

COMPETITIVE STRESS CAN MAKE THE HERBICIDE ROUNDUP® MORE DEADLY TO LARVAL AMPHIBIANS

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Abstract—Toxicity assessments on nontarget organisms have largely been addressed using short-term, single-species laboratory experiments. Although extremely helpful, these experiments inherently lack many pervasive ecological stressors found in nature. Though a substantial challenge, incorporating these ecological stressors in contaminant studies would shed light on potential synergistic effects. For the world's leading herbicide, glyphosate, we know little about how natural stressors affect the toxicity to nontarget organisms. To explore how the natural stress of competition might interact with a glyphosate-based herbicide, we used outdoor mesocosms containing three tadpole species that were exposed to a factorial combination of three glyphosate concentrations (0, 1, 2, or 3 mg acid equivalent (a.e.)/L of the commercial formulation Roundup Original MAX®) and three tadpole densities (low, medium, or high). We found that increased tadpole density caused declines in tadpole growth, but also made the herbicide significantly more lethal to one species. Whereas the median lethal concentration (LC50) values were similar across all densities for gray treefrogs (*Hyla versicolor*; 1.7–2.3 mg a.e./L) and green frogs (*Rana clamitans*; 2.2–2.6 mg a.e./L), the LC50 values for bullfrogs (*R. catesbeiana*) were 2.1 to 2.2 mg a.e./L at low and medium densities, but declined to 1.6 mg a.e./L at high densities. The large decrease in amphibian survival with increased herbicide concentration was associated with increases in periphyton abundance. We also found evidence that temperature stratification lead to herbicide stratification in the water column, confirming the results of a previous study and raising important questions about exposure risk in natural systems. *Environ. Toxicol. Chem.* 2011;30:446–454. © 2010 SETAC

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INTRODUCTION

Whereas most toxicity studies of nontarget organisms have focused on single-species tests, researchers have increasingly been exploring the impact of contaminants under more realistic ecological conditions. In doing so, we have developed a better appreciation not only of the direct and indirect effects that contaminants can have on food webs, but also of the synergistic and antagonistic interactions with natural stressors (reviewed in Relyea and Hoverman [1]).

For most contaminants, synergistic interactions with natural stressors have received limited empirical attention, yet in those studies where they have been examined, researchers have often discovered strong interactive effects, particularly on survival. For instance, a number of abiotic stressors—temperature, pH, and ultraviolet radiation—can alter the lethality of several pesticides [2–4]. Additionally, biotic stressors, such as chemical cues emitted by predators, can make a variety of pesticides more lethal to many species of larval amphibians [5,6]. Competition (i.e., reduced per-capita food) is another biotic stressor that can interact synergistically with pesticides. Invertebrates, for example, have higher mortality rates when exposed to contaminants under lower food conditions [7–9]. In amphibians, a variety of interactive effects on survival and growth have been found between competition and pesticides, although most of these appear to be driven by indirect effects through the food web rather than on direct synergistic interactions [10–12]. For most globally common contaminants, we still have a poor understanding of how competition might affect their toxicity.

Glyphosate-based herbicides are the most widely applied type of herbicide in the world, sold by several manufacturers under a variety of names, including Roundup® and Vision® (Monsanto). Despite the fact that terrestrial formulations end up in aquatic habitats, we know surprisingly little about how glyphosate formulations interact with natural stressors in aquatic systems [13,14]. However, we do know a good deal about the direct impacts of these formulations. In larval amphibians, for example, glyphosate formulations containing a common surfactant, polyethoxylated tallowamine (POEA), have been classified as moderately to highly toxic in laboratory, mesocosm, and pond enclosure experiments [15–21]. Among the many abiotic stressors that could interact with glyphosate, only pH has been manipulated. In these studies, glyphosate with POEA became substantially more lethal to larval amphibians at higher pH [15,16,18]. Among the many biotic stressors, one study has manipulated the stress of predation risk and found that glyphosate with POEA became more lethal in the presence of chemical cues emitted by predators [6]. Another study has manipulated the effect of competition and glyphosate with POEA and found a marginally nonsignificant effect in which low food abundance tended to make the herbicide more lethal [15]. Given that competition is pervasive in nature [22], and given Chen and colleagues' [15] suggestion that low food tends to make glyphosate-based herbicides more lethal to larval amphibians, examining the potential interaction between competition and glyphosate exposure is of paramount importance.

We examined how a range of glyphosate concentrations, as the formulated product Roundup Original MAX, impacted simple aquatic communities that included a range of larval amphibian densities. Using outdoor mesocosms containing three species of tadpoles, we tested the following hypotheses: Increased concentrations of Roundup Original MAX will negatively affect

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tadpole survival and growth; increased competition, manipulated by increasing tadpole density, will negatively affect tadpole survival and growth; and Roundup Original MAX and competitive stress will interact synergistically to increase the negative affect on tadpole survival and growth.

Pesticide background

Since coming to market in 1974, the use of glyphosate-based products has exponentially increased, particularly as a result of the introduction of genetically modified (i.e., Roundup-Ready[®]) crops that are resistant to applications of the herbicide. Glyphosate-based herbicides are now the most widely applied herbicides in the world [23]. Glyphosate functions by inhibiting the synthesis of aromatic amino acids that are critical for plant growth. Glyphosate alone is often ineffective at penetrating the waxy leaf cuticles of terrestrial weeds, so terrestrial formulations often contain a surfactant that lowers the surface tension of herbicide droplets, thereby improving leaf penetration. Numerous surfactants exist, but a common and effective surfactant is POEA. Interestingly, several studies show that the moderate-to-high toxicity of commercial glyphosate formulations to aquatic organisms is caused not by the regulated active ingredient, but by the surfactant [17,24].

Formulations with POEA and similar surfactants are designed for terrestrial use, but researchers have repeatedly found that the herbicide can be found in aquatic habitats from drift, inadvertent overspray, or vegetation wash-off. Aquatic concentrations of glyphosate can be influenced by environmental factors such as interception by vegetation, water depth, temperature, pH, and sorption of the chemicals by vegetation and soils [13]. Although extensive surveys of glyphosate concentrations in streams and rivers have been made, data on the concentrations of glyphosate in wetlands is scarce. Estimated worst-case scenarios of glyphosate contamination in water range from 1.4 to 7.6 mg acid equivalent (a.e.)/L depending upon initial concentration, number of applications, and interception by vegetation [13,24–26]. Observed worst-case concentrations of glyphosate in freshwater systems—including streams, lakes, and wetlands—range from 1.7 to 5.2 mg a.e./L [13,14,27]. In pond water, glyphosate can have wide-ranging half-lives, from 8 to 120 d, depending on site conditions, including the amount of particulate matter available for adsorption and the amount of microbial degradation [13,28].

MATERIALS AND METHODS

Mesocosm experiment

To examine the effects of Roundup Original MAX and tadpole density on wetland communities, we conducted an outdoor mesocosm experiment at the University of Pittsburgh's Pymatuning Laboratory of Ecology in northwestern Pennsylvania (USA). The experiment employed a randomized block design consisting of 12 treatments that were replicated twice within each of two spatial blocks for a total of 48 experimental units. The 12 treatments represented a factorial combination of four concentrations of the herbicide (0, 1, 2, or 3 mg a.e./L of glyphosate) crossed with three tadpole densities (low, medium, or high).

The mesocosms were 750-L round, cattle tanks filled with approximately 562 L of well water (pH = 8) on June 15, 2007. Water depth was approximately 0.4 m. On June 18, we added 15 g of rabbit chow to serve as an initial nutrient source, and 200 g of oak leaf litter (*Quercus* spp.) to serve as a substrate for periphyton growth. All mesocosms also received equal aliquots

of water, collected and pooled from multiple nearby ponds, to create seminatural pond communities consisting of periphyton, phytoplankton, and zooplankton. Invertebrate predators were removed prior to adding the pond water to the mesocosms. A single ceramic tile (15 × 15 cm) was placed into each tank (vertically, on the north side) to serve as a periphyton sampler. Mesocosms were covered with 60% shade cloth to prevent other organisms from ovipositing, and were allowed to develop algal and invertebrate populations for two weeks prior to adding the amphibians.

The amphibians used in this experiment were collected as egg masses from nearby ponds. We collected five clutches of American bullfrogs (*Rana catesbeiana*; collected on May 26 through June 4), 15 clutches of green frogs (*R. clamitans*; collected on May 26 through 28), and 14 clutches of gray tree frogs (*Hyla versicolor*; collected on May 26 through 29). The eggs were hatched in covered wading pools containing aged well water. Several hundred hatchlings were raised together, with water changes every few days once the animals became large enough to safely handle (after two weeks). After hatching, the tadpoles were fed rabbit chow ad libitum. Tadpoles used in the experiment were a mixture of all clutches from each species. These animals were early in development as indicated by stage (stage 25; [29]) and by mean initial mass (± 1 standard error; bullfrogs = 41 ± 4 mg, green frogs = 34 ± 3 mg, gray tree frogs = 75 ± 4 mg).

Amphibian larvae were added to the mesocosms on July 2 and 3 (defined as days 0 and 1 of the experiment). Tadpole density (i.e., competition) was manipulated by adding 20, 40, or 60 tadpoles of green frog and gray tree frog tadpoles to each mesocosm. Due to a limited number of bullfrog tadpoles, all mesocosms received an equal number of bullfrogs (20 per tank). Thus, to cause low, medium, and high competition, the three tadpole density treatments consisted of 60, 100, and 140 individuals per mesocosm. All three species consume periphyton, a form of attached algae. These densities per species (15, 30, and 45 tadpoles/m²) are within the natural densities of hatchling tadpoles (R.A. Relyea, personal observations). Twenty individuals of each species were held in the laboratory for 24 h to assess survival after handling; all three species exhibited 100% survival.

On day 7, the herbicide treatments were applied. Based on an estimate of 562 L of water in each mesocosm, we added 0, 1.041, 2.082, and 3.124 ml of Roundup Original MAX formulation to obtain nominal glyphosate concentrations of 0, 1, 2, and 3 mg a.e./L (reported to contain 48.7% glyphosate). These concentrations span the range of what has been observed in nature. The surfactant in Roundup Original MAX is a trade secret (S. Mortensen, Monsanto, personal communication), but past studies have found that its toxicity is essentially the same as formulated products that contain the POEA surfactant [6,30]. The measured amounts of Roundup were dissolved into 0.5 L of water and then drizzled over the top of the mesocosm. The surface water of the mesocosms was then agitated to encourage mixing.

Approximately 3 h after adding the Roundup, which we estimated was sufficient time for the water-soluble herbicide to diffuse down through the 40-cm-deep water column, water samples were taken. From each of the four replicates of each treatment, we sampled 200 ml of water from both the top (5 cm below the surface) and bottom (5 cm above the bottom) of the water column to test for Roundup stratification. Control (0 mg a.e./L) treatments were not sampled, but tests of our well water have found no detectable levels of glyphosate. Water samples

from each of the four replicate tanks were pooled and placed into a precleaned, glass amber jar and held at 2°C to prevent breakdown. Samples were later shipped to Mississippi State Chemical Laboratory (Mississippi State, MS, USA) where they were filtered and then analyzed to determine the concentration of dissolved glyphosate using high-pressure liquid chromatography.

The analysis of the water samples confirmed that the herbicide stratified in the water column. Glyphosate concentrations collected near the surface were higher than nominal, whereas concentrations near the bottom of the mesocosm were lower than nominal. However, the means of the top and bottom values were very close to the nominal concentrations (Table 1). For the highest concentration (3 mg a.e./L), the sample taken from the top of the water column broke during shipping. However, based on the precision of the other two measured concentrations relative to the nominal concentration, and based on the fact that similar patterns of stratification were found in another mesocosm study [31], it seems likely that the nominal concentration of 3 mg a.e./L, which had a measured bottom concentration of 2 mg a.e./L, had a top concentration of approximately 4 mg a.e./L. For simplicity, we will refer to the four herbicide treatments as 0, 1, 2, and 3 mg a.e./L.

Response variables

To assess the abiotic conditions in the experiment, water quality measurements of temperature, pH, and dissolved oxygen were measured twice (on days 7 and 16). To determine whether the water experienced stratification, temperature and pH values were measured at the top and bottom of the water column. Dissolved oxygen was taken in the middle of the water column. Water measurements were taken using a multiprobe system (Multiline P4 Universal Meter, Wissenschaftlich-Technische-Werkstätten) that was calibrated prior to each day's measurements.

Periphyton biomass was assessed on day 15. Ceramic tiles were removed from the tanks, scrubbed clean of any periphyton growth, collected in carbon-filtered, ultraviolet-irradiated water, and poured through a vacuum filter. Whatman® GF/C filters were dried and weighed earlier to obtain an initial filter weight, and were dried and weighed again after periphyton samples were collected. Filters used were dried for a period of 24 h, both before and after periphyton sampling, in an 80°C drying oven. The difference in initial and sample weights of the filter provided an estimate of periphyton abundance within the mesocosm tanks. Unlike estimates of chlorophyll *a* from such samples, which represent the relative abundance of one photosynthetic pigment of algae, measures of dry biomass represent periphyton, bacteria, and fungi, all of which can be consumed by the tadpoles.

The first gray tree frog emerged on day 17, and we checked all tanks for metamorphosing frogs from day 17 until the experiment was terminated on day 22 (Block A) and day 23 (Block B). Upon termination, we removed emerging metamorphs, drained the water from each tank, and sorted through

the leaf litter for surviving amphibians. Contaminated water and leaf litter was held in tanks until the following spring to allow pesticide breakdown. Amphibians collected, along with previously removed gray tree frog metamorphs, were euthanized with 2% tricaine methanesulfonate and preserved in 10% formalin. The preserved animals were later counted to assess survival, and weighed to assess mass. Our amphibian response variables for each of the three species were the proportion of surviving tadpoles and the mean individual mass from each mesocosm.

Statistical analysis

To determine how tadpole survival was affected by glyphosate concentration and tadpole density, we analyzed the data using multivariate analysis of variance (MANOVA). Initial analyses indicated that none of the species exhibited significant block effects or block interactions, so these terms were dropped from the analysis and the degrees of freedom were pooled with the error term. Most assumptions of the analysis were met, with the exception of homoscedastic variance. However, analyses of variance are generally robust to the violation of this assumption. To quantify the LC10, LC50, and LC90 values (the lethal concentration expected to kill 10, 50, and 90% of a population), we conducted standard probit analyses for each tadpole species within each level of competition. For each density, we adjusted for the low amounts of mortality in the controls using the procedures recommended by Finney [32]. Significant differences in estimates can be determined by nonoverlapping 84% confidence intervals; simulation studies find that this confidence interval approximates an $\alpha = 0.05$ [33].

Given that very few amphibians survived the 3 mg a.e./L exposure, we conducted a separate MANOVA on amphibian mass that only included the 0, 1, and 2 mg a.e./L herbicide treatments. Mass data were approximately normal and had equal variances. Although analyses of mass that include survival as a covariate can be useful when individual mass increases due to reduced competition, this was not the case in our data, so we did not include a survival covariate. The analysis of the mass data for larval bullfrogs and green frogs was straightforward because none of them had initiated metamorphosis at the time the experiment ended. The analysis of the mass data for gray tree frogs, however, was more complex because many of them had begun to metamorphose at the time the experiment ended. Among all gray tree frogs, 12.7% had not initiated metamorphosis (i.e., less than Gosner stage 39), 86.9% were in the process of metamorphosis (i.e., Gosner stages 39 through 45), and 0.3% had completed metamorphosis (i.e., Gosner stage 46). This is important because when amphibians metamorphose, an individual stops eating and its mass can decline 30 to 50% [34]. To resolve this problem, we used data on mass and developmental stage for all gray tree frogs \geq Gosner stage 39 to back-calculate the peak mass of metamorphosing gray tree frogs [35]. In this way, one can assess the impact of the treatments on the peak mass of a species prior to metamorphosis.

Table 1. Nominal and observed glyphosate concentrations (mg a.e./L) from the top and bottom of the water column after a single application^a

Nominal concentration	Observed top concentration	Observed bottom concentration	Mean observed concentration
1	1.40	0.79	1.09
2	3.30	0.83	2.07
3	—	2.04	—

^a a.e. = acid equivalent.

Means of the measured top and bottom values are very close to the nominal concentration. Concentrations and averages within the 3 mg a.e./L treatment are unknown due to a jar breaking during shipment for testing, but should be approximately 3 mg a.e./L.

To determine the peak mass of gray tree frogs, we weighed and staged [29] all individuals and then graphically determined the developmental stage at which peak mass was achieved (stage 39). Within each treatment, we then used the data from all individuals greater than stage 39 to obtain a regression line that indicated the rate of mass decline, as animals developed from stage 39 through complete metamorphosis (stage 46). Using the slope of each treatment's regression line, we used the mass and stage of each metamorphosing individual to back-calculate the estimated peak mass of each individual. We then averaged the final mass of all gray tree frog tadpoles combined with the estimated peak mass of all gray tree frog metamorphs to obtain a mean mass of gray tree frogs in each mesocosm. The mean mass of the bullfrogs, green frogs, and gray tree frogs for each mesocosm served as our growth response variables. Analysis of development stage in gray tree frogs confirmed that small changes in tadpole development mirrored the changes in tadpole growth (i.e., conditions that caused slower growth also caused slower development). As a result, we chose not to also report effects of the treatments on development.

The final analyses were on periphyton abundance, temperature, pH, and dissolved oxygen. The single periphyton sample was analyzed using an analysis of variance (ANOVA). Temperature, pH, and dissolved oxygen data, were analyzed using a repeated-measures ANOVA. Because temperature and pH were measured on two dates and at two depths (top or bottom of the mesocosm), these variables were analyzed by nesting sample depth within time. Because dissolved oxygen was also assessed on two sample dates but at a single location in the mesocosm, we used a repeated-measures ANOVA to analyze these data. Preliminary analyses indicated that all three water quality variables exhibited significant time-by-treatment interactions. Thus, for brevity, we simply report the result of the ANOVA analyses for each sample date. Periphyton abundance, temperature, pH, and dissolved oxygen data met the assumptions of normality and homogeneity of variances without needing to be transformed. When treatment effects were detected, we conducted mean comparisons using Tukey's tests. In all analyses, block main effects were never present and block-by-treatment interactions were rarely significant (only two instances out of 46 possible interactions). In these final analyses, block terms were not significant but they did explain some level of variation and were thus included.

RESULTS

Amphibian survival

The MANOVA on tadpole survival found significant effects of glyphosate concentration and marginal effects of tadpole density and the glyphosate-by-density interaction (Table 2). As

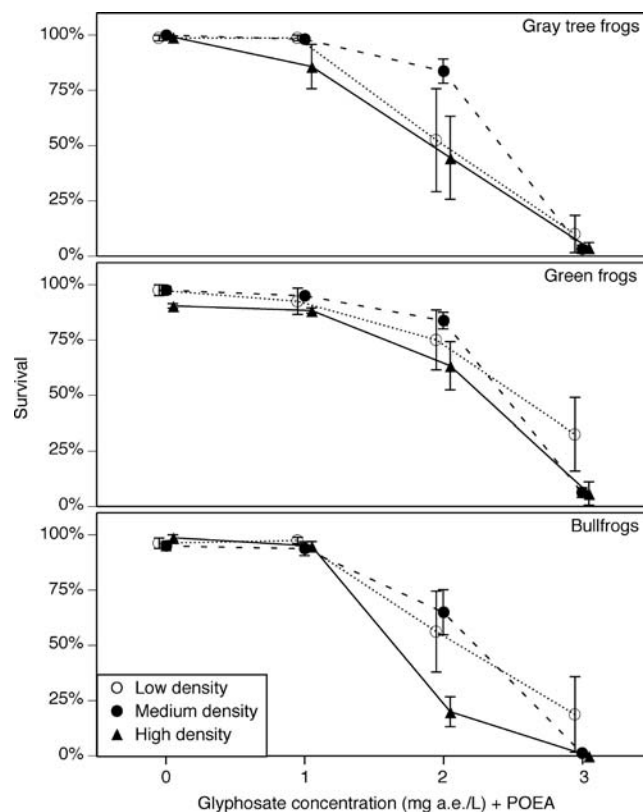


Fig. 1. Survival of three species of tadpoles when exposed to three tadpole densities and four concentrations of glyphosate (applied as the commercial formulation of Roundup Original MAX[®], Monsanto). Data points represent means \pm one standard error. To make the different error bars clearer, the low- and high-density values are shifted slightly along the x axis. a.e. = acid equivalent; POEA = polyethoxylated tallowamine.

a result, we examined the effect of the treatments on the survival of each species in separate ANOVAs.

Gray tree frog survival was affected by glyphosate concentration, but not by tadpole density or their interaction (Table 2, Fig. 1). Across all tadpole densities, survival in the 0 mg a.e./L treatments was high (99–100%). Mean comparisons were then conducted using Tukey's test. Compared to 0 mg a.e./L, exposure to 1 mg a.e./L had no effect ($p = 0.92$), but 2 and 3 mg a.e./L caused on average 40 to 95% death (both $p < 0.001$). Moreover, 3 mg a.e./L caused greater death than 2 mg a.e./L ($p < 0.001$). The LC50 values for gray tree frogs across the three density treatments ranged from 1.7 to 2.3 mg a.e./L (Table 3).

Green frog survival was affected by glyphosate concentration, marginally by tadpole density, but not their interaction (Table 2, Fig. 1). Across all tadpole densities, tadpole survival in the 0 mg a.e./L treatments was high (90–98%). Compared to

Table 2. Results of a multivariate analysis of variance (MANOVA) and three subsequent analyses of variance (ANOVAs) on the survival of larval gray tree frogs, green frogs, and bullfrogs when exposed to a factorial combination of four concentrations of glyphosate (formulated as Roundup Original MAX[®], Monsanto) crossed with three levels of tadpole density

Species	MANOVA	ANOVAs		
		Gray tree frog	Green frog	Bullfrog
Glyphosate	$F_{9,83} = 19.9$ $p < 0.001$	$F_{3,36} = 61.9$ $p < 0.001$	$F_{3,36} = 72.5$ $p < 0.001$	$F_{3,36} = 83.3$ $p < 0.001$
Density	$F_{6,68} = 2.0$ $p = 0.079$	$F_{2,36} = 1.8$ $p = 0.17$	$F_{2,36} = 2.6$ $p = 0.088$	$F_{2,36} = 3.0$ $p = 0.060$
Glyphosate · density	$F_{18,97} = 1.6$ $p = 0.067$	$F_{6,36} = 1.3$ $p = 0.30$	$F_{6,36} = 1.3$ $p = 0.29$	$F_{6,36} = 2.4$ $p = 0.048$

Table 3. Results of species-specific probit analyses used to estimate the lethal concentrations of Roundup Original MAX[®] (Monsanto) that cause 10, 50, and 90% death (LC10, LC50, and LC90, respectively)^a

Species	Competition	LC10	LC50	LC90
Gray tree frog	Low	1.41 (0.81, 1.70)	2.04 (1.70, 2.35)	2.96 (2.53, 4.63)
	Medium	1.85 (0.00, 2.20)	2.29 (1.56, 10.8)	2.83 (2.36, 24,000)
	High	1.00 (0.53, 1.29)	1.71 (1.36, 2.07)	2.96 (2.39, 4.84)
Green frog	Low	1.26 (0.42, 1.68)	2.58 (2.07, 3.86)	5.28 (3.64, 25.0)
	Medium	1.84 (1.09, 2.13)	2.35 (1.98, 2.84)	3.00 (2.58, 5.30)
	High	1.58 (1.25, 1.78)	2.18 (1.99, 2.37)	3.00 (2.69, 3.74)
Bullfrog	Low	1.38 (0.69, 1.72)	2.18 (1.77, 2.63)	3.46 (2.81, 6.76)
	Medium	1.63 (0.59, 1.91)	2.12 (1.70, 2.58)	2.76 (2.36, 7.20)
	High	1.18 (1.06, 1.28)	1.61 (1.52, 1.70)	2.21 (2.09, 2.36)

^aa.e. = acid equivalent.

Estimates are based on outdoor mesocosm experiments that crossed four concentrations of Roundup (0, 1, 2, or 3 mg a.e./L) with three levels of tadpole competition. Means are followed by 84% confidence intervals; nonoverlapping confidence intervals are significant at approximately $\alpha = 0.05$ [32]. All estimates adjust for low amounts of mortality in the controls.

0 mg a.e./L, exposure to 1 mg a.e./L had no effect on survival ($p = 0.92$), but 2 and 3 mg a.e./L caused on average 26 to 85% death (both $p < 0.001$). Moreover, 3 mg a.e./L caused greater death than the 2 mg a.e./L ($p < 0.001$). Across all glyphosate concentrations, tadpole survival was similar at low and medium tadpole densities ($p = 0.81$), but survival declined at high tadpole densities ($p = 0.041$). The LC50 values for green frogs across the three density treatments ranged from 2.2 to 2.6 mg a.e./L (Table 3).

Bullfrog survival was affected by glyphosate concentration, the glyphosate-by-density interaction, and marginally by tadpole density (Table 2, Fig. 1). The interaction occurred because the impact of increased glyphosate concentrations was larger under high competition than under low competition. At 0 and 1 mg a.e./L, survival was nearly identical (95–99%) across the density treatments. At 2 mg a.e./L, however, survival was 56 to 65% at low and medium densities, but only 20% at high densities. At 3 mg a.e./L, survival no longer differed among tadpole density treatments because very few animals survived at any density ($p > 0.88$). The LC50 values for bullfrogs reflected this competition effect; LC50 values were similar at low and medium densities (2.1 to 2.2 mg a.e./L), but both were significantly different from the LC50 values at high tadpole density (1.6 mg a.e./L; Table 3).

Amphibian mass

The second MANOVA examined treatment effects on tadpole mass with the highest glyphosate treatment removed due to low tadpole survival. We found no main effect of glyphosate concentrations, but an effect of tadpole density was noted, as well as a glyphosate-by-density interaction (Table 4). Thus, we

examined the effect of the treatments on the mass of each species in separate ANOVAs.

Gray tree frog mass exhibited no effect of glyphosate concentration, but an effect of density and a marginal glyphosate-by-density interaction occurred (Table 4, Fig. 2). Across all glyphosate treatments, low densities produced larger animals than medium or high densities ($p < 0.001$); the latter two treatments did not differ ($p = 0.14$). The marginal interaction between glyphosate concentration and density occurred because 1 mg a.e./L tended to cause a small reduction in growth at low density, but it caused an increase in growth at medium and high densities.

Green frog mass was affected by glyphosate concentration and tadpole density, but not their interaction (Table 4, Fig. 2). Across all densities, green frogs were larger as glyphosate concentration increased. Compared to 0 mg a.e./L, green frogs had similar mass with 1 mg a.e./L ($p = 0.98$) but greater mass with 2 mg a.e./L ($p = 0.040$); the latter two treatments did not differ ($p = 0.27$). Across all glyphosate concentrations, tadpoles were larger at low density than at medium and high density ($p < 0.001$); tadpoles had similar mass at medium and high densities ($p = 0.43$).

Bullfrog mass was not affected by glyphosate concentration, was affected by density, but not their interaction (Table 4, Fig. 2). Across all glyphosate concentrations, bullfrogs were larger at low density compared to medium and high density ($p < 0.001$); the latter two treatments did not differ ($p = 0.69$).

Periphyton

Periphyton biomass was significantly affected by glyphosate concentration ($F_{3,24} = 17, p < 0.001$), but not by tadpole density

Table 4. Results of a multivariate analysis of variance (MANOVA) and three subsequent analyses of variance (ANOVAs) on the mass of larval gray tree frogs, green frogs, and bullfrogs when exposed to a factorial combination of three concentrations of glyphosate (formulated as Roundup Original MAX[®], Monsanto) crossed with three levels of tadpole density^a

Species	MANOVA	ANOVAs		
		Gray tree frog	Green frog	Bullfrog
Glyphosate	$F_{6,30} = 2.0$ $p = 0.10$	$F_{2,17} = 2.6$ $p = 0.10$	$F_{2,17} = 5.1$ $p = 0.019$	$F_{2,17} = 1.9$ $p = 0.19$
Density	$F_{6,30} = 8.4$ $p < 0.001$	$F_{2,17} = 42$ $p < 0.001$	$F_{2,17} = 34$ $p < 0.001$	$F_{2,17} = 22$ $p < 0.001$
Glyphosate · density	$F_{12,40} = 2.1$ $p = 0.037$	$F_{4,17} = 2.6$ $p = 0.072$	$F_{4,17} = 1.1$ $p = 0.38$	$F_{4,17} = 0.4$ $p = 0.79$

^aa.e. = acid equivalent.

Due to substantial mortality, the highest glyphosate treatment (3 mg a.e./L) was not included in this analysis.

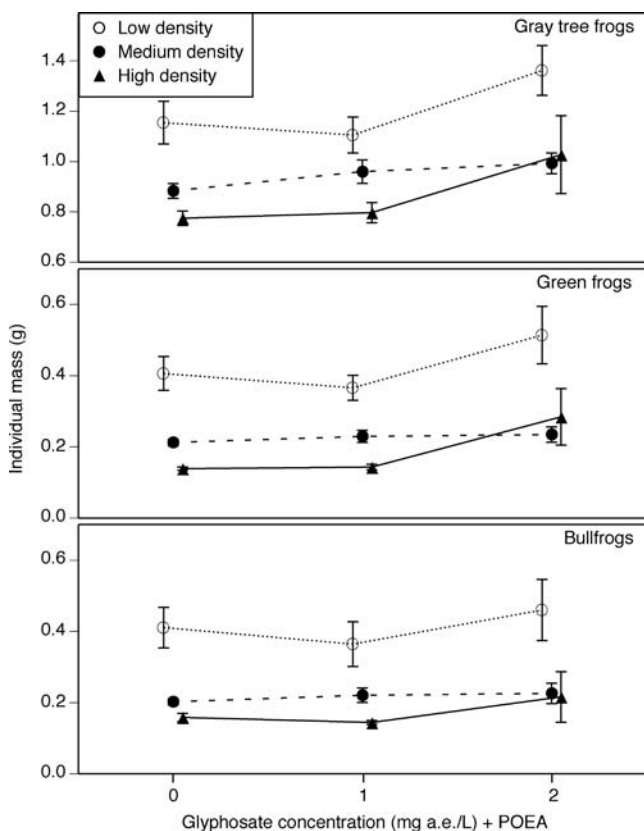


Fig. 2. Individual mass of three tadpole species when exposed to three tadpole densities and four concentrations of glyphosate (applied as the commercial formulation of Roundup Original MAX[®], Monsanto). Data points represent means \pm one standard error. To make the different error bars clearer, the low- and high-density values are shifted slightly along the x axis. a.e. = acid equivalent; POEA = polyethoxylated tallowamine.

($F_{2,24}=0.7$, $p=0.50$) nor their interaction ($F_{6,24}=1.6$, $p=0.20$; Fig. 3). Mean comparisons indicated that glyphosate concentrations of 0, 1, and 2 mg a.e./L had similar amounts of periphyton biomass ($p > 0.62$), whereas 3 mg a.e./L was associated with nearly twice as much periphyton ($p < 0.001$).

Water quality

The analysis of temperature revealed an effect of glyphosate concentration ($F_{3,24}=19$, $p < 0.001$), depth ($F_{1,24}=3,200$, $p < 0.001$), and time ($F_{1,24}=9,600$, $p < 0.001$), but no effect of density ($F_{2,24}=1.13$, $p=0.338$). The possible interactions included a depth-by-concentration ($F_{3,24}=8.9$, $p < 0.001$), a time-by-concentration ($F_{3,24}=22$, $p < 0.001$), a time-by-depth ($F_{1,24}=59$, $p < 0.001$), a time-by-concentration-by-depth

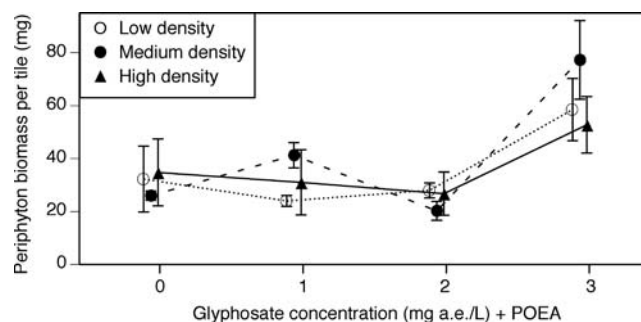


Fig. 3. Periphyton abundance on day 15 of the experiment across three tadpole densities and four concentrations of glyphosate (applied as the commercial formulation of Roundup Original MAX[®], Monsanto). Data points represent means \pm one standard error. To make the different error bars clearer, the low- and high-density values are shifted slightly along the x axis. a.e. = acid equivalent; POEA = polyethoxylated tallowamine.

($F_{3,24}=6.1$, $p=0.003$), and a time-by-density-by-depth ($F_{2,24}=3.4$, $p=0.049$) interaction. As a result, we analyzed the temperature data within each sample date.

On day 7, no effects of glyphosate concentration or tadpole density on temperature were observed, but a depth effect and a glyphosate-by-depth interaction were noted (Table 5; Fig. 4). Water near the bottom of the mesocosm was 2.6°C colder than the water near the top of the mesocosm. In addition, temperature at the top of the mesocosm had relatively small but significant variation that was related to the glyphosate concentrations (spanning 1.0°C; $F_{3,24}=4.3$, $p=0.014$), whereas temperatures at the bottom exhibited no effects of glyphosate ($F_{3,24}=1.1$, $p=0.400$).

On day 16, a depth effect on temperature continued to be noted, as well as a glyphosate effect, and interactions of glyphosate-by-depth, and density-by-depth (Table 5; Fig. 4). Water near the bottom of the mesocosm was 3.2°C colder than the water near the top of the mesocosm. The interactions occurred because temperatures near the bottom exhibited small (0.7°C) but significant glyphosate effects ($F_{3,24}=37$, $p < 0.001$), whereas temperatures near the surface experienced small effects (1.8°C) of glyphosate ($F_{3,24}=89$, $p < 0.001$) and density ($F_{2,24}=4.8$, $p=0.018$). Collectively, the data indicate that mesocosm temperatures were cooler near the bottom than the top, with small differences due to density and glyphosate concentrations at particular depths.

The analysis of pH showed a significant effect of depth ($F_{1,24}=250$, $p < 0.001$), and time ($F_{1,24}=887$, $p < 0.001$), but no effect of density ($F_{2,24}=1.9$, $p=0.17$) or glyphosate concentration ($F_{3,24}=2.6$, $p=0.077$). Among the interactions, density-by-depth ($F_{2,24}=3.6$, $p=0.043$), time-by-glyphosate concentration interaction ($F_{3,24}=32$, $p < 0.001$), time-by-depth

Table 5. Results of analyses of variance (ANOVAs) on each of two sample dates for temperature, pH, and dissolved oxygen (DO) in outdoor mesocosms exposed to a factorial combination of different glyphosate concentrations (using Roundup Original MAX[®], Monsanto) and three tadpole densities^a

Effect	Temperature day 7	Temperature day 16	pH day 7	pH day 16	DO day 7	DO day 16
Glyphosate	$F_{3,24}=2.0$ (0.15)	$F_{3,48}=120$ (0.001)	$F_{3,24}=4.9$ (0.008)	$F_{3,24}=8.9$ (0.0004)	$F_{3,24}=0.4$ (0.75)	$F_{3,24}=10$ (0.0002)
Density	$F_{2,24}=1.1$ (0.34)	$F_{2,24}=1.9$ (0.17)	$F_{2,24}=3.5$ (0.048)	$F_{2,24}=1.0$ (0.40)	$F_{2,24}=1.8$ (0.19)	$F_{2,24}=0.3$ (0.73)
Glyphosate · density	$F_{6,24}=1.1$ (0.37)	$F_{6,24}=0.4$ (0.88)	$F_{6,24}=1.5$ (0.21)	$F_{6,24}=0.7$ (0.65)	$F_{6,24}=1.6$ (0.20)	$F_{6,24}=0.6$ (0.73)
Depth	$F_{1,24}=1100$ (<0.001)	$F_{1,24}=6200$ (0.001)	$F_{1,24}=351$ (<0.001)	$F_{1,24}=27$ (<0.001)		
Glyphosate · depth	$F_{3,24}=3.7$ (0.026)	$F_{3,24}=24$ (<0.001)	$F_{3,24}=1.4$ (0.26)	$F_{3,24}=0.4$ (0.78)		
Density · depth	$F_{2,24}=0.6$ (0.54)	$F_{2,24}=6.0$ (0.008)	$F_{2,24}=4.7$ (0.019)	$F_{2,24}=0.5$ (0.61)		
Glyphosate · density · depth	$F_{6,24}=0.5$ (0.79)	$F_{6,24}=2.1$ (0.09)	$F_{6,24}=0.6$ (0.69)	$F_{6,24}=0.8$ (0.61)		

^a Values in parentheses are p values.

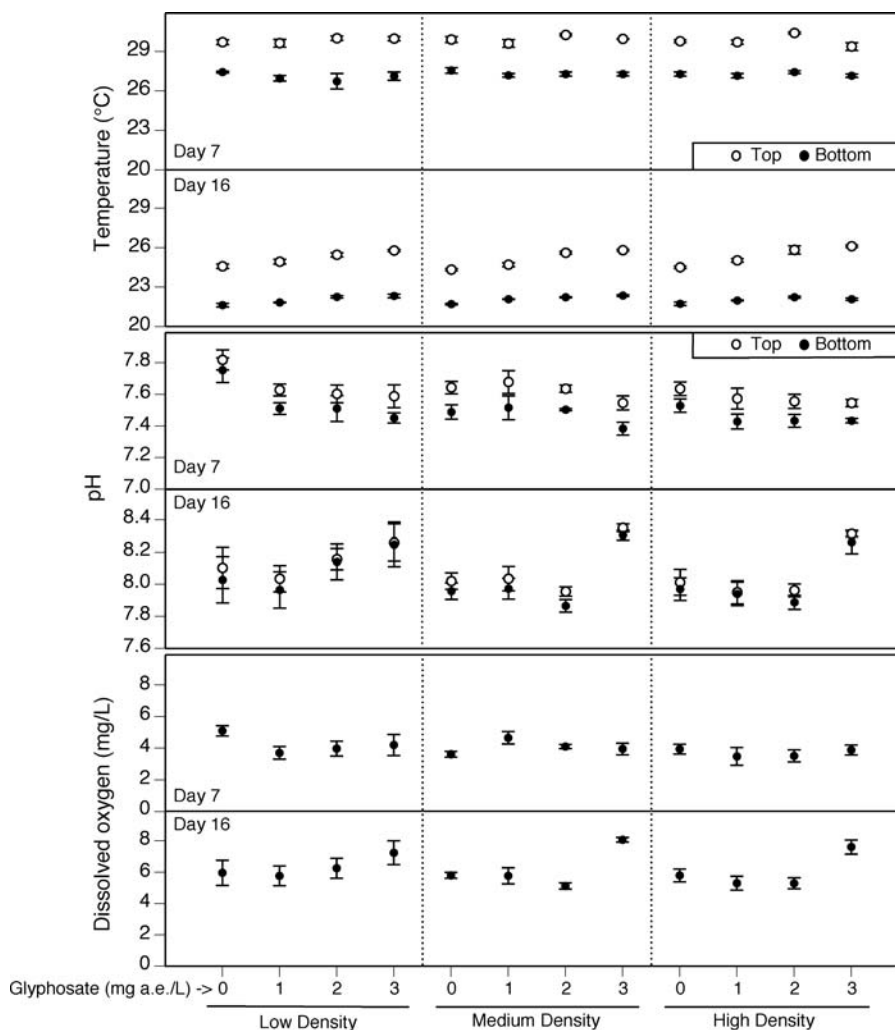


Fig. 4. Abiotic conditions of the mesocosm experiment; temperature and pH of mesocosms taken on days 7 and 16, assayed at the top and bottom of the water column. Dissolved oxygen measurements were taken in the middle of the water column. Data points represent means \pm one standard error. a.e. = acid equivalent.

($F_{1,24} = 35$, $p < 0.001$), and time-by-density-by-glyphosate concentration ($F_{6,24} = 19$, $p < 0.001$) were observed, but no glyphosate concentration-by-depth ($F_{3,24} = 0.15$, $p = 0.93$) or time-by-density interactions ($F_{2,189} = 0.05$, $p = 0.95$). Because of these interactions, we examined the data within each sample time.

On day 7, pH was affected by depth, density, glyphosate concentration, and a depth-by-density interaction (Table 5; Fig. 4). The treatments generated small effects on pH (< 0.2 pH units), with the biggest impact on pH occurring with a low density of tadpoles and no glyphosate present. On day 16, pH showed significant effects of depth and glyphosate concentration. The depth effects were again relatively small (< 0.1 pH units). The glyphosate effect occurred because 3 mg a.e./L caused higher pH (0.3 pH units) than the other three glyphosate concentrations ($p < 0.003$).

The analysis of dissolved oxygen revealed an effect of glyphosate concentration ($F_{3,24} = 3.9$, $p = 0.021$), and time ($F_{1,24} = 441$, $p < 0.001$), but not effect of density ($F_{2,24} = 1.92$, $p = 0.413$). Among the possible interactions were a time-by-concentration ($F_{3,24} = 23$, $p < 0.001$) and a time-by-density-by-concentration ($F_{6,24} = 3.6$, $p = 0.011$) interaction. As a result, we analyzed the dissolved oxygen data within each sample date (Table 5; Fig. 4). On day 7, no significant effects were noted. On day 16, however, the effect of glyphosate was

significant, with the 3 mg a.e./L treatment causing a 31% increase in dissolved oxygen compared to the three lower concentrations ($p < 0.003$). In general, dissolved oxygen concentrations increased 52% between days 7 and 16 and, by day 16, the highest glyphosate concentration had a higher dissolved oxygen concentration than the other glyphosate applications.

DISCUSSION

Our experiment indicated that increased competition not only reduces the growth of consumers in a simple food web, but can also make a globally common herbicide, Roundup Original MAX, significantly more lethal in one of the three species. In addition, we observed temperature stratification in the water column that led to an unequal distribution of the herbicide, with higher concentrations closer to the surface.

Increased tadpole density had a predictable impact on the growth of the tadpoles. For all three tadpole species, growth declined with increasing density, an outcome that is expected and reflects less per-capita consumption of periphyton in mesocosms with higher densities [34]. Given that increased density caused reduced growth but no reduction in the standing crop of periphyton, the data suggest that all densities of tadpoles were capable of consuming the standing biomass of periphyton to a similar level, as long as the herbicide did not cause substantial

mortality. When glyphosate concentrations did cause high mortality, all mesocosms had large increases in periphyton, suggesting a release from tadpole grazing pressure which simultaneously caused less periphyton consumption and allowed additional periphyton growth.

The impact of increased Roundup concentration was predictable from numerous past studies. In species of tadpoles from both North and South America, laboratory studies have found LC50 values to be in the range of 0.4 to 7.1 mg a.e./L [16,17,21,30]. For the three species used in the present study, Relyea [6] estimated that the LC50 values, based on 16-d lab experiments with reapplications of the herbicide every 4 d, were: bullfrogs = 1.55 mg a.e./L, green frogs = 1.63 mg a.e./L, and gray tree frogs = 1.01 mg a.e./L. These values are similar to the LC50 values from the current experiment, which ranged from 1.6 to 2.6 mg a.e./L. Of course, the current study lasted for 22 to 23 d and used a single application at the start of the experiment. This difference in protocol is the likely source of the slightly higher LC50 values in the present study. Collectively, the data suggest that the results of lab experiments using several different Roundup commercial formulations are good predictors of the LC50 values under more natural conditions.

Past mesocosm experiments using 3 mg a.e./L have also found substantial tadpole mortality. In diverse communities containing zooplankton, snails, predatory insects, and five species of tadpoles, Relyea [19] found that 3 mg a.e./L of glyphosate + POEA caused a 70% decline in amphibian species richness and an 86% decline in tadpole biomass relative to the controls. Due to the expectations that soil might ameliorate the lethal impacts of the herbicide, a second study that lacked predatory insects, and manipulated the presence and absence of different soils, found similar lethal effects on the tadpoles regardless of soil presence [20]. A third mesocosm experiment found that considerably less herbicide (1 mg a.e./L of glyphosate + POEA) had no impact on gray tree frogs (consistent with the current study), but caused 29% death in leopard frogs and 71% death in American toads [36]. These mesocosm studies are consistent with laboratory experiments using natural pond water in which 1.5 mg a.e./L of Vision (glyphosate + POEA) caused 35% mortality in leopard frogs at a pH of 5.5, but caused 80% mortality at a pH of 7.5 [15].

Given that estimated worst-case exposure scenarios of terrestrial glyphosate formulations in water range from 1.4 to 7.6 mg a.e./L [13,24–26], and observed worst-case scenarios range from 1.7 to 5.2 mg a.e./L [13,14,27], these experiments all suggest that expected environmental concentrations of glyphosate + POEA can cause substantial amphibian mortality in nature.

Interestingly, the herbicide became even more lethal to bullfrogs at higher levels of competition. Increased densities of the gray tree frogs and green frogs caused the mass of all three species to decline, as expected in a community in which all three species consume a shared resource (i.e., periphyton). While it is too early to know the mechanism underlying the synergy between Roundup and competition, it is clear that competition not only reduces an amphibian's rate of growth, but may also affect tadpole behavior, physiology, or endocrine function in ways that make tadpoles more susceptible to the herbicide. Given the observed synergy in bullfrogs, it is surprising that we did not observe the same synergy in green frogs or gray tree frogs. This suggests that species-specific traits may make some species inherently more sensitive to this combination of natural and anthropogenic stress than others. In the only previous exploration of glyphosate and food level on amphibians,

Chen et al. [15] examined leopard frog tadpoles (*R. pipiens*) and found a marginally nonsignificant effect of low food causing glyphosate + POEA (1.5 mg a.e./L) to become more lethal ($p = 0.079$). Thus, the phenomenon observed in our study in bullfrogs (but not in green frogs or gray tree frogs) may be a more common phenomenon than we currently appreciate, but more research is needed to assess this question.

Several studies have demonstrated interactions between competition and other pesticides. Many studies have examined interactions between tadpole competition and insecticides (carbaryl, malathion, and endosulfan) with a variety of different positive and negative effects on different species [10–12]. Such a wide range of effects is likely due to a combination of direct synergistic and antagonistic effects on the tadpoles, as well as indirect effects through the food web by eliminating many of the zooplankton and thereby affecting the availability of periphyton for the tadpoles. In studies focusing on invertebrates, it appears that increased competition can make both heavy metals (cadmium) and insecticides (carbaryl and esfenvalerate) more lethal through direct synergistic effects [7–9].

A prevailing idea in invertebrate research is that negative survival effects of contaminant-competition synergies on a population can be offset by increases in growth, postexposure survival, and fecundity of the individuals that are not killed by the contaminant [7,9]. In short, a compensatory effect may occur in which moderately lethal concentrations of pesticides can kill a substantial number of individuals, yet have no detrimental effect on the long-term persistence of the population. In the current study, gray tree frogs and green frogs exposed to 2 mg a.e./L showed moderate rates of mortality and increased growth rates, which likely reflects the increased per-capita food that became available when many of their competitors died. For amphibians, animals metamorphosing with greater mass typically experience improved survival and fecundity [37], but we lack the necessary data to understand how these magnitudes of increased tadpole growth might offset the mortality caused by pesticide exposure and, in turn, affect the long-term dynamics of the population.

The measurement of abiotic variables indicated that the mesocosms experienced a stratification of temperature. Thermal stratification is a common phenomenon in lentic water bodies, because surface waters warm faster than deeper waters. Thermal stratification makes the water column resistant to mixing of the water from top to bottom, and this kept the herbicide from mixing evenly throughout the water column [38]. Interestingly, we found that the upper water column contained two to four times as much glyphosate as the lower water column. On average, however, the top and bottom combined to produce a test value very close to our nominal concentration. Thermal stratification causing greater pesticide concentrations near the water's surface has been observed in previous studies [39,40], including a recent study examining the sensitivity of tadpoles to glyphosate over ontogeny [31]. Given that the present study only sampled stratification early in the experiment, future studies should examine how much this stratification persists over time.

The discovery of herbicide stratification has important implications for assessing the risk of glyphosate formulations in nature. First, samples taken from natural water bodies that stratify will likely show substantial differences in glyphosate concentrations at different water depths. Additionally, estimates of worst-case scenarios, which assume complete diffusion of the herbicide throughout the water column, may produce higher than expected concentrations near the water's surface and lower

than expected concentrations near the benthos. The true risk of the herbicide to aquatic organisms, therefore, depends upon their location in the water column. Attraction to the warmer surface by ectotherms to increase their metabolic rate would expose them to higher concentrations, whereas avoiding predators by seeking refuge in the benthos will expose them to lower concentrations. Thus, we need to consider our toxicological insights in concert with principles of limnology and the habitat preferences of nontarget organisms.

CONCLUSIONS

The examination of contaminant impacts under single-species laboratory conditions is an excellent first step in understanding the impact that contaminants could have on nontarget organisms. The present study highlights the new insights that can be gained by placing nontarget organisms into more natural conditions in which individuals can interact and temperature stratification can occur. Although the lethality of Roundup Original MAX was consistent with past laboratory and mesocosm experiments, the present study also identified that competition made this formulated product significantly more lethal to one species of tadpoles. Future work should examine the generality of this outcome, both by examining additional species of tadpoles and by determining the underlying mechanism. Given that several different biotic stressors can make a diversity of animals more susceptible to a range of pesticides, a common mechanism may underlie all of these studies.

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