Hematological, biochemical, antioxidant and histopathological alterations in kidneys of Wistar rat pups exposed to glyphosate herbicide during lactation period	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	Protocol corresponds to the guidelines of the National Research Center (NRC, 2011)
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Analytical grade glyphosate (96%) was purchased from Sigma Aldrich (St. Louis, MO, USA).
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA or other glyphosate metabolites is the tested substance	N	
Study		
Test species clearly and completely described	Y	8 pregnant albino rats of the Wistar strain weighting 220-245 g
Test conditions clearly and completely described	Y	Housing, diet and environmental conditions described.
Route and mode of administration described	Y	Glyphosate was dissolved in corn oil and administrated via the oral route to dams at a fixed volume of 0.5 ml/dam from the first day of parturition for 21 days (lactation period).
Dose levels reported	Y	0.3, 31 and 56 mg/kg body weight
Number of animals used per dose level reported	Y	8 adults divided in 4 groups of 2 rats that receive the vehicle or one of the 3 dose of glyphosate. Male and female pups in each litter were randomly divided into 5 male pups and 5 female pups.
Method of analysis described for analysis test media	N	No analysis performed
Validation of the analytical method	N/A	No analysis performed
Analytical verifications of test media	N/A	No analysis performed
Complete reporting of effects observed	Y	
Statistical methods described	Y	ANOVA with LSD post-hoc test, however, incorrect application of experimental unit to offspring rather than lactating mother. Therefore all statistical analyses are incorrect.
Historical control data of the laboratory reported	N	No HCD
Dose-effect relationship reported	Y	Yes
Overall assessment		
Reliable without restrictions	N	

Publication: Refaie 2020, Hematological, biochemical, antioxidant and histopathological alterations in kidneys of Wistar rat pups exposed to glyphosate herbicide during lactation period	**Criteria met? Y/N/?**	**Comments**
Reliable with restrictions	Y	No method of analysis, no HCD, and inappropriate statistical assumptions on the experimental unit.
Not reliable	N	

1. Information on the study

Data point:	CA 5.6
Report author	Zhao L. *et al.*
Report year	2021
Report title	Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of *StAR* expression in mouse Leydig cells
Document No	Science of the Total Environment, 2021, 785, 147323
Guidelines followed in study	None
Deviations from current test guideline	No guideline followed.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The involvement of NR1D1 in glyphosate-mediated inhibition of testosterone synthesis was investigated both in vitro and in vivo. Glyphosate exposure of TM3 cells significantly increased *Nr1d1* mRNA levels, but decreased *Bmal1*, *Per2*, *StAR*, *Cyp11a1*, and *Cyp17a1* mRNA levels. Western blotting confirmed elevated NR1D1 and reduced StAR protein levels following glyphosate exposure. Glyphosate exposure also reduced testosterone production in TM3 cells. In primary LCs, glyphosate exposure also upregulated *Nr1d1* mRNA levels and downregulated the mRNA levels of other clock genes (*Bmal1* and *Per2*) and steroidogenic genes (*StAR*, *Cyp17a1*, *Cyp11a1*, and *Hsd3b2*), and inhibited testosterone synthesis. Moreover, glyphosate exposure significantly reduced the amplitude and shortened the period of *PER2::LUCIFERASE* oscillations in primary LCs isolated from *mPer2*$^{\text{Luciferase}}$ knock-in mice. Four weeks of oral glyphosate upregulated NR1D1 at both the mRNA and protein levels in mouse testes, and this was accompanied by a reduction in StAR expression. Notably, serum testosterone levels were also drastically reduced in mice treated with glyphosate. Moreover, dual-luciferase reporter and EMSA assays revealed that in TM3 cells NR1D1 inhibits the expression of *StAR* by binding to a canonical RORE element present within its promoter. Together, these data demonstrate that glyphosate perturbs testosterone synthesis via NR1D1 mediated inhibition of *StAR* expression in mouse LCs.

Materials and methods

Animals

BALB/c wild-type (WT) mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.. *mPer2*^{Luc} knock-in reporter mice carrying the firefly *luciferase* (*Luc*) gene in exon 23 of the circadian clock gene *Per2* were obtained from the Jackson Laboratory (Sacramento, CA, USA). Mice (10-12 weeks old) were housed under a 12 h light/dark cycle (Zeitgeber time, ZT0: 0800 light on; ZT12: 2000 light off) for at least 2 weeks to synchronize their circadian clock system. All mice were provided with food and water ad libitum. The mouse house was maintained at a temperature of 23±2°C and a relative humidity of 50±10%.

Animal treatments

Pure glyphosate powder was purchased from Shanghai Yuanye Bio-Technology Co., Ltd.. Glyphosate was dissolved in distilled water to obtain a 0.5% solution, as previously described (5 g glyphosate/L water). Twenty WT mice were randomly assigned to two equally sized groups. Mice in one group were orally administered 0.5% glyphosate via daily drinking water (Glyphosate group). The control group received distilled water alone (CTRL group). Four weeks later, the mice from the Glyphosate and CTRL groups were euthanized at two representative time points (ZT0 and ZT12; n = 5 per group for each time point). Testes, epididymis, seminal vesicles, liver, and blood samples were collected. Body, testes, epididymis, seminal vesicles, and liver weights were recorded. The blood samples were stored at 4°C for 6-8 h to enable clotting. The serum was separated from the blood clot by centrifugation at 1000×g at 4°C for 15 min, collected, and stored at -80 °C.

Cell preparation and culture

TM3 cells were seeded on 35 mm collagen coated dishes and cultured in DMEM supplemented with 2.5% foetal bovine serum (FBS), 5% horse serum, and 1× antibiotic-antimycotic (AA, containing penicillin, streptomycin, and amphotericin B) in a humidified atmosphere of 5% CO_2 at 37°C. Primary LCs were isolated from 10 to 12-weeks-old adult *mPer2*^{Luc} knock-in mice or WT mice and cultured. Briefly, testes were de-capsulated and digested using 0.05% collagenase I in DMEM/F12 for 15 min at 34°C. The suspended LCs were collected by filtration using a 0.074 mm nylon cell strainer and purified over a discontinuous Percoll gradient (5%, 30%, 58%, and 70%). After centrifugation at 800×g at 4°C for 30 min, the majority of the purified LCs were found to be concentrated in the third layer. The LCs were seeded onto 35 mm collagen-coated dishes in 2 mL of DMEM/F12 containing 10% FBS supplemented with 1× AA and cultured in a humidified atmosphere of 5% CO_2 at 37°C for 2 days until they reached 70%-80% confluence. All cells were incubated with various concentrations of glyphosate at the indicated time points for further experiments.

TM3 cell viability assay

The effects of glyphosate on TM3 cell viability were assessed using the CCK-8 assay. TM3 cells were seeded in 96 well plates (1×10^4 cells per well) and cultured for 24 h. TM3 cells were then exposed to different concentrations of glyphosate (0, 0.0001, 0.001, 0.01, 0.1, 1, 5, 10, 50, 100, or 200 mM) for 24 h or 48 h, after which CCK-8 (10 μL) was added to each well, and the cells were incubated for 1 h at 37°C. The absorbance of each well at 450 nm was measured using a microplate reader. The data were expressed as a percentage of the untreated cells, which were assigned 100% viability.

TM3 cell apoptosis assay

TM3 cells were plated into 35 mm collagen-coated dishes at a density of 3×10^5 cells and incubated for 24 h prior to exposure with or without 0.1 mM glyphosate for 24 h or 48 h. TM3 cells were collected and washed twice with PBS. Apoptosis was evaluated using the Annexin V-FITC/PI Apoptosis Detection Kit. The cells were then stained with 5 μL Annexin V-FITC (FITC) and 5 μL propidium iodide (PI) in 100 μL binding buffer for 15 min at room temperature in the dark. Apoptotic cells were quantified by measuring the fluorescent signals using a flow cytometer.

Real-time monitoring of *PER2::LUC* oscillations

Primary LCs isolated from 10 to 12 weeks old adult *mPer2*^{Luc} knock-in mice were synchronized with 100 nM dexamethasone (DXM) for 2 h in serum-free DMEM/F12 medium containing 1× AA. The culture medium was changed to DMEM/F12 medium supplemented with 10% FBS, 1× AA, and 100 μM luciferin with or without 0.1 mM glyphosate. Luciferase activity was chronologically monitored at 37°C with a Kronos Dio AB-2550 luminometer, which was interfaced with a computer to continuously acquire data. The data are shown as photon counts per minute. The correction of the baseline was carried out using a 24 h moving average, while the first 12 h of data were excluded. The phase time was determined from the peak that appeared between 24 and 48 h after DXM synchronization. The amplitude and period of the *PER2::LUC* oscillations were determined using Cosinor Periodogram software.

RNA extraction and real-time quantitative PCR

Cultured cells or testes tissue samples were harvested at the indicated time points. Total RNA was extracted from cells or tissues using TRIzol reagent and subsequently treated with RNase-free DNase. cDNA was generated using the PrimeScript RT Reagent Kit. All qPCR reactions were performed in a final volume of 20 μL containing 10 ng of cDNA, the SYBR Premix Ex Taq II kit, and 200 nM specific primers. Amplification was performed using the CFX96™ qPCR system. All reactions were performed in triplicate. The relative expression levels of each sample were normalized to the average level of the constitutively expressed housekeeping gene *36b4*, which is a reliable and consistent standard for use in gene expression analysis.

Protein extraction and western blotting (WB)

Proteins were extracted using a protein extraction kit. Cultured cells were harvested 24 h or 48 h after exposure with 0.1 mM glyphosate. For in vivo analysis, 80 mg of testes samples were collected at the indicated time points (ZT0 and ZT12) after oral administration of Glyphosate or CTRL for 4 weeks. Protein concentrations were measured using a BCA Protein Assay Kit. Equal amounts of protein were resolved by 12% SDS-PAGE and then electrophoretically transferred onto PVDF membranes. The membranes were then incubated with primary antibodies diluted in Tris-buffered saline Tween (TBST) overnight at 4°C after blocking with 10% non-fat milk for 1 h at room temperature, and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) for 1 h. The primary antibodies used were anti-NR1D1 (1:2000), anti-StAR (1:2000), and anti-β-actin (1:5000). After washing five times in TBST for 5 min each time, peroxidase activity was measured using a Western Bright ECL HRP substrate kit. Immunoreactive bands were visualized using a gel imaging system (GBox-chemi-XRQ). β-actin was used as an internal control for normalization and quantitative analysis to compare the relative protein levels in a group of samples.

Haematoxylin and eosin (HE)

Testes samples collected at ZT0 and ZT12 were fixed with Blouin's fluid and then embedded in paraffin wax using standard protocols and sectioned at a thickness of 5 μm, which were deparaffinised with xylene and ethanol, stained with HE, dehydrated, and sealed with neutral gum for microscopic examination.

Measurement of secreted testosterone levels

Culture supernatants from LCs and TM3 cells were collected after incubation of the cells with or without 0.1 mM glyphosate for 24 h or 48 h. Serum was collected at ZT0 and ZT12 from mice orally administered with Glyphosate or CTRL for 4 weeks. The secreted testosterone levels were measured using an ELISA kit. The fluorescence intensity at 450 nm was determined using a microplate reader. The intra- and inter-assay coefficients of variability were <10%.

Dual-luciferase reporter

Genomic inspection revealed that there is a canonical sequence for the RORE cis-element (-859 to -864, +1 indicates the transcription start site) within the *StAR* promoter. A dual-luciferase reporter was first used to determine whether NR1D1 inhibits *StAR* transcription by binding to the *StAR* promoter. A 1773 bp fragment of the *StAR* gene (−1377 to +396, +1 indicates the transcription start site) was amplified from mouse liver genomic DNA using forward and reverse primers containing the KpnI restriction enzyme site and a 15 bp homologous arm (F, 5′-CTGGCCTAACTGGCCGGTACCTTCAAGGACAGACCAGGTTAGA-3′; R, 5′-AGGCTAGCGAGCTCAGGTACCGCGAGGAACATGCTGAGTG-3′). The 1773 bp fragment of the *StAR* gene was cloned into the *KpnI* site of the pGL4.10 vector using the seamless connection method (ClonExpress II One Step Cloning Kit), which was then named pGL4.10-*StAR*-Luc reporter plasmid (*StAR-Luc*).The target coding sequence (CDS) fragment of NR1D1 was amplified using PrimerSTAR HS DNA Polymerase using cDNA generated from mouse liver tissue as the template (F, 5′-CGATATCGGATCCGGTACCCTCGAGATGACGACCCTGGACTCCAATA-3′; R, 5′-GCGGTTTAAACTTACCGGTCTCGAGTCACTGGGCGTCCACCCGGAAG-3′). The CDS fragment of NR1D1 was cloned into the *XhoI* site of the pcDNA 3.1 plasmid, which was named pcDNA 3.1-*Nr1d1*. TM3 cells were plated in 24-well plates and transfected with *StAR*-Luc, pRL-CMV (an internal control plasmid), pcDNA 3.1-*Nr1d1*, and pcDNA 3.1 empty vector using Turbofect transfection reagent. Thirty-six hours after transfection, the cells were washed and lysed with 200 μL of reporter lysis buffer. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and the SPARK® Multimode Microplate Reader at 25°C. The light output, as a result of transcriptional activity, was divided by the light output from Renilla luciferase activity to normalize the samples.

Electrophoretic mobility shift assay (EMSA)

TM3 cells were transfected with the NR1D1 overexpression vector, pcDNA 3.1-*Nr1d1*. Nuclear proteins were isolated using a nuclear protein extraction kit. A 72 bp biotinylated probe was designed from the mouse *StAR* promoter, including the RORE cis-element (-859 to -864). The unlabelled and mutated probes were not biotinylated. All probes were synthesized by Sangon Biotech. Biotinylated and unlabelled probe sequences were: F, 5′-TTTCTCACCTTTGTGCACAGGTCAGGACCTGTAGCAGGGCAGGCCAGCCTTAGCTGC ATGAGGAAAGGGTGA-3′. Mutated probe: F, 5′-TTTCTCACCTTTGTGCACCCTAGCGGACCTGTAGCAGGGCAGGCCAGCCTTAGCTGC ATGAGGAAAGGGTGA-3′. EMSA was conducted using a chemiluminescent EMSA kit. A total of 0.04 pmol of biotinylated probe was added to each reaction. Unlabelled probes and mutated probes were added in a 15× excess in the competition assays. The probes were mixed with EMSA reagent and 3 μg of nuclear proteins, according to the manufacturer's instructions. The complexes obtained were separated by electrophoresis on 6% polyacrylamide gels and then electrophoretically transferred onto nylon membranes (Beyotime). The bands were visualized using a gel imaging system (GBox-chemi-XRQ).

Statistical analyses

All data are expressed as the mean ± standard error (SE) of at least three separate experiments, each performed in triplicate. Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparisons post-test or a two-way ANOVA with a Sidak's multiple comparisons post-test or a Student's t-test using GraphPad Prism Version 7.0. ANOVA assumptions were corroborated by the Kolmogorov-Smirnov test for normality and Bartlett's test for homogeneity of variances. The Durbin-Watson test was also used to evaluate the independence of observations. Differences were considered significant at $p < 0.05$.

Results

Determination of the optimal concentration of glyphosate exposure of TM3 cells

To investigate the effect of glyphosate on the cellular circadian clock and testosterone synthesis in LCs, the TM3 cell line was used as a representative LC model. A CCK-8 assay was used to determine the appropriate concentration of glyphosate that did not markedly affect cell viability. Exposure of TM3 cells to different concentrations of glyphosate for 24 h significantly reduced cell viability at concentrations of 5 mM or more. However, after incubation for 48 h, 1 mM glyphosate exposure markedly inhibited the viability of TM3 cells. Thus, 0.1 mM was the highest concentration of glyphosate, which did not significantly affect the viability of TM3 cells relative to the CTRL (0 mM) group at 24 h and 48 h. In addition, the cell apoptosis assay results confirmed that exposure with 0.1 mM glyphosate for 24 h and 48 h did not significantly affect the apoptotic rate of TM3 cells compared to the CTRL group. Therefore, 0.1 mM was selected as the optimal glyphosate exposure concentration for further analysis in this study.

Effect of glyphosate exposure on the expression of circadian clock genes and steroidogenic genes in TM3 cells

To investigate the effect of glyphosate on the circadian clock system and testosterone synthesis, the expression levels of several circadian clock genes and steroidogenic genes were examined in DXM-synchronized TM3 cells cultured in the presence or absence of 0.1 mM glyphosate for 24, 28, 32, 36, 40, and 44 h. Glyphosate exposure significantly downregulated the mRNA expression levels of circadian clock genes (*Bmal1* and *Per2*) (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0123$ and $p = 0.0002$, respectively). However, the mRNA expression levels of *Nr1d1* were markedly increased after glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Additionally, WB analysis verified the elevated expression levels of NR1D1 in TM3 cells after glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0021$) (Figure 2B-C). For steroidogenic genes, 0.1 mM glyphosate exposure significantly decreased the mRNA expression levels of *StAR*, *Cyp17a1*, and *Cyp11a1*, respectively, except for *Hsd3b2* (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$, $p = 0.0057$, and $p < 0.0001$, respectively). Glyphosate exposure also consistently inhibited *StAR* expression at the protein level (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0066$). In addition, the ELISA results revealed a decline in secreted testosterone levels at both 24 h and 48 h in TM3 cells after 0.1 mM glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). These results demonstrate that 0.1 mM glyphosate exposure alters the circadian clock system and attenuates testosterone production in TM3 cells.

Effects of glyphosate exposure on *PER2::LUC* oscillations in primary LCs

To further investigate the influence of glyphosate on the circadian clock system in LCs, primary LCs isolated from *mPer2*^{Luc} knock-in mice were treated with or without 0.1 mM glyphosate and changes in *PER2::LUC* oscillations were examined. Several *PER2::LUC* oscillations were clearly observed in DXM-synchronized primary LCs isolated from *mPer2*^{Luc} knock-in mice in the presence or absence of glyphosate. However, the amplitude of the *PER2::LUC* oscillations was significantly attenuated by exposure to 0.1 mM glyphosate. In addition, exposure to glyphosate significantly advanced the peak time and shortened the period of the *PER2::LUC* oscillations relative to the CTRL group. These data provide additional evidence that 0.1 mM glyphosate not only alters the circadian clock system in TM3 cells, but also does so in primary LCs.

Effect of glyphosate exposure on the expression of circadian clock genes and steroidogenic genes in primary LCs

To further determine the effect of glyphosate on the expression of circadian clock genes and steroidogenic genes in primary LCs, their mRNA expression levels were examined in DXM-synchronized primary LCs for 24, 28, 32, 36, 40, and 44 h in the presence or absence of glyphosate (0.1 mM). Glyphosate exposure upregulated the mRNA expression levels of *Nr1d1* in primary LCs (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Additionally, WB analysis confirmed that NR1D1 protein levels were consistent with elevated mRNA expression levels after glyphosate exposure ($p < 0.01$). In contrast, other circadian clock genes (*Bmal1* and *Per2*) were significantly decreased (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Notably, glyphosate exposure also decreased the mRNA expression levels of *StAR*, *Cyp11a1*, and *Cyp17a1* in primary LCs (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Moreover, the expression levels of *Hsd3b2* were markedly decreased in primary LCs treated with glyphosate, which was different from that observed in TM3 cells (two-way ANOVA, main effect for glyphosate exposure, $p = 0.0001$). A significant reduction in testosterone production was also observed at both 24 h and 48 h following glyphosate administration (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). These results further confirmed that glyphosate exposure perturbs the transcription of the circadian clock and steroidogenic genes and alleviates testosterone synthesis in LCs.

The effect of glyphosate on the expression of circadian clock genes, steroidogenic genes, and testosterone production in vivo

The above results revealed that the expression levels of circadian clock genes at the mRNA and protein levels were affected in both TM3 cells and primary LCs after glyphosate exposure. Notably, the mRNA and protein expression levels of *StAR* were significantly reduced in TM3 cells and primary LCs after glyphosate addition. To further investigate whether the influence of glyphosate on the testicular circadian clock and steroidogenic genes in vitro is consistent with that in vivo, glyphosate was orally administered to mice through drinking water for 4 weeks. No significant changes in body weight, relative testis weight, relative epididymal weight, relative seminal vesicle weight, or relative liver weight in glyphosate-treated mice compared to mice in the CTRL group were found. In addition, HE analysis of the testis sections revealed that exposure to glyphosate did not affect testis morphology. Consistent with the results in TM3 cells and primary LCs, the mRNA expression levels of *Nr1d1* were significantly increased in the testes of both ZT0 and ZT12 after glyphosate exposure for 4 weeks (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). WB analysis also confirmed the elevated NR1D1 expression at the protein level (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Compared with CTRL, the mRNA expression levels of *Bmal1* and *Per2* were significantly reduced in the testes after 4 weeks of glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). Declines in *StAR*, *Cyp17a1*, and *Hsd3b2* mRNA expression levels were observed in testes at ZT0 and ZT12 following glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). The mRNA expression levels of *Dbp* were increased at ZT0 and decreased at ZT12 following glyphosate administration. The protein levels of *StAR* were reduced in testes at ZT0 and ZT12 after 4 weeks of glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$). An ELISA verified that serum testosterone levels were significantly reduced at ZT0 and ZT12 after 4 weeks of glyphosate exposure (two-way ANOVA, main effect for glyphosate exposure, $p < 0.0001$).

NR1D1 inhibits *StAR* transcription by binding to a RORE cis-element within its promoter

Genomic inspection revealed that the canonical sequence for the RORE cis-element (-859 to -864) was located within the -2000 bp region from the transcriptional start site of the *StAR* gene in mice (NC_000074.7). To assess whether *StAR* expression is regulated by NR1D1 through its direct binding to the *StAR* promoter, the authors constructed a *StAR*-Luc reporter vector containing a 1773 bp fragment (-1377 to +396, +1 indicates the transcription start site) of the *StAR* gene. As indicated above, *StAR*-Luc, pRL-CMV (Promega, an internal control plasmid), pcDNA 3.1-*Nr1d1*, and pcDNA3.1 empty vector were transfected into TM3 cells for 36 h. As expected, the transcriptional activity of the *StAR* fragment was significantly inhibited following transfection with pcDNA 3.1-*Nr1d1* compared with the pcDNA 3.1-empty group ($p < 0.05$). To assess whether NR1D1 inhibits *StAR* transcription primarily through the canonical RORE cis-element located within its promoter, the authors performed an EMSA assay. A clear band representing a DNA-protein complex was detected when NR1D1 nuclear proteins from TM3 cells were incubated with a biotinylated probe encompassing RORE (-859 to -864). The band was absent after the addition of a competitor (unlabelled probe). However, the band reappeared in the presence of a mutated competitor. In summary, these results revealed that NR1D1 inhibits *StAR* expression by binding to a RORE cis-element within its promoter.

Conclusion

The authors concluded that this study is the first to examine the detrimental effects of glyphosate on the transcription of the circadian clock and steroidogenic genes, as well as testosterone production in TM3 cells, primary LCs, and in vivo. The authors also indicated they proved that *StAR* transcription can be directly repressed by elevated NR1D1 expression levels induced by glyphosate exposure in LCs, which is a reason for the dysregulation of testosterone synthesis in LCs. Finally, the authors stated that this study not only provides a basis for explaining the detrimental effects of glyphosate on the circadian clock system in LCs and mouse testes, but also expands the understanding of the underlying mechanisms of the reproductive toxicity of glyphosate in mammals.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article hypothesize a mode of action to perturb testosterone synthesis via NR1D1 mediated inhibition of StAR expression in mouse Leydig cells.

This study examined the effects of glyphosate on the expression of the circadian clock and steroidogenic genes, as well as testosterone production in TM3 cells and primary LCs. The authors also investigated the effects of glyphosate on PER2::LUCIFERASE (PER2::LUC) oscillations in mouse primary LCs isolated from mPer2Luciferase (mPer2Luc) reporter gene knock-in mice. Furthermore, the effects of glyphosate exposure on the expression of core clock and steroidogenic genes in mouse testes as well as serum testosterone levels were determined in vivo. Finally, dual-luciferase reporter and EMSA assays were used to uncover the underlying molecular mechanism of glyphosate impairment of testosterone production involving NR1D1 regulation of StAR expression in LCs. The lack of a concurrent and historical positive control data substantially diminishes confidence in the predictability of the methodology employed to probe the proposed mechanism.

The results indicate that glyphosate perturbs testosterone synthesis via NR1D1 mediated inhibition of StAR expression in mouse LCs. These results contradict a large body of high-quality data assessing endocrine disruption and specifically steroidogenesis. Furthermore, the results and conclusions of this publication are not consistent with Levine et al. (2007), which specifically investigated glyphosate & formulation in mouse Leydig cells and StAR protein expression.

The article is considered relevant (Category A) and reliable with restrictions for the following reason: No purity of the test material provided, no HCD/positive control reported. Only one dose evaluated/no dose-effect relationship for the in vivo experiment. The lack of a concurrent and historical positive control data substantially diminishes confidence in the predictability of the methodology employed to probe the proposed mechanism.

References:
Levine s. et al; Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis, Cell Biol Toxicol (2007) 23:385–400.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies

Publication: Zhao L. 2021, Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	All animal usage and experimental procedures were approved in accordance with the Guidelines for Animal Experiments by the Committee for the Ethics on Animal Care and Experiments of Northwest A&F University and performed under the control of the "Guidelines on Ethical Treatment of Experimental

Publication: Zhao L. 2021, Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells	Criteria met? Y/N/?	Comments
		Animals" (2006) No.398 set by the Ministry of Science and Technology, China
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Pure glyphosate powder, purchased from Shanghai Yuanye BioTechnology Co., Ltd. (Shanghai, China). No information on purity, content etc.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA or other glyphosate metabolites is the tested substance	N	
Study		
Test species clearly and completely described	Y	BALB/c wild-type (WT) mice (10–12 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China); No BW information was included
Test conditions clearly and completely described	Y	Standard laboratory conditions of temperature, humidity, and lighting described
Route and mode of administration described	Y	Mice in one group were orally administered 0.5% glyphosate via daily drinking water (Glyphosate group). No distinction if animals were group-caged or not. The control group received distilled water alone (CTRL group). Four weeks later, the mice from the Glyphosate and CTRL groups were euthanized. Cell preparation described, cytotoxicity assay performed.
Dose levels reported	Y	Glyphosate was dissolved in distilled water to obtain a 0.5% solution, as previously described (5 g glyphosate/L water). The pH levels of dose & control groups were not adjusted.
Number of animals used per dose level reported	Y	Twenty WT mice were randomly assigned to two equally sized groups.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N/A	
Analytical verifications of test media	N	pH of glyphosate acid test groups were not reported, which would be quite acidic compared to the control dose.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	No HCD, no positive control for the *in vitro* experiment to verify this novel method.
Dose-effect relationship reported	N	Only one dose
Overall assessment		
Reliable without restrictions	N	

Publication: Zhao L. 2021, Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells	**Criteria met? Y/N/?**	**Comments**
Reliable with restrictions	Y	No purity of the test material provided, no HCD/positive control reported. Only one dose evaluated/no dose-effect relationship for the *in vivo* experiment. The lack of a concurrent and historical positive control data substantially diminishes confidence in the predictability of the methodology employed to probe the proposed mechanism.
Not reliable	N	

1. Information on the study

Data point:	CA 5.9.4
Report author	Ferreira C. *et al.*
Report year	2021
Report title	Urine biomonitoring of glyphosate in children: Exposure and risk assessment
Document No	Environ Res 2021; 198: 111294 https://doi.org/10.1016/j.envres.2021.111294
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes (Relevance Category A)/Reliable with restrictions

2. Full summary of the study according to OECD format

Purpose: To determine the concentrations of glyphosate in the urines of 41 Portuguese children (2–13 years old) and to identify potential determinants of urine concentration. **Methods:** First morning spot urine samples were collected from study participants and glyphosate concentrations were estimated using the glyphosate ELISA Plate Kit® with a 0.6 μg/L limit of detection. **Results:** Glyphosate was detected in 95.1% of the samples with a mean concentration of 1.77 ± 0.86 μg/L and a maximum value of 4.35 μg/L. Glyphosate concentrations were higher in the urine of children aged 7–9 years, those living within 1 kilometer of agricultural areas, those with families that have a higher consumption of home produced foods, and those whose parents applied herbicides in the backyard. The authors' risk assessment estimated oral intake of glyphosate to range from 1% to 5.6% of the established Acceptable Daily Intake (ADI) (0.5 mg/kg bw/day). **Conclusions:** The results should be further analyzed considering the age of the participants, for whom no adjusted ADI exists.

Materials and methods

Study subjects and data collection: 41 healthy children, ages 2 to 13 years, were recruited from different regions of Portugal. Participants were 46% male (age 2 to 12 years) and 54% female (age 2 to 13 years). The participants were concentrated in the Coimbra region (82%, n = 32). The other 9 participants were distributed across 3 municipalities: Porto (n =1), Lisbon (n = 2), and Aveiro (n = 6). All of the children participating in the study lived in municipalities that subscribed to the "Municipalities without Glyphosate" campaign.

Parents completed a questionnaire about their child's anthropometric data and health including sex, age, weight, height, medication, and exposure to tobacco smoke. Parents also provided data concerning the family's sociodemographic status, parents' educational levels, distance from the family residence to an industrial zone or agricultural field or nearest highway, and residential use of agrochemicals.

First morning spot urine samples were collected between July 2018 and February 2019. Samples were collected in polystyrene containers and returned to the laboratory on the collection day, divided into aliquots, and stored at - 20 C until extraction for analysis.

Urine analysis: Urine samples were analyzed using the glyphosate ELISA Plate Kit®. The first step was to filter 500 uL of the urine samples through Millipore Amicon Ultra filters into 2 ml tubes. Samples were then centrifuged at 8000 g for 15 minutes. Then 300 uL of the filtrate was placed in a new tube and 200 uL of ethyl acetate was added. Then the sample was centrifuged again at 8000 g for 3 minutes. The initial preparation was completed by adding 350 uL of the sample diligent to 50 uL of the previous sample.

The next step was derivatization. To dilute the derivatization agent, 3.5 mL of the diluent were added to the derivatization reagent and centrifuged at 5444 g at 4 ◦C for 15 minutes. Then, 250 μL of each standard, control and sample was added to the respective labeled tubes having 1 ml of buffer. The tubes were centrifuged for approximately 2 seconds.

The previously prepared diluted derivatization reagent (100 µL) was added to each labeled tube (standard, control, and sample). Each tube was centrifuged for 15–30 seconds. Then, the tubes were incubated at room temperature for 10 minutes. To start the ELISA analysis, 50 µL of each standard concentration (0, 0.075, 0.2, 0.5, 1 and 4 ng/L), control (0.75 ± 0.2 ng/mL) and derivatized samples were added to the respective wells of the plate. Then 50 µL of the anti-glyphosate antibody was added to all wells.

The tubes were covered with aluminum foil and homogenized through rapid circular movements for 30 seconds. Then, they were incubated at room temperature for 30 minutes, protected from direct sunlight. Subsequently, 50 µL of enzyme conjugate were distributed in each individual well. The microplate was again covered, shaken, and incubated for 60 min at room temperature, protected from light.

Then 150 µL of substrate was added. The microplate was covered again, shaken, and incubated for 30 minutes at room temperature, protected from light. Finally, 100 µL of stop solution was added and the absorbance was read at 450 nm, using an ELISA microplate reader. According to the manufacturer, the detection limit of the kit corresponded to 0.6 µg/L.

Statistical analysis: Data were analyzed descriptively in Microsoft Excel. Other statistical analyses were performed using the GraphPad Prism 8 (CR) program. Where the data were found to be skewed, non-parametric tests were implemented. The Kruskal-Wallis test was used to compare variables with more than 2 categories and the Mann-Whitney test was used to compare variables with only two categories. A p value less than 0.05 was considered to be statistically significant.

The authors estimated the internal dose of glyphosate as the product of each child's urine concentration (in µg/L) times the average daily urinary volume for children of 1.5 L. The resulting internal dose was then divided by the average body weight (in kg) for the 4 age categories (14,4 kg for those < 3 years old, 20.5 kg for those 4 to 6 years old, 29.3 kg for those 7 to 9 years old, and 38.3 kg for those 10 to 13 years old) to yield dose per kg body weight (equation 1) or the probable daily intake (PDI).

$$\text{PDI (µg / kg bw)} = \frac{\text{concentration}_{urine} \text{ X volume}_{urine}}{\text{body weight (kg)}} \qquad \text{equation (1)}$$

Assuming that diet was the most likely route of exposure and assuming 1% absorption based on a human feeding study by Zoller et al. (2020), the amount ingested (or external dose) was estimated to be (equation 2 as in Niemann et al. 2015)

$$\text{External dose (µg / kg bw)} = \frac{\text{100 X internal dose}}{\text{1 \% oral absorption}} \qquad \text{equation (2)}$$

Results

Glyphosate was detected in 95% of samples (39 out of 41). The average urine concentration was 1.77 +- 0.86 ug/L. The maximum urine concentration was 4.35 ug/L. Urinary values were evaluated with respect to possible determinants of exposure. Average values were slightly higher for girls (2.00 +- 0.94 ug/L) than for boys (1.56 +- 0.57 ug/L). Average values were slightly lower for those less than 3 years of age (1.20 ug/L) compared with older children (4 to 6 years old 1.99 ug/L, 7 to 9 years old 2.12 ug/L, and 10 to 13 years old 1.74 ug/L) ($p = 0.063$). Glyphosate concentrations were slightly higher in the urine of those living within 1 kilometer of agricultural areas, those from families with a higher consumption of home-produced foods, and those whose parents applied herbicides in the backyard.

The authors conducted a risk assessment by taking the minimum, average, and maximum glyphosate urinary concentrations for each of 4 age groups, back calculating the amount of glyphosate ingested assuming 1% absorption, and then calculating estimated glyphosate intake as a percent of the Acceptable Daily Intake (ADI) of glyphosate (0.5 mg/kg bw) The following table summarizes those % ADI calculations. Values ranged from 1% to 5.6% of the ADI.

Glyphosate concentration	% ADI ≤ 3 years old	% ADI 4 to 6 years old	% ADI 7 to 9 years old	% ADI 10 to 13 years old
Minimum	1.8%	1.3%	1.0%	1.0%
Average	2.5%	3.0%	2.2%	1.3%
Maximum	3.6%	5.6%	4.5%	1.5%

Conclusion

The authors concluded that glyphosate was detected in 95% of the urine samples with a mean concentration of 1.77 µg/L and a maximum concentration of 4.35 µg/L. They also concluded that higher concentrations of glyphosate in urine were found among those children aged 7–9 years old, those living within 1 kilometer of agricultural areas, those who consumed higher amounts of home-produced foods, and those that had herbicides applied in the yard of their residences. Based on their risk assessment, the authors concluded that oral intake of glyphosate ranged from 1% to 5.6% of the established ADI for glyphosate (0.5 mg/kg bw). They considered the frequency of detection and the estimated oral intake to be of concern and, considering the vulnerability of children, encouraged that further biomonitoring studies should be conducted.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This is a small biomonitoring study with limited urine sample collection for each participant. The sampling strategy was not described, but the focus on recruiting participants from municipalities that subscribed to the "Municipalities without Glyphosate" campaign means that the results cannot be taken to be representative of the population of Portuguese children ages 2 to 13. Also, since 82% of the participants came from one region (Coimbra), and other regions had as few as 1 or 2 participants, it seems almost certain that the sampling strategy was a convenience sample. The results should be interpreted accordingly.

The authors concluded that age, living near agricultural areas, consumption of home-produced foods, and having herbicides applied in the backyard were predictors of urinary glyphosate concentrations. The actual differences in urinary concentrations across the categories of these factors were slight and none of the differences were statistically significant. Further, the analysis for each potential predictive factor was univariate and did not control for the other potential predictors. Thus, the authors' evaluation of potential predictors of urinary concentration is relatively uninformative.

The authors estimated that the study participants had glyphosate oral ingestion ranging from 1% to 5.6% of the glyphosate ADI (0.5 mg/kg bw). These estimates were based on assuming 1% oral glyphosate absorption as estimated by Zoller et al. (2020). One percent absorption is 20-fold lower than the absorption factor used previously in the literature (e.g., Niemann et al. 2015). However, the Zoller et al. (2020) study was a well-controlled human study and the 1% absorption is potentially representative for low oral exposures to glyphosate. Zoller et al. (2020) noted, with respect to the 1% absorption factor, that human glyphosate intake estimated from urinary concentrations could be approximately 20 times greater than previously estimated and that would be counterbalanced by

human systemic availability being 20 times lower than previously assumed. Assuming 1% absorption is valid, the estimated intakes in this study are 18-fold to 100-fold less than the ADI.

To conclude, the study by Ferreira and colleagues (2021) has several important limitations. The study participants are unlikely to be representative of children in Portugal. Urine collection per participant was limited to one spot sample, which requires assumptions about the daily volume of urine for each participant and about whether the single sample is representative of that participant's steady state throughout the year. The statistical analysis was superficial with respect to factors that might predict oral intake. Setting aside these limitations and taking the % ADI results at face value, the highest value seen in this study is a small percentage of the ADI.

References

Niemann L, Sieke C, Pfeil R, Solecki R. A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers. Journal für Verbraucherschutz und Lebensmittelsicherheit 2015;10(1):3-12.

Zoller O, Rhyn P, Zarn JA, Dudler V. Urine glyphosate level as a quantitative biomarker of oral exposure. International Journal of Hygiene and Environmental Health 228 (2020) 113526.

Publication: Ferreira C. et al. Urine biomonitoring of glyphosate in children: Exposure and risk assessment.	**Criteria met? Y/N/Uncertain**	**Comments**
Guideline-specific		
Study is in accordance to valid internationally accepted testing guidelines/practices.	n/a	
Study is completely described and conducted following scientifically acceptable standards.	Yes	
Test substance		
Exposure to formulations with only glyphosate as active substance.	Uncertain	Source of glyphosate exposure is not specified.
Exposure to formulations with glyphosate combined with other active substance(s).	Uncertain	Same comment as above
Exposure to various formulations of pesticides.	Uncertain	Same comment as above
Study		
Study design – epidemiological method is followed.	n/a	
Description of population investigated.	No	Portuguese children 2 to 13 years of age from municipalities that were participating in the "Municipalities without Glyphosate" campaign. The methodology within that sampling frame (e.g., random, convenience, etc.) was not specified
Description of exposure circumstances.	No	Also, it seems that the investigators did not ask pesticide users about which specific pesticides were used – especially

Publication: Ferreira C. et al. Urine biomonitoring of glyphosate in children: Exposure and risk assessment.	**Criteria met? Y/N/Uncertain**	**Comments**
		relating to glyphosate use among the herbicide users.
Description of results.	Yes	Superficial analyses
Have confounding factors been considered?	n/a	
Statistical analysis reported / described.	Yes	Analyses of exposure determinants were univariate and very limited. Most differences were small. Conclusions about factors that determine urinary levels were not based on multivariate evaluation and statistical conventions.
Overall assessment		
Reliable without restrictions	No	
Reliable with restrictions	Yes	This is a small study with only a single spot urine sample per participant. The sampling methodology and participation rate were not specified.
Reliability not assignable	No	
Not reliable	No	

グリホサートカリウム塩

要旨及び評価結果

（環境動態）

検索期間：2021 年 1 月 1 日～2021 年 5 月 14 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 7.3.1/009
Report author	Holtomo O. *et al.*
Report year	2021
Report title	Insight of UV-vis spectra and atmospheric implication for the reaction of •OH radical towards glyphosate herbicide and its hydrates
Document No	RSC Advances (2021), 11(27), 16404-16418
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Relevant (Category A acc. to EFSA GD, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The rate constant of the reactions of •OH radicals with glyphosate glyphosate and its hydrates (glyphosate$(H_2O)_{n=1-3}$) were evaluated using the dual method M06-2X/6-311++G(df,p)//6-31+G(df,p) over the temperature range of 200–400 K. The results served to estimate the atmospheric lifetime along with the photochemical ozone creation potential (POCP). The calculations yielded an atmospheric lifetime of 2.34 hours and a POCP of 24.7 for glyphosate. Upon addition of water molecules, there is an increase of lifetime and decrease of POCP for water monomer and water dimer. The POCP for water trimer is slightly above the gaseous glyphosate. However, the POCPs of glyphosate and its hydrates are comparable to that of alkanes. The glyphosate and its hydrates were found to be a potential reservoir of CO_2. The acidification potential (AP) of glyphosate was found to be 0.189 and decreases upon addition of water molecules. This shows negligible contribution to rain acidification as the AP is less than that of SO_2. The UV-vis spectra were attained using the M06-L/6-311++G(3df,3pd) method and cover the range 160–260 nm.

Materials and methods

The rate constant of the oxidative degradation of glyphosate were determined through H-abstraction mechanism in the presence of •OH radical. This mechanism is suspected to happen by five plausible channels as presented in reactions (R1a)–(R1f).

$$(OH)_2\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + {}^{\bullet}OH \rightarrow (OH)_2\text{–PO–}CH_2\text{–NH–}CH_2\text{–COO}^{\bullet} + H_2O \quad \text{(R1a)}$$

$$(OH)_2\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + {}^{\bullet}OH \rightarrow (OH)_2\text{–PO–}CH_2\text{–NH–}CH^{\bullet}\text{–COOH} + H_2O \quad \text{(R1b)}$$

$$(OH)_2\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + {}^{\bullet}OH \rightarrow (OH)_2\text{–PO–}CH_2\text{–N}^{\bullet}\text{–}CH_2\text{–COOH} + H_2O \quad \text{(R1c)}$$

$$(OH)_2\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + {}^{\bullet}OH \rightarrow (OH)_2\text{–PO–}CH^{\bullet}\text{–NH–}CH_2\text{–COOH} + H_2O \quad \text{(R1d)}$$

$$(OH)_2\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + {}^{\bullet}OH \rightarrow (HO)O^{\bullet}\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + H_2O \quad \text{(R1e)}$$

$$(OH)_2\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + {}^{\bullet}OH \rightarrow {}^{\bullet}O(HO)\text{–PO–}CH_2\text{–NH–}CH_2\text{–COOH} + H_2O \quad \text{(R1f)}$$

The thermochemistry of these five pathways was assessed. The enthalpy and free energy of these reactions were calculated in order to predict the feasibility and spontaneity of the reaction channels. The

bond dissociation enthalpy (BDE) of X–H (X = C, N, or O atom) was used to compare the heat required to break the X–H bonds in glyphosate and its hydrates. The BDE was calculated at five sites as presented in the reactions (R2a)–(R2f).

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COO^{\bullet} + H^{\bullet} \quad \text{(R2a)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH^{\bullet}\text{–}COOH + H^{\bullet} \quad \text{(R2b)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH_2\text{–}N^{\bullet}\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2c)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (OH)_2\text{–}PO\text{–}CH^{\bullet}\text{–}NH\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2d)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow (HO)O^{\bullet}\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2e)}$$

$$(OH)_2\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH \rightarrow {}^{\bullet}O(HO)\text{–}PO\text{–}CH_2\text{–}NH\text{–}CH_2\text{–}COOH + H^{\bullet} \quad \text{(R2f)}$$

The present work was completed by the means of the density functional theory (DFT) and the time dependent variant (TD-DFT). The exchange–correlation functionals M06-2X and M06-L combined with the appropriate split valence basis sets were employed, respectively. This is in order to yield accurate results of structures, electronic energies, UV-vis spectra, vibrational frequencies, enthalpies and free energies of the species in the reaction processes.

Computational details

Computations were carried out using the density functional theory (DFT) implemented in the Gaussian 09 suite package.

The time dependent DFT (TD-DFT) and DFT schemes through the meta-hybrid exchange correlation functional M06-2X were employed. The potential energy surfaces (PESs) of glyphosate were attained through the rotation of dihedral angles using the M06-2X/6-31G(df) method. The equilibrium geometries and frequencies of glyphosate and its hydrates were carried out using the dual method M06-2X/6-311++G(df,p)//6-31+G(df). However, for the kinetics of the reactions of •OH radical towards glyphosate and its hydrates, the M06-2X/6-311++G(df,p)//6-31+G(df,p) method was assigned. This composite basis set produced excellent results regarding the rate constant of •OH radical towards greenhouse gases. The single point energy calculations were done without orbital symmetry constraints. The direct inversion in the iterative subspace (DIIS) procedure was used for geometry optimizations. The equilibrium geometries of the different species of this study were validated based on the real frequencies obtained, except for the transition state equilibrium where only one imaginary frequency should be observed. The TD-DFT was performed based on the Runge–Gross scheme at the M06-L/6-311++G(3df,3pd) level of theory, in order to have the UV-vis spectra of glyphosate and its hydrates.

UV-vis spectral generation

The UV-vis spectrum of chemical species is turned out by the list of wavelengths and oscillator strengths employing the Gaussian broadening function. This function is the molar extinction coefficient ε(ω) that quantifies the ability of chemical species to absorb light at a given wavelength λ. This is expressed as follows:

$$\varepsilon(\lambda) = \sum_{k=1}^{N} \frac{A_k}{\sigma_k \sqrt{2\pi}} \exp\left(- \frac{(\lambda_k - \lambda)^2}{2{\sigma_k}^2} \right) \qquad (1)$$

where N indicates the number of excited states. The subscript k refers to the k^{th} absorption peak in the spectrum, λ_k is the wavelength at the origin, λ is any given wavelength, and σ_k is the dispersion that is related to the full width at half-maximum (Γ) by $\Gamma = 2\sigma\sqrt{2ln2}$. A_k indicates the absorption intensity of each band including the oscillator strength $f_k = 2m\omega_k|\mu_k|^2/3\hbar$ by the relation:

$$A_k = \frac{2\pi^2 e^2 N_A}{mc \ln(10)} f_k \qquad (2)$$

where m and e are the mass and electric charge of electron, respectively. N_A is the Avogadro's number, c is the speed of light, and μ_k is the transition dipole moment from ground state to k^{th} excited state.

Thermodynamics and kinetics of reactions

The H-abstraction mechanism presented in reactions (R2a)–(R2e) is characterized by the bond dissociation enthalpy (BDE) of all the X–H bonds of glyphosate molecule (C–H, O–H, and N–H). This is assessed using eqn (3).

$$\mathrm{BDE}_{298} = \Delta_f H^\circ(\mathrm{GPS–X^\bullet}) + \Delta_f H^\circ(\mathrm{H^\bullet}) - \Delta_f H^\circ(\mathrm{GPS–XH}) \qquad (3)$$

However, the H-atom transfer (HAT) from gaseous glyphosate to $^\bullet$OH radical is governed by the enthalpies and Gibbs free energies of reactions (R1a)–(R1e). These thermal parameters were calculated using the expression of eqn (4) and (5).

$$\Delta H_{r,298} = \Delta_f H^\circ(\mathrm{GPS–X^\bullet}) + \Delta_f H^\circ(\mathrm{H_2O}) - \Delta_f H^\circ(\mathrm{GPS–XH}) - \Delta_f H^\circ(^\bullet\mathrm{OH}) \qquad (4)$$

$$\Delta G_{r,298} = \Delta_f G^\circ(\mathrm{GPS–X^\bullet}) + \Delta_f G^\circ(\mathrm{H_2O}) - \Delta_f G^\circ(\mathrm{GPS–X–H}) - \Delta_f G^\circ(^\bullet\mathrm{OH}) \qquad (5)$$

where $\Delta_f H^\circ(Y)$ and $\Delta_f G^\circ(Y)$ are the enthalpy and Gibbs free energy of formation of species Y. The physical quantity $\Delta_f H^\circ(Y)$ is the sum of electronic energy, zero-point vibrational energy (ZPVE), and the thermal correction to enthalpy, while $\Delta_f G^\circ(Y) = \Delta_f H^\circ(Y) + RT$, where R represents the gas constant. The rate constants of the reactions (R1a)–(R1e) were calculated based on the transition state theory (TST). This theory is the most popular issue for assessing rate constants of chemical processes. Over the past two decades, significant progress has been made in developing methods for quantitative predictions of rate constants based upon the dynamical formulation of TST. The conventional TST rate constant at temperature T for a bimolecular reaction reads as:

$$k_{TST}(T) = \frac{\sigma}{2\pi\beta\hbar} \frac{Q_{TS}(T)}{\Phi_{React}(T)} \exp(-\beta V_0) \qquad (6)$$

$\beta = 1/k_B T$, k_B is the Boltzmann constant, $\hbar$ is the reduced Planck constant, and σ denotes the number of indistinguishable ways the reactants may approach the activated complex regions. $Q_{TS}(T)$ is the partition function at transition state, and $\phi_{React}(T) = Q_A Q_B$ is the total partition function of reactants A and B per unit volume at temperature T. V_0 is the barrier height of the reaction. Another variant of TST is the vibrational transition state theory (VTST) in which, $Q_{TS}(T)$ and V_0 varies with the reaction coordinate as $Q_{TS}(T,s)$ and $V_0(s)$. In VTST variant, the rate constant is expressed as the minimum of $k_{VTST}(T,s)$. This method was performed in the present work for the reaction of $^\bullet$OH radical towards glyphosate and its hydrates. In the total partition function of the reactants ($\phi_{React}(T) = Q_{glyphosate} Q_{OH}$), the contribution of electronic partition function of $^\bullet$OH radical takes into account the splitting of the ground state π^2 into $\pi_{1/2}^2$ and $\pi_{3/2}^2$. The separation 139.7 cm^{-1} of these sub-states was used in the calculations. The electronic partition of $^\bullet$OH radical is then written as follows $Q_{elec}(OH) = 2 + 2\exp((139.7\ cm^{-1})\hbar c/k_B T)$, where $\hbar$ and c are the Planck's constant and speed of light, respectively. It is worth mentioning that, the internal rotation modes of glyphosate and its hydrates were treated using the hindered rotor approximation, whereas the other vibrational frequencies were treated harmonically.

Considering the quantum tunnelling effects, the rate constant is multiplied by the quantum tunnelling factor η(T) as per eqn (7).

$$k(T) = \eta(T) k_{VTST}(T) \qquad (7)$$

The η(T) factor is defined as the ground state transmission coefficient at temperature T. This factor is quantitatively the ratio of the thermally averaged multidimensional semi-classical transmission probability to the thermally averaged classical transmission probability for scattering by the effective

potential.

Results

Structural study of molecular systems

To come out with the atmospheric implication of this study, the geometrical structures of glyphosate (glyphosate) and its hydrates was paramount challenges to deal with. A computational protocol was used to yield the low-energy conformers of glyphosate and its hydrates.

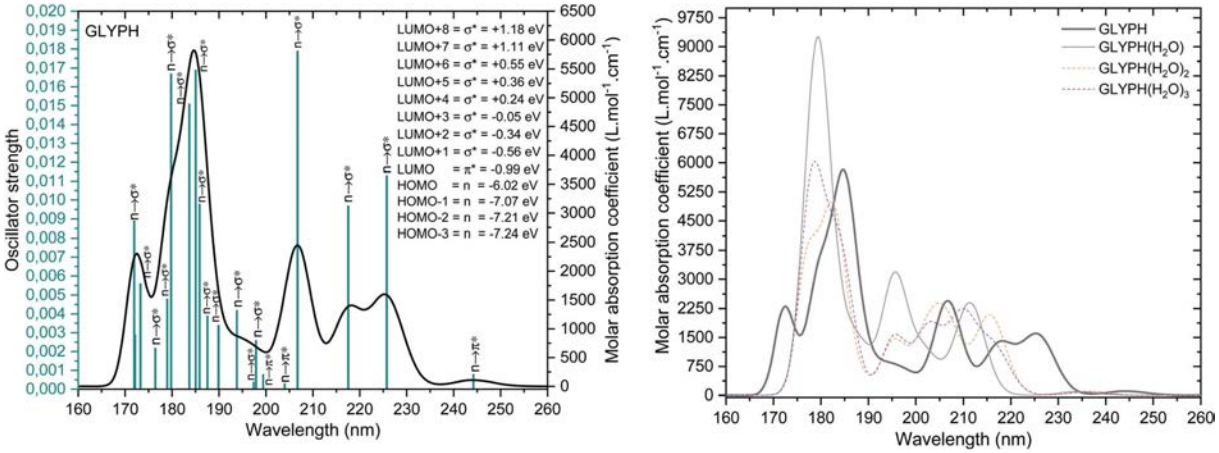
The order of degradation of glyphosate and its hydrates from the dipole moment analysis was stated as follows: glyphosate(H_2O) > glyphosate > glyphosate$(H_2O)_2$ > glyphosate$(H_2O)_3$. Thus, in humid air, glyphosate reduces its dipole moment and resist to degradation. This is in discordance with the average polarizability analysis where, the order of degradation follows as glyphosate$(H_2O)_3$ > glyphosate$(H_2O)_2$ > glyphosate(H_2O) > glyphosate. In this case the humid air favours the degradation of glyphosate.

UV-vis spectroscopies of glyphosate and its hydrates

In order to identify glyphosate in the atmosphere, the TD-DFT calculations were first performed at M06-2X/6-311++G(3df,3pd) level of theory. The results failed while comparing with experiment. To improve this result, a diagnostic based on the fact that HF/6-311++G(d,p) yielded good results, thus glyphosate molecule is a single reference system. Therefore, a pure DFT can be a good deal for the spectroscopy of glyphosate and its hydrates. Then the pure functional M06-L of the same family of Menesota University was selected to perform TD-DFT calculations. The obtained results gave satisfaction (206.74 nm).

The UV-vis spectra of the glyphosate and its hydrates are presented in Fig. 5. The main peak at 206.74 nm has a weak absorption coefficient value. This is due to the nearby peaks in which the overall Gaussian function accumulates the individual ones. The effects of water molecules impact seriously the spectrum of glyphosate. The blue shifts of peaks are noticeable along with the enhancement of the absorption peaks (hyperchromic effects). However, it is seen that, the spectra are localized in the UV window range 160–260 nm. This advises that, glyphosate and its hydrates are colourless.

Figure 5: UV-vis spectra of glyphosate its hydrates. The full width at half maximum was taken at 0.1 eV



Thermodynamics of reactions

In the presence of •OH radical, the reaction process of glyphosate can advent from six different issues as presented in the reactions (R1a)–(R1e). The bond dissociation mechanisms from the five channels are stated at equations (R2a)–(R2e). The enthalpies of these reactions (BDE) at 298.15 K advises on the amount of heat required to dissociate the C–H, N–H, and O–H bonds of glyphosate. The results are compared to the computational BDEs of other molecules as quercetin (282.8 kJ/mol at B3LYP/6-31++G*), caffeic acid phenethyl ester (312.0 kJ/mol at B3LYP/6-31+G**), 3,4-dihydroxyphenylpyruvic acid (304 kJ/mol at B3LYP/6-311++G**), *p*-phenylenediamine (340 kJ/mol at B3LYP/6-31++G**) and tetracyano-*p*-phenylenediamine (381 kJ/mol at B3LYP/6-31++G**), and the experimental BDE of 3,4-dihydroxycinnamic acid (285 kJ/mol is in excellent agreement with theory at B3LYP/6-31++G** fit). In these molecules, the BDE were obtained from the O–H bonds except for *p*-phenylenediamine and tetracyano-*p*-phenylenediamine where the BDE was calculated from the N–H

bonds. The computational results were assessed using the B3LYP functional combined with the Pople's basis sets 6-31++G*, 6-31+G**, 6-31++G**. It is seen, that the computational BDEs from the N–H bond are greater than those obtained from O–H bonds. This is consistent with what was observed in glyphosate and its hydrates. However, the BDEs of glyphosate increase in the following order: BDE (site C_6) < BDE (site O_{16}) <BDE (site C_3) < BDE (site N_1) < BDE (site O_{10}) = BDE (site O_{12}). For wetted glyphosate, it follows the order: BDE (site C_3) < BDE (site O_{16}) < BDE (site C_6) < BDE (site N_1) < BDE (site O_{12}) = BDE (site O_{10}). Therefore, the most favourable site of radical attacks is C_6 for gaseous glyphosate and C_3 for wetted glyphosate. In addition, the H-atom at site O_{10} and O_{12} are strongly attached; they require a great amount of heat for dissociation. The BDEs from the exothermic paths ((R1a)–(R1d)) are reliable with the molecules used for comparison, while the BDE from the endothermic path (R1e) is out of order. The glyphosate radical obtained after the dissociation of O_{16}–H bond involved in the carboxylic group, is thermodynamically unstable. It dissociates in turns into carbon dioxide CO_2 and $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$ radical. This mechanism is written as per equation (R3).

$$(OH)_2 - PO - CH_2 - NH - CH_2 - COOH \rightarrow (OH)_2 - PO - CH_2 - NH - CH_2^{\cdot} + CO_2 + H^{\bullet} \quad (R3)$$

In the calculation of the BDE of O_{16}–H, the enthalpy of the overall system $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$-CO_2 was considered as it takes into account the interaction between individual subsystems.

On the other side, the enthalpies $\Delta H_{r,298}$ and Gibbs free energies $\Delta G_{r,298}$ of reactions involving glyphosate (and its hydrates) and $^{\bullet}$OH radical were estimated at 298.15 K in order to predict the feasibility and spontaneity of the reaction channels.

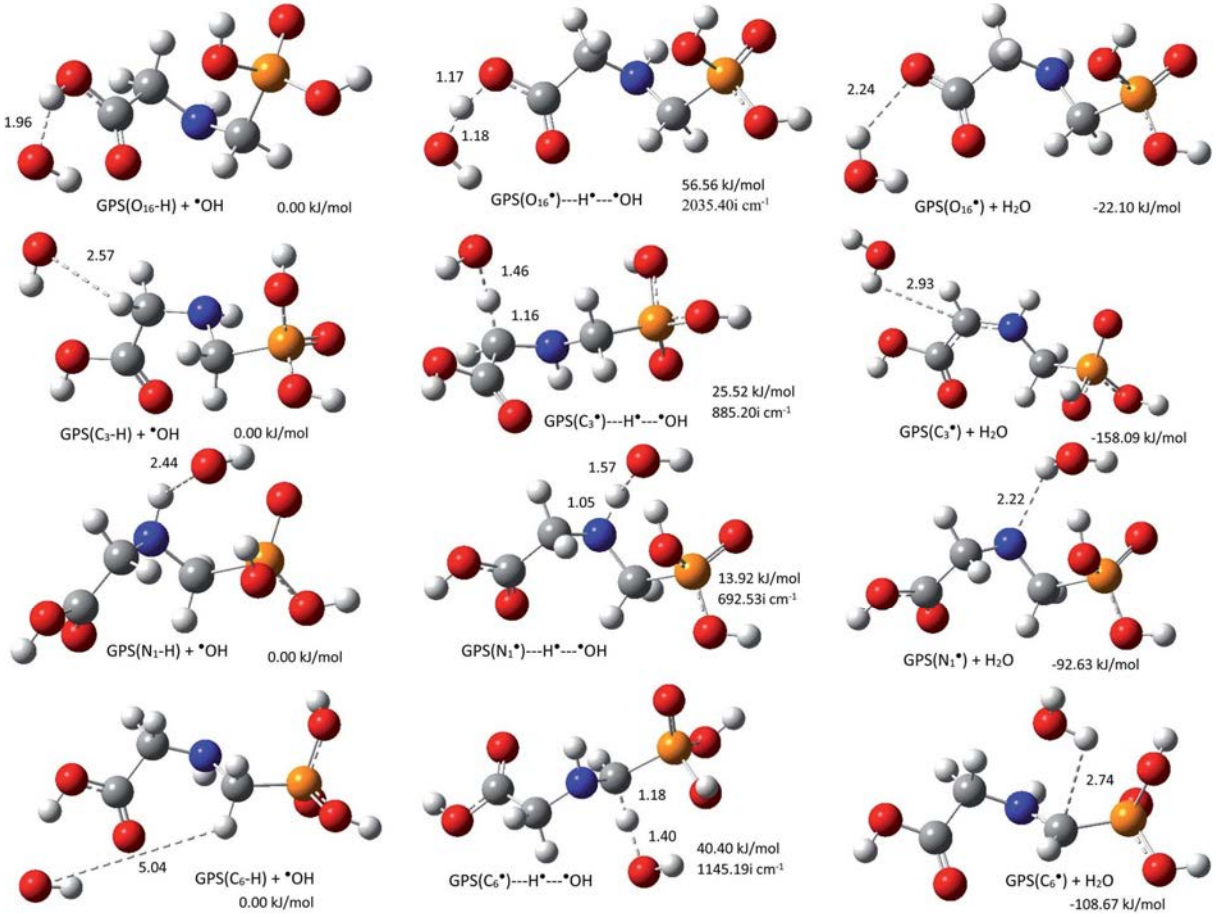
It turns out that, the reaction channels (R1a)–(R1d) are exothermic, while the channel (R1e) is endothermic. This is due to the nucleophilic character of the molecular systems at the level of P-atom. In fact, in the glyphosate molecule, the charge hole is made on P-atom and the electron lone pairs take place on the O-atoms and reinforce the enthalpy of the O–H bonds. It is noticeable that, this fact is correlated with the BDE and explains why channel (R2e) has the greatest value of BDE. Dealing with $\Delta G_{r,298}$, it comes out that, the reaction channels (R1a)–(R1d) are spontaneous, while the channel (R1e) is non-spontaneous. Thus, we can conclude that, the reaction channel (R1e) is unfeasible; therefore, an attention was paid only on the reaction channels (R1a)–(R1d) in the section devoted to kinetics of chemical reactions.

Moreover, in the presence of $^{\bullet}$OH radical, the reaction process (R3) yields three different products such as H_2O, CO_2 and $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$. As in the case of BDE calculation, the enthalpy and Gibbs free energy of formation of the overall system $(OH)_2$-PO-CH_2-NH-$CH^{\bullet}_2$-CO_2 was considered in the calculations of $\Delta H_{r,298}$ and $\Delta G_{r,298}$. In view of the production of CO_2 by the channel (R1a) and the feasibility of the reaction, one can assert that glyphosate is a reservoir of CO_2 in the lower atmosphere.

Kinetics of reactions

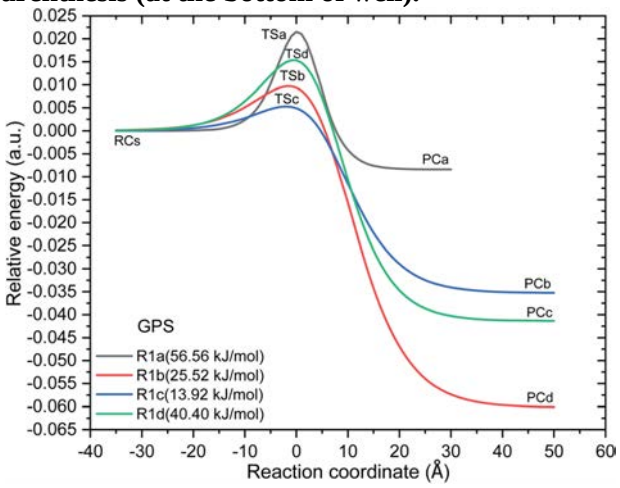
The rate constants k_{OH} of the reactions of $^{\bullet}$OH radical towards glyphosate and its hydrates were evaluated at all the thermodynamically feasible hydrogen sites. This includes the first four channels ((R1a)–(R1d)). The equilibrium geometries at reaction complex (RC), transition state (TS), and product complex (PC) for each pathway in gas phase are presented in Fig. 6 along with their relative energy (relative to RC). The stabilization of RCs and TSs for glyphosate and glyphosate(H_2O) were examined by the basis set superposition error (BSSE) corrected binding energies using the M06-2X/6-311++G(df,p) method. They were calculated from two fragments; $^{\bullet}$glyphosate/ $^{\bullet}$glyphosate(H_2O) and $^{\bullet}$OH radicals for RCs and three fragments; $^{\bullet}$glyphosate/ $^{\bullet}$glyphosate(H_2O), $H^{\bullet}$, and $^{\bullet}$OH radicals for TSs. As shown, the difference between binding energies of RCs and TSs are within 10^{-3} au, which can be ignored in the rate constant calculation.

Figure 6: **Reaction complex (RC), transition state (TS), and product complex (PC) for each reaction pathway of •OH radical towards glyphosate. The relative electronic energy of each step of the process are presented in kJ mol^{-1}. The internuclear lengths along the reaction coordinates and the imaginary frequency at TS are given in Å and cm^{-1}, respectively.**



As shown along the reaction coordinate, RC is first formed and as the reaction progresses, the intermediate (TS) is appeared and finally PC takes place. The three states along the minimum energy path (MEP) are stabilized by the interactions between glyphosate and •OH. All the species involved in the reaction paths (R1a)–(R1d), are verified by real vibrational positive frequencies, while TSs are confirmed by one imaginary frequency (IF) in the list of frequencies. The magnitude of these imaginary frequencies follows the order IF(R1a) > IF(R1d) > IF(R1b) > IF(R1c) for glyphosate. The larger the IF, the shorter is the width of the MEP. This is noticeable on the MEP presented in Fig. 7, where the narrowest and the largest potential barriers are along the (R1a) and (R1d), respectively. This behaviour is also observed for glyphosate hydrates. The relative energies of TSs are the energy barrier height (EBH) of the reaction path, which follows the order (R1a) > (R1d) > (R1b) > (R1c) for glyphosate. For glyphosate hydrates, one can read (R1a) > (R1b) > (R1d) > (R1c) for glyphosate (H_2O), (R1a) > (R1d) > (R1b) > (R1c) for glyphosate $(H_2O)_2$ and glyphosate $(H_2O)_3$. Thus, this order for glyphosate with water dimer and water trimer agrees with the one established for glyphosate in gas phase. Upon addition of water molecule, the EBH decreases in the reaction path (R1a), while the other paths show a disharmony. The EBHs of all the paths are further lowered or broken by water continuum. The continuum was assessed using the solvation model based on the quantum mechanical charge density of a solute molecule interacting with a continuum (SMD). In fact, the calculations show that the mechanism of H-abstractions from glyphosate in water continuum are spontaneous since the reaction complexes (RCs) are unstable. Once a RC is formed, the reaction moves to the product complex (PC).

Figure 7: **Minimum energy potentials of the reaction of •OH towards gas phase glyphosate. With the values of electronic energies at reaction complex (RC), transition state (TS), and product complex (PC), these potentials were interpolated by the Eckart unsymmetrical function. The barrier height from the reaction complex of each reaction path are presented in parenthesis (at the bottom of well).**



The calculated values of rate constant (298 K) are reported in Table 5 along with the branching ratio of each pathway. The internal rotation modes of glyphosate and its hydrates were used in place of vibrational modes using the hindered rotor approximation.

Table 5: **Rate constant (cm^3 per molecule per s) along with branching ratio (%) for each reaction path of glyphosate and its hydrates at 298 K from the processes of H-atom abstraction by •OH radicals. The total rate constant is given by $k_{OH} = k^a_{OH} + 2k^b_{OH} + k^c_{OH} + 2k^d_{OH}$**

Reaction	(R1a)	(R1b)	(R1c)	(R1d)	Total
Rate constant	k^a_{OH}	k^b_{OH}	k^c_{OH}	k^d_{OH}	k_{OH}
GPS	1.14×10^{-10}	1.74×10^{-12}	9.07×10^{-13}	8.83×10^{-14}	1.19×10^{-10}
GPS(H_2O)	7.56×10^{-12}	1.85×10^{-14}	2.96×10^{-11}	9.22×10^{-12}	5.56×10^{-11}
GPS(H_2O)$_2$	2.27×10^{-10}	4.09×10^{-15}	7.79×10^{-14}	6.76×10^{-11}	3.62×10^{-10}
GPS(H_2O)$_3$	5.20×10^{-13}	8.91×10^{-15}	5.10×10^{-14}	1.33×10^{-13}	8.56×10^{-13}
GPS(H_2O)$_\infty$					
Branching ratio	k^a_{OH}/k_{OH}	k^b_{OH}/k_{OH}	k^c_{OH}/k_{OH}	k^d_{OH}/k_{OH}	
GPS	96.15	1.47	0.76	0.08	—
GPS(H_2O)	13.60	0.033	53.20	16.60	—
GPS(H_2O)$_2$	62.60	0.001	0.022	18.70	—
GPS(H_2O)$_3$	60.80	1.04	5.96	15.60	—

The results show that, the dominant reaction is (R1a) with the highest value of branching ratio. The exception is seen for the hydrate glyphosate (H_2O), where the highest branching ratio appeared at channel (R1c). The discrepancy with the barrier height comes from the narrow and large widths. (R1a) is kinetically more favourable for its narrow barrier (small width). The available experimental result is $k_{OH} = (3.1 \pm 0.08) \times 10^{-13}$ cm^3 per molecules per s^{-1} obtained in water continuum. This result is affected by the complexation with iron. Nevertheless, the findings with the maximum size of water cluster (n = 3) fit the experimental results at 78.71%. The remaining percentage can be corrected by supplementing explicit water molecules to the hydrate glyphosate $(H_2O)_3$.

The fit of the total rate constant over the temperature range 200–400 K was completed for glyphosate and its hydrates (Fig. 8). The fit yielded the non-Arrhenius equation of the form $k^{env}_{OH}(T) = \exp(c + bT^{-1} + aT^{-2})$, where a, b and c are real constants of the fit, and T the temperature in K. The superscript 'env' indicates the environment of glyphosate, which can be gaseous state or explicit water. The equations of the rate constant as a function of temperature are given as per eqn (8)–(11).

$$k_{OH}^{gas}(T) = \exp\left(-2.263 - \frac{7268.6}{T} + \frac{338.63 \times 10^3}{T^2} \right) \quad (8)$$

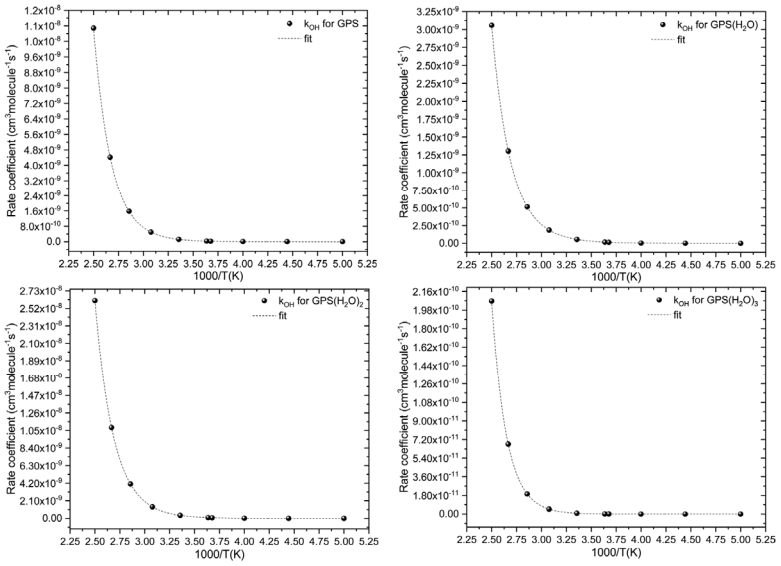
$$k_{OH}^{H_2O}(T) = \exp\left(-2.542 - \frac{8428.99}{T} + \frac{641.46 \times 10^3}{T^2} \right) \quad (9)$$

$$k_{OH}^{(H_2O)_2}(T) = \exp\left(-1.625 - \frac{7334.33}{T} + \frac{400.07 \times 10^3}{T^2} \right) \quad (10)$$

$$k_{OH}^{(H_2O)_3}(T) = \exp\left(-2.563 - \frac{9003.8}{T} + \frac{443.53 \times 10^3}{T^2} \right) \quad (11)$$

These equations were obtained with a coefficient of determination equal to unity.

Figure 8: **Temperature-dependent rate constant of the reaction between glyphosate (and its hydrates) with the •OH radical over the temperature range 200–400 K.**



Atmospheric lifetime

With the rate constants of the reactions of •OH radical towards glyphosate and its hydrates, the atmospheric lifetime τ_{OH} of glyphosate and its hydrates were estimated as the inverse of the product of rate constant and the concentration of •OH radical in the troposphere. This is expressed as per eqn (12).

$$\tau_{OH} = \frac{1}{k_{OH}[\bullet OH]} \quad (12)$$

where k_{OH} represents the rate constant of the reaction of •OH radical with glyphosate and its hydrates. The quantity [•OH] = 1.0×10^6 molecule cm^{-3} is the global average atmospheric concentration of the •OH radicals. In fact, other oxidants as O_3, $•NO_{x=1–3}$ radicals, and Cl atom also contribute to the degradation of the gas in the troposphere. However, they are less reactive compared to •OH radical, which is known as the 'atmospheric detergent'. This advises the use of •OH radical in the determination of atmospheric lifetime.

The results of atmospheric lifetime are reported in Table 6. They are of the order of hours and follow

the order glyphosate $(H_2O)_2$ < glyphosate < glyphosate (H_2O) < glyphosate $(H_2O)_3$. Thus, glyphosate and its hydrates are washed out from the troposphere once its release. However, the growth of the size of these hydrates may form aerosols and increase the atmospheric lifetime. However, glyphosate and its hydrates are not atmospherically well-mixed as they are short-live compounds. Therefore, they cannot be assigned to a unique atmospheric lifetime. The reported results represent the global atmospheric lifetime average. The local lifetime could significantly vary with the seasons, the physical and chemical conditions of atmosphere, and the location of emission.

Table 6: Atmospheric lifetime (hours), photochemical ozone creation potential (POCP), and acidification potential

	Atmospheric lifetime (hours)	POCP	Acidification potential
GPS	2.34	24.7	0.189
GPS(H_2O)	4.99	23.0	0.171
GPS$(H_2O)_2$	0.77	20.0	0.156
GPS$(H_2O)_3$	324.49(13.52 days)	32.7	0.144

GPS = glyphosate

Photochemical ozone creation potential

Photochemical ozone creation potential (POCP) has been suggested to quantify the ability of a gas to create ozone in the troposphere. In fact, the emitted gas reacts with the oxidants ($^{\bullet}OH$, $^{\bullet}NO_3$, Cl, etc.) in the troposphere, which yields the corresponding peroxy/alkoxy radicals. Further, these peroxy/alkoxy radicals react with $^{\bullet}NO$ to form $^{\bullet}NO_2$, which in turn regenerates $^{\bullet}NO$ along with the ozone molecule in the presence of light and oxygen. The procedure to estimate the POCP was recently improved and the new estimation procedure is written as per eqn (13).

$$\mathrm{POCP_X} = (A \times \gamma_s \times R \times S \times F) + P + R_{O_3} - Q) \qquad (13)$$

where the subscript X stands for the molecular system glyphosate$(H_2O)_{n=0-3}$. The quantities A, γ_s, R, and S are core parameters used for any compound, while F, P, R_{O_3} and Q are the parameters used for specific compound. These can take the default values of one (1) for F and zero (0) for P, R_{O_3} and Q. In North West European conditions, the parameter A = 100 is a multiplier. γ_s is the ozone formation index obtained from the structure of molecule X. it is expressed as per eqn (14).

$$\gamma_s = \frac{n_B}{6} \times \frac{28.05}{M_X} \qquad (14)$$

where n_B indicates the total number of reactive bonds (C–C, N–C, P–C, C–H, N–H, and O–H) in the molecule, M_X denotes the molecular weight of molecule X. R is a reactivity element that is related to the $^{\bullet}OH$ reactivity of X. It takes the following from $R = 1 - (B\gamma_R + 1)^{-1}$. The parameters $B = 4.0$ under North West European conditions and γ_R is the ozone formation index obtained from the reactivity of molecule X. It is expressed as follows.

$$\gamma_R = \frac{6}{n_B} \times \frac{k_{OH}^X}{k_{OH}^{ethene}} \qquad (15)$$

k^X_{OH} is the rate constant for the reaction of molecule X with $^{\bullet}OH$ radical at 298 K and 1 atm of air, and k^{ethene}_{OH} is the rate constant for the reaction of ethene with $^{\bullet}OH$ radical at 298 K and 1 atm of air. k^{ethene}_{OH} was found to be 8.64×10^{-12} cm^3 per molecule per s. The parameter S is related to the size of the molecule X and takes the form $S = (1-\alpha) \times \exp(-C^* n_c^{\beta}) + \alpha$, where n_c is the number of carbon of the gas molecule X. under North West European conditions, the values of α, C, and β are 0.56, 0.0038, and 2.7, respectively. The default values were assigned to the other parameters. As such, for glyphosate and its

hydrates, the difference between their POCP is seen from their structure and reactivity indexes.
The results of the estimated POCPs are reported in Table 6. It comes out that, the gaseous glyphosate and its hydrates are not efficient ozone producer, as their POCPs values are low. It is 24.7 for glyphosate and decreases with the water monomer (23.0) and water dimer (20.0). The POCP of water trimer is 32.7 larger than the other ones including gaseous glyphosate. It lies on the range of the ozone formation potential of alkane.

Acidification potential
Acid rain is one of the environmental concerns today. It impacts negatively on plants and animals on the earth's surface. When the substances containing the atoms N, F, Cl and S, get emitted into the atmosphere, they form the acid species such as HNO_3, HF, HCl, and H_2SO_4 which contributes naturally to the acid rain. In accordance with the above, the acidification potential (AP) is the number of acid equivalent potentials (H^+) per unit mass of a given compound X with respect to the number of H^+ per unit mass of the reference compound SO_2. It is thus a parameter to measure the ability of an emitted compound to contribute towards acid rain with respect to SO_2 in the local environment. It is expressed as per eqn (16).

$$\mathrm{AP_X} = \frac{M_{\mathrm{SO_2}}}{M_{\mathrm{X}}} \times \frac{1}{2}(n_{\mathrm{Cl}} + n_{\mathrm{F}} + n_{\mathrm{N}} + 2n_{\mathrm{S}}) \qquad (16)$$

where M_{SO_2} and M_X represent the molecular weights of SO_2 and molecule X = (glyphosate$(H_2O)_{n=0-3}$), respectively. The quantities n_{Cl}, n_F, n_N, and n_s are the number of Cl, F, N, and S atoms, respectively, in the molecular system X. The estimated results are compiled in Table 6. It turns out that, the AP of glyphosate is 0.189 and decreases upon addition of water molecules. This shows negligible contribution to rain acidification as the AP is less than that of SO_2.

Conclusion
The rate constant was fitted and expressed in non-Arrhenius model equation $k_{OH}^{env}(T) = \exp(c + bT^{-1} + aT^{-2})$ over the temperature range 200–400 K, where a, b, and c are real constants. The atmospheric lifetime was estimated from the rate constant along with the photochemical ozone creation potential (POCP). It turns out that, glyphosate and its hydrates are short-live compounds as their atmospheric lifetime are in the order of hours. glyphosate and its hydrates are inefficient ozone producers. The acidification potentials of glyphosate and its hydrates show negligible impact in the acidification of rain.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The aim of the study was the estimation of the atmospheric half-life of glyphosate considering reactions with OH-radical or Cl-atoms. The calculations yielded an atmospheric lifetime of glyphosate of 2.34 hours. The EU agreed method to determine the half-life of an active substance is the Atkinson approach. The calculation in the publication cannot be considered a common method and the endpoint should not supersede the endpoint calculated using the Atkinson method.
The study is considered reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.5
Report author	Geissen, V. *et al.*
Report year	2021
Report title	Cocktails of pesticide residues in conventional and organic farming systems in Europe - Legacy of the past and turning point for the future
Document No	Environmental Pollution (2021):278, 116827
Guidelines followed in study	SANTE/11813/2017
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Relevant (Category A acc. to EFSA GD, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, 340 EU agricultural topsoil samples were analysed for multiple pesticide residues (glyphosate and AMPA were among them). These samples originated from 4 representative EU case study sites, which covered 3 countries and four of the main EU crops: vegetable and orange production in Spain (abbreviated as S-V and S-O, respectively), grape production in Portugal (abbreviated as P-G), and potato production in the Netherlands (abbreviated as N-P). Soil samples were collected between 2015 and 2018 after harvest or before the start of the growing season, depending on the site.

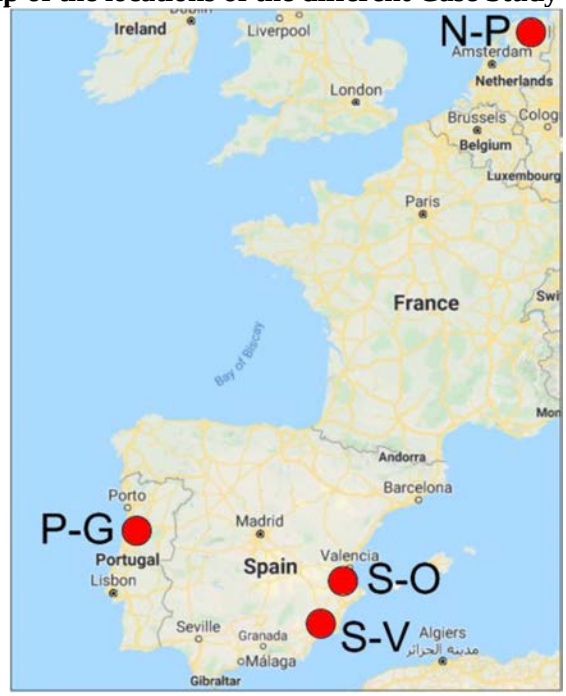
For glyphosate, the maximum amounts detected in conventional fields were 7.843, 1.3 and 0.18 mg/kg in grapes in Portugal, potatoes in the Netherlands and oranges in Spain, respectively. For AMPA, the maximum amounts detected in conventional fields were 4.294, 0.528 and 1.626 mg/kg in grapes in Portugal, potatoes in the Netherlands and oranges in Spain, respectively. In organic fields, glyphosate was detected only at S-O-O with a maximum amount of 0.105 mg/kg while AMPA was detected with maximum 0.014 and 0.593 mg/kg in N-P-O and S-O-O, respectively.

Materials and methods

Case study sites overview

For this study, data was compiled from 4 Case Study Sites (Figure 1) from 3 EC funded projects addressing soil quality (RECARE, iSQAPER and DIVERFARMING). In all three projects, pesticide application patterns and distribution of pesticide residues in agricultural soils were studied at a study site level. These case study sites represented typical cropping systems and covered different climate zones: vegetable production under plastic mulch in Southeast-Spain (S-V; Glyphosate was not determined in this site, therefore, no information or results of this site is summarised), orange production in Eastern Spain (S-O), grape production in Northern Portugal (P-G), and potato production in Northern Netherlands (N-P). The study site included both organic and conventional production systems, except for P-G (organic grape farms were not common in the sampled area of Portugal). The organic fields were converted more than 5 years ago (S-O) or more than 10 years ago (N-P). The conventional farms were managed as such for at least the last 10 years. Overall, 340 topsoil samples were collected and analysed (0-10/15 cm depth). Soil samples were collected between 2015 and 2018 at the end of the growing season (S-O, P-G) or before the growing season (N-P). Sampling at the end of the growing season aimed to assess the accumulative soil contamination scenario after the application period (expected to be the worst-case scenario) while sampling before the growing season tested the background situation. The characteristics of the study sites and the sampling pattern for each site are presented in Table 1. The soil samples were air-dried (at ambient temperatures, under dark conditions, and for a maximum of 1 week), sieved to 2 mm and frozen (-20°C) until the extraction and determination of pesticide residues could be carried out.

Figure 1: **Map of the locations of the different Case Study Sites**



S-V = vegetables production in Southeast-Spain (no glyphosate determination), S-O = orange production in Eastern Spain, P-G = grape production in Northern Portugal, and N-P = potato production in Northern Netherlands.

Table 1: **Characteristics of the case study sites and respective sampling details**

CSS code	Location	Crop	Climate	No. harvests (year period)	Soil type; Texture
P–G (P–G–C)	Bairrada, N-Portugal	Grapes	Temperate Mediterranean	1 (October)	Cambisols and Luvisols; clay, sandy loam, sandy, clay loam
N–P (N–P–C, N–P–O)	Groningen, N-Netherlands	Potatoes (in rotation with cereals)	Atlantic	1 (September)	Cambisols; sandy loam, clay
S–O (S–O–C, S–O–O)	Valencia, E-Spain	Oranges	Hot Mediterranean	1 (December/ January)	Cambisols; Sandy loam

CSS code	Location	Organic matter (mean ± SDev)	pH in H_2O (mean ± SDev)	Timing of soil sampling	No. of fields sampled	No. of samples per field	No. of samples per CSS
P–G (P–G–C)	Bairrada, N-Portugal	6.2 ± 1.8%	7.1 ± 1.3	October 2016	C: 9; O: 0	12	C: 108; O: 0
N–P (N–P–C, N–P–O)	Groningen, N-Netherlands	3.6 ± 0.9%	7.7 ± 0.4	April 2018	C: 9; O: 1	C: 3/4; O: 6	C: 28; O: 6
S–O (S–O–C, S–O–O)	Valencia, E-Spain	3.5 ± 1.5%	8.1 ± 0.2	February 2015	C:6; O: 6	C:9; O: 6	C: 54; O: 36

C = conventional, O = organic, SDev = standard deviation, No. = Number.

Analysis of pesticide residues in soil samples

The list of the pesticide residues tested in soil samples included 47 residues in P-G, 36 in N-P and 75 in S-O.

All soil samples were thawed and homogenised (hand mixed until a visual homogeneous sample was obtained) and split into two aliquots: one for the determination of basic soil properties (pH, organic matter and texture) and one (2 g) for determination of glyphosate and its main metabolite AMPA (in S-O, P-G and N-P). Since none of the parties interviewed for this study reported that glyphosate was applied in S-V, it was not analysed in those samples. Glyphosate and AMPA were determined using LC-MS/MS (liquid chromatography-tandem mass spectrometry; Instrument: Quattro Ultima from Micromass coupled to an Acquity UPLC system).

Analyses were performed according to the analytical quality control and method validation procedures for pesticides residues analysis in food and feed. The guidelines for the current version, at the time of analysis, of the SANTE document were applied. Analyses involved the use of calibration standards, reference standards and isotope labelled internal standards. The calibration standards were prepared from a mix solution that combined the reference standards of all the compounds that were going to be analysed. Isotope labelled internal standards were used for normalisation of response of glyphosate and AMPA. The reference standards were purchased from LGC Standards (Germany), HPC Standards (Germany) or Sigma-Aldrich (USA). The isotope labelled internal standards of glyphosate and AMPA were obtained from LGC Standards (Germany). Limits of quantification (LoQ) were used as reporting limits. The LoQ of glyphosate and AMPA was 0.050 mg/kg.

Data analysis - Residues in soil

The frequency of detection, the median and the range of concentrations for each compound from each organic and conventional farming system per study site was calculated. Furthermore, the range and the median number of pesticide residues found in organic and conventional soils for each site is presented. The content of the different pesticide residues found in each sample to obtain the total residues content per sample was added. Nonparametric Mann Whitney U tests were used to test significant differences in the number of residues and in the total residues content in soils between conventional and organic farms within the same site, and between sites within the same farming strategy. Statistical analyses were performed using STATISTICA, version 12. The significance level was set at 0.05.

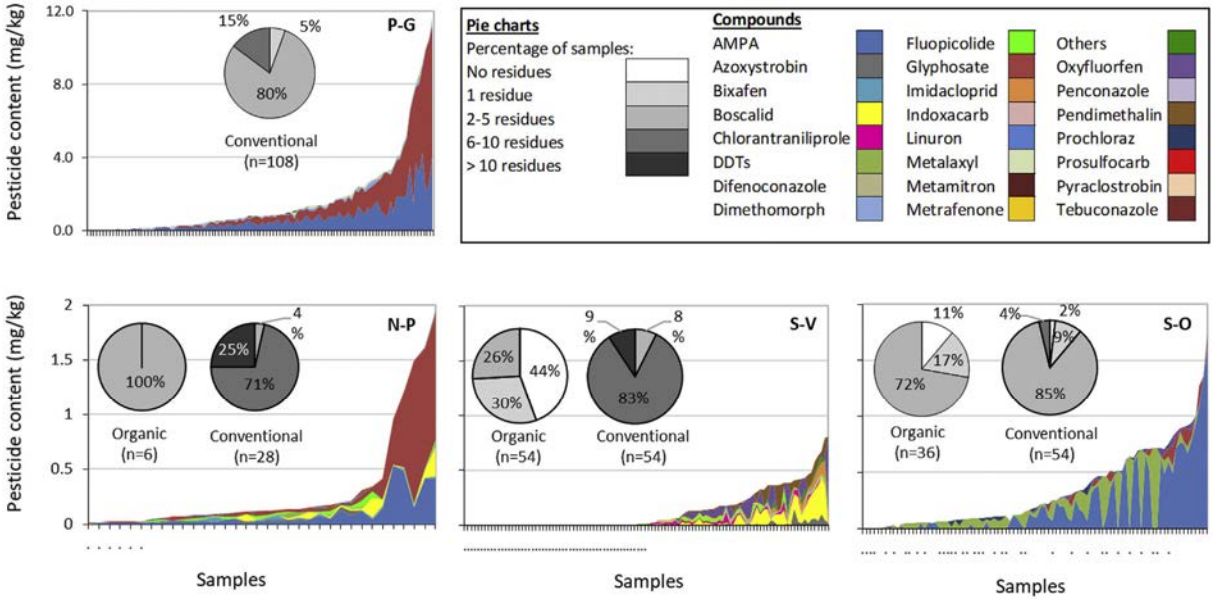
Results

Pesticide residues identified in the study sites - Frequency of detection

The only pesticide residue-free soils under conventional farming were identified in conventional orange farming in Spain (2% of all conventional soils; all other soils under conventional farming contained one or more pesticide residues. In conventional farming of grapes and oranges, more than 80% of the soils contained 2 to 5 residues, while most potatoe samples contained 6 to 10 different residues (71%). A substantial part of potato (25%) soil samples contained even more complex mixtures, with more than 10 residues. Soils from organic farms contained significantly fewer residues, with 11% of the soils in orange farming being free of tested pesticide residues. However, 100% of organic potato soils and 72% of organic orange soils contained mixtures of two to five residues. In total, 15 residues were detected with a frequency above 50% in one or more of the study sites and 7 residues with a frequency between 20 and 50% in one or more of the sites (Figure 2).

The detection frequency of glyphosate was 78% in P-G-C, 93% in N-P-C, 0% in N-P-O, 22% in S-O-C and 6% in S-O-O (Table 2).

Figure 2: **Number of pesticide residues and total pesticide content found in the organic and conventionally managed soils from P-G, N-P, S-V, S-O**



The number of residues in soils were aggregated per classes, being the distribution of each class is presented in the pie-charts. The total pesticide content is presented as a cumulative curve, being the topsoil samples from each site organized by increasing total pesticide content. The. below the x axis indicates soils under organic management, the remaining values are related to conventionally managed fields. Each colour under the curves represent a different pesticide residue.

Table 2: **Frequency, maximum value and median value of glyphosate and AMPA in the 3 tested study sites (in mg/kg)**

		P-G (conv)				N-P (conv/org)				S-O (conv/org)			
Compound	Persistence	% freq	Q1	Max	Median	% freq	Q1	Max	Median	% freq	Q1	Max	Median
AMPA (M-H)	VP	83	0.260	4.294	0.505	96/ 83	0.026/ 0.012	0.528/ 0.014	0.037/ 0.013	87/ 17	0.102/ 0.069	1.626/ 0.593	0.261/ 0.070
Glyphosate (H)	P	78	0.179	7.843	0.452	93/ 0	0.018/ <LoQ	1.300/ <LoQ	0.029/ <LoQ	22/ 6	0.070/0.076	0.180/ 0.105	0.090/ 0.090

known to be applied and detected

H = herbicide, M = metabolite; VP = very persistent, P = persistent. LoQ - Limit of quantification. Q1 - The first quartile concentration, i.e. 25% of the values above LoQ. orange production in Eastern Spain (S-O), grape production in Northern Portugal (P-G), and potato production in NorthernNetherlands (N-P), conv = conventional, org = organic. No reporting on glyphosate content in vegetable production in Spain (S-V).

Pesticide residues identified in the study sites - Pesticide residues content in the study sites

The highest pesticide content was found in P-G-C, with a total residue content of nearly 12 mg/kg, a value approximately 6 times higher than the maximum content in N-P-C and S-O-C fields (with a maximum content of 2 and 1.7 mg/kg, respectively; Figure 2). The residue content under organic farming in N-P did not exceed 0.2 mg/kg, i.e. 10% of the maximum content in the respective conventional fields. In S-O-O, the maximum residue content was 0.6 mg/kg, which was about 30% of the maximum residue content of the conventional fields. AMPA and glyphosate contributed among other the most to the total residue content of P-G, N-P and S-O (in S-O only AMPA contributed to the total residue content). In organically managed fields, AMPA and DDT metabolites had the highest contributions, and specially for S-O; in the other organic farming systems, the total content was low.

For glyphosate, the maximum amounts detected in conventional fields were 7.843, 1.3 and 0.18 mg/kg in P-G-C, N-P-C and S-O-C, respectively. For AMPA, the maximum amounts detected in conventional fields were 4.294, 0.528 and 1.626 mg/kg in P-G-C, N-P-C and S-O-C, respectively. In organic fields, glyphosate was detected only at S-O-O with a maximum amount of 0.105 mg/kg while AMPA was detected with maximum 0.014 and 0.593 mg/kg in N-P-O and S-O-O, respectively.

Conclusion

Mixtures of pesticide residues were present in all case study sites under conventional farming, both in samples taken at the start of the crops season, and samples taken post-harvest.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study several compounds, including glyphosate and AMPA, were analysed in 340 agricultural topsoil samples from 4 representative EU study sites in Spain, Portugal and the Netherlands. Soil samples were collected between 2015 and 2018 after harvest or before the start of the growing season.

No information on the sampling procedure and storage time of the topsoil samples is provided. This does not allow to assess the representativeness of the soil samples. Furthermore, only maximum and median values are reported and the results cannot be assigned to a respective sampling period. Therefore, the article is considered reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.5/
Report author	Piel S. *et al.*
Report year	2021
Report title	Understanding the origins of herbicides metabolites in an agricultural watershed through their spatial and seasonal variations
Document No	Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes, Part B (2021), Vol. 56(4), pp. 313-332
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Relevant (Category A acc. to EFSA GD, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of this study is to understand the spatial and seasonal variations of persistent herbicides metabolites and to determine their origins in the Vilaine River watershed, Britany-France. Data were collected at 13 sampling stations during five sampling campaigns in 2016 and 2017. The influence of land use and wastewater treatment plants (WWTP) on streams water quality has been identified. Aminomethylphosphonic acid (AMPA) is associated to anthropogenic urban contamination and nutrient loads. AMPA has its major sources in both glyphosate and phosphonate detergents issued from WWTP.

Among the studied compounds, glyphosate was observed at a detection frequency of 8 to 23% with concentration levels between 0.09 and 0.56 µg/L. AMPA was found with a detection frequency of 100% and reached its maximum concentration (2.48 µg/L) during summer downstream of the WWTP of the major urban area.

Materials and methods

River water sampling locations

The main River Vilaine crosses one major urban area (Rennes, 300,000 inhabitants) on the upstream part of the watershed. Redon city (10,000 inhabitants) in the south of the watershed is the only other major city crossed by the Vilaine River. At the mouth of this river is located a major drinking water treatment plant (DWTP), producing about 100k m^3/day of drinking water for approximately 1,000,000 inhabitants. The water treatment process is composed of a first step of sedimentation with ferric salts followed by sand filtration, a second step of interozonation followed by granular activated carbon filtration and a last step of disinfection with chlorine.

Vilaine River has four main tributaries: Oust, Meu, Seiche, and Isac (Figure 1). Meu and Seiche Rivers are located downstream of Rennes urban area, in the upper part of the watershed. Oust River joins the Vilaine River at Redon, south of the watershed. Oust river is known to be agriculturally influenced. Finally, Isac River is the last tributary to join the main river 25 km before the DWTP.

Thirteen sampling stations were chosen to describe influences based on previous work (mostly agricultural), land use and tributaries (Figure 1). Sampling stations were located on each major tributary before their confluence with the main river Vilaine (Meu (NW5), Seiche (NW7), Oust (NW10) and Isac (NW12)). Then, on the main river, stations upstream and downstream of river confluences were sampled (NW4, NW6, NW8, NW9, NW11 and NW13). NW0 is the “reference

station" corresponding to the source of Vilaine River. NW1 and NW4 are respectively located upstream and downstream of Rennes in order to measure urban impact on water quality.

NW4, corresponding to Rennes, shows 116.7 km^2 of urban land cover and 577.2 km^2 of dedicated agricultural land. NW10 corresponds to a bigger agricultural sub-watershed than NW4, with 185.6 km^2 of urban facilities and 3867.7 km^2 of dedicated agricultural land. NW1 station located upstream of Rennes shows a similar agricultural land use to NW4. The two main tributaries of the upper watershed, the Meu (NW5) and the Seiche (NW7) Rivers, show similar land use with small urban areas and agricultural area similar to NW4. NW6 and NW8 are the stations measuring the influence of Meu and Seiche tributaries on the River Vilaine water quality. NW9 station follows a long river stretch without major tributaries. Inhabitant-equivalent (IE) density is smaller than in the upper part of the watershed (Table 1). Consequently, this station shows a mix of agricultural (NW9) and urban (waters from upstream, NW4 to NW8) influences, depending on seasons and rainfalls.

DWTPi is located at the extreme downstream of the watershed; close to the inlet of the DWTP (Figure 1). An additional sampling point, the treated water of the DWTP, has been chosen to assess the treatment process efficiency for the removal of herbicides and metabolites of interest. Two major WWTP are located in this watershed. The largest - Beaurade (360,000 IE) is located just downstream of Rennes and upstream of sampling station NW 4. Beaurade WWTP is composed of low charge activated sludge and clarification processes. The second main WWTP is in Loudeac (175,000 IE) – in the upstream part of the Oust watershed and upstream of sampling station NW10. This WWTP receives only industrial effluents from slaughterhouses and laundry (effluents are more concentrated, for instance, in organic matter, grease, phosphorus) and consequently its influence on the Vilaine water quality is different from Beaurade WWTP which receives urban wastewater only. Loudeac WWTP is composed of low charge activated sludge, clarification and physicochemical dephosphatation processes.

Railways are concentrated in the upstream of Vilaine watershed and especially in NW4 drainage area (440.4 km). Moreover, railways are closely following Vilaine River course on the major part of the watershed. Railway lines in urban areas can be one of the major sources of glyphosate, used as weed controller.

Figure 1: Simplified Vilaine watershed map, with the main rivers, sampling stations, weather stations, and flow measurement stations.

Table 1: Land use characteristics of the sampling points sub-watersheds (*Inhabitant-Equivalent)

		Facilities			Urban	Agricultural					
Sampling station	Total area (km^2)	Total IE* (thousands of IE)	IE* density (IE/km^2)	Railways (km)	Area (km^2)	Cereals (km^2)	Including maize (km^2)	Oleaginous (km^2)	Proteaginous (km^2)	Pasture/ Meadow (km^2)	Other vegetables (km^2)
NW0 - Vilaine source	**93**	1	11	0,0	2,0	39,7	29,9	0,8	–	40,2	–
NW1 - Vilaine upstream Rennes	**698**	109	156	234	73,8	287,3	154,1	11,0	1,1	239,2	0,2
NW4 – Vilaine downstream Rennes	**784**	540	689	440	185,6	236,2	120,2	12,3	1,2	170,9	1,5
NW5 - Meu	**744**	122	164	123	41,2	311,2	149,1	14,3	1,7	163,4	1,3
NW6 - Vilaine downstream confluence Meu	**22**	11	500	0	2,3	5,4	2,0	0,6	–	3,9	–
NW7 - Seiche	**801**	110	137	217	53,9	356,1	156,4	28,8	2,7	215,2	0,8
NW8 - Vilaine donwstream confluence Seiche	**168**	16	95	23	12,4	70,1	26,1	6,8	0,4	51,3	0,3
NW9 - Vilaine upstream confluence Oust	**2173**	97	45	260	88,4	767,9	297,4	63,7	11,1	677,7	4,4
NW10 - Oust	**3554**	473	183	196	116,7	1309,8	453,2	75,4	10,3	785,8	43,2
NW11 - Vilaine downstream confluence Oust	**81**	1	12	27	26,2	19,9	8,7	1,4	0,3	31,4	0,0
NW12 - Isac	**632**	61	96	17	38,2	158,7	73,9	10,4	2,9	282,9	0,3
NW13 - Vilaine downstream Isac confluence	-	–	–	–	–	–	–	–	–	–	–
DWTPi - Férel	**388**	57	147	17	26,4	105,4	55,8	2,8	0,4	118,1	1,9

Hydro climatic conditions of sampling campaigns

In order to characterise hydroclimatic conditions of each sampling campaign, flows and meteorological data have been collected. River daily average flow measures at a limnimetric station are provided by the database Hydro (French Ministry for Ecology, Sustainable Development and Energy). The height flow measurement stations location is provided in Figure 1. Where limnimetric station does not exist, river flows have been calculated by the difference of measured flows at the upstream station and downstream station. The sampling stations with a calculated flow are NW4 (difference between NW1 and NW5 flows), NW6 (difference between NW4 and NW5 flows) and NW9 (difference between NW11 and NW10 flows).

The four weather stations are Ernee, close to the Vilaine source (NW0); Rennes-Saint Jacques, located in Rennes agglomeration (NW4); Saint Jacut-les-pins, located at the downstream of the Oust sub-watershed (NW10); and finally, Arzal, which is located few kilometres downstream of the DWTP (DWTPi).

Data for each sampling station are taken from the closest weather station (air temperature, rainfall, insolation duration and solar radiation). Those data are imported from the database “Publitèque”, powered by the French national service of meteorology (Meteo France). Acquisition frequency is one measure per hour.

Five sampling campaigns were conducted between 2016 and 2017 on the Vilaine river and its tributaries at 13 sampling stations (Figure 1), three campaigns during rain periods (C3 January 17, C4 May 17, C5 November 17) and two during warm and dry periods (C1 July 16 and C2 September 16). A sampling campaign is considered as rainy when conducted for a minimum rainfall of 10 mm/24h before sampling (according to the weather forecast).

Sampling campaigns C1 and C2 were characterised by low flow ($Q < 5$ m^3/s) and by low rainfalls (respectively < 1.8 and < 6.2 mm the week before sampling). Sampling campaigns C3 and C5 were characterised by high flow (respectively 16 and 11 m^3/s) and high rainfalls (respectively between 8.9 and 30.7 and between 12.9 and 32.9 mm the week before sampling). C3 and C5 corresponded to minimal period of pesticides application, and minimal degradation of pesticides in soils. Sampling campaign C4 was characterised by high flow (13 m^3/s) and significant rainfall (between 16.5 and 43.8 mm the week before sampling), C4 was conducted following a significant rainfall (22.8 mm 24 h before sampling) during the principal period of application of glyphosate. Details of the hydroclimatic parameters and the physiochemical characteristics are shown in Table 2 and Table 3.

Table 2: Hydroclimatic parameters (minimum, maximum, median) for the 5 sampling campaigns (C1 to C5).

Sampling campaigns	C1 (n = 13) July 16			C2 (n = 13) September 16			C3 (n = 13) January 17			C4 (n = 12) May 17			C5 (n = 13) November 17		
Parameters	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median
Rainfall (mm)															
Maximum daily, the week before the sampling day - RR (Vmax-1w)	0.2	1.6	0.4	0.4	2.2	1.3	7.1	12.3	10.7	7.9	22.8	16.7	5.7	13.9	7.9
Cumulative 1 week before sampling day - ∑RR (-1w)	0.0	1.8	0.3	1.0	6.2	2.9	8.9	30.7	25.7	16.5	43.8	32.8	12.9	32.9	20.2
Cumulative 2 week before sampling day - ∑RR (-2w)	0.3	7.9	4.2	15.5	44.0	27.8	12.7	37.2	29.5	17.9	45.0	34.2	14.1	34.3	21.2
Cumulative 4 week before sampling day - ∑RR (-4w)	6.9	14.2	9.6	28.1	47.4	35.9	36.2	53.7	45.9	18.7	51.2	39.8	34.1	82.5	49.1
Air temperature (°C)															
Average of the sampling day - TM(d)	16.0	18.0	16.9	15.6	17.7	17.5	0.0	1.3	1.1	11.4	12.4	12.0	3.1	6.4	4.9
Insolation duration															
The day of sampling - INST(d) (hour)	1.7	3.9	3.8	*NA*	*NA*	*NA*	7.8	8.3	8.3	4.9	9.1	5.2	4.3	5.0	4.6
Cumulative 1 week before sampling day - ∑INST(-1w) (days)	2.5	2.7	2.6	1.6	1.8	1.7	0.8	0.8	0.8	1.3	1.3	1.3	0.8	1.0	0.9
Cumulative 2 weeks before sampling day - ∑INST(-2w) (days)	5.7	5.8	5.8	2.3	2.4	2.4	1.0	1.4	1.1	4.3	4.8	4.8	1.6	1.6	1.6
Solar radiation (kilo joules/cm^2)															
Cumulative 1 week before sampling day - ∑GLOT(-1w)	17.2	17.3	17.2	9.2	9.9	9.8	2.5	2.7	2.5	9.8	10.1	9.8	3.1	3.3	3.2
Cumulative 2 weeks before sampling day - ∑GLOT(-2w)	34.5	34.7	34.7	15.7	16.9	16.8	4.3	4.8	4.4	26.1	26.1	26.1	6.4	6.4	6.4

Table 3: Physiochemical characteristics of the Vilaine River for 5 strategic sampling stations.

Sampling stations	NW1				NW4			NW9			NW10			DWTPi		Quality limits*
Parameters	Min.	Median	Max	Min.	Median	Max	Min.	Median	Max	Min.	Median	Max	Min.	Median	Max	
pH	7.6	**7.9**	8.3	7.6	**7.8**	8.4	7.5	**8.2**	8.8	7.3	**8.2**	8.6	7.8	**7.8**	8.4	**5.5 – 9**
Temperature of water - Tw (°C)	5.7	**11.2**	21.0	6.2	**11.8**	22.4	4.7	**13.6**	24.5	5.0	**13.3**	23.6	6.1	**15.1**	24.7	**25**
Dissolved oxygen - O_2 (%)	78	**90**	100	83	**89**	95	77	**86**	104	74	**90**	93	69	**90**	101	**> 30**
Conductivity - Cond (µS/cm) (25 °C)	361	**411**	448	322	**382**	517	394	**404**	478	250	**328**	393	298	**333**	498	**1 100**
Turbidity - Turb (NFU)	4.4	**5.8**	21.2	6.1	**7.8**	45.6	3.0	**6.2**	10.0	2.6	**3.1**	3.7	0.2	**2.8**	9.4	-
Fluorescence of dissolved organic matter - fDOM (RFU)	28.1	**29.3**	31.6	27.3	**41.0**	57.3	29.3	**34.0**	44.1	15.8	**24.9**	26.8	0.0	**27.2**	32.0	-
Dissolved organic carbon - DOC (C mg/L)	6.0	**6.5**	7.2	5.2	**6.2**	6.6	5.3	**5.9**	6.3	3.3	**5.0**	5.3	4.3	**5.7**	7.1	-
Total organic carbon - TOC (C mg/L)	6.4	**7.3**	8.7	6.2	**7.0**	7.2	5.8	**6.1**	6.5	3.6	**5.2**	5.6	4.4	**5.8**	7.6	**10**
Suspended matter - SM (mg/L)	8	**12**	34	8	**9**	36	6	**9**	12	5	**8**	16	2	**4**	9	-
Nitrate - NO_3 (NO_3 mg/L)	3.4	**10.0**	15.1	6.3	**8.1**	12.3	4.2	**9.3**	13.6	6.7	**16.8**	27.7	3.9	**12.0**	21.8	**50**
Ammonium - NH_4 (NH_4 mg/L)	0.02	**0.05**	0.32	0.15	**0.18**	0.37	0.03	**0.06**	0.09	0.01	**0.05**	0.10	0.04	**0.06**	0.08	**4.0**
Kjeldahl nitrogen KN (N mg/L)	0.8	**1.0**	1.6	0.9	**1.1**	1.3	0.7	**0.8**	1.1	0.5	**0.7**	1.4	0.5	**0.6**	1.0	-
Orthohosphate - PO_4 (PO_4 mg/L)	0.0	**0.1**	0.3	0.1	**0.3**	0.5	0.1	**0.3**	0.6	0.0	**0.1**	0.1	0.0	**0.1**	0.2	-
Total phosphorus - TP (P_2O_5 mg/L)	0.3	**0.3**	0.4	0.3	**0.4**	0.5	0.2	**0.3**	0.5	0.1	**0.2**	0.5	0.1	**0.1**	0.2	**0.7**

Sample collection

In each sampling station (NW), the samples were collected from a bridge using a stainless-steel bucket, according to the standardised method AFNOR FD T90-523-1. Before sampling, the bucket was rinsed 3 times with sampling water. The samples were carried out at a depth between 0.5 and 1 m in a homogeneous zone in the middle and upstream of the bridge. At the station DWTPi, raw water was sampled at the inlet of the drinking water treatment plant (DWTP). Temperature, pH, turbidity, conductivity (at 25°C), dissolved oxygen (% O_2), fluorescence of dissolved organic matter (fDOM) (excitation 365 nm – emission 480 nm) were analysed in situ using an EXO2 multi-parameters probe. Specific flasks were used according to laboratory analysis procedures. Samples were maintained at 5°C ± 3°C during transports, in coolers filled with ice blocks.

Analysis of herbicide compounds

Polypropylene flasks with sodium thiosulfate were used to store samples for glyphosate and AMPA analyses. For glyphosate and AMPA quantification, analyses were performed by Liquid Chromatography (LC)-Fluorescence after derivatisation with 9-fluorenylmethoxycarbonyl-chloride (FMOC-Cl) according to an internal method. This method has been developed in accordance with the

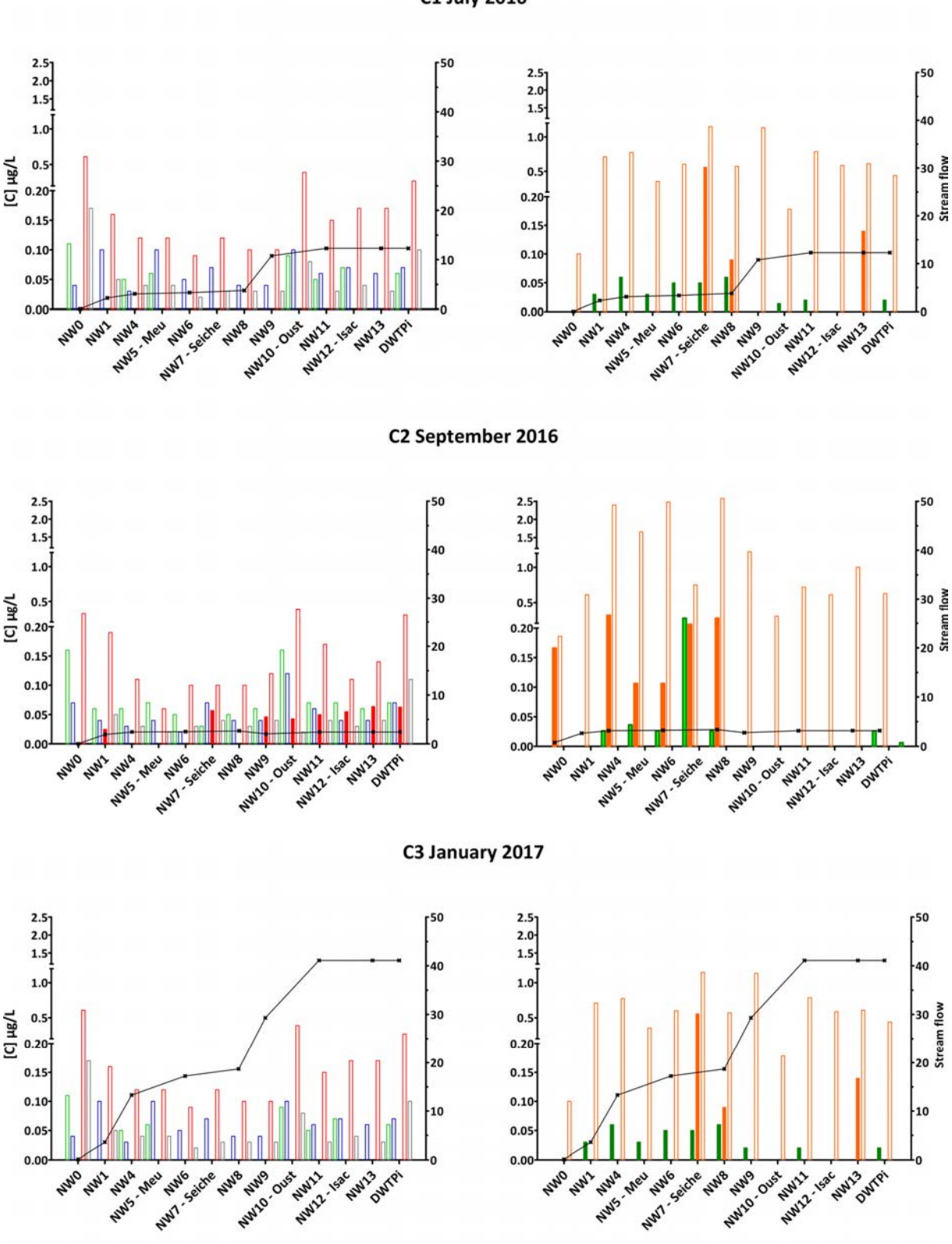
international standard ISO/IEC 17025:2005 and has been accredited by the French national accreditation committee (Cofrac). Analyses were performed using a LC system (Agilent 1200 Infinity) coupled to fluorescence detector (1200 G1321A). The limit of quantification (noted LoQ) is 0.05 µg/L for glyphosate and AMPA.
Chromatographic separation was performed at a flow rate of 0.4 mL/min on a X Bridge TM BEH C18 column (100 mm length × 2.1 mm I.D., 2.5 µm particle size) maintained at a constant temperature of 35°C. The binary mobile phase comprised ultra-pure water with 0.01% formic acid (solvent A) and CH_3CN with 0.01% formic acid (solvent B). The sample injection volume was 5 µL. Samples were dissolved in mobile phase at initial conditions (60% A) prior to injection. The gradient elution program was as follows: 0-2 min, 60% A; 2-13 min, 60-30% A; 13-15 min, 30-0% A; 15-27 min, 0-60% A (return to initial conditions); 27-28 min, 60% A (equilibration).

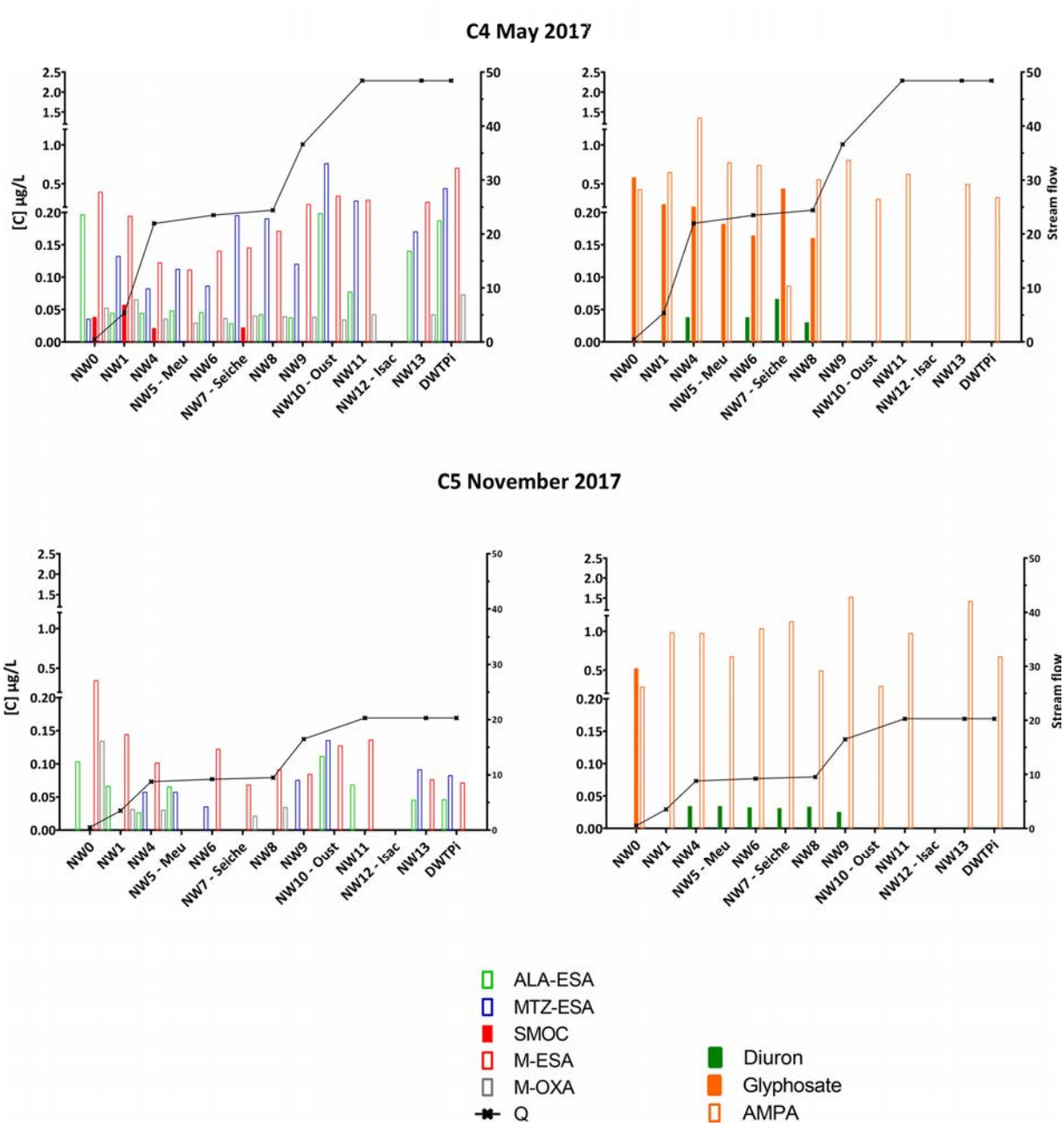
Risk assessment
A risk assessment was performed by first comparing the concentration levels with the environmental quality standards (EQS) in surface water for priority substances in the Directive 2013/39/EU. In France, AA-EQS and MAC-EQS have been established for AMPA (45 and 45,000 µg/L) and glyphosate (28 and 70 µg/L).
A second comparison could be performed with parametric values on the quality of water intended for human consumption. In Europe, the 98/83/EC European Council directive monitored the anthropogenic compounds and potential other pollutants in water. The directive fixed concentration limits in raw waters used to produce drinking water: 2 µg/L for any individual pesticide (including metabolites) and 5 µg/L for total pesticides concentration (including metabolites). In accordance with this directive, pesticides concentration in drinking water is limited to 0.1 µg/L for individual pesticides (included relevant metabolites) and to 0.5 µg/L for the sum of all detected and quantified individual pesticides and metabolites.

Results
Occurrence of herbicide compounds
Among the 15 compounds selected, glyphosate and AMPA have been frequently quantified (Frequency of Quantification (FoQ) > 30% of the samples). Figure 2 presents herbicides and their metabolites (glyphosate and AMPA) repartition in the watershed during the 5 campaigns.
Glyphosate was rarely observed in waters during cold months with 23% and 8% of FoQs during C3 and C5 campaigns, respectively. Concentration levels ranged from 0.09 to 0.56 µg/L.

Glyphosate was mainly found during spring and summer (C1, C2 and C4). During those seasons, it frequently occurred the same way that its metabolite AMPA in the upstream watershed (NW0 to NW8). Conversely, in the downstream watershed (NW9 to DWTPi), AMPA was found alone, indicating either that glyphosate had been already degraded in water (7 weeks < half-life < 10 weeks) or stored in the sediments. AMPA may also have a different source than glyphosate in this part of the watershed. AMPA can be derived from treated water of WWTPs representing 689,000 IE situated between NW9 and DWTPi.
AMPA was the compound with the highest concentrations (mean concentration 0.77 µg/L) and FoQ (100%). During C2 campaign (late summer - low flow), AMPA reached its maximum concentration of 2.48 µg/L (NW4). During this period WWTPs were the main contributors to the river flow, explaining the high AMPA concentrations in rivers.

Figure 2: **Herbicides and metabolites concentrations for each sampling campaign.**





Environmental and sanitary quality standards compliance in the Vilaine River watershed
Glyphosate and AMPA concentrations, up to 0.58 and 2.48 respectively, were systematically lower than their EQS (AA-EQS 28 and 45 µg/ L; MAC-EQS 70 and 45,000 µg/L).

The second comparison was performed with parametric values for raw waters intended for human consumption (2 µg/L quality limit for individual substance). Among the 15 studied herbicides and metabolites, only AMPA exceeded 2 µg/L during C2 campaign at NW4, NW6 and NW8 sampling stations (respectively 2.30, 2.38 and 2.48 µg/L). C2 campaign carried out during warm and dry period were characterized by low flow (Q < 5 m3/s) and low rainfalls (< 6.2 mm the week before sampling). The exceedance of threshold values during this campaign could be explained by the impact of WWTP whose discharges mainly contribute to the river flow and quality.

Concerning the drinking water sampled at the DWTP, AMPA was quantified with a concentration of 0.16 µg/L. The literature suggests that AMPA can be removed by clarification and sand filtration. AMPA is adsorbed on the flocs created by iron or aluminum salts and thus will settle down and be filtered with suspended particles. Ozonation and chlorination can also participate to the removal of AMPA depending on operation conditions, including dose, contact time and pH.

Spearman correlation
Two correlation matrices were built with Spearman correlation rank method (alpha = 0.05) on 64 samples (surface waters) of the 5 described campaigns. The first one was applied to herbicides and metabolites concentrations dataset and the second, on herbicides and metabolites concentrations and physicochemical water quality parameters datasets.

Diuron and AMPA were positively correlated together. The herbicide glyphosate and its metabolite (AMPA) showed no significant relationship. Glyphosate, diuron and AMPA were correlated with phosphorus forms (PO4 and TP) and nitrogen forms (KN and NH4). AMPA was also positively correlated with conductivity and negatively correlated with NO3. These physicochemical parameters are related to urban land use. This first group is primarily of urban origin.

Principal component analysis
First dimension (PC1) accounted for 26.28% of total variance, showed high positive loadings of turbidity, KN, DOC and TOC, SM, TP and glyphosate. There was no high negative loading for this factor. PC1 can be related to anthropogenic (wastewater) pollution sources.

AMPA showed a different behaviour: it is opposed to the agricultural factor PC2 and is orthogonally projected to glyphosate. Consequently, other activities, like food processing, laundry and milk industries, may be considered as potential sources of AMPA in the watershed. Indeed, AMPA is known to be a by-product and a major impurity of phosphonate detergents used in these industries.

Conclusion
Among the studied compounds, glyphosate was rarely observed (detection frequency of 8 to 23%) with concentration levels between 0.09 and 0.56 µg/L. AMPA reached its maximum concentration (2.48 µg/L) during summer downstream of the WWTP of the major urban area.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The aim of this study was to understand the spatial and seasonal variations of glyphosate and AMPA and to determine their origins in the Vilaine River watershed, Britany-France.
The sample storage time prior to analysis is not reported. Individual concentrations for glyphosate and AMPA assigned to sampling stations and sampling campaigns is presented graphically. Thus, no exact but narrative concentrations can be given for respective sampling locations and periods.
Therefore, the study is considered reliable with restrictions.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2021 年 1 月 1 日～2021 年 5 月 14 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 8.2.6.1 and CA 8.2.7
Report author	Tajnaiová L. *et al.*
Report year	2020
Report title	Determination of the Ecotoxicity of Herbicides Roundup® Classic Pro and Garlon New in Aquatic and Terrestrial Environments
Document No	Plants, 2020, 9, 1203
Guidelines followed in study	No OECD guideline mentioned. Modified EN ISO 8692, EN ISO 20079 and EN ISO 23753-1
Deviations from current test guideline	• No OECD guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities (no indication)
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The authors compared the effects of the glyphosate-containing herbicide Roundup® Classic Pro (Monsanto Canada, 28.85% w/v of glyphosate + surfactant ether alkylamine ethoxylate) to that of a metabolite of its active substance, aminomethylphosphonic acid (AMPA). Changes in the growth rates of two tested aquatic organisms, duckweed *Lemna minor* and green algae *Desmodesmus subspicatus*, the total chlorophyll content of duckweed fronds, and the levels of dehydrogenase activity (DHA) in soil were all analysed. The results of an algal growth test showed that AMPA has a 1.5× weaker inhibitory effect on the growth of *D. subspicatus* than the Roundup formula (IC_{50}AMPA = 117.8 mg/L). The authors concluded that AMPA was ecotoxic to the tested organisms.

Applicant's comment: *Glyphosate-based formulations that contain polyethoxylated tallow amine (POEA) surfactants or surfactants of similar chemical structure (e.g. ethoxylated ether alkylamine), are generally more toxic than the active substance itself. In addition, the composition of formulations is an important consideration when comparing the endpoints achieved in public literature with those achieved in regulatory studies conducted with either the technical material or studies conducted with the representative formulation MON 52276. Co-formulants may ameliorate or enhance potential effects on test organisms. For example, POEA-based surfactants (not permitted for use in Europe) or surfactants with similar structures, may lead to enhanced sensitivity.*

For this reason and the fact that the formulation tested contains ethoxylated ether alkylamine (similar chemical structure to POEA), only the results for the metabolite AMPA should be considered as relevant for the AIR5 glyphosate renewal dossier.

Materials and methods

Chemicals
The chemical substance aminomethylphosphonic acid (AMPA) was tested. AMPA was supplied by Tokio Chemical Industry co. Ltd., and was used to prepare a stock solution.

For aquatic tests, Bold's basal medium (BBM), pH 6.5 and Steinberg growth medium, pH 5.5 were prepared. Methanol was used to determine chlorophyll content. For the determination of DHA in soils, analytical grade ethanol (96%), hydrochloric acid (HCl, 1 mol/L), tris buffer solution (100 nmol/L, pH 7.6), substrate solution (TTC, 300 nmol/L), and triphenylformazan solutions (TPF) were used. All solutions for the determination of DHA were prepared according to ISO 23753-1.

Organisms

Two aquatic organisms were chosen for this experiment. The first one was green alga *Desmodesmus subspicatus* (R. Chodat), E. Hegewald et A. Schmidt, Brinkmann 1953/SAG 8681 BU AV Czech Republic, Třeboň. The second one was duckweed *Lemna minor* L., sterile culture, Steinberg: Federal Environmental Agency (FDA), Berlin, Germany.

Experimental design

The experimental design was carried out according to the following standards: EN ISO 8692, EN ISO 20079, EN ISO 23753-1, with modifications.

Algal growth inhibition test

The algal growth inhibition test was performed in 25 mL Erlenmeyer flasks. Pre-cultivation of green algae *D. subspicatus* in a 500 mL flat bottom flask was started three days prior to the inhibition test. Throughout the pre-cultivation, the flask was aerated by a tube with a microbiological filter. The cells were inoculated into the growth medium and subsequently cultured at 23±2ºC under continuous illumination provided by daylight lamps with an intensity of 8000 lx under sterile conditions. Test substances in different concentrations, BBM, and inoculum of a total volume of 15 mL were pipetted into the Erlenmeyer flasks under sterile conditions in a laminar flow box. Subsequently, the flasks were stoppered with pulp plugs and cultured under the same heat and light conditions as during pre-cultivation. The initial algal density of *D. subspicatus* depended on the concentration of the algal inoculum. During the test, the flasks were continuously shaken at 160 rpm by an orbital shaker. After 72 h, the cell concentration of *D. subspicatus* was determined with a light microscope. Before the application of testing organisms and the sterilization, the pH was changed using NaOH (0.1 mol/L) to pH 6.6, which is the standardized pH of an algae cultivation medium. For AMPA, the concentrations corresponded to the molar equivalent of glyphosate present in the respective dilutions of Roundup® Classic Pro. The highest concentration used was 2851 mg/L and other values used were as follows: 2138.6; 1425.7; 712.8; 356.4; 178.2; 128.3; 85.5; 64.1; 42.7; 21.3; 10.6; 5.3; 2.6; 1.3 mg/L and a control. In the pH-adjusted assay, decimal dilution was used and five concentrations plus a control were tested 2851.5; 285.1; 28.5; 2.8; and 0.2 mg/L. To determine the acute toxicity of the tested substance after 72 h, its IC_{50} value was calculated using the endpoint of growth inhibition. The specific growth rate was calculated as the logarithmic increase. The inhibition of growth rate in percent was calculated using the specific growth rate.

Duckweed growth inhibition test

The duckweed growth inhibition test was initiated by pre-cultivating *L. minor* in the Steinberg medium, whose pH was adjusted to pH 5.5±0.2, for 7 days. After the adaptation period, several healthy colonies with 2-3 fronds (12 fronds per beaker total) were transferred from the inoculum culture into 150 mL glass beakers containing 100 mL of modified Steinberg medium used for dilution of the prepared ready-to-use herbicide solution. All beakers were covered and placed in a cultivator under continuous cool fluorescent light at 24±1ºC. All fronds of *L. minor* were equidistant from the light (7000 lux). The conditions throughout the entire test were sterile. The test with the AMPA solution was complemented with a parallel test using a pH-adjusted AMPA solution of pH 5.5, the same pH as the modified Steinberg medium. Duckweed growth was determined by tallying the frond numbers (FN) and the area of the fronds. The frond numbers were taken at the first, fourth, and seventh day of the experiment. All visible fronds were counted. A photo of the surface of the fronds was taken simultaneously for area evaluation using the NIS-Elements 4.2 software. Fronds of *L. minor* were observed at the first, fourth, and seventh day of the experiment for toxicity symptoms, such as chlorosis, necrosis, and frond disconnection. For AMPA, 10 concentrations plus a control were used, each of which corresponded to the molar equivalent of glyphosate present in its respective concentration of Roundup® Classic Pro. The AMPA concentrations tested were as follows: 2851.6; 2138.7; 1425.8; 712.9; 356.4; 178.2; 85.5; 42.7; 21.3 and 10.6 mg/L. In the pH-adjusted assay, decimal dilution was used and 5 concentrations plus a control were tested: 2851.6; 285.1; 28.5; 2.8; and 0.2 mg/L. The specific growth rate for was calculated as the logarithmic increase. The inhibition of growth rate in percent was calculated using the specific growth rate. To verify the validity of the test, the doubling time of the frond area was calculated. Chlorophyll *a* (chl *a*), *b* (chl *b*) concentrations were determined by extraction in methanol. After 7 days of incubation, duckweed fronds were gathered and placed in a plastic vial. The roots of the fronds were removed. Next,

the plastic vials were filled up with 8 mL of methanol. The extraction was carried out in a cold environment (4ºC) and darkness. After 24 h of extraction, the samples were centrifuged at 5000 rpm for 15 min and then a spectrophotometric analysis of the supernatant was performed. To measure the concentration of chlorophyll *a* and *b*, the absorbance of the samples was measured at different wave lengths.

The determination of dehydrogenase activity in soil

A sample of soil was taken according to ČSN ISO 10381-6 from a depth of 0-15 cm in Dejvice, Prague. It is a loess aluminosilicate soil with an admixture of silicates on slate subsoil. After sieving through a 2 mm sieve, the soil was placed in a resealable plastic bag and stored in a refrigerator in the dark at 4ºC for 7 days. Prior to the DHA test, the pH was determined according to ČSN ISO 10390. The moisture content of the soil was gravimetrically measured according to ČSN ISO 11465. After an initial oven drying at 105 ◦C for water loss, the organic matter content of the soil was determined by the loss on ignition (LOI) method. The content of elements C, H, N, and S in the soil was quantified by burning the sample at 1200ºC. The gaseous combustion products (N_2, CO_2, H_2O, and SO_2) were purified, separated into individual components and analyzed on a TCD detector. The results of the analysis include all of the combustible sulfur, both organic and inorganic, as well as all of the combustible carbon, both organically bound and inorganically bound. The content of the elements was determined in two replication measurements. In this study, the effect of AMPA on DHA was compared to the effect of the herbicide Roundup on DHA. The highest AMPA concentration corresponded to the molar equivalent of glyphosate salt present in the Roundup formula, which is 2852 mg/L. The dehydrogenase activity, when AMPA was applied, was higher at all dilutions than when Roundup was applied. The soil was homogenized in a beaker and put in U-bottom tubes with 5 different concentrations of the herbicide, three per each concentration. The method used for measuring DHA in soil required that a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) as an artificial electron acceptor be added to the soil samples. Tubes with stoppers filled with 2 g of soil were covered with aluminum foil and placed in an incubator to be incubated in the dark at 25ºC for 24 h. Subsequently, methanol was added, and the tubes were vortexed to better homogenize the sample. The samples were then centrifuged for 10 min and the supernatant was pipetted off. Finally, the absorbance was measured by a UV-VIS spectrophotometer at 485 nm and the concentration of triphenylformazane was established.

Statistical analysis

All samples and controls were prepared in triplicate. The results are represented as the mean values ± their respective standard deviations. All statistical analyses were performed with the Data Analysis Tools in Microsoft Excel 2016. The standard deviations of the experimental data were calculated by means of the Microsoft Excel software. The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 8.4.3.

Results

After adding the test substances into the BBM medium, the pH of the medium decreased. AMPA caused the most significant change in the pH (see table below), so the authors adjusted the pH of a set of AMPA solutions.

Table 1. pH values

AMPA – pH value										
Concentration of AMPA(mg/L)	2852	2139	1426	713	356	178	86	43	21	11
pH of AMPA solution	3.67	3.75	3.80	3.90	4.08	4.16	4.30	4.43	4.64	4.81
Algae inhibition test, pH after 72 h	4.37	4.56	4.63	5.40	5.67	6.50	6.93	7.39	7.63	7.71
Duckweed inhibition test, pH after 7 d	4.49	4.56	4.75	4.82	5.07	5.62	5.75	5.85	5.91	5.85

Green algae acute toxicity test

In the case of AMPA, a direct metabolite of glyphosate, the half maximal inhibitory concentration (IC_{50}) was higher than that of the herbicide Roundup® Classic Pro, namely IC_{50} = 117.8 mg/L. In summary, AMPA alone is less toxic to green algae than Roundup® Classic Pro.

In the pH-adjusted solutions, the value of IC_{50} of AMPA was determined to be 192.1 mg/L, indicating a mild mitigation of its toxic effects. A pH-adjusted solution better represents in situ conditions where a pH buffering system, which would prevent the pH from decreasing in such an extreme way, is naturally present. The results of the toxicity tests are presented in the table below, showing the inhibition values corresponding to all of the tested concentrations:

Table 2. Algae toxicity test

Algae (*D. subspicatus*) toxicity test							
AMPA							
%	c (mg/L)	log c	I (%)	**%**	c (mg/L)	log c	I (%)
100.00	2,851.6	6.4	89.6	**0.78**	64.1	4.8	4.7
75.00	2,138.7	6.3	88.4	**0.39**	42.7	4.6	0.9
50.00	1,425.8	6.1	88.6	**0.19**	21.3	4.3	-5.7
25.00	712.9	5.8	87.0	**0.09**	10.6	4.0	-3.1
12.50	356.4	5.5	89.6	**0.04**	5.3	3.7	-3.7
6.25	178.2	5.2	81.7	**0.02**	2.6	3.4	-4.5
3.12	128.3	5.1	67.2	**0.01**	1.3	3.1	-5.6
1.56	85.5	4.9	6.2				

For *D. subspicatus*, the inhibition caused by AMPA in the pH-adjusted medium at a dose corresponding to the ready-to-use dose was higher than that caused by AMPA without pH adjustment. At the highest dose tested (2851.6 mg/L), the inhibition caused by AMPA with and without pH adjustment was 92.8% and 89.6%, respectively. Inhibition values corresponding to AMPA without pH adjustment were in the range of 87.0%-89.6% for the first five most concentrated doses tested. The first major difference in the inhibition effect was observed at the dose of 128.3 mg/L where inhibition dropped to 67.2%, and at the next dose of 85.5 mg/L, where inhibition was measured at 6.2%. Stimulation of growth occurred in AMPA without pH adjustments at a concentration of 21.3 mg/L. On the contrary, in AMPA with pH adjustment, inhibition effects persisted even at the concentration of 0.2 mg/L, which is over 10,000 times more diluted than the highest tested dose.

The criteria for the validity of the test according to the OECD guidelines for freshwater algae were met. The coefficient of variation of the daily growth rates in the control cultures during the test did not exceed 35% and the coefficient of variation of the average growth in the replicated control cultures did not exceed 15%. The pH of the control samples increased from the original 6.6 value in all three replicates, but it never exceeded the value of 8.0.

Duckweed acute toxicity test

The observed doubling time in the control was 2.4 days on average. The pH of the control samples increased from an initial value of 5.5 to an average value of 5.9. The table below shows the inhibition values corresponding to the AMPA concentrations used.

Table 3. Duckweed toxicity test

Duckweed (*L. minor*) toxicity test							
AMPA							
%	c (mg/L)	log c	I (%)	I (%)	c (mg/L)	log c	I (%)
100	2,851.6	6.4	100.0	6.2	178.2	5.2	86.1
75	2,138.7	6.3	100.0	3.1	85.5	4.9	85.2
50	1,425.8	6.1	100.0	1.5	42.7	4.6	84.7
25	712.9	5.8	100.0	0.7	21.3	4.3	70.7
12.5	356.4	5.5	99.2	0.3	10.6	4.0	36.8

At a concentration of 10.69 mg/L, duckweed growth inhibition for AMPA was 36.83%. When added to Steinberg's growth medium, the test substances lowered its pH, with the most significant decrease brought about by AMPA. For this reason, in addition to comparing pure AMPA, pH-adjusted AMPA was prepared as well. After adjusting the pH for the AMPA metabolite, a 10-fold dilution was used. The highest concentration corresponded with molar conversion to the glyphosate content in Roundup® Classic Pro and as such was determined to be 2851 mg/L. At this concentration, duckweed growth inhibition for AMPA with pH-adjustment was 95.3%. The measured data shows that after a pH adjustment in the medium with the tested AMPA, the inhibitory effect was lower than when using AMPA with no pH adjustment, but the differences were not significant ($I_{(c=285.1\ mg/L)}$ = 85.5%; $I_{(c=28.5\ mg/L)}$ = 80.4%). There was an increase in the total area of the duckweed fronds after four and seven days in the medium with the pH-adjusted AMPA. Furthermore, when the concentration of AMPA was lowered, no necrosis occurred; although partial chlorosis was present in all samples, even at the lowest concentration of 10.7 mg/L. Root growth, with the exception of controls, was not observed or only very stunted roots grew at the lowest concentrations. After seven days, all L. minor fronds turned white. The values of total chlorophyll in the fronds of the duckweed corresponded to the loss of biomass. None of the measured values exceeded the control samples. In contrast, at the four highest concentrations, no chlorophyll was detected after applying any of the three test substances. When diluted to 12.5%, a small amount of chlorophyll remained in the AMPA samples, and even at the lowest tested concentration, the decrease caused by AMPA was the least severe.

The determination of dehydrogenase activity in soil

A preliminary analysis of the soil sample showed that it was slightly acidic, with pH~6.34. The moisture of the soil was measured to be 72.27%. Ignition losses were determined at 15.84%. The total average sulfur content was set at 0.14% of the soil weight, hydrogen at 1.04%, nitrogen at 0.78%, and the total average carbon content was at 6.45%. The highest DHA was observed at the lowest dose tested, which corresponded to a dilution of 6.2%. The DHA value of the control was not exceeded at any tested dose of AMPA. At the highest tested AMPA concentration of 2852 mg/L, which corresponds to 100% of the measured concentration, the determined DHA was 1.6 higher than in the samples treated with Roundup® Classic Pro. This trend was maintained at all concentrations tested (in the range of 1.2-1.6). All values for both substances were lower than that of the control sample.

Conclusion

AMPA, being a metabolite of glyphosate, cause morphological changes in non-target organism *L. minor*. Furthermore, this substance exhibited toxicity to both duckweed *L. minor* and freshwater green algae *D. subspicatus* after short-term exposure, not only in application doses but also in much lower concentrations. When evaluating the effect of substances on the DHA of soil, the level of activity after the application of the test substances was shown as follows: AMPA > Roundup® Classic Pro.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study reports on endpoints with possible relevance to the EU-level risk assessment for aquatic organisms, specifically freshwater algae (*Desmodesmus subspicatus*) and aquatic plants (*Lemna minor*) for the glyphosate metabolite AMPA. For *D. subspicatus*, a regulatory relevant endpoint is reported (IC_{50} for inhibition of growth rate, usually described as ErC_{50}). For the *Lemna* test, no regulatory relevant endpoint was reported but the assessed parameters (growth rate, front area and chlorophyll content) could potentially serve as supporting information in a weight of evidence approach.
As the tests differed both in design as in the reported endpoints, assessments of reliability should be looked at separately.

Test with *D. subspicatus*: The report states that the test was conducted according to ISO 8692 with not further specified modifications. Furthermore, it is reported, that the test fulfilled the validity criteria laid down by OECD test guideline for freshwater algae. There are however important information missing from the report. Specifically, no data of the negative control are presented not even in the supplementary material. No raw data are provided so, the validity criteria could not be checked. Furthermore, the IC_{50} value was calculated without confidence intervals and no parameters to judge the fit of the curve used for calculating the IC_{50} are presented. The certainty of the level of protection can therefore not be fully assessed.

Test with *L. minor*: While relevant parameters were assessed (such as growth rate and front area), no regulatory relevant endpoints, such as ECx values, were calculated. No justifications are provided. Furthermore, control data are only presented for the parameters front area and chlorophyll content, and only in a graphical format. Due to the lack of raw data, no regulatory relevant endpoints can be calculated retroactively.

For both tests: The purity of the test material was not reported. Furthermore, exposure concentrations were not analytically verified. Especially for the *Lemna* test, which lasted 7 days without renewal of the test substance, actual exposure concentrations could vary significantly from the nominal concentrations.

Based on the above mentioned issues for both tests, the study can only be deemed reliable with restrictions.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		

1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	The study states that tests were conducted according to standardized test guidelines with modifications (not specified); EN ISO 8692 (growth inhibition of green algae) and EN ISO 20079 (duckweed growth inhibition test) with modifications. For algae: validity criteria according to OECD guidelines for freshwater algae were met For *Lemna*: no validity criteria mentioned
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Test cultures were obtained from research institutes in Germany and Czech Republic
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Uncertain	Not reported whether a solvent was used to prepare the test solutions
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Uncertain	Source of the test material was reported but no information on purity
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	Non-vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	Standardized test guidelines were inferred (see point 1.); validity criteria were met for the freshwater algae assay, for the *Lemna* assay validity was not discussed
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Uncertain	Glyphosate metabolite (AMPA) was tested but the preparation of test solutions was not described
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	No	Missing information on preparation of test solutions (no info on use of solvent)

11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes and No	Algae: Endpoints reported: IC_{50} *Lemna*: no regulatory relevant endpoint reported
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	*Lemna*: 10 levels and a control; 5 levels pH adjusted assay Algae: 15 levels and a control; 5 levels pH adjusted assay 3 replicates for all treatment and control levels
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Uncertain	Sufficient number of organisms and treatments was included but control results were not sufficiently reported for all endpoints (only for front area and chlorophyll content for the *Lemna* test)
17. Assessment of the statistical power of the assay is possible with reported data.	Uncertain	No raw data reported, however statistical test results are reported
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical methods were reported
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study

20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Uncertain	Medium type mentioned: Bolds Basel Medium (BBM) and Steinberg medium; composition not mentioned
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Algae: 23 ± 2 ◦C *Lemna*: 24 ± 1 ◦C.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	-
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	No residue measurements
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	IC_{50} value reported without C.I.

Overall assessment		
Reliable without restrictions	No	

Reliable with restrictions	Yes	The study reports on endpoints with possible relevance to the EU-level risk assessment for aquatic organisms, specifically freshwater algae (*Desmodesmus subspicatus*) and aquatic plants (*Lemna minor*) and the glyphosate metabolite AMPA. For *D. subspicatus,* a regulatory relevant endpoint is reported (IC_{50} for inhibition of growth rate, usually described as ErC_{50}). For the lemna test, no regulatory relevant endpoint was reported but the assessed parameters (growth rate, front area and chlorophyll content) could potentially serve as supporting information in a weight of evidence approach. As the tests differed both in design as in the reported endpoints, assessments of reliability should be looked at separately. Test with *D. subspicatus:* The report states that the test was conducted according to ISO 8692 with not further specified modifications. Furthermore, it is reported, that the test fulfilled the validity criteria laid down by OECD test guideline for freshwater algae. There are however important information missing from the report. Specifically, no data of the negative control are presented not even in the supplementary material. Furthermore, the IC_{50} value was calculated without confidence intervals and no parameters to judge the fit of the curve used for calculating the IC_{50} are presented. The certainty of the level of protection can therefore not be fully assessed. Test with *L. minor:* While relevant parameters were assessed (such as growth rate and front area), no regulatory relevant endpoints, such as ECx values, were calculated. No justifications is provided. Furthermore, control data are only presented for the parameters front area and chlorophyll content, and only in a graphical format. Due to the lack of raw data, no regulatory relevant endpoints can be calculated retroactively. For both tests: The purity of the test material was not reported. Furthermore, exposure concentrations were not analytically verified. Especially for the lemna test, which lasted 7 days without renewal of the test substance, actual exposure concentrations could vary significantly from the nominal concentrations. Based on the above listed limitations, the study can only be deemed reliable with restrictions.
Not reliable	No	

1. Information on the study

Data point:	CP 10.2.1
Report author	Gustinasari K. *et al.*
Report year	2021
Report title	Acute toxicity and morphology alterations of glyphosate-based herbicides to *Daphnia magna* and *Cyclops vicinus*
Document No	Toxicological Research, 2021, 37, 197-207
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities (no indication)
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, acute toxicity test of Glyphosate-Based Herbicides (GBHs) was conducted to *Daphnia magna* and *Cyclops vicinus*. Acute toxicity test was performed to both organisms at the Glyphosate concentrations of 20, 80, 160, 320, and 640 mg/L in exposure time of 12 h, 24 h, and 48 h, under static conditions. The mortality and morphology were observed to determine the LC_{50} and the effect of its morphology. The test showed that *D. magna* was more susceptible than *C. vicinus*. The LC_{50} of GBHs to *D. magna* and *C. vicinus* for its different exposure time were respectively show as follows: 76.67 mg/L and 207.89 mg/L (12 h); 36.2 mg/L and 159.8 mg/L (24 h); and 21.34 mg/L and 92.93 mg/L (48 h). There were no significant differences of the alteration of spin length, body length, and head length of *D. magna* to exposure of GBHs, except the head width. While body length alteration of *C. vicinus* was significantly different towards the increase in concentration.

Materials and methods

Test organism management

For this toxicity test, *D. magna* Straus, 1820 was obtained from cultures by the Institute of Biology, University of Szczecin, Poland. During the cultivation, *D. magna* was fed green algae, powder was weighed as much as 0.3 g, gently stir in order to dissolve with water in the container. Aeration was also supplied to provide oxygen requirements for *D. magna*. The culture was run for 5 days prior to the experiment. *C. vicinus* Uljanin, 1875 were obtained from Odra river, Poland and were sufficient in the amount and the size needed. A total of 180 specimens *D. magna* and *C. vicinus* were randomly assigned to give a loading of ten specimens per tank.

Experimental design:

The acute 12 h, 24 h, and 48 h static bioassay was performed in the Hydrobiology Laboratorium, Biology Institute of Szczecin University based on internal methods. The glyphosate-based herbicides used was from a brand named SUMIN ATUT 360 SL. It contained glyphosate in the form of isopropylamine salt at 360 g/L and 30.85% of detergent. Six different concentrations of glyphosate were made: 20 mg/L, 80 mg/L, 160 mg/L, 320 mg/L, 640 mg/L, and a control with no toxicant (0 mg/L). These concentrations were made by diluting the isopropylamine salt in distilled water. Each concentration was prepared in 100 mL volume and replicated three times on both *D. magna* and *C. vicinus*.

The ten individuals of *D. magna* and *C. vicinus* were introduced into each concentration in the container. There were three replicates for each group.

Observations:
The survival and mortality were counted for 12 h, 24 h, and 48 h. The experiments were kept at the same temperature without food. At the end of the test, the parameters of pH, temperature, EC (conductivity), TDS (Total Dissolved Solid), and DO (Dissolved Oxygen) were measured. Mortality was determined when the specimen did not respond to a repeated prodding.
Observations of the morphological response of *D. magna* and *C. vicinus* exposed to glyphosate were conducted after 48 h. The morphological indicators of *D. magna* were the spin length, body length, head width, and long head. While the morphological indicator of *C. vicinus* was body length. These indicators were observed using Zeiss Stereo microscope Discovery V12 (Germany). Each variable was measured from digital photographs, using the software Axio Vision. Responses were recorded if they differ from the controls.

Statistical analysis
As partial mortality in any replicate was observed, probit method was suitable to calculate the LC_{50}. The percentage of mortality was carried out for probit values against the logarithm of concentration using Microsoft excel. The LC_{50} was then calculated by substituting the probit value of 50% in the equation $y = b + ax$ in which variable x is known and a = unknown and b = intercept. The anti-logarithm value of "a" was taken as the LC_{50}. To determine the highest concentration value that does not cause an impact (NOEC), for this acute toxicity test the LC_{50} min/1000 was used.
The morphology indicators were analysed using one-way analysis of variance (ANOVA). The significant difference was level at $P = 0.05$. Further, the linear regression was also done. Regression correlation is divided into six level classifications, namely: $R^2 = 0$ (no correlation); $R^2 > 0$-0.25 (very low correlation); $R^2 > 0.25$-0.5 (moderate correlation); $R^2 > 0.5$-0.75 (strong correlation); and $R^2 > 0.75$-0.99 (very strong correlation); $R^2 = 1$ (perfect correlation).

Results

Acute toxicity
In the present work, acute toxicity test with GBHs (in the form of 360 g/L isopylamine salt) was performed using *D. magna* and *C. vicinus*. The table below shows the mortality percentage of *D. magna* and *C. vicinus* based on the influence of exposure time and concentration variation. All *D. magna* died at a concentration of 160 mg/L with an exposure time of 48 h. Exposure time had a significant effect on the death of *D. magna* at a concentration of 160 mg/L. All *D. magna* had died at 320 mg/L and 640 mg/L. Exposure time had a significant effect on death of *C. vicinus* at a concentration of 320 mg/L. At the highest concentration (640 mg/L), exposure time had no significant effect on death because almost all *C. vicinus* died. Concentration significantly affected the death of both *D. magna* and *C. vicinus*, at the time of exposure 12, 24, or 48 h.

Table 1. Percentage mortality of *Daphnia magna* and *Cyclops vicinus* exposed to glyphosate-based herbicides

Concentration (mg/L)	Species	Hours		
		12	24	48
0	*D. magna*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	C. vicinus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20	*D. magna*	10.00 ± 17.32	46.67 ± 25.17	53.33 ± 25.17
	C. vicinus	10.00 ± 17.32	10.00 ± 17.32	23.33 ± 15.28
80	*D. magna*	23.33 ± 40.41	53.33 ± 25.17	70.00 ± 26.46
	C. vicinus	16.67 ± 28.87	16.67 ± 28.87	26.67 ± 20.82
160	*D. magna*	63.33 ± 11.55	83.33 ± 15.28	100.00 ± 0.00
	C. vicinus	30.00 ± 0.00	36.67 ± 11.55	43.33 ± 11.55
320	*D. magna*	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	C. vicinus	46.67 ± 5.77	50.00 ± 10.00	73.33 ± 5.77
640	*D. magna*	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	C. vicinus	90.00 ± 10.00	93.33 ± 11.55	100.00 ± 0.00

The table below presents the value of LC_{50} for both *D. magna* and *C. vicinus* of the exposure of GBHs at the period bioassay of 12 h, 24 h, and 48 h. The LC_{50} of *D. magna* for each exposure time were 76.67 (12 h), 36.20 (24 h) and 21.34 (48 h), so the NOEC estimated 0.021 mg/L. While the LC_{50} of *C. vicinus* for each exposure time were 207.89 (12 h), 159.81 (24 h) and 92.93 (48 h), so the NOEC estimated 0.09 mg/L.

Table 2. The LC_{50} values of *Daphnia magna* and *Cyclops vicinus* exposed to glyphosate-based herbicides

Hours	Species	Equation for the regression analysis	LC_{50}
12	*D. magna*	y = 3.3246x–1.2657	76.67
	C. vicinus	y = 1.564x + 1.3749	207.89
24	*D. magna*	y = 2.4367x + 1.202	36.20
	C. vicinus	y = 1.8428x + 0.9393	159.81
48	*D. magna*	y = 2.4035x + 1.8052	21.34
	C. vicinus	y = 2.276x + 0.5205	92.93

The table below showed the environmental parameters of water as media exposure after 48 h period bioassay. The pH and DO decrease with increasing glyphosate concentration, from 6.84 to 4.83 and from 3.95 to 2.49 mg/L, respectively. While the conductivity and TDS increase with increasing concentration, from 26.87 to 424.8 S/m and from 0.02 to 0.27 mg/L, respectively. This proved that concentration influences pH, conductivity, TDS, and DO. However, temperature is not affected by glyphosate concentration.

Morphology alterations

The boxplot charts of concentration correlation to changes in morphological indicators comprise spin length, body length, dead width, and head length for *D. magna* and body length for *C. vicinus.* There was no significant differences on concentration of glyphosate to the spin length of *D. magna*. At a concentration of 80 mg/L, it appeared that the spin length was longer than the concentration of 20 mg/L. However, at a concentration of 160 mg/L the effects of shortening of the spin length began to be seen and continued to decline to 640 mg/L.

The difference in body length of *D. magna* after being exposed to glyphosate was not significant difference. The concentration of 20 mg/L until 160 mg/L almost had the same body length. Decrease in body length was only seen at a concentration of 320 mg/L and 640 mg/L. The head width alteration of *D. magna* had a significant difference.

The concentration of glyphosate to head length had no significant difference. At the concentration of 80mg/L, the head was longer than 20 mg/L, although there was a slight decrease to a concentration of 320 mg/L. At a concentration of 640 mg/L the head length was much shorter than at other concentrations. The morphological indicators of *C. vicinus* was the body length of *C. vicinus*. There was the significant difference for the body length alteration. There was a decrease in body length with each concentration.

The alteration of morphological with the measuring of body length, head length, head width, and spin length of *D. magna* were quantitative data. Visually, there were different appearance of both *D. magna* and *C. vicinus* at each concentration. *D. magna* changed colour to slightly white with increasing concentration. In addition, the internal organs of *D. magna* with increasing concentration loose from the outer layer of the body. *C. vicinus* changed colour as well. However, the internal organs of the *C. vicinus* could not be separated from their outer layers.

Conclusion

The findings showed that the toxicant effects on the *D. magna* and *C. vicinus* increased as the GBHs concentration of exposure and the longer period of exposure. The NOEC of GBHs of *D. magna* and *C. vicinus* were 0.021 mg/L and 0.092 mg/L, respectively. The alteration morphology occurred in both *D. magna* and *C. vicinus* as the concentration of GBHs increases. The test showed that *D. magna* was more susceptible than *C. vicinus*.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study provides regulatory relevant endpoints, specifically the LC_{50} for *D. magna* and *C. vicinus* after 48-hours exposure. However, the study cannot be deemed fully reliable due to the following aspects: First, the exposure concentrations were not analytically verified. Furthermore, previous exposure of *C. vicinus* to contaminants cannot be excluded as they were collected from a river with unknown contamination history. In addition, the exposure medium was not specified (possibly distilled water, but the phrasing is ambiguous) and important info on the culturing conditions is missing (i.e. temperature, etc.). Life-stage and size of the organisms at test start were not documented. Finally, for the morphological alterations, the control values were not reported.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes (*D. magna*) and No (*C. vicinus*)	*D. magna* were obtained from a culture, *C. vicinus* were sampled from a river

3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in distilled water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source and content reported
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Invertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	No	Reported: Source, feeding, aeration Not reported: Culturing conditions (incl. type of medium, O_2-content, temperature, pH, conductivity), life-stage or size of test organisms
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	Test with glyphosate formulation SUMIN ATUT 360 SL
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Uncertain	Doses are reported but constitution of exposure medium not clear, no dilution scheme provided
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoints reported: LC_{50} (12, 24 and 48-h) and NOEC (but not correctly determined)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	5 treatment levels and a negative control
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	3 replicates of 10 organisms per treatment group and control; control data reported
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data

18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	No	No information on test medium
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature roughly between 21-22 °C at the end of the test, not reported for acclimation period
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	No residue measurements
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported

27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Uncertain	Underlying equation of the probit curve was reported but no confidence intervals for the LC_{50} or raw data to calculate confidence intervals retroactively
Overall assessment		
Reliable without restrictions	No	
Reliable with restrictions	Yes	The study provides regulatory relevant endpoints, specifically the LC_{50} for *D. magna* and *C. vicinus* after 48-hours exposure. However, the study cannot be deemed fully reliable due to the following aspects: First of, the exposure concentrations were not analytically verified. Furthermore, previous exposure of *C. vicinus* to contaminants cannot be excluded as they were collected from a river with unknown contamination history. In addition, the exposure medium was not specified (possibly distilled water, but the phrasing is ambiguous) and important info on the culturing conditions is missing. Life-stage and size of the organisms at test start were not documented. For the morphological alterations, the control values were not reported
Not reliable	No	

1. Information on the study

Data point:	CP 10.3.1
Report author	Luo Q.H. *et al.*
Report year	2021
Report title	Effects of a commercially formulated glyphosate solutions at recommended concentrations on honeybee (*Apis mellifera* L.) behaviours
Document No	Scientific Reports, 2021, 11, 2115
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities (no indication)
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, a series of behavioural experiments were conducted to investigate the effects of Commercially Formulated Glyphosate (CFG) on honeybees after a chronic exposure. The results showed that there was a significant decline in water responsiveness at 1/2×, 1× and 2× the Recommended Concentration (RC) after 3 h of exposure to CFG for 11 days. The CFG significantly reduced sucrose responsiveness at 1/2× and 1× the RC. In addition, CFG significantly affected olfactory learning ability at 1/2×, 1×, and 2× the RC and negatively affected memory ability at 1/2× and 1× the RC. The climbing ability of honeybees also significantly decreased at 1/2×, 1×, and 2× the RC. Our findings indicated that, after they were chronically exposed to CFG at the RC, honeybees exhibited behavioural changes. These results provide a theoretical basis for regulating field applications of CFG, which is necessary for establishing an early warning and notification system and for protecting honeybees.

Materials and methods

Test organisms

Sampling sites were located at the Zhuifengshan Forest Apiary, Dachengzi town, Miyun district, Beijing. In order to avoid taking too many emerging bees at one time and affecting bee colonies, each experiment randomly selected six capped combs in six colonies from forty colonies and maintained in an incubator to take the emerging honeybees the next day (these combs were put back into the original colonies after collection). Each cage (15×15×10 cm, with mesh on two sides) contained 70 honeybees that were captured within 24 h. A total of 4 cages were randomly selected for four treatments in the LD_{50} experiment. The entire experiment was repeated for 6 times in total and thirty-six colonies involved. A total of 16 cages (70 bees/cage), 4 of which were randomly selected for water responsiveness assay (four treatment: 0, 1/2, 1, and 2× RC), and the rest for sucrose responsiveness, learning and memory and climbing assay (4 cages for four treatments in each behavioural experiment) respectively. Six combs were randomly selected again after such an experiment completed, and the entire experiment was repeated for a total of 3 times and 18 colonies involved (5 times for climbing assay).

LD_{50} experiment

LD_{50} was tested to assess the dose level used in this study. The sucrose solution contained 41% glyphosate isopropylamine salt (Monsanto Roundup Original; 356 g of glyphosate acid equivalent per liter) at concentrations of 0, 0.72, 3.6, 7.2 g(s) of glyphosate acid equivalent per liter respectively (abbreviated as g/L). Plastic feeder were inserted vertically into the cages and changed daily. On the

basis of the number of honeybees that survived daily, each honeybee was fed with an average of 33 μL of sucrose solution and then fed with sucrose solution 50% (w/w) and water ad libitum. The honeybees were fed in an incubator whose temperature was 30±1°C and whose relative humidity was 65%~70%. The insects in the control groups were fed 50% (w/w) sucrose solution and water. The total mortality rate was calculated at 48 h after treatment.

Test organisms preparation and treatment for experiments

At the beginning of the experiment, to keep the honeybees at the same age and adapt to the laboratory feeding, the honeybees were fed 50% sucrose for one week (during which each caged honeybee was fed with sufficient amounts of pollen, which was replaced daily). The recommended concentration of Monsanto Roundup Original was 50 mL-500 mL formulation solution in 30-40 L water according to different application. The formulation in our study was applied at a rate equivalent to 50 mL formulation solution in 40 L water, which was the lowest recommended concentration and equal to 445 mg a.i./L (1× recommended concentration). Beginning when they were 8 days old, the honeybees were continuously fed three concentrations of glyphosate (the 1/2× , 1× and 2× RC used in this study), which were equal to doses of 7, 14 and 28 μg/bee/day in 33 μL of 50% sucrose solution/day, on average.Tested honeybee were 18 days old. The control groups were fed with a 50% sucrose solution. All tested honeybees were starved for 4 h before the test. The honeybees were fixed in a tube such that the antennae and head could move freely.

Water responsiveness experiment

Water was placed on the antennae of the honeybees to detect the proboscis extension response (PER). The honeybees were tested twice: at both 1 h and 3 h after treatment. Thirty honeybees were tested per replicate, and three replicates were used per treatment (0, 1/2×, 1× and 2× RC).

Sucrose responsiveness experiment

Water was first put on the antennae of the honeybees to determine whether the insects sucked water, eliminating the proboscis extension response phenomenon due to thirst. The proboscis extension response was then used to test honeybee sucrose responsiveness to increasing concentrations of sucrose solution (0.3%, 1%, 3%, 10%, 30%; w/v). The sucrose solution was absorbed and placed on the antennae of the honeybees to detect the proboscis extension response. For each concentration, the percentage of proboscis extension response released by the honeybees was recorded.

Olfactory learning and memory ability experiment

Water was first placed on the antennae of the honeybees as described above. A 30% sucrose solution was used for the proboscis extension response assay, and the percentage of proboscis extension response was recorded. Linalool (Sigma, 95% purity) was used as the conditioned stimulus and was presented for 6 s first. During odour presentation, the proboscis extension response was elicited after 3 s by contacting the antennae with the sucrose solution (30%), and the same solution was immediately given as a reward. Conditioning trials were conducted three times (C1, C2, C3) at 20-min intervals. Five test trials (T1, T2, T3, T4, T5) were then conducted with linalool stimulation for 6 s without sucrose feedback. Proboscis extension responses were also recorded at 20-min intervals.

Climbing ability experiment

After 11 days of continuous feeding, the honeybees were starved for 1 h before testing. A box (65 cm in length, 35 cm in width and 4 cm in height) was divided into 10 lanes (50 cm×3.5 cm×4 cm) with a fluorescent lamp on the top and covered with glass. The tests were conducted in the dark and with the box tilted at 45°. One honeybee per lane was placed in the box, and the lamp was on. The time it took for a honeybee to climb 50 cm was recorded. Ten honeybees were tested in each trial (which was performed in five replicates) in the control and treatment groups.

Statistical analysis

All the data were processed by SPSS 19 (IBM Corp., Armonk, NY, USA). The ingestion LD_{50} was determined on the basis of the mortality of honeybees for each dose via Probit analysis. The effects on water responsiveness, sucrose responsiveness, learning and memory and climbing were analysed by ANOVA for both the Roundup-treated and control groups. And LSD (homogeneity of variance) or Tamhane's T2 test (heterogeneity of variance) was performed. A difference was considered significant when the P-value was <0.05.

Results

LD50

The mortalities were respectively 3.67±1.13%, 7.77±3.04%, 35.25±7.94%, and 75.00±2.85% at the concentrations of 0 g a.i./L, 0.72 g a.i./L, 3.6 g a.i./L, and 7.2 g a.i./L. The ingestion LD_{50} was 309 μg/bee ($y = 5.454x - 1.684$).

Roundup decreased water responsiveness

No significant effect of Roundup on water responsiveness was detected at 1 h after treatment ($P = 0.49$). Compared with the control solution, Roundup significantly decreased the proboscis extension response to water at 3 h after treatment at all tested concentrations. The highest percentage of proboscis extension response was 28.33% in the control groups, and the lowest was 6.67% in the Roundup treatment of 2× the recommended concentration.

Roundup affected sucrose responsiveness

The proboscis extension response percentage increased with increasing sucrose concentration in the control and treatment groups. A significant decrease in the proboscis extension response percentage was detected in response to the 0.03% and 0.3% sucrose solutions after treatment with Roundup at 1/2× the RC for 11 days. In honeybees treated with Roundup at 1× the RC, the proboscis extension response percentage in response to the 0.03%, 0.1% and 0.3% sucrose solutions was significantly lower than that of the control groups. Moreover, there was no significant effect of the proboscis extension response percentage for the ≥1% sucrose solution between the control and treatment groups. The groups of honeybees treated with 2× the RC of Roundup did not exhibit any significant effects on the proboscis extension response percentage in any of the treatments.

Roundup affected olfactory learning and memory ability

The olfactory learning and memory ability of honeybees was expressed as the proboscis extension response percentage. Fixed honeybees were evaluated in the training phases C1, C2, and C3 and then in the test phases T1, T2, T3, T4 and T5. The proboscis extension response percentage of honeybees treated with Roundup at 1/2× the RC indicated that the learning ability (the relationship between odour and sucrose feedback) during the second and third presentation of odour (C2 and C3) was significantly lower than that in the control groups. And compared with that in the control groups, the proboscis extension response percentage in the treatment group significantly decreased during all test phases. Compared with those in the control groups, the honeybees treated with Roundup at 1× the RC presented a significantly increased proboscis extension response percentage at C1 but a significantly decreased proboscis extension response at T1, T4 and T5. A significant reduction in olfactory learning performance was also noted during C2 and C3 in honeybees treated with the highest dose of Roundup (2× the RC). The T1-T5 tests revealed no significant effect of the highest concentration of Roundup.

Roundup significantly reduced climbing ability

The climbing ability of the honeybees was significantly affected by Roundup. Honeybees in the control groups required the shortest time to climb 50 cm, whereas those in the Roundup treatment groups at 2× the RC required the longest time. During the climbing activity tests, honeybees treated with Roundup at all tested concentrations took longer to walk through the 50-cm track than did those in the control groups after 11 days of exposure.

Conclusion

In this study, the authors provided new information on the influence of commercially formulated glyphosate at the recommended concentration on the behaviours of honeybees. The authors concluded that their findings showed that the water responsiveness, sucrose responsiveness, learning and memory ability and climbing ability of honeybees were affected by commercially formulated glyphosate at or below the recommended concentration. Also, the authors stated that additional research needs to be conducted to determine the effects of actual applications of commercially formulated glyphosate at the recommended or lower concentration rather than the pure glyphosate on honeybees.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study reports on possibly relevant endpoints for the acute bee risk assessment, specifically a 48-hour oral LD_{50} value, as well as behavioural assessments on bees after glyphosate exposure. However, the study has to be deemed reliable with restrictions based on the following aspects: The colonies used for this study were obtained from an apiary about which no further information was provided. This pertains especially to possible pre-existent contaminant exposure of the colonies as well as health aspects regarding bee specific illnesses and treatments. As stated in OECD Guideline 213 (Honeybees, Acute Oral Toxicity Test), "… bees treated with chemical substances, such as antibiotics, anti-varroa, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment". This aspect cannot be adequately assessed as information on the bee colonies health or possible contaminant contact points is missing from the study report. The reported temperature during the test was 30 ± 1°C, which is outside the temperature range of 25 ± 2 °C recommended in OECD 213. The study report states an observation timeframe of 48 hours, however exact time-points of observation are not reported. Test concentrations were not analytically verified. Lastly, the LD_{50} value was reported without confidence intervals. The certainty on the level of protection offered by the median LD_{50} value can hence not be assessed. Based on the above mentioned aspects, the reliability of the study is restricted.

Assessment and conclusion by RMS:

ECOTOXICOLOGY: Reliability criteria for the detailed assessment of full-text documents

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	No	Bees were acquired from a forest apiary, previous contact of the colony with contaminants is unknown
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	-	No carrier was used, test substance was directly dissolved in sucrose solution
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Uncertain	Content reported, Source and purity not reported

5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	Non-vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	Source, life-stage and haltering conditions reported
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	Roundup formulation; 356 g/L
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	An oral LD_{50} (48-hours) is reported
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	3 treatment levels and a negative control were tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	Increased mortality with increased test item concentration was observed
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	70 individuals per treatment group per experiment, experiment was repeated 6 times in total
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error as well as curve equation are reported but no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical methods were described incl. level of power
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Uncertain	Mortality was observed over 48-hours but exact time-points of observations not mentioned

20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, Bee study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, Bee study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, Bee study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, Bee study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, Bee study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, Bee study
20.6. Data on precipitation is recorded.	-	Not applicable, Bee study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	No	According to OECD 213 (Honeybees, Acute Oral Toxicity Test) the temperature should be 25 ± 2 °C; the reported temperature was 30 ± 1 °C
22. For bee studies, temperature of the study should be appropriate to species.	No	See above
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	-	Not applicable, Bee study
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	-	Not applicable, Bee study
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	No residue measurements
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical measurements conducted
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	LC_{50} reported but not the corresponding confidence interval; no raw data to re-calculate retroactively

Overall assessment		
Reliable without restrictions	No	

Reliable with restrictions	Yes	The study reports on possibly relevant endpoints for the acute bee risk assessment, specifically a 48-hour oral LD_{50} value, as well as behavioural assessments on bees after glyphosate exposure. However, the study has to be deemed reliable with restrictions based on the following aspects: The colonies used for this study were obtained from an apiary about which no further information was provided. This pertains especially to possible pre-existent contaminant exposure of the colonies as well as health aspects regarding bee specific illnesses and treatments. As stated in OECD Guideline 213 (Honeybees, Acute Oral Toxicity Test), "… bees treated with chemical substances, such as antibiotics, anti-varroa, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment". This aspect cannot be adequately assessed as information on the bee colonies health or possible contaminant contact points is missing from the study report. The reported temperature during the test was 30 ± 1 °C, which is outside the temperature range of 25 ± 2 °C recommended in OECD 213. The study reports states an observation timeframe of 48 hours, however exact time-points of observation are not reported. Test concentrations were not analytically verified. Lastly, the LD_{50} value was reported without confidence intervals. The certainty on the level of protection offered by the median LD_{50} value can hence not be assessed. Based on the above mentioned aspects, the reliability of the study is restricted.
Not reliable	No	