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COMMUNICATIONS

THE LETHAL IMPACT OF ROUNDUP ON AQUATIC AND TERRESTRIAL AMPHIBIANS

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Abstract. The global decline in amphibian diversity has become an international environmental problem with a multitude of possible causes. There is evidence that pesticides may play a role, yet few pesticides have been tested on amphibians. For example, Roundup is a globally common herbicide that is conventionally thought to be nonlethal to amphibians. However, Roundup has been tested on few amphibian species, with existing tests conducted mostly under laboratory conditions and on larval amphibians. Recent laboratory studies have indicated that Roundup may be highly lethal to North American tadpoles, but we need to determine whether this effect occurs under more natural conditions and in post-metamorphic amphibians. I assembled communities of three species of North American tadpoles in outdoor pond mesocosms that contained different types of soil (which can absorb the pesticide) and applied Roundup as a direct overspray. After three weeks, Roundup killed 96–100% of larval amphibians (regardless of soil presence). I then exposed three species of juvenile (post-metamorphic) anurans to a direct overspray of Roundup in laboratory containers. After one day, Roundup killed 68–86% of juvenile amphibians. These results suggest that Roundup, a compound designed to kill plants, can cause extremely high rates of mortality to amphibians that could lead to population declines.

Key words: amphibian decline; frog; glyphosate; pesticide; pollutants; Roundup; toad; toxicology.

INTRODUCTION

Many amphibian species around the world are experiencing population declines (Alford and Richards 1999, Houlihan et al. 2001, Kiesecker et al. 2001). A number of factors may affect amphibian populations, including degraded habitats, depleted ozone, emergent diseases and pathogens, invasive predators and competitors, and the presence of pollutants (Berger et al. 1998, Wake 1998, Alford and Richards 1999, Kiesecker et al. 2001, Davidson et al. 2002, Lips et al. 2003). Pesticides are one type of environmental pollutant that may be an important cause of mortality because some declines are associated with a proximity to pesticides and agricultural areas (Davidson et al. 2002). However, the few pesticides that have been tested on amphibians rarely reduce survival under conditions and concentrations that typically occur in nature (Bridges 1997, Boone and Semlitsch 2001, 2002, but see Relyea and Mills 2001, Relyea 2003, 2004, 2005*b*).

One of the most ubiquitous pesticides used around the world is glyphosate (commercial names: Roundup, Rodeo, Aqua Master; manufactured by Monsanto Company, St. Louis, Missouri, USA). Glyphosate is a broad-spectrum herbicide that inhibits the synthesis of essential amino acids. It is widely used to control undesirable weeds in agriculture (e.g., Roundup-Ready corn and soybeans), forestry, aquatic habitats, and residences. Roundup is the most commonly used formulation, containing both the active ingredient (glyphosate) as well as a surfactant (e.g., POEA; polyethoxylated tallowamine) that allows penetration of plant cuticles. Other formulations, such as Rodeo, do not come with a surfactant, but require that one be added prior to application. The use of glyphosate in the United States has rapidly grown during the past decade, from the 17th most commonly used pesticide in 1987 (3–4 million kg of active ingredient) to the second most commonly used pesticide in 1999 (30–33 million kg of active ingredient) with annual applications on more than eight million ha (Donaldson et al. 2002; National Pesticide Use Database, *available online*).[2] As classified by the USEPA, glyphosate formulations are considered practically nontoxic to birds and mammals, moderately to practically nontoxic to fish and invertebrates, and slightly to moderately toxic to amphibians (Giesy et al. 2000). Thus, the conventional wisdom has been that the application of glyphosate, a chemical designed to kill plants, has minor effects on any animals that might be present.

For larval amphibians, glyphosate has been tested on relatively few species (Mann and Bidwell 1999, Perkins et al. 2000, Lajmanovich 2003) including only four species of tadpoles in North America (Smith 2001, Chen et al. 2004, Edginton et al. 2004, Howe et al. 2004, Thompson et al. 2004, Wojtaszek et al. 2004). Collectively, this represents <0.2% of amphibian spe-

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[2] ⟨www.ncfap.org/database /default.htm⟩

PLATE 1. An adult wood frog traveling through an agricultural field. Amphibians such as the wood frog experience high rates of death when exposed to the application of Roundup both in the aquatic larval stage and in the adult terrestrial stage. Photo credit: Nancy Schoeppner.

cies in the world. To achieve a general understanding of glyphosate's impacts, we need to expand our information on amphibians both taxonomically and geographically.

When examining the impacts of any pesticide on amphibians, it is important that we make the transition from the foundational laboratory studies to more realistic and natural experimental venues. For other pesticides, this transition has been made by conducting experiments in outdoor aquatic mesocosms (i.e., cattle tank experiments; Boone and Semlitsch 2001, 2002). For glyphosate, most amphibian experiments have been conducted in the laboratory. Only three studies using outdoor mesocosms have been conducted, and they have reached different conclusions, likely due to differences in both experimental venues and glyphosate formulations (Thompson et al. 2004, Wojtaszek et al. 2004, Relyea 2005*a*). When conducting mesocosm experiments, it is important that we include natural components that exist in nature but are missing from laboratory experiments including algae, zooplankton, leaf litter, and soil. The addition of such components can be critical. For example, glyphosate is absorbed by soils and subjected to microbial breakdown. As a result, it is widely accepted that soil rapidly removes the herbicide from aquatic environments and any lethal impacts are restricted to a relatively brief window of time (Giesy et al. 2000, Thompson et al. 2004, Wojtaszek et al. 2004). However, the impact of soil on amphibian survival with glyphosate has never been tested.

When conducting laboratory and mesocosm experiments with pesticides and amphibians, the vast majority of our knowledge comes from experiments on the larval stage (i.e., tadpoles; Mann and Bidwell 1999, Perkins et al. 2000, Boone and Semlitsch 2001, 2002, Relyea 2003, 2004, 2005*a*, *b*). However, many amphibians spent a large fraction of their life in the terrestrial stage. Herbicides such as glyphosate are widely applied to terrestrial environments (primarily for weed control in forestry and agriculture), yet tests of its impact on terrestrial (post-metamorphic) amphibians appear to be restricted to only two Australian species that were exposed to glyphosate dissolved in water (Mann and Bidwell 1999). We need to determine how a direct terrestrial application of glyphosate affects post-metamorphic anurans.

In this study, I addressed these challenges by investigating the impact of a common commercial form of glyphosate (Roundup ''Weed and Grass Killer'') on several species of North American anurans in both the aquatic and terrestrial stage. I tested the following hypotheses: (1) adding Roundup to mesocosm communities containing tadpoles will cause tadpole mortality, (2) the addition of soil to these mesocosms will ameliorate the lethal effects of Roundup, and (3) applying Roundup to juvenile anurans will cause juvenile mortality.

Methods

I conducted separate experiments on aquatic and terrestrial amphibians at the University of Pittsburgh's Pymatuning Laboratory of Ecology, Pennsylvania, USA. In the aquatic experiment, I used outdoor pond mesocosms, which are well-accepted experimental venues for understanding amphibian ecology (Morin 1981, Wilbur and Fauth 1990, Werner and Anholt 1996, Relyea 2002). I used a completely randomized design with a factorial combination of herbicide treatments (Roundup present or absent) and soil treatments (no soil, sand, loam). The six treatments were replicated five times for a total of 30 experimental units. The experimental units were 1200-L cattle watering tanks filled with 1000 L of well water (pH = 8). Each tank received either no soil, 19 L of sand, or 19 L of loam soil. Sand was purchased as bagged sand, whereas the loam was collected from a nearby field; both soil additions were sufficient to cover the bottom of the tanks. Thus, the loam treatment accurately represented the soil present in ponds that form in field depressions where many amphibians deposit their eggs. The loam soil was tested for composition (29.2% sand, 21.4% clay, and 49.4% silt; University of Connecticut Soil Nutrient Analysis Laboratory, Storrs, Connecticut, USA), but it was not tested for the presence of pesticides. However, no pesticides had been applied to this soil for several years. After applying the soil treatments, all tanks received 300 g of deciduous leaves (primarily *Quercus* spp.), 25 g of rabbit chow (for an initial nutrient source), and pond water containing algae and zooplankton. The mesocosms were set up on 1 May 2003.

On 19 May, after a periphyton community was established (the tadpole resource base), I added three species of naturally coexisting tadpoles to each tank: 20 leopard frogs (*Rana pipiens*), 20 American toads (*Bufo americanus*), and 20 gray tree frogs (*Hyla versicolor*). These densities (8 individuals/1 m^2) are well within the range of densities found in natural ponds (R. A. Relyea, *personal observations*). Tadpoles were collected as newly oviposited eggs (8–10 egg masses per species) from nearby ponds and allowed to hatch in outdoor pools containing well water until they were used in the experiment. Tadpoles were early in their development (Gosner stage ~25; Gosner 1960) and initial tadpole mass (mean ± SE) was as follows: leopard frogs = 45 ± 3 mg, toads = 18 ± 2 mg, and gray tree frogs = 7 ± 1 mg. Individuals used in the experiment were haphazardly selected from a mixture of the hatched egg masses. A sample of 20 tadpoles of each species was set aside to assess 24-h survival due to handling. Survival of these samples in 10-L laboratory tubs was high: leopard frogs = 100%, toads = 100%, and gray tree frogs = 85%.

Two days after adding the tadpoles to the experimental tanks, I applied the herbicide treatment using a commercially purchased form of glyphosate (Roundup ''Weed and Grass Killer''; 25.2% glyphosate plus the POEA surfactant). This concentration was confirmed by the Mississippi State Chemical Laboratory (Mississippi State, Mississippi, USA) using high-pressure liquid chromatography. I applied the maximum amount likely to occur in natural wetlands by simulating a direct overspray for controlling aquatic weeds or a flooded depression in an agricultural field. I applied herbicide at the manufacturer's recommended rate (as listed on the container; 1.6 mL active ingredient [AI]/m^2). Thus, I added 15 mL of Roundup to each pesticide tank and 15 mL of well water to each control tank. This created a glyphosate concentration of 3.8 mg of AI/L (for consistency, I report all aquatic concentrations as milligrams of active ingredient per liter; mg AI/L). This concentration is similar to the maximum concentration expected for aquatic habitats in nature when spraying for terrestrial or aquatic weeds (3.7 mg AI/L; Giesy et al. 2000), but higher than concentrations that have thus far been observed in nature (up to 2.6 mg AI/L; Newton et al. 1984, Goldsborough and Brown 1989, Feng et al. 1990, Thompson et al. 2004; L. M. Horner, *unpublished manuscript*). Thus, the concentration used represents a worst-case scenario. Importantly, the concentration used is lower than most LC50 estimates for Roundup on tadpoles (the concentration estimated to kill 50% of a population = 3.9–15.5 mg AI/L, Mann and Bidwell 1999; 12.4 mg AI/L, Perkins et al. 2000; 1.7 mg AI/L, Lajmanovich et al. 2003).

The aquatic experiment was terminated on 11 June 2003 (20 d after herbicide application) because the toads in the no-pesticide treatments were approaching metamorphosis. All tanks were drained of their water and the tadpoles were removed, counted, and weighed. The proportion of survivors of each species had heteroscedastic errors (which could not be corrected by transformation), so I first ranked the data and then analyzed the survival data with a multivariate analysis of variance (MANOVA). Significant multivariate effects were followed by univariate ANOVAs; mean comparisons were conducted using Fisher's least significant difference (LSD) test.

The terrestrial experiment was conducted using juvenile frogs and toads in three separate laboratory experiments. In this experiment, the aim was to simulate the impact of amphibians receiving a direct terrestrial overspray in an agricultural field. As a worst-case scenario, I assumed no interception by vegetation during application (the actual amount of interception by terrestrial vegetation will vary in real-world applications). While most tests of post-metamorphic amphibians immerse the animals in water with different pesticide concentrations, the species used in this study spend very little time near water (except during breeding) and would more likely be directly sprayed on land. Thus, I placed the metamorphs in dry 10-L plastic tubs that were lined with damp paper towels to permit the animals to remain hydrated.

I used post-metamorphic animals that were collected after emergence from natural ponds originally containing dozens of egg masses (wood frogs, *R. sylvatica* (see Plate 1); and Fowler's toads, *Bufo woodhousii fowleri*) or collected after emergence from mesocosms containing tadpoles raised from a mixture of 10 egg masses (gray tree frogs, *H. versicolor*). These species were chosen due to their availability and because we have data on these species (or their close relatives) in previous studies (Relyea 2005*a*, *b*). I separately reared the three species at a density of seven frogs per tub. Initial juvenile mass (means ± SE) was as follows: wood frogs = 338 ± 11 mg, tree frogs = 425 ± 26 mg, and toads = 471 ± 40 mg. There were two treatments (Roundup presence or absence) replicated four times for a total of eight experimental units per species. Based on the same application rate as above (1.6 mg AI/m^2), I sprayed 6.5 mL of Roundup (using a second purchased bottle, concentration = 1.9% glyphosate) into each Roundup-assigned tub (after adding the animals) and 6.5 mL of water to each control tub. After 24 h, I counted the number of survivors in all tubs. Because the data were heteroscedastic (which could not be corrected by transformation), I first ranked the survivorship data and then analyzed each species using analyses of variance (ANOVA).

Results

In the aquatic experiment, there was a significant multivariate effect of Roundup (Wilks' $F_{3,22} = 164.2$, $P < 0.001$) and soil (Wilks' $F_{6,44} = 2.2$, $P = 0.046$) on the survival of the tadpole community, but there was no Roundup-by-soil interaction (Wilks' $F_{6,44}$ = 164, $P = 0.108$; Fig. 1). Soil type had no significant impact on the survival of toad tadpoles ($P = 0.925$) and leopard frog tadpoles (univariate $P = 0.093$), but did have a small effect on tree frog tadpoles (univariate $P = 0.023$). Loam soil caused a small reduction in tree frog tadpole survival (8–9% across pesticide treatments) compared to either no soil ($P = 0.007$) or sand ($P = 0.064$). Roundup caused a large reduction in the survival of all three species of tadpoles (univariate tests; $P < 0.001$). Across all soil types, Roundup reduced tree frog tadpole survival from 75% to 2%, toad tadpole survival from 97% to 0%, and leopard frog tadpole survival from 98% to 4%. Across all species,

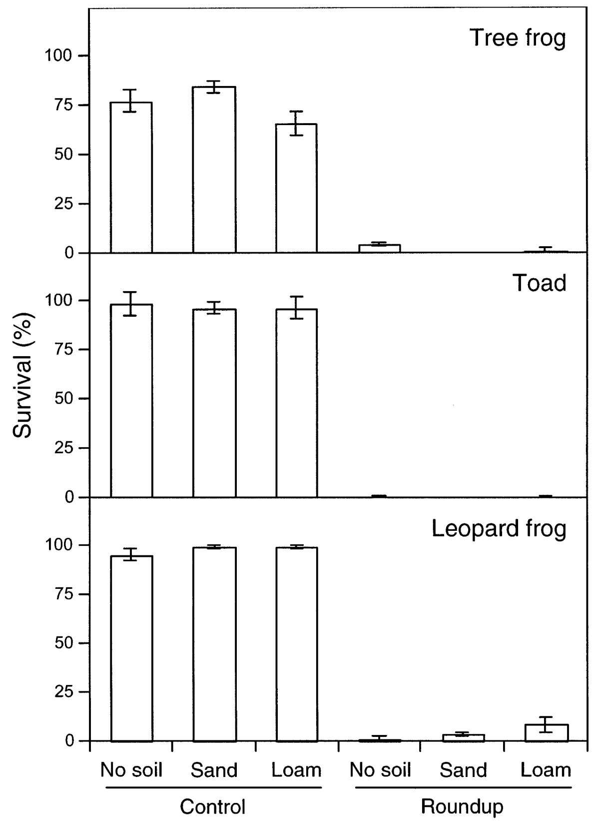

FIG. 1. The survival (mean ± SE) of three species of tadpoles (gray tree frog, *Hyla versicolor*; American toad, *Bufo americanus*; and leopard frog, *Rana pipiens*) reared in pond mesocosms when exposed to the presence or absence of Roundup (3.8 mg AI/L) crossed with three soil treatments (no soil, loam, or sand). The experiment lasted for 20 days.

only 2% of all tadpoles survived the Roundup application after three weeks.

In the terrestrial experiments, all three species suffered substantial mortality when exposed to Roundup (Fig. 2). After 24 hours, the application of Roundup reduced juvenile wood frog survival from 96% to 32% ($P = 0.002$), juvenile tree frog survival from 100% to 18% ($P = 0.001$), and juvenile toad survival from 100% to 14% ($P = 0.001$). Across all species, only 21% of all juvenile amphibians survived the Roundup application after one day.

Discussion

The most striking result from the experiments was that a chemical designed to kill plants killed 98% of all tadpoles within three weeks and 79% of all juveniles within one day. There have been only a few studies of Roundup's effects on tadpole mortality in the laboratory. Mann and Bidwell (1999) examined four species of Australian tadpoles (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis*, and *Litoria moorei*) and found $LC50_{48\text{-}h}$ values ranging from 3.9 to 15.5 mg AI/L for Roundup, 108 to 161 mg AI/L for technical grade glyphosate acid, and >450 mg AI/L for glyphosate isopropylamine salt (the latter two formulations of glyphosate lack the POEA surfactant). Perkins et al. (2000) examined the African tadpole *Xenopus laevis* and found $LC50_{96\text{-h}}$ values of 12.4 mg AI/L for Roundup, 6.8 mg/L for the POEA surfactant alone, and 9729 mg AI/L for Rodeo (an aquatic formulation of glyphosate that lacks the POEA surfactant). Lajmanovich et al. (2003) tested another formulation of glyphosate (GLYFOS, which also contains the POEA surfactant) on a South American tadpole (*Scinax nasicus*) and found an $LC50_{48\text{-h}}$ of 1.74 mg AI/L. In North America, $LC50_{96\text{-h}}$ values for glyphosate formulations containing POEA range from 1.5 to 9.4 mg AI/l in four species of tadpoles (*Bufo americanus*, *R. sylvatica*, *R. pipiens*, and *R. clamitans*), with lower lethality values found for glyphosate alone (Edginton et al. 2004, Howe et al. 2004). These studies suggest that, under laboratory conditions, ecologically relevant concentrations of Roundup can cause substantial mortality in some species of amphibian larvae and that this death is primarily due to the POEA surfactant.

I have recently completed static renewal studies of Roundup toxicity in the laboratory on six species of amphibians from the Midwestern United States (*Rana sylvatica*, *R. pipiens*, *R. clamitans*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolor*). Consistent with recent studies of tadpoles in Canada (Edginton et al. 2004), I found that $LC50_{16\text{-d}}$ values for these six species are relatively low, ranging from 0.6 to 2.5 mg AI/L (Relyea 2005*b*). Based on the LC50 probit analyses from the laboratory experiments, the 3.8 mg AI/L used in the mesocosm experiment predicted 93% mortality of leopard frog tadpoles, 94% mortality of American toads, and 92% mortality of gray tree frogs. These estimates are consistent with the mortality observed in the mesocosm experiment; mortality was 96% for leopard frogs, 100% for American toads, and 98% for gray tree frogs. Thus, under the more natural conditions of aquatic mesocosms, and with only a single application, Roundup can still be highly toxic to a variety of amphibian larvae.

The cause of the high Roundup-associated mortality appears to result from direct toxicity (possibly due to damaged epithelial cells in the gills; Edginton et al. 2004) rather than any indirect effect of Roundup-induced reduction of algal food resources in the mesocosms and subsequent tadpole starvation. Three pieces of evidence support this conclusion. First, I observed numerous dead tadpoles within the first 24 hours, which would not be expected if the cause of death were starvation (Audo et al. 1995; the exact amount of death could not be quantified without destructively sampling the mesocosms). Second, in a separate mesocosm experiment, Roundup actually increased, rather than decreased, periphyton biomass because there were so few tadpoles to consume the algae (Relyea 2005*a*). Third, in a laboratory study in which six species of North American tadpoles were fed ground fish flakes, Roundup still caused rapid death at 1 to 5 mg AI/L (Relyea 2005*b*).

Adding sand or loam soil did not reduce the toxic effects of Roundup. Although previous studies have demonstrated that glyphosate and the POEA surfactant can be absorbed by soil and broken down by soil microbes (Giesy et al. 2000), the current study suggests that the death of amphibians occurred before this breakdown could take place (typical half-life for glyphosate and POEA = 7–70 days depending on site conditions; USEPA 1992, Giesy et al. 2000). This is consistent with other studies of leopard frog tadpoles in Canada in which 2.0 mg AI/L caused 100% mortality under similar pH conditions (pH = 7.5; Chen et al. 2004). Moreover, it is reasonable to assume that these may be conservative estimates of mortality because the tadpoles in the mesocosm experiment were in relatively stress-free environments. In environments containing additional stressors (e.g., predation or competition), some pesticides can become even more lethal (Relyea and Mills 2001, Boone and Semlitsch 2002, Relyea 2003, 2004, 2005*b*). Curiously, however, in mesocosms placed in natural wetlands, 2.0 mg AI/L of the herbicide Vision (glyphosate plus POEA; Monsanto Company, Winnipeg, Manitoba, Canada) caused no significant mortality to larval leopard frogs or green frogs (Wojtaszek et al. 2004). These contradictory results suggest that there may be a number of important differences among experiments that can affect the effect of glyphosate products on amphibians including the formulation applied, the experimental venue used, and the amphibian population that is selected.

Rapid death also occurred in the terrestrial experiments. After only 24 hours, 79% of all juvenile frogs and toads died. There appear to be few studies of gly-

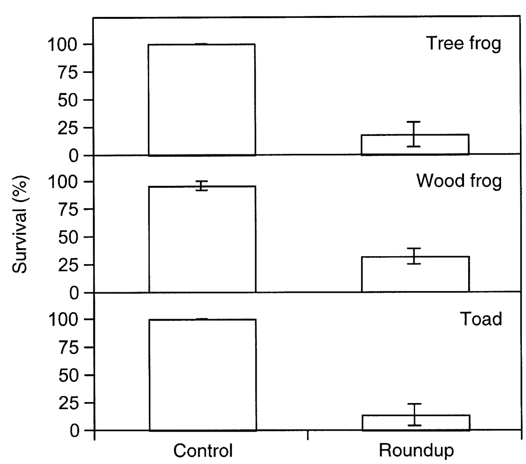

FIG. 2. The survival (mean ± SE) of three species of juvenile frogs and toads (gray tree frog, *Hyla versicolor*; wood frog, *Rana sylvatica*; and Fowler's toad, *Bufo woodhousii fowleri*) after 24 hours of exposure to a direct application of Roundup in laboratory tubs (1.6 mg AI/m^2).

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phosate's impact on terrestrial amphibians and all have been conducted by immersing animals in water containing glyphosate. Two Australian species of juvenile and adult frogs (*Crinia insignifera*, and *Litoria moorei*) have $LC50_{48\text{-h}}$ values ranging from 56 to 69 mg AI/L for Roundup (no experiments were conducted on glyphosate alone; Mann and Bidwell 1999). In the current study, I used amphibians that spend their post-metamorphic lives largely away from water and would experience Roundup as direct applications in the terrestrial environment (e.g., agricultural applications). My results suggest that a variety of North American amphibian species (from several families) are quite sensitive to the terrestrial application of Roundup. However, more natural field studies (using varying amounts of interception by vegetation) need to be conducted to better assess glyphosate's impact on juvenile amphibians in nature.

A critical question in interpreting the results of the aquatic and terrestrial experiments is whether the high rates of mortality observed were due to the active ingredient of Roundup (glyphosate) or whether they were due to the added surfactant (POEA). As discussed above, laboratory studies have shown that glyphosate alone has a low toxicity while the POEA surfactant can be highly toxic to a variety of taxa including amphibians (Mann and Bidwell 1999, Giesy et al. 2000, Perkins et al. 2000, Lajmanovich et al. 2003, Tsui and Chu 2003, Edginton et al. 2004, Howe et al. 2004). The current study did not isolate the impacts of glyphosate and the surfactant, so one cannot determine which component of Roundup caused the mortality, but it seems likely that the surfactant was the cause. What is clear is that the combination of ingredients present in the commercially applied form of Roundup ''Weed and Grass Killer'' can cause high rates of mortality in several species of North American amphibians. It is of interest to note that the manufacturer of Roundup (Monsanto Company) has recently released an additional formulation of glyphosate (Roundup Biactive) that is reported to have a less toxic surfactant (Tsui and Chu 2003). Initial tests on amphibians have supported this claim (Howe et al. 2004) and these new formulations should be the focus of future studies.

Conclusions

Natural communities are increasingly impacted by anthropogenic factors, and pesticides are one of several factors that have the potential to impact amphibian populations. While many pesticides can affect amphibian behavior, growth, and reproduction (Bridges 1997, 1999, Hayes 2002), it is often unclear whether such nonlethal effects will eventually translate into declines in amphibian populations. Further, when pesticides do reduce survival at relevant concentrations, unless the effects are substantial, the mortality may simply be compensatory and not lead to population declines (Boone and Semlitsch 2001, 2002). This appears to be the first study to document how a globally common pesticide can kill nearly every amphibian in an aquatic community (including amphibians from three different families). The elimination of 96–100% of tadpoles in the water, combined with the elimination of 68–86% of juvenile frogs and toads on land, could have a major negative impact on amphibian populations. In short, the current study suggests that applying Roundup formulations containing the POEA surfactant to amphibian habitats has the potential to cause substantial mortality in many amphibian species. However, the actual outcomes will likely be complex, depending on the timing of pesticide application and changes in amphibian sensitivity over ontogeny (e.g., Bridges 2000).

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