Bulletin of Environmental Contamination and Toxicology (2020) 104:173–179
https://doi.org/10.1007/s00128-019-02774-z

Developmental and lethal effects of glyphosate and a glyphosate-based product on *Xenopus laevis* embryos and tadpoles

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Received: 17 October 2019 / Accepted: 14 December 2019 / Published online: 13 January 2020

Abstract
Effects of pure glyphosate and a glyphosate-based product were evaluated comparatively using two embryonic development stages of *Xenopus laevis* as model system. When pure glyphosate was applied in pH adjusted media, lethal or developmental effects were not observed at concentrations up to 500 mg L^{-1}. The 96 h LC_{50} values for the commercial herbicide, in contrast, were 32.1 and 35.1 mg active ingredient L^{-1} for embryos and tadpoles, respectively. Since pure glyphosate has no effect on the selected biomarkers, it is thought that developmental toxic effects caused by glyphosate-based products are increased mainly due to formulation additives.

Keywords *Xenopus laevis* · Glyphosate · Roundup · Toxicity

Glyphosate (GLY) is an extensively used herbicide around the world and is application increased more than 12 times to 826 million kg between 1995 and 2014 (Benbrook 2016). The main target of GLY is to inhibit 5-enolpyruvylshikimate-3-phosphate phosphate synthase, the key enzyme in aromatic amino acid synthesis pathways in plants. The number of toxicity studies on GLY or products containing GLY also continues to increase due to differences in the composition of commercial formulations and variability in effects among test species (Lajmanovich et al. 2011; Iummato et al. 2018; Samanta et al. 2014). Moreover, GLY products are one suggested cause of amphibian population declines similar to some other pesticides (Relyea, 2011). At the same time, amphibians represent one of the most threatened and rapidly declining group of vertebrates worldwide. The International Union for Conservation of Nature (IUCN) database reports, for instance, that 32% of the 6771 amphibian species worldwide are considered endangered or vulnerable (IUCN 2019). Consequently, several studies evaluate effects of commercial herbicides such as glyphosate-based products (GBPs) on amphibian species (Bonfanti et al. 2018; Carvalho et al. 2019; Edginton et al. 2004; Güngördü 2013; Güngördü et al. 2016; Mann and Bidwell 1999; Moore et al. 2012; Relyea and Jones 2009; Smith 2001; Wagner et al. 2013). GLY is the active ingredient (AI) of more than 750 different GBPs formulated with various adjuvants (Guyton et al. 2015; Li et al. 2005). Among all GBP, Roundup® is the original GBP introduced by Monsanto in 1974. It is still one of the most widely used formulation (Monsanto 2019; Szekacs and Darvas 2018). Roundup® contains glyphosate isopropylamine salt in combination with the surfactant polyoxyethylene tallow amine (POEA). POEA is added to assist GLY penetration into plant surfaces and thereby improve its effectiveness (Williams et al. 2000). In animal species, it is believed that POEA disrupts cell membranes on respiratory surfaces (Brausch and Smith 2007).

Most studies on GBP toxicity to aquatic organisms, focused on lethal effects (Tush et al. 2013, 2018; Lajmanovich et al. 2011). In relatively few studies, pure GLY showed low toxicity to non-target species in the ecosystem (Benbrook 2019). The World Health Organization (WHO) database reports low aquatic toxicity of GLY. However, the toxicity of Roundup® is considered to be a consequence of the surfactant additive POEA (Hong et al. 2018). Therefore, studies have reported that various additives may cause different levels of toxicity of GBPs (Edge et al. 2014; Mann and Bidwell 1999). Moreover, commercial GBPs disrupt

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various biochemical processes, including respiration, protein and nucleic acid synthesis, and may cause genotoxicity (Gill et al. 2018; Yanniccari et al. 2012). GBPs may affect various non-target aquatic organisms, including invertebrates, fish, and amphibians (Hansen and Roslev 2016; Lajmanovich et al. 2013; Pereira et al. 2018; Silveira et al. 2019; Wagner et al. 2013). Many studies reported GLY to be responsible in the adverse effects on non-target organisms caused by GBPs. However, comparative assessments of effects caused by GLY and its different formulations is limited (Babalola et al. 2019; Bonfanti et al. 2018; Lanzarin et al. 2019). For example, while discussing the lethal effects of GLY and commercial GBP on fish and amphibians, evaluation of its effects on biochemical markers have not been adequately assessed. Perez et al. (2011) report that LC50 values of pure GLY, Roundup®, and POEA were 140, 8.3, and 2.0 L^{-1} for *Oncorhynchus mykiss*, respectively. Moreover, GBPs cause serious deterioration of the health of aquatic organisms with significant changes in biomarker (AChE, catalase, and GST) (Samanta et al. 2014). However, there is still uncertainty about the extent GLY contributes to GBH toxicity.

To address this gap amphibians were selected as test species, as they go through clear and distinct stages (Walsh et al. 2008) allowing to assess for age dependent effects. The latter is particularly relevant as different developmental stages of *Xenopus laevis* show different sensitivity to pollutants (Fort et al. 2004). Along this line, the aim of this study was to evaluate potential adverse effects of pure GLY and one of the widely used GBP on *X. laevis* from different developmental stages. Consequently, mortality, growth inhibition, and biomarker activity (metabolic, detoxification, and oxidative stress) were used as endpoints.

Materials and Methods

The commercial GBP (Roundup® Star, Belgium) was purchased from a local agricultural retailer. According to the information in the safety data sheet, 35.5% of its content is the potassium salt of GLY, 6% is ether alkylamine ethoxylate, and the remaining 58.5% are water and minor formulating ingredients. The GLY (*N*-(phosphonomethyl) glycine) concentration in the tested GBP is declared at 441 g L^{-1}. The density of tested GBP is 1.25 kg L^{-1} at 20 °C. GLY as pure active ingredient was purchased from Sigma-Aldrich (PESTANAL®, analytic grade, 45521).

The embryos and tadpoles used in the tests were obtained from male and female frog pairs from an adult *X. laevis* colony in our laboratory. *X. laevis* breeding and acquisition of embryos were performed according to ASTM-E1439-98 (ASTM 2003). All amphibian eggs, tadpoles, and adults were handled and cared for following animal use protocols reviewed and approved by Inonu University Research Animals Ethics Committee (Research Protocol No. 2013/A-44, 22 May 2013). Embryos and tadpoles were maintained in a standard Frog Embryo Teratogenesis Assay *Xenopus* (FETAX) test medium (ASTM 2003). Composition of the FETAX test medium was: 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4{\cdot}2H_2O$, 30 mg KCl, and 15 mg $CaCl_2$ per liter of distilled water.

Both exposure solutions of GLY and GBP were prepared fresh daily in the standard FETAX test medium (ASTM 2003). In order to perform the comparative experiments, the pH of GLY and GBP solutions were adjusted to 7.9, as recommended for FETAX tests, using NaOH (ASTM 2003). Embryos and tadpoles were exposed to test solutions under semi-static test conditions with a 12:12 h light:dark photoperiod at 23 °C (± 1 °C).

Before starting the FETAX test, glyphosate levels in the test medium were measured using high-performance liquid chromatography (HPLC) (1100 system, Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector and an auto-sampler. A reversed phase C18 column (5 µm, 4.6 mm internal diameter, and 250 mm length) was used. Ten microliters of the sample was injected. The column was eluted with a mobile phase of 10% acetonitrile (v/v) and 90% water (containing 0.1% TEA), with pH adjusted to 2.3 with phosphoric acid, and a flow rate of 1 mL min^{-1}. GLY was determined at 196 nm and was quantified using a standard curve (12.5–500 mg L^{-1}, $R^2 = 0.998$). The measured glyphosate concentrations in both GLY and GBH exposure media were determined to be at least 92% of the nominal concentrations (Table 1).

For the FETAX test, stage8 embryos were exposed to different GLY and GBP concentrations for 96 h. Four embryos were randomly selected, and placed with 2 mL of test medium into each well of 24-well plates, serving as one replicate per treatment. All concentrations were tested with eight replicates and thus a total of 32 embryos. In the FETAX test, seven concentrations of GLY (282–500 mg L^{-1}) and six

Table 1 Measured glyphosate concentrations in exposure water after HPLC analysis

GLY (mg L^{-1})		GBP (mg AI L^{-1})	
Nominal conc.	Measured conc.[a]	Nominal conc.	Measured conc.[a]
282	291.5 ± 7.96	31.0	32.2 ± 1.15
310	329.6 ± 25.73	34.2	33.7 ± 0.62
342	339.4 ± 12.93	37.6	35.6 ± 2.83
376	362.6 ± 30.12	41.3	40.3 ± 3.33
413	394.5 ± 36.09	45.5	41.9 ± 5.39
455	448.8 ± 22.16	50.0	47.6 ± 6.48
500	490.7 ± 12.28	-	-

[a]The measured concentrations are representing the mean ± standard deviation of two replicates

concentrations of GBP (31–50 mg AI L^{-1}) were tested plus controls. The test medium was changed every 24 h. Dead tadpoles were removed and incidences were recorded. The median lethal concentrations (LC_{50}) were determined after 24, 48, 72, and 96 h of exposure. At termination of the bioassays, surviving embryos were euthanized with 200 mg L^{-1} of tricaine methanesulfonate (MS222, suitably buffered with sodium bicarbonate) and fixed in 3% formalin for observation of developmental anomalies. Malformed embryos and malformation types were determined using a dissection microscope as suggested by Bantle (1995). Body length was measured using Euromex ImageFocus 4.0 software.

For the tadpole-toxicity bioassays, stage 46 tadpoles were exposed to different GLY and GBP concentrations for 96 h. Five randomly selected tadpoles were placed in each well of 12-well plates containing 3 mL of test solution. All concentrations were tested with 6 replicates resulting in a total of 30 tadpoles per concentration. In the tadpole-toxicity tests, seven concentrations of GLY (250–403 mg L^{-1}) and six concentrations of GBP (31–50 mg AI L^{-1}) were tested excluding control groups. For all other procedures the embryotoxicity assays procedures were followed.

For biochemical assays, stage 46 tadpoles were exposed to three GLY (3.5–17.6 mg L^{-1}) and GBP (50–250 mg AI L^{-1}) concentrations for an exposure period of 96 h. Fifteen randomly selected tadpoles and 10 mL of test solution were placed into 25 mL polycarbonate containers. All concentrations were tested with five replicates resulting in a total of 75 tadpoles per treatment. At the end of the exposure period, surviving tadpoles were euthanized by MS222 and placed in microcentrifuge tubes as soon as possible. Each vial was cooled on ice and tadpoles were stored at − 80 °C until enzyme activity assays.

Frozen tadpoles were thawed on ice and weighed before biochemical studies. All homogenization, centrifugation, and biochemical measurements followed protocols reviewed by Güngördü et al. (2016). The tadpoles were homogenized in ice-cold buffer [0.1 M K-phosphate buffer (pH 7.4) with 0.15 M KCl, 1 mM EDTA, and 1 mM DTT]. The homogenate was centrifuged at 16,000×*g* for 20 min at 4 °C. The supernatant was transferred to a clean microcentrifuge tube. The activities of enzymes were measured as soon as possible after collecting postmitochondrial supernatant without freezing. The samples were maintained on ice during analyses. Enzyme activity was determined spectrophotometrically using a microplate reader at appropriate wavelengths (VersaMax, Molecular Devices Corp., USA). The enzyme activities were then normalized to the organisms protein content.

Glutathione *S*-transferase (GST) activity was measured in the supernatant according to Habig et al. (1974). Glutathione reductase (GR) activity was measured following Stephensen et al. (2000). Carboxylesterase (CaE) activity was assayed using the procedure of Santhoshkumar and Shivanandappa (1999). Acetylcholinesterase (AChE) activity was measured following Ellman et al. (1961). All procedures were modified for a microplate reader system. Superoxide dismutase (SOD) activity was measured using a SOD Assay Kit (Sigma-Aldrich 19160, St. Louis, MO, USA) following the manufacturer's guidelines. Total protein concentration in the supernatant was measured using the Bradford method with bovine serum albumin (BSA) as standard (Bradford 1976). The protein content values were used to calculate the specific activities of each tested enzyme.

Graphpad Prism software (Version 5, USA) was used to calculate the average lethal concentration (LC_{50}) and 95% confidence intervals (CI) and for other statistical analyses. A log(dose)-normalized response curve ($Y = 100/(1 + 10^{(\log EC50 - x)} \times \text{hillslope})$ to fit mortality data. For statistical analysis of biomarkers, data were tested initially for homogeneity of variances and normality distributions by the Bartlett and Kolmogorov–Smirnov tests, respectively. Nonparametric data were analyzed using Kruskal–Wallis test followed by pairwise comparisons of groups using Mann–Whitney *U* tests. Parametric data were analyzed using the One-way Analysis of Variance (ANOVA) followed by the unpaired *t* test. A Bonferroni correction was applied ($0.05/3 = 0.016$). In order to determine growth inhibition, the head-to-tail lengths were measured and the lengths were compared using ANOVA (Dunnett's post hoc test, $p < 0.05$).

Results and Discussion

The LC_{50}s of different GBPs for frog larvae of 37 species were reported in the range from 0.2 to 494 mg AI L^{-1} (Wagner et al. 2013). These differences in toxicity may be attributed to the type of adjuvants (surfactants, solvents, and antifoaming compounds) present in different commercial formulations. This may be assumed as adjuvants may actually be more toxic than the active ingredient GLY. It has, moreover, been suggested that the toxicity by GBPs may be the result of synergistic effects between glyphosate and adjuvants (Cattani et al. 2014; Jacques et al. 2019). However, the contribution of GLY to the toxicity of GBPs has not yet been assessed comprehensively, likely due to a large number of different surfactants (Edge et al. 2014). We tested Roundup® containing alkylamine ethoxylate and POEA as a surfactant (Brausch and Smith 2007). POEA increases membrane permeability of cells and therefore allows increased absorption of the GLY (Giesy et al. 2000). Perkins et al. (2000) reported POEA being more toxic than Roundup® for *X. laevis* embryos (9.3 mg AI L^{-1}, LC_{50}). Our results also showed that the 96 h LC_{50} values of the GBP were 32.1 and 35.1 mg AI L^{-1} for embryos and stage 46 tadpoles, respectively (Table 2). Please note that one of the developmental stages tested in this study is the early embryonic stage

Table 2 The calculated LC_{50} values of GBP for *Xenopus laevis* at stage 8 embryos and stage 46 tadpoles after toxicity test according to measured concentrations

	Test range	LC_{50} (mg AI L^{-1})			
		24-h	48-h	72-h	96-h
8th stage	32.2–47.6		37.8 (36.8–39.0)	33.2 (32.4–33.8)	32.1 (30.9–32.6)
46th stage	32.2–47.6	45.1 (43.7–47.3)	37.6 (36.8–38.5)	36.3 (35.6–37.5)	35.1 (34.7–35.8)

Values in parentheses represent 95% confidence intervals

including gastrulation, neurulation and organogenesis, and the second was the premetamorphic tadpole stage. Consequently, the fact that similar LC_{50} values among developmental stages were reported here in combination with the absence of statistically significant malformations suggests that the toxicity is due to more general mechanisms such as oxidative stress rather than teratogenic effects. On the other hand, the calculated LC_{50} values in this study were relatively high compared to literature, even compared with a previous study that we conducted with the same GBP and *X. laevis* (the 96 h LC_{50} was 15 mg AI L^{-1}, Güngördü 2013). This may be related to the variability of the active substances and adjuvants present in commercial pesticide formulations. Nonetheless, even the highest GLY concentrations did not cause a lethality higher than 17% for both *X. laevis* embryos and tadpoles in this study suggesting a significant role of surfactants for GBP toxicity.

Moreover, the highest GLY concentrations (403 and 500 mg L^{-1}) caused no growth inhibition in embryos or tadpoles. However, nearly 15-fold lower concentrations of GBP resulted in significant inhibition of embryonic growth (Table 3). In contrast, another study reported both GLY (3–100 mg L^{-1}) and GBP (0.37–5.25 mg AI L^{-1}, Roundup Ultramax®) causing growth inhibition in *Leptodactylus latrans* embryos after pH adjustment to 7.7 (Bach et al. 2018). At the same time, Howe et al. (2004) found, similar to our study, that technical grade GLY had no acute or subchronic effect on tadpoles (96 h $LC_{50} > 17.9$ mg L^{-1}). However, different formulations of Roundup (Roundup Original) caused higher lethality and developmental abnormalities in tadpoles that resulted in 96 h LC_{50} levels for four frog species between 2.2 and 8 mg AI L^{-1} (Howe et al. 2004). These insights support the importance surfactant(s) for toxicity of GBPs (Howe et al. 2004). In the light of the high number of GBP, with likely a different set of surfactants, being registered (SERA 2011) calls for further studies to understand the underly mechanisms as a generalization seems difficult.

Table 3 Length of stage 8 embryos and stage 46 tadpoles of *Xenopus laevis* after 96-h exposure to different concentrations of GLY and GBP

GLY			GBP		
Conc. (mg L^{-1})	*n*	Length (mm)[a]	Conc. (mg AI L^{-1})	n	Length (mm)[a]
8th stage					
Control	32	7.14 ± 0.07	Control	32	7.31 ± 0.10
282	31	7.15 ± 0.08	31.0	14	6.45 ± 0.17*
310	31	7.14 ± 0.07	34.2	7	6.27 ± 0.09*
342	26	7.04 ± 0.08	37.6	–	–
376	32	7.22 ± 0.06	41.3	–	–
413	32	7.21 ± 0.08	45.5	–	–
455	30	7.38 ± 0.08	50	–	–
500	29	7.04 ± 0.10	–	–	–
46th stage					
Control[b]	30	10.13 ± 0.10	Control[b]	30	10.13 ± 0.10
250	25	10.52 ± 0.07	31.0	30	9.96 ± 0.07
275	29	10.36 ± 0.08	34.2	26	9.62 ± 0.08 *
303	26	10.44 ± 0.09	37.6	10	9.18 ± 0.16 *
333	29	10.28 ± 0.04	41.3	–	–
367	26	10.23 ± 0.08	45.5	–	–
403	25	10.48 ± 0.07	50.0	–	–

*Indicates groups that are significantly different from the control ($p < 0.001$)

[a]Lengths are expressed as mean ± standard errors. These values were obtained from the lengths of the surviving individuals (*n*)

[b]The average length of tadpoles at the beginning of this test is 7.30 ± 0.10 mm ($n = 32$)

Table 4 The enzyme activities of stage 46 tadpoles after 96-h exposure to different concentrations of GLY and GBP

Conc	*n*	GST[a]	GR[a]	CaE[a]	AChE[a]	SOD[b]
Control	5	130 ± 3.9	11.2 ± 0.43	167 ± 4.4	142 ± 3.2	0.65 ± 0.05
GLY (mg L^{-1})						
50	5	134 ± 4.9	11.3 ± 0.38	155 ± 3.9	128 ± 6.9	0.60 ± 0.02
100	5	131 ± 1.0	9.7 ± 0.47	154 ± 2.5	126 ± 5.4	0.60 ± 0.05
250	5	141 ± 3.5	10.7 ± 0.28	170 ± 3.1	132 ± 6.5	0.64 ± 0.03
GBP (mg AI L^{-1})						
3.5	5	137 ± 2.0	9.6 ± 0.17	142 ± 6.4	138 ± 7.0	0.61 ± 0.04
7.0	5	139 ± 4.9	8.6 ± 0.61	145 ± 3.3	123 ± 3.7	0.52 ± 0.01
17.6	5	115 ± 5.2	7.5 ± 0.24	127 ± 6.5*	110 ± 5.3	0.45 ± 0.03

*Significant differences from control are marked by asterisks (*t* test with Bonferroni correction, $p < 0.016$)

[a]The enzyme activity was expressed as nmol min^{-1} mg^{-1} protein ± standard error

[b]The enzyme activity was expressed as U mg^{-1} protein ± standard error

In addition to the effects on development and survival, GBPs can cause histopathological, teratogenic, and behavioral changes. Furthermore, GBP may affect the metamorphic success and sexual differentiation of tadpoles (Bach et al. 2018; Bonfanti et al. 2018; Miko et al. 2017; Navarro-Martin et al. 2014). The selected biochemical markers in embryos and tadpoles exposed to GLY did not show any statistically significant change in this study. Following exposure to GBP, however, GR, CaE, AChE, and SOD activities were inhibited in a dose-dependent manner (Table 4). Similar results were reported for CaE and AChE (also glutathione S-transferases) in *Rhinella arenarum* tadpoles after exposure to four GBPs (Lajmanovich et al. 2011). Since pure GLY does not cause statistically significant inhibitions in enzyme activities even at much higher concentrations suggests that GR, CaE, AChE and SOD inhibitions are associated with another factor such as adjuvants. In contrast to the present study, another GBP caused an increase of catalase and AChE activities and lipid peroxidation levels in fish (Samanta et al. 2014). In combination with the results above, the role of adjuvants and other additives in formations for the toxicity the AI is causing requires further investigations.

Finally, the data generated from this study provides important information for assessing the toxic effects GBPs in non-target aquatic organisms by relating the effects to pure GLY. It was highlighted that GBP cause higher toxicity than pure glyphosate in *X. laevis* tadpoles. Consequently, understanding the mechanisms behind this presumably synergizing effect of surfactants and other ingredients in GBP, or other pesticide formulations, seems of high relevance from a fundamental and applied perspective.

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