グリホサートカリウム塩

要旨及び評価結果

検索期間：2021 年 5 月 14 日～2021 年 8 月 31 日

評価対象：適合性区分 a に該当する文献

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1. Information on the study

Data point:	CA 8.1.5
Report author	Diaz-Martín R. D. *et al.*
Report year	2021
Report title	Short exposure to glyphosate induces locomotor, craniofacial, and bone disorders in zebrafish (*Danio rerio*) embryos
Document No	Environmental toxicology and pharmacology (2021), Vol. 87, Article No. 103700
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors used the zebrafish model to assess the effects of early life glyphosate exposure on the development of cartilage and bone tissues and organismal responses. The authors found functional alterations, including a reduction in the cardiac rate, significant changes in the spontaneous tail movement pattern, and defects in craniofacial development. These effects were concomitant with alterations in the level of the oestrogen receptor alpha osteopontin and bone sialoprotein. The authors also found that embryos exposed to glyphosate presented spine deformities as adults. These developmental alterations are likely induced by changes in protein levels related to bone and cartilage formation.

Materials and methods

Fish maintenance and embryo production

The authors followed all procedures and policies stated in the NOM-062-ZOO-1999 Mexican guideline (these guidelines apply mostly to mammalian species but the authors applied the same animal welfare principles to the fish in this study). The authors also followed the British guidelines for fish welfare. Zebrafish handling and maintenance complied with the guidelines of the Zebrafish Book. Wild-type adult zebrafish (*Danio rerio*) were obtained from commercial distributors and maintained in 6-L tanks (6 fish per tank) with dechlorinated water in a closed flow-through system at 27ºC, a 13:11 h light:dark cycle, and two feedings each day. Zebrafish embryos were generated by massive mating using 4 females and 2 males. Embryos were examined 2 h post-fertilization (hpf) under a Stemi 305 stereoscopic microscope to select normally developed embryos at the 16-cell stage. These embryos were then placed in Petri dishes containing EM buffer (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, and 0.7 mM $NaHCO_3$ at pH 7±0.2) for the exposure trials.

Glyphosate exposure

Fifty millilitres of stock solution (50 µg/mL) were prepared using 2.5 mg of glyphosate (99% purity, CAS Number 1071-83-6; Sigma-Aldrich, St. Louis, USA) dissolved in EM buffer. Glyphosate test solutions were then prepared at 0 (control), 1, 5, 10, and 50 µg/mL. The concentration of glyphosate in stock solution was determined by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS). The experiment was carried out with five completely independent replicates. Three replicates were used for craniofacial morphometrics. For each trial, 60 randomly distributed embryos were placed into 6-well plates (10 embryos per well) and exposed to glyphosate for up to 96 h. In all cases, after 48 h of exposure, 50% of the medium was replaced by a recently prepared solution. Plates

were incubated at 28.5°C, and the biological parameters of survival and hatching were evaluated every 24 h. No differences were observed in either survival or hatching rates among concentrations. After exposure, five embryos per group were selected and raised for 10 months until adulthood for skeletal assessment. Glyphosate exposure was performed according to the guidelines of the Institutional Animal Care and Use Committee of CIAD (Centro de Investigación en Alimentación y Desarrollo, A. C.).

Cardiac rate

The cardiac rate was measured at 48 and 72 hpf. Embryos were placed under the Stemi 305 stereoscopic dissection microscope coupled to a D3200 digital camera in a lateral position to visualize the heart, and heartbeats were recorded over 30 s and expressed in beats per minute (bpm). Three randomly selected embryos from each of the five repetitions were recorded for each glyphosate concentration.

Locomotor activity

Locomotor activity was measured by counting spontaneous tail movements at 24 hpf, with some modifications. Individual 1-min recordings were collected for 15 embryos per group. Each embryo was categorized depending on its motile capacity (i.e., number of tail movements within one minute) as follows: a) the non-motile embryos (NME) were those without spontaneous movements; b) moderately mobile embryos (MME) were those with a maximum of three spontaneous movements; and c) highly motile embryos (HME) were those that displayed more than four tail movements.

Craniofacial morphometrics

The morphological measurements of craniofacial cartilage were conducted following the method of Carvan et al. (2004). In addition, the authors incorporated Meckel's-Palatoquadrate (M-PQ) angle due to its relevance in analysing toxicity in zebrafish. Embryos at 96 hpf were anesthetized using tricaine and fixed with 4% formaldehyde overnight at 25ºC. Embryos were dehydrated stepwise in ethanol, stained with Alcian Blue solution (70% ethanol, 30% acetic acid, and 2 mg/mL Alcian Blue), and stored in glycerol-KOH. Fifteen stained embryos per group were photographed with the Stemi 305 Stereo Microscope coupled to the D3200 digital camera. The otic capsule length (OT), lower jaw length (LJL), ceratohyal cartilage length (CCL), hyosymplectic cartilage length (HS), intracranial distance (ID), the distance between Meckel's cartilage and the palatoquadrate cartilage (MPQ), and the distance between Meckel's cartilage and basihyal cartilage (MBH) were measured in mm and analysed with Fiji software.

Protein isolation and western blot analysis

Twenty embryos from the control and exposed groups were homogenized using a cellular homogenizer in lysis buffer (2% β-mercaptoethanol, 1% SDS, 20 mM EGTA, 2 mM Tris-HCl at pH 7.5, and protease inhibitors cocktail). The lysate was centrifuged at 6000×g for 10 min at 4ºC, and the proteins were precipitated with acetone at -20ºC overnight. The precipitated protein samples were dissolved in a lysis buffer. The total protein concentration was estimated using Bradford reagent. Protein samples were resuspended in sample buffer (0.1 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.2% bromophenol blue) and boiled for 5 min. The proteins (20 µg per lane) were separated by electrophoresis under reducing conditions with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly (vinylidene fluoride) (PVDF) membranes. For western blot analysis, the PVDF membranes were blocked with 6% milk in TBST (in mM: 10 Tris HCl and 75 NaCl, pH 8.0, 0.1% Tween 20) for 2 h and then incubated with the corresponding primary antibody. The anti-ERα antibody (SC-543) and anti-BSP antibody (SC-73634) were obtained from Santa Cruz Biotechnology. The anti-alpha tubulin antibody (T6199) and the anti-OPN antibody (ab166709) were obtained by Sigma-Aldrich and Abcam, respectively. After incubation with the primary antibody, the membranes were incubated with the corresponding secondary antibody conjugated to horseradish peroxidase (HRP). Detection was performed by chemiluminescence in a ChemiDoc XRS system using Image Lab Software.

Adult skeletal assessment

Adult fish (10 months) from the glyphosate exposed groups (n = 5 per group) were euthanized with tricaine and fixed with 4% formaldehyde overnight at 25ºC. Alizarin Red staining was performed. Samples were rinsed several times with phosphate buffered saline with 0.1% Tween 20 (PBST) and then dehydrated with ethanol overnight at 50%, 70%, and 100%. Subsequently, samples were incubated in

2 mg/mL of Alizarin Red in 1% potassium hydroxide (KOH) for 48 h. Pigmentation was bleached with H_2O_2 (3%) and KOH (0.5%) for 40 min. After which, fish were incubated with 25% glycerol/0.1% KOH for 48 h and rinsed in a 50% glycerol and 0.1% KOH solution to remove excess stain. Then, samples were kept at 4ºC in the same solution. Stained fish were observed and photographed to detect morphological alterations in the spine and skull using the Stemi 305 Stereo Microscope coupled to the D3200 digital camera. Craniofacial measurements of the length and height of the orbital cavity (LOC and HOC, respectively), skull length (SL), ID, LJL, width where the ceratohyal and palatoquadrate cartilage join (WCP), and the distance between the mandibular symphysis and the ceratohyal (DMSC), were measured in mm. The analysis of the data was performed using Fiji software.

Data analysis

Comparisons among groups with regard to the cardiac rate, craniofacial morphometrics, and protein level of embryos and adult skeletal measurements were evaluated by one-way analysis of variance (ANOVA) and post-hoc Tukey tests. Results were considered significant at $p < 0.05$. Shapiro-Wilk and Levene tests were used to evaluate normality and homoscedasticity. If the normality assumption was not fulfilled, a non-parametric Kruskal-Wallis test was used. Spontaneous tail movement comparisons at 24 hpf were conducted using Chi-square category tests. All analyses were performed in the R environment (R Core development team, 2018).

Results

Changes in heart rate and tail movements

At 96 hpf, no differences were observed in the survival rate (1 µg/mL = 98% and 100% in the other concentrations) or hatching rate (0 and 10 µg/mL = 98% and 100% of the embryos in the other groups hatched at 72 hpf). The heart rate showed a differential response related to the developmental stage. Compared to that of the control, lower heart rates were consistently observed in all exposed groups. Significant differences in heart rate were observed for the 10 µg/mL test concentration at 48 hpf, whereas at 72 hpf, significant reductions of ~10 and 12 bpm were observed with the 10 and 50 µg/mL test concentrations, respectively. A negative correlation between glyphosate concentration and the number of beats per minute was only observed at 72 hpf ($\rho = -0.36$, $p < 0.01$). Glyphosate appeared to alter the locomotor activity of zebrafish embryos. In general, most of the organisms were classified within the MME category, with significant differences observed with the 50 µg/mL test concentration (chi-square = 6, df = 2, $p = 0.04$).

Craniofacial alterations

Some craniofacial structures were responsive to glyphosate exposure. In particular, the CCL showed shortened lengths at all concentrations, with significant differences observed with the 50 µg/mL test concentration ($p < 0.05$). At this concentration, similar length reductions were observed for the MPQ, HS, and ID, although with higher variability and no significant differences. The M-PQ also shows significant differences and high variability.

Modification of proteins related to the ossification process

Levels of OPN were upregulated 4-fold in embryos exposed to glyphosate concentrations of 5 µg/mL or higher, with a significant difference observed at 50 µg/mL. A similar response was observed for BSP but with significant differences observed at concentrations of 5 µg/mL or higher. The level of ERα was also significantly higher at 10 and 50 µg/mL and was more than 2-fold higher in the 50 µg/mL exposed group compared to that of the control.

Early exposure to glyphosate generated bone defects in adulthood

Adult fish embryonically exposed to glyphosate showed distinctive bone deformities. Fifteen and 9.8% of the adults exposed to 50 and 10 µg/mL concentrations, respectively, presented marked curvatures in the spine as adults as well as deformities towards the caudal region of the column, which resembled the patterns of scoliosis. In contrast, the morphometric analysis of craniofacial measurements in adult fish exposed to glyphosate as embryos indicated that differences in the cranium were not present between the exposed groups and the control group.

Conclusion

The authors concluded that the changes induced by glyphosate regarding estrogenic activity and proteins related to the formation of bones and cartilage, such as OPN and BSP, could be related to the presence of skeletal deformities in both embryos and adult zebrafish. Evidence indicates that glyphosate is able to interfere with a variety of endocrine signalling systems, including steroid hormone signalling, while oestrogens could induce major disruptions in cartilage and bone formation, indicating that precise control of oestrogen signalling is required for the appropriate development of skeletal structures. Whereas further studies are necessary to corroborate the mechanisms linking the regulation of bone development proteins through ERα modifications, this study provides evidence that early and short exposure to glyphosate can produce skeletal alterations in zebrafish, which are likely to have ecotoxicological consequences on growth, reproduction, and survival.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This article used the zebrafish model to assess the effects of early life glyphosate exposure on the development of cartilage and bone tissues and organismal responses. The evidence suggests functional alterations, including a reduction in the cardiac rate, significant changes in the spontaneous tail movement pattern, and defects in craniofacial development. These effects were concomitant with alterations in the level of the oestrogen receptor alpha osteopontin and bone sialoprotein. Embryos exposed to glyphosate presented spine deformities as adults. These developmental alterations are likely induced by changes in protein levels related to bone and cartilage formation.

This article is of importance for the assessment of the endocrine disrupting properties of glyphosate. It reports the acute and chronic effects on zebra fish. The study seems to have been well conducted and reports a regulatory relevant and reliable endpoint: 96 h NOEC = 1 mg/L (based on the effects on bone sialoprotein (BSPII) relative expression).The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase. Temperature during the test is a bit high for the species tested.

Assessment and conclusion by RMS:

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Zebrafish embryos used for the present study were generated from individuals obtained from commercial distributors and cultured in the lab.

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in the buffer solution, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, purity and CAS number reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Batch specifications are not provided and the assessment of the ecotoxicological equivalence cannot be conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were analytically verified only in the stock solutions
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: NOEC (96-h)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	No positive control tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Concentrations were analytically verified only in the stock solutions
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Uncertain	Temperature set at 28.5°C for the tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	Concentrations were analytically verified only in the stock solutions, but not during the test
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	Concentrations were analytically verified only in the stock solutions, but not during the test
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical	Yes	The concentration of glyphosate

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
methods is included.		in-stock solution was determined by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS)
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No assessment of the ECX median values was conducted.

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This article is of importance for the assessment of the endocrine disrupting properties of glyphosate. It reports the acute and chronic effects on zebra fish. The study seems to have been well conducted and reports a regulatory relevant and reliable endpoint: 96-h NOEC = 1 mg/L (based on the effects on bone sialoprotein (BSPII) relative expression). The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase. Temperature during the test is a bit high for the species tested.
Not reliable	No	-

1. Information on the study

Data point:	CA 8.2.2, CP 10.2.2
Report author	Le Du -Carrée J. *et al.*
Report year	2021
Report title	Developmental effect of parental or direct chronic exposure to environmental concentration of glyphosate on the larvae of rainbow trout, *Oncorhynchus mykiss*
Document No	Aquatic Toxicology, 2021, 237, 105894
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

This study investigated the impact of parental and direct exposure to 1 µg/L of glyphosate using the active substance (AS) alone or one of two Glyphosate-Based Herbicide (GBH) formulations (i.e. Roundup Innovert® and Viaglif Jardin®) in the early developmental stages of rainbow trout. Three different modes of exposure on the F1 generation were studied: (1) intergenerational; (2) direct and (3) multigenerational. The impact of chemical treatments on embryo-larval development (survival, biometry and malformations), swimming behaviour, biochemical markers of oxidative stress equilibrium (TBARS and catalase), acetylcholine esterase (AChE) and energy metabolism (citrate synthase, CS; cytochrome-c oxidase, CCO; lactate dehydrogenase, LDH; glucose-6-phosphate dehydrogenase, G6PDH) was explored. Chemical exposure did not affect the survival of F1 embryos or malformation rates. Direct exposure to the AS induced some biometric changes, such as reduction in head size (with a 10% decrease in head length), independently of co-formulants. Intergenerational exposure to the AS or the Roundup GBH increased swimming activity of the larvae, with increase of between 78 and 102% in travel speeds. Viaglif co-formulants appear to have counteracted this behavioural change. The minor changes detected in the assayed biochemical markers suggested that observed effects were not due to oxidative damage, AChE inhibition or alterations to energy metabolism. Nonetheless, multi- and intergenerational exposure to Roundup increased CS:CCO and LDH:CS ratios by 46% and 9%, respectively, with a potential modification of the aerobic-to-anaerobic energy production balance. These biochemical effects were not correlated with those observed on individual level of biological organization. Therefore, further studies on generational toxicity of glyphosate and its co-formulants are needed to identify the other mechanisms of glyphosate toxicity at the cellular level.

Materials and methods

Ethics statement

Assays on fish were done in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). Experimental procedures were validated by the animal ethics committee ANSES/ENVA/UPC No. 16 and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS#2019010812403065). A lethal dose of 100 ppm of eugenol into tank water was used to euthanize fish.

Chemical compounds

We used the AS glyphosate (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6) and two GBHs, Roundup Innovert® (R) and Viaglif Jardin® (V). The purity of G was 98%, and the glyphosate concentration of Roundup and Viaglif were 360 and 420 g/L, respectively. For each product, aqueous concentrated solutions (4 mg/L) were prepared and stored under appropriate conditions (darkness, 4ºC). Pure glyphosate was diluted first in 10 mL of pure methanol (solvent concentration of concentrated solution was 10 mL/L so the final dose of methanol exposure was kept under 4 µL/L).

Fish

Specific pathogen free mature rainbow trout (3 years old; F0 generation) were exposed daily for 8 months to control (C) or to a mean glyphosate concentration of 123 ng/L using pure glyphosate, Roundup or Viaglif, before producing the F1 generation. Embryonic development was conducted in a shallow bottom tank containing approximately 300 L. All fish were maintained in filtered river water, with a water flow rate to ensure complete renewal once an hour and maintain appropriate physicochemical conditions and oxygen saturation greater than 60%. A photoperiod of 12 h of daylight was maintained throughout the experiments. Fish feed (Le Gouessant), adapted for fish size, was given ad libitum.

Reproduction and embryonic development

Reproduction of fish from F0 generation is described in the article of Le Du-Carrée *et al*. Embryonic development of the F1 generation until the eyed stage was conducted in tanks, containing approximately 300 L, continuously renewed with river water (at a flow rate of approximately 300 L/h). After the eyed stage, rainbow trout embryos were placed in 10 tanks (40 L) in a confined room. Temperature was maintained at 8º±2ºC throughout embryonic development.

Chemical exposure

Once the embryos reached the eyed stage, they were exposed to 10 conditions of chemical exposure. The name of each condition is composed of two letters separated by a slash, the first letter represents the chemical exposure of the F0 generation and the second letter represents the chemical exposure of the F1 generation. As an example, fish from non-contaminated parents, but directly contaminated make up the C/G, C/R, and C/V conditions. Chemical exposure of the F1 generation was conducted using the same methodology as that used for the F0: every working day (generally 5 days a week), 10 mL of the condition respective concentrated chemical solution was added to the experimental tanks in which water input was stopped for 1 h. After 1 h of contact, water flow was set to 13.5 L/h for the rest of the day, resulting in the gradual dilution of glyphosate. The theoretical kinetics of glyphosate concentrations was modelled and the integrated mean daily expected concentration was approximately 123 ng/L.

Samples and sampling dates

To measure hatching frequencies, egg survival was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes until all eggs were either dead 34 or hatched. The hatched eggs were placed in plastic breeding boxes 35 days after hatching.

For each condition, 25 larvae were sampled at 320 degree-day (DD; sampling date S1) after 14 days of direct exposure and at 328DD (S2) for intergenerational exposure. They were placed in a 3% glutaraldehyde solution, at 4ºC until biometric and malformation analyses. At 488DD (S3), 25 larvae were sampled in each tested condition to measure oxidative stress and metabolic markers. Whole larvae were flash-frozen in liquid nitrogen and stored at -80 ºC for future analyses. Proteins were extracted by homogenizing whole larvae in phosphate buffer (0.1 M, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor using the tissue homogenizer Precellys 24 (Bertin Technologies, France).

Determination of glyphosate concentrations in exposure tank

Glyphosate was quantified in water after two months of chemical contamination using a direct competitive ELISA assay (Novakits, ref. 1500086) at two time points in the daily experimental procedure (i.e. just before restarting the water flow [wfr] and 2 h after wfr). A total of 250 µL of filtered water was used to conduct the ELISA assay and final absorbance was read at 450 nm on a TECAN's Spark 10M microplate spectrophotometer. The four-parameter log-logistic function, LL.4, of the "drc" R package, was used to generate the standard curve. The OD value obtained for the sample was plotted on the quantification standard curve to determine the glyphosate concentration in each sample.

Biometric index measurements and malformation analyses

Biometric index measurements and malformations were analysed on images of individual larvae taken on a stereo microscope (Leica MZ75) combined with a ToupCam camera (U3CMOS05100KPA). Body and head length, eye diameter and yolk-sac surface were measured using the software ToupView 3.7

Swimming behaviour analysis

The protocol of photomotor assay was adapted from the study of Weeks Santos et al. (2019) and applied to swimming larvae (i.e. 855DD; S4) maintained at 11ºC throughout the experiment. To avoid perturbation due to the circadian rhythm, analyses were done during a maximum duration of 4 h per day for three days. An infrared camera coupled to the DanioVision (Noldus, version 12.1) system was used to record the behaviour of larvae placed in six-well cell culture plates (Nunc, ref. 140685) with each flat well containing 2 mL of water. For each assay, larvae were acclimated in the plate for 10 min in the dark before the measurements began. The 30 min recording was divided into three phases at different light intensities: 10 min of darkness (Dark 1), followed by 10 min of light (light 1), finally followed by 10 min of darkness (Dark 2). The distance (in meters) travelled by each larva during these three light-darks phases was then used to compare the effect of the different chemical treatments.

Assays for oxidative stress and metabolic markers and choline esterases

Colorimetric analysis was carried out on a TECAN Spark 10M microplate spectrophotometer. Choline esterases (ChE) were measured at 412 nm. Each assay was performed in duplicate or triplicate. Oxidative stress markers were assayed in whole larvae homogenates at 532 nm and 240 nm, respectively. Metabolic markers were measured in whole larvae samples at 412 nm, 550 nm, 340 nm and 340 nm. The slope of the optical density f time curve plotted on a calibration curve allowed the calculation of enzymatic activity. Calibration curves were generated using pure enzymes purchased at Sigma-Aldrich. Protein concentrations were measured using the Pierce BCA protein assay kit (ThermoFisher Scientific), and results were expressed as specific activity (IU/mg of protein).

Data processing and statistical analyses

Statistical analyses and data processing were done using R software (R Core Team, 2018). Data sets were tested for normality (Shapiro-Wilk) and homoscedasticity (test of Levene for parametric data and Fligner-Killeen for non-parametric data). When normal and homoscedastic data were confirmed, one-way ANOVA tests were used to compare means, followed by Dunnett's post-hoc test. For normal and heteroscedastic data, modified one-way ANOVA were employed to compare means, followed by Tamhane-Dunnett post-hoc test. For non-normal data, a Kruskal-Wallis test was used to compare means, followed by Dunn's post-hoc test. Differences between hatching and malformation rates were compared using a chi-squared test. Survival rates for the different chemical treatments were compared using the log-rank test in the "survival" R package. A p -value of 0.05 was used as the threshold for statistical significance.

Results

Glyphosate concentration in water

Glyphosate was never detected in the control tank during the experiment. Glyphosate concentrations between 1.18±0.036 and 1.95±0.086 µg/L were detected in all contaminated tanks 1 h after adding the chemical solutions. Two hours after restarting the water flow, measured concentrations were slightly below those predicted by the theoretical kinetics (from 0.30±0.015 to 0.34±0.021 instead of expected value of 0.51 µg/L).

Hatching and larvae survival, malformations and biometric indices

Neither the hatching percentage nor the larval survival rate during the 35 days after hatching was significantly affected by chemical exposure.

No yolk-sac oedemas were observed. Jaw appeared in larvae at frequencies varying from 0 to 4%, depending on the exposure condition. Spinal curvature was the most frequent malformation observed (12 to 24%). Chemical treatments had no impact on malformation rates.

No statistical significant differences between control and directly contaminated larvae were observed for body length, yolk-sac surface or eye:head length ratio. However, there were significant reductions in head ($p < 0.0001$, $df = 3$ and $f = 9.46$), eye diameter ($p < 0.0001$, $df = 3$ and $\chi^2 = 28.06$) and head:body length ratio ($p < 0.008$, $df = 3$ and $f = 6.51$). For head length, post-hoc tests revealed significant differences for the C/G (-11%), C/R (-11%) and C/V (-8%) conditions compared with the control ($p < 0.05$). For eye diameter, significant differences were observed for the C/G (-11%), C/R (-10%) and C/V (-7%) conditions ($p < 0.05$). For head:body length ratio, significant differences were observed for the C/G (-7%), C/R (-6%) and C/V (-6%) conditions compared with the control ($p < 0.05$). Intergenerational exposure induced no statistically significant differences between control and chemically contaminated larvae for most of the biometric indices considered. However, a significant effect of chemical concentration was observed in body length ($p < 0.0001$, $df = 3$ and $\chi^2 = 45.05$) and in the ratio between eye and head length ($p = 0.0004$, $df = 3$ and $f = 6.56$). Post-hoc tests revealed significant increases compared with the control, with +7% in body length for the R/C condition and +6% in eye:head length ratio for the V/C condition ($p < 0.05$).

Metabolic activity in whole larvae

No major significant change in mean activity was observed at 488DD for the oxidative stress markers (i.e. CAT and TBARS), or for AChE and the metabolic markers (i.e. CS, CCO, G6PDH) except for LDH ($p = 0.004$, $df = 9$ and $\chi^2 = 24.02$). A post-hoc test revealed a significant increase in LDH activity for the R/R condition compared with the control (+16%, $p < 0.05$).

Means of CS:CCO ratio values were $8.8 \times 10^{-3} \pm 5.2 \times 10^{-4}$ for the control condition and comprised between $8.0 \times 10^{-3} \pm 4.5 \times 10^{-4}$ and $9.7 \times 10^{-3} \pm 4.2 \times 10^{-4}$ for the other conditions except for the R/R condition which showed a ratio of $13 \times 10^{-3} \pm 1.7 \times 10^{-3}$. This latter ratio was significantly different to the control condition (global mean difference: $p = 0.004$, $df = 9$ and $\chi^2 = 24.27$; +46%, $p < 0.05$).

For the LDH:CS ratio, values were 458.98 ± 7.18 for the control condition, comprised between 458.56 ± 8.42 and 482.19 ± 13.62 for the other conditions, except for R/C and R/R, which showed values of 512.21 ± 10.57 and 501.93 ± 9.09, respectively. For the LDH:CS ratio, a statistical difference was observed among all the conditions ($p = 0.002$, $df = 9$ and $\chi^2 = 25.86$) and a post-hoc test revealed significant differences to the control for the R/C (+12%, $p < 0.05$) and R/R (+9%, $p < 0.05$) conditions.

Swimming behaviour of intergenerationally exposed trout

During the photomotor assay, larvae travelled approximately 4.95 ± 0.49 m and 6.86 ± 0.51 m in the first and the second period of darkness. Speed dramatically decreased in the presence of light with a mean travelled distance of 0.81 ± 0.12 m. No effect of chemical exposure was found in the presence of light, but global mean differences were observed between distance travelled by larvae in the first ($p < 0.0001$, $df = 3$ and $\chi^2 = 30.46$) and in the second period of darkness ($p < 0.0001$, $df = 3$ and $\chi^2 = 41.78$). Post-hoc tests revealed significant differences in means obtained for the G/C and R/C conditions compared with the control during the first period of darkness (+83 and 102%, respectively, $p < 0.05$) and during the second period of darkness (+78 and 83%, respectively, $p < 0.05$).

Conclusion

The authors studied the effect of parental and/or direct exposure to an environmental concentration of glyphosate, focusing on the embryo-larval development of the F1 generation. Although no effect was shown on embryo or larval survival rates regardless of the mode of exposure or the contaminants considered, some effects were observed at different levels of biological organization. Glyphosate, apparently independently of co-formulants and only in the case of direct exposure, appeared to induce developmental changes, such as reductions in head size and that of associated organs, but did not cause developmental malformations. Intergenerational exposure to glyphosate increased the swimming activity in larvae, without any correlation with AChE inhibition. The authors indicated that this behavioural change may potentially have an important impact on larvae survival in a stressful natural environment. Viaglif co-formulants seemed to counteract this behavioural change. No oxidative stress response or damage was detected in rainbow trout larvae. Nonetheless, parental exposure to one of the two tested GBHs may modify energy production by increasing the amount of energy produced via anaerobic metabolism compared with that produced by aerobic metabolism. Also, multigenerational exposure to the same GBH may potentially cause a reduction in the capacity of mitochondria for energy production. These effects, not detected with the AS alone, were probably not responsible for the other effects observed. Thus, there are likely other mechanisms of glyphosate toxicity at the cellular level and further investigations are needed to fully understand how they are responsible for both direct and generational toxicity of glyphosate and its co-formulants.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study investigates the impact of parental and direct exposure to 1 µg/L of glyphosate using the active subtance alone or one of two Glyphosaet-Based Herbicide formulations (i.e. Roundup Innovert® and Viaglif Jardin®) in the early developmental stages of rainbow trout. Three different modes of exposure on the F1 generation were studied: (1) intergenerational (i.e. fish only exposed through their parents); (2) direct (i.e. fish exposed only directly) and (3) multigenerational (i.e. fish both exposed intergenerationally and directly). The impact of chemical treatments on embryo -larval development (survival, biometry and malformations), swimming behaviour, biochemical markers. Chemical exposure did not affect the survival of F1 embryos or malformation rates. Direct exposure to the a.s. induced some biometric changes, such as reduction in head size (with a 10% decrease in head length), independently of co -formulants. Intergenerational exposure to the a.s. or the Roundup GBH increased swimming activity of the larvae, with increase of between 78 and 102% in travel speeds. Therefore, 1 generation LOEC = 1 µg/L.
The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.

Assessment and conclusion by RMS:

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline -compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non -guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals were cultured in the lab.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non -toxic) and a carrier control / positive control is considered in the test design.	Uncertain	Pre -dilution of pure glyphosate was done in pure methanol in a concentration so the final dose of methanol exposure was kept under 4 µL/L
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, purity, CAS number and content reported
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	The assessment of the ecotoxicological equivalence was not conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non -guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Concentrations were analytically verified just before and 2-h after restarting the water flow

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: LOEC (1 generation)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Just 1 concentration was tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	No	Only 1 concentration was tested
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time -points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5 -8.0), cation exchange capacity, organic carbon (0.5 -2 -5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A -horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/ - 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20 -25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	River water filtered
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15 -25ºC.	Uncertain	Temperature set at 8°C during the embryonic development and at 11°C for the swimming behaviour analysis. Main test temperature not reported
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	-
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		time was generated.
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	The concentration of glyphosate in water was determined by HPLC and fluorometric methods
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No assessment of the ECX median values was conducted.

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study investigates the impact of parental and direct exposure to 1 µg/L of glyphosate using the AS alone or one of two GBH formulations (i.e. Roundup Innovert® and Viaglif Jardin®) in the early developmental stages of rainbow trout. Three different modes of exposure on the F1 generation were studied: (1) intergenerational (i.e. fish only exposed through their parents); (2) direct (i.e. fish exposed only directly) and (3) multigenerational (i.e. fish both exposed intergenerationally and directly). The impact of chemical treatments on embryo -larval development (survival, biometry and malformations), swimming behaviour, biochemical markers. Chemical exposure did not affect the survival of F1 embryos or malformation rates. Direct exposure to the AS induced some biometric changes, such as reduction in head size (with a 10% decrease in head length), independently of co -formulants. Intergenerational exposure to the AS or the Roundup GBH increased swimming activity of the larvae, with increase of between 78 and 102% in travel speeds. Therefore, 1 generation LOEC = 1 µg/L. The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.
Not reliable	No	-

1. Information on the study

Data point:	CA 8.2.2, CP 10.2.2
Report author	Le Du-Carrée J. *et al.*
Report year	2021
Report title	Generational effects of a chronic exposure to a low environmentally relevant concentration of glyphosate on rainbow trout, *Oncorhynchus mykiss*
Document No	Science of the Total Environment, 2021, 801, 149462
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors investigated the impact of an environmentally relevant concentration of glyphosate and its co -formulants on an F2 generation issued from exposed generations F0 and F1. Trans, inter and multigenerational toxicity of 1 µg/L of the active substance was evaluated on early stages of development and juvenile rainbow trout (*Oncorhynchus mykiss*) using different molecular, biochemical, immuno-hematologic, and biometric parameters, behaviour analysis, and a viral challenge. Reproductive parameters of generation F1 were not affected. However, developmental toxicity in generation F2 due to glyphosate alone or co -formulated was observed with head size changes (e.g. head surface up to +10%), and metabolic disruptions (e.g. 35% reduction in cytochrome -c -oxidase). Moreover, larvae exposed transgenerationally to Viaglif and intergenerationally to glyphosate and Roundup presented a reduced response to light, potentially indicating altered escape behaviour. Overall methylation was, however, not altered and further experiments using gene -specific DNA methylation analyses are required. After several months, biochemical parameters measured in juvenile fish were no longer impacted, only intergenerational exposure to glyphosate drastically increased the susceptibility of rainbow trout to hematopoietic necrosis virus. This result might be due to a lower antibody response in exposed fish. The authors concluded that their results show that generational exposure to glyphosate induces developmental toxicity and increases viral susceptibility. Co -formulants present in glyphosate -based herbicides can modulate the toxicity of the active substance. The authors indicated that further investigations are required to study the specific mechanisms of transmission but their results suggest that both non -genetic mechanisms and exposure during the germinal stage could be involved.

Materials and methods

Ethics statement

Fish experimentation was carried out in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). Experimental procedures were validated by the animal ethics committee ANSES/ENVA/ UPC No. 16 and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS#2019010812403065). Euthanasia involved the addition of a lethal dose of 100 ppm of Eugenol into the tank water.

Chemicals

Three chemical compounds were tested: glyphosate active substance (G; Sigma -Aldrich, ref. 45,521, CAS Number 1071 -83 -6) and two GBHs: Roundup Innovert® (R; Agrilisa - for professional use) and

Viaglif Jardin® (V; Agrilisa - for home gardens). The purity of G was 98%, and the concentrations of R and V were 360 and 420 g/L of glyphosate, respectively.

Fish

Experiments were conducted using specific pathogen -free (SPF) rainbow trout reared in the protected and monitored fish facilities of the ANSES Plouzané Laboratory site (France).

Fish were maintained in tanks of 40 L (juveniles) and 400 L (adult) containing river water filtered with sand filter (approximately 20 µm), with a water flow rate to ensure complete renewal once an hour and maintain appropriate physicochemical conditions and oxygen saturation greater than 60%. The experimental facilities were supplied with river water with a mean pH of 7.8, a conductivity of 400 µS/m and a hardness of 12°TH. Concentrations of ammoniac, nitrate and nitrite are close to 0. Physicochemical analyses of the water were regularly carried out to guarantee excellent maintenance conditions for all the fish. A photoperiod of 12 h of daylight was maintained throughout the experiments. After the eyed stage period, embryos were placed in 8 tanks (40 L) positioned in a confined room. Temperature during embryonic development was maintained at 8±2°C. After this stage water temperature varied from 6 to 15°C. A trout -specific feed (Le Gouessant®), adapted to the fish size, and was given ad libitum.

Chemical exposure

For each product, concentrated aqueous solutions (4 mg/L in distilled water) were prepared and stored under appropriate conditions (darkness, 4ºC±2). A pre -dilution of pure glyphosate was done in 10 mL of pure methanol (concentration of solvent in concentrated solution was 10 mL/L so the final dose of methanol exposure was kept under 4 µL/L). The F0 and F1 generations were exposed daily for 10 days and 24 months, respectively, to a mean concentration of 123 ng/L of glyphosate using Glyphosate (G); Roundup (R); Viaglif (V). Unexposed control conditions (C) were included. F0 engendered the F1 generation. The F2 generation was produced by 2 to 5 females and 2 to 8 males of the F1 generation. Conditions C/V and V/V were lost during the experiment due to material dysfunction, so 8 conditions only could give birth to the F1 generation.

Reproduction

Procedure of trout reproduction is described in the article of Le Du-Carrée *et al* and embryonic development until the eyed stage was conducted in 300 L tanks containing two breeding boxes by tank and continuously renewed with river water (filtered with sand and cotton wool filters) with a flow rate of approximately 300 L/h.

Experimental design

Chemical exposure

Once the F2 larvae reached eyed stage, rainbow trout embryos were exposed to the 8 conditions. Fish produced from non-contaminated F0 but directly contaminated in F1 were C/G/C, and C/R/C and are considered intergenerationally exposed fish. Fish only contaminated through F0 are considered transgenerationally contaminated, and were G/C/C, R/C/C, and V/C/C. Finally, multigenerational exposure designated fish that were contaminated continuously for three generations. They are represented by the conditions G/G/G and R/R/R. F1 and F2 chemical exposure was conducted with the same methodology as for F0. Theoretical glyphosate concentration kinetics were modelled and the integrated mean daily theoretical concentration was approximately 123 ng/L.

Water sampling was performed in after approximately three months of chemical contamination to measure concentrations of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) in water tanks. Quantification of glyphosate and AMPA was done within 72 h after sampling using HPLC and fluorometric methods (Method ref. ANA -I10.MOA.69.B) by an external provider (Labocea, France).

Viral challenge

The virus used for viral challenge was the N61 strain (genotype E) of the infectious hematopoietic necrosis *IHN* virus (i.e. *IHNv*) isolated from diseased rainbow trout fry displaying typical signs of the disease. The infectious titre of the viral production, was $4x10^7$ $TCID_{50}$/mL. After approximately

6 months of chemical exposure, 280 F2 fish for each chemical treatment were randomly distributed to four 10 L tanks with constant water renewal (i.e. 70 fish per replicate of a condition). Three of these tanks were infected with *IHNv*, and one was used as the uninfected control. Non -infected EPC cell supernatant was used for uninfected control tanks. For 6 weeks after *IHNv* infection, general behaviour, the appearance of clinical signs (lethargy, darkening of the skin, exophthalmia), and mortality were recorded twice a day. Dead individuals were stored at -20°C for viral examination.

Sampling date

Fertility was considered to be the proportion of eggs surviving at 5 days post -fertilization. To perform this measurement, egg survival was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes. Larvae for biometric indices and malformation measurements were sampled at 350 DD (S1) and stored in a 3% glutaraldehyde solution at 4 DD until the analysis. Condition C/R/C was lost during storage and could not be analysed.

At 541 DD (S2), invasive sampling was done on 20 larvae exposed to the different conditions. They were flash -frozen in liquid nitrogen and stored at -80ºC for future analyses.

Eleven days before infection (S4), 96 h post -infection (96 hpi; S5), and 42 days post -infection (42 dpi; S6) to *IHNv*, invasive sampling was done on 20 juvenile rainbow trout aged approximately 6 months, with size and length (mean ± se) of 5.69±5.69 and 83.83±0.75, respectively. A blood sample was taken by withdrawing 10 µL of blood from the caudal vein with a lithium heparin haematocrit tube (Greiner ref. KG454244), and fish were euthanized. Then, at S4, gills were sampled, flash -frozen in liquid nitrogen and stored at -80ºC for future analyses. Between S3 and S4, condition G/G/G was lost due to an incident during fish maintenance and could not be sampled.

Biomarkers analysed

Biometric index measurements and malformation analysis

Biometric index measurements and malformation analysis were done on a picture of larvae taken with a binocular magnifier (Stemi 508) coupled to a camera (Canon DS126431) permitting 40x magnification. Body length (without tail fin), head surface, eye diameter, and yolk sac surface were analysed using ToupView software, version 3.7 on a total of 30 larvae per condition. Frequencies of malformation were determined on larvae considering jaw malformations, yolk sac oedema (not observed), and spinal curvature.

Swimming behaviour analysis

The analysis was done during a time window of seven days with free -swimming larvae (i.e. from 615 to 681 DD, S3 sampling date) maintained at 11 throughout the experiment.

Viral examination and immune parameters during the viral challenge

The presence and concentration of *IHNv* were checked individually from 3 dead fish collected at peak mortality, by replicate chemical condition. Extracted organs (kidneys, spleen, heart, and brain) were pooled and crushed using a mortar and pestle, diluted to 10^{-1} with Eagle medium, and centrifuged for 15 min at 2,000x g at 4°C. The supernatant was then diluted to 10^{-8}, and the virus concentration was determined for each fish. At S5, red blood cell (RBC) and white blood cell (WBC) counts were performed on a Thoma cell haemocytometer using whole blood diluted to 1/200 in Giemsa solution. At S6, detection and semi -quantification of anti -IHNv antibodies in the plasma of surviving fish were performed using a modified procedure.

Methylation

Extraction of total DNA was performed using a DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. Global DNA methylation was then measured on 10 ng of total DNA extracted from whole fish larvae sampled at S2, using a MethylFlash Global DNA Methylation (5 -methylCytosine or 5 -mC) ELISA Easy Colorimetric Kit (Epigentek), following the manufacturer's instructions. Absorbance of the products were measured on a TECAN Spark 10 M microplate spectrophotometer at 450 nm. Calculation of the percent methylated DNA for each sample was carried out by reporting the optical density values on the standard curve.

Oxidative stress and metabolic parameters

Choline esterases (ChE), oxidative parameters, namely thiobarbituric acid reactive substances (TBARS), catalase (CAT), and glutathione peroxidase (GPx), and metabolic parameters, i.e. citrate synthase (CS); cytochrome c oxidase (CCO); lactate dehydrogenase (LDH); and glucose -6 -phosphate dehydrogenase (G6PDH), were assayed in larvae (sampled at S2) and gills of juvenile (sampled at S4) following the same procedures described in a previous article (Le Du-Carrée et al., 2021).

Data processing and statistical analyses

Statistical analyses and data processing were performed with R software (R Core Team, 2018). Figures were generated using the ggplot2 package. Quantitative data sets were tested for normality (Shapiro-Wilk) and homoscedasticity (test of Levene for parametric data and Fligner-Killeen for non-parametric data). When normal and homoscedastic data were confirmed, one -way ANOVA tests were used to compare means, followed by a post -hoc test of Dunnett. In the case of normal and heteroscedastic data, modified one -way ANOVA tests were used to compare means, followed by a post -hoc test of Tamhane-Dunnett. In the case of non-normal data, a Kruskal-Wallis test was used to compare means, followed by a post -hoc test of Dunn. Differences between malformation rates were compared using a chi -squared test. Survival rates for the different chemical treatments were compared using the "survival" package. A p -value of 0.05 was used as the threshold for statistical significance. A test of correlations between variables was carried out using the lmrob R function (R package robustbase).

Results

Glyphosate concentrations in exposure tank water

Validation of the experimental chemical contamination procedure for the exposure of F0 and F1 generation trout in 400 L tanks was performed in a previous experiment. During this experiment, the river water supplying the tanks did not present any detectable concentration of glyphosate. After 1 h of exposure and just before water flow reopening, concentrations of glyphosate of 0.54 and 0.57 µg/L were quantified in tanks contaminated with glyphosate and Roundup. These concentrations were 46 and 43% below the theoretically expected value of 1 µg/L. One hour after water flow reopening, the concentration of glyphosate in the tanks contaminated with the active substance was close (2% variation) to the expected concentration of 0.71 µg/L, whereas it was 47% lower in the Roundup contaminated tanks. Two hours after restarting water flow, glyphosate concentrations were 45% below expected values for both tanks (0.28 instead of 0.51 µg/L theoretically). AMPA, the main metabolite of glyphosate, was not detected in any of the samples including those artificially contaminated with the active substance or Roundup.

Fertility and fecundity of the F1 generation

Relative fecundity of the F1 generation aged of two years varied between 1.79±0.61 and 3.05±0.40 eggs/g, regardless of the condition, with no detectable impact of the chemical contamination (Table 1). Fertility was calculated for control and intergenerationally contaminated fish of the F2 generation. It was greater than 97% regardless of the condition considered. No statistically significant difference was measured.

Table 1. Relative fecundity of the F1 generation (expressed in eggs/g; mean ± standard error, se) as a function of the chemical exposure conditions ($2 \leq n \leq 5$).

Mode of exposure	Condition	Relative fecundity	
		Mean	se
Control	C/C	2.17	0.44
Trans.	G/C	2.85	0.28
	R/C	2.10	0.33
	V/C	3.05	0.40
Direct	C/G	2.27	0.38
	C/R	1.79	0.61
Multi.	G/G	2.14	0.86
	R/R	2.53	0.44

Biometric indices and malformations observed on the F2 generation

No differences between control and chemically contaminated F2 larvae sampled at S1 were observed for body length and yolk sac surface (Table 2). Different chemical conditions induced significant changes in other biometric indices, such as an increase in head surface, df = 6 and $\chi^2 = 30.50$), eye surface ($p < 0.0001$, df =6 and $\chi^2 = 43.56$), and eye:head surface ratio ($p = 0.001$, df = 6 and $\chi^2 = 26.97$). For head surface, a post -hoc test revealed significant differences for the G/C/C (+10%) and G/G/G (+9%) conditions, compared to the control ($p < 0.05$). For eye surface, a post -hoc test revealed a significant increase (+9%) for the multigenerationnally exposed G/G/G condition ($p < 0.05$). For eye:head surface ratio, a post -hoc test revealed a significant reduction for C/G/C (-5%) and R/R/R (-9%) compared to the control ($p < 0.05$).

Table 2. Mean biometric indices measured at 350 (S1) in F2 larvae exposed directly or through their parents to glyphosate or GBHs. Standard errors are given in parentheses under each respective mean (n = 30). Lengths are expressed in, surfaces in and ratios in %. Numbers in bold with an asterisk indicate conditions significantly different ($p < 0.05$) from the control.

Parameter	Mode of exposure						
	Control	Transgenerational			Inter.	Multigenerational	
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	G/G/G	R/R/R
Body length	14.9 (0.121)	15.35 (0.148)	15.01 (0.113)	15.15 (0.117)	15.12 (0.091)	15.42 (0.09)	14.36 (0.186)
Head surface	6.25 (0.089)	**6.89*** (0.196)	6.32 (0.138)	6.39 (0.129)	6.47 (0.109)	**6.82*** (0.114)	5.85 (0.179)
Eye surface	1.17 (0.023)	1.34 (0.097)	1.21 (0.022)	1.2 (0.025)	1.15 (0.018)	**1.28*** (0.03)	1.01 (0.051)
Eye:head surface ratio	18.78 (0.29)	19.11 (0.71)	19.18 (0.27)	18.8 (0.25)	**17.89*** (0.2)	18.71 (0.4)	17.04 (0.67)

Spinal curvatures and jaw malformations were detected in F2 larvae (the analyses were done on a total of 89 113 larvae per condition). Low frequencies of jaw malformations were observed for all conditions, with proportions ranging from 0 to 7%. Spinal curvature was the most frequent malformation detected, with frequencies ranging from 0 to 11%. No yolk sac oedema was observed. Chemical treatments did not induce statistically significant induction of malformations compared to the non-exposed condition (i.e. the control).

Metabolic activity

Chemical contamination induced changes in certain enzymatic levels in F2 larvae sampled at S2 (Table 3). While no changes were observed for AChE, LDH, or TBARS regardless of the chemical condition considered, statistically significant reductions in enzymatic activities were detected for CAT, CCO, and CS. CAT activity was affected by chemical exposures ($p = 0.003$, df = 7 and f = 3.25), with a significant reduction of 13, 18, 15, and 23% found by a post -hoc test for the G/C/C, G/G/G, R/C/C and V/C/C conditions, respectively, compared to the control ($p < 0.05$). CCO and CS activities were also affected by chemical exposure ($p < 0.0001$, df = 7 and f = 12.30; $p = 0.04$, df = 7 and f = 2.14, respectively), with reductions for G/C/C and V/C/C comprised between 12 and 33%, compared to the control ($p < 0.05$). A reduction of 35% in CCO activity was also observed between the control and G/G/G ($p < 0.05$). The ratio between CS and CCO activities (CS:CCO ratio) was affected by chemical contamination ($p < 0.0001$, df = 7 and $\chi^2 = 53.60$). Increases of 33 and 31% were observed for G/C/C and G/G/G compared to the control, respectively ($p < 0.05$). The LDH:CS ratio was also altered by the chemical treatments ($p < 0.0001$, df = 7 and f=11.39). Conditions G/C/C, C/G/C, and V/V/V showed increased values of 19, 24, and 14% compared to the control, respectively ($p < 0.05$).

At S4, chemical contamination did not induce changes in LDH and GPx activities nor in CS:CCO and LDH:CS ratios in rainbow trout gills (see Table 4). However, changes were observed in CCO and CS activities ($p = 0.002$, df = 6 and f = 3.87 and $p = 0.003$, df = 6 and $\chi^2 = 19.94$), enzymes involved in aerobic metabolism.

Mean CCO activity ranged between 0.16±0.0089 and 0.26±0.011 IU/mg, while CS activities ranged between 0.32±0.0098 and 0.36±0.0082 IU/mg according to the considered treatment. A post -hoc test revealed that CCO and CS activities were 24% higher in the C/G/C condition compared to the control ($p < 0.05$) (see Table 4).

Table 3. Mean specific activities and TBARS levels measured in whole F2 larvae at 541 DD (S2) for the different chemical conditions. Specific activities are expressed in IU/mg of protein and MDA concentrations in nmol/mg of protein. Standard errors are given in parentheses under each respective mean ($11 \leq n \leq 20$). Values in bold with an asterisk are significantly different ($p < 0.05$) from the control condition.

Parameter	Mode of exposure							
	Control	Transgenerational			Intergenerational		Multigenerational	
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	C/R/C	G/G/G	R/R/R
AChE	0.31 (0.0094)	0.29 (0.007)	0.31 (0.0079)	0.28 (0.0099)	0.29 (0.0117)	0.31 (0.0105)	0.3 (0.011)	0.35 (0.0145)
CAT	7.46 (0.21)	**6.3*** (0.27)	**6.32*** (0.29)	**5.78*** (0.25)	6.46 (0.27)	6.31 (0.26)	**6.14*** (0.33)	6.96 (0.38)
CCO	0.25 (0.0173)	**0.16*** (0.0089)	0.23 (0.0076)	**0.2*** (0.0084)	0.23 (0.0113)	0.22 (0.0089)	**0.16*** (0.007)	0.26 (0.0109)
CS	0.36 (0.0082)	**0.32*** (0.0098)	0.34 (0.0092)	**0.32*** (0.0117)	0.32 (0.0128)	0.34 (0.0123)	0.32 (0.0116)	0.35 (0.013)
CS:CCO	1.56 (0.111)	**2.07*** (0.154)	1.54 (0.047)	1.59 (0.056)	1.47 (0.066)	1.6 (0.066)	**2.03*** (0.051)	1.38 (0.044)
LDH	13.4 (0.5)	14.05 (0.4)	14.41 (0.52)	13.5 (0.58)	14.89 (0.59)	12.54 (0.47)	12.07 (0.53)	13.03 (0.65)
LDH:CS	37.44 (1.53)	**44.65*** (1.24)	42.7 (1.34)	**42.68*** (1)	**46.56*** (1.28)	36.84 (0.84)	37.53 (0.95)	37.33 (0.87)
TBARS	0.92 (0.284)	0.57 (0.114)	1.18 (0.198)	0.54 (0.064)	1.07 (0.104)	0.97 (0.095)	0.65 (0.077)	0.82 (0.095)

Table 4. Mean specific activities measured in gills of F2 juveniles (S4) for the different chemical conditions. Specific activities are expressed in of protein. Standard errors are given in parentheses under each respective mean ($11 \leq n \leq 20$). The numbers in bold with an asterisk are significantly different ($p < 0.05$) from the values obtained for the control condition.

Parameter	Mode of exposure						
	Control	Transgenerational			Intergenerational		Multigenerational
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	C/R/C	R/R/R
CCO	1.7 (0.085)	1.8 (0.096)	1.62 (0.083)	1.66 (0.077)	**2.11*** (0.14)	1.95 (0.062)	1.71 (0.067)
CS	0.42 (0.018)	0.43 (0.014)	0.42 (0.015)	0.43 (0.021)	**0.52*** (0.026)	0.46 (0.011)	0.44 (0.016)
CS:CCO	0.25 (0.0084)	0.24 (0.0073)	0.26 (0.0085)	0.26 (0.0108)	0.26 (0.0119)	0.24 (0.0073)	0.26 (0.0139)
GPx	0.04 (0.004)	0.046 (0.0029)	0.038 (0.004)	0.045 (0.0035)	0.052 (0.006)	0.044 (0.0044)	0.042 (0.0041)
LDH	27.94 (1.08)	27.49 (1.57)	26.05 (1.07)	27.58 (1.1)	31.21 (1.31)	30.79 (1.09)	27.93 (0.87)
LDH:CS	64.9 (2.65)	66.24 (3.05)	61.67 (2.42)	65.44 (2.61)	61.14 (2.11)	67.36 (2.35)	64 (3.22)

Global methylation in whole F2 larvae

The proportion of 5 -methyl Cytosine (5 -mC) measured in total DNA of whole larvae, comprised between 2.68±0.26 and 3.42±0.21 5mc/total DNA, was similar among the different chemical exposure conditions.

Swimming behaviour

Distances travelled by larvae in darkness were comprised between 4.18±0.64 m and 6.89±1.11 m and between 7.97±0.69 m and 10.16±0.89 m (mean ± se) during the first and second periods. A drastic speed reduction was observed under light exposure, with distances travelled comprised between 1.87±0.24 m and 2.65±0.40 m. Chemical exposure did not induce statistically significant changes in the travelled distance for the different light intensity periods considered. However, a peak of swimming activity was observed during the minute following opening of the light, where larvae from the control condition presented the highest speed. A comparison of the speed of larvae during the minute following the opening of the light, for the different chemical exposure conditions, revealed a statistically significant effect of chemical exposure on the response to light ($p = 0.004$, $df = 7$ and $\chi^2 = 20.35$). A post -hoc test showed that control speed was 47, 40 and 42% higher than C/G/C, C/R/C, and V/C/C, respectively ($p < 0.05$).

Mortality induced by the viral challenge

Mortality was observed for all conditions following the viral challenge with *IHNv*. The lowest cumulative mortality rate was obtained for the non-chemically exposed larvae (i.e. 39.1±5.6%, mean ± se, n = 3), while values ranging from 46.0±1.6% to 83.0±5.7% were obtained for chemically exposed fish, with a significant difference between groups ($p < 0.0001$, $df = 6$ and $\chi^2 = 234$). A post -hoc test revealed a significant difference ($p < 0.05$) in mortality between the C/G/C, R/R/R, and C/R/C conditions, which presented restricted mean survival times (RMSTs) of 14.6±2.6, 23.0±3.9, and 25.3±2.5 dpi (mean ± se, n = 3), compared to 32.9±1.1, 33.8±0.5, 33.8±1.1, and 36.6±2.1 dpi for the control, V/C/C, R/C/C and G/ C/C conditions, respectively.

Maximum daily mortality (i.e. the mortality peak) was observed 6 to 10 dpi for all conditions. On the three pools of dead fish analysed at the mortality peak, *IHNv* was detected in 33.3 to 100% of the samples, depending on the exposure condition (Table 5). The mean viral titre of positive pooled fish was between $2.38x10^9 \pm 1.97x10^9$ and $4.88x10^3 \pm 1.76x10^3$ for the different conditions of chemical contamination, and was not correlated with the cumulative mortality observed.

Table 5. Proportion of *IHNv* -positive pools of fish per condition and mean viral titres at the mortality peak. Viral titres are expressed in 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) as means ± standard error (n = 3).

Condition	Parameters		
	Proportion of *IHNv* -positive fish	Mean titer ($TCID_{50}mL^{-1}$)	se
C/C/C	3/3	2.38×10^9	1.14×10^9
G/C/C	1/3	6.32×10^3	–
R/C/C	2/3	1.03×10^6	6.85×10^5
V/C/C	2/3	4.88×10^3	8.31×10^2
C/G/C	2/3	3.26×10^5	2.16×10^5
C/R/C	3/3	7.40×10^7	3.65×10^7
R/R/R	3/3	8.84×10^4	3.38×10^4

Immuno-hematologic parameters

After 96 h of viral infection (S4), no effects of chemical contamination were detected in the RBCCs or WBCCs of the F2 juvenile rainbow trout. RBCCs ranged between 0.32±0.031 and 0.56±0.11 TL, while WBCCs ranged between 15.69±1.55 and 25.5±3.97 GL.

At 42 dpi (S6), similar proportions of *IHNv* seropositive fish, ranging between 10/15 and 14/15 fish, were detected in the serum of the survivor fish for all exposure conditions (Table 6). However, the control and transgenerationally exposed fish presented higher proportions of highly seropositive fish (comprised between 60 and 85%), compared with fish intergenerationally exposed to glyphosate and Roundup and multigenerationally exposed to Roundup (comprised between 36 and 55%). No statistically significant differences in proportions of highly seropositive fish were found, but the p -value was just above the significance threshold ($p = 0.10$, $df = 6$ and $\chi^2 = 10.51$). Nevertheless, the highly positive individuals presented mean anti -*IHNv* antibody titres that were not significantly affected by chemical contamination.

Table 6. Proportions of *IHNv* -seropositive and highly seropositive survivors per condition and mean anti -*IHNv* antibody titres (mean ± standard error, n = 15) at 60 dpi (S5). High seropositivity is defined as specific antibody titres greater than ≥640. The proportion of highly seropositive fish per condition corresponds to the ratio between the number of fish with a titre greater than ≥640 and the total number of seropositive fish. Mean antibody titres were calculated for highly seropositive fish only.

Condition	Parameters			
	Seropositivity	High seropositivity	Mean anti-*IHNv*	se
		Frequency (%)	antibody titer	
C/C/C	11/15	73	1360	282
G/C/C	10/15	60	1280	286
R/C/C	12/15	83	1280	165
V/C/C	13/15	85	2153	369
C/G/C	13/15	54	3474	813
C/R/C	11/15	55	2560	859
R/R/R	14/15	36	1280	351

Conclusion

The authors concluded that their complex experimental design was an efficient approach to investigate generational effects on rainbow trout exposed to an environmentally relevant concentration of both pure glyphosate and two GBHs. Although no impact was observed on certain reproductive parameters of the F1 generation, the early development of the F2 generation was affected by chemical exposure of previous generations, with effects observed on metabolism, biometrics, and swimming behaviour. The intergenerational effects may be due to direct contact of the F2 organism with contaminants at the stage of germinal cells, while transgenerational effects could reflect epigenetic modifications inherited from the F0 generation. Biochemical parameters appeared to be restored as the fish develop. Intergenerational exposure to pure glyphosate drastically reduced the ability of rainbow trout to face a viral infection, potentially due to the inability of fish to elicit an efficient antibody response. The authors stated that their results demonstrated that glyphosate exposure induced both inter and transgenerational toxicity, sometimes with different effects depending on the physiologic functions considered. GBHs, particularly during early development, seemed to occasionally modulate the effects of the active substance. Re -exposure to glyphosate (i.e. multigenerational exposure) did not increase the toxicity compared to inter or transgenerational exposures. The authors indicated these results need to be strengthened by integrating more specific parameters allowing for an in -depth investigation of the mechanisms of glyphosate toxicity, the relationship between the active substance and the co -formulants, and also toxicity inheritance through generations, which will be helpful to adopt future regulations for the use of glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study investigates the impact of an environmentally relevant concentration of glyphosate on a F2 generation issued from exposed generations F0 and F1. Trans, inter and multigenerational toxicity of 1 µg/L of the active substance was evaluated on early stages of development and juvenile rainbow trout (*Oncorhynchus mykiss*) using different molecular, biochemical, immuno-hematologic, and biometric parameters, behaviour analysis, and a viral challenge. Reproductive parameters of generation F1 were not affected. However, developmental toxicity in generation F2 due to glyphosate alone or co -formulated was observed with head size changes (e.g. head surface up to +10%), and metabolic disruptions (e.g. 35% reduction in cytochrome -c -oxidase). Therefore, LOEC = 1 µg/L.
The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.

Assessment and conclusion by RMS:

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline -compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non -guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals were cultured in the lab.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Uncertain	Pre -dilution of pure glyphosate was done in pure methanol in a concentration so the final dose of methanol exposure was kept under 4 µL/L
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, purity, CAS number and content reported
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	The assessment of the ecotoxicological equivalence was not conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non -guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: LOEC (2 generations)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Just 1 concentration was tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Concentrations were analytically verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	No	Only 1 concentration was tested

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time -points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5 -8.0), cation exchange capacity, organic carbon (0.5 -2 -5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A -horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/ - 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20 -25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	River water filtered
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15 -25ºC.	Uncertain	Temperature varied from 6 to 15°C (it should have been 12 to 15.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	-
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate,	Yes	Concentrations were analytically

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
and where relevant its metabolites.		verified just before and 2 h after restarting the water flow approximately two months after the beginning of the experiment and a standard curve of the mean glyphosate concentrations as a function of time was generated.
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	The concentration of glyphosate in water was determined by HPLC and fluorometric methods
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No assessment of the ECX median values was conducted.

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study investigates the impact of an environmentally relevant concentration of glyphosate on a F2 generation issued from exposed generations F0 and F1. Trans, inter and multigenerational toxicity of 1 µg/L of the active substance was evaluated on early stages of development and juvenile rainbow trout (*Oncorhynchus mykiss*) using different molecular, biochemical, immuno-hematologic, and biometric parameters, behaviour analysis, and a viral challenge. Reproductive parameters of generation F1 were not affected. However, developmental toxicity in generation F2 due to glyphosate alone or co -formulated was observed with head size changes (e.g. head surface up to +10%), and metabolic disruptions (e.g. 35% reduction in cytochrome -c -oxidase). Therefore, 2 generations LOEC = 1 µg/L. The study is not considered fully reliable because although it presents analytical verifications of the tested item in the water in the exposure phase, it does not fully follow any agreed guidance and only one concentration was tested. Temperature of the test could have been a bit low for the tested species at some time points of the experiment.
Not reliable	No	-

1. Information on the study

Data point:	CA 8.2.6.1
Report author	Kaeoboon S. *et al*.
Report year	2021
Report title	Toxicity response of *Chlorella* microalgae to glyphosate herbicide exposure based on biomass, pigment contents and photosynthetic efficiency
Document No	Plant Science Today, 2021, 8(2), 293-300
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

In this study, the effects of glyphosate at different concentrations (50-500 µg/mL) on three *Chlorella* species including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* especially in relation to the biomass, pigment contents and photosynthetic efficiency were assessed. After treatment for 24 h, the acute toxicity results showed that *C. vulgaris* (IC_{50} = 449.34±6.20 µg/mL) was more tolerant to glyphosate than *C. ellipsoidea* (IC_{50} = 288.23±23.53 µg/mL) and *C. sorokiniana* (IC_{50} = 174.28±0.50 µg/mL). After a 72-h chronic toxicity treatment with glyphosate, glyphosate concentrations decreased to 400-500 µg/mL in *C. ellipsoidea*, 200-300 µg/mL in *C. sorokiniana* and 200-500 µg/mL in *C. vulgaris* respectively. During 24-h acute toxicity exposure to glyphosate, the pigment contents and maximum quantum efficiency of photosystem II (Fv/Fm) decreased as the concentration of glyphosate increased. Overall, the biomass, pigment contents and photosynthetic efficiency presented a high positive correlation. The authors indicated that their study provides detailed information on the toxicity and sensitivity of these *Chlorella* species to glyphosate.

Materials and methods

General chemicals and materials

The reagents and chemicals were purchased as follows: tris base ($H_2NC(CH_2OH)_3$ Tris (hydroxymethyl)-aminomethan; Carlo Eraba, France), NH_4Cl (FLUKA, Switzerland), $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, UK), $CaCl_2 \cdot 2H_2O$ (Ajax Finechem, Australia), K_2HPO_4 (Ajax Finechem, Australia), KH_2PO_4 (Merck, Germany), $Na_2EDTA \cdot 2H_2O$ (Fisher Scientific, UK), $ZnSO_4 \cdot 7H_2O$ (Ajax Finechem, Australia), H_3BO_3 (Merck, Germany), $MnCl_2 \cdot 4H_2O$ (Carlo Eraba, France), $FeSO_4 \cdot 7H_2O$ (Merck, Germany), $CoCl_2 \cdot 6H_2O$ (Ajax Finechem, Australia), $CuSO_4 \cdot 5H_2O$ (Merck, Germany), $(NH_4)6MoO_3$ (Mallinckrodt Chemical, USA), Acetic acid glacial (Carlo Eraba, France), Dimethyl sulfoxide (DMSO; Fisher Scientific, UK) and glyphosate (*N*-(phosphonomethyl)glycine; HPLC grade, Sigma-Aldrich, Germany). Spectrophotometric determinations were performed using a UV-1800 UV-visible spectrophotometer (Shimadzu, Japan). The effective quantum yield was determined by pulse amplitude modulation (PAM 2500, Walz, Germany).

Herbicide stock preparation

The glyphosate used in this study was analytical grade and prepared in sterile distilled water. Serial dilution was performed to achieve the required range of concentrations from 100-500 µg/mL for *C. ellipsoidea* and *C. vulgaris* and 50-300 µg/mL for *C. sorokiniana*. The culture medium was used as diluent.

Strains and culture conditions

C. ellipsoidea (TISTR 8260) and *C. vulgaris* (TISTR 8580) were purchased from the Thailand Institute of Science and Technology. *C. sorokiniana* strain KU.B2, which was isolated from an agricultural drainage in Nonthaburi Province, Thailand (July 2018), was obtained from the culture collection of the Department of Botany, Faculty of Science, Kasetsart University. *Chlorella* species was cultured in liquid TAP medium containing the following micronutrients: 2.42 gm tris base, 25 mL TAP-salt (15 gm/L NH_4Cl, 4 gm/L $MgSO_4 \cdot 7H_2O$ and 2 gm/L $CaCl_2 \cdot 2H_2O$), 1 mL phosphate solution (288 gm/L K_2HPO_4 and 144 gm/L KH_2PO_4), 1 mL trace elements solution (Hutner's trace elements; 50 gm/L $Na_2EDTA \cdot 2H_2O$, 22 gm/L $ZnSO_4 \cdot 7H_2O$, 11.4 gm/L H_3BO_3, 5 gm/L $MnCl_2 \cdot 4H_2O$, 5 gm/L $FeSO_4 \cdot 7H_2O$, 1.6 gm/L $CoCl_2 \cdot 6H_2O$, 1.6 gm/L $CuSO_4 \cdot 5H_2O$ and 1.1 gm/L $(NH_4)6MoO_3$) and 1 mL acetic acid. Then, the medium was adjusted to pH 7.0. *Chlorella* species were cultivated at 30±1°C, under controlled conditions using white cool fluorescent light lamps (330 µmol photons m^{-2} s^{-1}). The cultures were shaken five times per day during incubation. The concentration of each *Chlorella* mixture was quantified by cell counting with a Neubauer chamber. The regression equation for the relationship between cell density (y; $1.0x10^6$ cells/mL) and absorption (x; wavelength at 750 nm) was calculated as follows: $y = 33.944x - 0.8972$ ($R^2 = 0.9998$) for *C. ellipsoidea*, $y = 44.001x - 0.7281$ ($R^2 = 0.9999$) for *C. sorokiniana* and $y = 46.72x - 0.3799$ ($R^2 = 1$) for *C. vulgaris*. The same incubation conditions were used for the determination of IC_{50} and in the glyphosate toxicity treatments.

Toxicity experiments

The biomass was measured as the optical density (OD) at 750 nm. The exponential growth phase of samples was cultured in 150 mL culture medium in an Erlenmeyer flask with initial cell density of $1.0x10^6$ cells/mL for 24, 48 and 96 h. The biomass yield was calculated at 24, 48 and 72 h. Inhibition of growth was monitored as an index to determine the glyphosate toxicity and the IC_{50} value for biomass was calculated based on OD_{750}. In order to enable direct comparison of dose responses for IC_{50}, all responses were converted to % of control.

Photosynthetic pigment determination

The pigment contents were determined by spectrophotometry. 5 mL of each treatment was harvested then centrifuged at 3000 gm for 15 min. Subsequently, the supernatant was discarded and 5 mL DMSO was added to the extract of photosynthetic pigments. The samples were sonicated for 1 h and stored in the dark. After 24 h, each sample was centrifuged at 3000 gm for 15 min. The pigments of the *Chlorella* microalgae samples in the supernatant were analysed by using a spectrophotometer at appropriate wavelengths (470, 649 and 665 nm). The resulting absorbance measurements were obtained from Wellburn A.R. The equations used to calculate the pigment concentrations are as follows:

Chlorophyll *a* (Chl *a*) = 12.19 A665 - 3.45 A649
Chlorophyll *b* (Chl *b*) = 21.99 A649 - 5.32 A665
Total Chlorophyll = Chlorophyll *a* + Chlorophyll *b*
Total Carotenoids = (1000 A470 - 2.86 Chl *a* - 129.2 Chl *b*)/221

The pigment contents are represented as the concentrations in µg/mL.

Chlorophyll fluorescence

The effect of glyphosate on chlorophyll fluorescence was measured as the effective quantum yield using a pulse amplitude modulation (PAM) fluorometer with a suspension cuvette (KS2500; diameter of 7.5 mm, depth of 9.0 mm). Monitoring of the photoinhibition in microalgae was performed using standard method (Magnusson M. *et al*.). The effective quantum yield was determined by immersing the probe directly into the culture (measuring light intensity = 9, gain = 4) and one measurement was taken per replicate after 24 h treatment. The toxic response in each treatment was expressed as a percentage of control values.

Statistical analysis

The experimental treatments had three independent replicates. The results were analysed using GraphPad Prism 6 Software (San Diego, USA). P-values lower than 0.05 were considered as statistically significant with two-way analysis of Dunnett's test. Pearson's correlation was obtained for all treatments.

Results

Growth inhibition test to assess the glyphosate toxicity

The study investigated the glyphosate toxicity in *Chlorella* microalgae, including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* for further use as a biological indicator model. To examine the toxicity effects, *C. ellipsoidea*, *C. sorokiniana* and *C. vulgaris* were treated with 100-500, 50-300 and 100-500 µg/mL glyphosate, respectively. The growth inhibition test clearly manifested that glyphosate treatment induced a significant inhibitory effect on the acute toxicity in the three species after 24 h. Compared to the control, the biomass of *C. ellipsoidea* and *C. sorokiniana* was significantly inhibited by 50-500 µg/mL glyphosate and that of *C. vulgaris* was inhibited at 200 µg/mL. *C. vulgaris* (IC_{50} = 449.34±6.20 µg/mL) showed the greatest tolerance to glyphosate compared to *C. ellipsoidea* (IC_{50} = 288.23±23.53 µg/mL) and *C. sorokiniana* (IC_{50} = 174.28±0.50 µg/mL).

The glyphosate caused a decrease in biomass of all species as both concentration and time increased, leading to chronic toxicity. Between 24 and 48 h treatment, the biomass of *C. ellipsoidea* and *C. sorokiniana* did not exceed 300 µg/mL and 150 µg/mL respectively. After 72 h of treatment, the glyphosate concentration inhibit growth in the following concentrations: 400-500 µg/mL for *C. ellipsoidea*, 200-300 µg/mL for *C. sorokiniana* and 200-500 µg/mL for *C. vulgaris*.

Pigment contents after glyphosate exposure

After the 24 h treatment, the concentrations of total chlorophyll and carotenoids were significantly different among *Chlorella* sp. and the control. As indicated in Table 1, increased glyphosate concentration had a negative linear effect on the chlorophyll and carotenoids concentrations. For both *C. ellipsoidea* and *C. sorokiniana* inhibited growth, the value of total carotenoid content was relate similar to biomass result at the same concentration.

Table 1. Pigment contents of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), and carotenoid in (A) *Chlorella ellipsoidea*, (B) *Chlorella sorokiniana* and (C) *Chlorella vulgaris* after treated with different concentrations of glyphosate (50-500 µg/mL) for 24 h. The results are presented as the mean ± standard deviation in triplicate (n = 3).

(A)

Pigment content (µg ml^{-1})	Glyphosate (µg ml^{-1})					
	control	100	200	300	400	500
chl *a*	6.70 ± 0.06	4.81 ± 0.01	4.25 ± 0.01	2.06 ± 0.02	1.70 ± 0.01	1.96 ± 0.01
chl *b*	5.50 ± 0.04	3.99 ± 0.06	3.57 ± 0.16	1.72 ± 0.02	1.26 ± 0.02	1.42 ± 0.02
total chlorophyll	12.20 ± 0.04^{a}	8.80 ± 0.08^{b}	7.82 ± 0.15^{c}	3.78 ± 0.04^{d}	2.96 ± 0.03^{e}	3.37 ±.0.21^{f}
total carotenoid	1.84 ± 0.09^{a}	1.50 ± 0.01^{b}	1.24 ± 0.06^{c}	0.53 ± 0.01^{d}	0.37 ± 0.00^{d}	0.43 ± 0.01^{d}

(B)

Pigment content (µg ml^{-1})	Glyphosate (µg ml^{-1})					
	control	50	100	150	200	300
chl *a*	3.24 ± 0.01	2.36 ± 0.01	0.68 ± 0.01	0.44 ± 0.01	0.12 ± 0.00	0.09 ± 0.00
chl *b*	2.83 ± 0.01	2.08 ± 0.03	0.73 ± 0.00	0.55 ± 0.01	0.22 ± 0.01	0.20 ± 0.01
total chlorophyll	6.08 ± 0.02^{a}	4.44 ± 0.04^{b}	1.41 ± 0.01^{c}	0.99 ±0.02^{d}	0.34 ± 0.01^{e}	0.30 ± 0.01^{f}
total carotenoid	0.82 ±0.01^{a}	0.64 ± 0.00^{b}	0.26 ± 0.00^{c}	0.17 ± 0.01^{d}	0.10 ± 0.00^{e}	0.090 ± 0.00^{e}

(C)

Pigment content (µg ml^{-1})	Glyphosate (µg ml^{-1})					
	control	100	200	300	400	500
chl *a*	5.92 ± 0.19	4.69 ± 0.02	2.80 ± 0.01	1.98 ± 0.00	1.66 ± 0.01	1.54 ± 0.01
chl *b*	2.79 ± 0.11	3.41 ± 0.02	2.20 ± 0.01	1.71 ± 0.01	1.30 ± 0.01	1.16 ± 0.02
total chlorophyll	8.71 ±0.09^{a}	8.10 ± 0.01^{b}	5.00 ± 0.02^{c}	3.69 ± 0.01^{d}	2.96 ± 0.02^{e}	2.70 ± 0.01^{f}
total carotenoid	1.38 ± 0.02^{a}	1.26 ± 0.01^{a}	0.86 ± 0.01^{b}	0.67 ± 0.02^{cd}	0.55 ± 0.00^{d}	0.48 ± 0.00^{de}

Photosynthetic efficiency after exposed to glyphosate

The inhibition of effective quantum yield (Fv/Fm) using pulse amplitude modulation (PAM) fluorometer revealed similar patterns in the biomass and pigment contents of the three *Chlorella* species after 24 h cumulative glyphosate exposure. The results demonstrate that 400, 150 and 500 µg/mL glyphosate reached the level of highest inhibition of quantum yield of *C. ellipsoidea*, *C. sorokiniana* and *C. vulgaris*, respectively.Thus, the findings indicate that *C. vulgaris* was least influenced by glyphosate.

Correlation analysis

In this study, the obtained Pearson's correlation was utilized as a guide to evaluate the correlation coefficient (*r*). The correlation coefficients of the biomass, pigment contents and photosynthetic performance of three *Chlorella* species indicate that all species present high positive correlation ($r > 0.7$) after 24 h exposure.

Conclusion

The authors concluded from their experimental results that glyphosate exposure affects biomass, pigment contents and photosynthetic efficiency of *Chlorella* species. After 24-h acute toxicity glyphosate exposure in terms of biomass, *C. vulgaris* showed the greatest tolerance, while *C. sorokiniana* was the most sensitive. After 72-h chronic toxicity, the biomass yield of all *Chlorella* species was at a relatively low concentration level, which indicated, according to the authors, that the effect of glyphosate is both concentration and time-dependent. Similar to the biomass results, glyphosate exposure leads to reduced pigment contents and photosynthetic efficiency following 24-h exposure. This indicated to the authors that the relationship between the biomass, pigment contents and photosynthetic efficiency is significantly correlated in this study. The authors stated that these results could be beneficial to understanding the impact and potential risk of glyphosate toxicity on microalgae in aquatic environments.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This article reports the effects of glyphosate at different concentrations (50-500 mg/L) on three *Chlorella* species including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* in relation to the biomass, pigment contents and photosynthetic efficiency. The study seems to have been well conducted and reports regulatory relevant endpoints: 24-h acute EC_{50} = 449.34, 288.23 and 174.28 mg/L for *Chlorella vulgaris*, *Chlorella ellipsoidea* and *Chlorella sorokiniana*, respectively. Chronic endpoints at 72-h could also be calculated.
The study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium and the temperature during the tests and culture are higher than recommended for green algae. Purity of the test item is not given.

Assessment and conclusion by RMS:

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	-
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	The culture medium was directly used as diluent

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	No	The producer is provided, but not the purity
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Validity criteria cannot be assessed because no raw data are available
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	No analytical verifications of the concentration of glyphosate in the test media were conducted
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint reported: EC_{50} (24-h)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	5 concentrations tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Uncertain	No analytical verifications of the concentration of glyphosate in the test media were conducted
15. A clear concentration response relationship is reported - in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	3 replicates for each treatment group were tested
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation were provided. No raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented	Yes	Statistical analysis

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
(e.g., checking the plots and confidence intervals).		sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not relevant, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not relevant, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not relevant, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not relevant, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not relevant, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not relevant, aquatic study
20.6. Data on precipitation is recorded.	-	Not relevant, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not relevant, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	No bee study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	No	Temperature was 30ºC (should have been in the range 21-24ºC)
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Uncertain	No analytical verifications of the concentration of glyphosate in the test media were conducted
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Uncertain	No analytical verifications of the concentration of glyphosate in the test media were conducted
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported

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

Criteria		Criteria met? Yes / No / Uncertain	Comment / Justification
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.		No	No ECx assessment was conducted
Overall assessment			
Reliable without restrictions	No	-	
Reliable with restrictions	Yes	This article reports the effects of glyphosate at different concentrations (50-500 mg/L) on three *Chlorella* species including *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris* in relation to the biomass, pigment contents and photosynthetic efficiency. The study seems to have been well conducted and reports regulatory relevant endpoints: 24-h acute EC_{50} = 449.34, 288.23 and 174.28 mg/L for *Chlorella vulgaris*, *Chlorella ellipsoidea* and *Chlorella sorokiniana*, respectively. Chronic endpoints at 72-h could also be calculated. The study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium and the temperature during the tests and culture are higher than recommended for green algae. Purity of the test item is not given.	
Not reliable	No	-	

1. Information on the study

Data point:	CA 8.2.7
Report author	Mendes E. J. *et al.*
Report year	2021
Report title	Isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid on the physiology and water remediation capacity of *Salvinia molesta*
Document No	Journal of Hazardous Materials, 2021, 417, 125694
Guidelines followed in study	None (partially based on OECD TG 221)
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors evaluate the isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid (AMPA) and the potential of the aquatic macrophyte *Salvinia molesta* to remove these chemicals from contaminated water. Plants were exposed to environmentally relevant concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) for seven days. Then, based on the effective concentrations of glyphosate found to reduce photosynthetic rates by 10% (EC_{10}) and 50% (EC_{50}), the plants were exposed to combinations of 0, 16 and 63.5 µg glyphosate/L and 0, 5, 15, 25 µg AMPA/L. The $EC_{(10)}$ and $EC_{(50)}$ were lower for AMPA (6.1 µg/L and 28.4 µg/L respectively) than for glyphosate (16 and 63.5 µg glyphosate/L respectively). When occurring together, the deleterious effects of those chemicals to plants increased. *S. molesta* plants removed up to 74.15% of glyphosate and 71.34% of AMPA from culture water. Due to its high removal efficiency, *S. molesta* can be used in phytoremediation programs. It will be important to evaluate the combined effects of glyphosate and AMPA in any toxicological studies of the herbicide.

Materials and methods

Plant material

Salvinia molesta D. Mich (Salviniaceae) plants were collected at Barigui Park (Curitiba, PR, Brazil). Before initiating the experiments, the macrophytes were acclimated in plastic pots (50 L) containing autoclaved and reconstituted water under controlled conditions (temperature 20±2ºC, and a 12/12 h light cycle, at 100 µmol photons m^{-2} s^{-1}) for 60 days, for depuration. The plants were then washed for 3 min in a 0.5% hypochlorite solution, rinsed thoroughly in distilled water, and transferred to 5 L plastic pots containing autoclaved reconstituted water. The pots were kept in biological oxygen demand (B.O.D) chambers under the controlled condition mentioned above for 15 days until used. Tests were performed in 250 mL Erlenmeyer flasks stoppered with cotton wool containing 100 mL of autoclaved reconstituted water and the desired treatments. A density of 15 g plant/L was used in the bioassays.

Bioassays

Plants were submitted to environmental representative concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) in water for seven days following OECD guidelines. Those concentrations were chosen following worldwide occurrences of glyphosate and AMPA in surface waters, thus being environmentally representative concentrations. To investigate the interactive effects of glyphosate and AMPA, plants were exposed for seven days to glyphosate concentrations causing 10% (EC_{10}; 16 µg glyphosate/L) and 50% (EC_{50}; 63.5 µg glyphosate/L)

reductions in photosynthesis, plus increasing concentrations of AMPA (0, 5, 15, 25 µg/L). The AMPA concentrations used in the glyphosate AMPA assays were chosen based on concentrations known to cause photosynthetic reductions in those plants. The experiments were conducted under the same above-mentioned conditions of temperature and illumination. Analytical grade glyphosate and AMPA were used in all experiments. Parallel bioassays were conducted in flasks without plants under the same conditions to evaluate the degradation of chemicals by light, temperature, and hydrolysis.

Photosynthesis and pigment evaluations

After harvesting, the plants were thoroughly washed in distilled water (50 mL, three times) to remove unabsorbed chemical residues that might be present on plant surfaces. Photosynthetic parameters were evaluated using whole plants in a closed-infrared gas analyser system (CI-340 Hand-held Photosynthesis System; CID Bio-Science Inc., USA). Net photosynthetic rates (P_N) were evaluated three times/plant at -100 µmol photons m^{-2} s^{-1}. After the photosynthetic evaluations, the plants were flash-frozen in liquid nitrogen and subsequently stored at -80 ºC in aluminium foil until used for pigment and biochemical analyses. Chlorophyll and pheophytin *a* and *b* concentrations were measured in plants ground in 80% acetone.

Oxidative stress markers

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations were determined according to using 0.1 g of plants. Ascorbate peroxidase and catalase activities were evaluated after the determination of the total protein concentration in 0.1 g of plants ground in liquid nitrogen and homogenized in 1.0 mL of an extraction solution containing 100 mM phosphate buffer (pH 7.8), 100 mM EDTA, 1 mM *L-ascorbate*, and 2% polyvinylpyrrolidone (PVP) (m/v).

Chemical analyses

The concentrations of glyphosate and AMPA in the water medium and plants (initial and final concentrations) were determined using a LC-MS/MS system coupled to HPLC. Glyphosate and AMPA were extracted from 0.4 g of plants, previously thoroughly washed in ultrapure water, using 50 mL of acidified water. The plant extracts and samples of the growth solutions were filtered through C18 SPR cartridges previously conditioned with 15 mL of acidified water and 5 mL of methanol. The cartridges containing the samples were washed with 3 mL of 50% methanol in water (v/v). The eluate was then dried in a SpeedVac machine (RC1010, Thermo), and the residues resuspended in the mobile phase A. Before injection, the samples were filtered through nylon syringe filters.

Chromatographic separations were performed using an Ascentis® C18 column with a mobile phase consisting of 5 mM of ammonium acetate in water (phase A) and 5 mM of ammonium acetate in methanol (phase B), both pH 7.0. Mass spectrometry analyses were operated in a negative ion mode. In addition to glyphosate and AMPA, shikimate concentrations were also quantified in plants using the same LC-MS/MS technique. Analytical-grade glyphosate, AMPA, and shikimate were used to prepare the calibration curves. The six-point calibration curves showed good linearity for the analytes ($r^2 \geq 0.95$; $p < 0.0001$). Each sample batch included three blanks, three standards, and three fortified samples (for quality control). The recovery rates for all of the compounds were greater than 87%. The natural degradation of glyphosate and AMPA in the water (degradation) as well as the efficiency of plant removal of glyphosate and AMPA (removal efficiency) were calculated. as following:

$$Degradation(\%) = 100 - \left(\frac{C_7 \quad without\ plants}{C_0 \quad without\ plants} x \quad 100 \right)$$

$$Removal\ efficiency(\%) = 100 - \left(\frac{C_7 \quad with\ plants}{C_0 \quad with\ plants} x \quad 100 \right) - degradation$$

where C_0 and C_7 are the initial and final concentration of glyphosate or AMPA in the water.

Statistical analysis

The effective concentrations causing 10% (EC_{10}) or 50% (EC_{50}) photosynthetic reductions were determined from the nonlinear least square fits, using an inverse regression curve.

Statistical analyses were performed on JMP 7.0 software (SAS Institute Inc.). Data were tested for normality (Shapiro-Wilk) and homogeneity (Bartlett), and then statistically evaluated. Data from plants treated only with glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or only with AMPA (0, 10, 20, 30, 40 and 50 µg/L) were evaluated using one-way analysis of variance (ANOVA), and the means compared using a post hoc Tukey test ($P < 0.05$). Data from plants submitted to the factorial treatments with glyphosate (0, 16 and 63.5 µg/L) and AMPA (0, 5, 15, 25 µg/L) were evaluated using two-way ANOVA. Interactions between glyphosate and AMPA were included in the model. When differences were detected by ANOVA, the means were compared by a post hoc Tukey test ($p < 0.05$).

Results

Effects of glyphosate on plant physiology

Visible symptoms of injury (chloroses and some necrotic spots) were only observed in plants exposed to glyphosate concentrations ≥60 µg/L. Any symptoms of leaf injuries were restricted to the bases of the leaves and did not involve more than 30% of the leaf biomass, even among plants exposed to highest glyphosate concentrations. Plants exposed to glyphosate concentrations ≥40 µg/L showed decreased net photosynthetic rates (P_N; $F = 40.47$) and chlorophyll *b* concentrations ($F = 26.86$; $p < 0.001$), while their pheophytin *b*/chlorophyll *b* ratios increased ($F = 29.02$; $p < 0.0001$). The effective concentration causing 10% (EC_{10}) and 50% (EC_{50}) photosynthetic decreases were 16 and 63.5 µg/L respectively. Reductions of chlorophyll *a* concentrations were only observed in plants exposed to the highest glyphosate concentration (100 µg/L) ($F = 7.46$; $p < 0.01$), although their pheophytin *a*/chlorophyll *a* ratios were not significantly affected ($F = 1.16$; $p > 0.05$) by herbicide exposure.

Shikimate concentrations ($F = 57.39$) and ascorbate peroxidase activities (APX; $F = 33.26$) were greater in glyphosate-treated plants as compared to the control ($p < 0.0001$; Figure 3). Hydrogen peroxide (H_2O_2; $F = 36.85$) and MDA concentrations ($F = 47.66$) increased in plants treated with glyphosate concentrations ≥40 µg/L ($p < 0.0001$), while catalase activity (CAT) increased in plants treated with glyphosate at concentrations ≥60 µg/L ($F = 62.49$, $p < 0.0001$).

Effects of AMPA on plant physiology

Visible symptoms of injury (chlorosis) were only observed in plants exposed to AMPA at concentrations ≥30 µg/L. Similar to glyphosate-exposed plants, AMPA-exposed plants survived seven days of exposure to that compound. P_N ($F = 90.23$) and chlorophyll *a* concentrations ($F = 31.08$) significantly decreased ($p < 0.0001$) in plants exposed to AMPA. Chlorophyll *b* concentrations ($F = 9.67$) decreased in plants exposed to AMPA concentrations ≥20 µg/L in relation to the control ($p < 0.0001$). Pheophytin *a*/chlorophyll *a* ($F = 1.92$) and pheophytin *b*/chlorophyll *b* ($F = 1.00$) ratios were not significantly affected ($p > 0.05$) by AMPA treatments in relation to the control.

Shikimate concentrations were lowest in plants treated with 10 µg glyphosate/L ($F = 12.91$; $p < 0.0001$). H_2O_2 ($F = 130.86$) and MDA concentrations ($F = 46.91$), as well as APX ($F = 62.12$) and CAT ($F = 24.73$) activities significantly increased in AMPA-treated plants in relation to controls ($p < 0.0001$).

Interactive effects of glyphosate and AMPA on plant physiology

Chlorosis and necrosis were observed in plants when exposed to combined concentrations of glyphosate and AMPA, regardless of their concentrations. Although some leaves died, most of the leaf tissue appeared to remain viable. Significant interactions between glyphosate and AMPA addition were observed ($p < 0.01$) for all of the physiological parameters evaluated, except shikimate concentrations ($p > 0.05$). The addition of AMPA to plants exposed to glyphosate decreased their P_N and photosynthetic pigments and increased pheophytin/chlorophyll ratios – except among plants treated with 16 or 63 µg glyphosate/L + 5 AMPA/L, when chlorophyll *b* concentrations and pheophytin *b*/chlorophyll *b* ratios did not significantly differ from plants treated solely with equivalent glyphosate concentrations (16 or 63 µg glyphosate/L + 0 µg AMPA/L).

Shikimate concentrations in plants increased as glyphosate concentrations increased ($p < 0.0001$) but were not affected by AMPA addition ($p > 0.05$). Regardless of the glyphosate concentration used, the

addition of AMPA significantly increased plant H_2O_2 and MDA concentrations, as well as APX and CAT activities.

Glyphosate and AMPA degradation in the water

As can be seen in Table 1, no glyphosate or AMPA was detected in the water of flasks at the initial time (T_0) without their purposeful addition, indicating no inter-contaminations between treatments receiving only glyphosate or only AMPA. In systems without plants, glyphosate and AMPA degradation in water after seven days ranged from 0.93% to 4.58% and 1.55-3.57%, respectively, when the chemicals were applied alone. Residual concentrations of AMPA (0.49-1.30 µg/L) were observed after seven days in the water of flasks without plants and receiving only glyphosate. When combined, glyphosate and AMPA degradations ranged from 0.72% to 6.24% and 0.76 to 4.85% respectively. Once again, AMPA was detected in flasks with only glyphosate added.

Table 1. Glyphosate and AMPA concentrations in water media at the beginning of the experiments (T_0) and after seven days (T_7) and chemical degradation percentages in flasks without *Salvinia molesta* plants.

Treatment	Glyphosate (µg l^{-1})			AMPA (µg l^{-1})		
	T_0	T_7	Degradation (%)	T_0	T_7	Degradation (%)
0 µg l^{-1}	n.d.	n.d.	–	n.d.	n.d.	–
Glyphosate						
20 µg l^{-1}	20.12 ± 2.26	19.18 ± 1.97	4.58 ± 1.16	n.d.	0.77 ± 0.24	–
40 µg l^{-1}	41.67 ± 2.66	41.06 ± 2.64	1.45 ± 1.03	n.d.	0.49 ± 0.32	–
60 µg l^{-1}	61.94 ± 1.69	61.23 ± 1.36	1.12 ± 0.70	n.d.	0.56 ± 0.39	–
80 µg l^{-1}	79.84 ± 1.24	79.09 ± 0.97	0.93 ± 0.60	n.d.	0.54 ± 0.38	–
100 µg l^{-1}	102.96 ± 3.52	101.22 ± 4.04	1.70 ± 1.02	n.d.	1.30 ± 0.61	–
AMPA						
10 µg l^{-1}	n.d.	n.d.	–	10.33 ± 0.54	10.17 ± 0.52	1.95 ± 0.53
20 µg l^{-1}	n.d.	n.d.	–	20.39 ± 0.48	19.66 ± 0.43	3.57 ± 1.20
30 µg l^{-1}	n.d.	n.d.	–	30.83 ± 1.07	30.16 ± 0.43	3.29 ± 2.47
40 µg l^{-1}	n.d.	n.d.	–	41.05 ± 0.84	40.40 ± 0.77	1.55 ± 0.99
50 µg l^{-1}	n.d.	n.d.	–	51.17 ± 1.39	49.84 ± 0.93	2.58 ± 0.95
Glyphosate + AMPA						
0 µg glyphosate l^{-1} + 5 µg AMPA l^{-1}	n.d.	n.d.	–	5.20 ± 0.24	4.97 ± 0.15	4.35 ± 1.54
0 µg glyphosate l^{-1} + 15 µg AMPA l^{-1}	n.d.	n.d.	–	15.73 ± 0.58	15.15 ± 0.62	3.66 ± 2.26
0 µg glyphosate l^{-1} + 25 µg AMPA l^{-1}	n.d.	n.d.	–	26.20 ± 1.44	25.03 ± 1.22	4.40 ± 2.00
16 µg glyphosate l^{-1} + 0 µg AMPA l^{-1}	16.87 ± 1.16	16.12 ± 1.28	4.46 ± 1.73	n.d.	0.52 ± 0.15	–
16 µg glyphosate l^{-1} + 5 µg AMPA l^{-1}	16.96 ± 1.29	15.92 ± 1.53	6.24 ± 2.20	5.65 ± 0.28	5.42 ± 0.32	3.93 ± 5.52
16 µg glyphosate l^{-1} + 15 µg AMPA l^{-1}	15.47 ± 0.91	15.09 ± 1.05	2.51 ± 2.02	15.81 ± 0.92	15.22 ± 0.79	4.26 ± 6.73
16 µg glyphosate l^{-1} + 25 µg AMPA l^{-1}	16.74 ± 1.12	16.12 ± 1.28	3.70 ± 2.62	26.06 ± 1.34	25.09 ± 1.59	4.85 ± 6.88
63.5 µg glyphosate l^{-1} + 0 µg AMPA l^{-1}	63.93 ± 0.99	63.47 ± 1.13	0.72 ± 5.52	n.d.	0.58 ± 0.03	–
63.5 µg glyphosate l^{-1} + 5 µg AMPA l^{-1}	65.31 ± 1.71	64.62 ± 1.59	1.04 ± 0.26	5.85 ± 0.68	5.85 ± 0.61	0.76 ± 0.65
63.5 µg glyphosate l^{-1} + 15 µg AMPA l^{-1}	63.80 ± 1.06	62.65 ± 1.41	1.81 ± 0.61	13.00 ± 5.28	12.68 ± 4.58	3.19 ± 2.39
63.5 µg glyphosate l^{-1} + 25 µg AMPA l^{-1}	63.95 ± 1.24	62.90 ± 1.25	1.64 ± 0.17	25.53 ± 0.37	24.09 ± 0.91	1.82 ± 2.21

Values are means ± SD of four replicates. n.d. = not detected

Glyphosate and AMPA uptake by plants

No glyphosate or AMPA was detected in plants at the initial time (T_0). Among plants exposed to only glyphosate, plant glyphosate concentrations increased with the addition of glyphosate up to 80 µg/L, not differing between plants exposed to 80 and 100 µg glyphosate/L ($F = 105.79$; $p < 0.0001$). AMPA was also observed in plants exposed to only glyphosate, and increased as glyphosate addition increased (but did not differ between plants exposed to 60 and 80 µg glyphosate/L) ($F = 134.60$; $p < 0.0001$). In plants treated with glyphosate only, the effective removal of glyphosate ranged from 55% to 78%.

No glyphosate was detected in plants exposed to AMPA only. Among AMPA-treated plants, AMPA concentrations increased with the AMPA addition to the medium up to 30 µg/L, not differing between plants exposed to 30, 40 and 50 µg AMPA/L ($F = 144.33$; $p < 0.0001$). In plants exposed only to AMPA, its effective removal ranged from 62% to 71%.

Significant interactions ($p < 0.001$) between glyphosate and added AMPA were observed in terms of glyphosate and AMPA concentrations in the plants as well as for the removal efficiency of glyphosate ($F = 6.20$) and AMPA ($F = 43.03$). Increased concentrations of glyphosate were observed in plants exposed to 63.5 µg glyphosate/L when AMPA was added. In the absence of glyphosate, AMPA concentration was greatest in plants exposed to 25 µg AMPA/L, and did not differ between plants treated with 5 or 15 µg AMPA/L. In the presence of glyphosate, however, AMPA concentration in plants increased with AMPA addition. AMPA was observed in glyphosate-treated plants without any AMPA addition. The greatest AMPA concentrations were observed in plants treated with 15 and 25 µg AMPA/L and exposed to 16 µg glyphosate/L. The glyphosate removal efficiency of plants ranged

from 58% to 87%, being highest in plants treated with 16 µg glyphosate/L and 15 or 25 µg AMPA/L. AMPA removal efficiency did not differ among glyphosate-treated plants when AMPA was added to water; in the absence of glyphosate, AMPA removal was greatest in plants treated with 5 µg AMPA/L.

Conclusion

The authors concluded that they evidenced the deleterious effects that environmentally representative concentrations of glyphosate and AMPA have on the macrophyte *Salvinia molesta*. Based on photosynthetic rates, glyphosate showed lower EC_{10} and EC_{50} values (16 and 63.5 µg/L respectively) than AMPA (6.1 µg/L and 28.4 µg/L respectively), which was more toxic to the plants. The mechanisms by which glyphosate and AMPA affect photosynthesis were distinct: in addition to disrupting shikimic acid (and, thus, protein biosynthesis), glyphosate appears to induce chlorophyll degradation through ROS accumulation; AMPA, on the other hand, apparently disrupts pigment biosynthesis. Glyphosate and AMPA showed synergistic effects, increasing deleterious effects when occurring together in plant tissues. It will therefore be important to evaluate the combined effects of glyphosate and AMPA in toxicological studies of that herbicide. Despite the negative effects of the contaminants on its physiology, *S. molesta* plants were efficient at reducing glyphosate and AMPA concentrations in the surrounding water. When those compounds were added separately to the medium, the plants removed up to 74.15% of glyphosate and 71.34% of AMPA. Moreover, at a glyphosate concentration of 16 µg/L, the presence of AMPA increased the glyphosate-removal efficiency of *S. molesta*. Therefore, the use of the macrophyte *S. molesta* for the removal of glyphosate and AMPA from water can constitute an environmentally friendly technology, reducing the costs otherwise associated with physicochemical methods of contaminant removal.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study evaluates the isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid (AMPA) on the aquatic macrophyte *Salvinia molesta*. Plants were exposed to environmentally relevant concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) for seven days. Then, based on the effective concentrations of glyphosate found to reduce photosynthetic rates by 10% (EC_{10}) and 50% (EC_{50}), the plants were exposed to combinations of 0, 16 and 63.5 µg glyphosate/L and 0, 5, 15, 25 µg AMPA/L. The EC_{10} and EC_{50} were lower for AMPA (6.1 µg/L and 28.4 µg/L, respectively) than for glyphosate (16 and 63.5 µg glyphosate/L, respectively). When occurring together, the deleterious effects of those chemicals to plants increased.
The study is considered not fully reliable because it is not possible to identify the number of plants tested for each treatment and because plants were collected from the field in Brazil with no record of previous pesticide exposure (although they were maintained in the lab for 60 days for depuration).

Assessment and conclusion by RMS:

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

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

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	Study conducted according to OECD TG 221, but validity criteria cannot be verified because the measured variables (mainly photosynthetic rate) are different from those stated in the OECD GD (inhibition of growth).
2. No previous exposure to other chemicals is documented (where relevant).	Uncertain	Plants were collected from the field in Brazil with no record of previous pesticide exposure, but were maintained in the lab for 60 days for depuration
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Analytical grade glyphosate and AMPA (Pestanal®, Sigma-Aldrich, Canada) were used in all experiments
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	Study conducted according to OECD TG 221, but validity criteria cannot be verified
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of	Yes	-

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
glyphosate (or a glyphosate metabolite).		
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	The concentrations of glyphosate and AMPA in the water medium and plants (initial and final concentrations) were determined
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: EC_{50} (7-d) for both glyphosate and AMPA
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	-
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Analytical verifications were conducted at the beginning and the end of the test
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Uncertain	A density of 15 g plant/L was used in the bioassays, but the number of replicates is not known.
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature set at 20°C for the tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Yes	Analytical verifications were conducted at the beginning and the end of the test
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Analytical verifications were conducted at the beginning and the end of the test
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	Analytical methods were described
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	95% CI values were not reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study evaluates the isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid (AMPA) on the aquatic macrophyte *Salvinia molesta*. Plants were exposed to environmentally relevant concentrations of glyphosate (0, 20, 40, 60, 80 and 100 µg/L) or AMPA (0, 10, 20, 30, 40 and 50 µg/L) for seven days. Then, based on the effective concentrations of glyphosate found to reduce

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

Criteria		Criteria met? Yes / No / Uncertain	Comment / Justification
		photosynthetic rates by 10% (EC_{10}) and 50% (EC_{50}), the plants were exposed to combinations of 0, 16 and 63.5 µg glyphosate/L and 0, 5, 15, 25 µg AMPA/L. The EC_{10} and EC_{50} were lower for AMPA (6.1 µg/L and 28.4 µg/L, respectively) than for glyphosate (16 and 63.5 µg glyphosate/L, respectively). When occurring together, the deleterious effects of those chemicals to plants increased. The study is considered not fully reliable because it is not possible to identify the number of plants tested for each treatment and because plants were collected from the field in Brazil with no record of previous pesticide exposure (although they were maintained in the lab for 60 days for depuration).	
Not reliable	No	-	

1. Information on the study

Data point:	CA 8.2.8
Report author	Vera M. S. *et al.*
Report year	2021
Report title	First evaluation of the periphyton recovery after glyphosate exposure
Document No	Environmental Pollution, 2021, 290, 117998
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors evaluated the potential of freshwater periphyton to recover from glyphosate exposure using microcosms under laboratory conditions. Periphyton developed on artificial substrates was exposed to 0.4 or 4 mg/L monoisopropylamine salt of glyphosate (IPA) for 7 days, followed by translocation to herbicide-free water. The authors sampled the community 1, 2 and 3 weeks after the transfer. Dry weight, ash-free dry weight, chlorophyll *a*, and periphyton abundances were analysed. The periphyton impacted with the lowest IPA concentration recovered most of the structural parameters within 7 days in clean water, but the taxonomic structure did not entirely recover towards the control structure. Periphyton exposed to 4 mg IPA/L could not recover during 21 days in herbicide-free water, reaching values almost four times higher in % of dead diatoms and four times lower in ash-free dry weight concerning the control at the end of the study. Results suggest a long-lasting effect of the herbicide due to the persistence within the community matrix even after translocating periphyton to decontaminated water. The authors concluded that the exposure concentration modulates the recovery potential of IPA-impacted periphyton. The current research is the first to study the recovery in glyphosate-free water of periphyton exposed to the most commonly used herbicide in the world. Finally, the authors highlighted the need for more studies focused on the recovery potential of freshwater ecosystems and aquatic communities after glyphosate contamination.

Materials and methods

Periphytic community

Ad hoc devices (periphytometers) were set sub-superficially in a 3000 litres artificial outdoor pond ([chlorophyll *a*] = 1.6 µg/L; turbidity = 1.17 NTU) located in an experimental field of the University of Buenos Aires, to support artificial substrates for periphytic colonization. This pond has never been contaminated with glyphosate or any other pesticide. After four weeks, periphytometers with colonized substrates were removed and transferred from the experimental field to the laboratory in dark and cold. When periphytometers were removed, physical parameters were recorded in situ and water samples to determine glyphosate, phytoplanktonic chlorophyll *a* and nutrients concentrations were collected. Also, periphyton samples from colonized substrates were taken to determine periphyton chlorophyll *a* concentration.

Experimental design

Each microcosm (experimental unit, e.u.) consisted of an autoclaved glass beaker with 250 mL of filtered and autoclaved pond water with four colonized substrates vertically immersed. Pond water, where the periphytic colonization carried out, was filtered through a mesh and then autoclaved to remove other communities, which may modify and confound the responses of the periphyton.

Thirty-nine microcosms were placed randomly in a culture chamber with controlled photoperiod (12-h light/dark), light intensity (cool white fluorescent light of 30 µmol photon m^{-2} s^{-1}) and temperature (25±1ºC), and they were acclimatized to laboratory conditions for 48 h. Afterwards, 3 treatments were randomly assigned to the e.u. (by triplicate): two monoisopropylamine salt of glyphosate (IPA) concentrations (40 wt% in H_2O, CAS: 38641-94-0): (i) 0.4 mg/L, low glyphosate (LG), and (ii) 4 mg/L, high glyphosate (HG) treatments, and a (iii) control, without herbicide (C).
After the 48-h stabilization, two periods were considered, the exposure and the recovery. At the exposure period, samples were taken the day the experiment began (T0) and 7 days later (T7). On day 7, the periphyton was transferred by translocating the colonized substrates to beakers with herbicide-free water (filtered and autoclaved). Subsequently, samples were taken at 7, 14, and 21 days (R7, R14, and R21, respectively) after translocation to assess community recovery. At each sampling time, three microcosms per treatment were extracted from the chamber to determine physical, chemical and biological parameters. Also, at the start of the recovery period (R0), before translocating the community, physical and chemical parameters in water were measured to determine the initial recovery conditions of the medium.

Physical, chemical and biological variables

Conductivity, pH, temperature and dissolved oxygen concentration (DO) were measured. The temperature in microcosms was registered to check the correct functioning of the culture chamber. Nephelometric turbidity was assessed. Total phosphorus (TP) and nitrogen (TN), and soluble reactive phosphorus (SRP) were determined. TP and TN were determined from unfiltered water samples after acid digestion and SRP from filtered samples through Whatman GF/F filters.
Glyphosate concentration was measured by ion chromatography using a DIONEX DX-100©. The detection limit achieved was 0.2 mg/L. The standard deviation estimated for this analytical method was 3%. The periphyton was removed by scraping the surface of artificial substrates, brought to a known volume with distilled water, and sub-sampled for different analyses. All periphytic variables were expressed per unit area. Sample for quantitative analysis of the autotrophic fraction was fixed and preserved in cold and dark. Counts were performed using the inverted microscope technique. Periphyton classes' percentages and live and dead diatom abundance were considered. Another sample was used to determine dry weight (DW) and ash-free dry weight (AFDW) of periphyton. The periphytic chlorophyll *a* concentration (Chla) corrected by phaeopigments was estimated from the material retained in Whatman GF/F filters. Pigment extraction was carried out and Chla was quantified. The phytoplankton chlorophyll *a* was only determined in the outdoor pond as described for periphyton.

Statistical analyses

To evaluate the exposure and recovery responses in microcosms, periphytic variables were modelled using a general linear model (GLM) with treatment (three levels: C, LG, HG) and sampling time (five levels: T0, T7, R7, R14 and R21) as fixed effects factors. Instead, for each water variable, the two periods, exposure and recovery, were analysed separately. Water variables were also modelled using GLM with treatment (3 levels) and sampling time (2 levels for the exposure period -T0 and T7-, and 4 levels for the recovery period -R0, R7, R14 and R21-) as fixed factors. Tukey's post hoc tests were used for multiple comparisons of means. A p-value <0.05 was considered to be statistically significant. The recovery of a periphytic variable was considered as the time point with no significant differences between the control and any of the treatments with herbicide addition.
Normality and homoscedasticity were analysed. If homoscedasticity was not achieved, models were fitted using VarIdent with time or treatment as the modelling function. The best model was chosen by comparison of Akaike's Information Criteria (AIC). All analyses were performed using R Statistical Software version 3.6.3 (R Core Team, 2020) with nlme and emmeans packages.

Results

Outdoor pond variables

Physical, chemical and biological parameters were recorded in the outdoor pond, where substrates were colonized. In the pond, glyphosate concentration was under the detection limit.

Microcosms water variables

Mean physical and chemical water characteristics for each treatment and sampling time are shown in Table 1. The minimum mean conductivity value was registered at T0 (0.23±0.09 mS/cm) and the maximums at R21 (0.56±0.11 mS/cm, average between the three treatments). Conductivity only showed differences between times, both in the exposure ($F_{1,12}$ = 15.6615; p = 0.0019) and the recovery periods ($F_{3,24}$ = 69.2929; p <0.0001). Mean conductivity showed an average increase of 50% at T7 with respect to T0. pH was circumneutral to slightly basic throughout the study. The minimum value recorded was 7.60 at R0, and the maximum was 8.26 at R21 on the HG treatment. pH only registered differences in the recovery period due to the time-by-treatment interaction ($F_{6,24}$ = 4.2841; p = 0.0045). There were no differences in pH between C and LG throughout the recovery period (p >0.05). On the other hand, HG showed an increase in pH from 7.60 at R0 to 8.26 at R21, resulting in pH values higher than C at R21 (p <0.05).

Turbidity showed differences between times ($F_{1,12}$ = 15.8897; p = 0.0018) in the exposure period, with an almost two times increase from T0 to T7. On the other hand, there were differences in this variable due to the time-by-treatment interaction ($F_{6,24}$ = 15.7276; p <0.0001) in the recovery period. Mean comparisons revealed no differences in the control over time. Instead, treatments with IPA showed an increment in turbidity throughout the recovery. The three treatments showed different turbidity between them at R21 (p <0.05); LG and HG presented values 2.4 and 5.5 times higher than C (Table 1).

Dissolved oxygen, during the exposure, registered differences due to time-by-treatment interaction ($F_{2,12}$ = 17.697; p = 0.0003). Tukey's post hoc comparisons revealed a ~10% decrease in DO in both treatments with IPA (p <0.05), showing the lowest DO mean values (7.02±0.13 mg/L in LG and 6.83±0.11 mg/L in HG) of the entire experiment at T7 (Table 1). During the recovery, differences in DO were only between sampling times ($F_{3,24}$ = 56.5951; p <0.0001). DO decrease 6% from R0 to R7, and then there was a 20% increase until R21 in all the treatments.

The average SRP during the entire exposure period was 0.13±0.06 mg/L. The SRP only showed differences during the recovery between sampling times ($F_{3,24}$ = 7.7038; p = 0.0009). A marked SRP decrease at R14 revealed the lowest average values of the whole experience (0.07±0.03 mg/L, average between the three treatments) with a subsequent increase on the last sampling date (Table 1). Total phosphorus only displayed differences in the exposure period due to the treatment-by-time interaction ($F_{2,12}$ = 172.8995; p <0.0001). There was an increase in TP from T0, 0.24±0.02 mg/L, to the highest mean value of the experiment in HG at T7 (0.86±0.06 mg/L).

The mean glyphosate concentration at T0 was 0.40±0.02 mg/L and 4.09±0.04 mg/L in LG and HG, respectively. At the end of the exposure period, it was 0.42±0.04 mg/L in LG and 4.13±0.08 mg/L in HG. In controls and during the recovery, glyphosate concentrations were below the detection limit of the method.

Table 1. Physical and chemical water characteristics for each treatment and sampling time of the experiment. Data are presented as mean ± 1 SD. Grey shadow pattern represents the exposure period, white pattern represents the recovery period. When the time-by-treatment interaction was significant (p <0.05): differences between treatments within the same sampling date were indicated by different letters, and differences between consecutive times within a treatment were indicated by asterisks. Instead, when differences between sampling times were significant (p <0.05): differences between consecutive times were indicated by diamonds. n = 3. NTU: nephelometric turbidity.

Table 1.(continued)

	C	LG	HG
Conductivity (mS cm^{-1})			
T0 ♦	0.2±0.1	0.2±0.1	0.2±0.1
T7 ♦	0.4±0.0	0.3±0.0	0.3±0.0
R0	0.3±0.0	0.3±0.0	0.3±0.0
R7	0.4±0.1	0.3±0.0	0.3±0.0
R14 ♦	0.4±0.0	0.5±0.0	0.4±0.0
R21 ♦	0.5±0.0	0.6±0.2	0.6±0.1
pH			
T0	7.7±0.1	7.7±0.1	7.7±0.1
T7	7.7±0.0	7.7±0.0	7.8±0.1
R0	7.7±0.1	7.7±0.1	7.7±0.1
R7	7.9±0.1	7.9±0.1 *	7.8±0.0
R14	7.9±0.1	8.0±0.1	8.0±0.0
R21	7.7±0.1^{a}	7.9±0.1^{ab}	8.1±0.1^{b}

	C	LG	HG
Turbidity (NTU)			
T0 ♦	1.9±0.7	1.9±0.7	1.9±0.7
T7 ♦	3.3±1.0	4.4±1.5	3.1±0.5
R0	1.7±0.4	1.7±0.4	1.7±0.4
R7	1.4±0.5	3.8±2.2 *	6.7±1.1
R14	6.1±1.7	15.3±3.1 *	10.5±3.0
R21	7.9±4.4^{a}	19.1±3.7^{b}	43.2±8.0^{c} *
Dissolved oxygen (mg l^{-1})			
T0	7.7±0.1	7.7±0.1 *	7.7±0.1 *
T7	7.5±0.1^{a}	7.0±0.1^{b}	6.8±0.1^{b}
R0 ♦	7.7±0.0	7.7±0.0	7.7±0.0
R7 ♦	7.3±0.4	7.3±0.2	7.2±0.1
R14 ♦	8.2±0.1	8.2±0.6	7.5±0.1
R21 ♦	8.6±0.2	8.9±0.3	8.6±0.3

	C	LG	HG
Soluble reactive phosphorus (mg l^{-1})			
T0	0.1±0.1	0.1±0.1	0.1±0.1
T7	0.2±0.1	0.1±0.1	0.2±0.0
R0	0.1±0.0	0.1±0.0	0.1±0.0
R7 ♦	0.2±0.1	0.2±0.0	0.1±0.0
R14	0.1±0.0	0.1±0.0	0.1±0.0
R21	0.1±0.0	0.1±0.0	0.1±0.0
Total phosphorus (mg l^{-1})			
T0	0.2±0.0	0.2±0.0	0.2±0.0
T7	0.3±0.0^{a}	0.3±0.0^{a}	0.9±0.1^{b} *
R0	0.2±0.0	0.2±0.0	0.2±0.0
R7	0.2±0.0	0.2±0.1	0.2±0.1
R14	0.2±0.1	0.2±0.0	0.2±0.1
R21	0.2±0.0	0.3±0.1	0.2±0.0

Periphyton: glyphosate impact and recovery in microcosms

The controls, both for DW and AFDW, presented the minimum mean values at T7 (18.343±4.936 μg DW/cm^2; 16.040±2.461 μg AFDW/cm^2) and the maximum at R21 (45.879±3.910 μg DW/cm^2; 43.844±5.188 μg AFDW/cm^2). The mean DW throughout the experiment ranged between 14.351±4.148 μg/cm^2 (HG at R7) and 51.759±11.836 μg/cm^2 (LG at R7); and mean AFDW between 10.796±2.280 μg/cm^2 (HG at R21) and 46.816±11.333 μg/cm^2 (LG at R7). The interaction between treatments and time was significant for these two variables (DW: $F_{8,30} = 9.8840$; $p < 0.0001$; AFDW: $F_{8,30} = 11.0226$; $p < 0.0001$). For both, there was an increase at T7 in periphyton subjected to treatments with IPA, revealing values 2.3 (DW) and 2.6 (AFDW) times higher in HG than C. There were no differences between C and LG treatments, in DW or AFDW, during the recovery ($p > 0.05$). On the other hand, the periphyton exposed to HG presented a decrease at R7 concerning T7 (-66% DW and -73% AFDW). This community recorded the lowest DW and AFDW values during the recovery.

The mean periphytic chlorophyll *a* concentration in C for the whole experiment was 0.022±0.007 μg/cm^2. The Chla displayed differences due to the treatment-by-time interaction ($F_{8,30} = 6.3958$; $p = 0.0001$). There were no differences over time in controls ($p > 0.05$). The Chla increased from 0.015±0.002 μg/cm^2 (T0) to 0.036±0.006 μg/cm^2 in LG and 0.028±0.008 μg/cm^2 in HG (T7), when both treatments presented higher values than C (+147% LG and +93% HG). During the entire recovery period, there were no differences between the community subjected to LG and the control ($p > 0.05$). Instead, during the same period, periphyton exposed to the HG treatment did not differ from C until R14 ($p > 0.05$), but then Chla presented a 62% decrease reaching the lowest mean value of the whole experience (0.011±0.003 μg/cm2) at R21.

The mean periphyton total abundance registered the minimum values at T0 ($2.18x10^4 \pm 1.00x10^3$ ind/cm^2), and the maximums at R21 for all the treatments (C: $4.45x10^4 \pm 5.05x10^3$ ind/cm^2; LG: $5.34x10^4 \pm 1.37x10^4$ ind/cm^2; and HG: $1.08x10^5 \pm 2.71\times10^4$ ind/cm^2). Differences were observed due to the time-by-treatment interaction ($F_{8,30} = 24.2081$; $p < 0.0001$). The only significant difference was in HG at R21, when an increase of more than two times in the total abundance of the periphytic community was recorded concerning R14, resulting in higher values than C and LG at R21 ($p < 0.05$).

At T0, Chlorophyta accounted for 81.9±1.8%, followed by Bacillariophyceae (10.8±2.3%), Cyanobacteria (4.1±0.5%), Dinophyceae (2.9±0.5%) and Euglenophyceae (0.2±0.1%). There were differences due to the interaction between treatment and time for the relative percentage of chlorophytes ($F_{8,30} = 144.0479$; $p < 0.0001$). Chlorophyta proportion decreased ($p < 0.05$) at some point during the experiment in all the treatments. In C and LG, the decrease was recorded at R7 (-6% in C and -28% in LG, with respect to T7). Afterwards, in control, this percentage remained almost invariable ($p > 0.05$) until R21, and periphyton treated with LG underwent a significant increase at R14, reaching ~64% of the total periphyton abundance until R21. On the other hand, the high concentration of IPA generated a decrease in the percentage proportion of chlorophytes throughout almost the entire experience, from 81.9±1.8% at T0 resulting at R21 with only 33.3±0.1% of the total periphyton abundance. There were differences between the three treatments at R14 and R21 ($p < 0.05$).

The control treatment had the lowest values of mean relative abundance of total diatoms at all sampling times, with a minimum of 9.8±1.4% at T7. Differences were due to the treatment-by-time interaction ($F_{8,30} = 151.5796$; $p < 0.0001$), and comparisons revealed that the proportion of diatoms was 66% higher in LG than C at T7. In all the treatments, the transfer of colonized substrates to herbicide-free beakers triggered an increase in the percentage proportion of diatoms ($p < 0.05$), and at R7, the microcosms with IPA presented a percentage almost two times greater than the control ($p < 0.05$). Afterwards, the relative abundance of diatoms decreased (-7%) in LG and increased (+9.5%) in HG at R14 ($p < 0.05$), revealing differences between the three treatments at R14 and R21. The lowest mean relative percentage of Cyanobacteria was at T0 (4.1±0.5%) and the maximum at T7 in HG (24.5±1.6%). This percentage showed differences due to the time-by-treatment interaction ($F_{8,30} = 226.9154$; $p < 0.0001$). The cyanobacteria percentage proportion sharply increased from T0 to T7 in periphyton exposed to HG, reaching values more than five times higher than C and LG at T7 ($p < 0.05$). From R14 to the end of the recovery period, there were no differences in cyanobacteria relative abundance between the periphyton impacted with the LG treatment and the control ($p > 0.05$). On the other hand, during the same period, the community exposed to HG presented higher values than those exposed to the other treatments

($p < 0.05$), reaching at R21 an average proportion (24.4±0.2%) as high as in T7 and more than two times higher than C and LG.
Regarding dead diatoms, differences were registered in the treatment-by-time interaction ($F_{8,30} = 40.0396$; $p < 0.0001$). The control treatment registered the lowest values throughout the experience (15.6±2.0%, average for the entire experiment) without differences over time ($p > 0.05$). The percentage of dead diatoms increased from T0 (14.6±3.3%) to T7, more than two times and almost three times for LG and HG, respectively. At all sampling times during the recovery, there were differences in % of dead diatoms between the three treatments ($p < 0.05$). The HG treatment registered the highest percentages and the maximum value of the experiment at R21 (54.0±1.2%).

Conclusion

The authors concluded this was the first study to provide evidence of the capability of periphyton communities to recover after exposure to the most commonly used herbicide in the world. The authors suggested that the concentration of the herbicide during the exposure period will influence IPA effects on periphyton, and this exposure concentration will impact and play a decisive role in the recovery potential of the community. The authors highlighted the need for more studies focusing on the recovery potential of aquatic communities after glyphosate contamination, considering different scenarios and glyphosate-based commercial formulations, as would occur in natural freshwater ecosystems in agricultural landscapes. Microbial ecotoxicologists have been challenged to understand and predict how community recovery can reflect the recovery of an ecosystem. The authors suggested that the first step to answer this question is to increase the scientific literature on microbial communities' structural and functional recovery studies.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This is a higher tier study for aquatic organisms with two glyphosate concentrations tested (0.4 and 4 mg/L) on aquatic microcosms. The study is structured in two phases: 7 days of exposure to treated water and 21 days of recovery in clean water (both of them under static conditions). It evaluates the potential of freshwater periphyton to recover from glyphosate exposure using microcosms under laboratory conditions. Dry weight, ash-free dry weight, chlorophyll *a*, and periphyton abundances were analysed. The periphyton affected with the lowest concentration recovered most of the structural parameters within 7 days in clean water, but the taxonomic structure did not entirely recover towards the control structure. Periphyton exposed to 4 mg/L could not recover during 21 days in herbicide-free water, reaching values almost four times higher in % of dead diatoms and four times lower in ash-free dry weight concerning the control at the end of the study. Results suggest a long lasting effect of the herbicide due to the persistence within the community matrix even after translocating periphyton to decontaminated water. The study concludes that the exposure concentration modulates the recovery potential of impacted periphyton.
The study is considered reliable with restrictions. Analytical verifications of the tested item in the test medium were conducted at the beginning and the end of the exposure phase.

Assessment and conclusion by RMS:

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

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

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	The periphytic colonization was conducted in a pond that has never been contaminated with glyphosate or any other pesticide.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Uncertain	It's not clear whether the test item dissolved in distilled water, filtered pond water or a solvent was used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	Source, content and CAS number were reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoints reported: LOEC immediately after the exposure phase

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		(7-d) and after the recovery phase (28-d)
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Only 2 concentrations were tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	3 microcosms per treatment to determine physical, chemical and biological parameters
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	Water variables were continuously verified
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature set at 25°C for culture and tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Glyphosate concentration was measured at the beginning and the end of the exposure period
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	Analytical methods reported (ion chromatography)
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	LC_{50} 95% confidence interval is reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This is a higher tier study for aquatic organisms with two glyphosate concentrations tested (0.4 and 4 mg/L) on aquatic microcosms. The study is structured in two phases: 7 days of exposure to treated water and 21 days of recovery in clean water (both of them under static conditions). It evaluates the potential of freshwater periphyton (a complex of bacteria, fungi, algae and protozoa) to recover from glyphosate exposure using microcosms under laboratory conditions. Dry weight, ash-free dry weight, chlorophyll *a*, and periphyton abundances were analysed. The periphyton affected with the lowest concentration recovered most of the structural parameters within 7 days in clean water, but the taxonomic structure did not entirely recover towards the control structure. Periphyton exposed to 4 mg/L could not recover during 21 days in herbicide-free water, reaching values almost four times higher in % of dead diatoms and four times lower in

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

Criteria			Criteria met? Yes / No / Uncertain	Comment / Justification
		ash-free dry weight concerning the control at the end of the study. Results suggest a long lasting effect of the herbicide due to the persistence within the community matrix even after translocating periphyton to decontaminated water. The study concludes that the exposure concentration modulates the recovery potential of impacted periphyton. The study is considered reliable. Analytical verifications of the tested item in the test medium were conducted at the beginning and the end of the exposure phase.		
Not reliable	No	-		

1. Information on the study

Data point:	CP 10.1.3
Report author	Goodman R. M. *et al.*
Report year	2021
Report title	Influence of herbicide exposure and ranavirus infection on growth and survival of juvenile red-eared slider turtles (*Trachemys scripta elegans*)
Document No	Viruses, 2021, 13(8), 1440
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The authors exposed hatchling red-eared slider turtles (*Trachemys scripta elegans*) to herbicide and ranavirus treatments to examine direct effects and interactions on growth, morbidity, and mortality. Turtles were assigned to each herbicide treatments or a control group. Turtles were exposed to Roundup ProMax® or Rodeo® via water bath during the first 3 weeks of the experiment. After 1 week, turtles were exposed to either a control (cell culture medium) or ranavirus-infected cell lysate via injection into the pectoral muscles. Necropsies were performed upon death or upon euthanasia after 5 weeks. Tissues were collected for histopathology and detection of ranavirus DNA via quantitative PCR. Only 57.5% of turtles exposed to ranavirus tested positive for ranaviral DNA at the time of death. Exposure to environmentally relevant concentrations of herbicides did not impact infection rate, morbidity, or mortality of hatchling turtles due to ranavirus exposure. The authors also found no direct effects of herbicide or interactions with ranavirus exposure on growth or survival time. Results of this study should be interpreted in the context of the modest ranavirus infection rate achieved, the general lack of growth, and the unplanned presence of an additional pathogen in this study.
The results for combined herbicides (including Atrazine herbicide), reported in the original article, were not presented in this summary.

Materials and methods

The authors obtained 170 juvenile red-eared slider turtles from a commercial supplier (Reptile City; Dallas, TX, USA) in May of 2014. Ten turtles were immediately euthanized and decapitated 1 h after euthanasia. These turtles were necropsied to obtain the following tissues which genomic DNA was then extracted from: approximately 4 cm of mid- and distal intestine, the left kidney, and the left lobe of liver. Testing via quantitative polymerase chain reaction (qPCR; described below) for ranaviral DNA was performed to ensure that supplied animals were not infected with ranavirus. Upon confirmation of negative results for the subsample of 10 turtles, the remaining 160 turtles were placed individually in 5 L plastic containers containing 350 mL of filtered, dechlorinated tap water.

Laboratory temperatures were maintained between 19.5-28ºC with an average temperature of 24ºC. A natural photoperiod (approximately 6:00 a.m.-8:30 p.m. for May-June in Virginia) supplied by large windows in the room was supplemented by overhead fluorescent lights during 9:00 a.m.-6:00 p.m. Throughout the experiment, the authors fed turtles approximately 10% of their weight in Zoo Med natural aquatic turtle feed every other day. Turtle enclosures were placed on shelves in our laboratory and rotated weekly to avoid any bias due to position within the laboratory. Paper dividers were placed between each container and on the outside edge, to reduce possible stress.

After 3 days of acclimation to the laboratory conditions, turtles were assigned to one of eight experimental treatments (n = 20 for each) in a full factorial design with two factors: ranavirus exposure (control, ranavirus-exposed) and herbicide exposure (control, Roundup ProMax®, Rodeo®). At the initiation of the experiment, the authors collected initial weights and measurements of each turtle (mass in g; width and plastron and carapace length in mm using digital calipers) and collected 3-4 mm from each tail tip and sealed the wound. These tissue were collected to sample initial conditions in the event that any turtles not exposed to ranavirus during the experiment turned out to be ranavirus-positive at the conclusion. However, this scenario did not occur; thus, these tail tissues were not tested for ranavirus.

Herbicide exposure consisted of herbicide addition (or lack thereof, for controls) in 200 mL of water in housing containers during the first 3 weeks of the experiment. Herbicide-free dechlorinated water was used for all turtles during the remaining 2 weeks of the 5 week experiment. Levels of herbicide exposure were based on the higher end of but not maximal concentrations reported in the literature: 2000 µg a.e./L glyphosate for Roundup ProMax® and Rodeo® (Monsanto, Creve Coeur, MO, USA).

To constitute treatment water for glyphosate herbicides, 33.4 µL of Rodeo and 29.6 µL of Roundup ProMax® were each added to 500 mL of water and mixed on a stir plate on high for 10 min. The authors then added each 500 mL stock solution to 7500 mL of water in a large bucket and stirred each by hand for 5 min. In two different weeks, the authors took samples from the final herbicide solutions and shipped them overnight on ice to the laboratory to verify target concentrations. Actual concentrations of water samples were 2048-2223 ppb for glyphosate in Roundup Pro Max® and 1952-2292 ppb for Rodeo®.

Ranavirus exposure was executed at the start of the second week by injection of 100 µL of virus-infected cell lysate ($6.3x10^4$ $TCID_{50}$). Control turtles were each injected with 50 µL of Dulbecco's modified Eagle medium (used for fathead minnow cell culture) in each pectoral muscle.

Each week, turtles were weighed, measured, and placed in cleaned, disinfected tubs with reconstitutions of herbicide treatments for the first 3 weeks or herbicide-free dechlorinated water for the last 2 weeks. After virus exposures, turtles were checked visually every 12 h for external signs of ranavirus infection: lethargy, respiratory stress, cutaneous erythema, and ocular/nasal discharge. During weekly measurements and water changes, turtles were examined while in hand for ranaviral disease signs. When turtles expired before the completion of the experiment, they were examined and sampled as described below following euthanasia done at the conclusion of the experiment. Turtles were considered deceased when unresponsive to stimulation of the limbs and corneas. The authors weighed and measured, took note of and photographed any external signs of disease, and then decapitated and necropsied the turtles. The colour and condition of the liver was noted, and tissue samples were taken from the intestine, kidney, and liver, as previously described. Tissues were frozen at 80ºC until use in ranavirus testing. The remainder of the turtle was preserved in 10% buffered formalin for histopathological examination. The experiment was concluded after 5 weeks (4 weeks after virus exposures), when all remaining living turtles were euthanized, measured, and sampled as described above.

The authors extracted DNA from tissue samples with Qiagen DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany). The authors standardized the amount of genomic DNA used in each reaction with an Epoch spectrophotometer (Biotek, Winooski, VT, USA). The authors tested for presence of ranavirus DNA using quantitative polymerase chain reaction (qPCR) following the protocol of Picco *et al*. Each 25 µL PCR reaction contained: 7 µL volume of combined nuclease-free water and genomic DNA; 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems™, Foster City, CA, USA); 1.5 µL each of 10 µM primers: F 5'-ACA CCA CCG CCC AAA AGT AC-3', R 5'-CCG TTC ATG ATG CGG ATA ATG-3'; 2.5 µL of 2.5 µM probe: 5'-/56-FAM/CCT CAT CGT /ZEN/TCT GGC CAT CAA CCA /3IABkFQ/-3' (Integrated DNA Technologies, Coralville, IA, USA). All samples were tested in duplicate using Applied Biosystems™ StepOne Real-time PCR machine with two negative and two positive controls in each run. Samples with Ct values <30 for both runs were considered positive for ranavirus, according to standards established for this machine using known negative and positive controls from water, cultured ranavirus, and ranavirus-infected reptiles. If Ct values from two samples

for an individual were not both <30 or if one approximated 30, the authors ran two additional PCR reactions. Then, the consensus from three out of four total reactions was used.

The authors conducted histopathology on four turtles from each combination of herbicide and virus exposure (eight treatments, 32 turtles total). Four turtles with the lowest Ct values were selected from each of the ranavirus-exposed treatment groups, and the authors performed histological examinations of commonly infected tissues including the liver, spleen, pancreas, and gastrointestinal tract. All turtle tissues were processed at the University of Tennessee Veterinary Medical Center Diagnostic Laboratory where they were embedded in paraffin blocks, cut into 5 µm sections, mounted on glass slides, and stained with haematoxylin and eosin. The authors examined tissues under light microscopy for signs of ranaviral infection, as well as possible tissue lesions caused by herbicide exposure. All work in this study was approved by the Hampden-Sydney College Animal Care and Use Committee, under the protocol number 965 (approved 05 December 2014).

The authors used chi-squared tests of independence to compare ranavirus-positive and -negative PCR results among herbicide treatments and to compare incidence of external white growths (presumed fungus) and liver discoloration among herbicide treatments and ranavirus exposure treatments. To examine potential treatment effects of days survived and change in turtle mass, as well as plastron and carapace length, the authors used a weighted least squares regression model with herbicide and ranavirus exposure and an interaction effect as fixed factors, and time (day of measurement) as a fixed factor. The authors conducted their analyses in SPSS® Statistics version 23 (SPSS Inc., Chicago, IL, USA) and created Figures in R version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

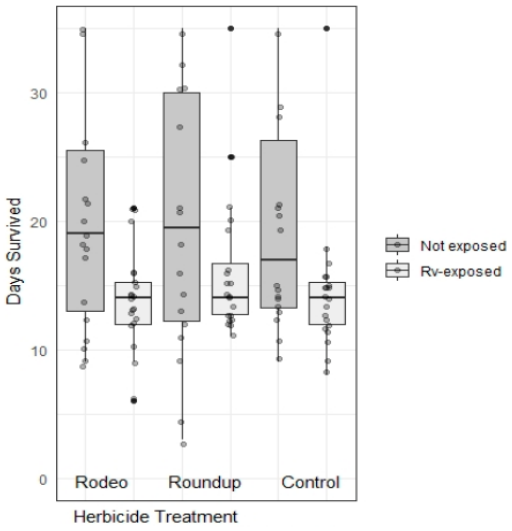
Herbicide treatment did not affect incidence of ranavirus infection among turtles exposed to ranavirus, although there was a non-significant trend for higher infection rates in the herbicide control group relative to other groups infected with ranavirus (Table 1). Neither variable influenced liver colour (tan or red), which was observed in some turtles at the time of necropsy (Table 1). Neither herbicide treatments nor ranavirus exposure treatments affected the incidence of flocculent white growths (typically circular; 1-3 mm in diameter), which were observed in some turtles at the time of necropsy (Table 1). These growths were not observed upon receipt of the shipment of turtles, but they were observed starting on the 10th day of the experiment and culminated in 1-23 growths per turtle, occurring on the skin but not the shells. We attempted to examine the white growths under microscopy but were unable to determine the causal agent. Histopathological changes in ranavirus-exposed turtles with the highest viral loads (lowest Ct values) included necrosis and haemorrhage in the liver, haematopoietic necrosis in the pancreas, and inclusion bodies consistent with ranavirus.

Table 1. Incidence of response variables at time of death among hatchling turtles (*Trachemys scripta elegans*) exposed to herbicide and ranavirus treatments.

Ranavirus Treatment	Herbicide Treatment	*N*	Incidence of Infection	Liver Discol-oration	White Growths
Rv-exposed	Roundup	20	9	6	8
Rv-exposed	Rodeo	20	9	8	11
Rv-exposed	Control	20	16	10	12
Control	Roundup	20	-	7	3
Control	Rodeo	20	-	10	6
Control	Control	20	-	7	7

Ranavirus exposure decreased the number of days survived; however, there was no effect of herbicide treatment (figure 1). The average starting sizes of hatchlings turtles were 7.26 g (SD = 0.97), 33.32 mm in carapace length (SD = 1.54), and 32.07 mm in plastron length (SD = 1.53).

Figure 1. Days survived for hatchling turtles (*Trachemys scripta elegans*) exposed to herbicide and ranavirus treatments.



Conclusion

The authors concluded that ranaviruses are increasingly recognized as significant pathogens worldwide, but their influence on health and survival in reptiles is understudied. The authors stated that their study provides an investigation of interactions between ranaviruses and herbicides, but the importance of many other pollutants and environmental stressors remains to be examined. As expected, ranavirus exposure decreased survival time and reduced mass and plastron length in juvenile turtles. However, contrary to the authors' expectations, they did not detect any direct effects of two formulations of glyphosate on growth or survival or any interaction with ranavirus exposure in terms of susceptibility, morbidity, or mortality. The authors urge caution in the interpretation of their results, because of the low growth rates and infection rates and the unplanned occurrence of another pathogen in our study system. In conclusion, the authors urge replication of ranavirus exposure studies in different study species and with the inclusion of other environmental pollutants and stressors.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study, hatchling red-eared slider turtles (*Trachemys scripta elegans*) were exposed to the formulated glyphosate herbicides Roundup ProMax® and Rodeo® to examine direct effects on growth and mortality. Turtles were exposed to herbicides via water bath during the first 3 weeks of a 5-week experiment. Exposure to a NOEC = 2 mg/L concentration of glyphosate (for both products) did not impact growth or survival time of hatchling turtles.
The study cannot be considered as fully reliable because it is uncertain whether and when the samples from the final herbicide solutions that were analysed to verify target concentrations, were taken from the experimental cages. In addition, the test items were not documented and only one concentration of each product was tested.

Assessment and conclusion by RMS:

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals used for the present study were purchased from a commercial supplier and acclimatized for 3 days to laboratory conditions
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	No	Just the name of the supplier was reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Batch specifications are not provided and the assessment of the ecotoxicological equivalence cannot be conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Uncertain	In two different weeks, samples from the final herbicide solutions were analysed to verify target

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		concentrations, but it is not clear whether and when the samples were taken from the experimental cages.
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint reported: chronic NOEC (35-d))
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	No	Only 1 concentration of each product was tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Uncertain	Samples from the final herbicide solutions were analysed to verify target concentrations, but it is not clear whether and when the samples were taken from the exposure cages.
15. A clear concentration response relationship is reported - in studies where the dose response test design is employed.	No	Only 1 concentration of each product was tested
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	20 replicates of 1 individual per treatment
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water	-	Not applicable, aquatic study

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
retention, microbial biomass (~1% of organic carbon).		
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Mean temperature set at 24°C (19.5-28°C).
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Uncertain	Samples from the final herbicide solutions were analysed to verify target concentrations, but it is not clear whether and when the samples were taken from the exposure cages.
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Uncertain	Samples from the final herbicide solutions were analysed to verify target concentrations, but it is not clear whether and when the samples were taken from the exposure cages.
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	No	No ECx assessment was conducted
Overall assessment		

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

Criteria			Criteria met? Yes / No / Uncertain	Comment / Justification
Reliable without restrictions	No	-		
Reliable with restrictions	Yes	This article reports the chronic effects of two glyphosate-based pesticides on growth and survival of juvenile Red-Eared Slider Turtles (*Trachemys scripta elegans*). The study seems to have been well conducted and reports a regulatory relevant endpoint: 35-d chronic NOEC = 2 mg/L (1.952 - 2.292). The study cannot be considered as fully reliable because it is uncertain whether and when the samples from the final herbicide solutions that were analysed to verify target concentrations, were taken from the experimental cages. In addition, the test items were not documented and only one concentration of each product was tested.		
Not reliable	No	-		

1. Information on the study

Data point:	CP 10.2.1
Report author	Fernandez C. *et al.*
Report year	2021
Report title	Toxic effects of chlorpyrifos, cypermethrin and glyphosate on the non-target organism *Selenastrum capricornutum* (Chlorophyta)
Document No	An Acad Bras Cienc, 2021, 93(4), e20200233
Guidelines followed in study	OECD TG 201 (2011) partially
Deviations from current test guideline	Deviation from OECD TG 201 (2011): • No analytical verifications of the tested item in the test medium during the exposure phase • The test item is not fully documented.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The toxic effects of the herbicide glyphosate on the growth, biovolume and ultrastructure of the green microalgae *Selenastrum capricornutum* were evaluated. Concentrations between 4.7-60 mg/L of glyphosate were tested along with a control culture. The tested concentrations were prepared using a commercial formulation. After 48 h, all tested concentrations of the pesticide reduced significantly the population growth. The 96 h effective concentration 50 (EC_{50}) was 15.60 mg/L for glyphosate. Cells exposed to the pesticide showed an increase in cellular size related to the increase in pesticide concentration and exposure time. The most significant damages observed on the ultrastructure of cells exposed to the pesticide included thylakoids and mitochondria disruption, formation of electrodense bodies, accumulation of lipids and increase in the size and number of starch granules. The present study demonstrates that the effects of the pesticide also extend to non-target organisms having significant ecological implications.

Materials and methods

Species test and toxicity bioassays

Toxicity tests were performed on the freshwater unicellular green algae *Selenastrum capricornutum* Printz, a standard test organism. Cultures of *S. capricornutum* were obtained from the Universidad Federal de São Carlos (São Paulo) and were kept in Bold´s Basic Medium.

Stock cultures were kept in liquid media during 7 days at a temperature of 24±2ºC under continuous illumination (4800 lux) in order to obtain a culture in an early phase of exponential growth. To avoid the settling of algae, the cultures were re-suspended for a few minutes three times a day on an orbital shaker.

The tested concentrations were prepared using the commercial formulation Round-up® (glyphosate 48% w/v). This formulation was diluted in sterile distilled water and added to sterile Bold´s Basic medium. In order to obtain the desired concentrations of the active substance, microalgae were exposed to one third-dilution in a range of 4.7-60 mg/L. These concentrations were selected through a preliminary assay in which the microalgae were exposed to a wider range of pesticide concentration. A toxicity tests with the reference substance potassium dichromate ($K_2Cr_2O_7$) using a one third-dilution in a range of

0.08-0.52 mg/L was performed to evaluate the physiological conditions of the organisms and hence the validity of the tests.

An initial inoculum of 5 x 10^4 cells m/L *S. capricornutum* was added to each concentration. All assays, i.e., reference toxicant tests, controls and pesticide treatments, were carried out in triplicate without medium replacement and under the same conditions of light, temperature and agitation as the stock cultures.

To evaluate the effects of pesticides on the population growth, the number of microalgae in each replicate and in the control culture was estimated by direct counting in Neubauer chamber every 24 h during the entire assay (96 h). To evaluate the effects of pesticides on cell volume, thirty cells from the control culture and from each replicate were measured every 24 h under a light microscope Olympus CX23 and the biovolume was calculated according to Sun & Liu (2003). Simultaneously, the shape, colour and presence of granules in the microalgae were observed.

Effective concentration 50 values (EC_{50}) and confidence intervals (95%) at each exposure time were calculated by means of a Probit analysis of the percent inhibition of algal growth rate (%I) using the Infostat software package (2017).

One-way analysis of variance (ANOVA) was used to evaluate whether there were significant differences between microalgae population growth (cell concentration and *r*) of the different treatments, when statistical differences among values were detected, Dunnett's test was applied. The non-parametric Kruskal-Wallis test was used to assess differences in microalgae cell volume between treatments since data did not attend criteria for parametric methods.

Electron microscopy techniques

At the end of the bioassay (96 h) the control culture and cultures exposed to the pesticide (60 mg/L of glyphosate) were centrifuged at 4000 rpm during 5 minutes.

Cells were fixed at 4ºC in 2.5% glutaraldehyde and post fixed in 1% OsO_4 using filtered culture medium as fixative vehicle. Fixed cells were subsequently dehydrated in an acetone series from 10% to absolute acetone. It was then embedded drop by drop in Spurr's low-viscosity resin and flat-embedded between glass slides coated with dry Teflon. Sections were cut with a Diatome 2.1 mm diamond knife, mounted on Formvar-coated grids and stained with uranyl acetate and lead citrate. They were examined under a Jeol 100 CX-II electron microscope at CCT-Bahia Blanca.

Results

Effects of the glyphosate on growth

Growth curves of *S. capricornutum* under different concentrations are shown in the figure 1 below, the results of the statistical analysis are listed in the table inside the figure. All tested concentrations inhibited the growth of *S. capricornutum* to some extent.

After 24 h of exposure only treatments with 36 mg/L ($p < 0.05$) and 60 mg/L ($p < 0.01$) significantly inhibited the growth, whereas with longer exposure times all concentrations inhibited the growth.

As shown in the table 1 below, the EC_{50} for the %I decreased with the increase of exposure time for glyphosate, indicating a higher toxicity as the exposure time increases.

Table 2 below shows the growth rates (*r*) of algal cultures exposed to different concentrations of pesticides after 96 h. The growth rate decreased as the concentration of the pesticide increased, and all concentrations assayed of the pesticide reduced significantly the growth rate compared to the corresponding control without pesticide.

Figure 1. 96 h growth curve of *Selenastrum capricornutum* in treatments with different concentrations of glyphosate. The table shows significant differences in cell number of treatments compared to the corresponding controls. One asterisk: p <0.05, two asterisks: p <0.01, ns: no significant differences. Adapted from Fernandez C. *et al*. An Acad Bras Cienc, 2021, 93(4), e20200233.

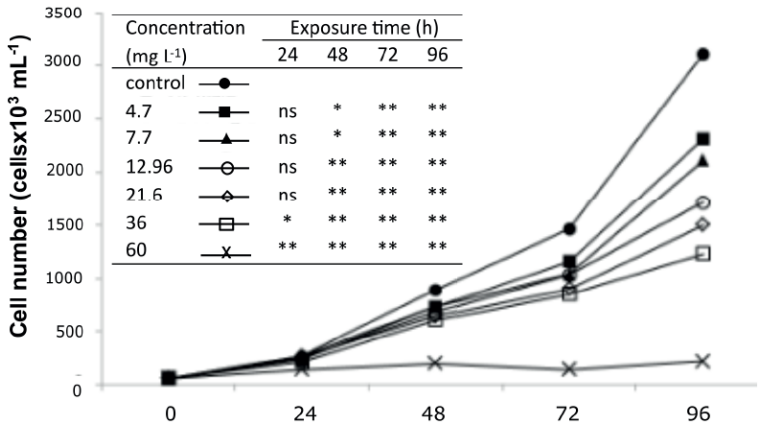


Table 1. Effective concentrations (EC_{50}, mg/L) and the respective 95% confidence limit (95% CI) for percent inhibition of algal growth rate (%I) of the pesticide to *Selenastrum capicornutum* in 24, 48, 72 and 96 h. Adapted from Fernandez C. *et al*. An Acad Bras Cienc, 2021, 93(4), e20200233.

		Glyphosate
24 h	EC_{50}	61.86
	95% CI	49.94-110.55
48 h	EC_{50}	39.02
	95% CI	28.97-62.13
72 h	EC_{50}	24.44
	95% CI	19.13-33.06
96 h	EC_{50}	15.60
	95% CI	12.27-19.67

The algal growth inhibition rate with respect to controls was calculated according to the following equation:

$$\%I = \frac{T - C}{C} \times 100$$

where T is the number of cells in each treatment and C is the number of cells in the control culture.

Table 2. Specific growth rate (r) (/h) of *Selenastrum capicornutum* cultures after 96 h of exposure to different concentrations of the glyphosate. One asterisk (p <0.05) and two asterisks (p <0.01) indicate significant differences compared to the corresponding control without pesticide. Adapted from Fernandez C. *et al*. An Acad Bras Cienc, 2021, 93(4), e20200233.

Concentration (mg/L)	**Growth rate (r)**
control	0.0420±0.0003
4.7	0.0388±0.0007**
7.7	0.0380±0.0005**
12.96	0.0358±0.0003**
21.6	0.0344±0.0008**
36	0.0324±0.0005**
60	0.0139±0.0004**

Exponential growth rate (r) was calculated through the following equation:

$$r = \frac{\ln N_1 - \ln N_0}{t}$$

where N_1 is the number of cells at the end of the assay, N_0 is the initial number of cells and t is the exposure time in hours.

Effects of the pesticide on cellular size and morphology

S. capricornutum cells exposed to the glyphosate evidenced an increase in the cellular size; such increase was related to the increase in concentration and exposure time. At the end of the bioassay (96 h), statistically significant differences were found between treatments (p <0.01). Cells exposed to 21.6, 36 and 60 mg/L showed a cell volume significantly higher than control cells (p <0.05).

With regards to morphology, at the end of the bioassay, normal cells were found only in cultures exposed to the lowest concentrations of the pesticide. At higher concentrations (glyphosate 21.6, 36 and 60 mg/L) different degree of anomalies were observed including deformed and chlorotic cells with abundant cytoplasmic granules and destroyed chloroplasts.

Effects of the pesticide on the ultrastructure

Control culture cells showed the characteristics that best typify Chlorococcales. The central nucleus exhibited condensed chromatin next to the nuclear envelope; it also exhibited a pronounced neckline where the large dictyosome was located. The parietal chloroplast occupied most of the cell volume and showed one conspicuous spherical pyrenoid. Thylakoids were arranged in an almost parallel pattern and starch granules were located between them. The cytoplasm was homogeneous, showing fine and regular granulation due to the presence of ribosomes. The cell wall was characterized by the presence of three layers, the outermost of which was involved in forming crests.

S. capricornutum cells exposed to the pesticide showed changes in the cell structure; the level of disturbance on the ultrastructure depended on the pesticide type and increased as the pesticide concentration increases.

In cells exposed to 60 mg/L of glyphosate the chloroplast and mitochondria were the organelles that underwent the greatest changes, with compacted thylakoids and a big development of the condriome. Abundant lipid globules and vacuoles were observed in the cytoplasm and in some cases occupied the entire cell. The nuclear chromatin was dispersed. The cell wall was not affected and the three- layer structure was still evident. At this concentration of glyphosate the formation of autospores could not be evidenced.

Conclusion

The authors concluded that the present study showed the toxic effects of the pesticide glyphosate on population growth and cell morphology and ultrastructure of *Selenastrum capricornutum*. Ultrastructural damage was produced by glyphosate. Besides, the EC_{50} for glyphosate was close to the concentrations found in some superficial waters indicating a higher ecological risk for this compound.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study evaluates the acute toxic effects of glyphosate on the growth, biovolume and ultrastructure of the green microalgae *Selenastrum capricornutum*. After 48 h, all tested concentrations reduced significantly the population growth. The regulatory relevant endpoint 96-h effective concentration 50 (EC_{50}) was 15.60 mg/L. Cells exposed to glyphosate showed an increase in the cellular size related to the increase in pesticide concentration and exposure time. The most significant damages observed on the ultrastructure of cells included thylakoids and mitochondria disruption, formation of electrodense bodies, accumulation of lipids and increase in the size and number of starch granules.
The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase and the test item is not fully documented.

Assessment and conclusion by RMS:

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	Study conducted according to OECD TG 201 and Environment Canada Series Report EPS 1/RM/25, but not all validity criteria can be checked.
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Cultures were obtained from a culture collection at the Universidad Federal de São Carlos (São Paulo) and were kept in Bold´s Basic Medium.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in the test medium, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	No	Only the name of the product and glyphosate content were reported.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	Study conducted according to OECD TG 201 and Environment Canada Series Report EPS 1/RM/25, but not all validity criteria can be checked.
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume	Yes	-

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.		
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Analytical verifications were not conducted
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint derived: EC_{50} (96-h))
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	-
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Analytical verifications were not conducted
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	-
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study

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Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	Yes	-
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Yes	Temperature set at 24°C for the tests.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	Analytical verifications were not conducted
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	Analytical verifications were not conducted
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	Analytical verifications were not conducted
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Yes	95% CI values were reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study evaluates the acute toxic effects of glyphosate on the growth, bio volume and ultrastructure of the green microalgae *Selenastrum capricornutum*. The 96-h effective concentration 50 (EC_{50}) was 15.60 mg/L. The study is considered reliable with restrictions because it lacks of analytical verifications of the tested item in the test medium during the exposure phase and the test item is not fully documented.
Not reliable	No	-

1. Information on the study

Data point:	CP 10.2.1, CP 10.2.2
Report author	Houssou A. M. *et al.*
Report year	2021
Report title	Acute and chronic effects of a glyphosate and a cypermethrin-based pesticide on a non-target species *Eucypris* sp. Vavra, 1891 (Crustacea, Ostracoda)
Document No	Processes, 2021, 9(4), 701
Guidelines followed in study	None
Deviations from current test guideline	• No guideline was used / followed
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1 / Reliable with restrictions)

2. Full summary of the study according to OECD format

The study aims to evaluate the acute and chronic sensitivities of a freshwater ostracod species (*Eucypris* sp.) to a glyphosate herbicide-based formulation. Lethal concentrations (LC_{50}) of the pesticide for the species at 24 and 48 h were determined. The chronic exposure allowed assessing the effects of low concentrations of the pesticide; firstly, on the parthenogenetic reproduction of *Eucypris* sp., and, secondly, on its population growth. Individuals of *Eucypris* sp. were exposed to 4.51 ppm and 9.03 ppm of glyphosate. These concentrations are respectively the 10% and the 20% of the 48-h LC_{50} (median lethal concentration) of the pesticide for the species. For glyphosate, the 24-h LC_{50} was 50.521 ppm, while at 48-h it was 45.149 ppm. After glyphosate chronic exposure, 60%, 50%, and 90% of individuals were able to reproduce at 10% of LC_{50}-48-h, 20% LC_{50}-48-h, and the control treatment, respectively. The population growth was also affected by the tested low concentrations of glyphosate. The low concentrations of glyphosate affected the species at the individual and population levels.

Materials and methods

Test species

A plankton sample (100 mL) was collected from the Oueme River (Benin) at Bétérou, with a plankton net of 50 µm mesh size. Since most ostracod species are benthic, the sampling was conducted during low flow by trailing the net on the substratum of the river. The sample was subdivided into two parts. A subsample was cultured in laboratory conditions, with a natural fertilizer (chicken droppings) at 0.6 g/L. The temperature of the culture medium was 26.9±0.1ºC, the pH was 7.1±0.0, and the dissolved oxygen was 4.2±0.3 ppm. The second subsample was fixed with formalin for ostracod species identification (just one ostracod species was identified in the sample, *Eucypris* sp.). The ostracod was identified at the genus level. After two weeks of culture, 10 specimens of *Eucypris* sp. were isolated and placed in a phytoplankton (*Scenedesmus* sp.) culture to allow the development of a monospecific culture, which was subcultured every two weeks. Individuals used for the present study were of the fifth generation. They may be considered as non-contaminated individuals by environmental pollutants.

Culture monitoring showed that the asexual female of *Eucypris* sp. reached sexual maturity with the deposit of the first eggs between 15 and 17 days of age, under the conditions presented above. Thus, neonates of 24-h of age, obtained from several females maintained individually, were grouped and bred to 14 days of age, when they were used in the experiment.

Chemicals properties

The test solutions were obtained by dissolving known concentrations of the commercial formulation of the pesticide in water. Kumark® 480 g/L (Kumark Company Limited, Kumasi, Ghana) was used. Glyphosate is a non-selective systemic herbicide with the chemical formula $C_3H_8NO_5P$. Its molecular weight is 169.07 g/mol. Glyphosate is not likely to volatilize (vapour pressure = $1.31x10^{-2}$ mPa). Its half-life in water is 9.9 at 20ºC.

Acute test

Twenty-eight glass cups composed the experimental design for the pesticide. Six acute concentrations were tested for the pesticide and one control (water only) was used. Each treatment had four replications. Seven adults of *Eucypris* sp. Vavra, 1891 (14 days old) were placed in each glass cup immediately after the distribution of the test solutions (28 individuals per treatment, and a total of 168 per test). To identify the nominal concentration to be tested for the pesticide, preliminary exposure tests with randomly selected concentrations were carried out (data not presented). For glyphosate, concentrations of 0 (control), 6, 12, 24, 36, 48, and 60 ppm were used in the definitive acute tests. Each test lasted 48 h. The test solutions were not renewed and *Eucypris* sp. individuals were not fed. Considering the chemicals properties, the loss of active ingredient concentrations in the 48-h was considered insignificant. During the experiment, the photoperiod was set at 16/8-h (light/darkness). Tests with glyphosate were performed at temperatures of 27.0±0.3ºC, pH of 7.2±0.1, and dissolved oxygen of 4.6±0.1 ppm. The mobility of individuals and mortality were monitored at 1-h, 24-h, and 48-h of exposure. Individuals were declared dead when a lack of movement was observed after a mild stimulus.

Chronic test

During chronic tests, the parthenogenetic reproduction, the survivorship of neonates, and population growth of *Eucypris* sp. under low concentrations of glyphosate conditions were tested. To assess the reproduction and neonate survival, the treatments (pesticide concentrations and one control) were conducted in glass containers. Each treatment was carried out in ten replications with a total of 30 containers. The tested concentrations were 10% of the estimated 48-h LC_{50} and 20% of the estimated 48-h LC_{50}. These concentrations were 4.51 ppm and 9.03 ppm. Twenty millilitres of the prepared test solutions were immediately placed in each glass container. One adult female of *Eucypris* sp. was placed in each cup. Individuals were fed ad libitum with phytoplankton (*Scenedesmus* sp.). The experiment lasted 10 days and the test solution was renewed every 96-h. The reproduction and survivorship of neonates were controlled daily.

Regarding the population growth test, it was conducted in polyethylene aquaria of 3 L each. The same concentrations were tested as in the reproduction test. Three replications were used with a total of 9 aquaria. 1 L of test solutions enriched with phytoplankton (*Scenedesmus* sp.) was used in each aquarium and 10 *Eucypris* sp. individuals were dropped. The test solution was renewed every 96-h. Once a week, 20 mL of the culture medium of each treatment was collected after homogenization and fixed with formalin in a pillbox. These were used for individual enumeration under a light microscope. The tests lasted 28 days. The photoperiod was 16/8-h (light/darkness), the temperature was 25.0±0.3ºC, the pH was 6.5±0.3, and the dissolved oxygen was 3.6±0.8 ppm.

Data analysis

The mortality values were used to estimate the median lethal concentrations (LC_{50}) at 24-h and 48-h for the pesticide. The LC_{50} were estimated using the probit method in the computing program PoloPlus v.1.0 (LeOra Software LLC, Northampton, UK). Reproduction rates and survival of neonates were calculated for each treatment. The one-way analysis of variance (ANOVA) was used to check differences among treatments (Statistica v.7 Software (StatSoft, Tulsa, OK, USA) was used). Before the ANOVA test, the normality of data was tested with the Kolmogorov–Smirnov (K-S) test, while the homogeneity of variance was assessed with Levene's test.

Results

Swimming ability

Eucypris sp. individuals exposed to acute doses of glyphosate showed a perturbation of their swimming

ability shortly after exposure. They became motionless with an erratic movement following a soft needle stimulus. This steady state preceded individual death in most of the cases, while some remained alive with the imperfection until the end of the study (48 h of acute exposure). Acute exposure, immobilization ranged from 13% to 82% after one hour. In 24 h, it ranged from 45% to 82%, while in 48 h, it was from 63% to 96%. The two highest concentrations were significantly different from control after 24h and 48h. The first four concentrations of glyphosate also showed significant temporal variations of individual immobilization ($p < 0.05$).

Individual mortality

The median 24 h lethal concentration was 50.525 ppm. The interval of confidence was 45.351-58.204 ppm. For exposure of 48 h, the glyphosate LC_{50} was estimated at 45.149 ppm (40.082-51.296 ppm).

Reproduction and neonate survival

After 10 days exposure to glyphosate, 60%, 50%, and 90% of parthenogenetic reproduction were obtained with the 10% LC_{50} (4.51 ppm), 20% LC_{50} (9.03 ppm), and the control (0.0 ppm), respectively. The survival of neonates was 98±0.1%, 83.7±0.5%, and 95±0.3%, respectively.

Population growth

A slowdown of the population growth was observed compared to the control in which an exponential profile was obtained. A maximum density of 1271.7±7.6 ind/L (28^{th} day) and 651.8±8.0 ind/L (21^{st} day) was observed with 4.51 ppm (10% LC_{50}) and 9.03 ppm (20% LC_{50}), respectively, against 2671.7±13.9 ind/L (28^{th} day) in the control.

Conclusion

The authors concluded that freshwater ostracod *Eucypris* sp. showed tolerance to glyphosate with 24-h LC_{50} of 50.525 ppm and 45.149 ppm in 48 h. Sub-lethal concentrations such as 9.03 ppm of glyphosate may affect the parthenogenetic reproduction of the species. These may also affect the survivorship of neonates and, consequently, the population growth over time.

The authors indicated that, in developing countries, where monitoring devices are absent for aquatic ecosystems and the use of pesticides is experiencing a rapid increase, high concentrations of glyphosate can quickly occur in aquatic environments. The risk of ecological disturbances would, therefore, be great with the corollary of a considerable reduction in services.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This article reports the acute and chronic effects of a glyphosate-based pesticide on the fresh-water Ostracoda species *Eucypris* sp. (aquatic invertebrate species other than *Daphnia magna*). The study seems to have been well conducted and reports regulatory relevant and reliable endpoints: 48-h acute LC_{50} = 9.03 mg/L and 28-d chronic LOEC = 0.903 mg/L (10% of the estimated 48-h LC_{50}). However, the study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium. In addition, the culture/test medium is not described.

Assessment and conclusion by RMS:

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	No	Non-guideline study
2. No previous exposure to other chemicals is documented (where relevant).	Yes	Individuals used for the present study are fifth generation of a lab culture from a natural sample. They may be considered as non-contaminated individuals by environmental pollutants.
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	Yes	Test item dissolved in distilled water, no solvent used
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Uncertain	Source and content reported, but not clear if the tested formulation Kumark® 480 g/L (Kumark Company Limited, Kumasi, Ghana) contains surfactants.
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	No	Batch specifications are not provided and the assessment of the ecotoxicological equivalence cannot be conducted
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Uncertain	Culture medium is not described
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	No	Non-guideline study
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when	Yes	-

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.		
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	No	Concentrations were not analytically verified
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoints reported: acute LC_{50} (24 and 48-h) and chronic LOEC (28-d))
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes (acute test) No (chronic test)	For the chronic test only 2 concentrations were tested
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	No	Exposure concentrations were not analytically verified.
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	Four replicates with seven adults per tested concentration (acute), ten replicates of 1 adult female per concentration (chronic reproduction) and 3 replicates of 10 individuals per concentration (chronic growth)
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard error provided; no raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	-	Not applicable, aquatic study
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Not applicable, aquatic study

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	-	Not applicable, aquatic study
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	-	Not applicable, aquatic study
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Not applicable, aquatic study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Not applicable, aquatic study
20.6. Data on precipitation is recorded.	-	Not applicable, aquatic study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	-	Not applicable, aquatic study
22. For bee studies, temperature of the study should be appropriate to species.	-	Not applicable, aquatic study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	No	No information on the composition of the culture/test medium
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	Uncertain	Temperature set at 27°C for culture and acute tests. No data of the temperature of the chronic tests are reported.
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	No	Exposure concentrations were not analytically verified
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	No	Exposure concentrations were not analytically verified
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	No	No analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Yes	LC_{50} 95% confidence interval is reported

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This article reports the acute and chronic effects of a glyphosate-based pesticide on the fresh-water Ostracoda species *Eucypris* sp. The study seems to

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

Criteria			Criteria met? Yes / No / Uncertain	Comment / Justification
		have been well conducted and reports regulatory relevant and reliable endpoints: 48-h acute LC_{50} = 9.03 mg/L and 28-d chronic LOEC = 0.903 mg/L (10% of the estimated 48-h LC_{50}). However, the study cannot be considered as fully reliable because it lacks of analytical verifications of the tested item in the test medium. In addition, the culture/test medium is not described.		
Not reliable	No	-		

1. Information on the study

Data point:	CP 10.4.2.1
Report author	Wee J. *et al.*
Report year	2021
Report title	Temperature and Aging Affect Glyphosate Toxicity and Fatty Acid Composition in *Allonychiurus kimi* (Lee) (Collembola)
Document No	Toxics, 2021, 9, 126
Guidelines followed in study	None (partially based on OECD TG 232)
Deviations from current test guideline	• OECD TG 232 validity criteria cannot be fully checked.
GLP/Officially recognised testing facilities	• No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

This study examined the toxicity of glyphosate with the temperature (20ºC and 25ºC) and aging times (0 day and 7 days) in soil using a collembolan species, *Allonychiurus kimi* (Lee). The degradation of glyphosate was investigated. Fatty acid composition of *A. kimi* was also investigated. The half-life of glyphosate was 2.38 days at 20ºC and 1.69 days at 25ºC. At 20ºC with 0 day of aging, the EC_{50} was estimated to be 93.5 mg/kg. However, as the temperature and aging time increased, the glyphosate degradation increased, so no significant toxicity was observed on juvenile production. The proportions of the arachidonic acid and stearic acid decreased and increased with the glyphosate treatment, respectively, even at 37.1 mg/kg, at which no significant effects on juvenile production were observed. The authors concluded that their results showed that the changes in the glyphosate toxicity with temperature and aging time were mostly dependent on the soil residual concentration. Furthermore, the changes in the fatty acid compositions suggest that glyphosate could have a chronic effect on soil organisms.

Materials and methods

Test animals

The test collembolan species, *A. kimi* (formerly known as *Paronychiurus kimi*), was collected from a paddy field in Korea in 1996. The *A. kimi* population was cultured in a plastic petri dish (9.0 cm in diameter and 1.5 cm in height) filled with approximately 0.5 cm depth of moist substrate comprised of plaster of Paris, activated charcoal, and distilled water at a ratio of 4:1:4 by volume in a dark growth chamber at 20±1ºC. Brewer's yeast was provided weekly as food. Age-synchronized collembolans were obtained. After the eggs hatched, the juveniles were reared under the same conditions and used for all subsequent experiments.

Chemical and toxicity test

A commercial formulation of glyphosate (Geunsami®) was obtained from Farm Hannong Ltd. (Seoul, Korea) and used throughout the experiments. The formulation contained glyphosate as an active ingredient (41%, w/w) in the form of isopropylamine salt.

Toxicity tests were conducted according to OECD guideline 232. The effects of glyphosate on the adult survival and juvenile production of *A. kimi* at 20ºC and 25ºC and aging times of 0 day and 7 days were investigated. The test concentrations were 0.0, 0.7, 3.7, 37.1, 74.1, and 370.5 mg/kg soil dry weight. The stock solution was prepared by dissolving the formulation in deionized water. Diluted stock solutions

adjusted to the test concentrations were mixed thoroughly with the soil to obtain the required moisture content of 50% water holding capacity. The spiked soil (30 g) was placed in a polystyrene vessel (55 mm in diameter and 60 mm in height). A total of 120 test vessels (6 concentrations 5 replicates 2 temperatures 2 aging times) were prepared at once, and 60 test vessels were separately stored in a dark growth chamber at 20ºC and 25ºC, respectively. After 0 day and 7 days of aging at each temperature, 30 test vessels (5 vessels per concentration) were randomly selected and used for the toxicity test.
At the beginning of each test, 10 *A. kimi* adults (42-46 day old) were introduced into each test vessel. The test vessels were kept under the condition that the aging proceeded at each temperature. The test vessels were aerated and weighed weekly to replenish the moisture loss by the addition of deionized water, if needed. Brewer's yeast was provided on the soil surface as food at the beginning of the test and biweekly. After 28 d, the surviving adults and juveniles produced in each vessel were counted by flotation with water and transferred to a petri dish filled with a moist substrate.

Fatty acid analysis

The effects of glyphosate treatment on the fatty acid composition of adults were determined at 20ºC and 25ºC. The test concentrations were 0.0 (control), 37.1, and 370.5 mg/kg. Because no significant effect on adult mortality was observed at any of the glyphosate concentrations, the tested concentrations were selected based on the lowest and highest concentrations at which a decrease in juvenile production was observed. After 28 days of exposure to 37.1 mg/kg and 370.5 mg/kg of glyphosate at 20ºC and 25ºC, the adults that survived the glyphosate treatments were transferred to and kept in the plastic petri dish for 1 d without feeding to excrete their gut contents. The adults exposed to the same concentration were pooled and transferred to a 2 mL microtube and then stored at 80ºC until fatty acid analysis.
The total cellular fatty acids in *A. kimi* adults were analysed. Briefly, 20 adults exposed to the same concentration were homogenized in 7.5 mL of a mixture of chloroform, methanol, and 0.05 M of phosphate buffer at a pH of 7.4 (1:2:0.8, v/v/v). After overnight shaking, 0.8 mL of distilled water and 0.8 mL of chloroform were added. The mixture was centrifuged at 1500 rpm for 5 min and then allowed to stand to separate into three layers of methanol, phosphate buffer, and chloroform. The lipids in the chloroform layer were saponified and methylated using the Sherlock Microbial Identification System to produce fatty acid methyl esters (FAMEs). Finally, the FAMEs were analysed using the Sherlock Microbial Identification System. This procedure was repeated three times. In addition, the total cellular lipids (TCLs) in *A. kimi* adults were estimated using an external standard curve of palmitic acid (16:0).

Glyphosate degradation in soil

To investigate the temperature-dependent degradation kinetics of glyphosate, 30 g of soil spiked with 370.5 mg/kg of glyphosate were prepared and stored at 20ºC and 25ºC. The glyphosate concentrations in 5 g of soil were measured at 0, 2, 4, 7, and 14 days after exposure with three replicates each. The control group was treated with distilled water only. In addition, a series of diluted glyphosate solutions (0-20 mg/L) was prepared by dissolving a standard glyphosate solution (1000 μg/mL) in 20 mL of 2 M ammonium hydroxide and used to obtain a standard curve.
The glyphosate concentration in the soil samples was determined after soil samples preparation. A linear relationship between the absorbance (measured at 435 nm) and concentration of glyphosate in standard solutions was obtained ($r^2 = 0.92$). No glyphosate was detected in the control soil, and the recovery rate of glyphosate was 79.59±12.75% in the glyphosate-treated soil. The limit of quantification of glyphosate was 2.08 mg/kg.

Data analysis

The degradation of glyphosate over time was fitted to a single first-order (SFO) kinetic model, as follows:

$$C_t = C_0 e^{-kt}, \qquad (1)$$

where C_t is the concentration of glyphosate at time t, C_0 is the initial concentration of glyphosate ($t = 0$), and k is the degradation rate constant. The degradation half-life time (DT_{50}), the time required for a 90% decline in concentration (DT_{90}), and k were estimated by fitting Equation 1 into a non-linear regression model.
To evaluate the effects of the temperature and aging time on the toxicity, an effective concentration of 50% (EC_{50}) on juvenile production and the corresponding 95% confidence intervals were estimated by

fitting the data to a logistic model. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) on juvenile production were determined by applying one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test ($p < 0.05$).
Prior to statistical analysis of the fatty acid profile, the data were arcsine-root trans- formed to normalize the distribution. The unsaturation index (UI; sum of the percentage of each unsaturated fatty acid multiplied by the number of double bonds) and the ratio of fatty acids with 16 carbons and 18 carbons (C16:C18) were calculated. To test whether the fatty acid profiles were affected by the glyphosate concentration and temperature, each fatty acid profile was compared using a two-way ANOVA with the temperature and concentration as independent variables. The Shapiro-Wilk test was used to confirm the normal distribution of data, and the Bartlett test was used to check for homogeneity of variance. A post hoc test for multiple comparisons was conducted using Tukey's test ($p < 0.05$). All the statistical data analyses were performed using SAS software (version 9.4). In addition, to visualize the differences in the fatty acid profiles across different glyphosate concentrations, principal component analysis (PCA) was conducted to evaluate the effects of glyphosate on the fatty acid composition. A permutational multivariate analysis (PERMANOVA) using the Euclidean distance as a similarity index was performed using the Adonis function in the Vegan package of RStudio version 1.2.5001.

Results

Toxicity test

The mean adult mortality rates in all controls were all less than 10%, and no significant difference was detected between the controls ($p > 0.05$). However, regardless of the aging time, the mean juvenile production per vessel in the control at 25ºC (135.8±31.4 for 0 d and 125.0±25.5 for seven days) was always significantly higher than that observed at 20ºC (82.5±6.5 and 76.0±12.3, respectively) ($p < 0.05$). The coefficient of variation calculated for the number of juveniles was less than 30% in all controls.
In all of the treatments, the adult survival within the entire test concentration range was not significantly different from that of the corresponding control, thereby indicating that glyphosate did not have a negative effect on adult survival. Thus, the median lethal concentration (LC_{50}) for adults could not be determined because all of the mortality values were below 50%. Meanwhile, juvenile production in all of the treatments was reduced in a concentration-dependent manner, but a significant difference in juvenile production from that of the control was observed only at 0 d of aging. At 20ºC with 0 day of aging, the EC_{50} value was 93.5 mg/kg and the NOEC and LOEC values were 3.7 mg/kg and 37.1 mg/kg, respectively. In the test at 25ºC with 0 day of aging, a significant difference from the control was observed only at the highest treatment concentration (370.5 mg/kg); thus, the EC_{50} values could not be estimated. In the seven-day aging test at both temperatures, no significant difference from the control was observed even at the highest concentration, thereby indicating that the toxicity of glyphosate to *A. kimi* was reduced as the aging time and temperature increased.

Fatty acid composition

At both temperatures, the TCL concentration per individual was lower in the glyphosate treatments than in the control, but no significant difference was observed between the treatments (Table 1). The TCL concentration in the control was 6.90±1.10 nmol/ind and 8.20±4.40 nmol/ind at 20ºC and 25ºC, respectively, but that in the treatments ranged from 3.10±0.60 nmol/ind to 3.80±1.90 nmol/ind.
Six fatty acids with a carbon chain length of 16 to 20 were identified as palmitoleic acid (16:1ω7), palmitic acid (16:0), linoleic acid (18:2ω6,9), oleic acid (18:1ω9), stearic acid (18:0), and arachidonic acid (20:4ω6,9,12,15). Each fatty acid composition is presented on a percentile scale in Table 1. Regardless of the temperature and glyphosate concentration, the most dominant fatty acids were oleic acid (39.65% to 44.63%) and palmitic acid (21.37% to 24.83%). The compositions of stearic acid and linoleic acid were very similar, ranging from 12.54% to 15.75% and 12.50% to 14.90%, respectively.
In addition, a significant interaction between the glyphosate concentration and temperature was detected in palmitoleic acid and oleic acid, but no significant differences in the proportion of each fatty acid were observed, except for palmitoleic acid, which was undetected at the highest concentration at 25ºC (Table 1).

Table 1. Proportions of fatty acids (mean ± standard deviation) in *Allonychiurus kimi* (Lee) adults after 28 day of exposure to glyphosate (0.0, 37.1, and 370.5 mg/kg) at 20ºC and 25ºC.

		20 °C			25 °C		
		Glyphosate Concentration (mg kg^{-1})			Glyphosate Concentration (mg kg^{-1})		
Fatty Acids		0.0	37.1	370.5	0.0	37.1	370.5
Palmitoleic acid	16:1ω7	5.77 ± 1.06	5.60 ± 3.68	5.48 ± 0.17	5.16 ± 0.18	6.72 ± 1.34	**0.00 ± 0.00**
Palmitic acid	16:0	21.37 ± 1.15	24.31 ± 0.43	23.79 ± 0.26	22.87 ± 0.71	22.84 ± 0.11	24.83 ± 1.30
Linoleic acid	18:2ω6,9	14.90 ± 0.95	13.41 ± 1.98	13.60 ± 0.93	12.50 ± 1.07	13.03 ± 0.81	13.70 ± 0.57
Oleic acid	18:1ω9	39.65 ± 0.07	44.63 ± 2.96	41.40 ± 1.11	40.18 ± 0.86	39.54 ± 1.68	43.46 ± 1.40
Stearic acid	18:0	12.84 ± 0.39	12.54 ± 1.87	**15.75 ± 0.11**	13.38 ± 0.17	**14.99 ± 0.37**	**15.49 ± 0.30**
Arachidonic acid	20:4ω6,9,12,15	5.49 ± 1.58	2.51 ± 3.55	0.00 ± 0.00	5.93 ± 0.47	**0.00 ± 0.00**	**0.00 ± 0.00**
Total cellular lipids (nmol·ind^{-1})		6.90 ± 1.10	3.80 ± 1.90	3.70 ± 1.20	8.20 ± 4.40	3.70 ± 0.20	3.10 ± 0.60
C16:C18 [a]		0.40 ± 0.04	0.39 ± 0.08	0.41 ± 0.00	0.42 ± 0.01	0.44 ± 0.00	**0.34 ± 0.01**
Unsaturation index [b]		0.97 ± 0.07	0.84 ± 0.11	0.74 ± 0.01	0.94 ± 0.03	**0.72 ± 0.05**	**0.71 ± 0.03**

The bold numbers indicate a significant difference from the control within the same temperature treatment (Dunnett's post hoc test; $p < 0.05$). [a] The ratio of fatty acids with 16 carbons and 18 carbons. [b] The unsaturation index was calculated as described by Haubert et al., 2004, Comp. Biochem. Physiol. B Biochem. Mol. Biol., 138, 41-52.

The two-way ANOVA results indicated that the proportions of palmitic acid, stearic acid, and arachidonic acid were significantly affected by the glyphosate concentration ($p < 0.05$), but no temperature effect on the six fatty acid profiles was observed (Table 2). The proportion of stearic acid, a long-chain saturated fatty acid, increased as the glyphosate concentration increased at 20ºC and 25ºC, but that of arachidonic acid, a polyunsaturated fatty acid (PUFA), decreased as the concentration increased and was not detected at the highest concentration at both temperatures.

Table 2. Two-way analysis of variance results on the effects of glyphosate and temperature on the individual fatty acid contents in *Allonychiurus kimi* (Lee) adults after 28 day of exposure to glyphosate.

Fatty Acids		Glyphosate (G)		Temperature (T)		G × T	
		F	*p*	*F*	*p*	*F*	*p*
Palmitoleic acid	16:1ω7	3.02	0.12	0.76	0.42	8.00	**0.02**
Palmitic acid	16:0	7.91	**0.02**	0.64	0.45	4.05	0.08
Linoleic acid	18:2ω6,9	0.21	0.82	1.78	0.23	1.35	0.33
Oleic acid	18:1ω9c	2.90	0.13	0.81	0.40	5.53	**0.04**
Stearic acid	18:0	9.41	**0.01**	3.72	0.10	2.82	0.14
Arachidonic acid	20:4ω6,9,12,15	14.14	**0.01**	0.78	0.41	1.05	0.41
C16:C18 [a]		1.15	0.38	0.00	0.98	3.03	0.12
Unsaturation index		11.74	**0.01**	2.28	0.18	0.29	0.76

The bold numbers indicate a significant effect of the factor on individual fatty acids ($p < 0.05$). [a] The ratio of fatty acids with 16 carbons and 18 carbons.

Owing to the increase in C18 and saturated fatty acids (stearic acid) and the decrease in C16 and unsaturated fatty acids (arachidonic acid and palmitoleic acid) due to glyphosate treatment, the UI and C16:C18 ratio at 25ºC decreased significantly as the glyphosate concentration increased. This was also evident from the biplot of the PCA, which clearly separated the fatty acid profiles exposed to different glyphosate concentrations according to the changes in the proportions of stearic acid and arachidonic acid. The PERMANOVA results also revealed significant influences of glyphosate application ($F = 3.45$; $p = 0.02$) on the fatty acid profiles.

Glyphosate degradation

The residual glyphosate concentration in the soil decreased exponentially over time at both temperatures. The lag phase was not observed in the initial degradation state and after two days of incubation, and the residual glyphosate concentration at 20ºC (51.8±9.0 mg/kg) was significantly higher than that at 25ºC (27.5±1.2 mg/kg) ($p < 0.05$). At the end of the experiment, the residual glyphosate concentration at 20ºC was 2.2±1.1 mg/kg, but no glyphosate was detected at 25ºC, thereby indicating that glyphosate was

rapidly degraded as the temperature increased.
Because the χ^2 error values for the SFO model were smaller than the validity criteria (15%) and their visual fits and residual plots were acceptable, the SFO model was used to estimate the DT_{50} and DT_{90} values (Table 3). The DT_{50} and DT_{90} values at 20ºC (2.38 day and 7.91 day, respectively) were longer than those at 25ºC (1.69 day and 5.63 day, respectively).

Table 3. Degradation kinetics parameter (k), DT_x values, and χ^2 error for glyphosate fitted with the single first-order model in soil at 20ºC and 25ºC.

Temperature (°C)	k	DT_{50} (d)	DT_{90} (d)	χ^2 Error (%)
20	0.291	2.38 (1.74–3.02)	7.91 (5.79–10.05)	9.55
25	0.409	1.69 (1.27–2.11)	5.63 (4.23–7.01)	14.02

DT_x values indicate the time required for the initial concentration to decrease x%. The degradation kinetics of glyphosate were investigated in soil contaminated with 370.5 mg/kg of glyphosate at both temperatures.

Conclusion

Glyphosate have been known to be toxicologically and environmentally benign. However, the authors concluded that their study showed that glyphosate can have negative effects on the juvenile production of *A. kimi* depending on the temperature and aging time. Glyphosate toxicity is largely determined by the amount of residue in the soil, which is dependent on the soil temperature. Thus, an understanding of the temperature-dependent half-life of glyphosate in various field soils in which the temperature fluctuates considerably is important to assess the ecologically relevant risk under field conditions. No glyphosate-induced adult mortality was observed in any of the treatment conditions, but significant changes in the fatty acid composition of *A. kimi* adults, which play a critical role in energy storage, cell structure, and regulatory physiology, were observed, thereby suggesting that glyphosate could have adverse effects on *A. kimi* at the sub-individual level. Considering the increasing application rate and repeated application of glyphosate in fields, glyphosate concentrations higher than the PEC could be present in the soil. If *A. kimi* is exposed to glyphosate for a long time above the PEC, then the ecological fitness of an individual may also be affected. Therefore, it is necessary to investigate the long-term impacts of glyphosate by conducting higher-tier tests, such as multigenerational tests.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study examined the toxicity of glyphosate with the temperature (20ºC and 25ºC) and aging times (0 day and 7 days) in soil using a collembolan species, *Allonychiurus kimi* (Lee). The degradation of glyphosate in soil was investigated. Fatty acid composition of *A. kimi* was also investigated. The half-life of glyphosate was 2.38 days at 20ºC and 1.69 days at 25ºC. At 20ºC with 0 day of aging, the EC_{50} and NOEC were estimated to be 93.5 and 3.7 mg/kg, respectively. As the temperature and aging time increased, the glyphosate degradation also increased, so no significant toxicity was observed on juvenile production. The proportions of the arachidonic acid and stearic acid decreased and increased with the glyphosate treatment, respectively, even at 37.1 mg/kg, at which no significant effects on juvenile production were observed. The study was conducted according to OECD TG 232 Collembolan Reproduction Test in Soil and is considered reliable with restrictions, the validity criteria cannot be fully checked.

Assessment and conclusion by RMS:

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
Key criteria		
1. For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.	Uncertain	As no raw data are provided not all validity criteria can be checked
2. No previous exposure to other chemicals is documented (where relevant).	Yes	The species has been cultured for years in the lab and the soil is according to guidance
3. For aquatic studies, the test substance is dissolved in water or where a carrier is required, it is appropriate (non-toxic) and a carrier control / positive control is considered in the test design.	-	No aquatic study
4. Glyphosate or its metabolite (test item) are sufficiently documented and reported (i.e. purity, source, content etc.).	Yes	-
5. For tests including vertebrates, there is a compliance of the batches used in toxicity studies compared to the technical specification.	-	No vertebrate study
6. Species used in the experiment are clearly reported, including source, experimental conditions (where relevant): strain, adequate age/life stage, body weight, acclimatization, temperature, pH, oxygen (dissolved oxygen for aquatic tests) content, housing, light conditions, humidity (terrestrial species) incubation conditions, feeding etc.	Yes	-
7. The validity criteria from relevant test guidelines can be extrapolated across different species but not necessarily across different test designs. If different, then the nature of the difference and impact should ideally be discussed.	Uncertain	As no raw data are provided not all validity criteria can be checked
8. Only glyphosate or its metabolite is the test substance (excluding mixture with other substances), and information on application of the test substance is described.	Yes	-
9. The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).	Yes	-
10. Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.	Yes	-
11. Analytical verifications are performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.	Yes	Analytical verifications of the concentration of glyphosate in soil samples were conducted
12. An endpoint can be derived. Findings do deliver a regulatory endpoint, and/or is useful as supporting information.	Yes	Endpoint reported: 28-d EC_{50} and NOEC
13. The test has been tested in several dose levels (at least 3) including a positive/negative control where relevant.	Yes	-
14. Suitable exposure throughout the whole exposure period was demonstrated and reported.	Yes	Analytical verifications of the concentration of glyphosate in

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		soil samples were conducted
15. A clear concentration response relationship is reported – in studies where the dose response test design is employed.	Yes	-
16. A sufficient number of animals per group was included to facilitate statistical analysis: mortality in control groups reported, observations/findings in positive/negative control clearly reported (where relevant).	Yes	5 replicates of 10 individuals for each treatment group
17. Assessment of the statistical power of the assay is possible with reported data.	Yes	Mean and standard deviation were provided. No raw data
18. If statistical methodology was applied for findings reported, then the data analysis applied should be clearly documented (e.g., checking the plots and confidence intervals).	Yes	Statistical analysis sufficiently described
19. Description of the observations (including time-points), examinations, and analyses performed, with (where relevant) dissections being well documented.	Yes	-
20. For terrestrial ecotox studies in the lab or the field, the substrates used should be adequately described e.g. nature of substrate i.e. species of leaf or soil type.	Yes	OECD substrate
20.1. Field locations are relevant/comparable to European conditions. Soils do not completely match the OECD criteria but are from Europe or to some extent representative for the European Agriculture.	-	Lab study
20.2. Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).	Yes	OECD artificial soil used
20.3. Other soils where information on characterization by the parameters: pH, texture, CEC, organic carbon, bulk density, water holding capacity, microbial biomass.	Yes	OECD artificial soil used
20.4. For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.	-	Lab study
20.5. For soil samples, sampling from A-horizon, top 20 cm layers; soils freshly from field preferred (storage max 3 months at 4 +/- 2°C).	-	Lab study
20.6. Data on precipitation is recorded.	-	Lab study
21. For lab terrestrial studies, the temperature was appropriate to the species being tested and generally should fall within the range between 20-25°C and soil moisture / relative humidity was reported.	Yes	2 temperatures (20 and 25°C) set
22. For bee studies, temperature of the study should be appropriate to species.	-	No bee study
23. For lab aquatic studies: 23.1. The source and / or composition of the media used should be described.	-	Not aquatic study
23.2. The temperature of the water should be appropriate to the species being tested and generally fall within the 15-25ºC.	-	Not aquatic study
24. The residue data can be linked to a clearly described GAP table appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).	Yes	Analytical verifications of the concentration

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

Criteria	Criteria met? Yes / No / Uncertain	Comment / Justification
		of glyphosate in soil samples were conducted
25. Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.	Yes	Analytical verifications of the concentration of glyphosate in soil samples were conducted
26. Analytical methods are clearly described and adequate statement of specificity and sensitivity of the analytical methods is included.	Yes	Analytical methods reported
27. Assessment of the ECX for the width of the confidence interval around the median value; and the certainty on the level of protection offered by the median ECX.	Yes	95% confidence intervals of the EC_{50} were calculated

Overall assessment		
Reliable without restrictions	No	-
Reliable with restrictions	Yes	This study examined the toxicity of glyphosate with the temperature (20ºC and 25ºC) and aging times (0 day and 7 days) in soil using a collembolan species, *Allonychiurus kimi* (Lee). The degradation of glyphosate in soil was investigated. Fatty acid composition of *A. kimi* was also investigated. The half-life of glyphosate was 2.38 days at 20ºC and 1.69 days at 25ºC. At 20ºC with 0 day of aging, the EC_{50} and NOEC were estimated to be 93.5 and 3.7 mg/kg, respectively. As the temperature and aging time increased, the glyphosate degradation also increased, so no significant toxicity was observed on juvenile production. The proportions of the arachidonic acid and stearic acid decreased and increased with the glyphosate treatment, respectively, even at 37.1 mg/kg, at which no significant effects on juvenile production were observed. The study was conducted according to OECD TG 232 Collembolan Reproduction Test in Soil and is considered reliable with restrictions, the validity criteria cannot be fully checked.
Not reliable	No	-