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THE TOXICITY OF ROUNDUP ORIGINAL MAX® TO 13 SPECIES OF LARVAL AMPHIBIANS

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Abstract—With the increased use of glyphosate-based herbicides (marketed under several names, including Roundup® and Vision®), there has been a concomitant increased concern about the unintended impacts that particular formulations containing the popular surfactant polyethoxylated tallowamine (POEA) might have on amphibians. Published studies have examined a relatively small number of anuran species (primarily from Australia and eastern North America) and, surprisingly, no species of salamanders. Using a popular formulation of glyphosate (Roundup Original Max®), the goal of the present study was to conduct tests of lethal concentrations estimated to kill 50% of a population after 96 h ($LC50_{96\text{-}h}$) on a wider diversity of species from both eastern and western North America. Tests were conducted on nine species of stage 25, larval anurans from three families (Ranidae: *Rana pipiens*, *R. clamitans*, *R. sylvatica*, *R. catesbeiana*, *R. cascadae*; Bufonidae: *Bufo americanus*, *B. boreas*; and Hylidae: *Hyla versicolor*, *Pseudacris crucifer*) and four species of larval salamanders from two families (Ambystomatidae: *Ambystoma gracile*, *A. maculatum*, *A. laterale*; and Salamandridae: *Notophthalmus viridescens*). For the nine species of larval anurans, $LC50_{96\text{-}h}$ values ranged from 0.8- to 2.0-mg acid equivalents per liter with relatively little pattern in differential sensitivity among the species or families. The four species of larval salamanders were less sensitive than the anurans, with $LC50_{96\text{-}h}$ values ranging from 2.7- to 3.2-mg acid equivalents per liter and no substantial differences among the species of salamanders. This work substantially increases the available data on amphibian sensitivity to glyphosate formulations that include either POEA surfactants or the equally moderately to highly toxic surfactants of Roundup Original Max and should be useful for improving future risk assessments.

Keywords—Tadpole Caudata Ecotoxicology Nontarget Pesticide

INTRODUCTION

Pesticides can provide tremendous benefits to society, including increased crop production and improved human health. These benefits occur by designing pesticides that target particular pest species, yet there is also the need to assess the potential impact on nontarget organisms. The herbicide glyphosate (marketed under names including Roundup® and Vision®; Monsanto) is an excellent example. It is the number one herbicide in the world because it is highly effective at promoting conifer release in forest management and controlling a diversity of agricultural weeds. Moreover, its use has recently surged due to the increased planting of genetically modified, glyphosate-resistant crops [1–2]. In recent years, however, there has been increased attention to the impact that glyphosate formulations containing the popular surfactant polyethoxylated tallowamine (POEA) might have on amphibians [3–12].

A number of risk assessments have been conducted on glyphosate-based products for nontarget animals. In the case of amphibians, because interest in the group is relatively recent, there are few amphibian data upon which to base these assessments [13–15]. A recent review of glyphosate impacts on amphibians conducted by the British Columbia Ministry of Environment [2] identified significant knowledge gaps, including a general lack of data for larval anurans from western North America and a complete lack of information for any species of salamanders. The present study addressed this knowledge gap by conducting toxicity tests on nine species of larval anurans and four species of larval salamanders from both eastern and western North America to determine any interesting species-, family-, or order-level patterns in amphibian sensitivity and to provide a substantial amount of comparative data for future risk assessments.

MATERIALS AND METHODS

We examined the toxicity of Roundup Original Max®, which contains 48.7% of active ingredient (i.e., glyphosate) and a surfactant (S. Mortensen, Monsanto, personal communication). The function of the active ingredient is to inhibit the synthesis of aromatic amino acids, whereas the function of the surfactant is to penetrate the waxy leaf cuticle and thereby allow the active ingredient to penetrate into the leaves. Different formulations of Roundup have different surfactants, and all surfactants are classified as inert ingredients that do not require testing or listing on the formulated product. As a result, manufacturers are continually developing new surfactants, and this calls for continued testing of formulated products.

To develop a relatively fine resolution on the estimates of the lethal concentration that would cause 10, 50, and 90% mortality (i.e., LC10, LC50, and LC90), we tested amphibian larvae under six nominal concentrations (0-, 1-, 2-, 3-, 4-, and 5-mg acid equivalents [a.e.] per liter). Given that previous studies suggested that the LC50 values were likely between 1- and 5-mg a.e./L [10], this series of evenly spaced concentrations better suited to our goal than using a geometric series of concentrations. All solutions were made in large batches (using carbon-filtered, ultraviolet-irradiated well water) and then distributed into the appropriate containers. A sample of each

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Table 1. The species of larval amphibians used in the experiments, including their initial mass (mean ± 1 standard error) and developmental stage[a]

Common name	Latin name	Family	Egg masses	Mass (mg)	Gosner stage
Wood frog	*Rana sylvatica*	Ranidae	20	95 ± 5	25
Leopard frog	*Rana pipiens*	Ranidae	1	52 ± 4	25
Cascades frog	*Rana cascadae*	Ranidae	5	110 ± 6	25
Green frog	*Rana clamitans*	Ranidae	15	23 ± 2	25
American bullfrog	*Rana catesbeiana*	Ranidae	5	34 ± 2	25
American toad	*Bufo americanus*	Bufonidae	10	25 ± 1	25
Western toad	*Bufo boreas*	Bufonidae	5	32 ± 1	25
Gray tree frog	*Hyla versicolor*	Hylidae	14	43 ± 2	25
Spring peeper	*Pseudacris crucifer*	Hylidae	26	14 ± 1	25
Northwestern salamander	*Ambystoma gracile*	Ambystomatidae	8	78 ± 6	NA[b]
Spotted salamander	*Ambystoma maculatum*	Ambystomatidae	24	39 ± 4	NA
Blue-spotted salamander	*Ambystoma laterale*	Ambystomatidae	10	109 ± 8	NA
Red-spotted newt	*Notophthalmus viridescens*	Salamandridae	30	207 ± 33	NA

[a] Mass data are based on a sample of 20 individuals.
[b] NA = not applicable. Gosner [16] stages do not apply to salamander larvae.

solution was saved in precleaned glass amber jars and shipped to the Mississippi State Chemical Laboratory for independent analysis of glyphosate concentration. To confirm the accuracy of the nominal concentrations, water was sampled from one of the large batches that represented seven species pooled together. These analyses determined that the five nominal concentrations of glyphosate produced actual concentrations of 1.12, 2.11, 3.03, 4.03, and 5.26 mg a.e./L (hereafter termed 1, 2, 3 4, and 5 mg a.e./L for simplicity). The zero concentration was not tested, but tests of the well water have indicated that glyphosate is not present.

The tested species spanned a range of amphibian phylogeny including true frogs (Ranidae), tree frogs (Hylidae), toads (Bufonidae), mole salamanders (Ambystomatidae), and newts (Salamandridae). When conducting such comparative studies, one can examine the different species at the same age, the same mass, or the same developmental stage and each approach is certainly defendable. We chose to test the sensitivity of the larvae to glyphosate when they were relatively early in development (e.g., anuran species at Gosner [16] stage 25; Table 1). All amphibians were collected from natural ponds and wetlands as newly oviposited eggs. For each species, an average of 13 egg masses from natural populations (Table 1) was collected. Experimental animals for all test concentrations were drawn from a mixture of larvae from all egg masses.

The tadpoles and salamanders were raised using static renewal in two types of containers. Tadpole experiments were conducted in 14-L plastic (i.e., polypropylene) tubs holding 8 L of water. For each tadpole species, we replicated the six nominal concentrations four times for a total of 24 experimental units. Each experimental unit contained 10 tadpoles. The larval salamander experiments were conducted in uncovered, 150-ml glass Petri dishes containing 100 ml of water at a density of one larva per dish. Raising larval salamanders singly is preferred because they commonly cannibalize one another when put together. For each salamander species, we replicated the six nominal concentrations eight times for a total of 48 experimental units. At 24-h intervals, we quantified survival, removed any dead animals, and conducted complete water changes. On either the first or second day of each of the experiments, the dissolved oxygen, temperature, and pH of the water were quantified in the anuran experiments. These values were similar among treatments and among species (e.g., among species, dissolved oxygen = 6.1–7.1 mg/L; temperature = 17.9–20.5°C; pH = 7.8–8.0). These measurements were difficult to measure in the shallow Petri dishes holding the salamanders, so we made the assumption that the values across nine species of anurans were representative of the values in the salamander dishes, given that both groups were simultaneously raised under the same laboratory conditions and test solutions were prepared from the same stock solutions.

Statistical analyses

The mortality data from the experiments were analyzed in two ways. For the anurans, the data were nonnormally distributed and had heteroscedastic errors. As a result, we analyzed the anuran mortality data using nonparametric Kruskal-Wallis tests. Following this test, we then conducted nonparametric Dunnett's tests for mean comparisons to determine the lowest concentration that caused significantly higher mortality than the controls [17]. Raising the salamander larvae singly precluded conducting a Kruskal-Wallis test on the four salamander species. For both the anurans and salamanders, we estimated $LC10_{96\text{-h}}$, $LC50_{96\text{-h}}$, and $LC90_{96\text{-h}}$ values, with 95% confidence intervals, using probit analyses.

RESULTS

Across all 13 species, survival in the control treatments remained high after 96 h. For the larval anurans (Fig. 1), survival in the controls was 100% for leopard frogs, American toads, western toads, green frogs, and bullfrogs; 97% for wood frogs, Cascades frogs, and gray tree frogs; and 90% for spring peepers. For larval salamanders, survival in the controls was 100% for the northwestern salamanders, blue-spotted salamanders, and red-spotted newts and 87% for spotted salamanders (Fig. 2).

For all nine species of larval anurans, the Kruskal-Wallis analyses detected significant effects of pesticide concentration on mortality ($p \leq 0.002$; Fig. 1). The subsequent mean comparisons, using Dunnett's tests, indicated the lowest concentrations that caused significantly greater mortality than the control ($p < 0.05$). For two species (bullfrogs and spring peepers), 1 mg a.e./L of glyphosate caused significantly greater mortality than the control. For the remaining seven species (green frogs, leopard frogs, wood frogs, Cascades frogs, American toads, western toads, and gray tree frogs), 2 mg a.e./L of glyphosate was the lowest concentration to cause significantly greater mortality than the control. Based on the probit analyses, the estimated $LC50_{96\text{-h}}$ values for the nine species of larval anurans ranged from 0.8 to 2.0 mg a.e./L (Table 2).

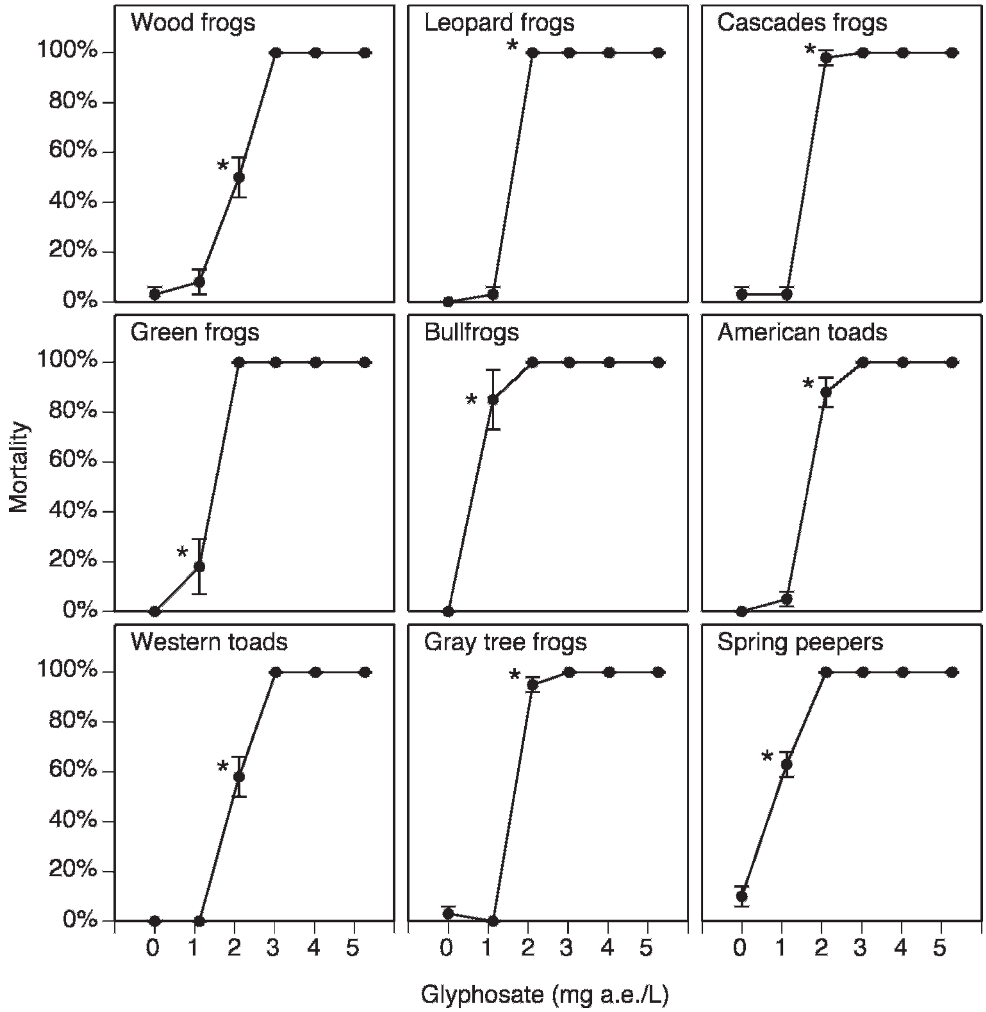

Fig. 1. Dose–response curves for nine species of larval anurans exposed to 0 to 5.26 mg acid equivalents per liter of Roundup Original Max. Asterisks indicate the lowest concentration at which mortality was significantly greater than the control treatment. Means ± 1 standard error are based on four replicates of each test concentration.

For the four salamander species, Kruskal-Wallis tests could not be conducted, but we were able to estimate $LC10_{96\text{-h}}$, $LC50_{96\text{-h}}$, $LC90_{96\text{-h}}$ values (Fig. 2 and Table 2). The three species of ambystomatid salamanders (northwestern, spotted, and blue-spotted) all had similar $LC50_{96\text{-h}}$ values: 2.8 mg a.e./L, 2.8 mg a.e./L, and 3.2 mg a.e./L, respectively. Although from a different family (Salamandridae), the red-spotted newt also had a similar $LC50_{96\text{-h}}$ value of 2.7 mg a.e./L.

DISCUSSION

The results of the present study provide toxicity estimates across a diverse group of larval amphibians for a popular formulation of a leading herbicide (Roundup Original Max). We found that 96-h exposures under static-renewal conditions resulted in LC50 estimates of 0.8 to 2.0 mg a.e./L for larval anurans and 2.7 to 3.2 mg a.e./L for larval salamanders. It was particularly striking that many of the species had a substantial increase in mortality with an increase of only 1 mg a.e./L (usually 1–2 mg a.e./L for larval anurans and 2–3 mg a.e./L for larval salamanders). Based on these data and toxicity categories defined by the U.S. Fish and Wildlife Service and U.S. Environmental Protection Agency, Roundup Original Max would be classified as moderately toxic ($1 < LC50 < 10$ mg a.e./L) to larval salamanders and moderately to highly toxic ($0.1 < LC50 < 1$ mg a.e./L) to larval anurans, depending on the species. Importantly, the present study used larvae that were all early in ontogeny. No data appear to examine whether early ontogenetic stages are more or less sensitive than much older ontogenetic stages (but see [18]). However, previous studies have demonstrated that the POEA surfactant (an inert ingredient that is not subject to testing or registration) is the component of Roundup Original Max that actually causes amphibian mortality [4,18].

The present study not only produced LC50 data on previously examined species [5,8,10,18] but also included one species of larval anuran from eastern North America (spring peepers) and two species of larval anurans from western North America (Cascades frogs and western toads) that had not been previously tested against Roundup formulations containing surfactants. The $LC50_{96\text{-h}}$ estimates from these species were in line with those from the other species, suggesting that all nine species of larval anurans (across three families) have similar sensitivities to other glyphosate formulations containing the POEA surfactant.

Despite increased efforts at testing agrochemical effects on amphibians, our work with salamanders addresses a critical gap focused on the sensitivity of larval salamanders to Roundup formulations. In contrast to frogs and toads, no

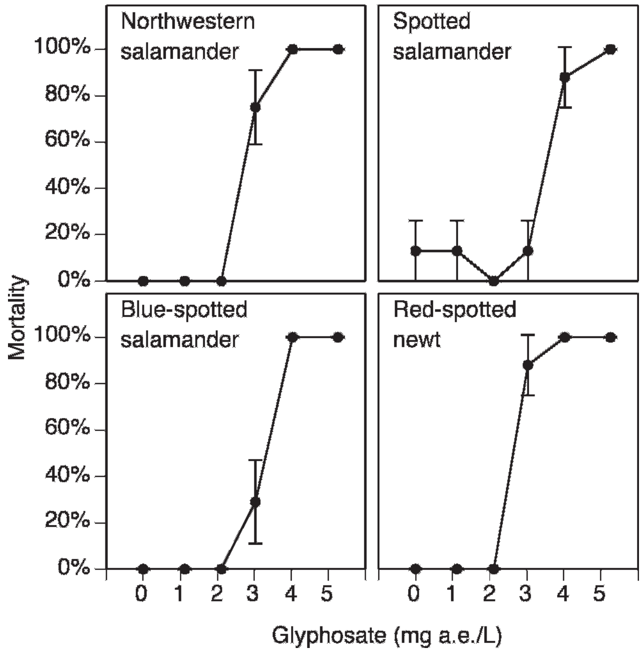

Fig. 2. Dose–response curves for four species of larval salamanders exposed to 0 to 5.26 mg acid equivalents per liter of Roundup Original Max. Means ± 1 standard error are based on eight replicates of each test concentration.

data have been published regarding the toxicity of Roundup formulations to salamanders. Results of the present study suggest that larval salamanders from two families are somewhat less sensitive than larval anurans. More studies on additional families of anurans and salamanders need to be conducted before we can draw strong conclusions about the sensitivity of amphibians in general.

A number of recent studies have examined the toxicity of Roundup formulations containing the POEA surfactant. In four species of Australian tadpoles (*Lymnodynastes dorsalis*, *Heleioporus eyrei*, *Crinia insigniferia*, and *Litoria moorei*), $LC50_{48\text{-h}}$ values ranged from 2.9 to 11.6 mg a.e./L [3–4]. Edginton et al. [6] conducted tests on four species of tadpoles from Africa and North America (African clawed frog [*Xenopus laevis*], American toads, green frogs, and leopard frogs) at a pH of either 6.0 or 7.5. Their $LC50_{96\text{-h}}$ estimates ranged from 1.8 to 3.5 mg a.e./L at the lower pH and 0.9 to 1.7 mg a.e./L at the higher pH. Howe et al. [18] examined the toxicity of glyphosate and POEA at two developmental stages in four species of North American tadpoles: leopard frogs, wood frogs, green frogs, and American toads. The $LC50_{96\text{-h}}$ estimates ranged from 6.5 to more than 8 mg a.e./L for the less developed tadpoles (Gosner stage 20) and ranged from 2.0 to less than 4 mg a.e./L for the more developed tadpoles (Gosner stage 25, the same stage used in the current study). Using longer exposure times (16 d), Relyea [10] exposed six species of North American tadpoles (bullfrogs, green frogs, leopard frogs, wood frogs, American toads, and gray tree frogs) to a range of concentrations (0–15 mg a.e./L of glyphosate and POEA) crossed with the presence or absence of predator cues. Despite the lower resolution that comes with widely spaced concentrations, the estimated $LC50_{16\text{-d}}$ values from Relyea [10] (0.4–1.8 mg a.e./L) were quite similar to those in the current study, which used shorter exposure times with more finely spaced concentrations. Collectively, these data suggest that glyphosate formulations containing the POEA surfactant have LC50 estimates that range from 0.4 to 11.6 mg a.e./L and that these estimates are strongly affected by pH, developmental stage, and phylogeny.

The toxicity estimates in the current study also are in general agreement with results from many experiments conducted under more natural conditions. For example, in outdoor mesocosms designed to simulate replicated natural ponds and wetlands (with pH of ~8), a diverse wetland community containing five species of tadpoles (spring peepers, American toads, wood frogs, leopard frogs, and gray tree frogs) and exposed to 0 or 3 mg a.e./L of glyphosate and POEA experienced a 70% decline in amphibian species richness and an 86% decline in tadpole biomass compared to the controls [9]. In a subsequent study, 0 mg a.e./L or 3 mg a.e./L was added to outdoor mesocosms and crossed with three soil treatments (no soil, sand, or loam). Adding soil had no ameliorating effect on tadpole survival, but adding 3 mg a.e./L of glyphosate and POEA caused mortality to increase from 25 to 98% in gray tree frogs, 2 to 96% in leopard frogs, and 3 to 100% in American toads [11]. In a mesocosm experiment that used one-third as much formulated product (1 mg a.e./L), gray tree frogs experienced no significant mortality, leopard frogs experienced 29% mortality, and American toads experienced 71% mortality [12]. In short, the magnitude of mortality observed in outdoor mesocosm experiments is within the range of expectations based on the estimated $LC50_{96\text{-h}}$ values, although there appear to be larger differences among species under more natural mesocosm conditions than under laboratory conditions.

Table 2. The estimated lethal concentrations values, $LC10_{96\text{-h}}$, $LC50_{96\text{-h}}$, and $LC90_{96\text{-h}}$ (italic font), from probit analyses (±95% confidence intervals) on 13 species of larval amphibians that were exposed to 0 to 5.26 mg acid equivalents per liter [a.e./L] of Roundup Original Max

Species	$LC10_{96\text{-h}}$ (mg a.e./L)	$LC50_{96\text{-h}}$ (mg a.e./L)[a]	$LC90_{96\text{-h}}$ (mg a.e./L)
Wood frog (*Rana sylvatica*)	*1.3* (1.0, 1.5)	*1.9* (1.7, 2.1)	*2.8* (2.5, 3.2)
Leopard frog (*Rana pipiens*)	*1.2* (1.1, 1.4)	*1.5* (1.3, 1.8)	*1.8* (1.5, 2.3)
Cascades frog (*Rana cascadae*)	*1.2* (0.9, 1.4)	*1.7* (1.5, 1.8)	*2.1* (1.9, 2.4)
Green frog (*Rana clamitans*)	*1.0* (0.8, 1.2)	*1.4* (1.3, 1.6)	*1.8* (1.7, 2.2)
American bullfrog (*Rana catesbeiana*)	*0.5* (−0.6, 0.7)	*0.8* (0.3, 1.0)	*1.2* (1.0, 1.4)
American toad (*Bufo americanus*)	*1.2* (0.9, 1.3)	*1.6* (1.5, 1.8)	*2.1* (1.9, 2.4)
Western toad (*Bufo boreas*)	*1.7* (−0.4, 1.9)	*2.0* (1.7, 2.2)	*2.4* (2.3, 4.0)
Gray tree frog (*Hyla versicolor*)	*1.4* (1.0, 1.6)	*1.7* (1.5, 1.9)	*2.0* (1.9, 2.2)
Spring peeper (*Pseudacris crucifer*)	*0.1* (−0.3, 0.3)	*0.8* (0.7, 1.0)	*1.6* (1.4, 2.0)
Northwestern salamander (*Ambystoma gracile*)	*2.4* (−1.7, 2.7)	*2.8* (1.8, 3.3)	*3.3* (3.0, 6.2)
Spotted salamander (*Ambystoma maculatum*)	*2.4* (−1.7, 2.7)	*2.8* (1.8, 3.3)	*3.3* (3.0, 6.2)
Blue-spotted salamander (*Ambystoma laterale*)	*2.7* (0.6, 3.1)	*3.2* (2.7, 3.9)	*3.7* (3.4, 6.3)
Red-spotted newt (*Notophthalmus viridescens*)	*2.3* (0.5, 2.6)	*2.7* (2.1, 3.1)	*3.1* (2.8, 4.4)

[a] For very low LC50 values, the lower 95% confidence interval can extend beyond zero.

Consistent with these mesocosm results are the results from other experiments that incorporate natural conditions in tests of formulations containing glyphosate and POEA. For example, Chen et al. [5] exposed leopard frog tadpoles to natural pond water at two levels of pH (5.5 or 7.5) and a range of concentrations. They found that 1.5 mg a.e./L of glyphosate and POEA caused 35% mortality at the lower pH but 80% mortality at the higher pH [5]. The authors note that these results have important implications to amphibians given that the Canadian government's expected environmental concentration is 1.43 mg a.e./L [19]. Using in situ pond enclosures with a range of concentrations at two ponds that had different pH levels (6.4 or 7.0), Wojtaszek et al. [8] found that green frog tadpoles exhibited an $LC50_{96\text{-h}}$ of 4.3 mg a.e./L at the lower pH but 2.7 mg a.e./L at the higher pH. In the same experiment, leopard frog tadpoles exhibited an $LC50_{96\text{-h}}$ of 11.5 mg a.e./L at the lower pH but 4.3 mg a.e./L at the higher pH. In an experiment that took advantage of aerial applications of glyphosate and POEA to Canadian forests, Thompson et al. [7] placed small mesh cages containing tadpoles into wetlands that were directly oversprayed, wetlands that were adjacent to sprayed areas, and wetlands that had a vegetation buffer from sprayed areas (pH range 5–9). The directly oversprayed areas had a mean concentration of 0.33 mg a.e./L (with one wetland having 1.95 mg a.e./L), whereas the buffered wetlands experienced a mean concentration of 0.03 mg a.e./L (effectively serving as a control). After 48 h, leopard frogs and green frogs experienced 14 and 36% mortality, respectively, in the directly oversprayed wetlands. However, leopard frogs and green frogs experienced 15 and 26% mortality, respectively, in the buffered (i.e., control) wetlands, which makes it difficult to attribute mortality to any specific additive or compensatory cause. What is clear from a large proportion of existing laboratory, mesocosm, and in situ experiments, and has been noted by several of the above investigators, is that glyphosate formulations containing the POEA surfactant and Roundup Original Max (which contains an undivulged surfactant) have the same potential to cause substantial amphibian mortality at environmentally expected concentrations.

CONCLUSION

By incorporating a greater diversity of species and orders of amphibians, one can arrive at more general and defendable assessments of the impact of pesticides on nontarget organisms. With the 13 species examined in the present study, toxicologists now have a better sense of the magnitude of differences in sensitivity among species, families, and orders of amphibians. Moreover, toxicologists now possess a substantially larger collection of studies upon which to base risk assessments for Roundup Original Max and other glyphosate formulations that contain the POEA surfactant. When combined with increased efforts to assess amphibian exposures to these formulations, these data will be important in helping to arrive at reliable risk assessments for amphibians.

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