Research Article

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Journal of Aquatic Pollution and Toxicology

2017
Vol. 1 No. 2: 8

Toxicity of Six Commercial Pesticide Formulations to Larvae of Two Tropical Frogs, *Rhinella* (Bufo) *marina* (Bufonidae) and *Engystomops* (Physalaemus) *pustulosus* (Leptodactylidae)

Nicole Sookoo, Adrian Hailey and Azad Mohammed

Department of Life sciences, Faculty of Science and Technology, The University of the West Indies, Trinidad, West Indies

Corresponding author: Azad Mohammed

azad.mohammed@sta.uwi.edu

Department of Life sciences, Faculty of Science and Technology, The University of the West Indies, Trinidad, West indies.

Tel: 868-6622002
Fax: 868-6635241

Citation: Sookoo N, Hailey A, Mohammed A. Toxicity of Six Commercial Pesticide Formulations to Larvae of Two Tropical Frogs, *Rhinella* (Bufo) *marina* (Bufonidae) and *Engystomops* (Physalaemus) *pustulosus* (Leptodactylidae). J Aquat Pollut Toxicol. 2017, 1:2.

Abstract

Pesticides are non-selective toxicants that can cause significant mortalities in non-target species such as amphibian. The acute toxicity of three commercial herbicide formulations (Roundup Ultra, Gramoxone Super, Karmex) and three commercial insecticide formulations (Revelo 350CS, Evisect S, BPMC) was determined for larvae of two tropical frog species, *Engystomops pustulosus* (Leptodactylidae) and *Rhinella marina* (Bufonidae). The 96 h LC_{50} for *E. pustulosus* ranged between 0.3 mg L^{-1} (Karmex) and 560 mg L^{-1} (Relevo 350CS) while for *R. marinus* it ranged between 0.8 mg L^{-1} (Evisect S) and 280 mg L^{-1} (Gramoxone Super) following exposure in the aqueous phase alone. Roundup was the only formulation in which LC_{50} did not vary significantly between the two species. In the presence of soil, the 96 h LC_{50} for *E. pustulosus* ranged between 0.8 mg L^{-1} (Evisect S) and 240 mg L^{-1} (Relevo 350CS), while the valued for *R. marinus* ranged between 1.4 mg L^{-1} (Karmex) and 620 mg L^{-1} (Relevo 350CS). *Engystomops pustulosus* was found to be less resistant to the pesticides in the aqueous phase alone, when compared to *Rhinella marina*. Since both responses varied, lit is unlikely that data collected for anyone species can be used to predict the toxicological responses in another species.

Keywords: Pesticides; Amphibian; Herbicide formulations; Insecticide formulations; Toxicological responses

Received: March 24, 2017; **Accepted:** May 03, 2017; **Published:** May 10, 2017

Introduction

Globally, the decline in amphibian population has been linked to several factors, habitat loss, diseases, climate change and pollutants such as pesticides [1-7]. Amphibians are highly susceptible to the effects of chemical because of their biphasic life cycle and permeable skin, which greatly increases the risk of exposure [8]. Various authors have reported on the acute toxic effects of pesticides on amphibian [9-11]. Sayim reported that Malathion had an LC_{50} value of 29 mg kg^{-1} to *Rana ridubunda* [12]. Profenofos, an organophosphate insecticide showed a 96 h LC_{50} value of 0.58 mg L^{-1} to *Rana spinosa* tadpoles [13]. Sparling and Fellers also reported that chlorpyrifos had an LC50 of 365 µg L^{-1} in *P. regilla* and 66.5 µg L^{-1} for *R. boylii* while endosulfan, had and LC_{50} of 15.6 µg L^{-1} for *P. regilla* and 0.55 µg L^{-1} for *R. boylii* [14]. However, effects of some commonly used pesticides in Trinidad and Tobago or the wider Caribbean are unknown for amphibians, especially during the larval stages [15]. Thus, further studies involving the knowledge of the toxicity of different pesticides in a variety of species are urgent and necessary for a better understanding of the risk to this group [16].

Amphibians account for 3.8% of the vertebrates used in toxicity test since 2000, a modest increase from 2.7% for studies between 1972 and 2000 [17,18]. Approximately half of the existing work on amphibians is on Ranidae, although amphibian species are known to differ in sensitivity [19-20]. Information is lacking, in particular, on the ecotoxicology of tropical amphibians, without which interspecies correlation estimation and other predictive models will not be useful for threatened tropical amphibians [21-23]. Therefore examining the acute toxicity of pesticides currently in use (three herbicides and three insecticides) provides valuable information about the potential impacts on amphibians in the Caribbean region. Identifying potential environmental threats can therefore be important in reducing the rate of decline of the species in region. Trinidad and Tobago imports approximately 2000 tons of pesticides annually, with an estimated usage of about 40 tons yr^{-1} km^{-2}. This gives Trinidad and Tobago an Environmental Vulnerability index score (EVI) scale of 7, which suggest that the country is very vulnerable to impacts from pesticides [24].

Engystomops pustulosus (tungara frog) and *Rhinella marina* (cane toad) are two native amphibian species widely distributed in Trinidad and Tobago. Tadpoles often inhabit ponds, rivers, and isolated pools which may be contaminated by surface runoff and spray drift from agricultural plots. *E. pustulosus* produces floating foam nests, from which larvae hatch into the water in a few days, *R. marina* eggs are laid in strings in water. *R. marina* was included in this study because toxicological data has been reported in the literature and would allow comparisons to be made with previous work. The other pesticides and amphibian taxon (the tungara frog, *Engystomops* (Physalaemus) *pustulosus*, Leptodactylidae) were selected since there was little information available on the ecotoxicology of this tropical family [25,26]. The pesticides chosen for this study were the most commonly sold formulations in Trinidad and Tobago and included Roundup (glyphosate) which has been well reported in the literature [27].

Methods

Study organisms

Engystomops pustulosus generally range in length from 30-36 mm and are widely distributed in dry forest, savanna, or grassland in low-lying areas, freshwater marshes, canals, ditches and ponds. Eggs are laid in in foam nests, generally under cover, in pools which prevent desiccation and predation [27]. Tadpoles hatch from eggs and leave the foam nest after about four days.

Rhinella marina (Bufonidae) is the largest species in the Bufonidae family. Females are considerably larger than males, attaining a snout-vent length of 15 cm. *R. marina* is a lowland toad inhabiting areas that are primarily below 1000 m; however altitude appears to be dependent on minimum temperature that the toad can tolerate. Females generally breed between April to September and can produces two clutches of about 8,000 to 35,000 eggs. These are laid in a double strand within a long thin string of gelatinous jelly and hatch within 24 to 72 h of laying, giving rise to tiny, shiny black tadpoles. Female can produce approximately 200,000 eggs during its reproductive life [28].

Biological material

Foam nests of *E. pustulosus* were collected from the University of the West Indies Field Station (10°38′14″N, 61°25′53″W) on days after heavy rain and transported individually in polypropylene containers to the laboratory. Nests were placed in 4 L polypropylene containers at 27°C and usually hatched on the second day after collection. *Rhinella marina* eggs were obtained from adults kept in captivity and induced to spawn by subcutaneous injection of 10 µg luteinizing hormone-releasing hormone [29,30]. Toxicity tests were initiated the day after hatching, when most tadpoles were at Gosner's stage 25 [31]. Each replicate was from a different nest (*E. pustulosus*) or male-female pairing (*R. marina*), with a sample size of six replicates for each formulation tested.

Experimental design and handling

Static non-renewal toxicity tests using the six pesticides formulations were conducted in 500 mL glass jars washed in 0.1 mol L^{-1} hydrochloric acid then thoroughly rinses with distilled water [32]. Tadpoles were exposed to both negative (dilution water only) and positive controls (concentration known to cause 100% mortality) and an ascending series of five nominal concentrations of each formulation. Test concentrations were prepared by volumetrically mixing aliquots of a freshly-prepared stock solution with dilution water. An initial log order range-finding test was conducted for each pesticide, to determine and approximate LC_{50} value so as to better estimate the range of concentrations in the definitive test. The final test ranges used for each of the pesticides. The active ingredients for each pesticide were also determined from the product formulation **(Tables 1 and 2)**.

Toxicity tests were conducted for 96 h at 25°C, pH of 7-8, total alkalinity 45 mg L^{-1}, total hardness 93 mg L^{-1} and dissolved oxygen >4 mg L^{-1}. Ten tadpoles were used per replicate (from one clutch of eggs) in 300 ml of test solution and six replicates (from separate clutches of eggs) included per test [33]. Tests results were deemed acceptable if the percentage mortality in the negative controls did not exceed 10%. The larvae were fed two pellets (mean pellet mass 18 mg) of Best Flake Cichlid-Gro Pellets (Worldwide Aquatics, Arvin, CA) daily, to eliminate starvation as a cause for mortality and uneaten food was removed [34]. The

Table 1 Pesticide formulations used in this study, the first three are herbicides, the last three are insecticides.

Pesticide	Chemical	Formulation	Concentration	Source
Glyphosate	Phosphonoglycine: N-(phosphonomethyl) glycine	Roundup Ultra	49%	Monsanto company, OH
Paraquat	Bipyridium: paraquat dichloride	Gramoxone Super	20%	Syngenta S.A., Colombia
Diuron	Urea: 3-(3,4-dichlorophenyl)-1,1-dimethylurea	Karmex	80%	Du Pont Ltd., New Zealand
Imidacloprid	Neonicotinoid: 1-[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine	Revelo 350CS	35%	Insecticidas Internacionales, Venezuela
Thiocyclam	Nereistoxin: thiocyclam hydrogen oxalate	Evisect S	50%	Jiangsu Lihua Chemical Co., China
Fenobucarb	Methyl carbamate: 2-sec-butylphenyl-N-methyl carbamate	BPMC	500g/L	Physta Chemical Co., China

Table 2 Test ranges (mg L^{-1}) of concentrations for the six commercial pesticide formulations used.

Formulation	Species	Test range
Roundup Ultra	*E. pustulosus*	0-20
	R. marina	0-75
Gramoxone Super	*E. pustulosus*	0-150
	R. marina	0-1200
Karmex	*E. pustulosus*	0-15
	R. marina	0-6
Revelo 350CS	*E. pustulosus*	0-2000
	R. marina	0-1200
Evisect S	*E. pustulosus*	0-15
	R. marina	0-15
BPMC	*E. pustulosus*	0-150
	R. marina	0-75

pellets were moistened in dechlorinated water for *E. pustulosus* (which is a benthic feeder) prior to use to allow the pellets to sink so that they were more readily available to the tadpoles.

Soil/water mixture

The tests were repeated using a soil/water mixture. Soil samples were collected from the University Field Station washed with three changes of distilled water and dried at 110°C for 15 h in a Precision Scientific Thelco laboratory Oven (Artisan Technology Group, Champaign Il). Five grams of soil was added to each glass jar (washed as described in the single phase testing) followed by 300 ml of pesticide mixture of the particular concentration (single phase concentrations). The mixture was stirred for 1 h and allowed to settle before the addition of the larvae. Six replicate chambers each with ten tadpoles (determined to be acceptable crowding levels) were used for each test concentration and negative and positive controls.

Data Analysis

The 96 h LC_{50} values were determined from the mortality data using Trimmed Spearman analysis. ANOVA and TUKEY HSD analysis (SYSTAT Ver. 5.0) was used to determine whether there was a significant difference (95%) between the LC_{50} values obtained for the two species. An interspecies toxicity was performed using Model II least squares methodology to determine the relative sensitivities of the two species to the different chemicals. In addition a 'Resistance Factor' (RF) (LC_{50}sp1/ LC_{50}sp2) was calculated for the various chemicals. Comparative analysis of toxicity of the selected pesticides used standard literature searches and the PAN pesticide database. Amphibian taxonomy was adjusted to current usage where necessary.

Results and Discussion

The 96 h LC_{50} for *E. pustulosus* ranged between 0.3 mg L^{-1} (Karmex) and 560 mg L^{-1} (Relevo 350CS) while for *R. marinus* it ranged between 0.8 mg L^{-1} (Evisect S) and 280 mg L^{-1} (Gramoxone Super) following exposure in the aqueous phase alone. Subsequent analysis used the equivalent pesticide (active ingredient, AI) concentration of each formulation. There was substantial variation in the sensitivity of the two species between the six pesticides. The pesticides generally showed higher toxicity to *E. pustulosus* when compared to *R. marinus* following exposure in both the aqueous phase and soil/water mixture. This would suggest that *E. pustulosus* may be more sensitive to the effects of pesticides than *R. marinus*. Relevo 350CS proved to be the least toxic to both species while Karmex and Evisect S were the most toxic in both test regimes **(Table 3)**.

The 96 h LC_{50} for the soil/water mixture for *E. pustulosus* ranged from 0.3 mg L^{-1} (Karmex) to 240 mg L^{-1} (Relevo 350CS) whereas for *R. marinus* values ranges from 1.4 mg L^{-1} (Karmex) to 620 mg L^{-1} (Relevo 350CS). These results suggest that the presence of soil had a significant effect on the toxicity of some of the pesticides. Three of the pesticides, Relevo 350CS, Roundup Ultra and Evisect S showed a significant increase ($P<0.05$) in toxicity to *E. pustulosus* in the presence of soil. However, for *R. marinus* the presence of soil resulted in a significant ($P<0.05$) decrease in toxicity of most of the pesticides **(Tables 3 and 4)**.

Relevo 350CS has a high water solubility (5.14×10^2 mg kg^{-1} at 20°C and pH 7) and low soil organic carbon-water partitioning coefficient (Koc=132-310) which means it would favour the aqueous phase. However in the presence of soil the toxicity to *E. pustulosus* increased but decreased for *R. marinus*. Cox et al. [35] reported that as the organic and clay content in soils increased there was an increase in the strength of adsorption of Relevo 350CS to the soil which may increase its bioavailability to *E. pustulosus*. Tadpoles of the genus Physalaemus are exotrophic and larvae feed on materials rasped from submerged surfaces [36,37]. Increased adsorption on soil particles would decrease the overall concentration in the aquatic phase, and consequently the amount that is bioavailable to *R. marinus* which generally feeds

Table 3 Mean 96 h LC_{50} values (mg L^{-1}) for exposure in the aqueous phase alone.

Pesticides	*Engystomops pustulosus*		*Rhinella marina*	
	Nominal	Active Ingredient	Nominal	Active Ingredient
Relevo 350CS	560 ± 63	196	187 ± 60	65
Roundup Ultra	7.9 ± 1.2	3.9	9.6 ± 1.0	4.7
Evisect S	3.3 ± 0.8	1.7	0.8 ± 0.2	0.4
BPMC	2.1 ± 1.3	1.1	30 ± 8	15
Gramoxone Super	1.0 ± 0.0	0.2	280 ± 34	56
Karmex	0.3 ± 0.0	0.2	1.4 ± 0.0	1.1

Table 4 Mean 96 h LC_{50} values (mg L^{-1}) for exposure in the soil/water mixture.

Pesticides	*Engystomops pustulosus*		*Rhinella marina*	
	Nominal	Active Ingredient	Nominal	Active Ingredient
Relevo 350CS	240 ± 110	84	622 ± 18	218
Roundup Ultra	4.6 ± 0.4	2.3	60 ± 2	29
Evisect S	0.8 ± 0.2	0.4	2.4 ± 0.9	1.2
BPMC	3.0 ± 2.4	1.5	27	13.5
Gramoxone Super	1.1 ± 0.3	0.2	462 ± 9	92
Karmex	0.3 ± 0.1	0.2	1.4 ± 0.1	1.1

at the surface of the water. Roundup Ultra showed increased toxicity to *E. pustulosus* and reduced toxicity to *R. marinus* in the soil/water test. Roundup Ultra has a high Koc value (24000 ml/g) and is therefore rapidly and tightly adsorbed to soil particle and organic matter. Evisect showed a similar increased toxicity to *E. pustulosus* in the presence of soil. Evisect is a white powder with a low Koc value (20 ml/g) and a low solubility (84000 ppm) which suggests that has low water solubility and high mobility in soils. Evisect has previously been reported to adsorb readily onto soil increasing its toxicity to the bog frog (*Rana limnocharis*) and tadpoles [38].

The toxicity of the BPMC, Gramoxone and Karmex showed some variability with the addition of soil, however, this was not significant. BPMC is very soluble in water and therefore remains in the aqueous phase. Gramoxone showed a significant ($P<0.05$) reduction in toxicity to *R. marinus* in the soil/water mixture. Gramoxone also readily binds to soils with high clay contents and becomes inactivated, showing no residual soil activity. Eisler reported that Gramoxone is quickly removed from the water phase with approximately 50% loss in 36 h in freshwater systems [39]. However, feeding on gramoxone contaminated soils and food can still result is significantly high mortalities as seen with *E. pustulosus* [40]. The toxicity of gramoxone was significantly higher ($P<0.05$) for *E. pustulosus* than *R. marinus* **(Tables 3 and 4)**.

There was no difference in the toxicity of Roundup Ultra to the two species following exposure in the aqueous phase. However there was a decrease in toxicity to *R. marinus* in the presence of the soil. Glyphosate pesticides such as roundup are one of the most-studied groups in relation to effects on amphibians and other non-target species. This is reflected in an analysis of the available data on the toxicity of commercial glyphosate formulations to larval amphibians. This analysis included 13 studies in which a total of 35 species of 10 families and two orders were tested from 24 h to 16 d exposure; a few observations at other time periods (6 h, 12 h, and 72 h) have been excluded from this compilation. The results show a variation in the toxicity of glyphosate formulations among species and over an extended time period from 1-16 days, with most LC_{50} values within a one order of magnitude 0.5-5.0 mg AI/L, typical of the results found in this study **(Table 5 and Figure 1)**.

The variation of toxicity of glyphosate formulations with exposure time corresponds to an approximate increase of toxicity of about 30% with each doubling of time. More data was available for 96 h than any other exposure period, so it was used in an assessment of variation among families and orders **(Table 5)**. Each species was only considered once in this analysis, where there were two or more values for a species at 96 h, the mean value was used. The mean (± S.D.) log LC_{50} for the 35 species was 0.302 ± 0.235, equivalent to a mean LC_{50} of 2.0 mg AI L^{-1}. There was no significant difference ($P>0.05$) between the two orders (one-way ANOVA of log LC_{50}, $F_{1,33}=1.42$, $P=0.24$), or among the ten families represented ($F_{9,25}=1.57$, $P=0.18$). Egea-Serrano et al [40] also found no significant effect ($P>0.05$) of family (n=8) or of developmental stage (embryo or larva), on survival in a meta-analysis of 48 studies on amphibian ecotoxicology, but showed a significant effect ($P<0.05$) of pollutant type (with pesticides as a single category). In contrast Hammond et al. did find significant phylogenetic variation among amphibians in sensitivity to a single pesticide (the insecticide endosulfan) [41]. We conclude that amphibians show no overall phylogenetic variation in sensitivity to pollutants, but they do show interspecific differences to some (but not all) pesticides. The lack of variation in toxicity of glyphosate formulations to amphibians should not lead to complacency in estimating the possible effects of pesticides on other species, however, as much greater variation was found in the other pesticides examined in this study.

Toxicity of other pesticides to amphibians

Though glyphosate pesticides (Roundup Ultra) are extensively studies, much less information is available about the toxicity of the other five formulations (BPMC, Gramoxone, Karmex, Revelo 350CS and Evisect S) to amphibians. There was insufficient data to examine variation of toxicity values among studies of the same species, within families or orders (indeed, only one order has been tested, anurans). It is possible, however, to quantify the variability of data for each pesticide, as an assessment of the need for further study and the reliability of extrapolation to unstudied, threatened taxa. This can be some using an appropriate safety factor which take account of the variation in sensitivity between species because we do not know whether the test species is the most sensitive one [43] **(Table 6)**.

A useful measure of the variability of toxicity is the standard deviation of the log transformed observed data (excluding interpolations and extrapolations), as shown in **Table 7**. For glyphosate formulations the mean log LC_{50} (of 83 values) was 0.297, similar to the inter-species mean given above (0.302). The S.D. was greater than for the inter-species results (0.282 vs. 0.235), an increase of variability of about 11%, due to the addition of a range of time periods. The variability of the other pesticides can be compared with those for glyphosate using a variance ratio test (i.e., rather than an ANOVA comparing means, as shown in **(Table 7)** [44].

The variability of the data for imidacloprid was not significantly different ($P>0.05$) to that from glyphosate, as would be predicted from inspection of the data in **Table 6**. The results of the only other study of this insecticide on amphibians were for two species of Ranidae, and were similar to those reported in this study [45]. Feng et al. [44] used technical grade imidacloprid rather than a commercial formulation, suggesting that the toxicity is largely due to the pesticide itself. Nevertheless, Puglis and Boone did find significant differences between technical grade and a commercial formulation of imidacloprid (19-150 mg AI L^{-1}) in effects on *Rana clamitans* larvae, however, no LC_{50} values were reported [46].

The other four formulations (BPMC, Gramoxone, Karmex and Evisect S) were all significantly ($P<0.05$) more variable than glyphosate in their toxicity to amphibians. Paraquat has a longer history of use than the other pesticides, but still ranks in the top 25 in current use in the USA [47,48]. It had the greatest variability of effect on amphibians (variance ratio>8). Toxicity to *R. marina* was similar to that of other species studied, with the LC_{50} at the

Table 5 Comparative toxicity data (LC_{50}, mg AI L^{-1}) for amphibian larvae to glyphosate formulations.

ORDER, Family and *Species*	Formulation	1 day	2 days	4 days	7-10 days	15-16 days	References
URODELA							
Ambystomatidae							
Ambystoma gracile	Roundup Original Max			2.8			Relyea and Jones [52]
Ambystoma gracile	Roundup				1.3	1.4	King and Wagner [53]*
Ambystoma laterale	Roundup Original Max			3.2			Relyea and Jones [52]
Ambystoma macrodactylum	Roundup			1.8	1.4	1.2	King and Wagner [53]*
Ambystoma maculatum	Roundup Original Max			2.8			Relyea and Jones [52]
Salamandridae							
Notophthalmus viridescens	Roundup Original Max			2.7			Relyea and Jones [52]
ANURA							
Bufonidae							
Anaxyrus americanus	Vision			1.7			Edgington et al. [54]
Anaxyrus americanus	Roundup Original	4.2					Howe et al. [55]
Anaxyrus americanus	Roundup					1.9	Relyea [56]
Anaxyrus americanus	Roundup Original Max			1.6			Relyea and Jones [52]
Anaxyrus boreas	Roundup Original Max			2.0			Relyea and Jones [52]
Anaxyrus boreas	Roundup	2.0			1.6	1.5	King and Wagner [53]*
Anaxyrus fowleri	Roundup			4.2			Fuentes et al. [57]
Rhinella arenarum	Roundup Ultra-Max	2.4	2.4	1.9			Lajmanovich et al. [58]
Rhinella granulosa	Glyphos & Cosmo-Flux			2.3			Bernal et al. [59]
Rhinella marina	Glyphos & Cosmo-Flux			2.7			Bernal et al. [59]
Rhinella marina	Roundup Ultra	4.3	3.7	3.5			This study*
Rhinella roqueana	Glyphos & Cosmo-Flux			1.5			Bernal et al. [59]
Centrolenidae							
Espadarana prosoblepon	Glyphos & Cosmo-Flux			2.4			Bernal et al. [59]
Dicroglossidae							
Euphlyctis cyanophlyctis	Roundup			3.8	2.1		Yadav et al. [60]
Hylidae							
Dendropsophus microcephalus	Glyphos & Cosmo-Flux			1.2			Bernal et al. [59]
Hyla chrysoscelis	Roundup			2.5			Fuentes et al. [57]
Hyla versicolor	Roundup					1.0	Relyea [56]*
Hyla versicolor	Roundup Original Max			1.7			Relyea and Jones [52]
Hyla versicolor	Roundup Original Max			2.0			Jones et al. [61]
Hypsiboas crepitans	Glyphos & Cosmo-Flux			2.1			Bernal et al. [59]
Litoria moorei	Roundup	3.1	2.9	2.2			Mann and Bidwell [49]
Pseudacris crucifer	Roundup Original Max			0.8			Relyea and Jones [52]
Pseudacris regilla	Roundup	0.32		0.26	0.24	0.23	King and Wagner [53]*
Scinax nasicus	Glyfos	3.6	2.7	2.0			Lajmanovich et al. [62]*
Scinax ruber	Glyphos & Cosmo-Flux			1.6			Bernal et al. [59]
Leptodactylidae							
Engystomops pustulosus	Glyphos & Cosmo-Flux			2.8			Bernal et al. [59]
Engystomops pustulosus	Roundup Ultra	4.3	4.0	2.9			This study*
Myobatrachidae							
Crinia insignifera	Roundup		3.6	2.8			Mann and Bidwell [49]
Heleioporus eyrei	Roundup	8.6	6.3	4.9			Mann and Bidwell [49]
Limnodynastes dorsalis	Roundup	4.6	3.0	2.3			Mann and Bidwell [49]
Pipidae							
Xenopus laevis	Vision			0.88			Edgington et al. [54]
Ranidae							
Rana cascadae	Roundup Original Max			1.7			Relyea and Jones [52]
Rana cascadae	Roundup	1.6			1.1	1.0	King and Wagner [53]*
Rana catesbeiana	Roundup					1.6	Relyea [56]*
Rana catesbeiana	Roundup Original Max			0.8			Relyea and Jones [52]
Rana catesbeiana	Roundup			2.8			Fuentes et al. [57]

ORDER, Family and *Species*	Formulation	1 day	2 days	4 days	7-10 days	15-16 days	References
Rana catesbeiana	Roundup Original Max			2.2			Jones et al. [61]
Rana clamitans	Vision			1.4			Edgington et al. [54]
Rana clamitans	Roundup Original	2.0		2.0			Howe et al. [55]
Rana clamitans	Roundup					1.6	Relyea [56]*
Rana clamitans	Roundup Original Max			1.4			Relyea and Jones [52]
Rana clamitans	Roundup			4.2			Fuentes et al. [57]
Rana clamitans	Roundup Original Max			2.6			Jones et al. [61]
Rana luteiventris	Roundup	1.2		0.9	0.81	0.74	King and Wagner [53]*
Rana pipiens	Vision			1.1			Edgington et al. [54]
Rana pipiens	Roundup Original	3.7		2.9			Howe et al. [55]
Rana pipiens	Roundup					1.8	Relyea [56]*
Rana pipiens	Roundup Original Max			1.5			Relyea and Jones [52]
Rana pipiens	Roundup			1.8			Fuentes et al. [57]
Rana sphenocephala	Roundup			2.1			Fuentes et al. [57]
Rana sylvatica	Roundup Original	5.6		5.1			Howe et al. [55]
Rana sylvatica	Roundup					1.0	Relyea [56]*
Rana sylvatica	Roundup Original Max			1.9			Relyea and Jones [52]

*Recalculated from mg AI L^{-1}. 4 d values in parentheses were estimated by interpolation or extrapolation, as described in the text

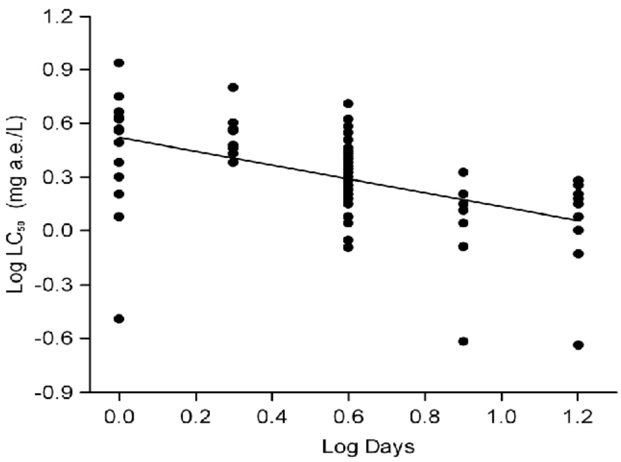

Figure 1 The slow decrease in LC_{50} for glyphosate formulations with increasing exposure time from 1-16 days, both log scales. The regression equation is log LC_{50}=0.519-0.385 log Days. Regression $F_{1,81}$=27.4, $P < 0.001$, r^2_{adj}=24.2%. The slope corresponds to a decrease of LC_{50} by a factor of 0.77 for a doubled exposure time.

upper end of the reported range, 207 mg/L at 24 h, compared to 320 and 204 mg/L for *Adelotus brevis* and *Limnodynastes peronii*, interestingly both members of the rarely-studied family Myobatrachidae. In contrast toxicity to the Leptodactylid *E. pustulosus* was greater than to any other amphibian studied. These results suggest that taxonomic variation of the effects of paraquat may be important in amphibians **(Tables 6 and 7)**.

Toxicity of (Karmex) diuron to amphibians is also apparently highly variable, although based on only two studies. Schuytema and Nebeker [48] reported much lower toxicity of diuron to amphibians than found here, although their study used technical grade pesticide, suggesting that additives in commercial formulations may be important in toxicity, as in glyphosate. Toxicity of thiocyclam and fenobucarb is also rather variable, although the only other reported values were obtained from the PAN pesticides database and original studies could not be located. For both these pesticides values of LC_{50} found here were above those reported previously (for two species of Ranidae), although not markedly so. We conclude that further data are required to assess the toxicity of all five pesticides and that work on these is more critical than further study of glyphosate [49-51].

Interspecies correlation

A comparison of the Resistance factors (RF) derived from the ratio of the 96 h LC_{50} between the two species is given in **Table**

Table 6 Comparative toxicity data (LC_{50}, mg AI L^{-1}) for amphibian larvae to the other five pesticides used in this study. The mean value is shown if >1 value was reported in a single study.

Pesticide and Species	Formulation	1 day	2 days	4 days	7-10 days	14+ days	Reference
Paraquat							
Adelotus brevis	Gramoxone	320	315	262			Johnson [63]
Anaxyrus fowleri	Chevron Co.	54	25	26			Sanders [64]
Bufo gargarizans	42% technical			8.4			Yin et al. [65]
Bufo gargarizans	-			20			PAN Pesticides Database
Bufo japonicus	-	9.6	14				PAN Pesticides Database
Engystomops pustulosus	Gramoxone Super	14	2.9	0.20			This study
Limnodynastes peronii	Gramoxone	204	153	100			Johnson [63]
Pseudacris triseriata	Chevron Co.	43	37	28			Sanders [64]
Rana pipiens	Paraquat Plus			0.5	1.6	1.6	Linder et al. [66]
Rana pipiens	Technical grade			1.3	4.2	3.1	Linder et al. [66]
Rhinella marina	Gramoxone Super	207	172	56			This study
Scinax nasicus	Gramoxone Super	39	30	22			Lajmanovich et al. [67]
Xenopus laevis	Paraquat Plus			8.1	4.2	3.2	Linder et al. [66]
Xenopus laevis	Technical grade				6.2	6.2	Linder et al. [66]
Diuron							
Engystomops pustulosus	Karmex	1.3	1.1	0.20			This study
Pseudacris regilla	99.8% pure					15	Schuytema and Nebeker [48]
Rana aurora	99.8% pure					22	Schuytema and Nebeker [48]
Rana catesbeiana	99.8% pure					13	Schuytema and Nebeker [48]
Rhinella marina	Karmex	4.4	3.0	1.1			This study
Xenopus laevis	99.8% pure					11	Schuytema and Nebeker [48]
Imidacloprid							
Engystomops pustulosus	Revelo 350CS	283	233	197			This study
Fejervarya limnocharis	95% pure	235	165	82			Feng et al. [44]
Pelophylax nigromaculatus	95% pure	268	219	129			Feng et al. [44]
Rhinella marina	Revelo 350CS	102	95	65			This study
Thiocyclam							
Engystomops pustulosus	Evisect S	7.5	4.1	1.6			This study
Fejervarya limnocharis	-		0.059				PAN Pesticides Database
Pelophylax porosus	-		1.0				PAN Pesticides Database
Rhinella marina	Evisect S	4.6	1.7	0.40			This study
Fenobucarb							
Engystomops pustulosus	BPMC	22	15	1.0			This study
Fejervarya limnocharis	BPMC		8.6				PAN Pesticides Database
Rhinella marina	BPMC	19	15	15			This study

Table 7 Variability of LC_{50} for pesticides (data from Tables 5 and 6) and variance ratio test comparing the variability with that of glyphosate.

Pesticide	Log LC_{50} ± SD	*n*	s^2	Variance ratio *F*; df	*P*
Glyphosate	0.297 ± 0.282	83	0.0795	-	-
Paraquat	1.223 ± 0.812	36	0.659	8.29; 35,82	<0.001
Diuron	0.529 ± 0.655	10	0.429	5.40; 9,82	<0.001
Imidacloprid	2.191 ± 0.219	12	0.0480	1.66; 82,11	>0.20
Thiocyclam	0.120 ± 0.679	8	0.461	5.80; 7,82	<0.001
Fenobucarb	1.012 ± 0.464	7	0.215	2.70; 6,82	<0.05

8 [52]. A RF>1 indicates that the species 1 is more resistant to the toxicant than species 2, while a value <1 indicates that the first species was less resistant than the second to toxicant. For *E. pustulosus* and *R. marinus* the RF ranged between <0.01 and 4.13 which suggests that in the aqueous phase, *E. pustulosus* was more sensitive to four of the six pesticides tested. However for the soil/water test, the RF ranged between <0.01 and 0.38 which suggests that the soil had a greater effect of increasing the toxicity of the pesticide to *E. pustulosus* which was then more sensitive to all six pesticides **(Table 8)** [53-58].

The interspecies toxicity performed between the two species for both the aqueous phase and the soil/water test gave an R^2 value of 0.21 for the aqueous phase while for the soil/water test the

Table 8 Resistance factors for both species following pesticide exposure under two conditions, aqueous phase and soil/water test (Species 1 PP*/ Species 2 BM*).

Pesticide	Aqueous phase	Soil/water test
Relevo 350CS	3.01	0.38
Roundup Ultra	0.82	0.08
Evisect S	4.13	0.33
BPMC	0.07	0.11
Gramoxone Super	<0.01	<0.01
Karmex	0.21	0.21

*PP=*Physalaemus pustulosus,* *BM=*Bufo marinus*

value was 0.50 [59-61]. Although there was a positive correlation in both the aqueous phase and the soil/water mixture, the low values of R^2 show that the relative sensitivity to the six pesticides differed between the two species [62-66]. There were complex interactions between species, type of pesticide, and environment (presence or absence of soil) among these data [67]. It suggests that both species show different responses and it is unlikely that data collected for anyone species can be used to predict toxicological responses in the other **(Figures 2 and 3)**.

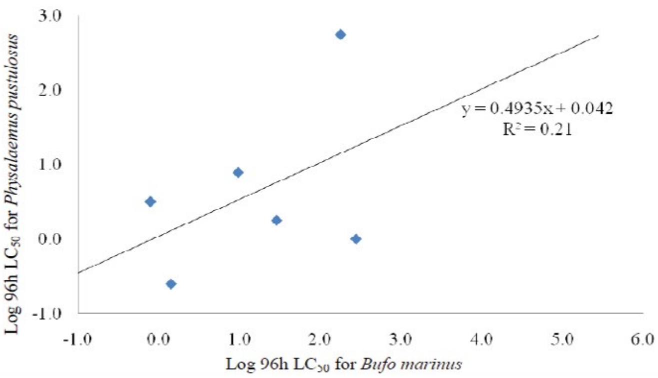

Figure 2 Interspecies correlation for *Physalaemus pustulosus* and *Bufo marinus* exposed in the aqueous phase.

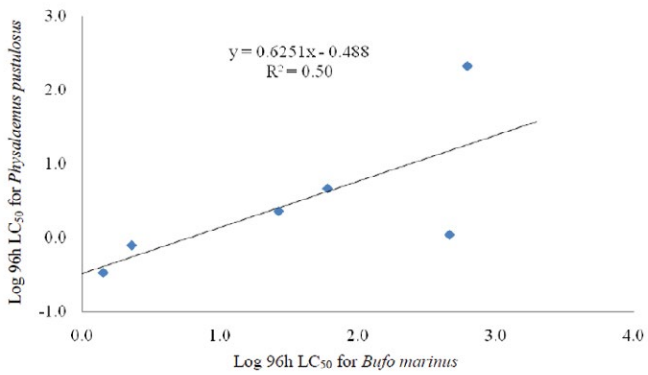

Figure 3 Interspecies correlation for *Physalaemus pustulosus* and *Bufo marinus* exposed in the soil/water mixture.

Conclusion

The toxicity of roundup to *E. pustulosus* and *R. marinus* were similar to those reported for other amphibian species. However gramoxone proved to be more toxic that other amphibians reported in the literature. Soil mixtures also increased the toxicity of the pesticides to *E. pustulosus* when compared to *R. marinus*. Responses of both species also differ, and it is unlikely toxicological data for one species can be used to predict responses in the other.

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