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THE TOXICITY OF GLYPHOSATE ALONE AND GLYPHOSATE–SURFACTANT MIXTURES TO WESTERN TOAD (*ANAXYRUS BOREAS*) TADPOLES

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Abstract: Pesticide choice based on toxicity to nontarget wildlife is reliant on available toxicity data. Despite a number of recent studies examining the effects of glyphosate on amphibians, very few have aimed to understand the toxicological effects of glyphosate in combination with surfactants as it is commonly applied in the field. Land managers interested in making pesticide choices based on minimizing impacts to nontarget wildlife are hindered by a lack of published toxicity data. Short-term acute toxicity trials were conducted for glyphosate in the form of isopropylamine salt (IPA) alone and mixed with 2 surfactants: Agri-dex and Competitor with western toad (*Anaxyrus* [*Bufo*] *boreas*) tadpoles. Glyphosate IPA mixed with Competitor was 6 times more toxic than glyphosate IPA mixed with Agri-dex, and both mixtures were more toxic than glyphosate IPA alone. The median lethal concentrations reported for 24-h and 48-h exposures were 8279 mg/L (24 h) and 6392 mg/L (48 h) for glyphosate IPA alone; 5092 mg/L (24 h) and 4254 mg/L (48 h) for glyphosate IPA mixed with Agri-dex; and 853 mg/L (24 h) and 711 mg/L (48 h) for glyphosate IPA mixed with Competitor. The present study indicates that the toxicity of a tank mix may be greatly increased by the addition of surfactants and may vary widely depending on the specific surfactant. *Environ Toxicol Chem* 2015;34:2791–2795. © 2015 SETAC

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INTRODUCTION

Aquatic invasive plants can have detrimental effects on aquatic ecosystems, altering native ecosystems and causing economic damage by clogging waterways and negatively impacting recreation. Management of unwanted vegetation often consists of the application of herbicides registered for use on aquatic systems directly to water bodies, where nontarget wildlife can potentially be negatively impacted.

Pesticides are most often applied as a mixture of an active ingredient and other additives. These mixtures are known as formulations if mixed by the manufacturer before purchase or tank mixes if mixed by the end user after purchase. Adjuvants are pesticide additives designed to improve the emulsifying, absorbing, spreading, sticking, and/or pest-penetrating properties of the spray mixture [1]. The specific type of adjuvant used in the present study is known as a surfactant, or surface-active agent, because of its spreading properties. By physically changing the surface tension of the formulation droplet, surfactants increase the surface area in contact with the vegetation [2].

In the United States, only 8 states (California, Washington, Idaho, Kentucky, Tennessee, Arkansas, Mississippi, and Utah) have registration requirements for adjuvants, and most do not require any toxicity data for registration. California and Washington are the only 2 states that require the disclosure of all adjuvant ingredients and the report of all efficacy, environmental, and toxicological trials for registration [3,4]. Federal registration requires toxicity data for active ingredients, but not for adjuvants or tank mixes and rarely for formulations [5]. Toxicity data requirements are thus limited for tank mixes, a widespread form of pesticide application.

Since its introduction by Monsanto Company in 1974, glyphosate has grown to be the most used herbicide active ingredient in the United States and other countries [6]. According to the most recent report by the US Environmental Protection Agency (USEPA), 180 to 185 million pounds were used in the United States in 2007 alone [7]. Glyphosate is a weak organic acid consisting of a glycine and a phosphonomethyl functional group [1], but is most commonly used to control broad-leafed plants and grasses in the form of isopropylamine salt (IPA) [8]. It functions as an herbicide by inhibiting the shikimate pathway, an enzymatic pathway that synthesizes 3 aromatic amino acids, resulting in the cessation of growth and subsequent death of the plant [9]. Glyphosate IPA is well known as the active ingredient of the formulated herbicide Roundup and is nearly always mixed with a surfactant before application.

Despite the widespread use of formulations and tank mixes and the species- and chemical-specific nature of toxicity, relatively few toxicity studies have been conducted with formulations and tank mixes. Even fewer studies have examined the toxicity of aquatic herbicides. A number of recent studies have examined the effects of glyphosate-based herbicides on amphibians [10–17], but did not examine how the toxicity changed when a surfactant was added. Previous studies conducted on nonamphibian test species suggest that glyphosate-based herbicide mixtures are more toxic than glyphosate alone [18–21]. Two studies have examined the differential toxicity of glyphosate IPA and formulated Roundup to amphibians, and both found the formulation to be more toxic than glyphosate IPA alone [22,23].

We determined the median lethal concentrations (LC50s) of 3 aquatic herbicide treatments to western toad (*Anaxyrus* [*Bufo*] *boreas*) tadpoles: glyphosate IPA alone, glyphosate IPA mixed with the surfactant Agri-dex, and glyphosate IPA mixed with the surfactant Competitor. Although amphibians are exposed to run off from herbicides such as Roundup that are not approved for aquatic use, it is especially important to examine

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the toxicity of herbicides that are directly applied to water bodies where amphibians spend much of their lives. The western toad is widely distributed in the western United States; populations occur in a variety of habitats from sea level to high elevations and are moderately common in urban areas [24]. Western toads breed in shallow, slow-moving water, and tadpoles remain in this aquatic environment for 30 d to 45 d until metamorphosis. The timing of herbicide application is not restricted in California for this species. Concentrations of herbicides are higher in shallow and slow-moving water than in deeper, faster moving water; and at times of warm weather, creeks containing western toad tadpoles can dry to small, stagnant pools.

The present study was not designed to understand how these herbicides are affecting amphibians in a natural setting, but to provide laboratory-based toxicity data that can be used to make better informed, conservation-based decisions in the absence of more conclusive data. The goal of the present study was 2-fold: 1) to compare the toxicity of 2 tank mixes, glyphosate IPA mixed with the surfactant Agri-dex compared with glyphosate IPA mixed with the surfactant Competitor; and 2) to compare both tank mixes with glyphosate IPA alone. Further research is necessary to determine the effects in a natural setting.

MATERIALS AND METHODS

Animal collection and care

We collected western toad tadpoles with mesh dip-nets from the Almaden–Calero canal in San Jose, California, in early May. After the tadpoles were transported from the field to the laboratory in an aerated cooler, they were immediately transferred into 50% fresh filtered tap water and 50% original canal water at a density of approximately 1 individual per liter. Twenty-four hours after field collection, tadpoles were transferred to 100% fresh filtered tap water, where they were held for 4 d with 1 water change after 48 h. Tadpoles were transferred 48 h before the test to the dilution water, 50% Holtfreter's solution, a mixture of 2 L distilled water, 3.5 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, and 0.2 g $NaHCO_3$ [25]. Altogether tadpoles were acclimatized to the laboratory for a total of 7 d before the experiments began. Tadpoles were fed Wardley Premium spirulina algae discs ad libitum.

Experimental design

We performed short-term (48 h) static toxicity tests on 3 aquatic herbicide treatments: glyphosate IPA alone, glyphosate IPA mixed with the surfactant Agri-dex, and glyphosate IPA mixed with the surfactant Competitor. Glyphosate IPA was sourced from commercially available Aquamaster (Monsanto), which consists of 53.8% glyphosate IPA and 46.2% water [26] for all treatments. The surfactant Agri-dex (Helena Holding) is a blend of heavy range petroleum-based oil, polyol fatty acid esters, and polyethylated derivatives and is designed to improve the wetting, spreading, and deposition characteristics of the pesticide [27]. The surfactant, Competitor (Wilbur–Ellis), is a modified vegetable oil (ethyl oleate sorbitan alkylpolyethoxylate ester, dialkyl polyoxyethylene glycol) and is designed to both enhance the ability of the pesticide to enter the cuticle of the plant and to increase the area that a droplet of spray mixture will cover [28]. We mixed 2 parts Aquamaster and 1 part surfactant by volume based on the aquatic vegetation management practices of the Santa Clara Valley Water District in San Jose, California.

To determine an initial estimate of the lethality of each of our 3 treatments, we conducted preliminary up–down tests [29]. We placed a single tadpole in a 4-L glass jar with a 1-L solution of artificial pond water (50% Holtfreter's solution) and an initial herbicide concentration. If the tadpole survived during the first 24 h, we repeated the 24-h trial with a new tadpole and increased the concentration 10-fold until a lethal concentration was found. If the initial concentration was lethal, we repeated the 24-h trial with a new tadpole and one-tenth the previous concentration until a concentration was found in which the tadpole survived. Up–down test results provided lethal and nonlethal concentrations of herbicide for each of the 3 treatments that allowed us to accurately design LC50 tests. For the LC50 tests, the glyphosate IPA-alone treatments consisted of 6 concentrations ranging from 5515 mg/L to 9084 mg/L glyphosate; the glyphosate IPA with Agri-dex treatments ranged in concentration from 649 mg/L to 5515 mg/L glyphosate; and the glyphosate IPA with Competitor treatments ranged in concentration from 195 mg/L to 1622 mg/L glyphosate (Table 1). We mixed treatment concentrations by volume in the laboratory and later converted to mg/L using molecular weights of the various treatment components for reporting.

The experimental units consisted of 4-L, wide-mouthed, glass jars filled with 3 L of solution (herbicide treatment mixed with artificial pond water) and 6 tadpoles per jar. The 6 concentrations were replicated 4 times, and each of the 3 treatments included a single control jar containing 6 tadpoles with artificial pond water and no herbicide. We randomly assigned tadpoles ranging from Gosner [30] stages 35 to 38 to each container. To minimize microclimate variations within the laboratory, we assigned jars to shelf positions using a randomized block design.

To reduce variation between the replicates, we mixed each of the herbicide concentrations in a single batch before filling the replicate jars. Two 40-mL samples were taken of each of the concentrations (including the controls) at the beginning of the present study to verify the intended concentrations of both glyphosate IPA and the surfactants. Sequoia Analytical Laboratory (Morgan Hill, CA, USA) verified the concentrations of glyphosate IPA, but the surfactant concentrations could not be verified because of a sample handling error.

The laboratory temperature was set to $19 \pm 1\,^{\circ}C$ for the course of the experiment. Water temperature was not recorded, but we mixed the Holtfreter's solution 24 h in advance to allow the solution to come to ambient temperature. Laboratory lighting was kept at a day to night ratio of 12:12-h [10,22,31]. Dead individuals were counted and removed at 12 h, 24 h, and 48 h to remove potential effects of the presence of dead tadpoles. Individuals that did not die during the present study were euthanized with a 1% solution of MS-222.

Statistical analysis

All data were analyzed using the statistical package SPSS Ver 20.0 (IBM). Glyphosate IPA concentrations were first converted from μL/L to mg/L. The 24-h and 48-h LC50 values were then determined using probit analysis based on mortality in each replicate and untransformed measured glyphosate IPA concentrations. Because there was no mortality in the control treatment after 48 h, observed mortality was not corrected for control mortality. Statistical significance between treatments was determined by examining 95% confidence interval ratios, which produces a p value closer to 0.005 than the standard 0.05 [32]. All statistical analyses were based on measured rather than nominal concentrations of glyphosate for all 3 tank mixes.

Table 1. Exposure concentrations and associated mortality at 24 h and 48 h for western toad (*Anaxyrus boreas*) tadpoles[a]

Herbicide treatment	Glyphosate IPA concentration (mg/L) Measured	Nominal	Surfactant concentration (mg/L) Nominal	% Mortality 24 h	48 h
Glyphosate IPA	5700	5515	N/A	25.0	37.5
	5800	5839	N/A	29.2	37.5
	6900	7137	N/A	45.8	66.7
	7000	7785	N/A	29.2	54.2
	7900	8434	N/A	41.7	75.0
	8800	9083	N/A	58.3	100.0
Glyphosate IPA with Agri-dex®	520	648	440	4.2	12.5
	1900	1946	1319	0.0	0.0
	2700	2595	1758	4.2	8.3
	3100	3244	2198	33.3	41.7
	5100	4541	3077	50.0	62.5
	5200	5515	3736	50.0	75.0
Glyphosate IPA with Competitor®	170	194	132	0.0	0.0
	370	389	264	4.2	4.2
	630	648	440	50.0	79.2
	820	973	659	58.3	58.3
	1500	1297	879	83.3	95.8
	1600	1622	1099	95.8	95.8
Control	ND	0	NA	0	0

[a]Percentage of mortality is reported on pooled results for each treatment.
IPA = isopropylamine salt; ND = not detected; NA = not available.

RESULTS

There was no mortality in the control treatment after 48 h. Glyphosate IPA alone was the least toxic of the 3 treatments after 24 h (LC50: 8279 mg/L glyphosate), followed by glyphosate IPA mixed with Agri-dex (LC50: 5092 mg/L glyphosate), and glyphosate IPA mixed with Competitor (LC50: 853 mg/L glyphosate; Table 2). The relative toxicity of the 3 treatments remained the same after 48 h, and the LC50 values were 16% to 22% lower than the 24-h values. The 48-h LC50 values of glyphosate IPA alone, glyphosate IPA mixed with Agri-dex, and glyphosate IPA mixed with Competitor were 6392 mg/L, 4254 mg/L, and 711 mg/L glyphosate, respectively. Both the 24-h and the 48-h LC50 values were significantly different among the 3 herbicide treatments, based on ratio tests [32]. The significance level is not reported in the ratio test technique, but is determined to be closer to 0.005 than the standard significance level of 0.05 [32].

DISCUSSION

In the present study, our results demonstrate that both herbicide tank mixes were more toxic than the active ingredient alone. The LC50 values for glyphosate IPA alone indicate that glyphosate IPA itself has low toxicity for western toad tadpoles. However, when mixed with the surfactant Competitor, the tank mix was almost 10 times as toxic. Glyphosate IPA mixed with Agri-dex was also more toxic than glyphosate IPA alone, but not as toxic as glyphosate IPA with Competitor.

Previous studies have also found glyphosate-based formulations to be more toxic than glyphosate alone to aquatic organisms [18–21,33]. In a study with 7 species of bacteria, algae, protozoa, and crustaceans, formulated Roundup was found to be on average 12 times more toxic than glyphosate IPA [33]. The same study found formulated Roundup to be more toxic than both glyphosate acid and glyphosate IPA, and also found that polyethoxylated tallow amine (POEA), the surfactant in Roundup, was more toxic than Roundup itself in 6 of 7 of the species tested [33]. Folmar et al. [20] found formulated Roundup to be more toxic than glyphosate acid to 4 species of fishes and POEA alone to be almost as toxic as formulated Roundup. In 24-h LC50 trials with *Litoria moorei* tadpoles, Mann and Bidwell [22], found formulated Roundup to be approximately 28 times more toxic than glyphosate acid alone, but they had no mortality after 48 h with glyphosate IPA to determine an LC50. To our knowledge, the study of Perkins et al. [23] is the only one that has determined the specific toxicity of glyphosate IPA alone and a glyphosate-based formulation using amphibians as test subjects. In that study, Roundup was found to be 700 times as toxic to the African clawed frog (*Xenopus laevis*) than glyphosate IPA alone.

Because the present study was limited as to test organism sample size, we designed the experiments to compare the relative toxicity of glyphosate-based tank mixes and did not test the toxicity of the surfactants alone. Therefore, we were not able to determine whether the higher toxicity of the tank mixes was because of the toxicity of the surfactants alone or because of an interaction of glyphosate IPA with the surfactants. Previous research has shown that certain surfactants on their own can be significant contributors to the toxicity of formulations [14,18–20,33], but further research

Table 2. Both 24-h and 48-h LC50 values for western toad tadpoles (*Anaxyrus boreas*) exposed to 3 herbicide treatments

Herbicide treatment	LC50 mg/L[a] (95% confidence limits) 24-h	48-h
Glyphosate IPA alone	8279 (7386–13 121)	6392 (5901–6754)
Glyphosate IPA with Agri-dex®	5092 (4498–6100)	4,254 (3757–4920)
Glyphosate IPA with Competitor®	853 (735–977)	711 (535–903)

[a]Acute toxicity values presented in terms of mg/L of glyphosate IPA.
LC50 = median lethal concentration; IPA = isopropylamine salt.

is necessary to understand how the surfactants Agri-dex and Competitor increase the toxicity of glyphosate tank mixes or formulations. However, because surfactants are never applied without an active ingredient, the most relevant information for assessing potential impacts on biota is toxicity data for tank mixes or formulations.

We found the glyphosate IPA with Competitor tank mix to be almost 6 times as toxic as glyphosate IPA with Agri-dex, but published toxicological studies of these tank mixes are lacking for direct comparisons. Only 1 published study was available that had tested the toxicity of the nonformulated surfactants Agri-dex and Competitor [34]. In that study, Competitor (96-h LC50: 95 mg/L) was found to be more toxic than Agri-dex (96-h LC50: >1000 mg/L) to rainbow trout. Although Agri-dex was not tested alongside Competitor, another study found Agri-dex to be the least toxic (96-h LC50: 271 mg/L) of 4 nonformulated surfactants (R-11, LI 700, Hasten, and Agri-dex) when tested with rainbow trout [35]. The results of the present study and the 2 studies mentioned above may indicate that Agri-dex has a relatively low toxicity.

However, many factors may interact with pesticide toxicity outside of the laboratory. For example, there may be synergistic effects from interactions with additional contaminants [36], species competition [13], predation [37], and environmental factors such as pH [33]. Sediment has been shown to both attenuate [16] and exacerbate [33] the toxicity of glyphosate-based herbicides. In addition, risks to nontarget wildlife are not limited to acute toxicity levels. Although our LC50 levels are several orders of magnitude above the estimated environmental concentration of 3.72 mg/L reported for glyphosate in the USEPA's Reregistration Eligibility Document [38], using a lesser toxic herbicide may lower the risk of currently undetermined sublethal impacts such as reduced growth, increased time-to-metamorphosis, and a variety of factors that may reduce reproductive fitness.

Because aquatic herbicides can be applied directly to aquatic systems, it is important to understand their toxicity to aquatic organisms such as amphibians. Amphibians play an essential role as both predators and prey; embryos are an important food item for many trophic levels, larvae are important herbivorous grazers, and adults can be both predators and prey for various species. Currently, neither US federal nor state regulations routinely require toxicological testing of pesticide active ingredients or adjuvants on amphibians [3,5], resulting in a paucity of toxicity data for amphibians. However, amphibians have experienced population declines around the globe [39], and pesticides have been specified as a possible contributing factor to declines in some populations [36,40]. However, because of the numerous variables capable of altering toxicity levels, LC50 values determined in a laboratory setting do not indicate impacts in a natural environment. The present study, and other laboratory-based toxicity studies like it, serve as the first step in understanding how chemicals may be affecting nontarget wildlife like amphibians.

The present study does not aim to illuminate the role of these chemicals in amphibian declines, but demonstrates the impact of surfactant choice in the toxicity of a tank mix. Whether or not pesticides are contributing to global amphibian population declines generally, the widespread declines in amphibian species have increased conservation concerns. Applicators concerned with potential, but not yet determined risks now have more information to steer data-driven, conservation-based decisions. In the absence of more conclusive data about negative impacts in a natural setting, the laboratory-based toxicity data produced in the present study, along with future studies on tank mixes, will allow for better informed aquatic vegetation management decisions that could improve the protection of amphibians and other nontarget wildlife.

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Data availability—Readers interested in receiving data should contact K. Vincent: kim.vincent@colorado.edu.

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