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A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities

Rick A. Relyea

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Abstract The ubiquity of anthropogenic chemicals in nature poses a challenge to understanding how ecological communities are impacted by them. While we are rapidly gaining an understanding of how individual contaminants affect communities, communities are exposed to suites of contaminants yet investigations of the effects of diverse contaminant mixtures in aquatic communities are rare. I examined how a single application of five insecticides (malathion, carbaryl, chlorpyrifos, diazinon, and endosulfan) and five herbicides (glyphosate, atrazine, acetochlor, metolachlor, and 2,4-D) at low concentrations (2–16 p.p.b.) affected aquatic communities composed of zooplankton, phytoplankton, periphyton, and larval amphibians (gray tree frogs, *Hyla versicolor,* and leopard frogs, *Rana pipiens*). Using outdoor mesocosms, I examined each pesticide alone, a mix of insecticides, a mix of herbicides, and a mix of all ten pesticides. Individual pesticides had a wide range of direct and indirect effects on all trophic groups. For some taxa (i.e., zooplankton and algae), the impact of pesticide mixtures could largely be predicted from the impacts of individual pesticides; for other taxa (i.e., amphibians) it could not. For amphibians, there was an apparent direct toxic effect of endosulfan that caused 84% mortality of leopard frogs and an indirect effect induced by diazinon that caused 24% mortality of leopard frogs. When pesticides were combined, the mix of herbicides had no negative effects on the survival and metamorphosis of amphibians, but the mix of insecticides and the mix of all ten pesticides eliminated 99% of leopard frogs. Interestingly, these mixtures did not cause mortality in the gray tree frogs and, as a result, the gray tree frogs grew nearly twice as large due to reduced competition with leopard frogs. In short, wetland communities can be dramatically impacted by low concentrations of pesticides (both separate and combined) and these results offer important insights for the conservation of wetland communities.

Keywords Amphibian decline · Pesticide mixture · Food web · Multiple pesticides · Synergistic

Introduction

Chemical contaminants are common in nature and ecologists are challenged to understand and predict the impacts that these contaminants have on natural communities (Relyea and Hoverman 2006). In aquatic systems, pesticides are a common type of contaminant. Indeed recent surveys in the US across different categories of land use have found that 30–60% of shallow groundwater and 60–95% of streams are currently contaminated with at last one pesticide (Gilliom et al. 2007). There is an increasing effort to examine the plethora of potential direct and indirect pathways by which aquatic organisms can be affected when embedded in a natural community (de Noyelles et al. 1994; Fleeger et al. 2003; Relyea and Hoverman 2006). Because of the substantial scale of this effort, the vast majority of current work in aquatic systems has focused on individual pesticides that are applied at a variety of amounts, times, and frequencies (Hanazato and Yasuno 1990; Havens 1995; Boone and Semlitsch 2001; Rohr et al. 2003; Relyea 2005).

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R. A. Relyea (✉)
Department of Biological Sciences,
University of Pittsburgh, 101 Clapp Hall,
Pittsburgh, PA 15260, USA
e-mail: relyea@pitt.edu

While understanding the effects of single contaminants on single organisms is daunting, even more sobering is the fact that communities of organisms in nature are typically exposed to suites of contaminants. Analyses of water from aquatic habitats and precipitation have revealed that pesticides commonly occur as complex mixtures (including acetochlor, atrazine, carbaryl, chlorpyrifos, diazinon, malathion, and metolachlor) and that the concentrations of the pesticides are often low (<1 p.p.m.; Zabik and Seiber 1993; Aston and Seiber 1997; McConnell et al. 1998; LeNoir et al. 1999; Battaglin et al. 2003; Hageman et al. 2006; Munn et al. 2006; Daly et al. 2007; Gilliom et al. 2007). Although we have data on the toxicity of most pesticides to a small number of model organisms, and a few studies have examined separate and pairwise combinations of pesticides (Howe et al. 1998; van Den Brink et al. 2002; Boone and Bridges-Britton 2006; Relyea 2004), we have a poor understanding of how more diverse mixtures of contaminants (e.g., four or more chemicals) affect individuals (Britson and Threlkeld 1998; Faust et al. 2003; Christin et al. 2003, 2004; Gendron et al. 2003; Hayes et al. 2006) or communities (Ridal et al. 2001; van Wijngaarden et al. 2004; Wendt-Rasch et al. 2004).

When examining the effects of pesticide mixtures, we can take one of two approaches. The first approach is to test each pesticide separately and in combinations that are increasingly diverse (combinations of two, three, four, etc.). While this approach can detect specific additive and synergistic effects across every pesticide combination (e.g., Relyea 2004), it creates prohibitively large experiments when dealing with more than a few chemicals (e.g., testing ten pesticides requires 1,024 treatment combinations). An alternative and time-saving approach is to test each pesticide separately and then select a few broad combinations to determine if any of the combinations cause important effects. If such effects are detected, one can subsequently dissect those combinations into smaller subsets to identify the key chemicals. In this study, I used the latter approach to examine how ten pesticides (five insecticides and five herbicides) present at low concentration would impact a wetland community composed of zooplankton, phytoplankton, periphyton, and larval amphibians. These communities are of important conservation interest not only because wetland habitats are in decline (Dahl 2000) but also because of concerns over the role that pesticides may be playing in global amphibian declines (Alford and Richards 1999; Davidson et al. 2001, 2002; Sparling et al. 2001; Stuart et al. 2004; Davidson and Knapp 2007). Surprisingly, no studies (to my knowledge) have examined how diverse mixtures of pesticides affect aquatic communities containing larval amphibians.

Materials and methods

The experiment was conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology in northwestern Pennsylvania (USA). The experiment employed outdoor mesocosms which have a long history of serving as useful experimental venues for ecotoxicology (see special features in Environmental Toxicology and Chemistry 1992, 1996; Hose and Van den Brink 2004). I applied a completely randomized design consisting of 15 treatments that were replicated 4 times for a total of 59 experimental units (only three replicates were conducted for the vehicle control). The treatments were composed of a negative control (water), a vehicle control (ethanol), one of five insecticides applied separately (carbaryl, malathion, chlorpyrifos, diazinon, and endosulfan), one of five herbicides applied separately (acetochlor, metolachlor, glyphosate, 2,4-D, and atrazine), a mix of the five insecticides, a mix of the five herbicides, and a mix of all ten pesticides (for each pesticide's mode of action, see Table 1). These ten pesticides were selected because they are among the most widely used and because many of them appear in surveys of aquatic ecosystems and atmospheric transport (Zabik and Seiber 1993; Aston and Seiber 1997; McConnell et al. 1998; LeNoir et al. 1999; Kiely et al. 2004; Hageman et al. 2006; Munn et al. 2006; Daly et al. 2007).

The experimental units were 1,300-l cattle watering tanks that served as pond mesocosms. The tanks were filled with approximately 1,000 l of well water (pH = 8) on 15–19 May 2006. On 7 June, I added water containing zooplankton, phytoplankton, and periphyton from a mixture of nearby ponds. The following day, I added 300 g of leaf litter (primarily *Quercus* spp.) and 25 g of commercial rabbit chow to serve as a source of algal nutrients and additional surface for algal growth in the tanks. On 16 June, I added two unglazed clay tiles (10 × 10 cm, oriented vertically) to serve as periphyton samplers.

Table 1 Mode of action of the ten pesticides used in the experiment

Pesticide	Type of pesticide	Mode of action
Carbaryl	Insecticide	Inhibits acetylcholine esterase
Malathion	Insecticide	Inhibits acetylcholine esterase
Chlorpyrifos	Insecticide	Inhibits acetylcholine esterase
Diazinon	Insecticide	Inhibits acetylcholine esterase
Endosulfan	Insecticide	Nervous system stimulant producing convulsions
Acetochlor	Herbicide	Inhibits cell division
Metolachlor	Herbicide	Inhibits cell division
Glyphosate	Herbicide	Inhibits amino acid synthesis
2,4-D	Herbicide	Auxin mimic
Atrazine	Herbicide	Inhibits photosystem II

I allowed the tanks to develop their algal and zooplankton communities for 18 days before adding tadpoles to the tanks (25 June). The tadpoles were collected as newly oviposited egg masses and hatched in 200-l wading pools (ten masses of leopard frogs, collected on 31 March, 23 masses of gray tree frogs, collected on 14 and 17 May). Hence, the two species were of different ages when they were added to the experiment. Thus, the experiment reflects a scenario in which two species that are oviposited at different times into a wetland experience an exposure to pesticides after both species are in the system. The potential impact of ontogeny on pesticide sensitivity is generally unknown in amphibians but there are some data suggesting greater sensitivity in older tadpoles (Howe et al. 1998). Prior to being added to the experiment, all tadpoles were fed rabbit chow ad libitum. To each tank, I added 20 tadpoles of each species from a mixture of all egg masses (initial mass ± SE: gray tree frogs = 77 ± 4 mg, leopard frogs = 134 ± 12 mg). This density of tadpoles (9/m^2 for each species) is well within natural densities for these two species (E. E. Werner, R. A. Relyea, D. K. Skelly, and K. L. Yurewicz, unpublished data).

Two days after adding the tadpoles, I applied the pesticides at nominal concentrations of 10 p.p.b. For most of the pesticides use in this experiment, this concentration is far below the maximum concentrations observed in natural water bodies and well below the maximum contaminant level or lifetime health advisory concentrations set by the US Environmental Protection Agency (EPA) (2006) (concentrations for aquatic communities that cause no unacceptable effect only exist for four of the ten compounds; Table 2). Thus, all mixture treatments were additive mixtures of pesticides such that the total nominal concentration of pesticide in a pesticide-mixture treatment was either 5 or 10 times higher than the nominal concentration of pesticide in any single-pesticide treatment. Setting the nominal concentrations of 10 p.p.b. was, of course, somewhat arbitrary and was not designed to mimic any specific mixture that has been observed in lentic systems. The goal was simply to assess the separate and combined impacts of the different pesticides at relatively low concentrations and the size of the experiment (59 mesocosms) required that only a single concentration could be used. The impacts of mixtures will undoubtedly differ under different concentration scenarios (whether one used equal concentrations or pesticide-specific concentrations).

All pesticides were purchased as technical grade chemicals (Chem Service, West Chester, Pa.) and therefore contained none of the inert ingredients that can be found in commercial formulations (Table 2). To achieve the nominal concentrations of 10 p.p.b. for a given pesticide, I dissolved the technical grade chemical in ethanol and then added the solution to the appropriate tanks. Because ethanol was used as the vehicle to carry the pesticides, I included a vehicle ethanol control to which I added the same amount of ethanol

Table 2 The purity of the ten pesticides used in the experiment, the actual concentrations achieved (nominal concentrations = 10 p.p.b.), and comparisons to current US Environmental Protection Agency (EPA) drinking water standards

Pesticide	Technical grade purity (%)	Actual concentration (p.p.b.)	Maximum concentrations observed in water bodies (p.p.b.)	Maximum contaminant level or lifetime health advisory (p.p.b.)[g]	Criteria for continuous concentration (p.p.b.)[h]
Carbaryl	99.5	6.9	2,500[c]	No standard	No standard
Malathion	99	5.8	583[e]	100	0.1
Chlorpyrifos	99.5	3.2	2[f]	2	0.041
Diazinon	99.5	2.1	33,000[f]	1	0.17
Endosulfan	99.3	6.4	9[b]	No standard	0.056
Acetochlor	98.0	10.0	21[d]	No standard	No standard
Metolachlor	97.1	7.4	124[d]	70	No standard
Glyphosate	98	6.9	5,200[a]	700	No standard
2,4-D	99	16.0	692[f]	70	No standard
Atrazine	98.1	6.4	172[d]	3	No standard

[a] Edwards et al. (1980)

[b] Muschal (1997)

[c] Norris et al. (1983)

[d] Battaglin et al. (2003)

[e] California Department of Fish and Game (1982)

[f] Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB)

[g] EPA (2006)

[h] Defined by the EPA as "the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect" (http://www.epa.gov/waterscience/criteria/wqcriteria.html#cmc)

as was added to the tanks receiving a mixture of all ten pesticides for an ethanol concentration = 0.003%. Once all pesticides were applied and the surface water was mixed using a 500-ml container, I waited 1 h and then collected water samples from all single-pesticide tanks midway in the water column and pooled the four samples from each treatment. Samples were collected in pre-cleaned amber jars, frozen, and then shipped to the Mississippi State Chemical Laboratory for concentration analysis using high pressure liquid chromatography. These analyses indicated that the actual concentrations were 2–16 p.p.b. (Table 2) and often within the drinking water standards of the US EPA. Moreover, variation from the nominal concentration did not hinder the objective of the experiment. The control tanks in the experiment were not tested for contaminants, but tests of the well water used for filling the tanks have indicated no detectable concentrations of any of the ten pesticides used in the experiment.

Response variables

To understand how the food web was affected by the separate and mixed pesticides, I quantified several response variables. The first data collected were the abiotic conditions of the mesocosms. On days 10 and 35, I quantified temperature, pH, and dissolved oxygen in each tank using a digital water meter (WTW, Wareham, Mass.).

On days 16 and 36, I sampled the zooplankton of each tank by plunging a 0.2-l tube sampler at the four cardinal directions and in the center of the tank (all midway in the water column). The five samples from each tank were combined into a single, 1-l sample for each tank, filtered through a 62-μm Nitex screen, and preserved in 70% ethanol. The zooplankton collected from each tank were classified to species. Twelve taxonomic groups of cladocerans and copepods were detected, but the assemblage was dominated (91%) by two species of cladocerans (*Daphnia pulex* and *Ceriodaphnia* sp.) and two species of copepods (*Skistodiaptomus oregonensis* and *Leptodiaptomus minutus*). Hence, I analyzed the abundance of these four groups.

On days 16 and 35, I sampled the phytoplankton. From each tank, I collected 500 ml from the middle of the water column and vacuum filtered the water through a Whatman GF/C filter. To assess the amount of chlorophyll *a* from each filter, I followed the protocols of Arar and Collins (1997) including the acidification step. Chlorophyll *a* concentrations (μg/l) were determined with a calibrated fluorometer (model TD-700; Turner Instruments, USA).

On days 25 and 36, I sampled the periphyton that had grown on the unglazed tiles. On each date, I scrubbed the periphyton from one side of the tile into a tub of filtered water and then vacuum filtered the slurry through Whatman GF/C filters that had been previously dried for 24 h at 80°C. After filtration, I dried the filters again at 80°C for 24 h and then reweighed them to determine the biomass of algae present on each tile.

Because gray tree frogs have an inherently shorter time to metamorphosis than leopard frogs, the gray tree frogs were the first to emerge from the experiment. On day 21 (17 July), the first gray tree frog metamorph was observed. From that date to the end of the experiment (day 57; 22 August), I conducted daily searches of each tank to collect metamorphs. The last gray tree frog emerged on day 39 and the first leopard frog metamorph emerged on day 30. Metamorphs were removed when they had at least one emerged forelimb (stage 42; Gosner 1960). All collected metamorphs were held in the laboratory in 1-l tubs containing moist sphagnum moss until tail resorption was complete (stage 46). Once this stage was achieved for an individual frog, I recorded the number of days that had passed since the start of the experiment to the completion of metamorphosis (which I defined as time to metamorphosis). Each animal was then euthanized in 2% MS-222, preserved, and later weighed. The amphibian response variables for each tank and species of frog were percent survival, mean time to metamorphosis, and mean mass at metamorphosis.

Once most of the metamorphs of both species had emerged (later determined to be 97.3% of all live tadpoles), I began removing water from the tanks from day 50 up to and including day 57 to simulate the pond drying that occurs in wetlands where these species occur. Each day, I removed approximately 120 l of water from each tank to simulate natural pond drying, which accelerates metamorphosis (Denver et al. 1998). Thus, any individuals that remained in the tanks would have the opportunity to metamorphose. After 8 days of gradually removing water, there was little water left in the tanks so I defined day 57 as a "dry pond". I then recovered all remaining amphibians from each tank to determine how many animals had died versus how many animals had simply not emerged due to slow growth and development. Any tadpole possessing at least one emerged forelimb on the final day was considered a successful metamorph and was held until metamorphosis was complete as described above.

Statistical analyses

Because I measured a number of response variables, I analyzed the data using multivariate ANOVA (MANOVA). The multivariate analysis was composed of the second measurements of all abiotic conditions, cladoceran and copepod abundance, phytoplankton abundance (as measured via chlorophyll *a*), periphyton biomass, the survival of gray tree frogs and leopard frogs, and size at and time to metamorphosis of the gray tree frogs. Due to nearly 100% mortality in two of the pesticide treatments for leopard

frogs, there were no life history data in these treatments. Thus, to prevent the first MANOVA from excluding all of the other food web data from these two treatments, the leopard frog life history responses were analyzed in a separate MANOVA. Following significant multivariate results, I examined each univariate response variable using either an ANOVA (for responses that were measured at the end of the experiment: tree frog mass, tree frog time to metamorphosis, tree frog survival, and leopard frog survival) or a repeated-measures ANOVA [for responses measured twice during the experiment: temperature, dissolved oxygen, and pH as well as the abundance of cladocerans (*D. pulex* and *Ceriodaphnia* sp.), copepods (*S. oregonensis* and *L. minutus*), phytoplankton, and periphyton]. When necessary, data were log-transformed. Because the zooplankton data had heteroscedastic errors, these data were ranked prior to analysis. The multivariate analyses controlled the experiment-wise error rate at $\alpha = 0.05$, to maximize their power, post hoc comparisons were conducted using Fisher's LSD test which preserves the comparison-wise error rate at $\alpha = 0.05$.

Results

The MANOVA on all final response variables (excluding the two life history traits of leopard frogs) revealed a significant effect of the treatments (Wilks' λ, $F_{182,313} = 2.5$, $P < 0.001$). Hence, I subsequently examined the response variables using ANOVAs.

Amphibians

For the two species of amphibians, I analyzed survival, mass at metamorphosis, and time to metamorphosis. Leopard frogs exhibited large effects of the pesticide treatments. For survival, there were treatment effects (Table 3; Fig. 1). While survival was 96% in the control, survival was 76% with diazinon, 16% with endosulfan, and 1% with either the mix of five insecticides or all ten pesticides ($P \leq 0.005$). Compared to the endosulfan treatment, the mix of five insecticides or all ten pesticides caused higher mortality ($P = 0.031$). If we combine the survival of metamorphs plus all remaining tadpoles that failed to metamorphose when the tanks dried, we can assess whether the treatments caused the leopard frogs to die during the experiment or simply caused slower growth and development that prevented some of the animals from metamorphosing prior to tank drying. An ANOVA on these combined data still indicated an effect of treatment ($F_{14,44} = 93.6$, $P < 0.001$); compared to the control, the diazinon treatment was no longer different ($P = 0.247$), but the results of endosulfan, the mix of five insecticides, and the mix of all ten pesticides were unchanged because no tadpoles remained in any mesocosms exposed to these latter three treatments and no tadpoles remained in the controls.

The high rates of death in the mix of five insecticides and the mix of all ten pesticides (mean = 99%) prevented the life history traits of the leopard frog from being included in the first MANOVA. Thus, these traits were analyzed in a second MANOVA that revealed a significant effect (Wilks' λ, $F_{24,70} = 4.5$, $P < 0.001$). Mass at metamorphosis was affected by the treatments (Table 3; Fig. 2). Compared to the control, metamorphs were smaller with diazinon ($P = 0.011$) but larger with endosulfan ($P < 0.001$). Time to metamorphosis was marginally affected by the treatments (Table 3; Fig. 2), but none of the pesticide treatments differed from the control ($P > 0.07$). Had there not been pond drying near the end of the experiment, leopard

Table 3 Results of ANOVAs and repeated-measure ANOVAs that examined how the pesticide treatments affected a number of biotic and abiotic response variables. *F*-values are given; *P*-values are *in parentheses*

Response variable	Treatment	Time	Treatment × time
Tree frog survival	1.6 (0.103)		
Tree frog mass at metamorphosis	4.5 (<0.001)		
Tree frog time to metamorphosis	2.3 (0.019)		
Leopard frog survival	56.1 (<0.001)		
Leopard frog mass at metamorphosis	7.4 (<0.001)		
Leopard frog time to metamorphosis	2.0 (0.053)		
Daphnia pulex abundance	13.9 (<0.001)	<0.1 (0.895)	1.3 (0.243)
Ceriodaphnia abundance	4.1 (<0.001)	<0.1 (0.982)	1.4 (0.212)
Skistodiaptomus oregonensis abundance	11.0 (<0.001)	<0.1 (0.971)	1.1 (0.421)
Leptodiaptomus minutus abundance	8.5 (<0.001)	<0.1 (0.999)	1.7 (0.098)
Phytoplankton (chlorophyll *a*)	3.9 (<0.001)	158 (<0.001)	1.1 (0.385)
Periphyton biomass	3.4 (<0.001)	6.0 (0.019)	2.5 (0.012)
Temperature	1.1 (0.404)	655 (<0.001)	1.1 (0.411)
pH	5.1 (0.001)	23.8 (<0.001)	3.0 (0.003)
Dissolved oxygen	9.5 (<0.001)	11.4 (0.002)	3.5 (0.001)

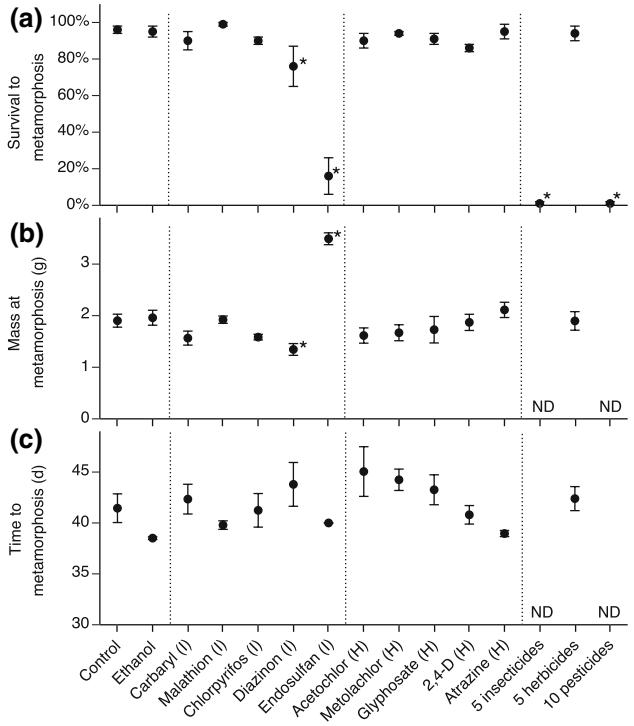

Fig. 1 Changes in the **a** survival, **b** mass at metamorphosis, and **c** time to metamorphosis of leopard frog tadpoles (*Rana pipiens*) in outdoor mesocosms exposed to no pesticides (control), solvent only (ethanol), five separate insecticides (*I*) (carbaryl, malathion, chlorpyrifos, diazinon, and endosulfan), five separate herbicides (*H*) (acetochlor, metolachlor, glyphosate, 2,4-D, and atrazine), a mixture of the five insecticides, a mixture of the five herbicides, and a mixture of all ten pesticides. In two of the treatments, there were no life history data (*ND*) due to a lack of surviving animals. Data are mean ± 1 SE. *Asterisks* indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test)

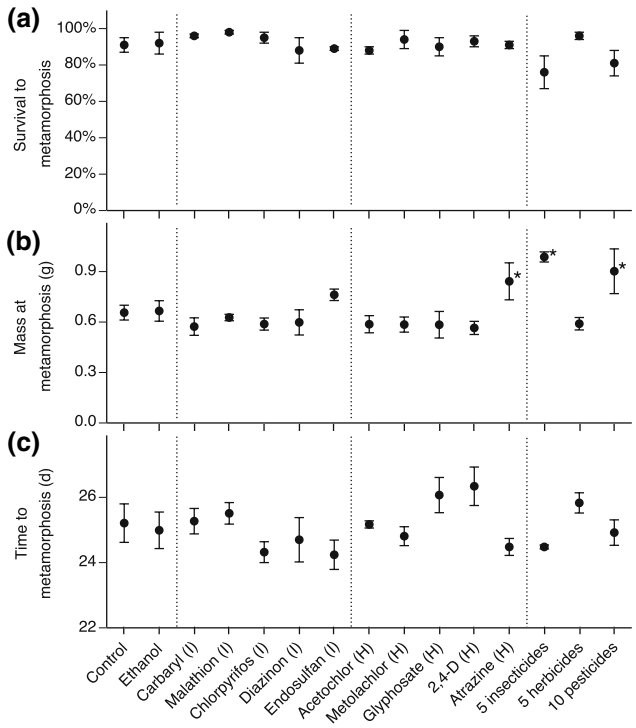

Fig. 2 Changes in the **a** survival, **b** mass at metamorphosis, and **c** time to metamorphosis of gray tree frog tadpoles (*Hyla versicolor*) in outdoor mesocosms exposed to no pesticides (control), solvent only (ethanol), five separate insecticides, five separate herbicides, and a mixture of all ten pesticides. Data are mean ± 1 SE. *Asterisks* indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). For abbreviations, see Fig. 1

frogs in the diazinon treatment would have obviously exhibited a longer mean time to metamorphosis. In summary, leopard frogs experienced low mortality with diazinon (24%), high mortality (84%) with endosulfan, and very high mortality (99%) with a mix of insecticides or all ten pesticides; metamorphs were smaller with diazinon but larger with endosulfan. Including survival as a covariate in the analyses of growth and development did not alter these results.

For gray tree frogs, there was no effect of treatment on survival (Table 3; Fig. 2). However, there were effects on mass at metamorphosis. Compared to the control, tree frogs were larger with atrazine ($P = 0.045$), the mix of insecticides ($P = 0.001$), and the mix of all ten pesticides ($P = 0.009$). The mix of insecticides caused a greater mass at metamorphosis than any of the five insecticides alone ($P \leq 0.017$) and the mix of herbicides caused a smaller mass at metamorphosis than atrazine alone ($P = 0.008$). There also were effects on time to metamorphosis, but none of the pesticide treatments differed from the control ($P > 0.06$). In summary, gray tree frogs exhibited no differences in survival or time to metamorphosis, but they did emerge larger with atrazine, the mix of insecticides, and the mix of all ten pesticides.

Zooplankton

The zooplankton exhibited dramatic responses to the pesticide treatments. For all four taxa, there were significant effects of the treatments, but no effects of time or treatment-by-time interactions (Table 3; Fig. 3). Subsequent mean comparisons identified which treatments differed from the controls. For example, compared to the controls, *D. pulex* was much less abundant with chlorpyrifos, diazinon, the mix of insecticides, and the mix of all ten pesticides ($P < 0.001$). *Ceriodaphnia* was more abundant in the vehicle control (i.e., ethanol) treatment ($P = 0.019$) but absent in any treatment containing a single insecticide or multiple insecticides ($P \leq 0.049$). Among the copepods, *S. oregonensis* exhibited moderately reduced abundance when exposed to a mixture of the five herbicides ($P = 0.021$) but a sharp reduction in abundance when exposed to endosulfan alone, the insecticide mix, and the mixture of all ten

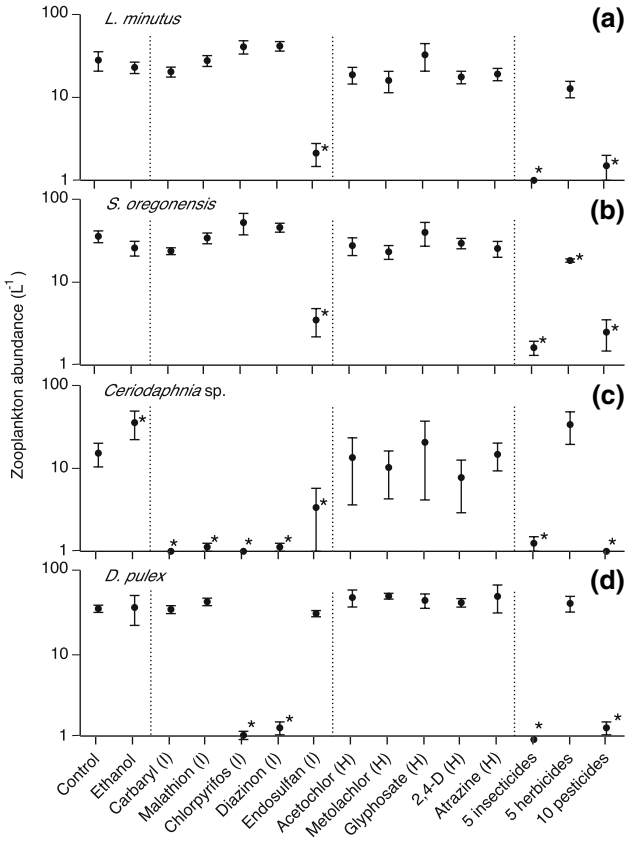

Fig. 3 Changes in the abundance of copepods (**a** *Leptodiaptomus minutus* and **b** *Skistodiaptomus oregonensis*) and cladocerans (**c** *Ceriodaphnia* sp. and **d** *Daphnia pulex*) in outdoor mesocosms exposed to no pesticides (control), solvent only (ethanol), five separate insecticides, five separate herbicides, and a mixture of all ten pesticides. Data are mean ± 1 SE, averaged over two sample periods (day 16 and 36), on a log scale. *Asterisks* indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). For abbreviations, see Fig. 1

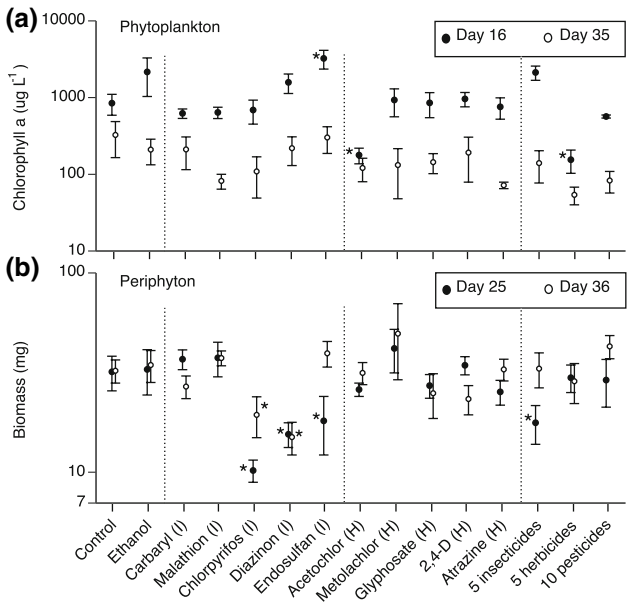

Fig. 4 The abundance of **a** phytoplankton (measured as chlorophyll *a* concentration) and **b** periphyton (measured as dry biomass on a clay tile) over time in outdoor mesocosms exposed to no pesticides (control), solvent only (ethanol), five separate insecticides, five separate herbicides, and a mixture of all ten pesticides. Data are mean ± 1 SE on a log scale. Within each sample date, *asterisks* indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). For abbreviations, see Fig. 1

pesticides ($P < 0.001$). For the other dominant copepod (*L. minutus*), there was a sharp reduction in abundance when exposed to endosulfan alone, the insecticide mix, and the mixture of all ten pesticides ($P < 0.001$). In summary, *Ceriodaphnia* was sensitive to all five insecticides, *D. pulex* was sensitive to chlorpyrifos and diazinon, and both copepod species were sensitive to endosulfan. In all cases, the mix of insecticides and the mix of all ten pesticides impacted the zooplankton to a degree that was nearly identical to the individual effects of one or more of the individual insecticides.

Phytoplankton and periphyton

The two types of algae also responded to the treatments. The analysis of chlorophyll *a* from phytoplankton indicated significant effects of treatment and time, but no treatment-by-time interaction (Table 3; Fig. 4). Chlorophyll *a* was higher on the first sample date than the second sample date. Compared to the control, there was more chlorophyll *a* with endosulfan ($P = 0.043$) but less chlorophyll *a* with acetochlor and the mix of herbicides ($P \leq 0.014$). The mix of insecticides was not different from any insecticide alone ($P > 0.06$) while the mix of herbicides was different from metolachlor, glyphosate, 2,4-D, and atrazine alone ($P < 0.02$), but not different from acetochlor alone ($P = 0.248$). Thus, phytoplankton increased with endosulfan and decreased with acetochlor and the effects of mixing either insecticides or herbicides were predictable from those compounds that had significant individual effects.

The analysis of periphyton revealed significant effects of treatment, time, and a treatment-by-time interaction (Table 3; Fig. 4). On the first sample date, there was an effect of the treatments ($F_{14,44} = 4.3$, $P < 0.001$). Compared to the control, periphyton was less abundant with chlorpyrifos, diazinon, endosulfan and the mix of insecticides ($P \leq 0.028$). Periphyton abundance with the mix of insecticides was different from carbaryl and malathion alone ($P \leq 0.006$) but not different from chlorpyrifos, diazinon, endosulfan alone ($P > 0.06$); the mix of herbicides was not different from any of the five herbicides alone ($P > 0.2$). On the second sample date, there also was an effect of the treatments ($F_{14,44} = 2.3$, $P = 0.020$). Compared to the control, periphyton was less abundant with chlorpyrifos and

diazinon ($P \leq 0.043$). Periphyton abundance with the mix of insecticides was similar to the individual effects of carbaryl, malathion, chlorpyrifos, and endosulfan ($P \geq 0.06$) but greater than diazinon alone ($P = 0.015$). In summary, periphyton was often less abundant with chlorpyrifos, diazinon, endosulfan and the impact of mixing the five insecticides was largely predictable from the individual pesticide effects.

Abiotic effects

The analysis of temperature indicated an effect of time, but no effect of treatment or a treatment-by-time interaction (Table 3). The mean temperature (±1 SE) was 20.5 ± 0.1°C on day 10 and 28.3 ± 0.3°C on day 35. This simply reflects the warmer ambient outdoor temperatures over time.

The analysis of dissolved oxygen found effects of treatment, time, and a treatment-by-time interaction (Table 3; Fig. 5). On the first sample date, there was an effect of the treatments ($F_{14,44} = 8.1$, $P < 0.001$). Compared to the control, oxygen concentrations were higher with endosulfan ($P = 0.005$), but lower with ethanol, acetochlor and the mix of five herbicides ($P \leq 0.013$). On the second sample date, there also was a treatment effect ($F_{14,44} = 3.6$, $P = 0.001$). Compared to the control, oxygen concentrations were higher with diazinon, endosulfan, the mix of five insecticides, or the mix of all ten pesticides ($P \leq 0.035$).

The analysis of pH detected effects of treatment, time, and a time-by-treatment interaction (Table 3; Fig. 5). On the first sample date, there was a treatment effect (univariate $F_{14,44} = 5.4$, $P < 0.001$). Compared to the control, tanks with endosulfan had higher pH ($P \leq 0.001$), whereas tanks with ethanol, acetochlor, or the mix of herbicides had lower pH ($P \leq 0.050$). On the second sample date, there was still a treatment effect ($F_{14,44} = 4.1$, $P < 0.001$). Compared to the control, tanks with diazinon, endosulfan, the mix of insecticides, and the mix of all ten pesticides had higher pH ($P \leq 0.035$).

Given that blooms of phytoplankton should increase pH and dissolved oxygen, I also examined correlations among these variables. Using regressions of phytoplankton abundance on pH and dissolved oxygen, I found that this positive relationship existed on both sample dates for pH ($P = 0.001$, $r = 0.433$; $P = 0.005$, $r = 0.362$) and on both sample dates for dissolved oxygen ($P = 0.021$, $r = 0.299$; $P = 0.014$, $r = 0.318$).

In summary, two of the insecticides (diazinon and endosulfan alone and in mixtures) generally caused higher pH and higher oxygen concentrations while acetochlor (alone and in the herbicide mixture) generally caused lower pH and lower oxygen concentrations. In general, all of these abiotic conditions are nonlethal to aquatic organisms.

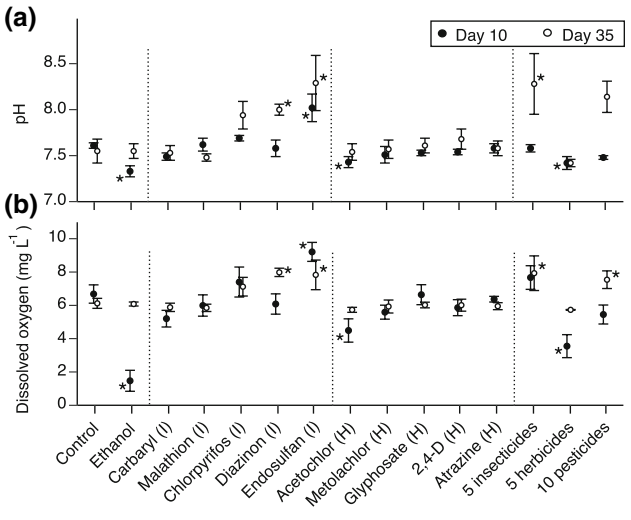

Fig. 5 Changes in **a** pH and **b** dissolved oxygen over time in outdoor mesocosms exposed to no pesticides (control), solvent only (ethanol), five separate insecticides, five separate herbicides, and a mixture of all ten pesticides. Data are mean ± 1 SE. Within each sample date, *asterisks* indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). For abbreviations, see Fig. 1

Discussion

The results of the experiment demonstrate that separate and combined pesticides can have dramatic direct and indirect effects on aquatic communities including the zooplankton, phytoplankton, periphyton, and larval amphibians (Fig. 6). Interestingly, the extent of the indirect effect was pesticide dependent. Among the five insecticides, carbaryl and malathion appeared to impact the cladocerans with no further impact on the community. Chlorpyrifos, however, reduced the abundance of cladocerans, increased the abundance of phytoplankton and reduced the abundance of periphyton. Diazinon caused a similar chain of events, but the indirect negative effect on the periphyton extended to reduce leopard frog tadpole growth and development and ultimately caused 20% of the leopard frogs to not metamorphose before the environment dried. Finally, endosulfan reduced the abundance of copepods and caused a similar negative indirect effect on the periphyton early in the experiment, but as the leopard frog tadpoles appeared to die of direct toxicity the periphyton rebounded and the grey tree frogs actually experienced greater growth. The individual herbicides showed occasional impacts on individual taxa, but there was no clear indication of any indirect effects from the addition of the herbicides. The mixtures of the five insecticides or all ten pesticides caused both cladocerans and copepods to decline; however, the expected cascade to reduce the periphyton was likely opposed by the severe

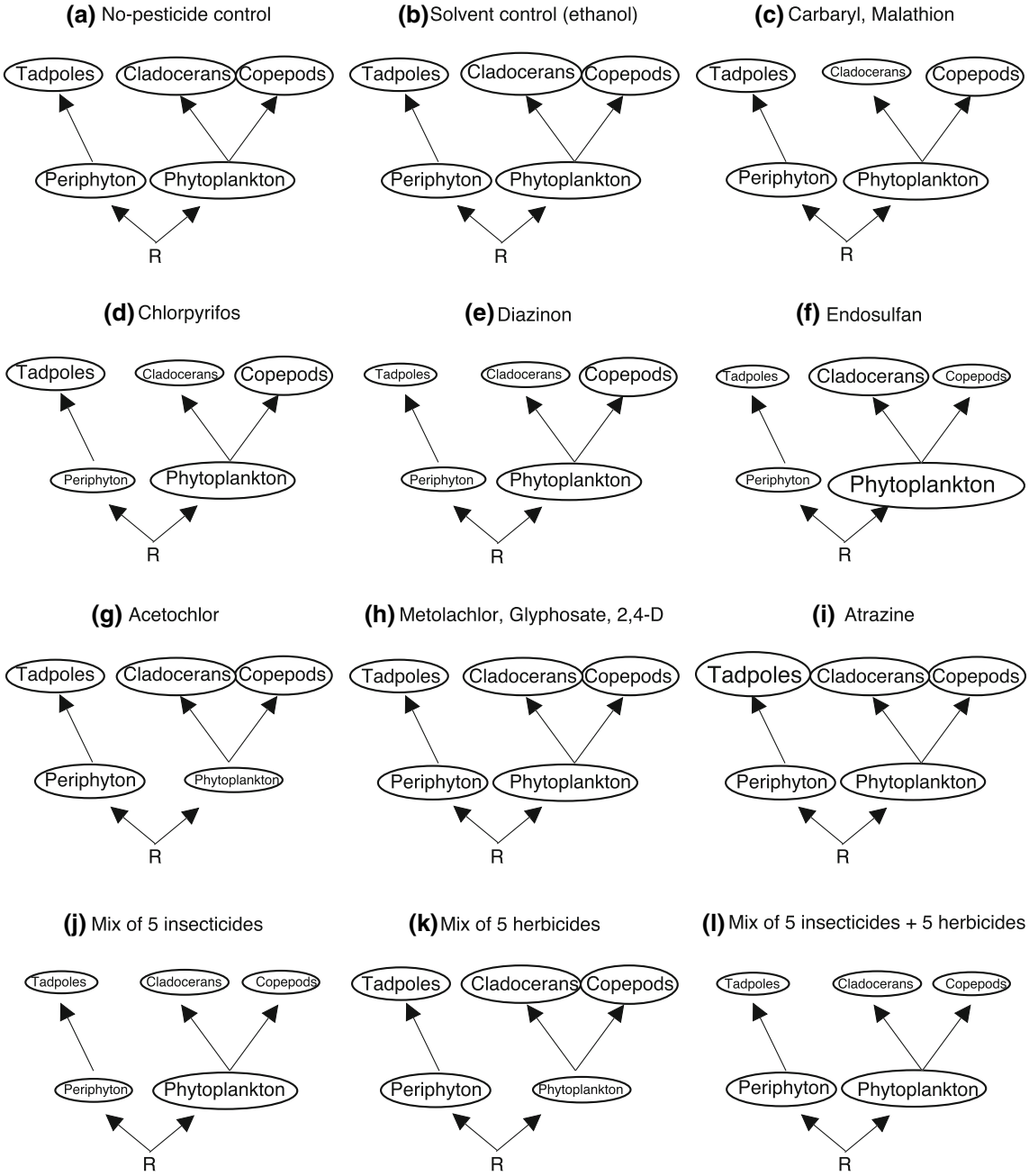

Fig. 6 Changes in biomass of trophic groups in the food web as a result of exposure to **a** no pesticide (control), **b** solvent control (ethanol), insecticides (**c** carbaryl, malathion; **d** chlorpyrifos; **e** diazinon; **f** endosulfan), herbicides (**g** Acetochlor; **h** metolachlor, glyphosate, 2,4-D; **i** atrazine) and pesticide mixtures (**j** five insecticides; **k** five herbicides; **l** five insecticides plus five herbicides). The size of the *circle* and *font* for each trophic group indicates the qualitative change in the biomass of that trophic group. *R* Basal resources for the two types of algae; for other abbreviations, see Fig. 1

decline in leopard frog tadpoles that normally would have consumed the periphyton. Moreover, the high mortality of the leopard frogs caused a competitive release of the gray tree frogs. For many taxa (zooplankton and algae), the effects of the mixtures simply reflected the effect of an individual chemical in the mix whereas, in other taxa (tadpoles), the mixture of several pesticides caused larger impacts than each pesticide alone. In the latter scenario, the design employed cannot determine whether these larger impacts were due to synergistic effects among pesticides or whether the impacts are due to a greater overall concentration of pesticides in the system.

The insecticides had large negative effects on the zooplankton; all five insecticides eliminated *Ceriodaphnia*, chlorpyrifos and diazinon eliminated *D. pulex*, and endosulfan eliminated most of the copepods. These results are

consistent with previous studies on these chemicals. For example, Fernandez-Casalderrey et al. (1994) found a $LC50_{24\text{-h}}$ value of 1 p.p.b. for cladocerans with diazinon and van Wijngaarden et al. (2005) found that 10 p.p.b. of chlorpyrifos eliminated cladocerans but had no effect on copepods. Past studies with endosulfan have found that 10 p.p.b. reduced cladocerans but completely eliminated copepods (Barry and Logan 1998), which is in agreement with endosulfan's relatively high $LC50_{24\text{-h}}$ value for cladocerans (620 p.p.b.; Fernandez-Casalderrey et al. 1994). Interestingly, 6.9 p.p.b. of carbaryl and 5.8 p.p.b. of malathion reduced the abundance of one cladoceran (*Ceriodaphnia*) but not the other (*D. pulex*), suggesting species-level differences in sensitivity. Past research on these two insecticides, using much higher concentrations (320–3,500 p.p.b.), has shown dramatic declines in zooplankton (especially for cladocerans; Bridges and Boone 2003; Mills and Semlitsch 2004; Relyea 2005). Collectively, this suggests that cladocerans are generally more sensitive to carbaryl, malathion, chlorpyrifos, and diazinon, whereas copepods are more sensitive to endosulfan. Moreover, the elimination of the cladocerans by chlorpyrifos and diazinon allowed an increase in copepods, likely via competitive release. This is consistent with outcomes from several other studies (Hanazato and Yasuno 1987, 1989, 1990; Havens 1994; Mills and Semlitsch 2004; Relyea 2005; van Wijngaarden et al. 2005).

In one zooplankton taxon (*Ceriodaphnia*), the mixture of five herbicides caused an increase in abundance. Little is known about the effects of these herbicides on zooplankton at the concentration used here. The few existing studies for three of the herbicides (atrazine, 2,4-D, and acetochlor) suggest that 6–16 p.p.b. of these herbicides should either have no effect on cladoceran survival or cause increased reproduction in cladocerans (Solomon et al. 1996; Kashian and Dodson 2002). The vehicle control (i.e., ethanol) showed a similar effect on *Ceriodaphnia* as the mixture of all five herbicides, suggesting that this increase was somehow related to the solvent and not the herbicides themselves.

Based on the separate pesticide effects, the mixtures of pesticides caused rather predictable effects on the zooplankton assemblage. The mix of the five insecticides led to very low numbers of both cladocerans and copepods, which is what one would expect based on the result that diazinon or chlorpyrifos alone killed nearly all of the cladocerans and endosulfan alone killed nearly all of the copepods.

When insecticides cause large decreases in zooplankton abundance, one would expect to observe a bloom in phytoplankton due to reduced herbivore pressure (assuming top–down control; Havens 1994, 1995; Barry and Logan 1998; Bridges and Boone 2003; Fleeger et al. 2003; Boone et al. 2004; Mills and Semlitsch 2004). Although I observed phytoplankton blooms with these three insecticides as well as in the mix of five insecticides and the mix of all ten pesticides (as indicated by the water turning green with low light transmittance as well as the observed increases in pH and dissolved oxygen with diazinon and endosulfan), only the endosulfan treatment exhibited a significant increase in the concentration of chlorophyll *a* from phytoplankton. The lack of an effect on chlorophyll *a* in the other treatments (despite an observed bloom of phytoplankton) can occur when there is a compositional change in the taxa composing the phytoplankton or if there is a shift in the relative proportions of photosynthetic pigments that are being employed (e.g., chlorophylls *b*, *c*, or other pigments; Wetzel and Likens 2000). The addition of acetochlor caused a reduction in phytoplankton that was also observed in the herbicide-mixture treatment. There appear to be no studies examining the direct impacts of acetochlor on phytoplankton, but the mechanism may be due to direct toxicity. For the mixture of all ten pesticides, the abundance of phytoplankton (on day 16) was intermediate to the increased phytoplankton found in the insecticide mix and the decreased phytoplankton found in the herbicide mix. This further suggests that the insecticides and herbicides had opposing effects on the phytoplankton (i.e., reduced herbivore pressure vs. direct toxicity to phytoplankton).

Given that phytoplankton and periphyton can compete for resources (e.g., light), insecticides that cause a phytoplankton bloom should cause a subsequent decline in the amount of periphyton (Mills and Semlitsch 2004; Relyea and Diecks 2008). This prediction was upheld on day 25 when I found a reduction in periphyton with chlorpyrifos, diazinon, and endosulfan (Fig. 6). This reduction in periphyton also occurred in the mixture of insecticides but, for reasons that are unclear, not when the five insecticides were combined with the five herbicides. This might simply reflect opposing effects on the competing phytoplankton from insecticides (indirectly favoring phytoplankton via killing zooplankton) and herbicides (directly killing phytoplankton). Later in the experiment (day 36), the reduction in periphyton with chlorpyrifos and diazinon persisted but the endosulfan treatment contained higher amounts of periphyton that resembled the controls. This rebound in periphyton with endosulfan was likely the result of the endosulfan treatment eliminating most of the leopard frog tadpoles which would have otherwise consumed the periphyton. The same rebound in periphyton occurred with the mix of insecticides in which even more leopard frogs did not survive. Interestingly, the decline in phytoplankton with acetochlor (relative to the control) was not associated with an increase in periphyton, suggesting that the periphyton already had sufficient resources for maximal growth.

The percentage of leopard frogs that were able to metamorphose prior to pond drying was pesticide dependent

(Fig. 6). In the case of diazinon, it is clear that the reduced percentage of metamorphs was not due to direct toxicity; when we summed the number of emerging metamorphs (76%) with the number of tadpoles that remained in the tanks at the end of the experiment (14%), the total (90%) was not significantly different from the number of emerging metamorphs in the control treatment (96%). Thus, the reduction in emerging leopard frog metamorphs with diazinon was due to the indirect effect involving the zooplankton, phytoplankton, and periphyton that caused a reduction in leopard frog growth and development such that these animals could not metamorphose by the time the environment dried. The two insecticides that did not cause an indirect effect (malathion and carbaryl) also did not affect the survival or life history of the leopard frogs. Interestingly, chlorpyrifos did cause an indirect effect but there was no observed impact on leopard frog survival or life history. It may be that the chlorpyrifos cascade was of shorter duration than that of diazinon and this minimized the negative impact, but the experiment lacked the temporal resolution to address this possibility.

Similar impacts of indirect effects have been observed in recent studies applying either: (11) low concentrations that are toxic to zooplankton but not expected to have any lethal or sublethal effects on tadpoles (Relyea and Diecks 2008), or (2) high concentrations and allowing the chemical to degrade before adding the tadpoles to the tanks (to allow only indirect effects; Mills and Semlitsch 2004). In both scenarios, indirect effects are observed that cause reduced growth and development of tadpoles which can ultimately lead to death when the environment dries. In contrast, when high concentrations of insecticides are applied after the tadpoles are added to a community, researchers have observed short-term increases in periphyton (likely due to a short-term, pesticide-induced reduction in tadpole foraging) and an increase in tadpole mass at metamorphosis (Boone et al. 2005; Boone and Bridges-Britton 2006). Hence, sublethal pesticide concentrations can have both density- and trait-mediated indirect effects on aquatic food webs (Relyea and Hoverman 2006).

The low concentration of endosulfan alone caused a high amount of leopard frog mortality (80% more than the control treatment). This low survival was not a reflection of an indirect effect retarding leopard frog development and preventing metamorphosis prior to the environment drying (no tadpoles remained in the tanks), but rather most likely due to direct toxicity since no tadpoles remained in the dried endosulfan tanks. Indeed, the few metamorphs emerging from the endosulfan treatment were nearly twice as large as metamorphs emerging from the control treatment, suggesting that these few surviving animals had abundant per capita food resources and were not dying of starvation. Given that no assessment of metamorph health was conducted, one cannot determine whether these larger metamorphs were more or less healthy than the control animals.

Because no amphibian testing is required for pesticide registration, there are few data on the toxicity of endosulfan on amphibians. The existing studies have mostly been conducted as single-species, laboratory experiments in which pesticides are reapplied to maintain a given concentration. For example, *Litoria citropa* tadpoles exposed to 0.8 p.p.b. of endosulfan experienced 11–34% mortality (Broomhall 2002) and salamanders (*Ambystoma barbouri*) exposed to 10–100 p.p.b. of endosulfan experienced 10–30% mortality (compared to the solvent control; Rohr et al. 2003). In another study that exposed three species of tadpoles (*Rana sylvatica*, *Rana clamitans*, and *Bufo americanus*) to a range of endosulfan concentrations (68–364 p.p.b.), Berrill et al. (1998) found low mortality during the 4-day exposure, but much higher mortality in the subsequent 11-day post-exposure period (~60% mortality at 68 p.p.b. of endosulfan compared to controls). In our own subsequent laboratory experiments to estimate LC50 values for endosulfan across ten species of larval anurans, we have LC50 values as low as 1 p.p.b. (R. A. Relyea et al., unpublished data), In contrast, Rohr and Crumrine (2005) found that wood frogs exposed to 10 p.p.b. of endosulfan experienced no mortality. Thus, the few existing studies of endosulfan's effects on amphibians suggest that some species can be highly sensitive to quite low concentrations. As is often the case, the high toxicity to many amphibians parallels the high toxicity of endosulfan to freshwater fish (reviewed in Berrill et al. 1998).

The concentration of endosulfan that killed 86% of leopard frogs was quite low (6.4 p.p.b.; Table 2) and, therefore, ecologically relevant. Endosulfan cannot only be inadvertently applied to aquatic habitats when applied to control crop pests (particularly during aerial applications), but can also be transported to more distant wetlands via atmospheric transport (LeNoir et al. 1999; McConnell et al. 1998; Hageman et al. 2006; Daly et al. 2007). Moreover, in countries such as Costa Rica, endosulfan is being increasingly used on crops with ~40 t imported each year from 2000 to 2004 (Daly et al. 2007).

Even greater than the 86% mortality caused by endosulfan was the 99% mortality caused by the mixture of the five insecticides or the mixture of all ten pesticides. Given that no leopard frog tadpoles remained in these treatments at the time of tank drying, this effect was not the result of the animals suffering from slowed growth and development but was the result of direct toxicity. Hence, the reduced survival is also not merely the additive effect of endosulfan (which killed tadpoles) and diazinon (which slowed tadpole growth and reduced emergence prior to pond drying but did not kill the tadpoles). Because of the experimental design employed, one cannot determine whether this mortality occurred due to synergistic interactions among the pesticides or because of the greater total concentration of pesticides (i.e., nominal concentrations of 50 or 100 total p.p.b.).

However, it is clear that mixtures of pesticides, each at quite low concentrations (2–16 p.p.b.), can have a decimating effect on leopard frog survival. Given that the mixture of the five insecticides and the mixture of all ten pesticides produced a similar outcome, the most parsimonious explanation is that the mixture of the five insecticides was responsible for both outcomes.

Understanding the effects of pesticide mixtures on aquatic organisms is still in its early stages. A few recent studies have examined how mixtures of pesticides affect individuals, with a focus on sublethal effects. For example, Hayes et al. (2006) exposed tadpoles of two species (*Xenopus laevis* and *R. pipiens*) to separate and combined pesticides in the laboratory (0.1–10 p.p.b. of up to nine chemicals) and found a variety of sublethal effects (e.g., reduced growth and development, immunosuppression) but no mortality effects. Christin et al. (2003, 2004) and Gendron et al. (2003) exposed *X. laevis* and *R. pipiens* to a single mix of six pesticides (each at a single representative concentration, 0.02 p.p.t.–56 p.p.b.) and found compromised immune function. At much higher concentrations (1–2 p.p.m.) and under laboratory conditions, Relyea (2004) examined the separate and pair-wise effects of four pesticides on tadpoles of five species and found additive negative growth and survival effects. Under community mesocosm conditions, our understanding of pesticide mixtures is quite limited. Boone and Bridges-Britton (2006) exposed gray tree frog tadpoles to mixtures of atrazine (20 p.p.b.) and carbaryl (2.5 p.p.m.) in mesocosms and found no effects on survival. It is clear that we have a long way to go to understand how pesticide mixtures impact amphibians, particularly when embedded in an ecological community.

In contrast to leopard frogs, gray tree frogs suffered no reduction in survival, no change in time to metamorphosis, and increased mass at metamorphosis when exposed to atrazine, the mix of the five insecticides and the mix of all ten pesticides (compared to the control treatment). The mechanism underlying the atrazine effect is unclear, but the mechanism underlying the effect in the two mixture treatments is likely due to the reduced density of surviving leopard frogs. It is also important to note that the lack of negative effects on the gray tree frogs is restricted to the response variables that were measured. The current data do not address additional sublethal impacts that may have occurred (Hayes et al. 2006) and no data were collected on amphibian health. However, these results do highlight the importance of testing multiple species of amphibians because the sensitivity of each species within the community can be dramatically different. Moreover, only by testing the species within a community context can we observe previously unknown increased growth effects on less sensitive species within the community.

Conclusion

The typical approach taken in order to understand how contaminants in natural habitats affect non-target organisms has been to expose organisms to one contaminant at a time under single-species, laboratory conditions, whereas the common scenario in nature is an exposure to suites of chemicals and within the context of an ecological community. By taking a mechanistic approach, we can identify the likely direct and indirect pathways by which different taxa embedded within a food web are affected (Fig. 6). The results of this study demonstrate that a single application of insecticides and herbicides (alone and in combination at low concentrations) can have dramatic effects on several taxonomic groups. For many of the taxa (zooplankton and algae) the effects of the pesticide mixtures were largely predictable from the individual pesticide effects. In contrast, mixtures of globally common pesticides (driven by the mixture of the insecticides) can cause up to 99% mortality in larval amphibians, and this effect was not completely explained by the individual pesticide effects. Given the constraints of the design when examining ten different pesticides, one cannot determine whether these combined effects are due to additive or synergistic interactions among the pesticides, but it is clear that the impact can be caused by the five insecticides alone. Thus, future work that examines interactions within this subset of pesticides could determine the underlying mechanisms of leopard frog death. Although the subsequent impact on the terrestrial population of frogs was not determined (nor estimated via modeling), the sheer magnitude of the larval amphibian mortality would have negative impacts on amphibian populations over time, particularly if these exposures occurred repeatedly. This is a key point in light of amphibian declines occurring throughout the world, including at sites that appear to be relatively pristine but are subjected to atmospheric transport of pesticides at low concentrations from distant areas. These results argue for much more research to address the impacts of pesticide mixtures on aquatic communities in general and on amphibians in particular.

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