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TOXIC EFFECTS OF THE HERBICIDE ROUNDUP® REGULAR ON PACIFIC NORTHWESTERN AMPHIBIANS

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ABSTRACT—One of the most widely used herbicides for commercial and home use is glyphosate, the active ingredient in Roundup® Regular. We examined toxicity of the herbicide Roundup® on 6 amphibian species: *Ambystoma gracile*, *Ambystoma macrodactylum*, *Anaxyrus* [*Bufo*] *boreas*, *Pseudacris regilla*, *Rana cascadae*, and *Rana luteiventris*. Larvae were exposed to 6 different Roundup® Regular treatments (0 (control), 0.1, 0.5, 1.0, 2.0, and 5.0 mg AI/L dilutions of glyphosate) and monitored for 16 d. Estimated acute lethal concentrations at 24 h (LC50) varied significantly among species (ANOVA, $F_{(3,\ 56)} = 3.54$, $p < 0.0202$), with concentrations ranging from 0.43 mg AI/L of Roundup® for *P. regilla* to 2.66 mg AI/L for *A. boreas*. Bufonid and ambystomatid larvae were less sensitive than Ranid and Pseudacrid species tested, with no salamander larval mortality occurring at 24 h. Mean time-to-death varied from 1 d for *P. regilla* to 8.3 d for *A. gracile*, respectively (ANOVA, $F_{(5,\ 971)} = 108$, $p < 0.0001$). For exposure times longer than 24 h, the *A. boreas* was not significantly different than the salamanders for time-to-death, based on Tukey-Kramer comparisons. Results suggest Roundup® Regular is highly toxic to the amphibians at levels below EPA standards for drinking water and at concentrations they may be exposed to during overspray. We recommend the use of less toxic glyphosate-based herbicides in aquatic systems, if applications are necessary, or made during times of year when amphibian larvae are not present.

Key words: amphibian decline, anurans, herbicide, Pacific Northwest, pollutants, Roundup®, salamanders

Global decline of amphibians has been attributed to a wide variety of environmental stressors including habitat loss and fragmentation, ultraviolet radiation, climate change, virulent pathogens, and pollutants (Wake 1998; Alford and Richards 1999; Houlahan and others 2000; Blaustein and others 2003; Stuart and others 2004). The pattern of worldwide decline suggests individual amphibian species respond differently to various environmental stressors (Semlitsch and others 2000; Bridges and Semlitsch 2001; Bridges and others 2002). As a result, toxicological studies of potential environmental stressors are important for risk assessment and conservation management of amphibians.

Glyphosate-based herbicide formulations have been demonstrated to be highly toxic to amphibians (Bidwell and Gorrie 1995; Mann and Bidwell 1999; Bridges and Semlitsch 2000). Based on field observations of mortality after herbicide applications and the results of toxicological studies of glyphosate-based herbicides on amphibians, the Australian National Registration Authority for Agricultural and Veterinary Chemicals (NRA) restricted the use of 84 glyphosate herbicides in aquatic systems because of their harmful effects (National Registration Authority 1996; Tyler and Williams 1996; Mann and Bidwell 1999).

Toxicological studies of a few North American amphibians have demonstrated them to be highly sensitive to glyphosate-based herbicides, for both acute and chronic exposures (Howe and others 2004; Relyea 2005a). Acute lethal concentration (LC50) estimations for some eastern North American species are very low, ranging between 0.5 to 4.7 mg AI/L (AI refers to the concentrations of active ingredient glyphosate: Edginton and others 2004; Relyea 2005b). In addition to acute toxicity, which results in direct mortality, chronic exposure has had sublethal effects on amphibians by compromising survivability through indirect effects. For example, *Lithobates* [*Rana*] *pipiens* larvae exposed to the glyphosate-based herbicide Roundup® exhibited tail damage, gonadal abnormalities, decreased size, and a delayed rate of metamorphosis (Howe and others 2004). A similar study on *Rana cascadae* resulted in an increased rate of metamorphosis and decreased mass in newly metamorphed juveniles (Cauble and Wagner 2005). In addition, indirect impacts of herbicides may alter community structure and decrease species richness (Relyea and others 2005).

In the United States, glyphosate-based herbicides are used widely for home and commercial application, and restoration projects. In addition, many glyphosate-tolerant crop plants (Roundup® Ready) have been genetically engineered to enhance weed removal in agricultural systems by aerial spraying (Reddy 2001). Glyphosate-based herbicides are advertised as environmentally friendly because of glyphosate's specific targeted design to kill plants and its presumed low environmental persistence times of 40–60 d (Feng and others 1990; Giesy and others 2000). However, the combined herbicide formulation, which includes glyphosate (the active ingredient) and other inert compounds such as polyethoxylated tallowamine (POEA, the common surfactant in Roundup®), can have effects on non-targeted species and may persist long enough in the environment to potentially affect amphibians (Mann and Bidwell 1999).

Given the potential risks of glyphosate-based herbicides to amphibians, we investigated the toxicological effects on 6 different pond-breeding amphibian species. Our experiments consisted of static tests using potentially relevant environmental exposures with concentrations up to 5.0 mg AI/L. Previous studies of aerial applications to control plants in small wetlands demonstrated concentrations up to 2.6 mg AI/L, and glyphosate has been detected in other habitats up to 2.3 mg AI/L (Newton and others 1984; Goldsborough and Brown 1989; Feng and others 1990; Thompson and others 2004). Further, calculations of maximum application rates of Roundup® per the manufacturer's recommendation for a body of water with a mean depth of 15 cm suggest concentrations can reach 3.7 mg AI/L (Giesy and others 2000). In addition, this experiment was conducted using dilutions of Roundup® Regular, the commonly available formulation for home and garden use. This study provides results to evaluate and compare the potential sensitivity of the herbicide Roundup® on Pacific Northwestern amphibians.

METHODS

We exposed larvae of 6 amphibian species endemic to the Pacific Northwest to the herbicide Roundup® Regular: Long-toed Salamander (*Ambystoma macrodactylum*), Northwestern Salamander (*Ambystoma gracile*), Western Toad (*Anaxyrus* [*Bufo*] *boreas*), Pacific Tree Frog (*Pseudacris regilla*), Cascades Frog (*Rana cascadae*), and the Columbia Spotted Frog (*Rana luteiventris*). Embryos were collected at localities throughout Kittitas County, Washington (UTM Zone 10,WGS84) during the Spring of 2005 and transported to the laboratory: Lion's Rock (682866 E, 5235779 N; *A. macrodactylum*); Swamp Lake (628517 E, 5239904 N; *A. gracile, A. boreas, P. regilla*, and *R. cascadae*); and Engelhorn Pond (686317 E, 5206226 N; *R. luteiventris*). Embryos were kept in separate glass aquaria for each species, aerated in pond water until 24 h after hatching, and then randomly assigned and exposed to treatments. All experiments were conducted in a controlled animal laboratory room at 18°C with a 12-h photoperiod.

Solutions were mixed from a commercially available formulation (Roundup®, active ingredient 50.2% glyphosate isopropylamine salt, Monsanto Company). All concentrations are reported as milligrams of active ingredient per liter (mg AI/L). Larvae were placed into one of 6 treatments, with 10 individuals/treatment and 3 replicates/treatment, consisting of a control 0, 0.1, 0.5, 1.0, 2.0, and 5.0 mg AI/L. Concentration of glyphosate in the stock solution and serial dilution was confirmed by Columbia Analytical Services (Kelso, WA) using high-pressure liquid chromatography (Cauble and Wagner 2005). Dilutions were made in spring water treated to buffer the pH. Treatment concentrations were chosen to represent concentrations of glyphosate that have been measured in natural bodies of water and include a worst case scenario of a direct overspray at maximum application rate (Giesy and others 2000). Dilutions were mixed in a common large 5L container and then 150 mL/experimental treatment replicate was dispensed to clean 250 mL beakers. Three replicates of each treatment were prepared for a total of 18 containers for each species. Larvae were randomly assigned to each treatment. We

TABLE 1. Lethal concentration 50% mortality (LC50) estimates calculated using the Trimmed Spearman-Karber method. Ninety-five percent confidence intervals of point estimates are in parentheses. NA represents no estimate due to lack of mortality.

Species	$LC50_{24\text{-h}}$	$LC50_{7\text{-d}}$	$LC50_{15\text{-d}}$
P. regilla	0.43 (0.38–0.51)	0.32 (0.26–0.38)	0.30 (0.24–0.36)
R. luteiventris	1.65 (1.09–1.91)	1.08 (0.91–1.23)	0.98 (0.84–1.10)
R. cascadae	2.11 (2.01–2.28)	1.40 (0.95–1.78)	1.33 (1.16–1.48)
A. [*Bufo*] *boreas*	2.66 (2.37–3.06)	2.08 (1.63–2.39)	1.95 (1.65–2.21)
A. macrodactylum	NA	1.85 (1.40–2.17)	1.55 (1.15–1.87)
A. gracile	NA	1.73 (1.43–2.02)	1.83 (1.36–2.21)

monitored and recorded larval mortality at the same time every day over a 16-d period.

Mortality was compared among species by analyzing the mean time-to-death and the LC50 in each treatment. The mean time-to-death calculations for each species and replicates were determined using analysis of variance statistical testing performed with NCSS software (Hintze 2001) and significant differences were analyzed using the Tukey-Kramer multiple comparisons test. LC50 is the concentration at which 50% of a test population dies when exposed to the chemical. Tests for LC50 at 24 h, 7 d and 15 d were determined for each species using the trimmed Spearman-Karber method (Hamilton and others 1977, 1978).

RESULTS

The 6 species of amphibians varied greatly in their toxicity responses to exposure of Roundup® Regular. Anurans were more directly sensitive than salamanders at 24 h with a higher incidence of mortality. The salamanders $LC50_{24\ h}$ could not be calculated for *A. macrodactylum* or *A. gracile* because less than half of the larvae died (Table 1). However, there were significant differences in toxicity for anuran larvae $LC50_{24\ h}$ (ANOVA, $F_{(3,\ 56)} = 3.54$, $p < 0.0202$; Fig. 1, Table 2), which varied from 0.43 mg AI/L for *P. regilla* to 2.66 mg AI/L for *A. boreas* (Fig. 1, Table 1). The toxicity for *P. regilla* at 24 h was significantly different (Tukey-Kramer, $p < 0.01$) than for the other anurans. No *P. regilla* tadpoles survived above concentrations of ≥1.0 mg AI/L during the initial 24-h exposure, while at the same concentration *A. boreas* and *A. gracile* had no mortality.

For longer exposure times, the 7-d LC50 showed no evidence for a difference among species (ANOVA, $F_{(5,\ 54)} = 1.26$, $p < 0.0754$; Fig 2, Table 2). However, for the 15-d LC50 values (ANOVA, $F_{(5,\ 54)} = 2.124$, $p < 0.0305$; Fig. 3, Table 2), there was a significant difference among *P. regilla* LC50 compared to the other species (Tukey-Kramer, $p < 0.001$). The LC50s for the longer exposure times of 7 and 15 d were lower compared to the 24-h LC50s for all species, and this allowed for LC50 calculations of salamander species.

Overall, there was a trend for decreased mean time-to-death with increasing Roundup® Regular concentration. For mean time-to-death, there was strong evidence for significant differences among treatments (ANOVA, $F_{(5,971)} = 790$; $p < 0.000001$; Fig. 4, Table 3). Most notably, all the anurans died within 24 h at the highest concentration.

There was also strong evidence for differences among species in their mean time-to-death (ANOVA, $F_{(5,\ 971)} = 108$, $p < 0.0001$; Fig. 4, Table 3). The responses clustered into 2 significantly different groups. The 1st group consisted of *P. regilla*, *R. cascadae*, and *R. luteiventris*,

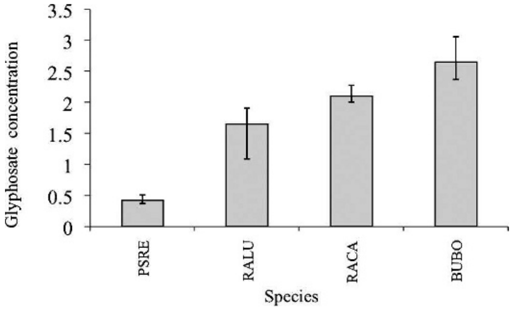

FIGURE 1. Lethal concentration (LC50) estimates at 24 h for amphibian larvae exposed to the herbicide Roundup® Regular (mg AI/L). $LC50_{24\text{-h}}$ estimates were calculated using the Trimmed Spearman-Karber method. Error bars are 95% confidence intervals of point estimates. Key: RACA *Rana cascadae*; RALU *Rana luteiventris*; PSRE *Pseudacris regilla*; BUBO *Anaxyrus* [*Bufo*] *boreas*.

TABLE 2. Analysis of variance of estimated LC50 for Roundup® Regular treatments at 24 h, 7 d, and 15 d.

Source of variation	MS	df	F	p
24 h	396.4	3	3.54	<0.0202
7 d	526.6	5	1.26	<0.0754
15 d	785.2	5	2.12	<0.0305
Error	512	1033		

with short mean time-to-death compared to the salamanders and *A. boreas* (Tukey-Kramer, $p < 0.05$). *Psuedacris regilla* was the most sensitive species with a mean time-to-death of 24 h for the 2.0 mg AI/L Roundup® treatment (Tukey-Kramer, $p < 0.001$), and *A. boreas* was the least sensitive (Tukey-Kramer, $p < 0.001$) with a mean time-to-death of 8.5 d for the 2 mg AI/L Roundup® treatment. In contrast, the mean time-to-death was 3.3 d for *R. cascadae* and 2.1 d for *R. luteiventris* for the 2 mg AI/L (Fig. 2).

DISCUSSION

All amphibians evaluated were sensitive to Roundup® Regular at low and potentially ecologically relevant concentrations. Roundup® Regular was particularly toxic to the anurans, directly causing mortality at low concentrations ($LC50_{24\text{-h}}$) ranging from 0.43 to 2.66 mg AI/L. Our calculated LC50 values were lower when compared to other studies. For example, acute estimated $LC50_{24\text{-h}}$ for *Lithobates* [*Rana*] *clamitans* and *Anaxyrus* [*Bufo*] *americanus* ranged from 2.0 to 4.2 mg AI/L (Howe and others 2004). Mann and Bidwell (1999) examined the toxic effects of different formulations of glyphosate on 6 different Australian species and found $LC50_{48\text{-h}}$ estimates ranging from 3.9 to 15.5 mg AI/L.

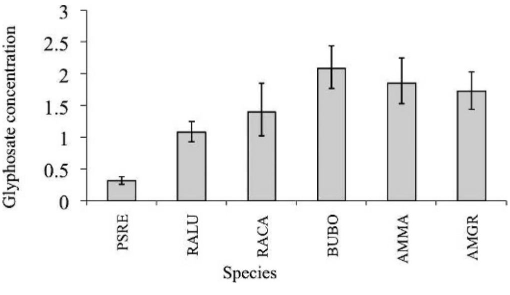

FIGURE 2. Lethal concentration (LC50) estimates at 7 d for amphibian larvae exposed to the herbicide Roundup® Regular (mg AI/L). $LC50_{24\text{-h}}$ estimates were calculated using the Trimmed Spearman-Karber method. Error bars are 95% confidence intervals of point estimates. Key: RACA *Rana cascadae*; RALU *Rana luteiventris*; PSRE *Pseudacris regilla*; AMGR *Ambystoma gracile*; AMMA *Ambystoma macrodactylum*; BUBO *Anaxyrus* [*Bufo*] *boreas*.

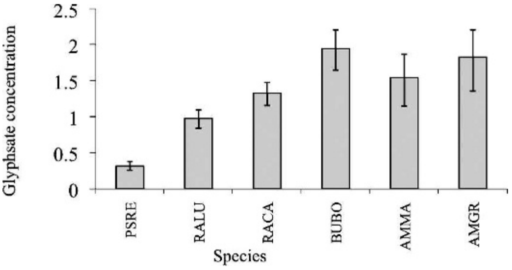

FIGURE 3. Lethal concentration (LC50) estimates at 15 d for amphibian larvae exposed to the herbicide Roundup® Regular (mg AI/L). $LC50_{24\text{-h}}$ estimates were calculated using the Trimmed Spearman-Karber method. Error bars are 95% confidence intervals of point estimates. Key: RACA *Rana cascadae*; RALU *Rana luteiventris*; PSRE *Pseudacris regilla*; AMGR *Ambystoma gracile*; AMMA *Ambystoma macrodactylum*; BUBO *Anaxyrus* [*Bufo*] *boreas*.

Our study assessed the toxicity of one of the most commonly available and used formulations of glyphosate-based herbicides, Roundup® Regular. Studies suggest glyphosate and associated surfactants in different formulations are the factors responsible for amphibian toxicity (Mann and Bidwell 1999; Howe and others 2004). Surfactants aid in the effectiveness of glyphosate as an herbicide. For example, Polyethoxylated tallowamine (POEA), the surfactant for Roundup® Regular, breaks down the cuticle on plant leaves and enhances the adsorption of glyphosate. POEA has been implicated in endocrine disruption, tail abnormalities, and intersex conversion in young metamorphs of *L. pipiens* (Howe and others 2004). Some studies suggest that the toxicity is mostly accounted for by POEA (Mann and Bidwell 2000; Tsui and Chu 2003); however, most available formulations in the United States use surfactants that may be toxic to amphibians. The Roundup® Regular formulation we used had a surfactant included which is proprietary and unknown.

Based upon risk assessment of the toxic effects of some glyphosate-based herbicides on amphibians and non-target species, the Austra-

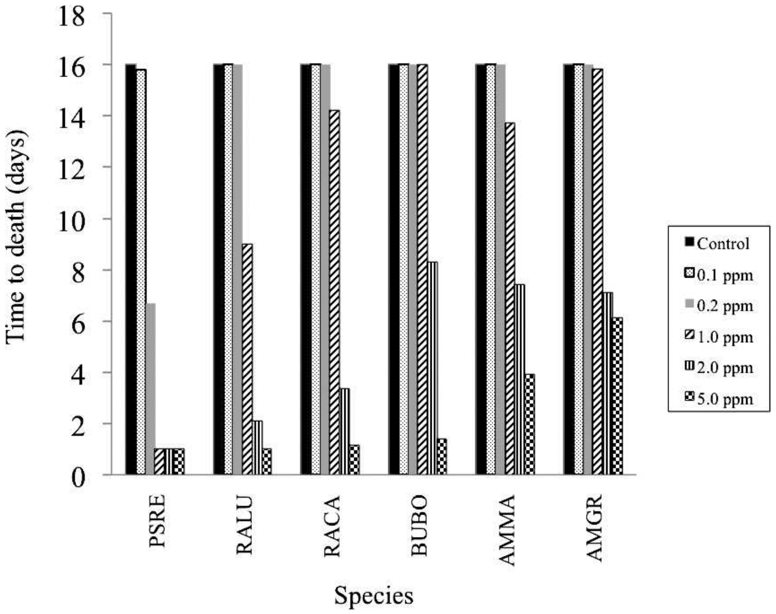

FIGURE 4. Mean Time to death in days for larvae exposed to Roundup® at increasing glyphosate concentrations from 0.1 mg AI/L to 5 mg AI/L. Key: RACA *Rana cascadae*; RALU *Rana luteiventris*; PSRE *Pseudacris regilla*; AMGR *Ambystoma gracile*; AMMA *Ambystoma macrodactylum*; BUBO *Anaxyrus* [*Bufo*] *boreas*.

lia NRA restricted their use in aquatic systems (National Registration Authority 1996). One formulation approved for use in Australia in aquatic systems is Roundup BiActive®, which in some cases is 100 times less toxic to amphibian larvae (Mann and Bidwell 1999). Roundup BiActive®, however, is not available for purchase in the United States and there exists no regulations for use of different glyphosate-based herbicide formulations or their surfactants in aquatic systems or terrestrial areas.

Roundup® Regular formulations toxic to amphibians are not intended for use in aquatic systems, but with overspray and the persistence time of the surfactants, toxic levels could accumulate in shallow ponds where amphibians breed (Relyea and others 2005). In addition, because of the biphasic life history of amphibians they can also be exposed in terrestrial environments. Indirect effects have been observed in simulated overspray experiments using pond mesocosms that resulted in direct killing of amphibians, significant shifts in larval food sources, and loss of predatory insects (Relyea and others 2005). Further evidence suggests glyphosate concentrations in surface waters can range from 0.1 to 2.3 mg AI/L (Newton and others 1984; Goldsborough and Brown 1989; Feng and others 1990), well within the range of acute $LC50_{24\text{-h}}$ values for our study amphibians. In addition, our results suggest that the $LC50_{24\text{-h}}$ for *P. regilla* of 0.43 mg AI/L is below EPA standards for drinking water (Environmental Protection Agency 2009).

Increasing evidence suggests synergistic factors rather than single factors most likely mediate amphibian declines (Blaustein and others 2003). Therefore, sublethal effects resulting from herbicide exposure may compromise individuals. Interaction experiments of the ubiquitous water mold pathogen, *Saprolegnia diclina*, and glyphosate increases embryo mortality and decreases hatching success (King and Wagner unpubl. data). Glyphosate-based herbicides are highly toxic to Pacific Northwestern amphibians and have the potential for syner-

TABLE 3. Analysis of variance for the time-to-death with respect to species and Roundup® treatments.

Source of variation	MS	df	F	p
Species	844	5	108.37	<0.00001
Treatment	6160	5	790.07	<0.00001
Error	7.78	971		

gistic interactions with other environmental stressors; therefore, we urge that less toxic formulations should be made widely available to the consumer. In addition, we suggest that in order to mitigate for potential harm caused by use of glyphosate-based herbicides, they be applied, if necessary, during seasonal periods when amphibian larvae are not present.

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