グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2010 年 1 月 1 日～2019 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	KCA 8.2.1
Report author	Antunes, A. M. *et al.*
Report year	2017
Report title	Gender-specific histopathological response in guppies *Poecilia reticulata* exposed to glyphosate or its metabolite aminomethylphosphonic acid
Document No	Journal of applied toxicology, 2017; 37:1098-1107
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Ecotoxicity of glyphosate (GLY) and its metabolite aminomethylphosphonic acid (AMPA) was investigated in guppies, *Poecilia reticulata*. The median lethal concentration after 96 hours of exposure (LC_{50}, 96 h) of both test item was determined in male and female guppies

Both genders showed similar median lethal concentration (LC_{50}) at 96 hours for glyphosate and AMPA. The acute 96 hour-LC_{50} of glyphosate obtained for male and female guppies *P. reticulata* were 68.78 mg/L (95 % C.I.: 64.59–73.24 mg/L and 70.87 mg/L (95 % C.I.: 65.91–76.26 mg/L), respectively. The 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L (95 % C.I.: 175.12–184.54 mg/L) and 164.3 mg/L (95 % C.I.: 160.6–168.54 mg/L), respectively.

Materials and methods

Tested products; GLY and AMPA 96% and 99%, respectively, were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). The stock solutions of GLY and AMPA were prepared in ultrapure water with a nominal concentration of 250 mg l^{-1}.

Animal collection and maintenance; *P. reticulata* used in the experiments was part of the animals group kept in the Aquatic Animal Biotery of the Cell Behavior Laboratory (Institute of Biological Sciences IV, Universidade Federal de Goiás, Goiânia, Brazil). All of them were 3-month-old F1 generation animals born in the biotery from a wild parental generation. 318 mature male and 318 mature female guppies (vitellogenic oocyte occurred) of an average weight of 252 ± 20 mg and 178.6 ± 14.4 mg, and total average length of 2.98 ± 0.3 cm and 2.47 ± 0.2 cm, respectively.

Toxicity test: LC_{50}; For each experimental condition, eight males or eight females guppies were maintained in 2 liter tanks (4 fish L) and exposed to different nominal concentrations of GLY (50, 55, 60.5, 66.5 and 73.2 mg/L) or AMPA (86.8, 104.2, 125, 150 and 180 mg/L) during 96 h in the static test under 12: 12 h light/dark cycles. These concentrations were determined in the preliminary tests. The control group that consisted of eight fish kept in dechlorinated water. All treatments were performed in a triplicate design and the fish were not fed during the experimental period (USEPA, 1993). The mortality was reported at different exposure times (2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h). Physical and chemical parameters of water were analyzed every morning and did not show any changes over the experimental period, such as temperature 24 ± 1 °C, dissolved oxygen 8 mg/L, ammonia 0.002 mg/L, pH 7.0 ± 1, nitrite 0.025 mg/L and nitrate 0.5 mg/L. Cumulative mortality data obtained at the end of the experiments (96 h) were analyzed by the trimmed Spearman–Karber method to estimate the LC_{50} of a 96 h exposure to GLY and AMPA.

Statistical analysis; All statistical analyses were performed using the Statistica 7.0 software (Statsoft Inc., 2005, Tulsa, OK, USA). The differences between the treatments of the analyzed variables were identified using parametric tests (two-way ANOVA, followed by the Tukey's test) and/or non-parametric tests (Kruskal–Wallis), depending on the distribution of the data and homogeneity of variances (Shapiro–Wilk and Levene's tests). Linear and non-linear regression analyses were also applied to verify the relationship between variables.

Results

Median lethal concentration (LC_{50}); No mortality was observed for both genders in the control group during the experimental period of 96 h. The LC_{50} results showed that the GLY is more toxic to the guppies than to its metabolite AMPA, whereas no significant difference was observed between the genders ($P > 0.05$). The GLY LC_{50} values obtained for male and female *P. reticulata* were, respectively, 68.78 mg/L (95% confidence interval = 64.59–73.24 mg/L) and 70.87 mg/L (95 % confidence interval = 65.91–76.26 mg/L). The AMPA LC_{50} in turn, were 180 mg/L (95 % confidence interval = 175.12–184.54 mg/L) and 164.3 mg/L (95 % confidence interval = 160.6–168.54 mg/L), respectively. The GLY and AMPA toxicity increased linearly with the increasing concentration for females (GLY: $y = 0.6281x – 30.141$, $r = 0.96$, $P < 0.05$; AMPA: $y = 0.150x – 11.193$, $r = 0.93$, $P < 0.05$) and males (GLY: $y = 0.666x – 30.653$, $r = 0.93$, $P < 0.05$; AMPA: $y = 0.168x – 11.898$, $r = 0.88$, $P < 0.05$).

Discussion

The results of the LC_{50} values of GLY (male 68.78 mg/L and female 70.87 mg/L) and AMPA (male 180 mg/L and female 164.3 mg/L) based on the mortality test indicated a low sensitivity of *P. reticulata* in comparison to the other teleost species, as reported by the USEPA. In addition, it was observed that the AMPA is less toxic to *P. reticulata* than GLY (male 2.6-fold, female 2.3-fold).

Conclusion

The present study determined the acute 96 hour-LC_{50} of glyphosate and AMPA. The glyphosate LC_{50} values obtained for male and female guppies *P. reticulata* were 68.78 mg/L (95 % C.I.: 64.59–73.24 mg/L and 70.87 mg/L (95 % C.I.: 65.91–76.26 mg/L), respectively.
The 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L (95 % C.I.: 175.12–184.54 mg/L) and 164.3 mg/L (95 % C.I.: 160.6–168.54 mg/L), respectively.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The acute 96 hour-LC_{50} values for male and female guppies *P. reticulata* after exposure to glyphosate were 68.78 mg/L and 70.87 mg/L, respectively. The acute 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L and 164.3 mg/L, respectively.
In the material and methods part some important is missing. No information on preparation of test solution and application is given. Source and composition of media are unclear. Furthermore, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.8
Report author	Daam M.A *et al.*
Report year	2019
Report title	Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae
Document No	Ecotoxicology (2019) 28:707–715
Guidelines followed in study	OECD (2015) Test No. 241: the larval amphibian growth and development assay ASTM (2013) Standard guide for conducting the frog embryo teratogenesis assay-Xenopus (FETAX). ASTM E1439-12
Deviations from current test guideline	Not reported
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

The aim of this study was to evaluate the acute toxicity of the active ingredient glyphosate to tadpoles of two tropical frog species: *Physalaemus cuvieri* and *Hypsiboas pardalis*. The calculated 96 h LC_{50} (median lethal concentration; in mg a.s./L) values for *P. cuvieri* and *H. pardalis* were 115 and 106 mg a.s./L, respectively.

Materials and methods

Test species

Three or more egg masses from different parents of *Physalaemus cuvieri* and *Hypsiboas pardalis* were collected from ponds at the Estação Biológica de Boracéia in Salesópolis, South-East Brazil (23°37′59”S, 45°31′59”W), which is located within a non-polluted, protected watershed. Egg masses were transported in sealed plastic bags containing water from the collection site to the laboratory of the School of Arts, Sciences and Humanities in the University of São Paulo. Hatched larvae were kept in 50 L plastic tanks filled with tap water filtered through an activated carbon granular filter. Tank water was renewed every other day. The temperature in the laboratory was controlled at 25 ± 2 °C with natural photoperiod. Larvae were fed daily with a 3:1 ground mixture of rabbit chow (Purina Mills, LLC, USA; ~16% protein) and Tetra Min Fish Flakes (Tetra Werke, Melle, Germany; ~45% protein) ad libitum until the beginning of the experiments. The bioassays were conducted with Gosner stage 25 tadpoles. Only healthy individuals, as judged by external morphology and behavior, were selected for the experiments.

Lethality tests

Acute (96 h) bioassays were conducted to evaluate the sensitivity of *P. cuvieri* and *H. pardalis* to the pure active ingredients glyphosate (CAS Number 1071-83-6; Purity 99.2%; Sigma-Aldrich). A semi-static design was adopted, in which test solutions were renewed 48 h after the start of the experiment. The tests were conducted under the same conditions as those described above, except that animals were not fed during the test. Based on the results of range-finding tests, five logarithmically-spaced test concentrations (all in mg a.i./L) were determined: Glyphosate: 84; 97; 112; 130; 150.

Test concentrations were prepared with stock solutions. Each treatment was conducted in quadruplicate, in which each replicate consisted of a glass jar containing 10 tadpoles in 1 L test solution. Every 24 h, water quality parameters (pH, temperature, conductivity, DO) were recorded using a multi-parameter meter (YSI 556), and dead individuals counted and removed.

Data analysis

The 96h LC_{50}, LOEC (lowest observed effect concentration) and NOEC (no observed effect

concentration) were calculated based on the % mortality rates in the different treatments using the statistical programs PROBIT 1.5 and TSK 1.5. In all cases, the most appropriate statistical test was defined depending on the experimental design and the nature of the available data, following the recommendations of EPA. To test for interspecies differences in sensitivity, LC50 values for each compound and species were compared with a Z test using the formula proposed by EPA. Analyses of Variance (ANOVA) followed by post hoc tests were employed to test for treatment effects on physical-chemical variables (mean values for each treatment over the experimental period) using the software PAST.

Results

Survival was 100% in all control treatments. Water quality parameters were comparable in control replicates with a cofficient of variation of less than 4% for all para- meters (pH, temperature, conductivity and DO).
The 96h LC_{50} values generated and is presented in Table 1, whereas the mortality levels of the individual treatments for *P. cuvieri* and *H. pardalis* are visualized in Figure 1 and 2, respectively.

Table 1: LC_{50} (median lethal concentration; in mg/L) and the 95% confidence interval as determined for larval *Physalaemus cuvieri* and *Hypsiboas pardalis* after 96-h exposure to glyphosate.

	Physalaemus cuvieri	Figure	*Hypsiboas pardalis*	Figure
Glyphosate	115 (112–119)[b]	1	106 (103–109)[b]	2

[b]Probit test

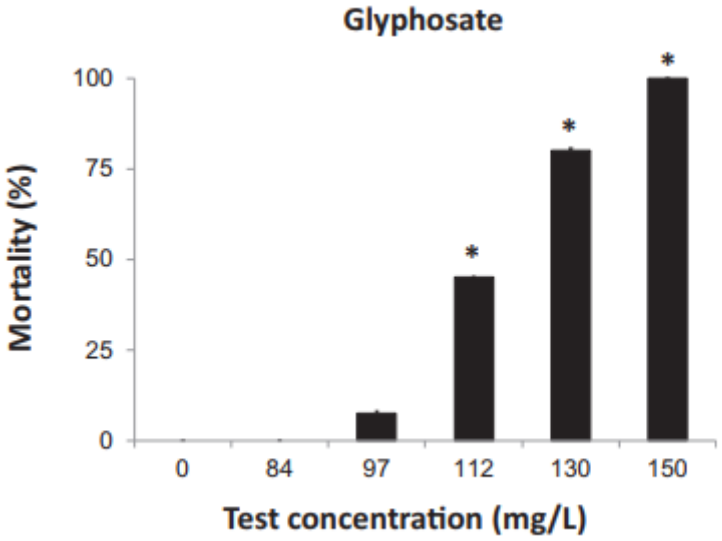

Figure 1: Mortality (in %) of *Physalaemus cuvieri* at the end of the 96 h laboratory tests evaluating the toxicity of glyphosate. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control.

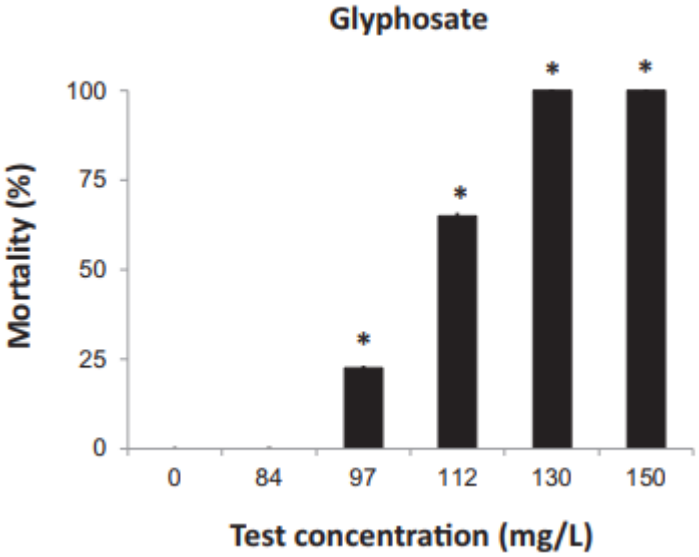

Figure 2: Mortality (in %) of *Hypsiboas pardalis* at the end of the 96 h laboratory tests evaluating the toxicity of glyphosate. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control.

Conclusion

The LC_{50} for *Physalaemus cuvieri* and *Hypsiboas pardali* was determined to be 115 mg a.s./L and 106 mg a.s./L, respectively.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study investigated the acute toxicity of glyphosate to larvae of *Physalaemus cuvieri* and *Hypsiboas pardalis.* The LC_{50} for *Physalaemus cuvieri* and *Hypsiboas pardali* was determined to be 115 mg a.s./L and 106 mg a.s./L, respectively.

The study was conducted according to portions of OECD 241. However, validity criteria were not reported. It is unknown if the larvae were exposed to any other chemicals as no analysis of watershed water was provided.There was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.2.1
Report author	Rodrigues L. B. *et al.*
Report year	2019
Report title	Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms
Document No	Mutat Res Gen Tox En 842 (2019) 94-101
Guidelines followed in study	OECD 236
Deviations from current test guideline	Not reported
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The present study assessed the acute toxicity of glyphosate, as well as the main metabolite aminomethylphosphonic acid (AMPA) on non-target aquatic organisms. The toxic effects of these chemicals were evaluated in a zebrafish (*Danio rerio*) embryo-larval toxicity test according to OECD Test Guideline 236 at 6 concentrations between 1.7 and 100 mg/L. Three replicates with 20 fertilized eggs per concentration were used.

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L).

Materials and methods

Test chemicals; Technical-grade glyphosate (GLY; Glyphosate PESTANAL®; purity 99%, CAS No. 1071-83-6) and aminomethylphosphonic acid (AMPA, purity 99%, CAS No. 106651-9) were purchased from Sigma-Aldrich.

Zebrafish maintenance and egg production; Adult male and female zebrafish (D. rerio) were provided by the zebrafish facility (ZebTec Tecniplast) at the Institute of Biology, University of Brasília and kept in separate tanks (ethical approval UFG Nº 102/2014). Fish were maintained in a Rack Hydrus (Alesco) recirculating system using water filtered by reverse osmosis, where water passes through several levels of filtration (activated carbon filters and biological filters), is then disinfected by ultraviolet (UV) light and automatically adjusted for pH and conductivity. The temperature was maintained at 26 ± 1 °C, conductivity at 750 ± 50 μS, pH at 7.5 ± 0.5 and dissolved oxygen of 8 ppm. Nitrate, nitrite and ammonia were regularly monitored. This water was used in preparing the test solutions of all assays performed. Adult organisms were fed with commercial dry flake food (TetraColor Flakes®) and live brine shrimp. On the day of the test, zebrafish eggs were collected about 30 min after natural mating, rinsed in water and examined under a stereomicroscope (Bel Photonics STM PRO). Unfertilized or damaged eggs were discarded. The fertilization success was checked, and only batches of eggs with a minimum fertilization rate of 90% were used.

Fish embryo acute toxicity (FET) test; The zebrafish embryo-larval toxicity test was carried out according to OECD Test Guideline 236. Twenty fertilized eggs per concentration were randomly selected and carefully distributed in a 24-well plate, filled with 2 mL of GLY, AMPA at 1.7, 5, 10, 23, 50 and 100 mg/L and controls (negative control – NC: maintenance water and positive control – PC: 3,4-dichloroaniline at 4.5 mg/L). Tests were performed in triplicates (three independent experiments) in a climate chamber at 26 ± 1 °C and 12 h light under static conditions. Neither food nor aeration was provided during the bioassays. Embryo development was assessed at 24, 48, 72 and 96 h post-fertilization (hpf), using a stereomicroscope (Bel Photonics STM PRO) with 3x magnification. The distinction between the normal and abnormal development of embryos was established according to the zebrafish development descriptions reported previously. Lethal (egg coagulation, no somite formation, nondetachment of the tail from yolk sac and no heart beating) and sublethal (effects on the eye and body pigmentation, absorption of the yolk sac, hatching rate, swimming bladder inflation, otolith, presence of edemas and blood accumulation, tail deformities) parameters were observed and reported.

Statistical analysis; The FET and Comet data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Each experimental value was compared to its corresponding negative control and the statistical difference was considered significant when $p < 0.05$. With respect to the FET, the toxicity was expressed as the lethal concentration (LC50), which was calculated using GraphPad Prism software (version 5.0, GraphPad Software, San Diego, CA, USA) with 95% confidence interval.

Results

Acute effects for zebrafish early-life stages; The present study investigated the effects of active ingredient GLY and its metabolite AMPA on the zebrafish embryonic development (survival and malformations) at 24, 48, 72 and 96 h of exposure. According to Fig. 1, no significant mortality was observed in zebrafish early-life stage after exposure to different concentrations (1.7–100 mg/L) of GLY and AMPA (Fig. 1), which presented survival rate ≥90% in all exposure periods.

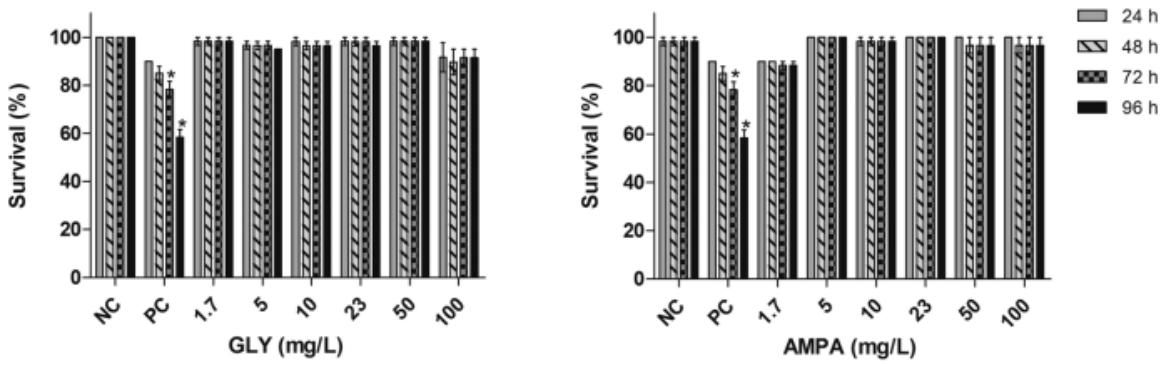

Fig. 1. Survival rate of zebrafish at different developmental stages exposed to GLY and AMPA for 24, 48, 72 and 96 h. Twenty fertilized eggs per experimental group were evaluated. Bars represent the mean ± standard error of the mean of three independent experiments. *$p < 0.05$ statistically different from the respective negative control (NC) based on one-way ANOVA and Dunnett's post hoc test. PC = positive control (3,4-dichloroaniline at 4.5 mg/L after 24, 48, 72 and 96 h of exposure).

In relation to sublethal effects, Fig. 2 shows that GLY induced some morphological abnormalities, however, these malformations were not statistically significant when compared to their respective negative control.

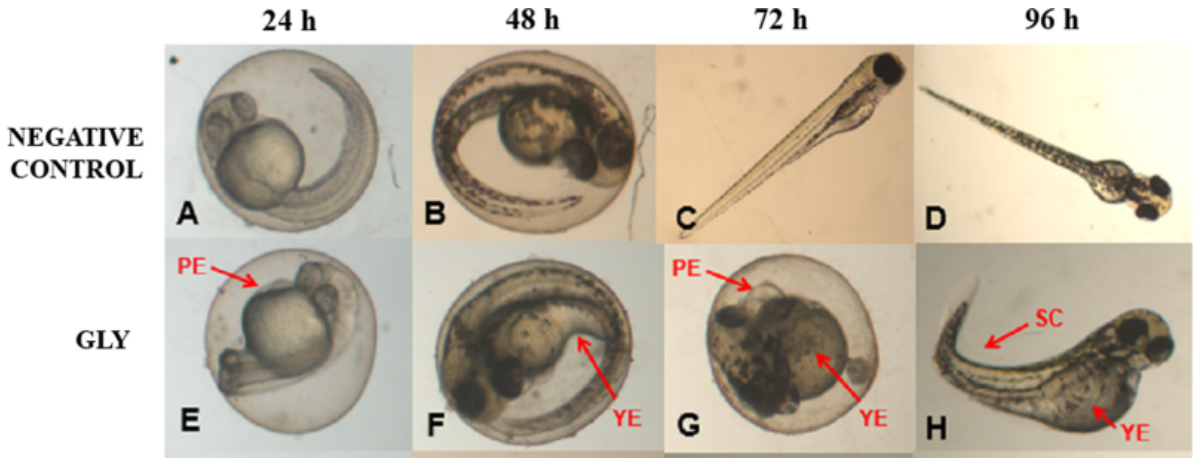

Fig. 2. Zebrafish embryos and larvae abnormalities after GLY exposure: pericardial edema (PE), yolk sac edema (YE), spinal curvature (SC). Embryos control after 24 h and 48 h of exposure, respectively (A–B); larvae control after 72 h and 96 h of exposure, respectively (C–D), embryos exposed to GLY at 23 mg/L and 100 mg/L for 24 h and 48 h, respectively (E–F); non-hatching embryo exposed to GLY at 10 mg/L for 72 h (G); larvae exposed to GLY at 100 mg/L for 96 h (H).

Discussion

The current results showed that glyphosate and AMPA did not induce acute toxicity in zebrafish early-life stage with LC_{50}-96 h > 100 mg/L. Similar effect was observed by researchers in assessing the acute effects of glyphosate (0.005; 0.05; 5; 10 and 50 mg/L) on early-life stages of zebrafish and common carp (Cyprinus carpio) for 120 h. The authors demonstrated that all tested concentrations, except the highest concentration (50 mg/L), induced cumulative mortality ≤ 10% after 96 h of exposure. Glyphosate at 50 mg/L caused the highest cumulative mortality, reaching 17.5% after 120 h of exposure while in this study, there were no significant differences between this group (glyphosate at 50 mg/L) and control with 1.7% of larvae mortality after 96 of exposure. It is worth noting that according to OECD 236, the survival of embryos in the NC must be ≥ 90% (validation criterion of the test), and therefore mortality ≤ 10% in the experimental groups is acceptable.

Conclusion

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L) in zebrafish.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The acute toxicity of technical glyphosate and its metabolite aminomethylphosphonic acid (AMPA) to zebrafish embryos was investigated.

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L) in zebrafish.

The study was stated to have been conducted according to OECD guideline 236, but there is no information on hatching rates in the treatment and control groups, so exposure of the embryo without a potential barrier function of the chorion cannot be confirmed.

Concerning the validity of the study, four of the six validity criteria from the test guideline are mentioned in the paper (fertilization rate of embryo batches used was >90%, survival in the negative control group was > 90%, temperature was maintained at 26 ±1ºC and dissolved oxygen was at an

acceptable level 8ppm). There is no information presented on the performance of the positive control group (3, 4-dichloroaniline) and no information provided on the hatching rates in the negative control group at 96 hours, which for the control group should exceed 80%. As these information are not presented and the fact that there was no analytical verification of test concentrations reported, this study considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.1 / KCP 10.2.1
Report author	Gabriel U. U. *et al.*
Report year	2010
Report title	Toxicity of roundup (a glyphosate product) to fingerlings of *Clarias gariepinus*
Document No	Animal Research International (2010) 7(2):1184-1193
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Acute static renewal bioassays were conducted on fingerling and adult of *Clarias gariepinus* (mean weight, 1.22 ± 0.6g; mean total length, 5.25 ± 1.25 cm) using the herbicide, Roundup (glyphosate). In the acute study, fingerlings were exposed in triplicate to 0.0, 14.0, 16.0, 18.0, 20.0 22.0, and 24.0 mg/l of the herbicide for 96 hours to determine general behavioural responses.

The 96 hour LC_{50} of Roundup on the fish was 19.58 mg/l.

Materials and methods

The fingerlings of *C. gariepinus* (mean weight 1.22 ± 0.6g; mean total length 5.25 ± 1.25 cm) were obtained from a private farm, Comsystem, Kpite, Rivers State and transported in 25 litre jerry can to the Wet Laboratory, Department of Fisheries and Aquatic Environment, Rivers State University of Science and Technology, where they were distributed 60 fish per aquarium in four rectangular aquaria filled with 20 litre borehole water (dissolved oxygen, 0.01 ± 0.05 mg/l, pH- 7.5 ± 1.3; conductivity, 410 ± 20.4 µS/cm; total dissolved solid 400 ± 10.25 ppm). They were fed at one percent biomass, half at 0900 and 1600 hours for a week. Cleaning of the tanks and water exchange were done daily. Mortality during acclimation period was less than one percent. Mucus accumulation on the skin as well as gills and skin pigmentation were recorded.
Range finding test and trial runs were done. Twenty litres of each of the following concentrations: 14, 16, 18, 20, 22 and 24 ppm of Roundup containing 360 g/l glyphosate (in the form of 480g/l isopropylamine salt) and a control were prepared in triplicate in glass aquaria. Ten fish was randomly distributed into each of the tanks. The general behaviours, opercular beat frequency, OBF, tail beat frequency, TBF and mortality (%) were recorded at 12, 24, 48, 72 and 96th hour, respectively. The exposure lasted for 96 hours. Data obtained from the experiments were subjected to ANOVA using Statistical Package for the Social Sciences, SPSS version 15 and differences among means were separated by Duncan Multiple Range test at 0.05%. The dependent variables in the trials (OBF, TBF and cumulative mortality) were regressed on concentration of the toxicant to obtain the regression lines of best fit for predicting the values of the dependent variables with changes in that of the independent with Microsoft Excel®. Correlation analysis was used to determine the degree of association among the dependent and independent variables. Lethal concentrations (LC50) values for the 24, 48, 72 and 96 hour and the median lethal times (MLT50) for the various concentrations of herbicide were done with Probit Analysis. Safe concentration of the herbicide at the various time intervals were obtained by multiplying the lethal concentration by a factor, 0.1. The interaction effects of the behavioural responses (TBF and OBF) with exposure duration and concentrations of the herbicides were presented graphically.

Results

On introduction into the toxicant the fish showed initial hyper-excitability, stress responses such as increased opercular ventilatory rate, dash and erratic swimming and gasping for air within the first two hours. As exposure time increased before death occurred they "hung" on the surface of the solution gulping air, fell steadily to the aquaria bottom. This was usually followed by dash swimming. This sequence was repeated several times before the fish lost balance, lay flat on the bottom (exertion), tail beat stopped, followed by cessation of opercular movement and then death (non-response to tactile stimuli).

Table 1: Tail and opercular beat frequency (TBF and OBF) and cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

Variable	Time of exposure (hours)				
	12	**24**	**48**	**72**	**96**
TBF/min.	5.45 ± 2.73^{b}	15.49 ± 4.41^{ab}	7.87 ± 4.41^{ab}	21.12 ± 5.22^{b}	4.49 ± 5.40^{b}
OBF/min.	113.50 ± 7.23^{a}	113.10 ±7.60^{a}	115.23 ±7.23^{a}	10.79 ± 8.99^{a}	65.15 ± 9.15^{b}
Cum. mortality	6.67 ± 6.86^{e}	37.227 ± 31.21^{d}	56.11 ± 36.16^{c}	63.89 ± 32.39^{b}	73.33 ±29.31^{a}

	Concentration of Roundup (mg/l)						
	0.0	**4.0**	**16.0**	**18.0**	**20.0**	**22.0**	**24.0**
TBF/min.	15.73 ±4.41^{a}	5.79 ±4.41^{a}	16.46 ±4.41^{a}	9.00 ±4.63^{a}	7.04 ±5.92^{a}	0.00 ±0.00	0.00 ±0.00
OBF/min.	96.71 ±7.60^{bc}	123.27 ±8.00^{ab}	108.58 ±8.00^{a-c}	100.93 ±8.00^{a-c}	73.42 ±10.19^{c}	124.72 ±16.99^{ab}	136.99 ±16.99^{a}
Cum. Mortality	0.00 ±0.00	22.67 ±12.80^{d}	24.00 ±24.43^{d}	32.67 ±19.81^{c}	50.67 ±37.70^{b}	77.33 ±36.15^{a}	77.33 ±37.89^{a}

Means with the same superscript in the row are not significantly different (p>0.05)

Table 2: Regression lines of best fit for the prediction of the values of OBF/min., TBF/min. and cumulative mortality of C. gariepinus exposed to acute levels Roundup for 96 hours

Dependent Variable	Independent variable	Prediction equation	Curve type	r^2
TBF	Time	$Y=2.8415e^{0.221x}$	Exponential	0.9828
TBF	Concentration	$Y=0.0017x^2 +0.0192x$	Power	0.9442
OBF	Time	Y=23.314Ln(x) +16.325	Logarithmic	0.9186
OBF	Concentration	Y=25.117Ln(x) +19.269	Logarithmic	0.9812
Mortality	Time	Y=0.513x	Linear	0.9922
Mortality	Concentration	$Y=0.0021x^2 + 0.2451x +4.4278$	Polynomial	0.9722

Where x = independent variable, y= dependent variable

Cumulative mortality of exposed fish was very variable relative to the concentration of the herbicide (Figure 1).

The cumulative mortality differed with the time of exposure ($p < 0.01$), concentration of toxicant ($p < 0.001$) and interactions between exposure duration and herbicide concentration ($p < 0.01$, Figure 1). Exposed fish produced copious amount of mucus on the gill and skin which appeared to be concentration-dependent in exposed fish with minimal amount on the control group. Pigmentation of the skin of the fish was not noticed in any of the exposure concentrations.

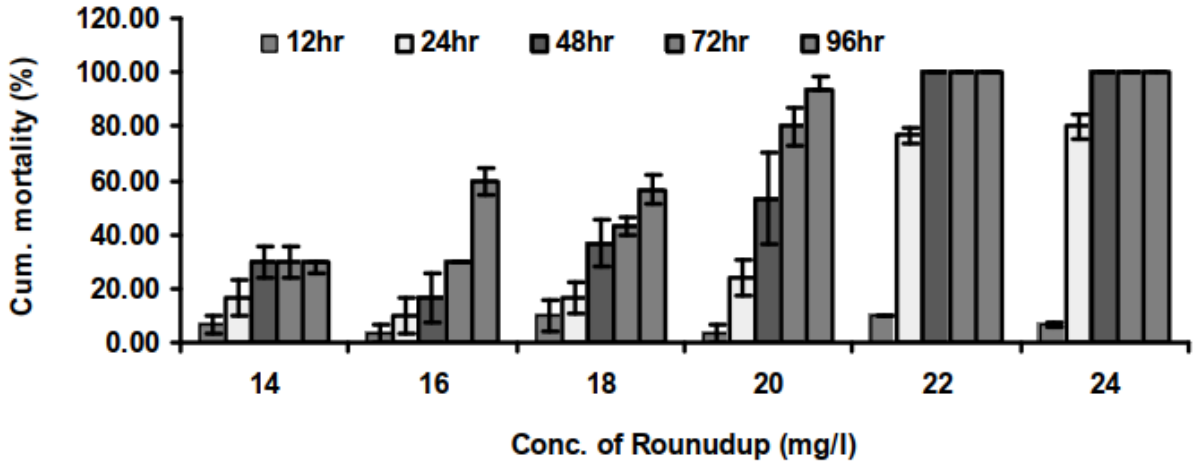

Figure 1: Percentage cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

The 24, 48, 72 and 96 hour LC_{50} and associated 95% confidence limits of the herbicide concentrations shown below indicated that the range of the values between the 24 hour and 96 hour LC_{50} (4.93 mg/l) as very narrow. Safe concentrations of Roundup to fingerlings of C. gariepinus were very low (2.08 mg/l for 24 hour and 1.59 mg/l for 96 hour). The time it took for half of the exposed fish to die at the various exposure concentrations decreased with time with the highest concentration (24 mg/l) killing half of the exposed fish at about one sixth the time it took for 14 mg/l of the herbicide.

Table 3: Lethal concentrations and associated 95% confidence limits of Roundup to *C. gariepinus* fingerling exposure to Roundup for 96 hours

Time (hours)	Lethal Concentration	Safe concentration	Probit model estimation equation
24	LC_{50}- 20.81 (19.58-22.48)	2.08	y= -4.73+0.23x
24	LC_{90}- 26.44 (24.45-31.47)	2.64	
48	LC_{50}-18.50 (16.67-19.40)	1.85	y= -5.18+0.29x
48	LC_{90}-22.54 (20.88-26..09)	2.25	
72	LC_{50}-17.11 (16.30-17.84)	1.71	y= -5.30+0.31x
72	LC_{90}-21.44 (20.59-23.31)	2.14	
96	LC_{50}-15.88 (14.99-16.64)	1.59	y= -5.06+5.06x
96	LC_{90}-19.91 (18.97-21.42)	1.20	

Where y=dependent variable, x= independable variable

Discussion

The threshold concentration causing 100% mortality in this study was 22 mg/l which is lower than that reported for other toxicants tested on any of the clariid species 7 suggesting that it may be more toxic than other tested toxicants. Half of the exposed fish (50%) were killed by 15.88 mg/l of herbicide in 19.69 hours, hence the herbicide can be classified as being slightly toxic. Besides, in the wild where the agro-chemical is indiscriminately used the impact of the exposure stress caused by the herbicide, may be protracted, following the survivors throughout life and may affect various aspects of their lives.

Conclusion

The 24, 48, 72 and 96 hour LC_{50} and associated 95% confidence limits indicated that the range of the values between the 24 hour and 96 hour LC_{50} (4.93 mg/l) as very narrow. Safe concentrations of Roundup to fingerlings of C. gariepinus were very low (2.08 mg/l for 24 hour and 1.59 mg/l for 96 hour). The 96 hour LC_{50} of Roundup on the fish was 19.58 mg/l.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effects of Roundup containing 360 g/l glyphosate (equivalent to 480g/L isopropylamine salt) were tested in an acute test with *C. gariepinus* fingerlings. The 96 hour-LC_{90} was determined to be 19.91 mg prod./L.

There is no analytical verification of test concentrations reported and thus the reliability of the endpoint is questionable. The appearance of mucus accumulation on the skin and gills and skin pigmentation recorded in fish in the holding / stock vessels is a clear indicator of stress. Therefore, the condition of the fish used in the test is questionable. The study was not conducted in accordance with a recognised test guideline and was not performed under conditions of GLP. Furthermore, the purity of the formulation roundup is not clearly given as the specification in the full text contains some typing errors. The study is considered reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.2 / KCA 8.2.5
Report author	Levine S.L. *et al.*
Report year	2015
Report title	Aminomethylphosphonic acid has low chronic toxicity to *Daphnia magna* and *Pimephales promelas*
Document No	Environmental Toxicology and Chemistry (2015), Vol. 34, No. 6, pp. 1382-1389
Guidelines followed in study	OECD 211, OECD 210
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

The purpose of the present study was to assess the potential for chronic toxicity of AMPA to fathead minnow (*Pimephales promelas*) and *Daphnia magna*. Chronic toxicity to *P. promelas* was evaluated in a fish early–life stage study. The primary endpoints were larval survival, growth, and development. The chronic toxicity to *D. magna* was evaluated in a *Daphnia* reproduction test. The primary endpoints were survival, growth, and reproduction.

The NOAEC for *P. promelas* was determined to be 12 mg/L, the highest concentration tested. The no-observed-effect concentration for *D. magna* was determined to be 15 mg/L.

Materials and methods

Test substance

Synthesis of AMPA was performed by Chemir, and it had a purity of 98.7%. The water solubility for AMPA is reported to be 10 500 mg/L (based on glyphosate acid solubility data [RMS Germany. 2013]); therefore, solvent (to aid the dissolution of AMPA into water) was not required for the aquatic exposures. Stock solutions for waterborne exposures were prepared in well water, appeared clear and colorless after mixing, and were stored under refrigerated conditions (~ 4 ± 1 °C).

For the *D. magna* reproduction study, primary stocks and test solutions were prepared every 2 d to 3 d during the test. A primary stock solution was prepared in ultraviolet sterilized dilution water at a nominal concentration of 120 mg AMPA/L, equivalent to the highest concentration tested. Proportional dilutions of the primary stock solution were made in dilution water to prepare test solutions at nominal concentrations of 7.5 mg AMPA/L, 15 mg AMPA/L, 30 mg AMPA/L, and 60 mg AMPA/L.

For the fish early–life stage study, stock solutions were delivered using syringe pumps into mixing vessels and mixed with diluent water in a continuous diluter system to prepare nominal test concentrations of 0.75 mg AMPA/L, 1.5 mg AMPA/L, 3.0 mg AMPA/L, 6.0 mg AMPA/L, and 12 mg AMPA/L. Delivery of the test solutions was started 7 d prior to the initiation of the test to achieve equilibrium of the test substance in the test chambers.

Daphnia magna reproduction study—Culturing, exposure, and observations

Daphnia magna are the required cladoceran test species under the Organisation for Economic Co-operation and Development (OECD) 211 guideline [OECD 2008]. *Daphnia magna* was tested because it is representative of an important group of freshwater invertebrates and has a long and successful history as a test organism in the laboratory. Neonates (juveniles) <24 h old were used to initiate the test and were obtained from established cultures. Parental daphnids were cultured in well water that was

filtered with a 0.45-µm filter and passed through an ultraviolet sterilizer. The source of well water was characterized as moderately hard water with an average specific conductance of 362 µS/cm, hardness of 132 mg/L as $CaCO_3$, alkalinity of 173 mg/L as $CaCO_3$, and pH of 8.2 during the 4-wk period immediately preceding the test.

During the 2-wk period preceding the test, culture temperatures ranged from 19.6 °C to 20.8 °C, pH from 8.1 to 8.7, and dissolved oxygen from 7.6 mg/L to 9.5 mg/L. During culturing and testing, daphnids were fed daily with a mixture of yeast, cereal grass medium, and trout chow, as well as a suspension of the freshwater green alga *Pseudokirchneriella subcapitata*. During the test, organisms in each test chamber were fed 0.5 mL of yeast–cereal–trout chow and 1.0 mL of algae, which represented 0.60 mg C/daphnid/d. Although this amount of feed exceeded the OECD guideline recommended amount of 0.1 mg C/daphnid/d to 0.2 mg C/daphnid/d, an excess amount was fed to maintain sufficient feed in the system to support acceptable reproduction rates, which is an acceptable deviation from the testing guideline.

The 4 adult daphnids used to supply neonates for the test were held for 19 d prior to collection of the juveniles for testing and had each produced at least 1 previous brood. Adult daphnids in the culture had produced an average of at least 3 young per adult per day over the 7-d period prior to the test. The adults showed no signs of disease or stress, and no ephippia were produced during the holding period. To initiate the test, juvenile daphnids were collected from the cultures and indiscriminately transferred 1 or 2 at a time into the transfer chambers that were impartially assigned to a control or treatment group until each transfer chamber contained 10 daphnids. All animals were released from the transfer chambers into the assigned test chambers below the water surface (to avoid air contact) using wide-bore pipettes to not harm the neonates.

We tested AMPA in a semistatic renewal design with the renewal of test solutions every 2 d or 3 d. Concentrations of AMPA were measured on 3 occasions during the test: at the beginning and end of the first renewal cycle, at the beginning and end of the longest renewal cycle during the second wk of the test, and at the beginning and end of the last renewal cycle. Test chambers were 250-mL glass beakers that contained approximately 200 mL of test solution and were loosely covered with plastic Petri dishes. Beakers were impartially positioned in an environmental chamber that was programmed to maintain the target water temperature (20 ± 1 °C) throughout the test period. A 16:8-h light:dark photoperiod was used with a 30-min transition period of low light intensity when lights went on and off to avoid sudden changes in lighting. Lighting was provided by fluorescent light bulbs that emit wavelengths similar to natural sunlight. At test initiation the light intensity at the water surface of 1 representative test chamber was 296 lux (measured with a SPER Scientific Model 840006C light meter).

Temperature was measured continuously in 2 replicate test chambers in each treatment group, and measurements rotated among replicates in each group. Dissolved oxygen and pH were measured in the newly prepared solutions for each treatment group at test initiation and on renewal days and in the old solutions from 2 replicate test chambers in each treatment and control group on renewal days and at test termination. When a first-generation daphnid was found dead, measurements of temperature, dissolved oxygen, and pH were taken in the replicate at that time and then discontinued. Hardness, alkalinity, and specific conductance were measured in batch solutions of the negative control; the highest test concentration at test initiation and on 1 renewal day each week (day 7 and day 14); and pooled replicate solutions at test termination. Total organic carbon (TOC) was measured in the dilution water at test initiation and termination using a Shimadzu model TOC-VCSH analyzer and following the *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association]. Hardness and alkalinity were measured by titration based on procedures in the *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association].

First-generation daphnids were observed daily during the test for immobility, the onset of reproduction, and clinical signs of toxicity. Following the onset of reproduction, the second- generation daphnids were counted 3 times per week and at test termination (day 21). Body lengths and dry weights of the surviving first-generation daphnids were measured at the end of the exposure period.

Fish early–life stage study—Culturing, exposure, and observations

Test methodology followed the procedure outlined in the OECD 210 test guideline for *P. promelas* with the exception of doubling the required level of replication [OECD 1992]. We selected *P. promelas* for the early–life stage study based on past use and ease of handling in the laboratory. Embryos (Chesapeake Cultures) were examined under a dissecting microscope to select healthy, viable specimens at approximately the same stage of development (<24 h). Embryos collected for use in the test were from 10 individual spawns and were <24 h old when the test initiated. Test chambers were 9-L glass aquaria filled with approximately 7 L of test solution and contained an embryo incubation cup attached to a reciprocating rocker arm (2 rpm) for water circulation during embryo incubation. To initiate the test, groups of 1 to 3 embryos were impartially distributed among incubation cups until each cup contained 20 embryos. A single incubation cup constructed from 50-mm-diameter glass cylinders with 425-µm nylon screen mesh attached to the bottom was placed into each test chamber. The incubation cup with the embryos was impartially assigned to each of the control and treatment groups.

The test was conducted in a temperature-controlled environmental chamber designed to maintain the target test temperature of 25 ± 1 °C throughout the test period. Temperature was measured in each test chamber at the beginning of the test, weekly during the test, and at the end of the test using a liquid-in-glass thermometer. Temperature also was monitored continuously in 1 negative control replicate using a Fulscope ER/C Recorder. Fluorescent light bulbs that emit wavelengths similar to natural sunlight were used on a 16:8-h light:dark photoperiod. A 30-min transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting.

The negative (dilution water) control and AMPA test concentrations were delivered in a continuous-flow diluter. Syringe pumps (Harvard Apparatus) delivered the stock solutions (at a rate of 30 µL/min) into mixing chambers and mixed with dilution water (at a rate of 125 mL/min) to achieve the target test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters that were calibrated prior to test initiation and verified at weekly intervals during the test. The flow of test water from each mixing chamber was split and directed into 4 replicate test chambers. The proportion of the test water that was split into each replicate was checked prior to the test and at approximately weekly intervals during the test to ensure that flow rates varied by no more than ± 10% of the mean for the 4 replicates. The diluter flow rate was adjusted to provide approximately 6 volume additions of test water in each test chamber per day. The general operation of the diluter was checked visually at least 2 times/d during the test and at least once at the end of the test.

According to the OECD guideline, concentrations above the 96-h lethal concentration for 50% of the population or 10 mg/L, whichever is lower, need not be tested. To assure that a mean measured concentration ≥10 mg/L was tested, the highest nominal test concentration of 12 mg/L and the lower concentrations of 6 mg/L, 3 mg/L, 1.5 mg/L, and 0.75 mg/L were selected. Stock solutions were stored under refrigerated conditions, and fresh aliquots were placed in the syringe pumps daily during the test. Water samples were collected from 1 test chamber of each treatment and control group 4 d prior to test initiation to confirm the operation of the diluter. Water samples were collected from alternating replicate test chambers of each treatment and control group on day 0, day 7, day 14, day 21, day 28, and day 33 (test termination) to determine concentrations of the test substance in the test chambers. All samples were collected at mid-depth in the test chambers, placed in glass vials, and processed immediately for analysis.

Dissolved oxygen and pH were measured in alternating replicates of each treatment and control group at the beginning of the test, weekly during the test, and at the end of the test. Hardness, alkalinity, and specific conductance were measured in alternating replicates of the negative control (dilution water) and the highest concentration treatment group at the beginning of the test, weekly during the test and at the end of the test. Hardness and alkalinity were measured by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association], and specific conductance was measured using an Acorn Series Model CON6 Conductivity-Temperature meter.

During the first day of exposure, embryos were observed twice for mortality and fungal infection. Thereafter, until hatching was complete, observations of embryo mortality and the removal of dead embryos were performed once daily. When hatching reached >90% in the control groups on day 5 of the test, the larvae were released to their respective test chambers and the posthatch period began. During the 28-d posthatch exposure period, the larvae were observed daily to evaluate the mortality and the numbers of individuals exhibiting clinical signs of toxicity or abnormal behavior. From these observations, time to hatch, hatching success, and posthatch growth and survival were evaluated. Hatching success was calculated as the percentage of embryos that hatched successfully. Posthatch survival was calculated from the number of larvae that survived to test termination as a percentage of the number of embryos that hatched successfully.

Newly hatched larvae were fed live brine shrimp nauplii (*Artemia* sp.) 3 times/d during the first 7 d of the posthatch period. Thereafter, they were fed live brine shrimp nauplii 3 times/d on weekdays and at least 2 times/d on weekends. Fish were not fed for approximately 48 h prior to the termination of the test to allow for clearance of the digestive tract before weight measurements were made. To ensure that the feeding rate per fish remained constant, rations were adjusted at least weekly. The test chamber loading rate (the total wet wt of fish per liter of water in the tank) at the end of the test was 0.32 g fish/L.

Posthatch growth of *P. promelas* was evaluated at the conclusion of the 28-d posthatch exposure period. Total length for each surviving fish was measured to the nearest 1mm using a metric ruler, with wet and dry weights measured to the nearest 0.1 mg using an analytical balance. Fish were placed in an oven at 60 °C for up to approximately 48 h to obtain dry weight data.

Analytical method for detection of AMPA

Samples were diluted, as appropriate, with freshwater. The 2.0 mL of diluted sample and/or external calibration standards were placed into the 15-mL test tube. Then, 1.0 mL of 0.37 M aqueous potassium tetraborate was added to each test tube, followed by 2.0 mL of 0.025 M NBD-C1 (methanolic) for derivatization. Solutions were capped, mixed, and heated at approximately 80 °C for 40 min. Next, 1.0 mL of 1.2 M HCl was added to each test tube, and samples were then left undisturbed for approximately 10 min prior to analysis. Samples (25 µL injection volume) were analyzed on an Agilent Series 1100/1200 high performance liquid chromatograph equipped with an Agilent Series 1100 variable wavelength detector at 500 nm. Chromatographic separations were achieved using a YMC-Pack ODS-AM (150 mm × 4.6 mm, 3 µm particle size) analytical column at a temperature of 40 °C and eluted over a gradient of 0.1% H_3PO_4 (solvent A) and CH_3CN (solvent B). The retention time for AMPA was approximately 6.5 min to 7.3 min, and the method limit of quantitation for these analyses was defined as 0.4 mg AMPA/L.

Statistical and power analyses

Test endpoints analyzed statistically in the *Daphnia* test for first-generation daphnids were survival, reproduction (the number of live young produced per 21-d surviving adult), and growth (length and dry wt). Neonates produced by those first-generation daphnids that did not survive the full 21 d were excluded from analysis of reproduction.

Test endpoints analyzed statistically in the fish early–life stage test were hatching success, larval survival, and growth (total length, wet wt, and dry wt). Data on time to hatch were evaluated by visual interpretation.

Discrete-variables data were analyzed using Fisher's exact test to identify treatment groups that showed a statistically significant difference ($p \leq 0.050$) from the negative control. All continuous-variable data were evaluated for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test ($p = 0.010$). When the data passed the assumptions of normality and homogeneity of variance, those treatments that were significantly different from the control means were identified using the 1-tailed Dunnett's test ($p \leq 0.050$). All statistical tests were performed using a personal computer

with SAS software. The results of the statistical analyses were used to aid in the determination of the no-observed–adverse effect concentration (NOAEC), defined as the greatest test concentration that produced no significant treatment-related adverse effects on survival, reproduction, or growth.

Results

Daphnia magna survival, growth, and reproduction

Water temperatures were maintained within the targeted range of 20 ± 1 °C, dissolved oxygen concentrations remained ≥76% of saturation (6.8 mg/L), and pH ranged from 7.1 to 8.6 during the test. Specific conductance, hardness, and alkalinity were similar between the control and treatment groups and did not appear to be influenced by AMPA. The TOC in the dilution water at test initiation and termination was <1 mg C/L.

Table 1: Means and ranges of water quality measurements taken during the 21-Day *D. magna* exposure to AMPA

Mean Measured Concentration (mg AMPA/L)	Mean ± Std. Dev. and Range of Measured Parameters					
	Temperature (°C)	Dissolved Oxygen [1] (mg/L)	pH	Hardness [2] (mg/L as $CaCO_3$)	Alkalinity [2] (mg/L as $CaCO_3$)	Conductivity [4] (µS/cm)
Negative Control	19.8 ± 0.55 (19.1 – 20.8)	8.2 ± 0.58 (7.2 – 9.1)	8.4 ± 0.10 (8.3 – 8.6)	135 ± 2 (132 - 136)	167 ± 5 (160 – 171)	339 ± 46 (280 – 391)
7.4	19.9 ± 0.48 (19.2 – 20.7)	8.0 ± 0.60 (7.1 – 9.1)	8.4 ± 0.09 (8.3 – 8.6)	--	--	--
15	19.9 ± 0.50 (19.1 – 20.8)	7.9 ± 0.65 (6.9 – 9.1)	8.4 ± 0.08 (8.2 – 8.6)	--	--	--
30	19.9 ± 0.54 (19.0 – 20.7)	7.9 ± 0.66 (6.8 – 9.1)	8.3 ± 0.10 (8.2 – 8.5)	--	--	--
57	20.0 ± 0.52 (19.1 – 20.6)	7.9 ± 0.60 (7.0 – 9.1)	8.2 ± 0.23 (7.7 – 8.5)	--	--	--
120	20.1 ± 0.54 (19.1 – 20.7)	8.0 ± 0.54 (7.1 – 9.1)	7.9 ± 0.48 (7.1 – 8.4)	139 ± 2 (136 – 140)	164 ± 6 (156 – 170)	340 ± 46 (274 – 381)

[1] A dissolved oxygen concentration of 9.1 mg/L represents 100% saturation at 20ºC in freshwater. Any recorded dissolved oxygen measurement greater than 100% saturation is reported as 9.1 mg/L.
[2] -- = no measurements scheduled.

Measured concentrations of AMPA for the *D. magna* study were close to nominal concentrations throughout the renewal periods. Concentrations of AMPA in the new test solutions prepared and sampled on day 0, day 9, and day 19 ranged from 92.5% to 106% of the nominal concentrations. Concentrations of AMPA in the old test solutions sampled immediately prior to renewal on day 2, day 12, and at test termination on day 21 ranged from 78.6% to 117% of the nominal concentrations. When the measured concentrations of the samples collected during the test were averaged for each treatment group, the mean measured test concentrations were 7.4 mg AMPA/L, 15 mg AMPA/L, 30 mg AMPA/L, 57 mg AMPA/L, and 120 mg AMPA/L.

There was no significant effect of AMPA on individually exposed first-generation daphnids across the treatments, and survival was ≥80%. A summary of adult survival is presented in Figure 1A. After 21 d of exposure, survival in the negative control, 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L groups was 75%, 80%, 100%, 70%, 100%, and 90%, respectively. Although survival in the negative control group was slightly below the 80% criterion in OECD guideline 211, it is not considered to have impacted the validity of the present study because of the small difference and with the final mortality occurring near the end of the study. In addition, there was ≥80% survival in all treatment groups. The surviving daphnids in the control replicates appeared normal and healthy through the end of the test, indicating that the mortality observed was attributed to incidental death and not the health of the organisms. In addition, the percentage survival of the control replicates was within the control criterion of 70% as specified in the American Society for Testing and Materials standard guide E 1193-97 [ASTM International. 1997]. Survival in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups at test termination did not follow a concentration–response pattern and was 80%, 100%, 70%, 100%, and 90%, respectively. No significant differences in survival were detected in any of the AMPA treatment groups in comparison

with the control ($p > 0.05$, Fisher's exact test). Consequently, the no-observed-effect concentration (NOEC) for survival was 120 mg AMPA/L.

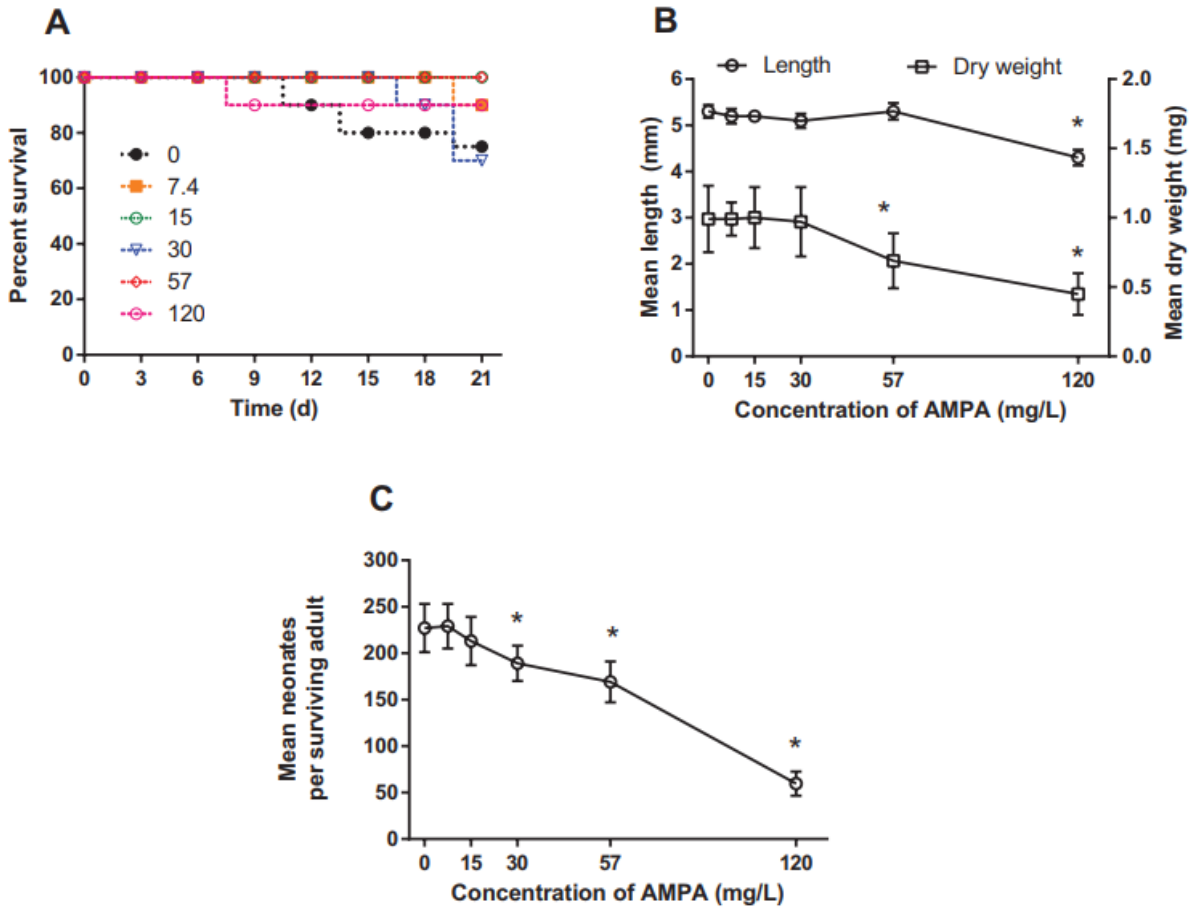

Fig. 1. (A) Survival (percentage) of *Daphnia magna* exposed to increasing concentrations of AMPA for 21 d. (B) Sublethal endpoints in 21-d chronic *D. magna*: body length and dry weight. (C) Reproductive endpoint neonates per surviving adults. * Statistically significant difference ($p < 0.05$) from the control (0 mg/L).

Daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, and 57–mg AMPA/L treatment groups that survived until test termination generally appeared normal. In the 120–mg AMPA/L treatment group, all surviving first-generation daphnids appeared pale in comparison with the control organisms from day 5 through the end of the test. Daphnids in this treatment group were also observed to be smaller than the control organisms from day 7 through the end of the test. All surviving daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, and 57–mg AMPA/L treatment groups were normal in appearance throughout the test and at test termination, with the exception of 1 daphnid in the 57–mg AMPA/L treatment group that appeared pale on day 6 of the test but appeared normal from day 7 to the end of the test.

A summary of production of neonates by surviving first-generation daphnids is presented in Figure 1C. The first day of brood production in the negative controls and in all AMPA treatment replicates was day 7, day 8, or day 9 of the test, indicating that there was no apparent delay in the onset of production at any concentration of AMPA tested. Immobile neonates were noted in the control, 7.4–mg AMPA/L, and 57–mg AMPA/L treatment groups. However, the mean number of immobile neonates per surviving adult in these replicates was less than 1. No aborted brood or aborted eggs were present in the control or any of the AMPA treatment replicates. No males or ephippia were produced during the test.

Summaries of the mean lengths and dry weights of surviving first-generation daphnids are presented in Figure 1B. Daphnids in the negative control group averaged 5.3 mm in length and 0.99 mg in dry weight. Daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups had mean lengths of 5.2 mm, 5.2 mm, 5.1 mm, 5.3 mm, and 4.3 mm, respectively, and mean dry weights of 0.99 mg, 1.0 mg, 0.97 mg, 0.69 mg, and 0.45 mg, respectively. There were significant decreases in length in the 30–mg AMPA/L and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$) but not in the 57–mg AMPA/L treatment group. There were significant decreases in dry weight in the 57–mg AMPA/L and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$). Consequently, the NOEC for growth was 30 mg AMPA/L.

Adult daphnids in the negative control group produced an average of 227 live young per surviving adult (coefficient of variance of 11.6%), well above the validity criterion of ≥60 live young per surviving adult. Adult daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/ L, and 120–mg AMPA/L treatment groups produced an average of 229, 213, 189, 169, and 59.6 live young per surviving adult, respectively. There was a significant decrease in mean neonate production in the 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$). Consequently, the NOAEC for reproduction is 15 mg AMPA/L.

Pimephales promelas embryo hatching success, growth, and survival

Samples of the test solutions collected during the test had measured concentrations that ranged from 82.5% to 117% of nominal concentrations. When the measured concentrations of test solution samples collected on day 0, day 7, day 14, day 21, day 28, and day 33 of the test were averaged for each treatment group, the mean measured test concentrations were 0.73 mg AMPA/L, 1.5 mg AMPA/L, 2.9 mg AMPA/L, 6.0 mg AMPA/L, and 12 mg AMPA/L, which represented 97%, 100%, 97%, 100%, and 100% of nominal concentrations, respectively. Therefore, the results of the present study have been based on mean measured concentrations. The analytical results are summarized in Supplemental Data, Table S4.

Hatching success of the *P. promelas* embryos is summarized in Figure 2A. Daily observations of the embryos indicated that there were no apparent differences in time to hatch between the negative control group and any of the AMPA treatment groups. All *P. promelas* embryos in the control and treatment replicates hatched by day 5 of the test. Hatching reached >90% in the control groups on day 5 of the test, at which time the larvae were released to their respective test chambers.

Hatching success in the negative control, 0.73–mg active ingredient (a.i.)/L, 1.5–mg a.i./L, 2.9–mg a.i./L, 6.0–mg a.i./L, and 12–mg a.i./L treatment groups was 99%, 100%, 100%, 100%, 100%, and 99%, respectively (Figure 2A). There were no statistically significant differences in hatching success in any of the AMPA treatment groups in comparison with the negative control ($p > 0.05$). Larval survival in the negative control, 0.73–mg a.i./L, 1.5–mg a.i./L, 2.9–mg a.i./L, 6.0–mg a.i./L, and 12–mg a.i./L treatment groups was 91%, 91%, 93%, 90%, 91%, and 92% (Figure 2A), respectively; and there were no statistically significant differences in hatching success in any of the AMPA treatment groups in comparison with the negative control ($p > 0.05$). In addition, there were no statistically significant reductions in total length, wet weight, and dry weight (Figure 2B) among fish in the AMPA treatment groups in comparison with the negative control ($p > 0.05$). Based on an evaluation of each of these endpoints, the NOAEC for growth was 12 mg a.i./L.

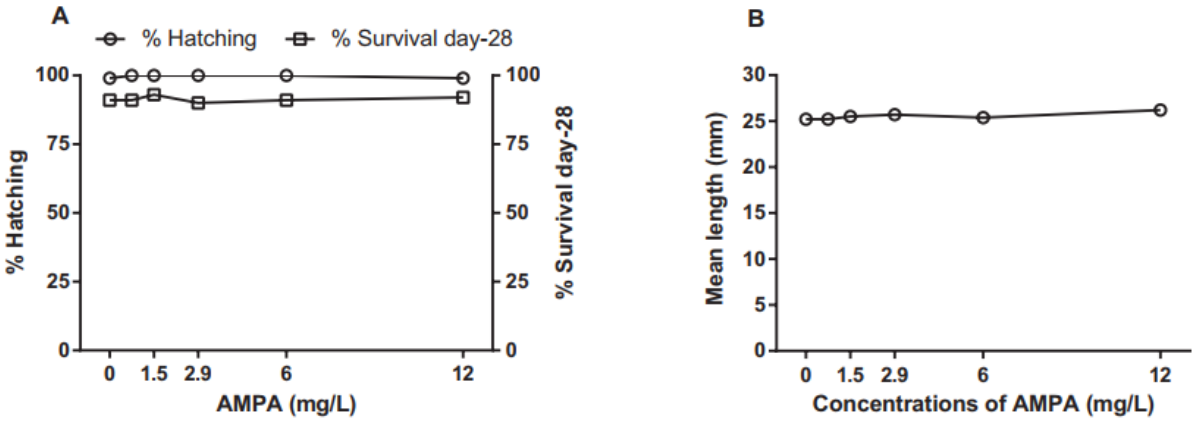

Fig. 2. (A) Hatching success (percentage) and survival (percentage) at day 28 of *Pimephales promelas* in an early–life stage study with aminomethylphosphonic acid (AMPA). (B) Body length (millimeters) of *P. promelas* exposed to AMPA.

Table 2. Means and Ranges of Water Quality Measurements Taken During the 33-Day *P. promelas* Exposure to AMPA

Mean Measured Concentration (mg AMPA/L)	Mean ± SD and Range of Measured Parameters					
	Temperature (°C)	DO[2] (mg/L)	pH	Hardness[2] (mg/L as $CaCO_3$)	Alkalinity[2] (mg/L as $CaCO_3$)	Conductivity[2] (µS/cm)
Assay Control	24.8 ± 0.27 (24.4 – 25.4)	7.9 ± 0.40 (7.4 – 8.2)	8.1 ± 0.10 (8.0 – 8.2)	136 ± 3 (132 – 140)	172 ± 5 (166 – 178)	378 ± 11 (361 – 393)
0.73	24.7 ± 0.59 (23.7 – 25.5)	7.9 ± 0.39 (7.4 – 8.2)	8.1 ± 0.09 (8.0 – 8.2)	-- --	-- --	-- --
1.5	24.9 ± 0.66 (23.9 – 25.7)	7.9 ± 0.39 (7.3 – 8.2)	8.1 ± 0.10 (8.0 – 8.2)	-- --	-- --	-- --
2.9	25.0 ± 0.52 (24.2 – 25.6)	7.9 ± 0.33 (7.5 – 8.2)	8.1 ± 0.12 (7.9 – 8.2)	-- --	-- --	-- --
6.0	25.0 ± 0.46 (24.3 – 25.7)	7.9 ± 0.34 (7.5 – 8.2)	8.0 ± 0.12 (7.9 – 8.2)	-- --	-- --	-- --
12	25.1 ± 0.48 (24.3 – 25.7)	7.9 ± 0.37 (7.4 – 8.2)	7.9 ± 0.13 (7.8 – 8.1)	138 ± 2 (136 – 140)	174 ± 4 (170 – 180)	379 ± 11 (365 – 395)

[1] A dissolved oxygen concentration of 4.9 mg/L represents 60% saturation at 25°C in freshwater. Any recorded DO measurement greater than 100% saturation is reported as 8.2 mg/L.
[2] -- = no measurements scheduled.

Conclusion

For *D. magna* exposed to concentrations ranging from 7.4 mg AMPA/L to 120 mg AMPA/L for 21 d, reproduction was the most sensitive endpoint with significant treatment-related effects noted at 30 mg AMPA/L, 57 mg AMPA/L, and 120 mg AMPA/L. Consequently, the NOAEC based on reproduction was 15 mg AMPA/L. No impact was noted on hatching success, survival, or growth in *P. promelas* embryos exposed to concentrations ranging from 0.73 mg AMPA/L to 12 mg AMPA/L for 33 d. Consequently, the NOAEC was 12 mg AMPA/L, the greatest concentration tested. These values exceed the worst-case water concentrations from conservative modelling and surface water monitoring data by 2 to 3 orders of magnitude, indicating no unacceptable chronic risk for vertebrate and invertebrate aquatic organisms from environ mental exposure to AMPA.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Chronic toxicity tests of the glyphosate environmental metabolite aminomethylphosphonic acid (AMPA) were performed with fathead minnow (*Pimephales promelas*) and *Daphnia magna*.
During a 21-d exposure period under semi-static test conditions the effects on survival, growth, and reproduction of the cladoceran *Daphnia magna* were determined resulting in a no-observed-effect concentration (NOEC) of 15 mg AMPA/L. During a 33-d exposure period under continuous renewal test conditions the effects on time to hatch, hatching success, posthatch growth and survival of the fish *Pimephales promelas* were assessed resulting in an NOAEC of 12 mg AMPA/L, the highest tested concentration. Test methodology followed the procedure outlined in the OECD 210 test guideline for *P. promelas*. For the chronic test on *Daphnia magna* the OECD 211 guideline is mentioned in the full text.

The study is well documented and all relevant information, e.g. information on the test item, test design, application method and implementation of the study, is available. In addition, a chemical analysis of test solutions was performed. All information for evaluation of the study is given. The study is considered as reliable.

1. Information on the study

Data point:	KCA 8.2.1
Report author	Schweizer M. *et al.*
Report year	2019
Report title	How glyphosate and its associated acidity affect early development in zebrafish (*Danio rerio)*
Document No	PeerJ, (2019) Vol. 7, pp. e7094
Guidelines followed in study	OECD Guideline 236
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Zebrafish (*Danio rerio*) embryos exposed to concentrations between 10 µM and 10 mM glyphosate (corresponding concentrations between 1.69 and 1690.7 mg glyphosate/L) in an unbuffered aqueous medium, as well as at pH 7, for 96 hours post fertilization (hpf). Furthermore, for investigations of the influence of pH, the test concentration 1 mM glyphosate (169.07 mg glyphosate/L) was tested at different pH values ranging between pH 3 and 8 vs. the respective pH controls. A total of 32 embryos were used per treatment with 8 replicates of 4 embryos each. The observed endpoints included mortality, the hatching rate, developmental delays at 24 hpf, the heart rate at 48 hpf, hatching success from 60 to 96 hpf and malformations at 96 hpf. $LC_{10/50}$, EC_{10} and, if reasonable, EC_{50} values were determined for unbuffered glyphosate.

In unbuffered glyphosate medium the lethal concentrations were calculated to be 385 mM (LC_{10}) and 582 mM (LC_{50}) at 96 hpf. Regarding heart rates the EC_{10} was 43 mM. Concerning the hatching rate, EC_{10} and EC_{50} levels at 96 hpf were 155 and 224 mM, respectively. For developmental delays at 24 hpf the EC_{10} was 126 mM.

Materials and methods

Glyphosate; Glyphosate (N-(phosphonomethyl)glycine, 96% pure substance, molecular weight: 169.07 g/mol, CAS: 1071-83-6; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used to prepare the test solutions. A stock solution with a concentration of 25 mM was prepared as follows: glyphosate was diluted in reconstituted water (0.23 g KCl, 2.59 g $NaHCO_3$, 4.93 g MgS_4O x 7 H_2O and 11.76 g $CaCl_2$ x 2 H_2O were dissolved separately in one L double-distilled water, then 25 mL of each stock solution was added to 900 mL double-distilled water). The stock solution was then diluted to the following test concentrations: 10, 50, 100, 250, 500, 750 mM, one and 10 mM glyphosate. All those concentrations were tested unbuffered and at pH 7. For pH adjustments, 1M HCl and NaOH solutions were used as recommended in the Organisation for Economic Co-operation and Development (OECD) 236 (2013) guideline. For investigations of the influence of pH, 1 mM glyphosate was tested at different pH values ranging between pH 3 and 8 vs. the respective pH controls. Due to preliminary results from the broad-scale pH testing, particular attention was paid to the range between pH 3 and 4. Measurements of pH were conducted with a pH meter (SevenCompactDuo; Mettler Toledo, Gießen, Germany) directly prior to the exposure.

Maintenance of zebrafish and test procedure; The embryos used in this study stem from our own breeding stock of the D. rerio West aquarium strain established in the Animal Physiological Ecology group, Tübingen University. Adult zebrafish were kept in 90 L aquaria filled with a 1:1 mixture of purified water and filtered tap water (AE-2L water filter with an ABL-0240-29 activated carbon filter, 0.3 mm; Reiser, Seligenstadt, Germany) at 26 ± 1 °C and an oxygen saturation of 100% ± 5%. Conductivity ranged from 260 to 350 mS/cm, nitrite and nitrate concentrations from 0.025 to 0.1 mg/L, one and five mg/L, respectively, and total water hardness from eight to 12 dH. Fish were subjected to

an artificial 12:12 h day/night cycle and fed three times daily with flake food (TetraMin; Tetra GmbH, Melle, Germany) supplemented with frozen black mosquito larvae and glass worms (Poseidon Aquakultur Freeze, Ruppichteroth, Germany) prior to spawning to ensure sufficient dietary protein. The day before the test, pre-exposure and test Petri dishes (90 and 30 mm in diameter) were filled with the respective solutions and stored at 26 ± 1 °C overnight to saturate the glass (the same was done with the Schott flask used for the stock solution, beforehand). On the morning of the test, Petri dishes were emptied and refilled with 70 mL (pre-exposure) and three mL (test Petri dishes) solution. For spawning, Plexiglas boxes 20 x 20 x 6 cm in size and covered with a mesh grid to keep zebrafish from feeding on their own eggs were used as breeding boxes. They were topped with artificial sea grass acting as an optical spawning stimulus and were placed into the fish tanks the evening before the start of the test. Zebrafish spawn at sunrise; therefore, spawning in the laboratory starts with the onset of light the next morning. Eggs were collected with a sieve, rinsed with tepid tap water, transferred into pre-exposure Petri dishes and incubated for 2 h at 26 ± 1 °C. Following the pre-exposure, eggs for the test were chosen with regard to their age and developmental stage (0 hours post fertilization (hpf) ≙ 8 a.m.), placed into the small 30 mm Petri dishes and stored in a heated cabinet at 26 ± 1 °C. A total of 32 individuals were used per treatment, that is, four per Petri dish and eight replicates each. Embryos were checked every 12 to 24 h. Endpoints investigated under a stereo microscope (Stemi 2000-C; Zeiss, Oberkochen, Germany) included mortality, developmental delays at 24 hpf, heart rate at 48 hpf, hatching success from 60 to 96 hpf and malformations at 96 hpf. Except for mortality, analysis of all endpoints, including hatching success, was based on living embryos/larvae at the respective time point of evaluation.

Table 1. Overview of observed lethal and sublethal endpoints at respective time points.

Endpoint	12 hpf	24 hpf	48 hpf	60 hpf	72 hpf	96 hpf
Mortality	✓	✓	✓	✓	✓	✓
Developmental delays		✓				
No somites		✓				
Non-detachment of the tail		✓				
No development of the eyes		✓				
Heart rate			✓			
Hatching success				✓	✓	✓
Malformations						✓
Oedema						✓
Eye/brain defects						✓
Deformation of the spine						✓
Light pigmentation						✓

Heart rates were determined from two out of four individuals per Petri dish for 20 s, and values were extrapolated to 1 min. Coagulated eggs, dead larvae and empty egg shells were removed from the Petri dishes to avoid depletion of oxygen due to biological degradation processes. The embryo test was run three times and conducted according to Organisation for Economic Co-operation and Development (OECD) 236 (2013). The compound 3,4-dichloraniline (98%, CAS: 95-76-1; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at a concentration of four mg/L served as a positive control and reconstituted water, as a negative control. According to the Directive 2010/63/EU of the European Parliament and the Council on the protection of animals for scientific purposes, D. rerio embryos and larvae that do not feed independently are not regarded as animals, thus regulations and permissions for animal testing do not apply. Nevertheless, all embryos in our tests were handled in the least stressful way possible and with the utmost care. After test termination embryos/larvae were euthanized with MS222.

Statistics; All statistical analyses were conducted in JMP 11.2.0 (SAS Institute Inc., Cary, NC, USA). Mortality, hatching success and the malformation rate at 96 hpf, as well as developmental delays at 24 hpf, were analysed with a likelihood-ratio w2 test, followed by Fisher's exact test. Finally, the sequential Bonferroni-Holm method was applied accounting for multiple testing. A Cox regression was used to assess mortality and hatching success over time. For the analysis of heart rate, the data were averaged

per Petri dish and checked for a normal distribution and homogeneity of variances. Subsequently an ANOVA with Tukey's HSD or Dunnett's test was conducted. If data did not meet the criteria for an ANOVA and transformation of the data did not lead to the desired result, a non-parametric Steel-Dwass test was conducted instead. Additionally, for assessing the pH range in which pH control and glyphosate treatments differed in heart rate across the whole span of tested pH, non-linear regression analysis, including calculation of 95% confidence intervals (TableCurve 2D v5.01; SYSTAT Software Inc., San Jose, CA, USA), was applied. Non-linear regression analysis by TableCurve was also used for determining LC10/EC10 and LC50/EC50 values of endpoints in unbuffered glyphosate treatments.

Results

After 96 hpf, mortality and hatching success were 0% and above 80%, respectively, in control embryos. The 3,4-dichloraniline positive control induced high mortalities, with rates consistently above 80% after 96 hpf. Thus, the validity criteria according to Organisation for Economic Co-operation and Development (OECD) 236 (2013), including sensitivity of zebrafish, were met.

Unbuffered glyphosate: At the two highest concentrations tested (1 and 10 mM), it was already difficult to select well-developed eggs after the 2 h pre-exposure period. The yolk sac, which usually has a regular spherical shape, was found to be asymmetric and partly oval, and the chorion fluid, which is naturally clear, was murky in some cases and contained indefinable streaks.

As early as 12 hpf, all individuals, without exception, in the 10 mM treatment died. Mortality in the 1 mM exposure experiment was beyond 85% at 12 hpf and reached 100% within the first 24 h. Within the 750 mM glyphosate treatment, only six out of a total of 96 individuals survived until the end of the test at 96 hpf, whereas concentrations of 250 mM and below resulted in negligible or no mortality (3.125%). Regarding mortality at 96 hpf, all treatments 500 mM were highly significantly different from the control (likelihood ratio w2, $p < 0.001$). Lethal concentrations were calculated to be 385 mM (LC10) and 582 mM (LC50) at 96 hpf. Heart rates showed a concentration-dependent relationship, decreasing with increasing glyphosate concentration. The mean heart rate was 149 beats per minute (bpm) for the control and between 130 and 140 bpm for low (10, 50 mM), 120 and 130 bpm for medium (100, 250 mM) and 110 and 120 bpm for the higher (500, 750 mM) concentrations. Thus, differences between the control and the 750 mM concentration ranged between 30 and 40 bpm. The treatments with the highest concentrations of glyphosate (one mM, 10 mM) could not be evaluated due to 100% mortality at that time point. Only two individuals out of those exposed to one mM glyphosate survived until 60 hpf and seemed to continue the observed relationship between glyphosate and heart rate by showing even lower rates (93 and 96 bpm). As single individuals, they were not included in the statistical analysis. All remaining treatments were significantly different from the control (ANOVA with Tukey's HSD, $p < 0.001$) and the relationship between glyphosate concentration and heart rate could be described by linear regression analysis ($R2 = 0.546074$, $p < 0.001$). The EC10 was 43 mM. Concerning the hatching rate, we observed a clear division between a cluster of treatments that comprised the control treatment and lower concentrations of glyphosate (10, 50, 100 mM) and another treatment cluster comprising higher concentrations (250, 500, 750 mM).

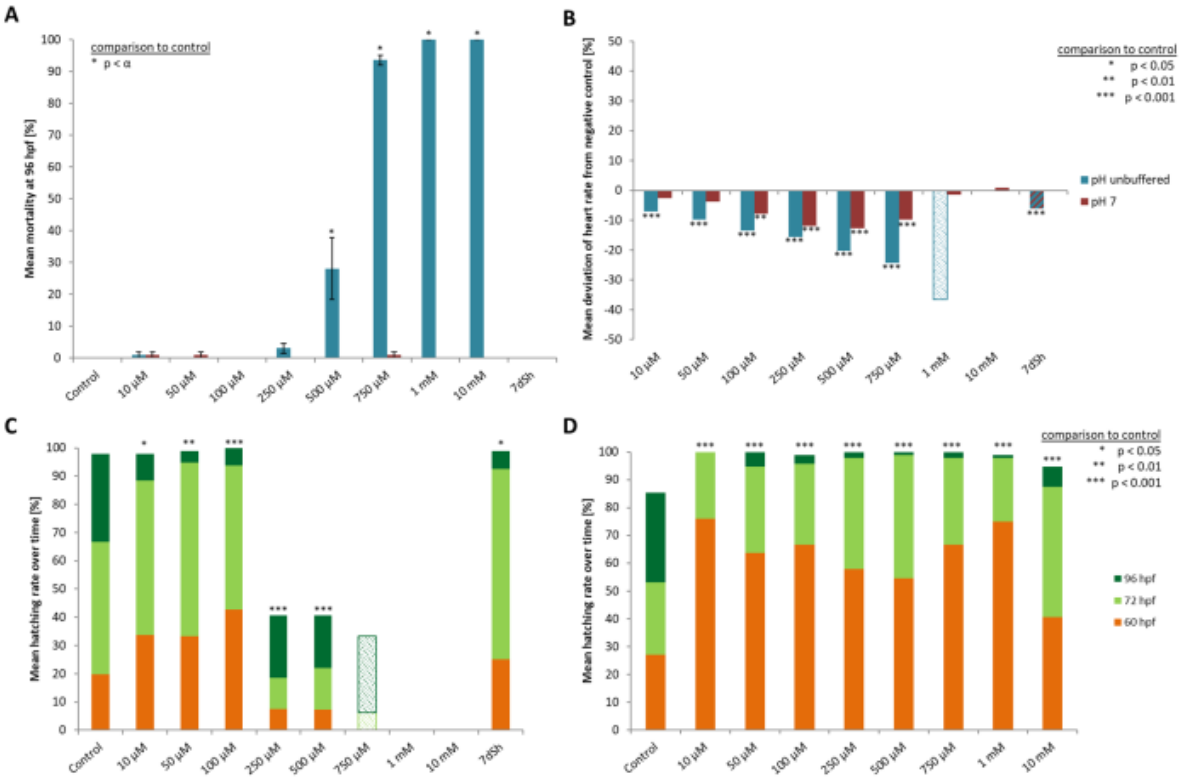

Figure 1. Mortality, heart rate and hatching success in percentage of unbuffered and pH 7 treatment. (A) Mortality after 96 hpf (likelihood ratio $\varkappa^2$, Fisher's exact test, Bonferroni-Holm, $p < \alpha$), (B) heart rate at 48 hpf relative to the negative control (Steel-Dwass, $p < 0.01$), (C) hatching rate over time in unbuffered treatments (Cox regression, $p < 0.05$), (D) hatching rate in pH 7 treatments over time (Cox regression, $p < 0.001$); shaded bars mark treatments with $n < 5$ that show tendencies but are not included in the statistical analyses.

Embryos exposed to lower concentrations hatched in 98–100% of cases, whereas hatching success in the experiments with 250 and 500 mM glyphosate was approximately 40%. All glyphosate treatments showed significant differences compared with the control (Cox regression, $p < 0.05$). EC10 and EC50 levels at 96 hpf were 155 and 224 mM, respectively. There were no developmental delays at 24 hpf for glyphosate concentrations between 10 and 100 mM, whereas in treatments with 250 to 750 mM, rates varied from 15% to 25%. The EC10 for this endpoint was 126 mM. Results for all concentrations 250 mM were highly significant (likelihood ratio w2, $p < 0.001$) compared with the control. A direct concentration dependency could not be observed. Rather, it seemed that a distinct concentration threshold had to be exceeded to induce those developmental delays and failures, which later approached the same level. Prevalent defects were a lack of tail detachment, sometimes combined with apically curved tails; a lack of somite formation and an impairment of eye development was not detected. Occasionally, embryos were fully developed but either the complete tail or just the posterior end of their tails remained attached to the yolk sac. Under normal conditions, movement begins after tail detachment. Yet, even the embryos in glyphosate treatments that lacked tail detachment, overall development had progressed to a point at which muscular contractions were already visible. But due to the undetached tails, embryos were unable to turn around and their movement was very limited. Additionally, some embryos had the posterior end of their tails detached but displayed severe spine deformations.

Those embryos could not move their tails in the same fluid manner as normally developed embryos could. Malformations could be found in embryos of all glyphosate treatments but with rates below 20%. All glyphosate treatments were significantly different from the control. Among the malformations recorded, lightly pigmented embryos and larvae were particularly frequen). Furthermore, reduced eye size occurred regularly, and some individuals suffered from cardiac or yolk sac oedemas. Two

individuals showed a notable shortening of the tail. Deformations of the spine at 96 hpf were observed surprisingly rarely, despite the high rates of tail and spine malformations at 24 hpf.

Table 2. Results for concentration-dependent glyphosate treatments, as well as for pH-dependent control and glyphosate treatments, as percentages.

	Mortality		Hatching		HR	D	M
	96 hpf (%)	Over time	96 hpf (%)	Over time	48 hpf (bpm)	24 hpf (%)	96 hpf (%)
Unbuffered							
Neg. control	0	–	97.92	–	148.75	0	0.26
10 µM	1.04	n.s.	97.92	*	**138.38*** **	0	**2.36***
50 µM	0	n.s.	98.96	*	**134.19*** **	0	**5.47***
100 µM	0	n.s.	100	***	**128.69*** **	0	**4.69***
250 µM	3.13	n.s.	**40.65***	***	**125.56*** **	**22.23***	**13.57***
500 µM	**28.13***	*	**40.58***	***	**118.63*** **	**20.36***	**16.06***
750 µM	**93.64***	***	**33.33***	n.a.	**94.50*** **	**19.04***	**18.06***
1 mM	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
10 mM	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
LC_{10}/EC_{10}	385 µM		155 µM		43 µM	126 µM	179 µM
LC_{50}/EC_{50}	582 µM		224 µM		–	–	–
7dSh	0	n.s.	98.93	*	**139.75*** **,†**	0.35	**0.27**†
Neutral (pH 7)							
Neg. control	0	–	85.42	–	148.04	0	0.52
10 µM	1.04	n.s.	**100***	***	144.25	0	1.04
50 µM	1.04	n.s.	**100***	***	142.56	1.04	1.87
100 µM	0	n.s.	**98.96***	***	**136.57****	0	2.60
250 µM	0	n.s.	**100***	***	**130.31*** **	0	0.26
500 µM	0	n.s.	**100***	***	**129.19*** **	0	1.56
750 µM	1.04	n.s.	**100***	***	**133.50***	0	2.35
1 mM	0	n.s.	**98.96***	***	145.94	0.69	**6.56***
10 mM	0	n.s.	**94.79***	***	149.38	0	**7.29***
pH range—control							
Neg. control	**0**	–	94.69	–	160.38	0	0.26
pH 3	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	**84.03***	***	**61.11***	***	**140.19*** **	**9.36***	**4.17***
pH 3.4	**51.04***	***	**77.78*** **,†**	***	**141.38*** **	**6.93***	**3.51***
pH 3.5	**8.33***	n.s.	**15.77*** **,†**	***,†	**144.25*** **	**8.32***	**2.0***
pH range—glyphosate							
pH 3	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	**72.57***	***	83.33	***	**136.46*** **	**13.33***	0
pH 3.4	**28.47***	***	**60.61*** **,†**	***	**141.49*** **	**4.54***	**3.80***
pH 3.5	**17.36***	n.s.	**66.57*** **,†**	***,†	**143.50*** **	**10.31***	**2.04***

Notes:
Asterisks (*) and bold indicate statistically significant differences from the negative control (Cox regression, ANOVA. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Likelihood ratio χ^2, Fisher's exact test, Bonferroni-Holm: *$p < \alpha$).
Crosses (†) denote additional statistical significances between pH control and glyphosate within the same pH range or, in the case of 7dSh, differences from 1 mM glyphosate at pH 7. For unbuffered glyphosate concentrations, endpoint-related LC_{10}/EC_{10} and LC_{50}/EC_{50} values are given.
HR, heart rate; D, developmental delays; M, malformations; n.s, not significant; n.a., not available (no sufficient sample sizes for statistical analysis).

Glyphosate at pH 7; When the glyphosate solutions were adjusted to pH 7, almost no mortality or developmental delays occurred, and malformation rates were below 10% but were still significantly elevated in 1 and 10 mM treatments (likelihood ratio w2, $p < 0.001$). In the concentration range of 10 to 500 mM, heart rates showed a similar trend to those in unbuffered treatments but at a lower level: bpm decreased with increasing concentration. Still, treatments between 100 and 500 mM differed significantly from the negative control (Tukey's HSD, $p < 0.01$). At 750 mM, heart rates increased again, with a higher frequency than at 250 and 500 mM. At the two highest concentrations (1, 10 mM), heart rates were, on the one hand, marginally decelerated (1 mM) and on the other hand, marginally accelerated (10 mM) compared with the negative control. Thus, it seems that there is a turning point between 500 and 750 mM, at which the relationship between increasing concentration and heart rate shifts from deceleration to acceleration in comparison with the negative control. As already seen for

lower concentrations in unbuffered treatments, glyphosate tends to induce early hatching, even at the lowest concentration and independently of concentration. This effect unfolded to its true extent in the pH-neutral treatments. At least twice as much larvae had hatched across all glyphosate treatments at 60 hpf compared with the negative control. After 72 hpf, all larvae were hatched in glyphosate treatments, except for single individuals that hatched at 96 hpf or did not hatch at all, whereas in the negative control, only 53% of the embryos were hatched at 72 hpf and even about 15% remained unhatched at 96 hpf.

pH range , In a first step, one mM glyphosate was tested at pH 3, 4, 5, 6, 7 and 8 in comparison with negative controls at the respective pH but without the pesticide. Mortality was 100% for both treatments at pH 3, independent of the presence of glyphosate. Only a single individual survived the first 12 hpf. In contrast, only one individual died throughout all other exposures within 96 hpf. Morphological aberrations described for high glyphosate concentrations under unbuffered conditions also applied to low pH treatments, independent of glyphosate addition. Concerning sublethal endpoints, results between different acidities in the range of pH 4 to 8, as well as between control and glyphosate within the same pH range, were inconspicuous for the most part. Thus, the pH 3 to 8 series was tested just once, and subsequent testing concentrated on the range from pH 3 to 4. Thus, in the next step, pH 3, 3.25, 3.5, 3.75 and 4 were investigated in detail. As embryos exposed to pH 3.75 and 4 did not show any prominent effects, only a single run was conducted, and the final testing scheme was determined from pH 3 to 3.5 in 0.1 increments. Additionally, a test with unbuffered glyphosate at a one mM concentration (which resulted in a pH of 3.2 in the test solution) was included for direct comparison. Mortality decreased with increasing pH. Treatments with a pH of 3.2 and lower induced 100% mortality after 96 hpf. Whereas embryos exposed to pH 3 and 3.1 died within 48 hpf at the latest, embryos in pH 3.2 treatments survived considerably longer.

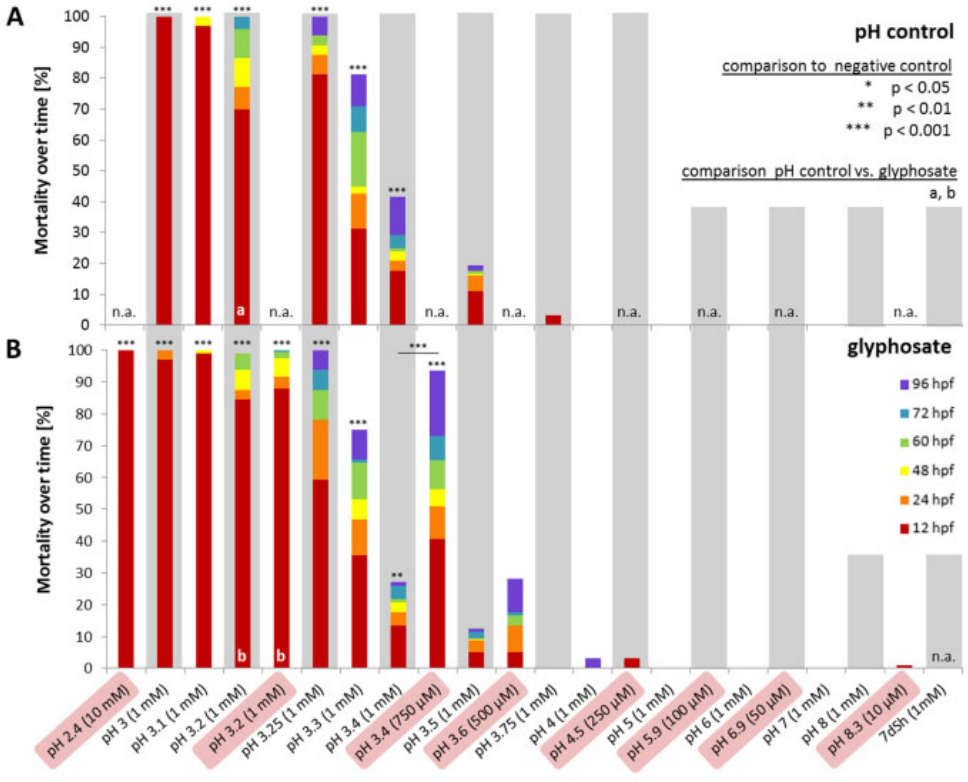

Figure 2. Mortality over time as percentages of embryos exposed to the pH control (A) and glyphosate (B). Respective concentrations of glyphosate are given in brackets. Results from unbuffered treatments (50 µM–10 µM glyphosate; highlighted in red) are combined with pH range results and positioned according to their measured pH. Treatments not conducted in the pH control or glyphosate scheme are labelled n.a. (not available). Significant differences from the negative control are marked with asterisks (*), except glyphosate pH 3.4 with an additional significant comparison between unbuffered and pH 7 treatment. Significances between pH control and glyphosate treatments within respective pH ranges are denoted with letters (a and b) (Cox regression, $p < 0.01$).

Apart from pH 3.5 without glyphosate, all treatments showed elevated mortality rates compared with the negative control (Cox regression, $p < 0.05$). There were no differences between control and glyphosate treatments with corresponding pH values, except for the elevated mortality in unbuffered glyphosate compared with the respective pH 3.2 control. Compared with the negative control, hatching was significantly delayed and also reduced in both glyphosate and pH control treatments (Cox regression, $p < 0.001$). Whereas 30% of the control embryos hatched at 60 hpf, in the pH control and glyphosate exposures, the hatching rate at 60 hpf was consistently below 5% (see Supplementary File, hatching rate). The tendency toward glyphosate-induced premature hatching at 60 hpf that was observed in pH-neutral treatments was not evident at low pH. Although not statistically significant (except for pH 3.5: Cox regression, $p < 0.001$), embryos exposed to glyphosate tended to hatch earlier and more frequently than embryos in the respective pH controls. Heart rates were significantly lowered by glyphosate at pH 3.3 to 3.5, as well as by the corresponding control pH treatments (Steel-Dwass, $p < 0.001$). Differences between glyphosate and the respective controls at the same pH value could only be detected when the full pH range dataset (including results for pH 3 to 8) was analyzed. At a pH between 5.55 and 6.02, glyphosate elevated the embryonic heart rate significantly compared with pH controls (TableCurve 2D v5.01). Developmental delays and malformations occurred in the low pH treatments, but they did not vary in a pH-dependent manner, and there was no detectable difference between glyphosate and the respective pH controls.

Comparison; When datasets for the unbuffered glyphosate treatment and the pH range were merged regarding mortality in relation to pH, interestingly, embryos exposed to unbuffered glyphosate showed higher mortalities at 500 and 750 mM compared with their 1 mM counterparts at pH 3.5 and 3.4, respectively. The unbuffered 750 mM treatment with a pH of 3.4, in particular, resulted in a mortality rate more than twice as high as that in the glyphosate pH 3.4 treatment (1 mM), mirroring mortality effects seen in treatments ranging rather between pH 3.25 and 3.3.

Conclusion

In unbuffered glyphosate medium the lethal concentrations were calculated to be 385 mM (LC_{10}) and 582 mM (LC_{50}) at 96 hpf. Regarding heart rates the EC_{10} was 43 mM. Concerning the hatching rate, EC_{10} and EC_{50} levels at 96 hpf were 155 and 224 mM, respectively. For developmental delays at 24 hpf the EC_{10} was 126 mM.

3. Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

For Zebrafish (*Danio rerio*) embryos acutely exposed to glyphosate at concentrations between 1.69 and 1690.7 mg glyphosate/L (10 µM to 10 mM) for 96 hours post fertilization (hpf) the LC_{10} and LC_{50} values (96 hpf) were calculated to be 65.1 mg a.s./L (385 µM) and 98.4 mg a.s./L (582 µM), respectively (in unbuffered glyphosate medium). Regarding heart rates the EC_{10} was 7.27 mg a.s./L (43 µM). Concerning hatching rate, 96 hpf -EC_{10} and EC_{50} values were 26.2 mg a.s./L (155 µM) and 37.9 (224 µM), respectively. For developmental delays at 24 hpf the EC10 was 21.3 mg a.s./L (126 µM). The test was conducted according to OECD 236 test guideline.

Concerning the validity criteria in the OECD 236, despite the stated > 80% mortality in the positive control (>30% required) there are no details presented to confirm the level of mortality. The fertilisation rate of the batch of eggs used was not reported. Finally, acute endpoints based on developmental delay and heart rate are not relevant to an EU level risk assessment for Annex I renewal purposes.

The test design is adequately described, however, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.2.1
Report author	Gholami Syedkolaei S.J. *et al*. (Seyed Jalil Gholami)
Report year	2013
Report title	Toxicity evaluation of Malathion, Carbaryle and Glyphosate in common carp fingerlings (Cyprinus carpio, Linnaeus, 1758).
Document No	Journal of Veterinary Research (2013), Volume 68, Number 3, pp. 257-267
Guidelines followed in study	OECD 203
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities.
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Fingerlings of the common carp (*Cyprinus carpio*, Linnaeus, 1758) are often exposed to a wide range of pesticides when they are released introduced into the sea at the estuaries of the rivers flowing into the Caspian Sea. The present study investigated effects of lethal concentrations (expressed as 96-hour LC_{50s}) and sublethal concentrations (determined by acetylcholinesterase assay) of glyphosate on these fingerlings.

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

Materials and methods

Chemicals: Sodium carbonate, sodium hydroxide, copper sulfate, potassium sodium tartrate, bovine serum albumin, phosphoric acid, tris and hydrochloric acid were purchased from the official representative of the German Company Merck in Iran. Glyphosate was purchased from Bazargan Kala (Iran). Absorbance was read using an ELISA Microplate Reader (ELx 808, BioTek).

Reactants: 0.1 mol phosphate buffer solution (PBS) (pH 7 with no Tritone), the Folin–Ciocalteu reagent (FCR) (diluted with an equal volume of distilled water), DTNB (dissolved in TRIS/HCl buffer) and acetylthiocholine iodide were used in the experiments.

Two thousand fingerlings with the mean weight of 2±0.4 g were obtained from the Shahid Rajaee Fish Breeding and Rearing Center, Sari, Mazandaran Province, and were transferred to the Fish Breeding and Rearing Research Center in the Department of Fisheries at the College of Agriculture & Natural Resources (UTCAN) in University of Tehran (Karaj). In order to adapt to the new environmental conditions, the fish were kept in two 1000-liter fiberglass tanks for 15-20 days. The physicochemical parameters of water were controlled as follows: pH=7, total water hardness ($CaCO_3$) =175 mg/l, dissolved oxygen=more than 7 ppm and temperature=20±2°C. The stock solution of glyphosate was prepared with the concentration of 10,000 ppm.

Lethal concentration experiments (bioassays): To perform bioassay, the range of concentrations of glyphosate and the logarithmic distances were determined in a pilot test and then the main experiment was carried out. Based on the results of this pilot test, the fingerlings were exposed to the following concentrations of glyphosate for 96 hours: 5.5, 6.5, 7.5, 8.5, and 9.5 mg/l. Effects and LC_{50s} were

determined in accordance with the OECD Guidelines for the Testing of Chemicals (No. 203) in static water. Bioassay for each pesticide was performed on 150 fingerlings (a total of 450) that were randomly and equally put in fifteen 100-N fiberglass tanks (three replicates for each concentration with 10 fish in each tank). The experimental conditions were close to those during the adaptation period. The fingerlings were not fed during the experiment. All experimental groups were monitored twice a day and the behavior of the fingerlings was studied. Moreover, the number of deaths was recorded at 24, 48, 72, and 96 hours after the toxin was added.

Sublethal toxicity experiment: The fingerlings were randomly placed in nine 100-N fiberglass tanks. Each tank contained 40 fingerlings, and the experimental conditions were the same as in the previous experiments. As in the rearing and adaptation periods, the subjects were fed 2% of their body weight and the feeding was stopped 24 hours before they were killed. In the sublethal toxicity experiment, the fingerlings were exposed to three different concentrations of glyphosate, each with three replicates for 15 days. The treatments were as follows: 0 (control), 0.6, and 1.2 mg/l of glyphosate. These concentrations were determined based on the LC_{50} values. About 10% of the water in each tank was siphoned off every day in order to remove waste materials and reduce ammonia levels in the water. To maintain the stability of experimental conditions, the removed water was replaced by an equal volume of water with the initial concentrations of the pesticide.

Sampling and extract preparation (upper layer): A number of fingerlings from each treatment were sampled on the fifth, tenth, and fifteenth days after their first exposure to pesticides. Because of the very small size of the fingerlings, it was not possible to take blood or tissue samples. Therefore, they were beheaded and both parts (head and trunk) were frozen at -70°C to be later used for extract preparation. The obtained tissues were manually homogenized in 0.1mmol PBS (pH 7 and containing 1% of Tritone X-100). The samples were centrifuged and the resulting extract (upper layer) was removed to be used as the enzyme source.

Total protein assay and ACHE activity measurement: Total protein concentration in the tissues was measured by using the Lowry method at 540 nm utilizing an ELISA microplate reader. In this method, FCR was used as the color reagent. Protein concentration in tissue samples was then determined using the resulting curve and its linear equation. The specific activity of cholinesterase (in μU/min/mg protein) was measured based on Ellman's method at 420 nm using a microplate reader. To this end, a mixture of the extract (upper layer), 0.1 mol PBS, DTNB (Ellman's reagent) and acetylthiocholine iodide were added to each tube. Finally, 100 ml of the final solution was poured into each well of the microplate and absorbance per minute (O.D. /min) was read.

Calculations and statistical analysis: The data obtained from the bioassay and mortality rate of the fingerlings determined by using the probit model were analyzed. The values obtained from bioassays were then estimated using the POLO-PC 2002 software (under license of the University of Tehran). The specific activity of the enzyme (in μU/min/mg protein) was calculated as the dependent variable. The data were statistically analyzed using two-way ANOVA. The concentrations of the pesticides and the durations of exposure to them were the independent variables. The difference between means was also evaluated using Duncan's test with type-I error level of 0.05.

Results

Bioassay results: No mortality was observed during the adaptation period of the fingerlings. The results showed that their mortality rate increased with at the higher concentrations. Based on the mortality rates in the bioassays, the mean LC_{10}, LC_{50}, and LC_{90} values of glyphosate for the fingerlings at 24, 48, 72, and 96 hours were calculated (α=0.95) (Table 1).

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

Table 1: The mean values obtained from bioassays in Caspian Sea common carp fingerlings

Chemical's name	Lethal concentration (mg/l)	24-hour	48-hour	72-hour	96-hour
Glyphosate	LC_{10}	5.995	5.976	5.865	5.548
	LC_{50}	7.202	7.172	6.985	6.753
	LC_{90}	8.651	8.606	8.319	8.168

Conclusion

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The acute 96 hours- LC_{50} for common carp fingerlings was determined to be 6.75 mg/L by static exposure to glyphosate at 5 test concentrations between 5.5 and 9.5 mg/L.

The test was conducted according to OECD 203, but validity criteria are missing. No information on the test item such as purity is given. The results for the control are not stated. Furthermore, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.3.1.3 / KCP 10.3.1.5
Report author	Thompson H. M. *et al.*
Report year	2014
Report title	Evaluating Exposure and Potential Effects on Honeybee Brood (*Apis mellifera*) Development Using Glyphosate as an Example
Document No	Integr Environ Assess Manag (2014), 10: 463-470
Guidelines followed in study	Oomen et al. 1992
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

This study aimed to develop an approach to evaluate potential effects of plant protection products on honeybee brood with colonies at realistic worst-case exposure rates. The approach comprised 2 stages. In the first stage, honeybee colonies were exposed to a commercial formulation of glyphosate applied to flowering *Phacelia tanacetifolia* with glyphosate residues quantified in relevant matrices (pollen and nectar) collected by foraging bees on days 1, 2, 3, 4, and 7 post-application and glyphosate levels in larvae were measured on days 4 and 7. Glyphosate levels in pollen were approximately 10 times higher than in nectar and glyphosate demonstrated rapid decline in both matrices. Residue data along with foraging rates and food requirements of the colony were then used to set dose rates in the effects study. In the second stage, the toxicity of technical glyphosate to developing honeybee larvae and pupae, and residues in larvae, were then determined by feeding treated sucrose directly to honeybee colonies at dose rates that reflect worst-case exposure scenarios. There were no significant effects from glyphosate observed in brood survival, development, and mean pupal weight. Additionally, there were no biologically significant levels of adult mortality observed in any glyphosate treatment group. Significant effects were observed only in the fenoxycarb toxic reference group and included increased brood mortality and a decline in the numbers of bees and brood. Mean glyphosate residues in larvae were comparable at 4 days after spray application in the exposure study and also following dosing at a level calculated from the mean measured levels in pollen and nectar, showing the applicability and robustness of the approach for dose setting with honeybee brood studies. This study has developed a versatile and predictive approach for use in higher tier honeybee toxicity studies. It can be used to realistically quantify exposure of colonies to pesticides to allow the appropriate dose rates to be determined, based on realistic worst-case residues in pollen and nectar and estimated intake by the colony, as shown by the residue analysis. Previous studies have used the standard methodology developed primarily to identify pesticides with insect-growth disrupting properties of pesticide formulations, which are less reliant on identifying realistic exposure scenarios. However, this adaptation of the method can be used to determine dose–response effects of colony level exposure to pesticides with a wide range of properties. This approach would limit the number of replicated tunnel or field-scale studies that need to be undertaken to assess effects on honeybee brood and may be of particular benefit where residues in pollen and nectar are crop- and/or formulation-specific, such as systemic seed treatments and granular applications.

Materials and methods

Technical grade glyphosate (62.27% w/w glyphosate isopropylamine [IPA] salt corresponding to 46.14% w/w glyphosate acid equivalent [a.e.]) and the soluble concentrate formulation of glyphosate (MON 52276) (30.68% glyphosate a.e. as the IPA salt, batch no GLP-0810-19515-A), supplied by Monsanto (St. Louis, MO) were used in the study. All honeybee colonies were obtained from National Bee Unit, FERA, (York, UK) apiaries and were confirmed as having low incidence of adult bee diseases, viruses, and varroa with no clinical signs of brood diseases.

Exposure assessment

Two 180 m^2 well-ventilated but insect-proof glasshouses were used for the study so as to be as representative as possible of the outdoor situation (e.g., polytunnel) but without direct rainfall. *Phacelia* was planted directly into the soil in the glasshouses and no pesticides were used during its cultivation. Application was performed when *Phacelia* flowers were at 100% of full bloom.

Three days before the application, 2 small honeybee colonies comprised of 4 to 6 frames of brood and 6000 to 12 000 adult bees were located on opposite sides of each glasshouse and allowed to fly freely. At the time of installation, each colony was fitted with a pollen trap and provided with a limited amount of stores to ensure that feeding on the crop was encouraged. This was done by removing as many frames as possible which contain only nectar or pollen, while ensuring survival and a maximum foraging activity. A supply of clean water, with provision to prevent bees from drowning, i.e., a sponge, was provided and replenished as required (it was removed during spray application).

To confirm that bees were foraging on the flowering *Phacelia*, foraging assessments were carried out each day during times when peak activity was expected. The assessments were performed by marking a 5 m × 1 m wide transect within the crop and counting the number of bees foraging within the marked area during a 1 min period once each day during the peak activity period (between 10.00–15.00 h in this study, based on previous experience). In addition, the number of bees returning to each hive and the number carrying pollen loads were counted during a 30 s period. These 2 counts provided information on the level of foraging activity of each hive within each glasshouse. Daily assessments of the crop were undertaken by visual assessment of the quality of the forage available, e.g., % plants with wilted flowers, wilted leaves.

The glyphosate formulation was applied at a rate equivalent to 8 L/ha (2.88 kg a.e./ha) in 400 L water/ha achieving an application efficiency of between 102% to 104% of the target rate, in both glasshouses. The application rate of 2.88 kg a.e./ha is the highest single application rate recommended for glyphosate, whereas the typical single application rate is 2.16 kg a.e./ha. The final treatment solution was prepared by adding the required quantities of test item—measured by weight, to measured volumes of tap water and thoroughly mixing in the field immediately before use to give the final treatment solution. The application was made during a period when the bees were actively foraging, using a 3 nozzle lunch box sprayer unit with a hand-held boom fitted with Lurmark 03 F110 nozzles. Direct spray drift onto the colonies was avoided by directing the spray away from the hives, and no direct overspray of the colonies occurred.

Pollen traps were activated 24 h before pollen collection, and the content of the pollen trap fitted to each hive was collected on days −1 (i.e., the day before application), 1, 2, 3, 4, and 7 after the application. The content of the traps was discarded on day 6 so as to only collect a sample from days 6 to 7. Each day and hive sample was kept separate unless they were too small for residue analysis, in which case samples from the same glasshouse were combined. All samples of pollen, nectar, and larvae were stored at −20° C.

On days 0 (before application), 1, 2, 3, 4, and 7 after the application samples of approximately 40 returning forager bees were collected from each colony by blocking the entrance of the hives with a foam bung and collecting returning foraging bees directly into collection jars. The nectar was collected from the honey stomachs of individual honeybees by removal of the stomach by dissection and placed in a preweighed tube. Samples were combined to produce samples large enough for residue analysis (minimum 200 mg).

On days 4 and 7 after the application, samples of 10 4–5-day-old larvae were taken from each colony using a forceps and stored at −20° C. Each day and hive sample was kept separate. On day 7, an additional sample of nectar was taken from the combs using a syringe in each colony and each hive sample was kept separate.

Residue analysis

Residues of glyphosate were extracted from larvae, pollen, nectar, and sucrose solution samples with acetonitrile/water (1:4, v/v). Recovery samples were fortified by spiking blank samples after weighing. For larvae, pollen, and nectar, the whole sample was accurately weighed into a single-use centrifugation tube. The sample was then homogenized, extracted with acetonitrile–water (1:4) with a high speed laboratory mixer, separated by centrifugation followed by solid-phase extraction of the supernate using a C18 column. All samples were then derivatized with fluorenylmethyl-chloroformate (FMOC-Cl). For derivatization, internal standard (1.0 µg/mL), borate buffer (0.2 mol/L sodium tetraborate decahydrate in water), and FMOC-Cl (5 g/L in acetonitrile) were added to the diluted extract. The samples were closed, mixed, and incubated at ambient temperature for at least 1 h. Finally, pH 3 water was added.

A second cleanup was carried out by applying the derivatized product to an Oasis HLB SPE column (equilibrated with dichloromethane followed by methanol and pH 3 water) and then rinsed with dichloromethane and the glyphosate-FMOC was eluted with methanol. The eluate was evaporated to dryness using a vacuum rotary evaporator. The residue was reconstituted in 5% acetonitrile solution and transferred into a glass vial for high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) analysis.

The samples were analyzed using high-pressure liquid chromatography (Shimadzu LC-System) coupled with a triple quadrupole mass spectrometry detector (Sciex API4000). A Phenomenex Synergi column 2.5 µm Max-RP, 20 × 2.0 mm, 2.5 µm (No. 00M-4372-B0-CE) + 4 mm guard column was used. The column temperature was 40°C and a 30 µL injection volume was used. The mobile phase comprised A: water + 0.1% acetic acid (80%), B: methanol + 0.1% acetic acid (15%), and C: 100 mM ammonium acetate solution in methanol (5%) with a linear gradient over 5 min to comprise A: water + 0.1% acetic acid (0%); B: methanol + 0.1% acetic acid (95%) and C: 100 mM ammonium acetate solution in methanol (5%). Glyphosate-FMOC was quantified using the transition 390.0 to 149.8 with an internal standard glyphosate 1,2-^{13}C2 15N-FMOC transition 393.0 to 152.8.

At the start of the analytical sequence, the detector linearity was confirmed over the calibration range of interest by constructing a calibration function of peak area versus concentration within the range from 2.0 ng/mL to 5000 ng/mL for larvae and nectar samples, 1.0 ng/mL to 3500 ng/mL for pollen samples, and from 2.0 ng/mL to 4000 ng/mL for sucrose solution samples. Injections of sample extracts were interspersed with injections of quality control standards after 2 to 4 samples to verify the detector response.

The methods were validated before use and showed 92%–102% recovery with relative standard deviation (RSD) <15% with sucrose samples spiked at 1 and 400 mg a.e./kg, larval samples spiked at 1 and 200 mg a.e./kg, pollen samples spiked at 1, 500 and 700 mg a.e./kg and nectar samples spiked at 1 and 500 mg a.e./kg. Calibrations were linear within the range. Unless otherwise specified the limit of detection (LOD) was 0.3 mg a.e./kg, denoted as not detected (n.d.), and the limit of quantitation (LOQ) was 1.0 mg a.e./kg. Where data were used to generate mean values residues less than the LOQ were ascribed a value of 0.6 mg a.e./kg.

Effects assessment

Two approaches were made to assess exposure levels to be used in the effects study: one based on generic published data on the requirements for nectar and pollen by larvae (generic data) and the other based on the observations made in the exposure study (study data).

Generic data. The calculations were based on a daily brood requirement of 30 mg nectar (based on 40% sugar in nectar) and 1mg pollen per worker larva (Rortais et al. 2005). Based on a brood frame being 3600 cells (British Standard frame) and 5 frames of brood (4–6 were used in this study), there are 18 000 brood cells. The brood is unsealed for 25% of the time (hatch day 3 to sealed day 8 with emergence day 21, empirically determined in this study) therefore 4500 larvae have a requirement for 135 g/d nectar

and 4.5 g/d pollen.

Study data. The second approach was to assess the amount of pollen and nectar returning to the hive over the time course of exposure using the data on the numbers of returning foragers in the study and the amounts of pollen and nectar collected from bees by using the pollen trap and individual bee samples.

The maximum pollen collected per colony was 2.9 g on day 1 and the traps were estimated to be approximately 50% efficient based on calculated pollen collection (Levin and Loper 1984; Delaplane et al. 2013). Thus 6 g of pollen per day was returned to the hive (the colony was using approximately 4.5 g of this based on the study by Rortais et al. [2005]).

The nectar collection was more difficult to directly assess but with a mean of 18 foragers returning to the hive per 30 s (observed in this study) and approximately 50 µL per load (max) this gives 18 trips/30 s × 60 s/min × 60 min/h × 12 h max foraging/d = 25 920 trips/d × 0.050 mL = 1296 mL/day (of which the colony was using 135 g, based on Rortais et al. [2005]). Because the assessment is brood exposure, the conservative collection estimate is justified. Therefore, as a worst case example considering the colony size used in the exposure study, the colony collected 6 g pollen and 1296 mL (i.e., 518 g sugar, assuming 40% sugar content) nectar and of this the brood consumes 4.5 g pollen and 135 g nectar (Rortais et al. 2005) that allowed the excess to be stored for later consumption.

Considering that bee colonies used in the brood study were up to 50% bigger than those used in the residue study, an additional calculation for the expected total daily intake of glyphosate residues was undertaken assuming that such colonies would collect 9 g pollen and 1944 mL nectar. Furthermore, the determined residue content based on a worst-case application rate of 2.88 kg a.e./ha for spot treatments in orchards and vines and was adjusted to reflect the more realistic maximum application rate of 2.16 kg a.e./ha for preplanting, preemergence of crops, and preharvest applications.

The brood feeding study was undertaken using glyphosate as the technical grade IPA salt. Three dose levels of the test item were used based on the residues identified in pollen and nectar in a glass house study performed before the initiation of the bee brood study. The lowest dose was based on the mean residue concentrations achieved over the first 3 days following the residue study spray application (75 mg glyphosate a.e./L). The mid-dose was based on the highest residue concentrations following the spray application (150 mg glyphosate a.e./L) and the highest dose was equivalent to twice this latter rate (301 mg glyphosate a.e./L). The test item was introduced into each hive in equivalent volumes of 50% sucrose (w/v) solution (1 L) for each treatment group. Hence, the range could also be expressed in terms of concentration in the introduced dosing solution (mg glyphosate a.e./L and mg glyphosate a.e./kg). Control colonies were supplied with 50% w/v sucrose solution in deionized water and the toxic reference, fenoxycarb, (750 mg a.s./L as the formulation Insegar WG 250 g a.s./kg, batch no SM01A406) reported to have significant adverse effects on honeybee brood, was used to ensure that the study had the ability to detect effects of the test substance if they occurred (de Ruijter and van der Steen 1987).

Twenty standardized honeybee colonies each consisting of a single wooden Smith hive with British Standard frames and a queen were used; each of the queens used in the study was of similar age and lineage. The colonies were divided into 5 groups of 4 colonies. Each colony had a dead bee trap fitted to the front and the contents were counted daily during the brood assessment period (Imdorf et al. 1987). The colonies contained a mean of 14 250 to 19 500 adult bees, 1.5 to 2.5 frames of brood, 1.0 to 1.9 frames of stores, and 0.2 to 0.7 frames of pollen. The test colonies were allowed to fly freely, there were no nearby flowering crops and few flowering weeds (clover). Colonies were assembled according to treatment and groups were placed at least 20 m apart from each other. Two colonies (one control colony and one of the highest exposure rate colonies) (301 mg glyphosate a.e./L) became queenless after dosing but were retained in the study as the marked brood was viable and this was therefore not considered to have a significant impact on the study. All colonies were generally assessed within 1 week before dosing and again within weeks 1, 2, and 3 after dosing (day 0). Each assessment was carried out on every frame within each colony, and included counts of the number of combs of adults, brood (sealed and unsealed), and stores (nectar and pollen) as well as any behavioral or physical abnormalities.

The processes during the study followed the method for honeybee brood feeding test with insect growth regulating compounds (Oomen et al. 1992). Up to 24 h before dosing, 100 brood cells containing eggs, 100 cells containing 1- to 2-day-old larvae and 100 cells containing 3- to 4-day-old larvae were selected in each colony and marked using the standard Oomen et al. (1992) acetate overlay sheet method.

On day 0, one group was an untreated control, i.e., fed 1 L 50% sucrose solution, 3 groups were treated with glyphosate IPA salt (added to 1 L of 50% sucrose to achieve doses of 301, 150 and 75 mg glyphosate a.e./L), and one group was treated with the toxic reference, fenoxycarb, dispersed in 1 L of 50% w/v sucrose (750 mg a.s./L). Doses were administered by removing frames of stores from the colonies and placing a 1 L glass container containing the treated or control sucrose within the brood chamber. The container contained a cork float to allow access to the sucrose solution. Samples of each concentration of test item treated sucrose solution were retained for analysis by subsampling 5 mL from each of the prepared solutions and combining to a single sample (total 4 samples; control and 3 doses of glyphosate). The uptake of each sucrose solution was checked daily and the container removed when empty or after 5 days whichever was later.

On day 7, the marked brood cells (eggs, young, and old larvae) were assessed for mortality and appearance in each test colony. The final assessment for each larval was undertaken at day 13 for brood cells marked as containing old larvae, day 15 for cells containing young larvae, and day 16 for cells containing eggs. The cells were uncapped, the bee removed carefully with forceps, and the age of the bee assessed, weighed, and any deformities noted.

On days 4 and 7 (when the marked brood cells were assessed), samples of ten 4- to 5-day-old larvae were sampled from each treated colony (not from an area in which marked brood cells were located) for residue analysis. For the purpose of this study, mortality was defined as the total number of cells in any one group at any one observation period that were empty (other than recently emerged), contained dead larvae or pupae or contained larvae or pupae that were considered unhealthy (sick) and unlikely to survive. Brood mortality was statistically analyzed using a generalized linear model linked to a logit distribution for the brood mortality data and an analysis of variance for pupae weight data to determine the no observed effect concentration (NOEC) (equivalent to the no observed adverse effect level [NOAEL]) statistically, using the software Genstat v12 (VSN International). The study was considered valid if there were significant effects of the toxic reference (>40% effects on all stages) during the detailed brood assessment when compared to the control. The performance of the colonies in the control group were comparable with historical control data for the testing facility (10%–30% larval mortality overall), and demonstrate that the control colonies had performed correctly.

Results

Exposure study

Daily assessments were made of the percentage of the plants that had wilted leaves or flowers. The crop started to show significant effects of the treatment from day 4 onward in both glasshouses and this coincided with the decreased foraging activity in glasshouse 2 although less pronounced effects on foraging were observed in glasshouse 1.

Foraging assessments showed foraging activity on the crop at the start of the study and this continued throughout the exposure period in glasshouse 1 with a peak on day 4; lowest foraging activity was on day 5 at 38% of the mean prespray activity. In glasshouse 2, the foraging activity declined throughout the assessment period and reached <10% of the mean prespray activity on days 5 to 7. The weights of pollen collected from the traps fitted to each hive ranged from 0.37 to 1.8 g per colony per day.

Samples of honeybee products (nectar and pollen) and larvae were analyzed for residues of glyphosate acid equivalents. Glyphosate residues in nectar samples taken from forager bees before the application were not detectable (<0.3 mg a.e./kg). Residues in nectar samples taken at various time points after the application and originating from forager honeybees ranged from 2.78 to 31.3 mg a.e./kg and declined

over time (Figure 1A). Residues in nectar samples taken from the colonies 7 days after the application ranged from below the LOQ (1.0 mg a.e./kg) to 1.30 mg a.e./kg.

Residues in pollen samples taken from the pollen trap before the application were not detectable (<0.3 mg a.e./kg). Residues in pollen samples taken at various time points after the application and originating from the trap ranged from 87.2 mg a.e./kg to 629 mg a.e./kg and declined over time (Figure 1B). Residues in larvae samples at 2 time points (day 4 and day 7) after the application ranged from 1.23 mg a.e./kg to 19.50 mg a.e./kg.

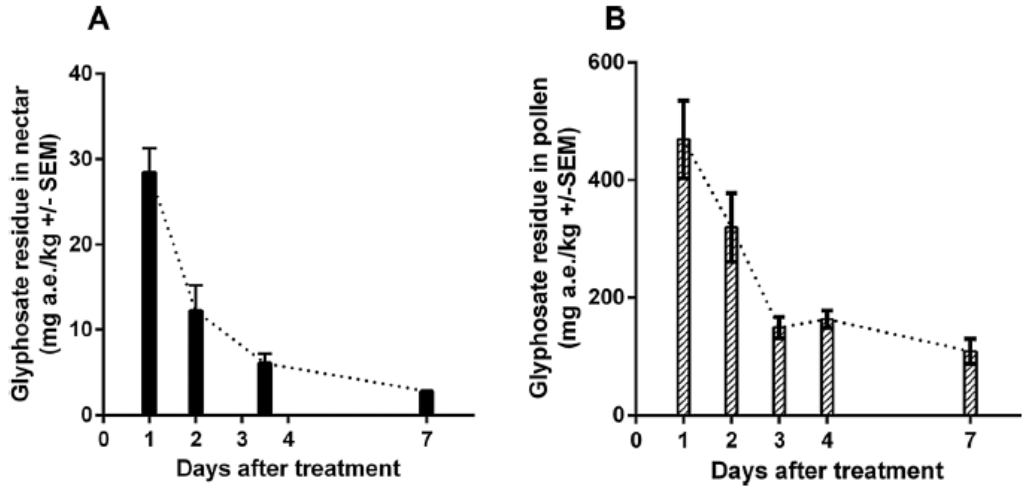

Fig. 1. Decline of glyphosate residues (mg a.e./kg ± SE). (A) Nectar collected from foragers. The nectar sample from days 3 and 4 were combined due to the small amount collected for analysis. (B) Pollen collected in pollen traps in mg a.e./kg matrix.

Effects study

Consumption of treated sucrose. Analysis of the dosing solutions showed they were within 11% of the nominal doses. The control colonies consumed between 0.63 and 1.0 L of untreated sucrose. In the glyphosate-treated colonies, at least 3 of the 4 colonies in each group consumed the total volume of treated sucrose fed to each of them. There was no statistically significant difference in sucrose consumption in comparison to control for the 301 mg a.i./L group ($p = 0.438$), 150 mg a.i./L group ($p = 0.212$), the 75 mg a.i./L group ($p = 0.054$), which was slightly higher than the control, and the positive control fenoxycarb ($p = 0.151$).

In the 301 mg glyphosate a.e./L group, one colony consumed 0.39 L and the other 3 each consumed 1.0 L resulting in mean exposure to 255 ± 26 mg glyphosate a.e. In the 150 mg glyphosate a.e./L group, one colony consumed 0.67 L and the other 3 each consumed 1.0 L resulting in mean exposure to 130 ± 12 mg glyphosate a.e. In the 75 mg glyphosate a.e./L group one colony consumed 0.90 L and the other 3 each consumed 1.0 L resulting in mean exposure to 73 ± 2 mg glyphosate a.e. In the fenoxycarb treated colonies, consumption rates ranged from 0.45 to 0.88 L resulting in mean exposure to 510 ± 72 mg fenoxycarb. Exposure at the 150 mg a.i./L dose was significantly lower than at the 301 mg a.i./L dose ($p = 0.049$) and exposure at the 75 mg a.i./L dose was significantly lower than at 150 mg a.i./L dose ($p = 0.002$).

Brood mortality. Figure 2 summarizes the survival of marked brood stages at day 7 after dosing and just before emergence. There were no significant treatment-related effects except in the fenoxycarb toxic reference treated colonies, in which overall survival of marked cells was 20% for marked eggs ($p < 0.001$), 0% for marked young larvae ($p < 0.001$) and 12% for marked old larvae ($p < 0.001$), meeting the established validity criterion for the toxic reference (>40% effects at all stages). This can be compared with overall survival of 85% for marked eggs, 96% for marked young larvae, and 96% for marked old larvae in controls and 82%–87% for marked eggs (300 mg a.i./L: $p = 0.435$, 150 mg a.i./L:

p = 0.310, 75 mg a.i./L: p = 0.250), 87%–94% for marked young larvae (300 mg a.i./L: p = 0.185, 150 mg a.i./L: p = 0.060, 75 mg a.i./L: p = 0.254), and 94%–95% for marked old larvae (300 mg a.i./L: p = 0.434, 150 mg a.i./L: p = 0.202, 75 mg a.i./L: p = 0.291) in the glyphosate-treated colonies. The control mortality is similar to historical levels in studies conducted at the Food and Environmental Research Agency (FERA) (10%–30%). Deformities were observed in the fenoxycarb-treated colonies where discolored heads, thorax, and abdomens were noted. No deformities were observed in of the control or any glyphosate-treated colonies. Additionally, there were no significant effects on the mean weight of the exposed pupae (Table 1) compared to controls in the 300 mg a.i./L group (p = 0.424), the 150 mg a.i./L (p = 0.207), or the 75 mg a.i./L (p = 0.292). The fenoxycarb-treated colonies showed significant effects on weight of surviving pupae marked as old larvae (p = 0.003). The only dead pupae observed in any significant number were those in the fenoxycarb treated group where a mean of up to 190 pupae/day was observed and a mean of 600 pupae were recovered from the colonies over the 17-day period after dosing compared with 2.0 pupae/d in the control and 1.3 to 1.8 pupae/d in the glyphosate-treated colonies. The only adverse effects on colony development were observed in the fenoxycarb-treated colonies where declines in the numbers of bees and brood were observed in the latter stages of the study compared to controls for the 300 mg a.i./L group (p = 0.401), the 150 mg a.i./L group (p = 0.414), the 75 mg a.i./L group (p = 0.360), or the positive control fenoxycarb (p = 0.070).

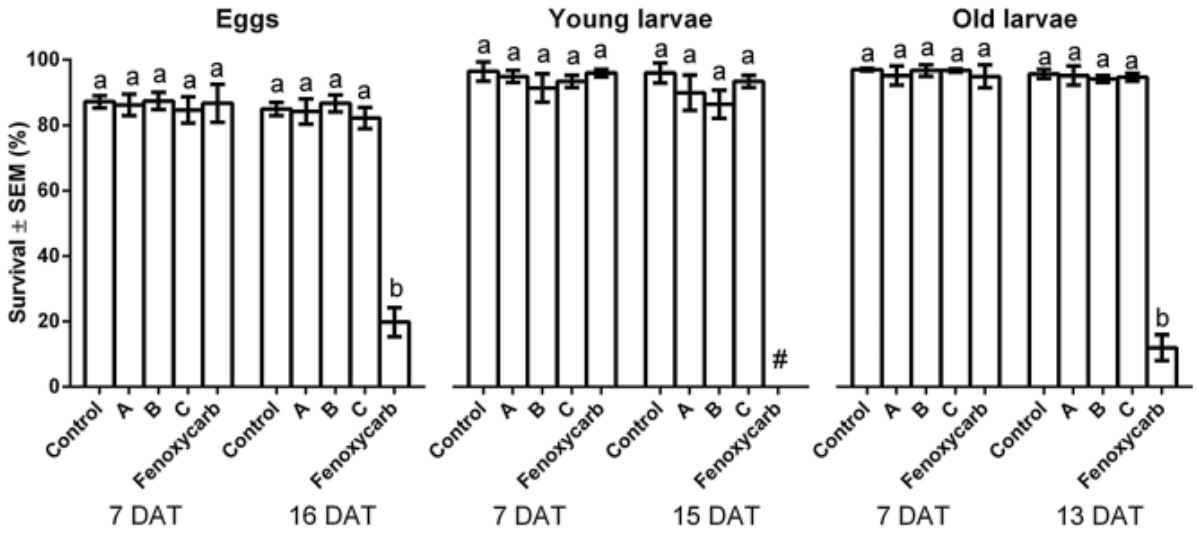

Fig. 2. Survival (% ± SE) of Eggs (7 and 16 Days After Treatment, DAT), Young Larvae (7 and 15 DAT) and Old Larvae (7 and 13 DAT) for treatment groups (mean consumption) Control (0 mg glyphosate a.e.), A (255 ± 46 mg glyphosate a.e.), B (138 ± 12 mg a.e.), C (73 ± 2 mg glyphosate a.e.), and Fenoxycarb (510 ± 72 mg). Different letters above the bars indicate statistical difference (p < 0.05) from the respective control. # no statistical analysis as no variance due to 100% mortality.

Table 1. Mean pupae weight with SE at final assessment including dead and sick in the fenoxycarb treatment

Treatment	Dose rate mg/L	Mean dose consumed mg (SE)	Weight-surviving pupae marked as eggs (mg)	Weight-surviving pupae marked as young larvae (mg)	Weight-surviving pupae marked as old larvae (mg)
Control	0	0	127.5 ± 0.7	128.4 ± 0.6	128.9 ± 0.4
Glyphosate	301	255 ± 46	135.7 ± 0.6	125.4 ± 0.6	125.6 ± 0.4
Glyphosate	150	138 ± 12	126.7 ± 0.6	124.4 ± 0.8	122.6 ± 0.5
Glyphosate	75	73 ± 2	124.7 ± 0.8	128.3 ± 1.0	121.2 ± 0.5
Fenoxycarb	750	510 ± 72	125.9 ± 0.9	128.8 ± 1.3	115.4 ± 1.0[a]

SE = standard error

[a] Statistically different effect ($p < 0.01$)

Adult bee mortality. No biologically significant adult mortality was observed in any treatment group with a mean total of 73 to 25 dead adult workers were recovered from dead bee traps over the entire 17-day period after dosing.

Residue analysis. The residues in larvae sampled at 2 time points (day 4 and day 7) after dosing of the colonies (Figure 3) ranged from below the LOQ (1.0 mg a.e./kg) to 82.1 mg a.e./kg (at the highest dose rate) confirming that larvae were exposed to test item provided in the sucrose solution and consumed it. There was a linear relationship between dose level and glyphosate levels in larvae on days 4 and 7. Levels of day 7 were considerably lower than on day 4 and are likely the result of larval growth and glyphosate exposure ending after 5 days of exposure. Notably, these residue levels are comparable with values from the exposure study which ranged from 2.9 to 19.5 mg a.e./kg with a mean of 11.5 mg a.e./kg on day 4 to 1.2 to 10.6 mg a.e./kg with a mean of 5.3 mg a.e./kg on day 7 after the glyphosate application.

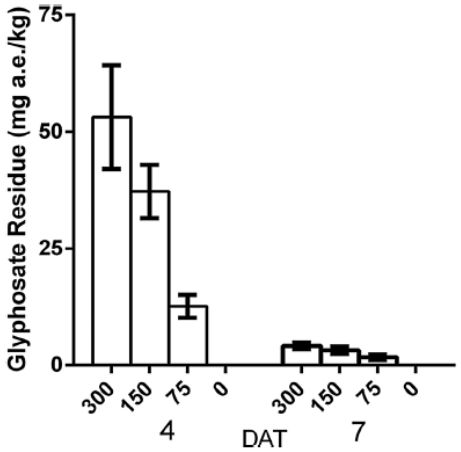

Fig. 3. Residues (mg a.e./kg ± SE) in larvae 4 and 7 days after treatment (DAT) for dose groups with dose rate of 300, 150, 75, and 0 mg a.e./kg sucrose solution.

Conclusion

There were no significant effects from glyphosate observed in brood survival, development, and mean pupal weight. Additionally, there were no biologically significant levels of adult mortality observed in any glyphosate treatment group. Significant effects were observed only in the fenoxycarb toxic reference group and included increased brood mortality and a decline in the numbers of bees and brood. Mean glyphosate residues in larvae were comparable at 4 days after spray application in the exposure study

and also following dosing at a level calculated from the mean measured levels in pollen and nectar, showing the applicability and robustness of the approach for dose setting with honeybee brood studies.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The Oomen et al. (1992) approach was used to quantify at residues in relevant matrices (pollen, nectar, and larvae) following application of glyphosate at 2.88 kg a.e./ha (400 L water/ha) to flowering *Phacelia tenacetifolia* in large glasshouses. Then brood feeding tests following the Oomen approach, were conducted by feeding 1 L treated sucrose solution at 75 / 150 and 301 mg glyphosate a.e./L directly to honeybee colonies.

The study is adequately described and all information to evaluate the study are available. At the time the study was conducted, there were no field level test guidelines adopted for use in the EU. The test did follow a recognised approach and is considered fit for purpose. The study is considered as reliable.

1. Information on the study

Data point:	KCA 8.2.7
Report author	Tian Y. *et al.*
Report year	2015
Report title	Growth inhibition of two herbicides on Spirodela polyrhiza
Document No	Nongyao kexue yu guanli (Pesticide Science and Administration), 2015, Vol. 36 (issue 3), pp. 61-65
Guidelines followed in study	OECD 221
Deviations from current test guideline	Not reported
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The inhibitory activities of glyphosate on the aquatic macrophyte *Spirodela polyrhiza,* were studied in the laboratory by using quantity of the thallus as test indicator. The effects of glyphosate were tested in a semi-static exposure of 7 days at concentrations between 8.4 and 20.902 mg/L. The results showed that glyphosate had remarkable effects on the growth inhibition of *Spirodela polyrhiza,* and the inhibitory rate increased with higher concentrations. The 168 hour-EC_{50} value was determined to be 12.817 mg/L.

Materials and Methods

Test materials and culture

The tested organism, *Spirodela polyrhiza*, was introduced from Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

The *Spirodela polyrhiza* was placed in a crystal dish with the volume of 300 mL (10 × 5cm). The Swiss standard (SIS) culture medium was added (Table 1). The light was 9000 - 10 000lx incandescent light, the temperature was 24 ± 2℃, and the culture medium was replaced every 7 days to maintain the stability of the concentration of the nutritional ingredients in the solution. It can only be used in the experiment after 14 days of continuous pre-culture. Before the experiment, enough 4-leaf *Spirodela polyrhiza* with good shape and similar shape and size were selected to carry out the experiment. The above experimental operations should be carried out in an ultra-clean work table to prevent culture medium pollution.

Main instruments and test reagents for the test

Main instruments and reagents

Intelligent artificial climate box PRX-350B (Ningbo Saifu Experimental instrument Co., Ltd.), super clean worktable VS-1300L-U (Sujing Antai), biosafety cabinet BHC-1300 II A/B3 (Suzhou Antai); Glyphosate 96.8% original drug (provided by Ministry of Agriculture Pesticide Inspection Institute), Dimethylformamide (Analytical Reagent, Beijing Chemical Plant), Twin 80 (Analytical Reagent, Beijing Chemical Plant).

Table 1: (SIS) culture medium component*

Storage solution serial No.	Reagent	Storage solution concentration (g/L)	Concentration of culture medium (mg/L)
A	$NaNO_3$	8.5	85
	KH_2PO_4	1.34	13.4
B	$MgSO_4\ 7H_2O$	15	75
C	$CaCl_2{\cdot}2H_2O$	7.2	36
D	Na_2CO_3	4	20
E	$Na_2EDTA{\cdot}2H_2O$	0.28	1.4
	$FeCl_3{\cdot}6H_2O$	0.17	0.84
F	H_3BO_3	1	1
	$CuSO_4\ 5H_2O$	0.005	0.005
	$ZnSO_4\ 7H_2O$	0.05	0.05
	$MnCl_2\ 4H_2O$	0.2	0.2
	$Na_2MoO_4\ 2H_2O$	0.01	0.01
	$Co(NO_3)_2\ 6H_2O$	0.01	0.01

* All storage solutions shall be kept in refrigerated and dark conditions, and the storage solution AE can be kept for 6 months, while the reserve liquid F can only be kept for 1 month. Prepare 1LSIS medium, take 10 mL stock solution A, 5 mL storage solution B~E, 1 mL stock solution F into volumetric flask, add 900 mL distilled water, adjust pH to 6~7 with 1 mol/L HCl, and then use distilled water to 1L.

Experimental Methods

Allocation of test mother liquid: 0.1 g glyphosate was obtained by dissolving it in aseptic water, and the volume was fixed to 100 mL capacity bottle. And then 1 000 mg/L glyphosate mother liquid could be obtained. After sealing the above liquid with sealing film, put it in the refrigerator at 4℃ for further test.

Experimental Design

On the basis of the pre-test, a series of concentration gradients are set according to the equal ratio difference. The concentrations of glyphosate were 8.4, 10.08, 12.096, 14.515, 17.418, 20.902 mg/L and solvent control group and blank control group. 200 mL (height > 2cm) culture solution containing different concentrations of glyphosate was added to the crystal dish with diameter 10 cm. Three selected *Spirodela polyrhizas* were put into the above toxic solution, sealed with an aseptic culture container ligated with a rubber band. 3 repeats were set up in each treatment, and finally they were randomly placed in an artificial climate box. The experimental conditions were consistent with the pre-culture conditions. In order to maintain the concentration of the test solution, semi-static culture was used in this experiment. PH was measured before replacing the culture test solution on the 3rd and 5th day, respectively. All the above operations should be operated under aseptic conditions to prevent culture medium pollution. The test period was seven days. After the experiment was over, the average specific growth rate μ of the blank control was calculated, and the growth inhibition percentage of each treatment group was also calculated.

Test Index

The number and growth condition of *Spirodela polyrhiza* in each treatment group were recorded every 2 days, and whether the culture medium was normal or not was also recorded. All clearly visible leaves should be counted. The increase of the number of *Spirodela polyrhiza* leaves indicated its growth, and the difference between each concentration group and the control group indicated the toxic effect.

Data Processing

The average specific growth rate (μ)

The average specific growth rate in a specific period is to calculate the growth variables (leaf number, total leaf area) during the logarithmic growth period, and the following formula is used to calculate each repetition of the control and treatment.

$$I = \frac{\mu_c - \mu_t}{} \times 100\%$$

μ_c

In this: I - Average specific growth inhibition rate, %;

μ_c - control group μ mean value;

μ_t - control group μ mean value

Results

In the process of effectiveness analysis, the solvent control group grew well and the solvent content was less than 100 μL/L; In addition, the pH variation range (0.6 - 1.2) was not more than 1.5 before and after the replacement of the *Spirodela polyrhiza* culture solution. The average specific growth rates of leaf bodies in each blank treatment group were calculated to be 0.294 d^{-1} and 0.317 d^{-1}, respectively, both > 0.275 d^{-1}. The average specific growth rate of leaves in each blank treatment group was 0.294 d^{-1} and 0.317 d^{-1}, respectively. The above test results meet the requirements of *Spirodela polyrhiza* growth inhibition test in OECD, and the test system is effective.

Effect of glyphosate on the growth of *Spirodela polyrhiza* can be seen from Figure 1. Within a certain range, the herbicide can inhibit the growth of *Spirodela polyrhiza*, and with the increase of the concentration of the test solutions, the inhibition effect is strengthened.

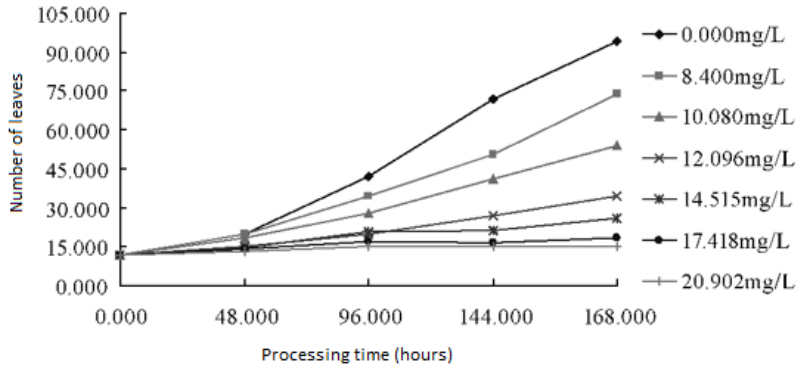

Figure 1: Inhibition of different concentrations of glyphosate on the growth of *Spirodela polyrhiza*

The growth inhibition rates of glyphosate on the leaves of *Spirodela polyrhiza* can be seen from Table 2. The coefficient of variation of each treatment group changed a bit, and the growth inhibition rates on the leaves of *Spirodela polyrhiza* showed significant differences at different concentrations of the test solution. Within a certain range, the growth inhibition rates on the leaves of the *Spirodela polyrhiza* increased with the increase of the concentration of the test solution.

Table 2: Inhibition rate of different concentrations of glyphosate on the growth of *Spirodela polyrhiza*

Treatment concentration (mg/L)	Coefficient of variation (%)	Inhibition rate of growth* I (%)
0.000	1.359	0.000 ± 0.231g
8.400	2.707	11.650 ± 0.406f
10.080	1.231	26.926 ± 0.153e
12.096	5.600	48.512 ± 0.489d
14.515	4.980	62.456 ± 0.317c
17.418	7.070	78.548 ± 0.257b
20.902	15.230	88.113 ± 0.307a

* indicates growth inhibition rate ± standard error. In the same column of data, the same letter indicates that there is no significant difference at 0.05 level (P = 0.05).

The EC_{50} of glyphosate on the leaves of duckweed was calculated by using "SPSS Statistics 17.0" software. The EC_{50}, 95% confidence interval and linear equation of glyphosate for *Spirodela polyrhiza* were calculated (Table 3). It can be seen from the correlation coefficient of the linear equation that the growth inhibition rate of the two herbicides on the *Spirodela polyrhiza* is a good linear relationship with the concentration of the test solution. The EC_{50} of glyphosate to the *Spirodela polyrhiza* was 12.817 mg/L.

Table 3: Inhibitory medium concentration of glyphosate

Test solution	EC_{50} (mg/L)	EC_{50} 95% confidence interval	Linear equation
Glyphosate	12.817	12.256 - 13.388	$y = 5.928x – 6.567$ $R^2 = 0.993$

Conclusion

The results showed that glyphosate had remarkable effects on the growth inhibition of *Spirodela polyrhiza*, and the inhibitory rate increased with higher concentrations. The 7 day-EC_{50} value was determined to be 12.817 mg/L.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effects of glyphosate to the aquatic macrophyte *Spirodela polyrhiza* was tested in a semi-static exposure of 7 days at concentrations between 8.4 and 20.902 mg/L. The 7 day-EC_{50} value was determined to be 12.817 mg/L.

This study was conducted to guideline but not to GLP. The test concentrations were not analytically verified and thus the exact exposure concentrations of the aquatic macrophyte are unknown. Therefore, the study should considered as reliable with restrictions.

1. Information on the study

Data point:	KCA 8.4.1 / KCA 8.4.2.1 / KCA 8.5
Report author	von Mérey G. *et al.*
Report year	2016
Report title	Glyphosate and aminomethylphosphonic acid chronic risk assessment for soil biota
Document No	Environmental Toxicology and Chemistry (2016), Vol. 35, No. 11, pp. 2742-2752
Guidelines followed in study	OECD 222; OECD 226; OECD 232; OECD 216
Deviations from current test guideline	Earthworm cocoons were not counted, in accordance with OECD 222.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable

2. Full summary of the study according to OECD format

The exposure risk from glyphosate and the primary soil metabolite aminomethylphosphonic acid (AMPA) on representative species of earthworms, springtails, and predatory soil mites and the effects on nitrogen-transformation processes by soil microorganisms were assessed under laboratory conditions based on internationally recognized guidelines. For earthworms, the reproductive no-observed-effect concentration (NOEC) was 472.8 mg glyphosate acid equivalent (a.e.)/kg dry soil, which was the highest concentration tested, and 198.1 mg/kg dry soil for AMPA. For predatory mites, the reproductive NOEC was 472.8 mg a.e./kg dry soil for glyphosate and 320 mg/kg dry soil for AMPA, the highest concentrations tested. For springtails, the reproductive NOEC was 472.8 mg a.e./kg dry soil for glyphosate and 315 mg/kg dry soil for AMPA, the highest concentrations tested. Soil nitrogen-transformation processes were unaffected by glyphosate and AMPA at 33.1 mg a.e./kg dry soil and 160 mg/kg dry soil, respectively. Comparison of these endpoints with worst-case soil concentrations expected for glyphosate (6.62 mg a.e./kg dry soil) and AMPA (6.18 mg/kg dry soil) for annual applications at the highest annual rate of 4.32 kg a.e./ha indicate very low likelihood of adverse effects on soil biota.

Materials and methods

Test substances

Glyphosate (N-phosphonomethylglycine) is an acidic substance, which is manufactured and formulated as a salt to increase the solubility in water and compatibility with other formulation components. In water, AMPA is highly soluble (56 g/L at 20 °C), whereas neither glyphosate nor AMPA is significantly soluble in common organic solvents. Therefore, no cosolvent was required, and both stock solutions of glyphosate and AMPA test items were prepared in deionized water (5 - 20 Mohm at 25 °C). Two batches of AMPA analytical reference standards with purity of 98.7% (synthesized by Chemir) and 99.7% (Acros Organics BVBA) were dissolved in deionized water. For soil nitrogen-transformation tests, stock solutions of glyphosate acid technical grade (96.59% purity; Monsanto Europe) were prepared by direct addition of test item to deionized water. For all other tests, glyphosate isopropylamine salt (nominal purity 62% w/w, measured purity 63.81 ± 0.29% w/w; MON 0139), corresponding to 45.9% w/w glyphosate a.e. (measured 47.28 ± 0.21% w/w; Monsanto Europe), were prepared in deionized water.

Earthworm reproduction tests

The earthworm reproduction test with glyphosate was conducted according to OECD guideline 222. For AMPA, an earthworm reproduction test was conducted according to the OECD 222. Both testing guidelines are equivalent in terms of the procedures employed during the tests (soil pH, temperature, lighting regime, soil composition and humidity, rearing, feeding quantities, test design, endpoints, number of replicates, growth stage of worms at test initiation, and so on). Therefore, to avoid repetition,

the procedures used in the glyphosate study only are described.

Glyphosate - Earthworm reproduction test. In the earthworm reproduction glyphosate study *Eisenia fetida* (Haplotaxida: Lumbricidae, Savigny, 1826) were used as the test species. Mature adult *E. fetida* (~3 mo old with clitellum), weighing between 300 mg and 600 mg, were obtained from an age-synchronized stock culture from the test facility and reared under ambient laboratory conditions in the test facility. The original breeding animals were purchased from W. Neudorff. A detailed description of earthworm culturing is provided in Annex 4 of OECD 222. The *E. fetida* were reared in the laboratory on standard breeding medium (1:1:1 mixture of straw, horse manure, and peat; straw and horse manure were purchased from farmers, and peat was purchased from Torfwerk Moorkultur Ramsloh); no exposure to the test item was allowed prior to use in testing. Testing was conducted in artificial soil, equivalent to the soil in which the worms were originally cultured. The test aims to evaluate effects on adult body weight and survival percentage (according to treatment) during an initial 4-wk adult exposure period. Effects on juvenile production were then assessed at the end of a 4-wk period that followed directly after adult removal from the test. Behavior (including feeding activity) and pathological symptoms (e.g., lethargy, morphological alterations) of adults and juveniles were also assessed.

On the day before the test start, earthworms (from aged-synchronized batches, to ensure that similar-sized earthworms were used) were acclimated to test conditions in a separate batch of artificial soil supplemented with pasteurized horse manure, purchased from farmers and collected from horses not treated with growth promoters, nematicides, or other veterinary products - also used as the food source during testing. On test start day, volumes of the test solution (prepared by direct addition of glyphosate isopropylamine to deionized water) were mixed into bulk samples of artificial soil, to achieve nominal glyphosate soil concentrations of 14.18 mg a.e./kg dry soil, 23.64 mg a.e./kg dry soil, 47.28 mg a.e./kg dry soil, 236.4 mg a.e./kg dry soil, and 472.8 mg a.e./kg dry soil. Glyphosate test concentrations were selected to cover the range and exceed field exposure concentrations. A toxic reference test was also performed in a separate test with carbendazim (Nutdazim 50 Flow, SC 500) at concentrations of 5 mg/kg dry soil and 10 mg/kg dry soil.

Test vessels were filled with the appropriate treated soil (810 g wet wt corresponding to 600 g dry wt). Groups of 10 individually weighed earthworms were randomly assigned to replicates within each treatment group, with a total of 40 earthworms used per treatment group divided equally between 4 replicates. For the control group (water only), 80 worms were used, divided equally between 8 replicates. Groups of 10 earthworms were placed onto the assigned replicate soil surface and closed with perforated transparent lids (following a brief burrowing period) to reduce evaporative water loss, allow gaseous exchange, and prevent worms from escaping the replicate vessels. Test vessels were then randomly positioned in an environmental test chamber under continuous light (to maintain worms in the soil). On day 1 and weekly thereafter for the 4-wk adult exposure period, 5 g of air-dried finely ground horse manure was scattered on the soil surface of each test vessel and wetted with 5mL of deionized water. The amount of manure applied each week (up to 5 g) was dictated by feeding activity.

After 4 wk, adult earthworms were removed from the vessels by emptying the contents of each replicate vessel onto a tray and removing the adult worms. Care was taken not to remove any cocoons from the soil. Cocoons were not counted, in accordance with OECD 222. It can be reasonably assumed that effects on cocoon numbers would lead to effects on numbers of juveniles; hence, the endpoint number of juveniles accounts for effects at earlier life stages of earthworm progeny. All worms were rinsed with deionized water and dried on filter paper before recording body weights (by replicate and by treatment). Behavioral (including feeding activity) and pathological symptoms were also recorded during the exposure period and at the time of adult removal. The adult worms were then discarded. The soil in each replicate vessel was then mixed carefully with 5 g of manure, and the mixture was returned to the vessels. The test continued for a further 4 wk. At test termination (8 wk after adult addition) the number of surviving juveniles in each test vessel was recorded on manual inspection of the substrate. Soil was emptied on the lower edge of a white tray (30 cm × 40 cm). Subportions of the soil were spread in the middle of the tray, resulting in a thin layer of soil of approximately 10 cm × 10 cm. The subportion was examined thoroughly for juvenile worms, after which it was moved to the upper edge of the tray. This procedure was repeated until the entire soil from a vessel was examined. The entire procedure was

repeated until there were no additional juvenile counts in 2 consecutive counting procedures, resulting in an average of 5 counting procedures per vessel. The counting tray and soil samples were illuminated using a fiber-optic light source connected with a double gooseneck light guide. The water content and pH of the artificial soil were determined. Adult body weights and the effects on reproduction (juvenile numbers) were analyzed using a lower-tailed Dunnett's multiple comparisons test ($\alpha = 0.05$). The Kolmogorov-Smirnov test and Cochran's test procedure were used, respectively, to test the biomass data for normality and homogeneity of variance. Survival was analyzed with a 1-sided Fisher's exact binomial test with Bonferroni correction ($\alpha = 0.05$).

AMPA - Earthworm reproduction tests. The procedures used during the AMPA earthworm study are considered equivalent to those employed in the glyphosate earthworm reproduction study described above in Glyphosate—Earthworm reproduction test. Mature adult *E. fetida* (~3mo old with clitellum), weighing between 300 mg and 600 mg, were obtained from an age-synchronized stock culture from the test facility and reared under ambient laboratory conditions in the test facility. A detailed description of earthworm culturing is provided in Annex 4 of OECD 222.

In the AMPA earthworm reproduction study, mature (clitellated) adult *E. fetida* were exposed to AMPA (99.7% purity; Acros Organics BVBA) mixed into artificial soil at nominal soil concentrations of 58.6 mg AMPA/kg dry soil, 87.8 mg AMPA/kg dry soil, 131.9 mg AMPA/kg dry soil, 198.1 mg AMPA/kg dry soil, 297.1 mg AMPA/kg dry soil, 445.5 mg AMPA/kg dry soil, 668.5 mg AMPA/kg dry soil, and 1002.5 mg AMPA/kg dry soil. A control group was prepared using deionized water only. A toxic reference test was also performed in parallel using earthworms from the same batch, exposed to carbendazim at concentrations of 1.0 mg active substance (a.s.)/kg dry soil, 2.2 mg a.s./kg dry soil, and 5.0 mg a.s./kg dry soil. For effects on biomass and production of juveniles, homogeneity was tested with the Brown-Forsythe and Bartlett tests. Dunnett's multiple comparison test was conducted using GraphPad Prism, Ver 6.03, because a continuous response could not be observed for all the test concentrations, as recommended by the OECD 222 test guideline and the OECD statistical guidance. The 50% effect rate on reproduction was calculated using GraphPad Prism.

Soil predatory mite reproduction test

The soil predatory mite reproduction tests for glyphosate and AMPA were both conducted according to OECD guideline 226 predatory mite (*Hypoaspis* [Geolaelaps] *aculeifer*) reproduction test in soil. The procedures used in the 2 studies were identical. Full details of the procedures are presented for glyphosate only. The *Hypoaspis* used in these studies were originally purchased from Katz Biotech and reared in the test facility under ambient conditions since June 2005.

Glyphosate - Soil predatory mite reproduction test. The glyphosate soil predatory mite reproduction test was conducted using glyphosate isopropylamine salt (MON 0139). Survival of mites (*H. aculeifer*) and their reproductive performance were evaluated at 4 nominal concentrations, equivalent to 50 mg MON 0139/kg dry soil, 100 mg MON 0139/kg dry soil, 500 mg MON 0139/kg dry soil, and 1000 mg MON 0139/kg dry soil (= 23.64 mg a.e./kg dry soil, 47.28 mg a.e./kg dry soil, 236.40 mg a.e./kg dry soil, and 472.80 mg a.e./kg dry soil, respectively). A negative control with deionized water only was also included. A toxic reference test was performed in parallel using dimethoate EC400 (422.4 g/L; Perfekthion) at concentrations of 4.1 mg active ingredient (a.i.)/kg dry soil, 5.12 mg a.i./kg dry soil, 6.4 mg a.i./kg dry soil, 8.0 mg a.i./kg dry soil, and 10 mg a.i./kg dry soil. Mites were reared in the laboratory under ambient conditions on a mixture of plaster of paris, activated charcoal, and deionized water (8:1:9). Adults with no more than a 3-d age difference were used at the start of the test. No exposure of the mites to glyphosate was allowed prior to the test. Each treatment group contained 40 mites divided equally between 4 replicate vessels, with the control group comprising 8 replicates, each containing 10 mites. In addition, 2 test vessels without mites were included with each test concentration and in the control group for soil pH measurements. Glass bottles (100mL nominal volume) with screw tops were filled with 20 g (dry wt) artificial soil at the required test concentrations. Cheese mites were added as a food source to the surface of the soil, and vessels were then covered to prevent mites from escaping. Bottles were opened every second day during the 14-d test for the addition of food and to allow aeration. At the end of the

test (day 14), the parental mites and juveniles were counted, after extraction using a MacFayden high-gradient extractor (heat/light extraction method). This was achieved by adding the soil substrate from each test vessel into a canister placed inverted onto the extraction system. Soil substrate was retained within the canister using a plastic net (2mm mesh size) on the bottom. Beneath the canister was a funnel attached to a collecting flask with 25mL of a fixing liquid. A temperature gradient was created between the upper and the lower parts of the system, by circulating heated air in the canister area and cooled air in the collection area. Over the 48-h extraction time, the following regime was applied: 25 °C for 12 h, 35 °C for 12 h, and 45 °C for 24 h. During this time, adults and juveniles moved down through the soil away from the heat source and fell through the funnel into the fixing liquid. Extraction efficiency was determined to be 95% in a separate extraction using vessels containing a known number of juvenile and adult mites in untreated substrate. Water content and pH were determined at test start and end. Statistical analysis was performed with the software ToxRat Professional 2.10. A 1-sided Fisher exact binomial test with Bonferroni-Holm correction for mortality and a 1-sided Dunnett multiple comparisons test for reproduction ($\alpha = 0.05$) were used to compare the control with independent test item groups. Abbott's formula was used to correct for control mortality.

AMPA - Soil predatory mite reproduction test. The soil predatory mite reproduction test with AMPA was conducted at 5 nominal application rates, equivalent to 40 mg test item/kg dry soil, 80 mg test item/kg dry soil, 160 mg test item/kg dry soil, 240 mg test item/kg dry soil, and 320 mg test item/kg dry soil. A negative control (deionized water only) group was also included. All procedures and observations in the test with AMPA were as described for the mite (OECD 226) test with glyphosate in Glyphosate—Soil predatory mite reproduction test. A reference test was performed with dimethoate EC400 (414.8 g/L) at test concentrations of 0 mg a.i./kg dry soil, 4.1 mg a.i./kg dry soil, 5.12 mg a.i./kg dry soil, 6.4 mg a.i./kg dry soil, 8.0 mg a.i./kg dry soil, and 10 mg a.i./kg dry soil.

Springtail reproduction tests

The springtail reproduction tests for glyphosate and AMPA were both conducted according to OECD guideline 232. The procedures used in the 2 studies were identical. Full details of the procedures are presented for glyphosate only. Springtails used in these studies were originally purchased from Biologische Bundesanstalt in May 2000 and reared in the laboratory of the test facility under ambient laboratory conditions.

Glyphosate - Springtail reproduction test. The springtail reproduction test conducted for glyphosate was conducted using glyphosate isopropylamine salt. Survival of springtails (*Folsomia candida*) and their reproductive performance were evaluated at 5 nominal application rates of 32 µL MON 0139/kg dry soil, 50 µL MON 0139/kg dry soil, 100 µL MON 0139/kg dry soil, 500 µL MON 0139/kg dry soil, and 1000 µL MON 0139/kg dry soil (= 15.1 mg a.e./kg dry soil, 23.6 mg a.e./kg dry soil, 47.3 mg a.e./kg dry soil, 236.4 mg a.e./kg dry soil, and 472.8 mg a.e./kg dry soil, respectively). A negative control with deionized water only was also included. In a reference toxicity test with Betosip (15.7% phenmedipham), concentrations of 50 mg/kg dry soil, 100 mg/kg dry soil, 200 mg/kg dry soil, and 400 mg/kg dry soil were tested. Each treatment group, including the control group, comprised 50 mites divided equally between 5 replicate vessels. For each treatment group and for the control group, 2 test vessels without springtails were provided for pH measurement purposes. Glass containers (150mL nominal volume) were filled with 30 g (wet wt) of the required treated or control soil. Springtails were reared in the laboratory under ambient conditions on a mixture of plaster for stucco, activated charcoal, and water (8:1:9). No exposure to the test item was allowed prior to testing. Juvenile springtails, 10 d to 12 d old and from a synchronized cohort, were added to each test vessel and then covered with a glass lid for 28 d, following which the surviving adults and juveniles were counted. Water content and pH were determined at test start and end. Adult and juvenile springtails were counted at test end. Statistical analysis was performed with the software ToxRat Professional 2.10. A 1-sided Fisher exact binomial test with Bonferroni correction ($\alpha = 0.05$) and Welch's t test ($\alpha = 0.05$), because of non-heterogeneity of variance, were used to compare the control with the independent test item groups for significance of parental mortality and reproductive reduction, respectively. Abbott's formula was used to correct for control mortality.

AMPA = Springtail reproduction test. The springtail reproductive test for AMPA was conducted with AMPA (98.7% purity) mixed into artificial soil at 5 nominal application rates, equivalent to 30 mg/kg dry soil, 54 mg/kg dry soil, 97.2 mg/kg dry soil, 175 mg/kg dry soil, and 315 mg/kg dry soil. The negative control used deionized water only. In a separate toxic reference test with 100% crystalline boric acid (BDH Prolabo) mixed with the soil, also included in the test design, the sensitivity of the population was determined with test concentrations of 0 mg/kg dry soil, 44 mg/kg dry soil, 67 mg/kg dry soil, 97.2 mg/kg dry soil, 150 mg/kg dry soil, and 225 mg/kg dry soil. The procedures used during the Springtail reproduction study were essentially equivalent to those used in the springtail test with glyphosate (described in Glyphosate - Springtail reproduction test) with the following exceptions. Each treatment group comprised 40 springtails (10 per test vessel), whereas the control group comprised 8 replicates. Statistical evaluation was performed with ToxRat Professional 2.10. A 1-sided Fisher exact binomial test with Bonferroni correction and a 1-sided Dunnett test were used to compare the control with independent test item groups. Mortality of adult springtails

Soil nitrogen-transformation tests

Soil nitrogen-transformation tests were conducted with glyphosate and AMPA according to OECD guideline 216 and performed according to good laboratory practice. The procedures used in the 2 tests were identical, although tested rates differed. Full details of procedures used are presented for glyphosate only.

Glyphosate - Soil nitrogen-transformation test. The soil nitrogen-transformation test for glyphosate was conducted using glyphosate acid (96.59% purity; Monsanto Europe) applied at 2 soil concentrations, 6.62 mg a.e./kg dry soil and 33.1 mg a.e./kg dry soil. The tested rates were equivalent to 1 and 5 times the maximum predicted environmental concentration in soil following a worst-case application of glyphosate to bare soil in the EU. Each treatment group and the control comprised 3 replicate test vessels. The control was treated with water only. Field-collected soil was used (LUFA standard soil, type 2.3). On collection, the soil was manually cleared of large objects, such as stones and parts of plants, and then moist-sieved to a particle size ≤ 2 mm. The soil was stored under aerobic conditions in the dark at 4 ± 2 °C until required for use.

Glyphosate was prepared in deionized water and then mixed into a bulk sample of soil at the start of the test. The soil moisture content was 40% (± 5%) of the maximum water holding capacity. During the test, the weight of a moisture control vessel maintained under the same test conditions was used as a guide to correct for test vessel water loss. Control and treated bulk samples of soil were amended with ground lucerne meal (0.5%) as a nitrogen source with a C to N ratio of 16:4:1. Bulk samples were then subsampled (~500 g) into replicate vessels and incubated at 20 ± 2 °C for 28 d. All containers were covered with a perforated lid to avoid evaporative water loss and stored in the dark. Soil (10 g) was taken from 1 replicate from each treatment for pH (water) determination at the start and end of the Glyphosate - Soil nitrogen-transformation study. An additional soil sample was taken from 1 replicate per treatment for moisture and dry matter content determination at the end of the study. As soon as possible after dosing (day 0) and after 7 d, 14 d, and 28 d, a 50-g soil sample (based on dry wt) was removed from each replicate to determine NH_4^+, NO_2^-, and NO^3. Soil extracts were prepared by adding 250mL of 2 M KCl, then shaking for 2 h and centrifuging for 15 min. The supernatant was analyzed using a Bran+Luebbe Autoanalyzer AA3 system. Effects below 25% deviation from control were not considered to be biologically significant.

AMPA - Soil nitrogen-transformation test. In the soil nitrogen-transformation test conducted for AMPA, the bulk samples of field-sampled soil were prepared at AMPA (98.7% purity) soil concentrations of 40 mg/kg dry soil, 80 mg/kg dry soil, 160 mg/kg dry soil, 320 mg/kg dry soil, and 640 mg/kg dry soil. In addition, a negative control (nontreated soil) was tested. Although conducting reference tests and use of positive controls are not guideline requirements, in a separate reference test with dinoterb (2-tert-butyl-4,6-dinitrophenol, 99.9% purity; Sigma-Aldrich Chemie), test concentrations of 6.8 mg/kg dry soil, 16 mg/kg dry soil, and 27 mg/kg dry soil were applied, in addition to the control (0 mg/kg dry soil), with 3 replicates per treatment group.

Results

Glyphosate - Earthworm reproduction test

There was 0% mortality of adult *E. fetida* at glyphosate concentrations of 14.18 mg a.e./kg dry soil, 236.4 mg a.e./kg dry soil, and 472.8 mg a.e./kg dry soil. Mortality (2.5%) was observed at 23.64 mg a.e./kg dry soil and 47.28 mg a.e./kg dry soil, which is considered incidental background mortality given the 10% validity criterion for adult mortality in the control ($p > 0.3$). No statistically significant differences were detected for adult biomass ($p > 0.05$; Figure 1A) and for the numbers of juveniles produced at each of the treatment groups when compared to the control ($p > 0.05$; Figure 1A). Adult and juvenile feeding behavior was also not adversely affected over the duration of the test (56 d). The resulting no-observed-effect concentration (NOEC) for effects on reproduction was determined therefore to be the maximum test concentration of 472.8 mg a.e./kg dry soil.

In the reference test with carbendazim, juveniles were reduced by 65% and 92% at 5 mg reference item/kg dry soil and 10 mg reference item/kg dry soil, respectively. The control treatment had a mean number of 143 juveniles, whereas 5 mg/kg dry soil and 10 mg/kg dry soil treatments with carbendazim had a mean number of 51 juveniles and 11 juveniles, respectively. These reference test values show that the test system was appropriate to detect toxic effects on earthworm reproduction. The validity criteria, namely adult mortality < 20% and number of juveniles per replicate > 30 in the control treatment, and coefficient of variance between control replicates < 30% were all met. The guideline requirements for water content, temperature, and pH were all met.

AMPA - Earthworm reproduction test

In the earthworm reproduction study with AMPA, there were no significant effects on *E. fetida* adult mortality across concentrations compared to the control ($p > 0.22$). In all treatment groups, all 10 adults survived the treatments, except for 1 mortality in a single replicate of the 668.5 mg/kg dry soil treatment (Figure 1B). Adult earthworm biomass was significantly lower compared to the control at the 445.5 mg AMPA/kg dry soil, 668.5 mg AMPA/kg dry soil, and 1002.5 mg AMPA/kg dry soil test concentrations ($p < 0.0001$; Figure 1B). Adult biomass at 198.1 mg AMPA/kg dry soil was also significantly lower than the control ($p = 0.007$), but at 297.1 mg AMPA/kg dry soil there was no significant difference ($p > 0.802$) because the biomass (in percentage of control) was 88.5% and 88.2% in the 131.9 mg AMPA/kg dry soil and the 297.1 mg AMPA/kg dry soil treatment groups, respectively. The effect at 198.1 mg AMPA/kg dry soil is therefore considered to not be treatment-related. Juvenile production was not significantly affected at concentrations up to 198.1 mg/kg dry soil ($p > 0.342$). At 297.1 mg AMPA/kg dry soil and higher concentrations juvenile *E. fetida* numbers decreased significantly compared to the control ($p = 0.0013$). The resulting NOEC for effects on reproduction therefore was concluded to be 198.1 mg/kg dry soil, with a reproductive lowest-observed effect concentration (LOEC) at 297.1 mg AMPA/kg dry soil. The calculated 50% effective concentration (EC50) value for AMPA on earthworm survival was > 1000 mg/kg dry soil. The reproduction EC50 value was calculated at 654.7 mg AMPA/kg dry soil (95% confidence interval 610.9 - 705.5 mg/kg dry soil). The resulting regression equation was $y = - 0.1108 (\pm 0.005)$ AMPA mg/kg $+ 122.6 (\pm 2.271)$, with an R^2 of 0.92. The reference test item carbendazim resulted in decreased biomass of 33.3% at 5.0 mg/kg dry soil and no reproduction, showing that the test system was sensitive to pesticide application. The validity criteria and guideline requirements were all met.

Glyphosate - Soil predatory mite reproduction test

No significant effects were observed on soil mite survival ($p > 0.3$) or reproduction ($p > 0.05$) up to and including the highest test concentration (472.8 mg a.e./kg dry soil; Figure 1C) after 14 d of continuous exposure. All validity criteria and guideline recommendations were met. In the reference test with dimethoate the EC50 on reproduction was determined to be 4.9 mg a.i./kg dry soil. The reference test demonstrated the sensitivity of the test system to detect reproductive toxicity in soil mites. The NOEC was therefore set at the highest test concentration.

AMPA - Soil predatory mite reproduction test

No significant effects were observed on soil mite survival ($p > 0.1$) or reproduction ($p > 0.05$) up to and including the highest test concentration (320 mg AMPA/kg dry soil; Figure 1D). All validity criteria and guideline recommendations were met. The reference test with dimethoate showed that the test was sensitive at detecting reproductive toxicity in soil mites. The NOEC for AMPA was therefore concluded to be at the highest test concentration, 320 mg/kg dry soil.

Glyphosate - Springtail reproduction test

No significant effects were observed on springtail survival ($p > 0.5$) or reproduction ($p > 0.05$) up to and including the highest test concentration (472.8 mg a.e./kg dry soil; Figure 1E). The validity criteria and guideline recommendations were all met. In the reference test with phenmedipham, the EC50 on reproduction was determined to be 28.4 mg phenmedipham/kg dry soil, which demonstrates that the test system was sensitive for reproductive toxicity. The NOEC for glyphosate was therefore concluded to be the highest test concentration.

AMPA - Springtail reproduction test

No significant effects were observed on springtail survival ($p > 0.5$) or reproduction ($p > 0.06$, $\alpha = 0.01$) up to and including the highest test concentration (315 mg AMPA/kg dry soil; Figure 1F). The validity criteria and the guideline recommendations were all met. In the reference test with boric acid, the EC50 for reproduction was determined to be 108.6 mg/kg dry soil, demonstrating sensitivity to reproductive toxicity of the test system. The NOEC for AMPA was therefore concluded to be the highest test concentration.

Glyphosate - Soil nitrogen transformation test

Nitrogen-transformation rates in the soil treated at glyphosate rates equivalent to 6.62 mg a.e./kg dry soil and 33.1 mg a.e./kg dry soil were - 0.13% and 2.13% different compared to the control between day 14 and day 28, respectively (Figure 1G). The validity criterion of < 15% variation between control treatments was met in the test. As the rates of nitrate formation between the control and the treated groups were < 25% on day 28, glyphosate can be evaluated as having no long-term influence on nitrogen transformation in LUFA soils at concentrations ≤ 33.1 mg a.e./kg dry soil. No reference test was conducted, in line with the OECD guideline.

AMPA - Soil nitrogen-transformation test

Stimulation of nitrogen-transformation rates was observed across all treatments on day 7 and day 14, which was possibly linked to the high levels of nitrogen and phosphorus released from the degradation of AMPA in the biologically active soil. Only in the 2 highest test concentrations did the increase exceed 25% compared to the control at 28 d. The test was therefore prolonged from 28 d to 56 d for the 2 highest test concentrations, 320 mg/kg dry soil and 640 mg/kg dry soil (Figure 1H). At 56 d, the deviation from the control was 26.7% at 320 mg/kg dry soil and 43.1% at 640 mg/kg dry soil. The reference test results with dinoterb showed increases of 37.6% at 6.8 mg/kg dry soil, 51.4% at 16.00 mg/kg dry soil, and 27.1% at 27 mg/kg dry soil. The validity criterion of < 15% variation between controls was met at all sampling intervals.

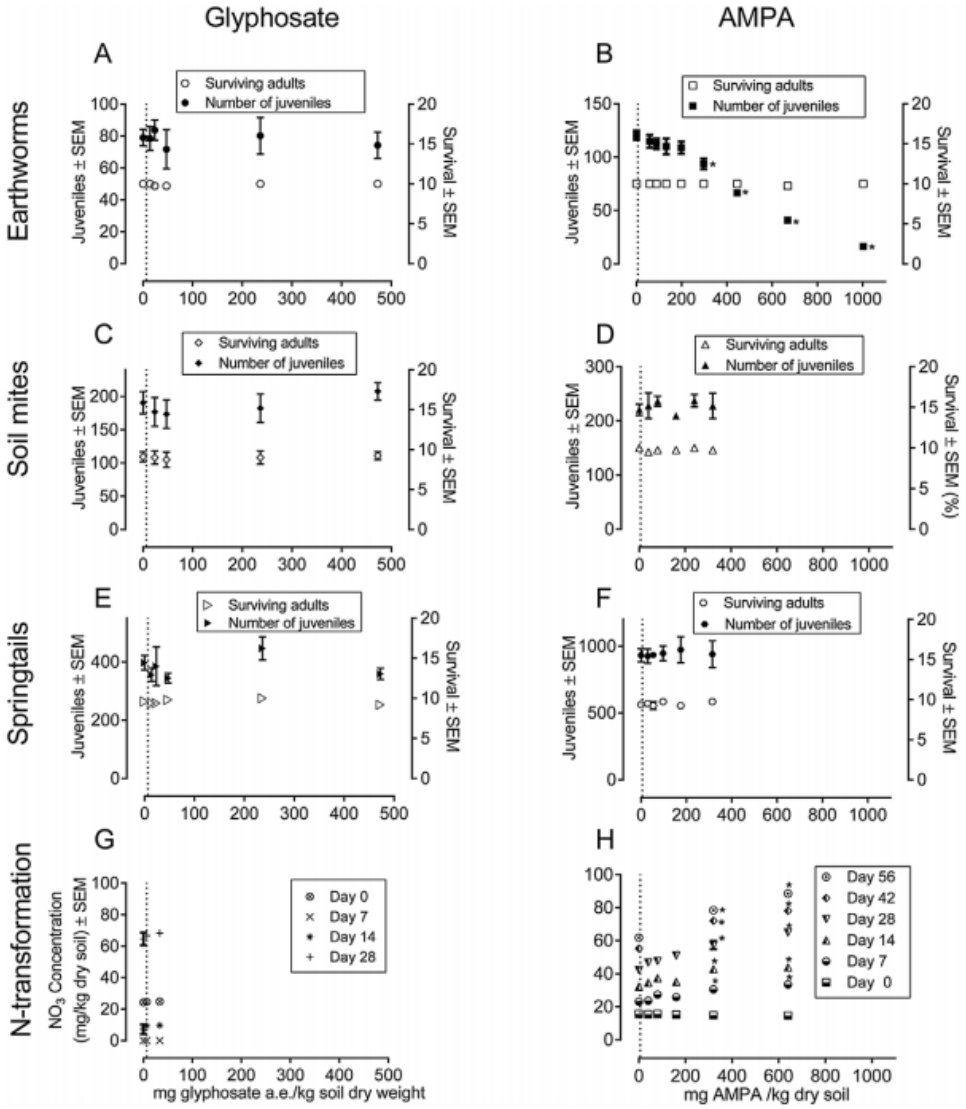

Fig. 1. Chronic risk assessment for glyphosate (left) and aminomethylphosphonic acid (AMPA; right) in soil. Number of surviving adults (28d) and number of juveniles (56d) in earthworms exposed to glyphosate (A) and AMPA (B), (* statistically significant effect [$p \leq 0.05$] compared with control treatment), in soil predatory mites (*Hypoaspis aculeifer*) exposed to glyphosate (C) and AMPA (D) for 14d, and in springtails (*Folsomia candida*) exposed to glyphosate (E) and AMPA (F) for 28 d. Effects on nitrogen transformation in soil treated with glyphosate (G) and AMPA (H) for 0 d, 7 d, 14 d,28 d, 42 d, and56 d(* > 25% effect compared with control treatment). Vertical dotted line in each graph indicates the worst-case predicted environmental concentration of glyphosate/AMPA. Vertical bars indicate standard error of the mean (SEM).

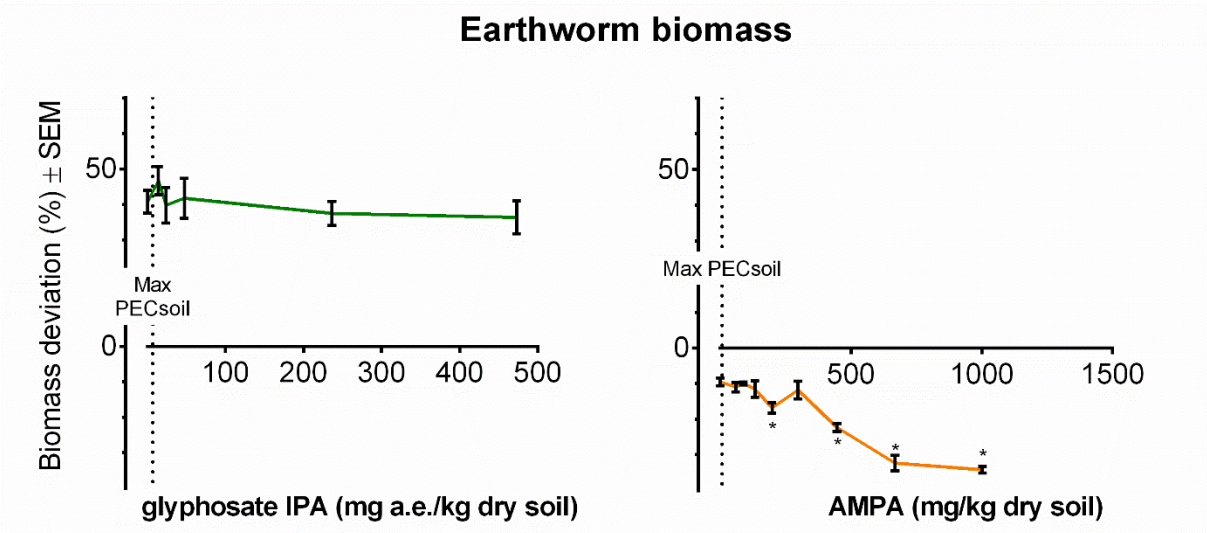

Fig. 2. Effects of glyphosate (A) and AMPA (B) on earthworm biomass after 28 d of exposure. The vertical dotted line indicates the predicted environmental concentration for AMPA and glyphosate. An asterisk next to a data point indicates a significant difference ($P < 0.05$) when compared against the control treatment.

Risk assessment

The chronic effects of exposure to glyphosate and the major soil metabolite AMPA to representative taxonomic groups of soil macroorganisms and nitrogen transformation were assessed following standard practices outlined under Annex VI Uniform Principles of the EU's Plant Protection Products Regulation (EC) No 1107/2009. At soil concentrations relevant to recommended glyphosate field application rates, no significant adverse effects were observed in any of the test species or systems exposed to glyphosate or AMPA. The risk assessment for soil macroorganisms in the EU compares the lowest NOEC achieved for each of the taxonomic groups with worst-case initial predicted soil concentrations (soil $PEC_{initial}$) achieved directly following a bare soil application and the potential for accumulation in soil following applications over multiple years to the same field (soil PEC_{accu}) The ratio of the endpoint to the predicted soil concentration is determined (toxicity exposure ratio = NOEC - $PEC_{initial}$) and compared against trigger values in accordance with Annex VI Uniform Principles of the EU's Plant Protection Products Regulation 1107/2009. Where trigger values are exceeded, a low exposure risk may be concluded. The long-term trigger value of 5 using NOECs derived from laboratory tests accounts for uncertainty related to interspecies sensitivity, predicted exposure estimates, and extrapolation from laboratory to field exposure.

For glyphosate and AMPA, the initial soil concentration ($PEC_{initial}$) at a soil depth of 5 cm has been determined based on a bare soil application (without foliar/crop interception), at the maximum cumulative annual application rate of 4.32 kg glyphosate a.e./ha for the EU. The risk of glyphosate and AMPA residues accumulating in soil over multiple years is considered by deriving the PEC_{accu} value. This is the sum of the $PEC_{initial}$ and plateau concentrations in soil, achieved in the top 5 cm (tillage depth for permanent crops) soil layer, following applications to bare soil at the maximum cumulative annual application rate (4.32 kg a.e./ha) each year for 10 yr.

It is important to mention that a single application rate of 4.32 kg glyphosate a.e./ha is not supported in the representative use rate but rather represents the recommended maximum cumulative (total) annual application rate for all uses and, therefore, a very conservative worst-case approach.

For exposure of soil mites, springtails, and earthworms to glyphosate in soil, the achieved chronic endpoints exceed the worst-case predicted glyphosate $PEC_{initial}$ and PEC_{accu} soil concentration by factors of 82 and 71, respectively.

For exposure of soil mites, springtails, and earthworms to AMPA in soil, the achieved chronic endpoints exceed worst-case AMPA $PEC_{initial}$ soil concentrations by factors of between 97 and 491, whereas the chronic endpoints exceed the PEC_{accu} soil concentrations by factors of between 32 and 162.

For soil nitrogen transformation, the endpoints achieved for glyphosate and AMPA (33.1 mg a.e./kg dry soil [glyphosate] and 160 mg a.e./kg dry soil [AMPA]) both achieved a < 25% effect on nitrogen-transformation rates following a 28-d soil exposure to either glyphosate or AMPA. These soil exposure rates exceed the worst-case predicted $PEC_{initial}$ soil concentrations by factors of 6 (glyphosate) and 78 (AMPA). The achieved endpoints also exceed the PEC_{accu} soil concentrations, by factors of 5 for glyphosate and 26 for AMPA.

For the soil mite, springtail, and earthworm reproduction chronic endpoints, the toxicity exposure ratio values exceed the EU Regulation No 546/2011 Annex VI trigger (5), indicating that for the ecotoxicologically relevant endpoints achieved for survival and reproduction, the use of glyphosate according to label recommendations is unlikely to result in adverse effects inside the treated area for soil biota - from exposure to both glyphosate and AMPA.

For the soil microbial community, relative to expected field application rates for exposure to glyphosate there is at least a 5-fold safety margin. For exposure to AMPA, a 26-fold safety margin applies. The observed increases in nitrate concentrations at the higher test concentrations are expected to be related to the large quantity of nitrogen and phosphate provided to the microbes via degradation of AMPA in the biologically active soil.

Table 1. Glyphosate and aminomethylphosphonic acid chronic risk assessment for soil organisms[a]

Test species	Test item	Test duration (d)	Endpoint type	NOEC (mg a.e. or AMPA/kg soil)	$PEC_{initial}$ (mg a.e./kg soil)	PEC_{accu} (mg a.e./kg soil)	$TER_{initial}$	TER_{accu}
Earthworm	Glyphosate IPA salt	56	Adult mortality	472.8	5.76	6.62	82	71
			Biomass	472.8			82	71
			Reproduction	472.8			82	71
	AMPA	56	Adult mortality	1002.5	2.04	6.18	491	162
			Biomass	297.1			146	48
			Reproduction	198.1			97	32
Soil mite	Glyphosate IPA salt	14	Adult mortality	472.8	5.76	6.62	82	71
			Reproduction	472.8			82	71
	AMPA	14	Adult mortality	320	2.04	6.18	157	52
			Reproduction	320			157	52
Springtail	Glyphosate IPA salt	28	Adult mortality	472.8	5.76	6.62	82	71
			Biomass	472.8			82	71
	AMPA	28	Adult mortality	315	2.04	6.18	154	51
			Biomass	315			154	51
N-transformation	Glyphosate acid	28	Effect <25%	33.1	5.76	6.62	6	5
	AMPA	28	Effect <25%	160	2.04	6.18	78	26

a.e. = acid equivalent; AMPA = aminomethylphosphonic acid; IPA = isopropylamine; NOEC = no-observed-effect concentration; PEC_{accu} = accumulative predicted environmental concentration, cumulative worst-case application of 4.32 kg a.e./ha of glyphosate for 10 yr; $PEC_{initial}$ = initial predicted environmental concentration, assuming single worst-case application of 4.32 kg a.e./ha of glyphosate; TER_{accu} = toxicity to exposure ratio (= NOEC - PEC_{accu}); $TER_{initial}$ = toxicity to exposure ratio (= NOEC - $PEC_{initial}$).

Conclusion

The risks from exposure to glyphosate and the primary soil metabolite AMPA at levels that exceed commercial application rates were evaluated against a battery of representative soil macroorganisms and microorganisms under controlled laboratory conditions. Results from the present studies demonstrate that the potential impact to beneficial soil macro-organisms and nutrient cycling soil microorganisms under environmentally relevant exposure scenarios is low.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The aim of the paper was to evaluate potential effects of Glyphosate, Glyphosate salt and AMPA on earthworm, soil mites, springtails and soil micro-organisms.

The studies have been conducted according to recognised guidelines and validity criteria were presented. Test substance information, test organism origin, study designs and toxicity effects were adequately described. The study is considered reliable.