



# Interactive effects of predators and a pesticide on aquatic communities

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Chemical contaminants are ubiquitous in nature and a major goal of ecologists has been to understand and predict their impacts on natural communities. While direct toxic effects can be garnered from single-species laboratory studies, the full suite of possible effects can only be observed when organisms are embedded within a community. In this study, we manipulated the concentration of malathion (a broad-spectrum insecticide) to determine the impacts on aquatic communities containing phytoplankton, periphyton, and 27 species of animals (16 zooplankton, 5 snails, 3 tadpoles and 3 predatory insects). Using relatively low concentrations (0.13 to 0.46 mg l<sup>-1</sup>), we found important direct (and interactive) effects of predators and malathion on the food web as well as a number of apparent density- and trait-mediated indirect effects. Malathion initiated an indirect effect by decreasing zooplankton diversity and abundance, which propagated an increase in phytoplankton, a decrease in periphyton, and a subsequent decrease in the growth of leopard frog tadpoles. There also was an apparent trait-mediated indirect effect whereby increased amounts of the pesticide reduced predation rates on amphibians without affecting the survival of the primary amphibian predator (larval *Anax* dragonflies). In contrast, snail survival and growth was unaffected by the pesticide but there were strong, species-specific effects from their primary predator (adult *Belostoma* water bugs). This is one of few studies to examine the impacts of malathion on aquatic communities across a range of concentrations, despite the fact that it is currently the most commonly applied insecticide in the United States, it is applied around the world, and it can be legally directly sprayed over aquatic habitats to control the mosquitoes that carry malaria and West Nile virus. Our results suggest several mechanisms by which a wide variety of pesticides with similar modes of action might impact aquatic communities.

As humans increasingly alter the environment, we struggle to understand the impacts that these alterations may have on natural populations, communities and ecosystems. In the arena of contaminants, the traditional approach to predicting impacts has been to test contaminants under highly controlled conditions using a small number of model organisms and then extrapolating the results to a wide range of taxa and ecological situations (Walker et al. 2006). However, it is increasingly apparent that this approach prevents us from understanding the complex array of outcomes that can only be observed under more complex, natural conditions (deNoyelles et al. 1994, Fleeger et al. 2003, Van Wijngaarden et al. 2005, Relyea and Hoverman 2006).

The transition from single-species laboratory experiments to more natural community-level experiments can be quite challenging because we will often need to quantify changes in all of the taxonomic groups that compose the community and understand the mechanisms by which the taxa interact (Hurlbert 1971, Hanazato and Yasuno 1989, Pratt et al. 1997, Mills and Semlitsch 2004, Relyea 2005, Rohr and Crumrine 2005). However, we can meet this challenge by drawing upon a wealth of single-species

toxicology studies to understand direct lethal and sublethal effects of pesticides and then combining this with knowledge of basic community ecology. In this way, we can make a priori predictions about which taxa will be directly killed by a given pesticide concentration and which taxa will be indirectly affected due to the trophic connections in the food web that propagate density-mediated (via changes in the abundance of a particular trophic group) and trait-mediated indirect effects (via changes in the traits of a particular trophic group; Relyea and Hoverman 2006). In addition, we might be able to generalize our insights to other communities and to other contaminants with similar modes of action.

We examined how a range of concentrations of a globally common insecticide affected freshwater communities containing a diversity of phytoplankton, periphyton, and 27 species of animals (zooplankton, snails, larval amphibians and predatory insects). Although the entire food web was of interest, there was particular interest in understanding the impact on the amphibians due to growing conservation concerns over declining amphibian populations around the world (Alford and Richards 1999, Stuart et al. 2004). Among a number of putative causes

for these declines, exposure to pesticides has received attention because investigators have documented patterns between declining populations and the upwind use of pesticides (Davidson et al. 2001, 2002). Of the many classes of pesticides that are used, patterns thus far implicate carbamates and organophosphates, which are insecticides that inhibit acetylcholine esterase (Sparling et al. 2001, Davidson 2004). Of these insecticides, malathion is one of the few that frequently appears in surveys on wetland habitats (McConnell et al. 1998, LeNoir et al. 1999). Surprisingly, there are very few studies of malathion's effects on aquatic communities despite the fact that it is globally applied, it can be legally applied directly over water, and it is currently the most used insecticide in the United States (Kiely et al. 2004). Previous studies of malathion community effects have been limited to experiments that compared the effects of

several pesticides, each applied at a single concentration (Relyea 2005, Relyea et al. 2005). The goal of the current study was to examine community effects using a range of concentrations that are sublethal to larval amphibians but likely lethal to some invertebrates in the community. Our hypothesis was that lethal effects on zooplankton and sublethal effects on invertebrate predators would respectively generate density- and trait-mediated indirect effects on the community (sensu Abrams 1995; Fig. 1). Specifically, we predicted that direct toxic effects of the pesticides on the zooplankton would initiate an indirect effect that would cause an increase in phytoplankton, and declines in periphyton and the herbivores that consume periphyton (snails and tadpoles). Moreover, if the pesticide concentrations are not lethal to the invertebrate predators but are capable of altering predator-prey interactions (i.e. predator behavior), we predicted that

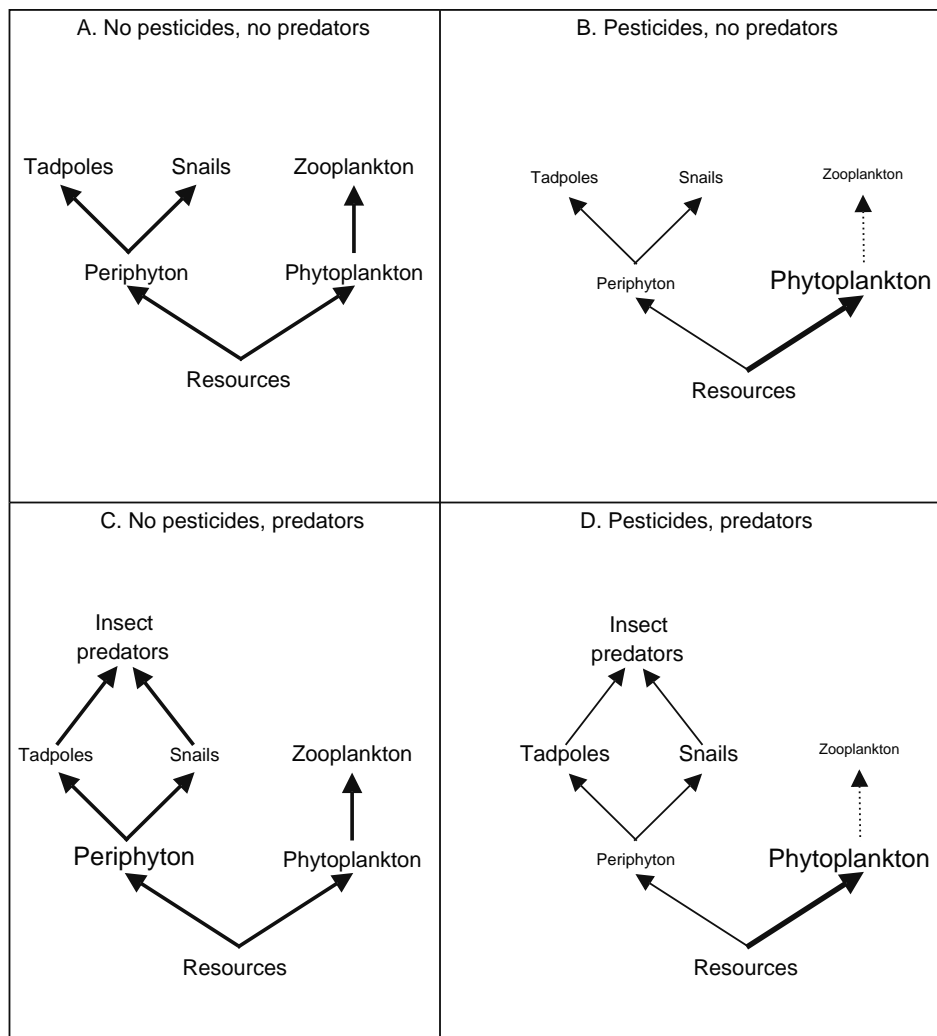


Figure 1. The aquatic food webs that were used in the experiment. Using the food web in panel A as the basis for comparison, the addition of a pesticide (B) should induce a trophic cascade through the zooplankton that ultimately impacts the biomass of the snails and tadpoles. The addition of insect predators (C) should reduce the survival of the snails and tadpoles and thereby have a positive impact on the biomass of periphyton. The addition of pesticides and predators (D) should cause a more complex outcome, including a zooplankton-mediated indirect effect on the periphyton and a pesticide-induced reduction in the per-capita predation rate between the insect predators and their prey (snails and tadpoles).

more of the herbivores would survive predation under greater pesticide concentrations.

## Malathion background

Malathion is a globally applied insecticide that is effective against a broad spectrum of invertebrates including mosquitoes that carry malaria and the West Nile virus. It has a relatively short half-life, ranging from 2 days at pH = 8 to 26 days at pH = 6 (Guerrant et al. 1970, Wang 1991). As a point of reference, natural freshwater communities typically possess a pH range 5–8 (Mitch and Gosselink 1986). Malathion is moderately toxic to larval amphibians ( $LC_{50_{16-d}} = 1.3\text{--}5.9 \text{ mg l}^{-1}$ ; Relyea 2004) and highly toxic to aquatic invertebrates (US EPA Ecotox Database, <<http://cfpub.epa.gov/ecotox>>). It is currently the most commonly applied insecticide in the United States with  $9.1\text{--}11.3 \times 10^6$  kg of active ingredient applied annually to nearly  $1 \times 10^6$  ha of cropland and an additional  $1.3\text{--}3.2 \times 10^6$  kg of active ingredient applied in the home, garden, industrial and governmental sectors (Kiely et al. 2004, National Pesticide Use Database, <[www.ncfap.org/data-base/default.htm](http://www.ncfap.org/data-base/default.htm)>). Based on current application rates, expected concentrations in a 30-cm deep wetland range up to  $1.6 \text{ mg l}^{-1}$  (Relyea 2004) and surveys of natural wetlands have found concentrations up to  $0.6 \text{ mg l}^{-1}$  (California Fish and Game 1982, USDA 1997, McConnell et al. 1998, LeNoir et al. 1999). Few geographically broad surveys exist for pesticide concentrations in lentic habitats, with most survey work being conducted on streams and rivers or ground water (USGS 2000). However, the US EPA has recently completed a major effort at assessing the risk of various pesticides to the California red-legged frog (*R. aurora draytonii*; Odenkirchen and Wentz 2007) that include the estimated environmental concentrations (EEC) of malathion in lentic habitats. The mean ( $\pm 95\%$  CI) EEC based on >50 categories of crop spraying is  $0.009 \pm 0.027 \text{ mg l}^{-1}$  with a range of 0.001–0.097  $\text{mg l}^{-1}$ . However, considerably higher concentrations are expected during direct applications over water including applications for mosquito control (EEC = 0.539  $\text{mg l}^{-1}$ ) and applications to control the pests of rice and water cress (EEC = 1.404–1.797  $\text{mg l}^{-1}$ ).

## Methods

We conducted the experiment at the Univ. of Pittsburgh's Pymatuning Laboratory of Ecology over a 21-d period during the summer of 2005. Using a completely randomized design, we employed a factorial combination of predatory insects (present or absent) crossed with four nominal pesticide concentrations (0, 0.25, 0.50 and 1.00  $\text{mg l}^{-1}$  of malathion). The eight treatment combinations were replicated four times for a total of 32 experimental units. The experimental units were 1000 l mesocosms (i.e. cattle watering tanks) filled with approximately 800 l of well water on 26–27 May (pH = 8). To each mesocosm, we added 300 g of dry leaf litter (primarily *Quercus* spp.) and 25 g of commercial rabbit chow on 27 May to serve as initial nutrient sources and surfaces to grow periphyton (a resource

for tadpoles and snails). Three days later (30 May), we collected pond water from seven nearby ponds, removed all predatory insects, and added a homogenized mixture of this water to all mesocosms to serve as an initial source of zooplankton, periphyton and phytoplankton. Each mesocosm was also equipped with an unglazed clay tile (10 × 10 cm, oriented vertically against the north side of the mesocosm), which was used to sample periphyton.

The experiment used a simple aquatic food web that is common to many ponds and wetlands (Fig. 1). We used three species of tadpoles in the experiment: wood frogs *Rana sylvatica*, leopard frogs *R. pipiens* and American toads *Bufo americanus*. All the species were collected as newly oviposited egg masses in early April from single populations in nearby ponds (wood frogs = 30 clutches, leopard frogs = 5 clutches, toads = 10 clutches). The eggs were hatched in covered, 200 l outdoor wading pools containing well water and fed rabbit chow ad libitum until used in the experiment. Twenty tadpoles of each species were added to each mesocosm on 10 June from a mixture of all egg masses (initial mass  $\pm 1$  SE: wood frogs =  $106 \pm 13$  mg; leopard frogs =  $62 \pm 5$  mg; toads =  $85 \pm 8$  mg). A sample of 20 tadpoles was set aside to assess short-term survival due to handling; 24-h survival of these samples was 100% for all three species.

We used five species of freshwater snails in the experiment: *Helisoma trivolvis*, *H. anceps*, *H. campanulata*, *Physa gyrina* and *Stagnicola elodes*. The snails were all originally collected from nearby ponds, although two species (*H. trivolvis* and *S. elodes*) were being held as cultured populations prior to the start of the experiment. Twenty snails of each species were haphazardly selected from our available animals and added to each mesocosm on 10 June (initial mass  $\pm 1$  SE: *H. trivolvis* =  $94 \pm 10$  mg; *H. anceps* =  $78 \pm 4$  mg; *H. campanulata* =  $318 \pm 13$  mg; *P. gyrina* =  $194 \pm 12$  mg; *S. elodes* =  $111 \pm 9$  mg). An additional sample of 20 snails was set aside to assess short-term survival due to handling; 24-h survival was 95 to 100%.

For those mesocosms assigned the predator treatment, we added three predator species: larval dragonflies *Anax junius*, larval predaceous diving beetles (*Dytiscus* sp.) and adult water bugs *Belostomatidae flumineum*. To reflect natural densities of these predators (E. E. Werner et al. unpubl.), we added one beetle, two dragonflies and two water bugs. A single *Dytiscus* beetle was used because previous studies found that when two *Dytiscus* beetles are added to a mesocosm, only one survives due to cannibalism (Relyea et al. 2005).

After all of the animals were added to the experiments, we dosed the mesocosms with the appropriate amount of commercial malathion (Malathion Plus, reported to contain 50% active ingredient; Ortho Corp., Marysville, OH, USA). Based on the volume of water in the mesocosms and the reported 50% active ingredient (plus 50% inert ingredients), we added 0.414 ml, 0.828 ml and 1.656 ml of malathion to achieve the nominal concentrations of 0.25  $\text{mg l}^{-1}$ , 0.50  $\text{mg l}^{-1}$  and 1.00  $\text{mg l}^{-1}$ , respectively. Because we used a commercial formulation with undisclosed inert ingredients, we did not include a vehicle control of the inert ingredients alone. We collected water from all mesocosms within 1 h of the applications to determine the actual concentrations achieved.

Approximately 200 ml was collected from each mesocosm (collected in the middle of the water column) and all samples for a given treatment were pooled. Water samples were frozen ( $-29^{\circ}\text{C}$ ) and then shipped to an independent laboratory for analyses (lower detection limit = 0.2 ppb). These analyses indicated that our three actual concentrations (0.14, 0.30 and  $0.46\text{ mg l}^{-1}$ ) were approximately half the nominal concentrations. The underlying cause of this disparity is unknown, although it is possible that some of the pesticide may have precipitated out of the water column after the applications. As a result, we report all concentrations in terms of the actual concentrations. As a point of reference, these actual concentrations are higher than the EEC when pesticides drift into lentic habitats during crop spraying ( $0.009 \pm 0.027\text{ mg l}^{-1}$ ) but less than the EEC for direct applications of malathion over water for mosquito control and aquatic crop protection ( $0.539\text{--}1.797\text{ mg l}^{-1}$ ; Odenkirchen and Wente 2007).

We took multiple samples of the zooplankton and phytoplankton because these taxa were expected to experience rapid population responses to our treatments. Zooplankton were sampled on days 5, 10, 15 and 20 using a 0.2 l tube sampler that was plunged into the middle of the water column at five locations in the mesocosm and then filtered through  $62\text{ }\mu\text{m}$  Nitex screening. The pooled samples for each mesocosm were preserved in 70% ethanol and later counted and identified to species. There was a total of 16 zooplankton species: eight species of copepods (*Acanthocyclops vernalis*, *Eucyclops agilis*, *Leptodiaptomus ashlandi*, *L. sicilis*, *Skistodiaptomus oregonensis*, *S. pallidus*, *Diacyclops thomasi*, *Senecella calanoides*) and eight species of cladocerans (*Scapholeberis* sp., *Simocephalus* sp., *Daphnia pulex*, *D. galeata mendotae*, *D. parvula*, *D. ambigua*, *D. retrocurva*, *Ceriodaphnia* sp.). Because our primary interest was in the indirect effect that might be caused by exposure to malathion, we analyzed the pesticide's impact on overall zooplankton species richness and then analyzed the abundance of the 16 species when pooled as either copepods or cladocerans because species within each group appear to have similar sensitivities to insecticides (Relyea 2005).

Phytoplankton were sampled on days 10, 15 and 20 by removing a 500 ml sample of water from each mesocosm and filtering the water through a Whatman GF/C filter. The filters were then wrapped in foil and placed in a freezer for subsequent analysis. The filters were extracted in 90% acetone and steeped in the dark at  $4^{\circ}\text{C}$  for 12 h. After steeping, the samples were centrifuged for 5 min at 1000 g and total chlorophyll was determined using spectrophotometry (Arar 1997).

Periphyton was sampled on day 20 by removing the clay tile from each mesocosm. Each tile was brushed clean of algae into a tub of filtered water and this water was then filtered through a Whatman GF/C filter that had been previously dried for 24 h at  $80^{\circ}\text{C}$  and weighed. After filtration, the filters were dried again for 24 h at  $80^{\circ}\text{C}$  and reweighed to determine the biomass of periphyton that had grown on each tile.

After 21 days, we terminated the experiment. During the experiment, most of the toads (98%, beginning on day 11) and a few of the wood frogs (2%, beginning on day 18) metamorphosed. Beginning on day 11 and every day following, we searched all mesocosms for metamorphs,

euthanized metamorphs in MS-222, and preserved them in 10% formalin. On day 21, we drained the mesocosms, sorted through the leaves, and removed all tadpoles, snails and predators. Predators were enumerated on site whereas tadpoles and snails were preserved in 10% formalin for enumeration and weighing at a later date (tadpoles were first euthanized in 2% MS-222). Because most of the wood frogs and all of the leopard frogs were recovered as tadpoles and the few wood frogs that metamorphosed exhibited no pattern among treatments, we used the mass of the tadpoles to examine treatment effects on wood frog and leopard frog mass. In all cases, the amphibian response variables represented mesocosm means.

## Statistical analyses

We analyzed the data using analyses of variance. For taxa that we measured multiple times during the experiment (zooplankton sampled four times and phytoplankton sampled three times), we conducted repeated-measures analyses of variance (rm-ANOVAs). For those taxa that were measured at the end of the experiment (amphibians, snails and periphyton), we conducted a multivariate analyses of variance (MANOVA) on the total biomass from each of the three groups. By first requiring significance at the multivariate level, we ensured that the analysis used an experiment-wise significance level of  $\alpha = 0.05$ . This protected us against type I errors before conducting subsequent univariate analyses of variance (ANOVAs) on the species composing each group to determine the underlying cause of the multivariate effects. In all cases of detecting significant univariate effects, we conducted mean comparisons using Fisher's LSD test. The survival of predators in the experiment did not meet the assumptions of parametric analyses, so we tested these data using non-parametric Kruskal-Wallis tests.

## Results

### Zooplankton diversity

To determine how the species richness of zooplankton was affected by the treatments, we conducted a rm-ANOVA and found effects of pesticides, time, and a time-by-pesticide interaction (Table 1, Fig. 2). Because of this interaction, we conducted subsequent univariate analyses within each sample period. On all four sample dates, there were significant univariate effects of the pesticides ( $p \leq 0.002$ ) but no effect of predators or pesticide-by-predator interactions ( $p > 0.05$ ). Moreover, mean comparisons among the four pesticide treatments (within each sample period) indicated that the control treatment always had more species of zooplankton than any of the three treatments exposed to malathion ( $p < 0.05$ ).

### Zooplankton abundance

To understand how the abundance of zooplankton changed with the treatments over time, we conducted rm-ANOVAs on cladoceran and copepod abundance measured on days 5,

Table 1. Results of repeated measures-ANOVAs on the species richness of zooplankton and the abundance of cladocerans, copepods and phytoplankton in mesocosms treated with a factorial combination of predators and pesticide concentrations. Bold p-values are significant at  $p < 0.05$ .

Source	Species richness			Cladoceran abundance		Copepod abundance		Phytoplankton abundance		
	DF	F	p	F	p	F	p	DF	F	p
Predators	1,24	0.0	1.0	9.5	<b>0.005</b>	0.1	0.765	1,24	1.6	0.212
Pesticides	3,24	67.9	<b>&lt;0.001</b>	285.1	<b>&lt;0.001</b>	19.0	<b>&lt;0.001</b>	3,24	5.4	<b>0.006</b>
Predators × pesticides	3,24	0.3	0.799	10.1	<b>&lt;0.001</b>	0.5	0.688	3,34	1.5	0.246
Time	3,72	12.8	<b>&lt;0.001</b>	5.0	<b>0.003</b>	16.4	<b>&lt;0.001</b>	2,48	4.1	<b>0.023</b>
Time × predator	3,72	2.5	0.064	6.5	<b>0.001</b>	1.3	0.297	2,48	0.4	0.691
Time × pesticide	9,72	8.8	<b>&lt;0.001</b>	4.7	<b>&lt;0.001</b>	9.5	<b>&lt;0.001</b>	6,48	1.3	0.290
Time × predator × pesticide	9,72	1.0	0.420	6.5	<b>&lt;0.001</b>	1.2	0.280	6,48	0.3	0.945

10, 15 and 20. For cladocerans, all of the main effects and interactions were significant (Table 1, Fig. 3), indicating that pesticides and predators interacted in different ways over the course of the experiment. However, the interactions simply reflected changes in cladoceran abundance with  $0 \text{ mg l}^{-1}$  of malathion; there were few cladocerans in any treatments containing malathion. When malathion was absent, cladocerans were equally abundant on day 5 in the two predator treatments ( $p = 0.564$ ). Cladocerans became more abundant with predators by day 10 d ( $p = 0.010$ ) and day 15 ( $p = 0.010$ ). By day 20, however, cladocerans became less abundant in the predator environment ( $p = 0.028$ ). Hence, malathion eliminated most of the cladocerans whereas the presence of predators was associated with dynamic shifts in cladoceran abundance in the absence of malathion.

For copepods, there were effects of time, pesticides, and a time × pesticide interaction (Table 1, Fig. 3). Subsequent univariate analyses within each sample date always revealed significant effects of pesticides ( $p \leq 0.002$ ) but never revealed effects of predators or the interaction ( $p > 0.2$ ). Early in the experiment (day 5), there was a steady decline in copepods with increased concentrations of malathion relative to the control (mean comparisons:  $p = 0.086$ ,  $p < 0.001$  and  $p < 0.001$ , respectively). However, as time passed, copepods became more abundant in mesocosms exposed to the low concentration of malathion ( $0.14 \text{ mg l}^{-1}$ ). On days 10, 15 and 20, copepods were more

abundant with  $0.14 \text{ mg l}^{-1}$  of malathion than any of the other malathion treatments (all mean comparisons:  $p < 0.05$ ). In summary, malathion generally had a strong negative effect on zooplankton abundance, although copepods did show some ability to rebound at the lowest concentration of malathion.

### Phytoplankton abundance

When we sampled phytoplankton on three dates (day 10, 15 and 20), we found significant effects of pesticides and time (Table 1, Fig. 4). While phytoplankton abundance declined over the course of the experiment, mean comparisons indicated that the pesticide effect occurred because all treatments containing malathion had a greater concentration of phytoplankton (i.e. total chlorophyll concentration) than the control treatment ( $p \leq 0.005$ ).

### MANOVA of periphyton, snails and amphibians

The MANOVA on the taxonomic groupings of biomass and abundance exhibited a multivariate effect of predators, pesticides, and their interaction (Table 2, Fig. 5). These multivariate effects were caused by several of the univariate responses. Periphyton biomass was affected only by the pesticides; across the three treatments containing malathion, periphyton was reduced by an average of 75% compared to the no-pesticide control ( $p \leq 0.001$ ). Snail biomass was reduced by an average of 24% with predators, but was unaffected by the pesticides and the interaction. Tadpole biomass experienced an interactive effect of pesticides and predators. Predators reduced tadpole biomass by 49% when malathion was absent ( $p < 0.001$ ) but had no effect when malathion was present at any concentration ( $p > 0.15$ ). Compared to the controls, any addition of malathion with predators absent caused a decrease in tadpole biomass ( $p = 0.001$ ) whereas malathion additions at the highest two concentrations with predators present caused an increase in tadpole biomass ( $0.30 \text{ mg l}^{-1}$ ,  $p = 0.019$ ;  $0.46 \text{ mg l}^{-1}$ ,  $p = 0.023$ ). In summary, pesticides were associated with a decline in periphyton biomass, predators were associated with a decline in snail biomass, and the two factors had interactive effects on amphibian biomass.

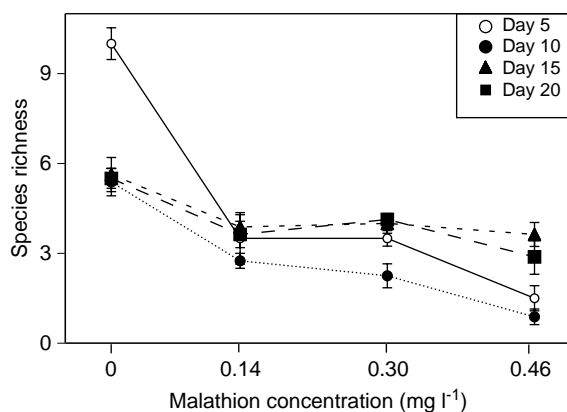


Figure 2. The effect of malathion on the species richness of zooplankton averaged across predator treatments (means  $\pm 1$  SE).

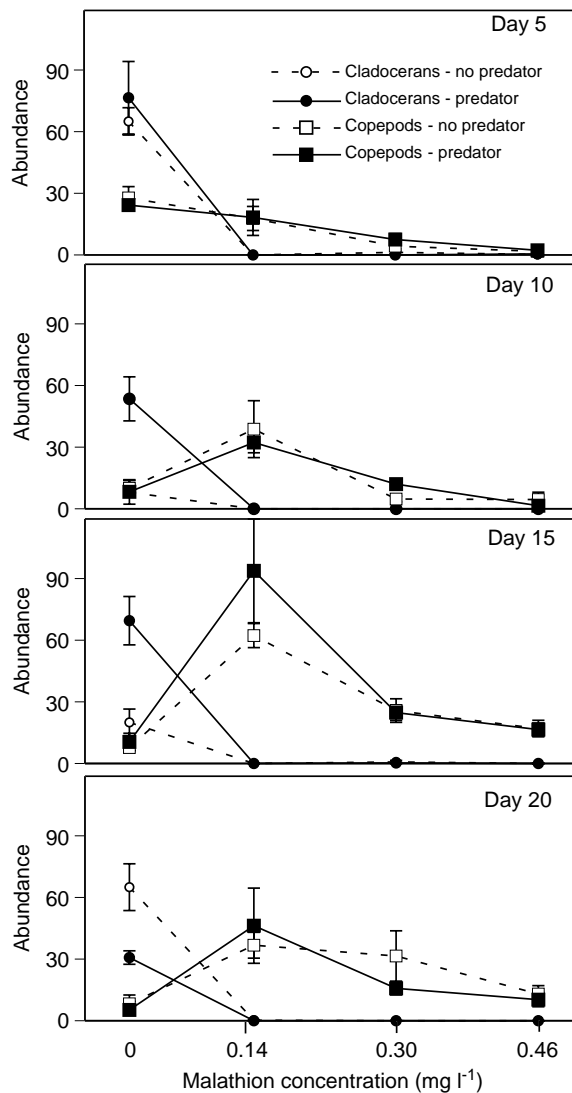


Figure 3. The effect of malathion crossed with either predators or no predators on the abundance of cladocerans and copepods (means  $\pm 1$  SE).

### Snail survival and growth

To understand the predator effects on total snail biomass, we conducted subsequent ANOVAs on the survival and growth of each species (Table 3, Fig. 6). A major contributor to the decline in total snail biomass was the reduced survival experienced by all five species of snails with predators. The reduction in survivorship was small for *H. campanulata*, *H. trivolvis* and *P. gyrina* (8–16%) but large for *H. anceps* and *S. elodes* (57–59%). Although predators caused a reduction in survival, predators caused individual snail mass to increase for *H. trivolvis* and *S. elodes* (11–19% larger). Unfortunately, survival of *P. gyrina* (with predators) was too low to provide an adequate test of growth. Thus, the reduction in total snail biomass with predators was driven by a reduction in the survival of all five species and partially countered by increases in the growth of two of the five species.

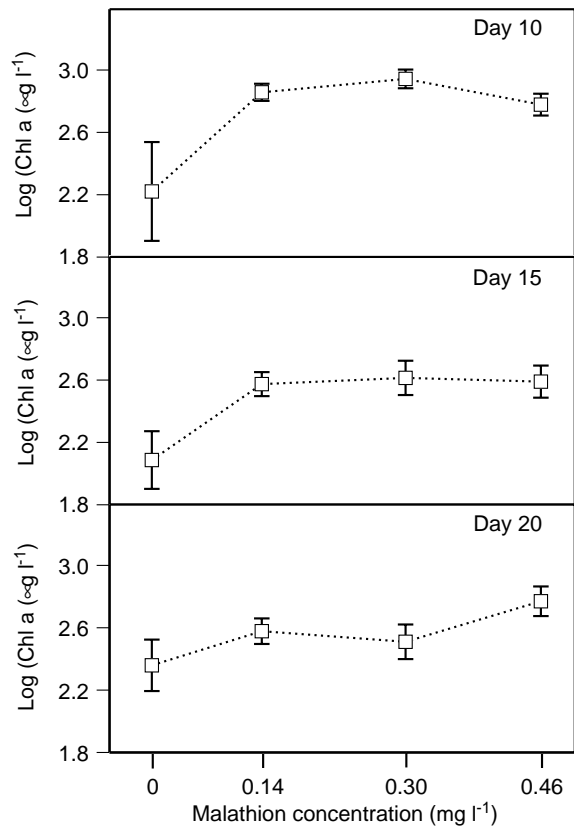


Figure 4. The effect of malathion on the abundance of phytoplankton (measured as Chl a concentration) averaged across predator treatments (means  $\pm 1$  SE).

### Tadpole survival and growth

To understand the interactive effects of predators and pesticides on total tadpole biomass, we conducted subsequent ANOVAs on the survival and growth of each species. For wood frogs, survival was reduced by predators but not by pesticides. However, the predator-by-pesticide interaction approached significance (Table 3, Fig. 7). Without predators, survival was high across all malathion concentrations. With predators, wood frog survival was reduced by

Table 2. Results of a MANOVA on the effects of predators and pesticide concentrations on the biomass or abundance of major taxonomic groupings. Univariate tests (p-values) are shown for both main effects and their interaction. Bold p-values are significant at  $p < 0.05$ .

A. Multivariate	DF	F	p
Predators	3,22	11.6	<b>&lt; 0.001</b>
Pesticides	9,54	3.3	<b>0.003</b>
Predators $\times$ pesticides	9,54	2.3	<b>0.028</b>
B. Univariate	Predators	Pesticides	Predators $\times$ pesticides
Tadpole biomass	<b>0.006</b>	0.578	<b>0.001</b>
Snail biomass	<b>&lt; 0.001</b>	0.623	0.604
Periphyton biomass	0.255	<b>&lt; 0.001</b>	0.837

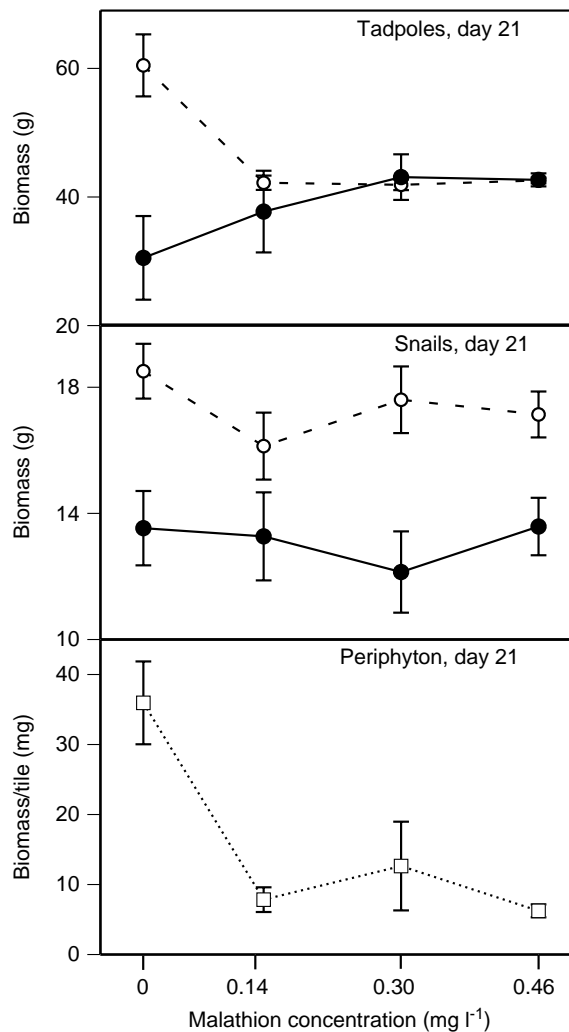


Figure 5. The effect of malathion crossed with either predators (closed symbols) or no predators (open symbols) on the biomass of tadpoles, snail, and periphyton. Periphyton data are averaged across predator treatments (means  $\pm$  1 SE).

51% with 0 mg l<sup>-1</sup> of malathion ( $p < 0.001$ ) but the reductions in survival became smaller as the concentration of malathion increased (30%,  $p = 0.059$ ; 15%,  $p = 0.309$ ; and 19%,  $p = 0.079$ ; respectively). Individual wood frog mass was increased by 22% with predators but mass exhibited no effect of pesticides or a predator  $\times$  pesticide interaction.

For leopard frogs, survival was affected by predators, pesticides and their interaction (Table 3, Fig. 7). The interaction occurred because survival was high in the absence of predators regardless of malathion concentrations, but the reduction in survival due to predators diminished with increased concentrations of malathion. For example, predators reduced leopard frog survival by 69% with 0 mg l<sup>-1</sup> of malathion ( $p < 0.001$ ), 49% with 0.14 mg l<sup>-1</sup> of malathion ( $p = 0.023$ ), 29% with 0.30 mg l<sup>-1</sup> of malathion ( $p = 0.098$ ), and 19% with 0.46 mg l<sup>-1</sup> of malathion ( $p = 0.002$ ).

The individual mass of leopard frog tadpoles was affected by predators and the pesticides but not by their interaction (Table 3, Fig. 7). Averaged across pesticide environments, tadpoles were 69% larger with predators than without predators. Averaged across predator environments, mass was highest with no pesticide and mass continually declined with higher concentrations of malathion (26% with 0.14 mg l<sup>-1</sup>,  $p = 0.038$ ; 31% with 0.30 mg l<sup>-1</sup>,  $p = 0.014$ ; and 45% with 0.46 mg l<sup>-1</sup>,  $p < 0.001$ ).

Toad survival was generally low across all treatments and was affected by predators and pesticides but not the interaction (Table 3, Fig. 7). Our anecdotal observations suggested that many of the toads died while trying to metamorphose in the mesocosms. Averaged across pesticide environments, predators caused an 11% decrease in toad survival. Averaged across predator environments, toad survival was 9 to 24% higher without pesticides than with any of the pesticide treatments ( $p \leq 0.05$ ). The lack of any surviving toads in four of the 12 treatments prevented an analysis of toad mass.

Table 3. The results of ANOVAs on the survival and individual mass of three species of tadpoles and five species of freshwater snails when exposed to a factorial combination of predators and pesticide concentrations. p-values are listed with F-statistics in parentheses; bold p-values are significant at  $p < 0.05$ . American toads and *P. gyrina* exhibited very low survival which precluded any analysis of individual mass.

Species	Response	Predator	Pesticide	Predator $\times$ pesticide
Wood frogs	survival	< <b>0.001</b> (29.5)	0.176 (1.8)	0.096 (2.4)
	mass	< <b>0.001</b> (15.9)	0.495 (0.8)	0.728 (0.4)
Leopard frogs	survival	< <b>0.001</b> (48.4)	<b>0.035</b> (3.4)	<b>0.031</b> (3.5)
	mass	< <b>0.001</b> (21.8)	<b>0.007</b> (5.2)	0.487 (0.8)
American toads	survival	<b>0.004</b> (10.3)	<b>0.001</b> (7.4)	0.420 (1.0)
	mass	—	—	—
<i>H. anceps</i>	survival	< <b>0.001</b> (73.7)	0.435 (0.9)	0.276 (1.4)
	mass	0.773 (0.1)	0.873 (0.2)	0.337 (1.2)
<i>H. trivolis</i>	survival	<b>0.012</b> (7.5)	0.649 (0.6)	0.812 (0.3)
	mass	<b>0.001</b> (13.9)	0.603 (0.6)	0.925 (0.2)
<i>H. campanulata</i>	survival	<b>0.035</b> (5.0)	0.359 (1.1)	0.177 (1.7)
	mass	0.683 (0.2)	0.218 (1.6)	0.794 (0.3)
<i>P. gyrina</i>	survival	< <b>0.001</b> (18.4)	0.230 (1.5)	0.469 (0.9)
	mass	—	—	—
<i>S. elodes</i>	survival	< <b>0.001</b> (104.5)	0.787 (0.4)	0.459 (0.9)
	mass	<b>0.005</b> (9.9)	0.852 (0.3)	0.525 (0.8)

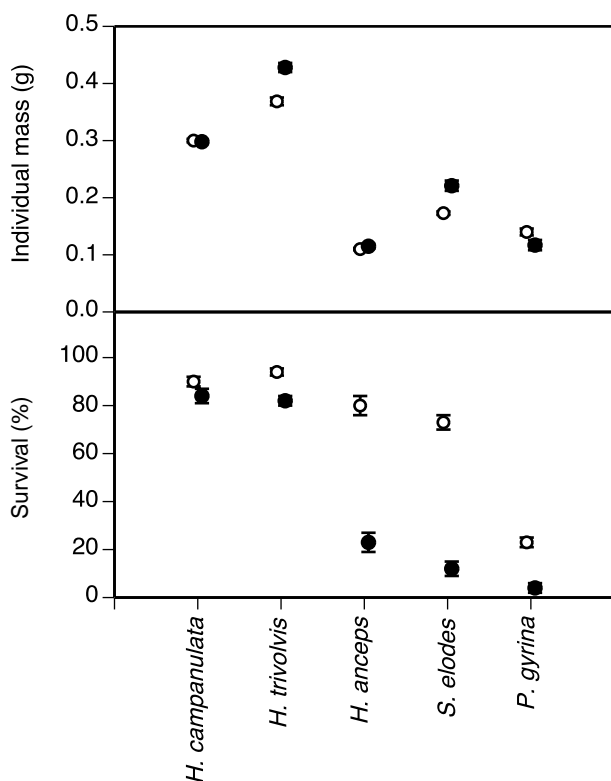


Figure 6. The effect of predators (closed symbols) versus no predators (open symbols) on the survival and mass of five snail species (averaged across malathion treatments; means  $\pm$  1 SE).

### Predator survival

The Kruskal–Wallis tests on predator survival indicated that there were no differences among the pesticide treatments. On average, 35% of the predatory insects were recovered at the end of the experiment. Only one of the 24 beetle larvae survived ( $\chi^2 = 3.0$ , DF = 3,  $p = 0.392$ ). There was a tendency of lower dragonfly survival and higher water bug survival with increased malathion concentrations, but the data were too variable to detect any treatment effects ( $\chi^2 = 5.1$ , DF = 3,  $p = 0.162$ ;  $\chi^2 = 2.6$ , DF = 3,  $p = 0.458$ ).

### Discussion

The results of our experiment demonstrate that malathion can alter aquatic communities and have a number of direct and indirect effects on a food web. One of the most striking effects was the dramatic reduction in zooplankton, an effect that appears to be common among different insecticides (carbaryl – Hanazato and Yasuno 1987, 1989, Relyea 2005; diazinon – Giddings et al. 1996; endosulfan – Barry and Logan 1998, Rohr and Crumrine 2005; esfenvalerate – Fairchild et al. 1992, Lozano et al. 1992; pyridaben – Rand et al. 2000). At intermediate and high concentrations of malathion, both cladocerans and copepods were eliminated. However, at the low concentration, the zooplankton assemblage rebounded in abundance but was converted from being dominated by cladocerans to being dominated by copepods. Similar impacts on zooplankton have been observed in a previous study using a single concentration of

malathion (nominal concentration =  $0.32 \text{ mg l}^{-1}$ ; Relyea 2005) and in many studies using carbaryl. For example, higher concentrations of carbaryl ( $0.5$  to  $6.0 \text{ mg l}^{-1}$ ) dramatically reduce all groups of zooplankton whereas lower concentrations ( $0.1 \text{ mg l}^{-1}$ ) kill the cladocerans but not the copepods (Hanazato and Yasuno 1987, 1989, 1990, Havens 1994, Mills and Semlitsch 2004, Relyea 2005). As noted by Van Wijngaarden et al. (2005), because the results of single-species, laboratory studies on zooplankton sensitivity to insecticides are typically consistent with the results of community mesocosm studies, “this makes it probable that in microcosm and mesocosm experiments, observed reductions in densities of microcrustaceans . . . can generally be considered as direct toxic effects.” The mechanism for malathion favoring the copepods appears to be pesticide-mediated competitive release. Because copepods are more resistant to insecticides than cladocerans at lower concentrations and because cladocerans and copepods compete for intermediate size phytoplankton (with cladocerans generally preferring smaller phytoplankton and copepods generally preferring larger phytoplankton), malathion can cause an indirect positive effect on copepods (Havens 1994, Mills and Semlitsch 2004, Relyea 2005).

With large reductions in the diversity and abundance of zooplankton, one would expect a bloom of phytoplankton which should, in turn, decrease light transmission and reduce periphyton biomass (Fig. 1). Our results supported this prediction, with phytoplankton (total chlorophyll concentration) increasing with the addition of malathion. This indirect effect subsequently decreased the abundance of periphyton (the resource of tadpoles and snails), following a similar dose–response relationship that was observed in the zooplankton. As one might expect, other insecticides that are highly toxic to zooplankton have shown the same indirect effect (with effects that can last for several months) including carbaryl (Havens 1994, 1995, Boone et al. 2004, Mills and Semlitsch 2004) and endosulfan (Barry and Logan 1998). In a study using endosulfan (Rohr and Crumrine 2005), the cascade to periphyton was not observed, but the experimental units were small 11 l tubs in which sufficient light could reach the periphyton.

The pesticide-mediated reduction in periphyton had a negative impact on the growth of leopard frogs but not wood frogs. The 26–45% reduction in mass experienced by leopard frogs is important because slower growth can have lasting impacts on the size at metamorphosis, post-metamorphic survival, time to reproduction, the ability to attract mates, and fecundity (Berven 1981, Smith 1987, Semlitsch et al. 1988, Gerhardt 1994, Altwegg and Reyer 2003). The lack of an effect on wood frog tadpoles suggests at least three non-exclusive mechanisms: 1) wood frogs were either foraging on periphyton that was higher up on the sides of the mesocosm (analogous to shallower water that would be less shaded by phytoplankton blooms), 2) wood frogs were able to sustain themselves on lower quality periphyton than leopard frogs (Schiesari 2006), or 3) wood frogs responded to the reduction in periphyton by improving their foraging and digestive efficiency to prevent substantial mass loss (Relyea 2000, 2002, Relyea and Auld 2004, 2005). Additional experiments are necessary to assess the relative role of these mechanisms.

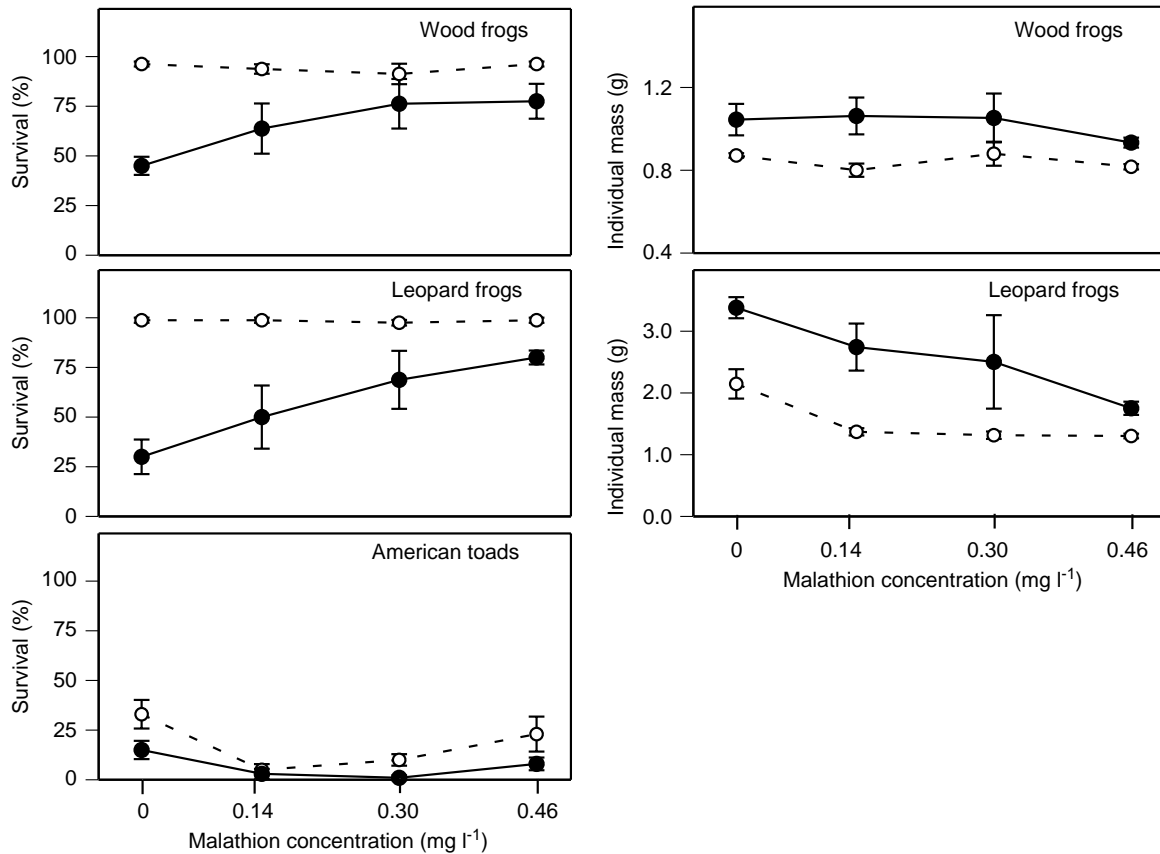


Figure 7. The effect of malathion crossed with either predators (closed symbols) or no predators (open symbols) on the survival and mass of three tadpole species (means  $\pm$  1 SE).

While there are no published investigations of malathion's indirect impacts on tadpole growth, reductions in tadpole growth have been described in previous studies using the insecticide carbaryl, although at concentrations that were an order of magnitude higher than the concentrations of malathion used in the present study (1.9–6.0 mg l<sup>-1</sup>, Mills and Semlitsch 2004). In the Mills and Semlitsch (2004) study, carbaryl was added to mesocosms, the chemical was allowed to degrade, and then the tadpoles were added; there was a striking indirect effect of reduced zooplankton, increased phytoplankton, decreased periphyton and slowed tadpole growth. In contrast, Boone and colleagues (Boone et al. 2001, 2004, Boone and Semlitsch 2001, 2002, 2003) used a similar range of carbaryl concentrations (3.5–7.0 mg l<sup>-1</sup>), but added the insecticide after the tadpoles were added. These latter studies have observed both positive and negative effects on tadpole growth, possibly because of this difference in protocol from Mills and Semlitsch (2004), may have caused a combination of both indirect effects and possible effects on tadpole foraging behavior. Because these latter studies have not quantified periphyton biomass (the food resource of tadpoles), it is difficult to identify the mechanisms underlying the variety of growth outcomes. Collectively, however, these studies suggest that insecticides that eliminate cladocerans might be expected to commonly cause an indirect effect that reduces the growth of some tadpole species.

Contrary to our predictions (Fig. 1), the application of the insecticide had no effect on snail growth despite the fact

that the snails also consume periphyton. One possibility is that the snails were simply not resource limited, so a reduction in periphyton would have no effect on their growth. However, when predators were present, the total number of surviving snails declined by 31% and the growth of two snail species increased (*H. trivolvis* and *S. elodes*). This suggests that at least these two species were resource limited. The most likely explanation for the lack of reduced snail growth with malathion present was that the snails were foraging more on the upper sides of the mesocosms and less on the bottom of the mesocosm where the shading effects of phytoplankton would be maximized. However, we did not collect habitat use data on snails to evaluate this hypothesis.

More surprising was that not one of the five species of snails suffered any significant mortality when exposed to malathion, despite the fact that insecticides such as malathion are generally highly toxic to invertebrates. There are few laboratory tests on the toxicity of malathion to snails, but the limited data suggest similar outcomes. For example, Tchounwou et al. (1991) conducted single-species toxicity tests with two species of snails and estimated LC50<sub>48-h</sub> values of 289 mg l<sup>-1</sup> for *H. trivolvis* and 126 mg l<sup>-1</sup> for *Biomphalaria havanensis*. Similarly, Frumin et al. (1992) found that *Lymnaea stagnalis* snails exhibited an LC50<sub>48-h</sub> of 13.3 mg l<sup>-1</sup> and *Planorbis corneus* exhibited an LC50<sub>48-h</sub> of 81.3 mg l<sup>-1</sup>. Further support can be found in a mesocosm study in which aquatic communities were exposed to a variety of pesticides (including 0.32 mg l<sup>-1</sup> of malathion; Relyea 2005) with no impact on snail survival.

Collectively, these data suggest that unlike most other invertebrates, snails are highly resistant to direct toxic impacts of malathion. The data on insect predators was quite variable, but it was clear that our concentrations of malathion did not cause widespread extermination of the insects, suggesting that adult water bugs and larval dragonflies may be considerably less sensitive to malathion than the zooplankton (Relyea 2005).

The predators also affected the survival of the snails, but not in any way that interacted with the addition of pesticides. Whereas water bugs are poor predators of tadpoles, they are specialist predators on freshwater snails and were likely the primary predator on snails (Hoverman et al. 2005, Hoverman and Relyea 2008). Of the four species that had high survival without predators (*H. campanulata*, *H. trivolvis*, *H. anceps* and *S. elodes*), the former two species experienced little to no reduction in survival (6–12%) whereas the latter two species experienced substantial reductions in survival (57–61%). For the three *Helisoma* species, we have a good understanding of snail defenses against their predators and how shell morphology (i.e. relative shell width) in particular helps to determine vulnerability to water bugs. For example, *H. campanulata* has a relatively wide shell that appears to be a specialized defense against water bugs, *H. trivolvis* has an intermediate shell width that is rapidly inducible to become wider and less vulnerable to water bugs, and *H. anceps* has a relatively narrow shell that appears to be a specialized defense against predatory crayfish at the cost of vulnerability to water bugs (Hoverman et al. 2005, Hoverman and Relyea 2007a, 2007b, 2008, Hoverman and Relyea unpubl.). Based on this functional knowledge of snail morphology and a priori expectations of species-specific predation rates by water bugs, the results strongly suggest that the primary predation threat on snails came from water bugs. Moreover, because this predation risk did not interact with the pesticide treatments, the data suggest that predation rates between water bugs and snails were not altered by the presence of malathion. This conclusion is consistent with past studies documenting that malathion does not alter the capture efficiency of water bugs (Relyea and Edwards unpubl.).

The interactive effect of predators and pesticides on the survival of leopard frogs and wood frogs was particularly intriguing. In previous community toxicology experiments, investigators have observed an increase in herbivore survival as a result of pesticides eliminating many or all of the herbivore predators (i.e. a density-mediated indirect effect; Mills and Semlitsch 2004, Relyea 2005, Relyea et al. 2005). In our experiment, however, the pesticide had no effect on predator survival, yet, in mesocosms containing insect predators, prey survival continually improved as we increased the concentration of malathion. The most likely explanation for this observation is that the predators' ability to successfully capture prey was compromised with increased pesticide concentrations. Recent studies have demonstrated that sublethal concentrations of pesticides (particularly those that inhibit acetylcholine esterase) can alter the behavior of predators and prey (e.g. decreased capture ability of predators and decreased movement of prey) and cause a reduction in predation rates (reviewed by Weis et al. 2001, Relyea and Hoverman 2006). Thus, the pesticide can cause a trait-mediated indirect effect that improves prey survival without

any change in predator density. Of the two predators that survived the experiment (dragonflies and water bugs), dragonflies are a much more voracious predator of tadpoles than water bugs (Relyea 2001) and low concentrations of malathion substantially reduce the capture success of dragonfly larvae (Relyea and Edwards unpubl.). Hence, it is likely that the interactive effects between predators and pesticides on tadpole survival were mediated through the behavior of the dragonfly predators.

The negative impact of the predators on tadpole survival were simultaneously associated with increases in the individual mass of the surviving tadpoles, suggesting that the survivors experienced competitive release for their resources (i.e. periphyton). When we consider the combined effects of amphibian survival and growth, we can see that both factors contributed to the changes in total amphibian biomass. Without predators, increased malathion was associated with decreases in total amphibian biomass. Because amphibian survival was generally unaffected, the decline in total amphibian biomass without predators was simply a reflection of the reduced growth of leopard frogs. With predators, increased malathion was associated with increases in total amphibian biomass due to increased leopard frog and wood frog survival. However, the positive contribution of this higher survival to total amphibian biomass was partially counteracted by negative contribution of the leopard frog tadpoles growing slower.

## Conclusions

In this study, we found that low concentrations of malathion were capable of interacting with the presence of predators to affect the entire community. Adding malathion caused a large decrease in zooplankton, an increase in phytoplankton, and a decrease in periphyton that impacted the growth of the leopard frog tadpoles but not the growth of the other periphyton-consuming herbivores. Adding predators reduced the survival of both the snails and tadpoles and the resulting decline in competition for periphyton was not exhibited in the biomass of periphyton but rather in the increased biomass of the snails and tadpoles. Adding malathion to communities containing predators continued to cause the zooplankton-mediated indirect effect but also reduced the predator's negative effect on tadpole survival in what appears to be a trait-mediated indirect effect. Collectively, this suggests that the insecticide's effect on many trophic groups can only be understood when embedded within the natural food web containing a nexus of species interactions.

Hence, to understand the impacts of contaminants on natural communities, we must address both the direct toxic effects on organisms (as determined from single-species laboratory studies) and the indirect effects that occur when organisms are embedded within a natural community. Such an understanding is particularly important considering that low concentrations of contaminants are much more common in nature and because single-species tests would suggest that low, sublethal concentrations should have little or no impact on the organism in question. The diversity of impacts caused by predators and the pesticide demonstrate that although single-species laboratory experiments offer

an important starting point in assessing the impacts of contaminants on non-target organisms, removing the organism from their communities prevents us from identifying additional pathways through which these contaminants can have positive and negative on the organisms. Understanding the complete set of effects is especially critical when considering the impacts of contaminants on groups such as amphibians because the answers have immediate conservation implications.

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