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Toxicity of Formulated Glyphosate (Glyphos) and Cosmo-Flux to Larval Colombian Frogs 1. Laboratory Acute Toxicity

M. H. Bernal[1], K. R. Solomon[2], and G. Carrasquilla[3]

[1]*Laboratory of Herpetology, Eco-Physiology & Ethology, Universidad del Tolima, Barrio Santa Elena, Ibagué, Tolima, Colombia,* [2]*Centre for Toxicology and Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada, and* [3]*Facultad de Salud, Universidad del Valle, Cali, Colombia*

The spraying of coca (*Erythroxylum coca*) with glyphosate in Colombia has raised concerns about possible impacts on amphibians. There are few toxicity data for species other than those from temperate regions, and these have not been generated with the combination of formulated glyphosate (Glyphos) and the adjuvant, Cosmo-Flux (coca mix) as used in coca control in Colombia. In order to characterize toxicity of the spray mixture to frogs from Colombia, Gosner stage-25 tadpoles of *Scinax ruber, Dendropophus microcephalus, Hypsiboas crepitans, Rhinella granulosa, Rhinella marina, Rhinella typhonius, Centrolene prosoblepon,* and *Engystomops pustulosus* were exposed to the coca mix at concentrations of glyphosate ranging from 1 to 4.2 mg a.e./L diluted in dechlorinated tap water in glass containers. Cosmo-Flux was added to Glyphos in the proportion of 2.3% v/v, as used in aerial application for coca control. Exposures were for 96 h at 23 ± 1.5°C with 12:12-h light/dark cycle. Test solutions were renewed every 24 h. Concentrations, measured within the first hour and at 24 and 96 h using enzyme-linked immunosorbent assay (ELISA) (Abraxis, LLC), ranged from 70 to 130% of nominal values. LC50 values ranged from 1200 to 2780 µg glyphosate acid equivalents (a.e.)/L for the 8 species tested. Data suggest that sensitivity to Roundup-type formulations of glyphosate in these species is similar to that observed in other tropical and temperate species. In addition, sensitivity of larval amphibians to Roundup-type formulations spans a relatively narrow range. Finally, toxicity of the mixture as used to spray coca was likely driven by the surfactant in the glyphosate formulation, as the addition of Cosmo-Flux did not enhance toxicity above those reported for Vision = Roundup®.

Extensive reviews of the effects of glyphosate and its formulated products on aquatic organisms concluded that glyphosate presents a negligible risk to aquatic organisms (World Health Organization International Program on Chemical Safety, 1994; Giesy et al., 2000; Solomon & Thompson, 2003). Although amphibians are physiologically unique and ecologically important, no regulatory agencies currently require amphibian toxicity data as part of their registration requirements. Determining direct and indirect effects of agrochemicals on amphibian species continues to be identified as a general research need (Linder et al., 2003).

Several recent publications reported that glyphosate (active ingredient) exerts low toxicity to larval amphibians. The 48-h LC50 values of technical-grade glyphosate isopropylamine (IPA) salt to larval Australian frogs (*Litoria moorei, Crinia insignifera, Lymnodynastes dorsalis*, and *Heleioporus eyrei*) were reported to range from >343,000 to >466,000 µg glyphosate acid equivalents (a.e.)/L (Mann & Bidwell, 1999). The 96-h LC50 of glyphosate IPA in *Rana clamitans* was reported to be >38,900 µg a.e/L from a static exposure study (Howe et al., 2001). From this limited data set, it appears that glyphosate IPA is essentially nontoxic to amphibians.

The toxicity of some formulated glyphosate products to amphibians is greater than that of the active ingredient. A study by Mann and Bidwell (1999) examined the acute toxicity of Roundup herbicide (MON 2139) for *C. insignifera, H. eyrei, L. dorsalis,* and *L. moorei* tadpoles and reported 48-h LC50 values ranging between 2900 and 11,600 µg a.e./L glyphosate. Using a formulation of glyphosate (Vision containing glyphosate and ethoxylated tallowamine surfactant [POEA] and equivalent to Roundup), 96-h LC50 values as low as 880 µg a.e./L were reported for tadpoles of *Xenopus laevis*, *Bufo*

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Address correspondence to Dr. M. H. Bernal, Laboratory of Herpetology, Eco-Physiology & Ethology, Universidad del Tolima, Barrio Santa Elena, Ibagué, Tolima, Colombia. E-mail: mhbernal@ut.edu.co

americanus, Rana clamitans, and *Rana pipiens* (Edginton et al., 2004). Embryo stages were less sensitive than Gosner stage 25 larvae, and toxicity was affected by the pH of the exposure medium, although not in a consistent manner. A study on *R. clamitans, R. pipiens, Rana sylvatica*, and *B. americanus* (Howe et al., 2004) reported 96-h LC50 values for Roundup Original of 2200, 2900, and 5100 µg a.e./L, respectively. A study on *Rana cascadae* reported a 48-h LC50 for Roundup of 2336 µg a.e./L using static exposures in glass tanks (Cauble & Wagner, 2005). In a study carried out with *R. catesbeiana, R. clamitans, Hyla versicolor, R. pipiens*, *B. americanus,* and *R. sylvatica*, 384-h LC50 for Roundup were reported to range from 977 to 1865 µg a.e /L (based on the assumption that the reported concentration of the AI was as the IPA; Relyea 2005). It is not clear why only the 16-d LC50 values were calculated when it appeared that mortality occurred early in the exposure period; however, the reported LC50s were not greatly different from those reported by other authors (discussed earlier).

The toxicity of some other formulations of glyphosate is less than that of Roundup. Roundup Biactive (MON 77920) was practically nontoxic to tadpoles, producing 48-h LC50 values of 328,000 µg a.e./L for *L. moorei* and >360,000 µg a.e./L for *C. insignifera*, *H. eyrei*, and *L. dorsalis* (Mann & Bidwell, 1999). It is clear that components of the formulation other than the active ingredient are drivers of acute toxicity.

Aerial applications of glyphosate to control illicit coca (*Erythroxylum coca*) and poppy (*Papaver sominferum*) crops have been made in Colombia since 1997. Since 2006, poppy has not been grown to a significant extent in Colombia and is no longer sprayed. It has been pointed out that the glyphosate–Cosmo-Flux mixture as used in the spray program in Colombia could present a risk to native frog species (Solomon et al., 2007). The 96-h LC50 for the spray mixture as used in Colombia to larvae of *X. laevis* was 1300 and 1100 µg a.e./L for the poppy and coca mixtures (Solomon et al., 2007), respectively (Wildlife International, 2006a, 2006b). This was not greatly different from the values reported in the literature for Roundup in the same species, suggesting that the addition of the adjuvant Cosmo-Flux did not alter the toxicity of the mixture. However, there are few data in the literature on the susceptibility of tropical frog species to formulations of glyphosate and there are no data for species native to Colombia. Because of this, acute laboratory tests on larvae of native Colombian species of frogs were conducted. This study describes the acute toxicity of the Glyphos and Cosmo-Flux mixture to tadpoles (Gosner stage 25) of the frog species, *Hypsiboas crepitans* (Wied-Neuwied, 1824), *Rhinella granulosa* (Spix, 1824), *Engystomops pustulosus* (Cope, 1864), *Rhinella marina* (Linnaeus, 1758), *Scinax ruber* (Laurenti, 1768), *Dendropsophus microcephalus* (Cope, 1886), *Rhinella typhonius* (Linnaeus, 1758), and *Centrolene prosoblepon* (Boettger, 1892), during a 96-h exposure period under static-renewal test conditions in the laboratory.

MATERIALS AND METHODS

Test Organisms

Species that occur in locations where coca is grown (≤ 1000 m a.s.l.) were the focus of the study. Embryos of the test species were collected in the locations shown in Table 1 and transported to the University of Tolima where they were raised to Gosner stage 25 at a temperature of 23–25ºC in tanks containing city water that was dechlorinated by continuous aeration for at least 48 h prior to use. Embryos were not fed while they developed to stage 25. Only the tadpoles of *R. typhonius* and *S. ruber* were caught directly in field in stage 25. Tadpoles were not fed for 24 h before or during the test.

Testing Procedures

Formulated glyphosate (Glyphos, a product sold in Colombia but similar to Roundup in terms of active ingredient and POEA surfactant) and Cosmo-Flux as used in the spray program were obtained and stored separately at room temperature in the dark. Glyphos contains 354 g glyphosate a.e./L (as the IPA) and between 10 and 15% ethoxylated tallowamine (POEA) surfactant. Cosmo-Flux contains a mixture of linear and aryl polyethoxylates

TABLE 1
Species of Larval Frogs Used in the Acute Toxicity Studies and Their Location of Collection

Species	Stage collected	Location	Altitude (m a.s.l.)
Hypsiboas crepitans	Gosner 10–11	Potrerillo (4º14'N; 74º58'W)	430
Rhinella granulosa	Gosner 10–11	Payandé (4º19'N; 75º06'W)	630
Engystomops pustulosus	Gosner 10–11	Ibagué (4°21'N; 75°06'W)	827
Rhinella marina	Gosner 10–11	Payandé (4º19'N; 75º06'W)	630
Scinax ruber	Gosner 25	Potrerillo (4º14'N; 74º58'W)	430
Dendrosophus microcephalus	Gosner 10–11	Potrerillo (4º14'N; 74º58'W)	430
Rhinella typhonius	Gosner 25	Ibagué (4º25'N; 75º12 W)	1200
Centrolene prosoblepon	Gosner 10–11	Falan (5°07'N; 74°58'W)	1100

(17% w/v) and isoparaffins (83% v/v) (Cosmoagro, 2004). The water used for testing was the same as that used for raising the tadpoles (described earlier). Specific conductance, hardness, alkalinity, and pH of the water were measured.

Test chambers were 2-L glass jars containing 1 L of test solution. The chambers were indiscriminately positioned by treatment group in an air-conditioned area of the laboratory designed to maintain the test temperature and day length throughout the test period. A primary stock solution was prepared by dissolving the Glyphos and Cosmo-Flux in dilution water to obtain a nominal concentration of 100,000 µg glyphosate a.e./L. All solutions were prepared using a positive displacement pipette. Cosmo-Flux was added in the proportion of 0.023 µl per 1 µl of Glyphos (2.3% v/v) to obtain the proportion of spray mix as used in the field. Nominal test concentrations were selected based upon the results of exploratory range-finding toxicity tests. Two replicates of each test solution were prepared by serial dilution of the stock solution with dilution water to yield a range of nominal concentrations in 1 L of solution of 4200 to 1000 µg glyphosate a.e./L. Each solution was mixed by inversion. Ten tadpoles were impartially placed in each of the two test chambers for a total of 20 tadpoles per concentration. The rate of biomass loading (defined as total wet weight of 10 tadpoles/L test water) was below 0.6 g/L as recommended in ASTM guidelines (ASTM, 1998), with the exception of *S. ruber*, which were about 0.7 g/L. Test solutions were renewed daily by transferring the test organisms to freshly prepared solutions. Mean measured test concentrations were determined from samples of test water collected from the pooled replicates for each treatment and control group at the beginning of the test (0 h) and from the test solutions for each treatment and control group at 24 h and at test termination (96 h). All samples were collected at mid-depth and were analyzed immediately without storage.

Fluorescent lights that emit wavelengths similar to natural sunlight (Phillips TLT 20W/54RS) were used for illumination of the test chambers. A photoperiod of 12:12-h light/dark cycle was controlled with an automatic timer. The temperature during the study was 23 ± 1.5°C. Temperature was measured in each test chamber at the beginning and end of the test and at approximately 24-h intervals during the test, including before and after renewals, using a liquid-in-glass thermometer. Temperature also was measured continuously during the test in one negative control test chamber using a maximum and minimum digital thermometer. Dissolved oxygen and pH were measured in each test chamber at the beginning and end of the test and at approximately 24-h intervals during the test, including before and after renewals. Dissolved oxygen was measured using a portable dissolved oxygen meter Hanna HI 8043, and measurements of pH were made using a Hanna HI 8314 membrane pH meter. Observations of mortality and other signs of toxicity, such as unusual swimming activity, were observed approximately 4, 24, 48, 72, and 96 h after test initiation. The cumulative percent mortality observed in the treatment groups was used to estimate LC50 values at 96 h.

Analytical Methods

The ELISA test kit manufactured by Abraxis, LLC (Warminster, PA), was used to measure the concentration of glyphosate in the test solutions. Calibration standards of glyphosate solutions, ranging in concentration from 0.15 to 5 µg/L, were prepared from the standard supplied with the test kit and used to construct the standard curve. A standard curve was prepared with each set of samples analyzed. The standard curve was constructed by plotting the %B/Bo (absorbance value for each standard/absorbance value for the zero standard) against the corresponding glyphosate concentration. Concentrations of glyphosate were determined by interpolation from the standard curve. Final concentration of glyphosate in the exposure solution was calculated by correcting for the mean quality control (QC) percent recovery based on analyses of two replicates of one concentration (0.5 µg/L) of the standard. The Abraxis glyphosate assay has an estimated minimum detectable concentration based on a 90% B/Bo of 0.1 µg/L. The method limit of quantitation (LOQ) for these analysis was defined as the lowest calibration standard, 0.15 µg/L. Two matrix blank samples were analyzed to determine possible interferences. No interferences were detected above the LOQ during the samples analysis.

It was not logistically possible to measure concentrations of Cosmo-Flux; however, the proportions of Glyphos and Cosmo-Flux were kept constant and were the same as those used in the aerial spraying of coca. The results are thus representative of realistic field exposures.

Statistical Analyses

For consistency with other studies, the mortality data were analyzed using the U.S. EPA Probit Program Version 1.5 (U.S. EPA, 1994). The LC1 was estimated as a regression-derived approximation of the no-observed-effect concentration, and the LC50 was calculated for comparison to other literature values. The LC1, which is derived from the response data, is preferred as an indicator of the low effect concentration as it is independent of the experimental design (Crane & Newman, 2000). Where insufficient data were available for the Probit program (no or one concentration with a response between 0 and 100%), LC50 values were estimated by interpolation from a graph of percent concentration versus response.

RESULTS AND DISCUSSION

Measurement of Test Concentrations and Water Quality

Samples collected at test initiation had measured concentrations of glyphosate that ranged from 75 to 125% of the nominal concentrations. Samples collected prior to the renewal of the test solutions at 24 h contained measured concentrations that ranged from 74 to 112% of the nominal concentrations. Samples

collected at test termination contained measured concentrations that ranged from 71 to 130% of the nominal concentrations. When the measured concentrations of samples collected at 0, 24, and 96 h were averaged, the mean measured concentrations ranged from 85 to 105% of nominal concentrations. Because measured values were close to nominal, the nominal concentrations were used to determine the LC1 and LC50 values in order to compare responses of larval anurans reported under field conditions (Bernal et al., 2009) and those in other studies. Water temperatures were within the 23 ± 1.5°C range established for the test. Mean dissolved oxygen concentrations were about 6.85 mg/L. The 95% confidence interval of measurements of oxygen concentration, hardness, alkalinity, specific conductance, and pH in the dilution water at test initiation are summarized in Table 2.

Signs of toxicity, such as slow swimming and remaining on bottom with no movement, were generally noted at lower exposure concentrations, and uncontrolled fast swimming and remaining in a vertical position were more evident at concentrations close to and exceeding the LC50 concentration. In general, most of the toxic responses were expressed within 24 to 48 h of test initiation.

TABLE 2
Hardness, Alkalinity, Specific Conductance, and pH in the Dilution Water at Test Initiation

Parameter	Mean	95% Confidence interval
Oxygen concentration(mg/L)	6.85 (n = 96)	6.60–7.10
Hardness (mg/L as $CaCO_3$)	112 (n = 6)	97.1–126.8
Alkalinity (mg/L as $CaCO_3$)	89.3 (n = 6)	70.2–108.4
Specific conductance (mS/cm)	263.2 (n = 6)	197.4–328.9
pH	8.23 (n = 96)	8.20–8.25

Toxicity values for the eight species of frogs (Table 3) are presented in terms of glyphosate concentration (a.e.) to allow for comparison to data from the literature. The most sensitive species was *D. microcephalus* and the least sensitive was *E. pustulosus*. The slopes of the concentration-response relationships ranged from 7.18 to 13.47. These are large slopes and are consistent with literature data where such slopes have been reported for formulated glyphosate (Perkins et al., 2000) and surfactants (Dorn et al., 1993; Wong et al., 1997).

The LC50 values for these eight species of frogs were combined with those from the literature (Brain & Solomon, 2009) in a species sensitivity distribution (SSD). Plotting positions were calculated using standard procedures (Solomon & Takacs, 2002). The SSD data (Figure 1) illustrate that the larvae of frogs from Colombia are not more nor are they less sensitive than other frogs tested in other locations are to glyphosate formulations such as Roundup and Vision. This is consistent with the observed toxicity of the mixture to *X. laevis* (Wildlife International, 2006a, 2006b) where values were similar but slightly greater (less toxic) than those reported with the Vision formulation of glyphosate (Edginton et al., 2004). The 5th centile of the toxicity distribution was 692 µg a.e./L, suggesting that overall, 95% of larval frogs would have LC50s greater than this value.

CONCLUSIONS

The acute toxicity values determined in Colombian species of frogs suggest that sensitivity to Roundup-type formulations of glyphosate in these species is similar to that observed in other species tested in other locations (Brain & Solomon, 2009). There is no underlying assumption that would suggest that tropical species, such as those tested in Colombia, would have different sensitivity to pesticides such as those containing glyphosate and our observations are consistent with other observations on tropical and temperate species (Maltby et al., 2005). These data add to those currently in the literature and suggest that sensitivity of larval amphibians to Roundup-type formulations

TABLE 3
Toxicity Values for Colombian Frog Species Exposed to Formulated Glyphosate and Cosmo-Flux

Species	Slope[b]	Intercept[b]	LC1 (µg a.e./L)	95% Confidence interval (µg a.e./L)	LC50 (µg a.e./L)	95% Confidence interval (µg a.e./L)
D. microcephalus[a]	–	–	–	–	1200	–
R. typhonius[a]	–	–	–	–	1500	–
S. ruber	13.47	2.09	1103	716–1294	1642	1470–1783
H. crepitans	7.23	2.72	984	645–1225	2064	1835–2285
R. granulosa	9.09	1.62	1300	737–1632	2348	2036–2588
C. prosoblepon	7.18	2.24	1145		2414	
R. marina	9.75	0.74	1578	1122–1874	2733	2473–2982
E. pustulosus	8.78	1.09	1514	1040–1827	2787	2510–3057

[a]LC50 values estimated from a graph of concentration vs. percent response. Slope could not be calculated.
[b]Slope and intercept in log probit units.

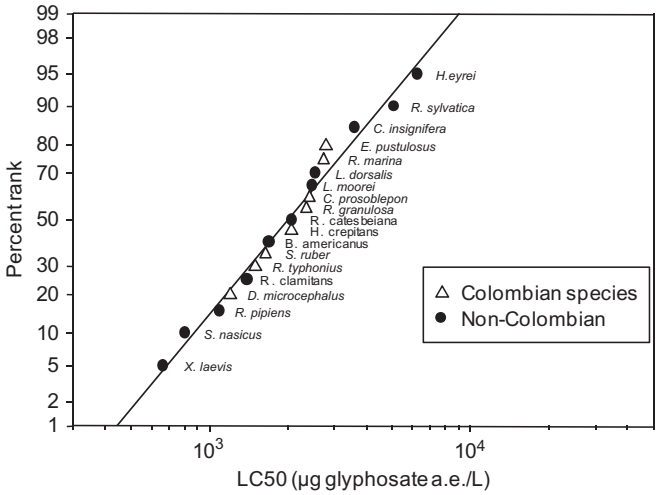

FIG. 1. Species sensitivity distribution of LC50 values for glyphosate plus Cosmo-Flux as used in the spraying of coca in Colombia in larval amphibians from Colombia and LC50 values for frogs from other locations to Roundup and Vision. Data for Roundup and Vision are from (Brain & Solomon, 2009).

spans a relatively narrow range. Toxicity of the mixture as used to spray coca was likely driven by the surfactant in the glyphosate formulation as the addition of Cosmo-Flux did not increase toxicity above those reported for Vision = Roundup (discussed earlier). The relatively low toxicity of Cosmo-Flux is consistent with the LC50 values reported in the fish, *Piaractus brachypomus*, which was 4,417 mg formulation/L in juveniles of 40 g mass (Rondon-Barragan et al., 2007).

Extrapolation of these toxicity values directly to the environment is inappropriate except for simple hazard ranking. In realistic environmental conditions where sediments and organic detritus are present in pools inhabited by amphibian larvae, concentrations of glyphosate and the POEA surfactant will decrease rapidly due to binding to sediments (Solomon et al., 2007) and this will likely reduce exposures and risks to amphibians. These hypotheses were tested and results are reported in a companion article (Bernal et al., 2009).

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