

APPLIED ISSUES

A tale of two pesticides: how common insecticides affect aquatic communities

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SUMMARY

1. Recent ecotoxicology studies show that pesticide exposure can alter community composition, structure and function. Generally, community responses to pesticides are driven by trait- and density-mediated indirect effects resulting from sublethal and lethal effects of pesticide exposure on vulnerable taxa. These effects depend upon the concentration of the pesticide and the frequency of exposure.
2. While more research is needed to understand community-level responses to pesticide exposure, testing the effects of multitudes of registered chemicals on ecologically relevant communities is overwhelming. Recent reviews suggest that contaminants with similar modes of action should produce comparable community-level responses because they have similar direct effects and, as a result, similar indirect effects; this hypothesis remains largely untested.
3. We subjected pond communities [containing zooplankton, phytoplankton, periphyton and leopard frog tadpoles (*Rana pipiens*)] to several applications (single applications of medium or high concentrations or weekly applications of a lower concentration) of two acetylcholine esterase inhibiting insecticides, malathion and carbaryl that have comparable toxicity for aquatic organisms.
4. We found that both insecticides cause comparable trophic cascades that affect zooplankton and phytoplankton abundances; however, their effects on amphibians diverged, especially when exposed to higher concentrations of insecticides. Malathion caused a trophic cascade beginning with a decline in cladocerans followed by increases in phytoplankton. At a medium concentration, this cascade also caused a subsequent decrease in periphyton. Carbaryl caused a similar trophic cascade with the highest application, a weak trophic cascade with the medium application and no cascade with smallest application. Malathion directly reduced tadpole survival at all concentrations. Survivors in the two higher treatments were larger at metamorphosis while survivors in the lowest treatments were smaller and developed slowly. In contrast, carbaryl was not directly toxic to tadpoles, but indirectly reduced survival because slow growth and development prevented some tadpoles from metamorphosing before the mesocosms dried at medium and low applications.
5. These results suggest that these common pesticides, which share the same mode of action, have similar effects on zooplankton and algae, but differences in the strength and timing of their effects on tadpoles reduce the generality of responses at higher trophic levels. Overall, general predictive models of contaminant effects could be improved by incorporating the relative timing of direct and indirect effects of exposure.

Keywords: amphibian, community, food webs, temporary pools, toxins

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Introduction

Understanding how anthropogenic contaminants affect ecological communities is a major modern challenge. We face the task of understanding the effects of nearly 80 000 registered contaminants in the United States, including hundreds of pesticide active ingredients that come in thousands of commercial formulations (Jones *et al.*, 2004; Giagnesi & Reigner, 2006a,b). Moreover, the effects of contaminants on communities depend upon many factors including the concentration of the contaminant, the timing of the exposure and the number of exposures (Relyea & Diecks, 2008).

Recent reviews have suggested that many impacts of contaminants on communities can be predicted by applying the same conceptual framework used to understand community composition and structure in uncontaminated systems. In particular, knowledge of direct lethal and sublethal effects of contaminants and the density- and trait-mediated indirect effects that they cause provides a useful framework for making predictions about changes in community structure (Van den Brink *et al.*, 2002; Fleeger, Carman & Nisbet, 2003; Traas *et al.*, 2004; Relyea & Hoverman, 2006; Rohr, Kerby & Sih, 2006; Clements & Rohr, 2009). This community ecology approach to ecotoxicology suggests that pesticides with similar toxicity and modes of action should have comparable effects on communities even when contaminants are in different chemical classes.

While many reviews have suggested these patterns should occur, there are few direct tests that examine the effects of similar contaminants on communities (but see Relyea, 2005; Boone & Bridges-Britton, 2006; Relyea, 2009) and fewer that make comparisons across several application regimes (Farmer, Hill & Maund, 1995; Williams & Semlitsch, 2009). Different application regimes of pesticides yield diverse results. For example, Relyea & Diecks (2008) found that a press treatment consisting of 7 weekly applications of $10 \mu\text{g L}^{-1}$ of malathion caused larger impacts on many of the response variables than single pulse applications that were 25 times higher in concentration. It would be useful to know if such patterns occur with similar pesticides; however, finding such patterns by comparing studies across the literature is challenging as studies vary widely in community assemblage, concentrations of contaminants used and

the timing and frequency of applications. Thus, experiments comparing the roles of contaminants at several concentrations and application regimes are needed to test whether and when generalisations can be made about their community effects.

Among contaminants, pesticides have received recent attention for their potential to alter population and community dynamics (Relyea & Hoverman, 2006) and contribute to amphibian population declines (Blaustein *et al.*, 2003). Data from California show that amphibian populations downwind of applications of carbamate and organophosphate insecticides that inhibit acetylcholine esterase are in decline (AchE; Davidson, Shaffer & Jennings, 2001, 2002). Moreover, Pacific tree frogs (*Pseudacris regilla* Baird and Girard) with decreased AchE activity are found in sites where co-occurring species are declining (Sparling, Fellers & McConnell, 2001). Pesticides in these wetlands are found at concentrations that would be considered sublethal according to traditional short-term laboratory experiments designed to determine lethal concentrations to target populations (i.e. LC50 tests). This suggests that these pesticides may be affecting populations ways other than through direct toxicity (e.g. Relyea & Diecks, 2008).

Our study tested whether pesticides with similar modes of action have similar impacts on aquatic communities and which application regimes produce these similarities. We chose to use the insecticides malathion and carbaryl because they share the same mode of action (i.e. AchE inhibition), are in different chemical classes, are among the most commonly used insecticides in the United States (Kiely, Donaldson & Grube, 2004) and have been associated with amphibian declines (Davidson *et al.*, 2001, 2002). We tested the effects of each pesticide using a low concentration applied weekly and medium and high concentrations applied once. Based on LC50 values for various taxa and knowledge of trophic interactions within this community, we hypothesised that the two insecticides would cause similar direct and indirect effects on aquatic communities. Specifically, we predicted that (i) malathion and carbaryl would both kill zooplankton, (ii) reductions in zooplankton would cause a trophic cascade in which phytoplankton would increase and subsequently shade periphyton, (iii) as a result of decreased periphyton (the tadpoles' main food source), tadpole growth and development would be reduced and (iv) low, weekly applications of the

insecticides would cause a stronger trophic cascade than larger, single applications, although the cascade could take longer to develop. While other studies have examined community-level effects across a range of pesticide applications, none have made comparisons across such a large range with more than one pesticide (Relyea & Diecks, 2008).

Methods

Pesticide background

Both carbaryl and malathion are commonly used broad-spectrum insecticides that act by inhibiting AchE, thereby disrupting nervous impulses and chemical signalling at neurotransmitters. Comparisons between these insecticides can be made without adjusting for toxicity because the concentrations of each chemical that are toxic to amphibians are quite similar. Both chemicals reach waterbodies through aerial spray, run-off and direct application when used to control mosquitoes or aquatic crops such as rice (Garber, Jones & Steeger, 2007; Odenkirchen & Wente, 2007).

In the United States, a total of 1.8 million kg of carbaryl, a carbamate insecticide, is sold annually. Approximately 50% goes to agricultural uses and another 50% to non-agricultural uses (e.g. gardening, pet care), making it the second most common insecticide sold for non-agricultural use (Kiely *et al.*, 2004). Application intervals are between 3 and 14 days depending on use (Garber *et al.*, 2007). Peak expected environmental concentrations (EECs) for carbaryl in aquatic systems in maximum use scenarios range from 0.47 to 166 ppb, although spraying for rice has an EEC of 2579 ppb (Garber *et al.*, 2007). The United States national water quality assessment program surveys have found carbaryl in nearly 50% of water bodies surveyed with maximum concentrations of 1.06 ppb (Garber *et al.*, 2007). Breakdown rates of carbaryl in water are pH-dependent; it degrades in distilled water with a half-life of 3 h at a pH of 9 and 12 day at a pH of 7 (Wolfe, Zepp & Paris, 1978). Estimates of lethal concentrations for larval anurans (LC50s) vary between 2.5 and 20.6 ppm (Marian, Arul & Pandian, 1983; Relyea, 2003).

Malathion is an organophosphate insecticide, which, unlike carbaryl, binds irreversibly to AchE. In the United States, it is the most commonly used

insecticide, with 9–11.3 million kg of active ingredient used annually for agriculture (Kiely *et al.*, 2004). Malathion is also used for gardening and public health pest-control programmes. Spraying occurs every month of the year with 2- to 14-day intervals between applications (Odenkirchen & Wente, 2007). The U.S. Environmental Protection Agency's EECs for malathion in surface water range from 9 to 27 ppb when sprayed on terrestrial crops and 1404–1797 ppb when sprayed on aquatic crops (rice and watercress, Odenkirchen & Wente, 2007). Breakdown rates of malathion increase with temperature and alkalinity; it has a half-life >4 years at a pH of 4. At a pH of 8, malathion has a half-life of 1 h at 40 °C and a half-life of 40 day at 0 °C (Wolfe *et al.*, 1977). Malathion is highly toxic to aquatic invertebrates and moderately toxic to amphibians, with LC50 values for larval anurans between 1.3 and 5.9 ppm (Relyea, 2004, USEPA Ecotox database, available online <http://cfpub.epa.gov/ecotox/>).

Experimental design

Our experiment was conducted at the University of Pittsburgh's Pymatuning Lab of Ecology (Linesville, PA, U.S.A.). We used a completely randomised design employing seven treatments. In addition to a 0-ppb control, each pesticide was applied at nominal concentrations of 25 ppb applied weekly, 250 ppb applied once or 2500 ppb applied once. Hereafter, we refer to these treatments as 'control', 'weekly low', 'single medium' and 'single high'. Since we were interested in both direct and indirect effects of pesticides on amphibians, we chose treatment concentrations that ranged from sublethal to lethal for many amphibians (Relyea, 2004).

Each of the seven treatments was replicated four times for a total of 28 experimental units. The experimental units were 1200-L mesocosms filled with *c.* 1055 L of well water between 21 and 23 April 2007 and covered with 60% shade-cloth lids to prevent colonisation by ovipositing animals and escape by metamorphosing frogs. Oak (*Quercus* spp.) leaf litter (300 g) and rabbit chow (25 g) were added to each tank on 30 April to provide nutrients and substratum to the mesocosms. Aliquots of water collected from several local ponds were screened for predators and added on 1, 4 and 24 May to provide a natural source of algae and zooplankton to the

mesocosms. Two unglazed clay tiles (225 cm²) were added to all tanks on 7 and 10 May to serve as periphyton samplers. All tiles were positioned vertically on the north side of each mesocosm.

Leopard frogs (*Rana pipiens* Schreber) were collected from one egg mass located in northwest PA and hatched in covered 200-L wading pools. While we typically would use tadpoles from more than one egg mass to increase genetic diversity, we were unsuccessful in locating additional egg masses in 2007. On 24 May, 40 tadpoles (initial mass 59.3 ± 2.4 mg) were added to each tank. This density of leopard frog tadpoles was used to ensure competition for resources (periphyton; Relyea & Diecks, 2008). We examined handling stress on the tadpoles by holding 20 individuals for 24 h. The resulting survival was 100%. Tadpoles in mesocosms were allowed to recover from handling for a week before applying the pesticide treatments.

Pesticides were first applied to the mesocosms on 30 May (defined as day 1 of the experiment). For the weekly low applications, we reapplied the pesticides after weeks 1, 2, 3 and 4. Weekly low applications were stopped after week four because frogs began to metamorphose. To achieve the nominal pesticide concentrations, we added 52.75, 527.5 and 5275 μL of commercial grade malathion (50% active ingredient, Malathion Plus; Ortho Corp. Marysville, OH, U.S.A.) and 117.2, 1172 and 11722 μL of commercial grade carbaryl (22.5% active ingredient, GardenTech Sevin[®] Ready-to-Spray Bug Killer; Aventis Cropscience, Research Triangle Park, NC, U.S.A.) to the appropriate mesocosms. To ensure that pesticides were mixed into the treatments, we stirred each mesocosm with a 0.5-L cup for several minutes after adding pesticides and then allowed 4 h to pass before collecting test samples to let the pesticides further diffuse into the water column. Past mesocosm studies have found this amount of mixing sufficient to disperse pesticides evenly in mesocosms (Relyea & Diecks, 2008). Both carbaryl and malathion degrade quickly in mesocosms, such that weekly applications at low concentrations do not increase the total concentration of pesticides over time (Boone & Semlitsch, 2002; Relyea & Diecks, 2008). Water samples were collected from throughout the water column in all mesocosms and pooled across replicates. Samples were stored at 4 °C and later shipped for analysis by an independent laboratory using high-pressure liquid chromatogra-

phy (Mississippi State Chemical Laboratory, MS, U.S.A.).

Of the six water samples sent for testing, two samples were damaged during shipment (250 ppb malathion and 2500 ppb carbaryl). The two tested malathion samples had nominal concentrations of 25 and 2500 ppb but actual concentrations of 3.1 and 384 ppb, respectively. The two tested carbaryl samples had nominal concentrations of 25 and 250 ppb but actual concentration of 13.5 and 141 ppb, respectively. Thus, compared to the nominal concentrations, the actual concentrations were 54–56% for carbaryl and 12–15% for malathion. Based on these measured concentrations, we estimated the actual concentration of the two damaged samples to be *c.* 34 ppb (for the 250 ppb nominal malathion treatment) and 1380 ppb (for the 2500 ppb nominal carbaryl treatment). While actual concentrations deviated considerably from the nominal concentrations, the deviation was consistent among tested concentrations within each pesticide. Variation from nominal concentrations is frequently found in mesocosm experiments (reviewed in Brock, Van Wijngaarden & Van Geest, 2000) and is thought to arise from pesticide precipitation, volatilisation, binding to substrata or degradation of stored samples before mailing (Farmer *et al.*, 1995; Brock *et al.*, 2000); thus, low measured values do not necessarily reflect error in the application of the pesticide.

Response variables

To quantify the responses of primary producers and consumers to the treatments, we sampled periphyton, phytoplankton and zooplankton on days 7 and 21. Periphyton was collected by scrubbing one side of an unglazed ceramic tile into a tub of filtered well water. We then vacuum-filtered the slurry onto a pre-dried (80 °C) and pre-weighed Whatman GF/C filter. The filter was dried again at 80 °C for 24 h and weighed to determine the biomass of periphyton that had grown on the tile.

Phytoplankton density measured as chlorophyll *a* (chl *a*) was quantified by collecting 250 mL of water at the bottom and top of the water column in the centre of each mesocosm. Water was vacuum-filtered onto a Whatman GF/C filter. Filters were wrapped and frozen at –80 °C. Chl *a* was extracted with dimethyl formamide and then quantified using a fluorometer (Model TD-700; Turner Designs, Sunnyvale, CA,

U.S.A.). Chl *a* concentrations were adjusted for breakdown products (pheophytin) that were also quantified with a fluorometer.

Zooplankton abundance was quantified by collecting 200 mL of water from five standardised locations within each mesocosm using a tube sampler plunged into the water column. The sample was filtered through a 62- μ m Nitex screen, and the zooplankton were preserved in 70% ethanol. Zooplankton were subsequently identified and counted using a dissecting scope. Past studies using the same zooplankton assemblages have found that sensitivity to insecticides differs between cladocerans and copepods, but sensitivity is very similar among species belonging to each group. As a result, we only identified zooplankton to the level of cladocerans and copepods (Relyea & Diecks, 2008).

Once the first tadpole metamorphosed on day 31, we added pieces of wooden lath to each mesocosm to serve as perches for metamorphs. From this date onward, we checked for metamorphs daily and collected all individuals that had both forelimbs emerged (Gosner stage 42; Gosner, 1960). We considered metamorphosis complete when the tail was resorbed to >3 mm. All collected individuals that were not fully metamorphosed were placed in 1-L plastic containers with wet sphagnum moss and checked daily until metamorphosis was complete. Each individual was weighed after completing metamorphosis. For each tank, the mean time to metamorphosis, mean mass at metamorphosis and survival to metamorphosis were used as our amphibian response variables.

Pond drying is a common phenomenon in habitats where leopard frogs breed. Tadpoles detect pond drying via reductions in water volume and consequently increase their development rate to metamorphose before the pond dries (Denver, Mirhadi & Phillips, 1998). To examine how tadpoles exposed to pesticides respond to pond drying, we removed 225 L of water from each mesocosm every third day beginning on day 88, 60 days after the last weekly pesticide application. Drying was completed on day 97. Tadpoles that had not metamorphosed by day 97 were preserved in ethanol and considered mortalities since they would not have survived in a dry pond. Individuals with at least one emerged forelimb were considered survivors and held until metamorphosis was complete (e.g. Relyea & Diecks, 2008). The remaining tadpoles were counted, so that we could

differentiate between individuals that died because of slow development and pond drying from tadpoles that died during the experiment.

To determine abiotic conditions and effects of treatments on water quality, we measured dissolved oxygen (DO), temperature, pH and light extinction. DO, pH and temperature were measured between 11:00 and 13:00 hours on days 7 and 20 with a calibrated Wissenschaftlich-Technische Werkstätten MultiLine P4 meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Light extinction was measured on days 7 and 21 to quantify the shading effect of phytoplankton. We measured photosynthetic active radiation at 10 and 30 cm below the surface using a photometer (LI-COR, Lincoln, NE, U.S.A.). We calculated light extinction (*k*) using the formula:

$$K = \frac{\ln(L_{10}/L_{30})}{d}$$

where L_{10} and L_{30} are the photons detected at 10 and 30 cm, respectively, and *d* is the difference in depth between the two measurements.

Statistical analyses

The data were analysed with one-way analyses of variance (ANOVA). Water quality data (pH, DO, temperature and light extinction) and biotic data (periphyton mass, chl *a*, cladocerans and copepod) were measured at two time points. We initially analysed each of these response variable with separate repeated-measure ANOVAs (rm-ANOVAs). Since most response variables had significant time-by-treatment interactions, we then conducted separate multivariate one-way ANOVAs (MANOVAs) on the abiotic variables within each time point.

A separate one-way MANOVA was used to analyse amphibian data (leopard frog mass at metamorphosis, time to metamorphosis and survival) since these data were not repeatedly sampled. One replicate (single medium carbaryl treatment) was excluded from the analysis as it was colonised by libellulid dragonfly larvae late in the experiment and had much higher mortality of tadpoles than other replicates of this treatment. This treatment was not excluded from the other analyses, because those data were collected prior to colonisation by the dragonflies.

Where necessary, response variables were log- or arcsine-transformed to meet the assumption of normality. For the water quality, algae and zooplankton

Table 1 Analyses of pesticide effects on water quality response variables [pH, dissolved oxygen (DO), temperature and light extinction]

Response variable	Treatment (d.f. = 6, 21)	Time (d.f. = 1, 21)	Treatment × Time (d.f. = 6, 21)	Treatment on day 7 (d.f. = 6, 21)	Treatment on day 21 (6, 21)
pH	13.2 (<0.001)	2.0 (0.177)	5.0 (0.003)	15.5 (<0.001)	1.9 (0.128)
DO	2.6 (0.049)	3.1 (0.092)	4.5 (0.005)	15.2 (<0.001)	0.6 (0.766)
Temperature	4.9 (0.003)	6286 (<0.001)	2.4 (0.067)	4.5 (0.005)	0.8 (0.563)
Light extinction	4.7 (0.003)	13.6 (0.001)	4.0 (0.008)	4.7 (0.004)	4.3 (0.006)

Results are from rm-ANOVAS followed by ANOVAS conducted within each sample date. For each response variable, *F*-values are listed first followed by *P*-values in parentheses. Bold *P*-values are significant ($\alpha = 0.05$)

data, the assumption of homogeneity of variances was met for all data except the cladoceran and periphyton data for the first sample date and copepods and light extinction data for the second sample date. For the amphibian data, all response variables except for mass at metamorphosis met the assumption of homogeneous variances. ANOVAS are robust to violations of only one assumption (Quinn & Keough, 2002, p. 191). When evaluating MANOVAS, we used Pillai's trace, which is robust to violations of the assumption of homogeneous variances (Quinn & Keough, 2002, p. 434).

Whenever there were significant main effects in MANOVAS, we performed ANOVAS on each response

variable. When there were significant ANOVAS, pairwise comparisons were made between pesticide treatments and the control using Fisher's LSD test. All pair-wise comparisons were two-tailed, with the exception of amphibian survival.

Results

Water quality

Water quality was affected by the insecticides on day 7 and 21. Repeated-measures ANOVAS for pH, DO, temperature and light extinction revealed significant

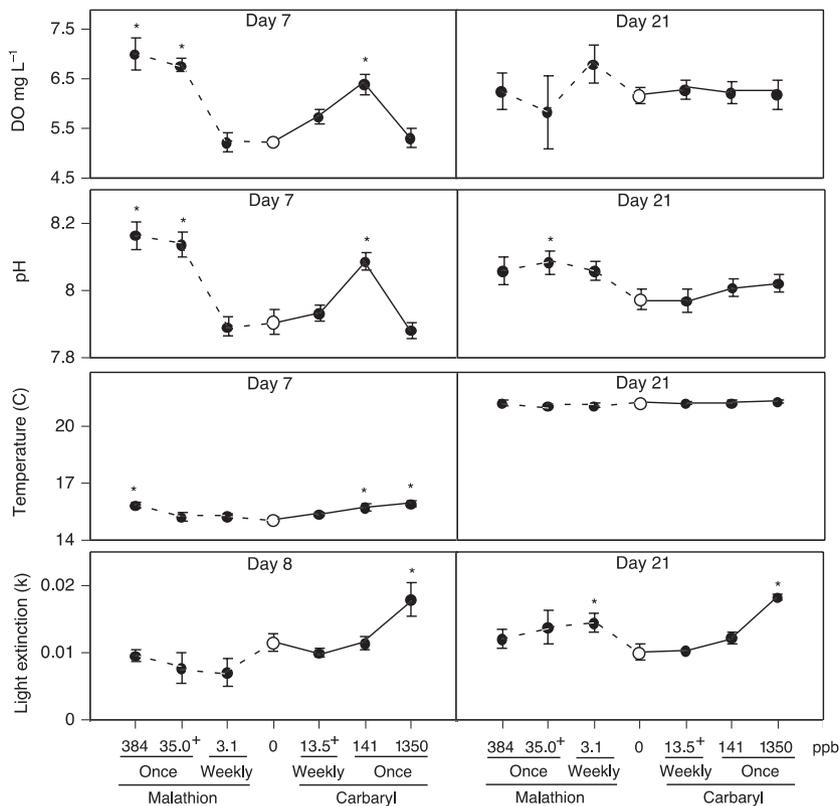


Fig. 1 The effects of pesticide concentration, type (carbaryl or malathion) and timing of application (delivered weekly or once) on water dissolved oxygen, pH, temperature and light extinction 7 or 8 days and 21 days after the experiment was initiated. Data are means \pm SE. *Treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). ⁺Actual concentrations were not available, values were estimated.

Table 2 Results of analyses of pesticide effects on cladocerans, copepods, phytoplankton (assayed as chlorophyll *a*) and periphyton

Response variable	Treatment (d.f. = 6,21)	Time (d.f. = 1,21)	Time × treatment (d.f. = 6,21)	Treatment on day 7 (d.f. = 6,21)	Treatment on day 21 (d.f. = 6,21)
Cladocerans	24.2 (<0.001)	4.2 (0.054)	5.6 (0.001)	8.3 (<0.001)	22.3 (<0.001)
Copepods	5.1 (0.002)	28.5 (<0.001)	1.5 (0.225)	1.6 (0.180)	5.1 (0.002)
Chlorophyll <i>a</i>	1.9 (0.120)	4.5 (0.045)	2.6 (0.048)	0.5 (0.812)	3.0 (0.029)
Periphyton	4.0 (0.008)	80.0 (<0.001)	14.5 (<0.001)	4.5 (0.030)	7.0 (<0.001)

Results are repeated-measures ANOVAS followed ANOVAS conducted on each sample date. *F*-values are listed first, followed by *P*-values in parenthesis. Bold *P*-values are significant at ($\alpha = 0.05$)

(or marginally non-significant) time-by-treatment interactions on all response variables (Table 1, Fig. 1). As a result, we ran separate MANOVAS for each sample date and found significant multivariate effects on day 7 (Pillai's Trace $F_{18, 84} = 3.494, P < 0.001$) and 21 (Pillai's Trace $F_{18, 84} = 1.78, P = 0.029$).

The multivariate effect on day 7 was driven by all four water quality variables (Table 1, Fig. 1). For malathion, the weekly low treatment did not differ from the control for any water quality variables. The single medium treatment had higher pH and DO, and the single high treatment had higher pH, DO and

temperature (all $P < 0.005$). For carbaryl, the weekly low treatment was not different from the control for any of the water quality variables. The single medium treatment had higher pH, DO and temperature (all $P \leq 0.009$). The single high treatment had a higher temperature and 56% greater light extinction (both $P \leq 0.012$).

The multivariate effect on day 21 was driven by light extinction and pH. For malathion, relative to the control, the weekly low treatment had 50% greater light extinction ($P = 0.032$), and the medium treatment had 40% greater light extinction, although the latter

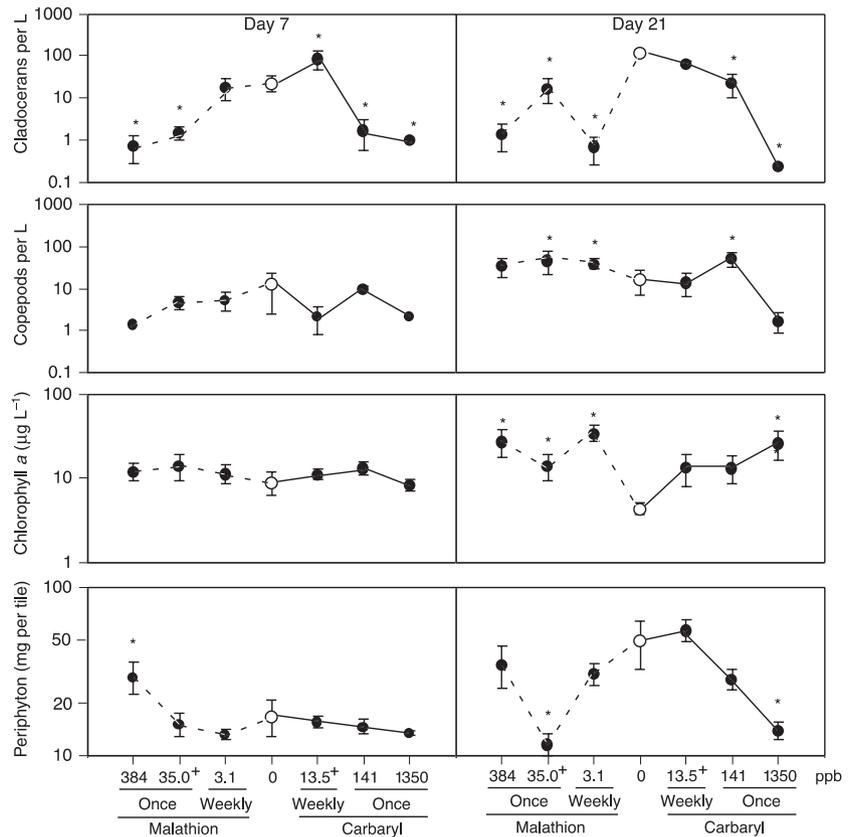


Fig. 2 The effects of pesticide concentration, type (carbaryl or malathion) and timing of application (delivered weekly or once) on chlorophyll *a* concentration, periphyton mass and copepod and cladoceran abundances 7 and 21 days after the experiment was initiated. Data are means \pm SE. *Treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). +Actual concentrations were not available, values were estimated.

difference was marginally non-significant ($P = 0.067$). The single medium malathion treatment also had a higher pH than the control ($P = 0.025$). The single high malathion treatment did not differ from the control in any of the water quality variables. For carbaryl, only the single high treatment differed from the control in water quality variables. Mesocosms exposed to single high concentrations of carbaryl had 80% greater light extinction ($P < 0.001$).

Algae and zooplankton

Pesticide treatments affected both algal and zooplankton populations. Repeated-measures ANOVAs on cladocerans, copepods, phytoplankton (measured as chl *a*) and periphyton showed significant time-by-treatment interactions for three of the four response variables (Table 2, Fig. 2). As a result, we ran a separate MANOVA for each sample date and found significant multivariate effects of treatment on day 7 (Pillai's Trace $F_{18, 84} = 2.464$, $P = 0.001$) and day 21 (Pillai's Trace $F_{18, 84} = 4.149$, $P < 0.001$).

The multivariate effect on day 7 was driven by changes in cladocerans and periphyton relative to the control (Table 2, Fig. 2). For cladocerans in the malathion treatments, the weekly low treatment did not differ from the control, whereas the single medium and single high treatments reduced cladocerans by 93 and 97%, respectively (both $P < 0.021$). For cladocerans in the carbaryl treatments, the weekly low treatment increased cladocerans by 380% ($P < 0.032$), while the single medium and single high treatments reduced cladocerans by 92 and 98%, respectively (both $P < 0.05$). At this early stage of the experiment, there were no effects on copepods. Periphyton biomass was only affected in one treatment; the single high malathion treatment had 20% more periphyton than the control ($P = 0.013$).

All four taxonomic groups contributed to multivariate effect on day 21 (Fig. 2, Table 2). For cladocerans, the three malathion treatments had 86–99% fewer individuals than the control (all $P < 0.001$). The weekly low carbaryl treatment did not differ from the control, but the single medium and single high carbaryl treatments caused cladoceran populations to decline by 81 and 99.9%, respectively (both $P < 0.001$).

By day 21, the pesticide treatments also affected the abundance of copepods. For malathion, the weekly low and the single medium treatments had 235–288%

more copepods relative to the control (both $P < 0.05$). The single high malathion treatment had 216% more copepods, but this was not-significant ($P = 0.079$). For carbaryl, the weekly low treatment did not differ from the control, the single medium carbaryl treatment had 315% more copepods ($P < 0.043$), and the single high carbaryl treatment did not differ from the control ($P = 0.083$).

On day 21, all malathion treatments and the single high carbaryl treatment had more phytoplankton than the control (Table 2, Fig. 2). For the malathion treatments, these increases ranged between 320 and 790% (all $P < 0.05$). For carbaryl, the weekly low and single medium treatments did not differ from the control (both $P \leq 0.111$), whereas the single high carbaryl treatment caused a 600% increase in chl *a* ($P = 0.007$).

Some of the pesticide treatments also affected periphyton biomass on day 21. For malathion, the single medium treatment reduced periphyton by 70% ($P < 0.001$). For carbaryl, the single high treatment reduced periphyton by 76% ($P < 0.001$).

Leopard frogs

The effects of malathion and carbaryl on tadpole survival and life history depended upon the application amount and frequency. The MANOVA on the leopard frog response variables showed a multivariate effect of treatment (Pillai's Trace $F_{18, 60} = 6.028$, $P < 0.001$). This multivariate effect was driven by univariate effects on survival ($F_{6, 27} = 3.390$, $P < 0.019$), time to metamorphosis ($F_{6, 27} = 12.156$, $P < 0.001$) and size at metamorphosis ($F_{6, 27} = 10.456$, $P < 0.001$).

Exposure to pesticides affected frog survival (Fig. 3). For malathion, relative to the control, survival was 8% lower in the weekly low and single medium treatments (both $P < 0.05$) and 22% lower in the single high treatment ($P < 0.001$). For all three treatments, nearly all the mortality occurred during the experiment; few tadpoles remained when the mesocosms were dried. For carbaryl, relative to the control, survival was 10% lower in the weekly low treatment and 22% lower in the single medium treatment (both $P < 0.05$), but there was no effect of the single high treatment ($P = 0.994$). In the weekly low and single medium treatments, most of the mortality was because of animals not completing metamorphosis before the mesocosms dried. These mortalities were observed as live tadpoles that

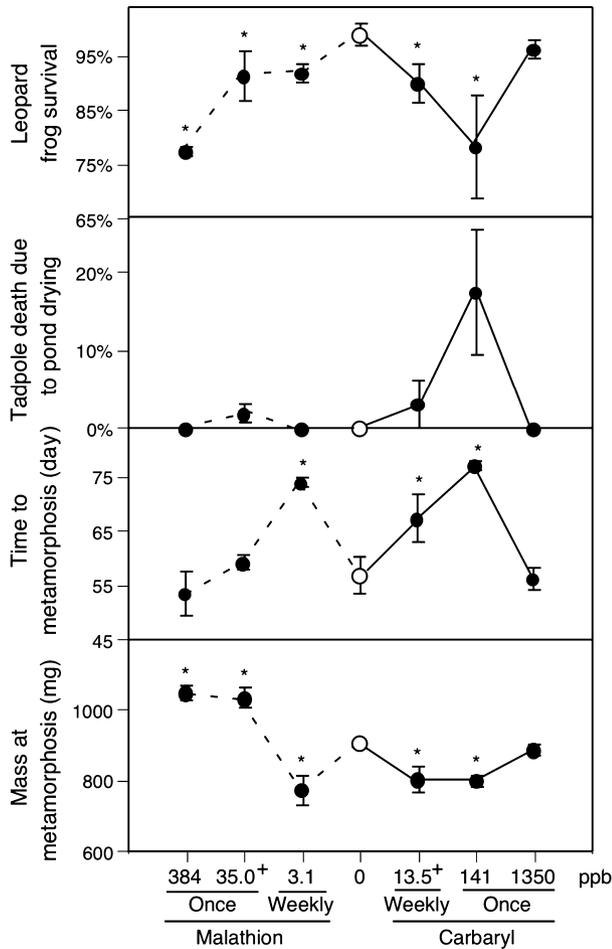


Fig. 3 The effects of pesticide concentration, type (carbaryl or malathion) and timing of application (delivered weekly or once) on survival of leopard frogs (*Rana pipiens*), proportion of leopard frog tadpoles that did not metamorphose before simulated pond drying as a result of slow development, time to metamorphosis and mass at metamorphosis. Data are means \pm SE. *Treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). †Actual concentrations were not available, values were estimated.

had not metamorphosed by day 97 when the mesocosm drying was finished.

Time to metamorphosis was also affected by pesticide treatments (Fig. 3). For malathion, tadpoles in the weekly low treatment took an average of 17 day longer to metamorphose ($P < 0.001$), while tadpoles in the single medium and single high treatments did not differ from the control. For carbaryl, relative to the control, tadpoles in the weekly low and single medium treatments took longer to metamorphose by 10 and 20 days, respectively (both $P < 0.01$). Tadpoles in the single high treatment did not differ from the control.

Mass at metamorphosis varied with treatment. For malathion, relative to the control, tadpoles in the weekly low treatment metamorphosed with 15% lower mass ($P = 0.011$). Conversely, tadpoles in the single medium and single high malathion treatments metamorphosed with 13–15% greater mass (both $P < 0.018$). For carbaryl, tadpoles in the weekly low and single medium treatments metamorphosed with 12% less mass (both $P < 0.05$), while tadpoles in the single high treatment had similar mass to the control.

Discussion

This experiment is one of only a few tests of the hypothesis that exposure of communities to insecticides with similar modes of action and toxicity should result in comparable community level responses (Van den Brink *et al.*, 2002; Fleeger *et al.*, 2003; Traas *et al.*, 2004; Relyea & Hoverman, 2006; Rohr *et al.*, 2006; Clements & Rohr, 2009). We found that trophic cascades leading to phytoplankton blooms occurred in all malathion treatments and the single high carbaryl treatment. The insecticides also differed in their effects on tadpoles. In the malathion treatments, strong trophic cascades and early death of tadpoles led to slower growth and development in the weekly low treatment and fast growth in the single medium and single high treatments. In contrast, weak trophic cascades and strong direct effects of carbaryl on development caused slower development and growth in the two lower carbaryl concentrations, while tadpoles in the highest concentration were unaffected. Thus, while both insecticides had similar effects on zooplankton and algae, they had different effects on amphibian survival and life history. As a result, only limited generalisations about the community effects of these insecticides can be made at any application.

Both insecticides caused decreases in cladoceran populations. This was likely a direct effect of the insecticides' toxicity. Previous research has found direct lethal effects of malathion on cladocerans at concentrations as low as 5 ppb in laboratory studies (Wong, Chu & Shum, 1995) and 10 ppb in mesocosm studies (Relyea & Diecks, 2008). Toxic effects in the weekly low malathion treatment, which had concentration lower than this threshold (3 ppb each week for 4 weeks), took longer to manifest. Direct lethal effects of carbaryl on cladocerans have been recorded for concentrations as low as 15 ppb for *Daphnia* sp. in

laboratory studies (Takahashi & Hanazato, 2007) and 20 ppb for *Daphnia* sp. in mesocosm studies (Havens, 1995). The concentration of carbaryl in our weekly low treatment (estimated at 13.5 ppb each week) appears to have been too low to have direct toxic effects on cladocerans. The observed increase in cladocerans in this treatment was driven primarily by a single replicate that had more than four times more cladocerans than the average of the other three replicates for this treatment, though it was not a statistically significant outlier.

Copepods were also affected by some of the insecticide treatments. At weekly low and single medium exposures to both pesticides, whenever the abundance of cladocerans decreased, the abundance of copepods increased by day 21. This pattern has been observed in other insecticide studies and has been attributed to the lower sensitivity of copepods to insecticides combined with a competitive release of copepods caused by the decrease in cladocerans, which overlap with copepods in their consumption of phytoplankton (Hanazato & Yasuno, 1987, 1989; Havens, 1995; Mills & Semlitsch, 2004; Relyea, 2005; Relyea & Diecks, 2008). In contrast to the weekly low and single medium treatments, the highest concentrations of each pesticide caused copepod mortality (Relyea & Diecks, 2008). Indeed, direct toxic effects of carbaryl on all species of zooplankton are observed at concentrations above 1000 ppb (Hanazato & Yasuno, 1989). This mortality likely dampened or prevented any competitive release of copepods, resulting in no significant rebounds in copepod abundance in the single high treatments. Compositional shifts of zooplankton assemblages are a common response to insecticides including diazinon (Giddings *et al.*, 1996), azadirachtin (Kreutzweiser *et al.*, 2004), endosulfan (Barry & Logan, 1998; Rohr & Crumrine, 2005), esfenvalerate (Fairchild *et al.*, 1992; Lozano *et al.*, 1992) pyridaben, carbaryl (Hanazato & Yasuno, 1987, 1989) and malathion (Relyea, 2005). To our knowledge, there are no studies exploring mechanisms underlying the high sensitivity of cladocerans to insecticides relative to the lower sensitivity of copepods.

We predicted that zooplankton decreases would result in higher phytoplankton densities if phytoplankton growth was limited by herbivory. Indeed, chl *a* concentrations increased in all malathion treatments by day 21, suggest that zooplankton herbivory

did limit phytoplankton growth prior to the trophic cascade. Similar results were observed in the single high carbaryl treatment, but not in the weekly low and single medium carbaryl treatments, which caused no declines or weak declines in cladocerans by day 21. Phytoplankton blooms associated with insecticide applications have been previously documented with both organophosphate (Hurlbert, 1972; Relyea & Diecks, 2008) and carbamate insecticides (Mills & Semlitsch, 2004).

Phytoplankton blooms are generally expected to reduce the amount of light that can pass through the water column; however, our measures of light extinction were not always associated with phytoplankton (i.e. chl *a* concentration). We found that the greatest light extinction coefficients occurred in the weekly low malathion treatment and the single high carbaryl treatment, which is consistent with the increases in phytoplankton observed in these treatments. The single medium and single high malathion treatments, which also had increases in phytoplankton, both had non-significant trends of increasing light extinction. Light extinction may also have been affected by factors other than phytoplankton, such as dissolved organic matter.

If the periphyton living on the bottom of a pond is light limited, shading from a phytoplankton bloom should decrease periphyton abundance. On the other hand, if periphyton growth is limited by herbivory from grazing tadpoles, increased mortality of tadpoles should increase periphyton as a result of decreased grazing pressure and increased nutrient inputs from decomposing tadpoles. For malathion, a small (but non-significant) reduction in periphyton in the weekly low treatment and a large reduction in the single medium treatment demonstrated a consistent negative effect of phytoplankton shading. Interestingly, the high malathion treatment displayed a rapid increase in periphyton early in the experiment. This is inconsistent with the time period (several weeks) that a trophic cascade would require to affect periphyton abundance (Relyea & Hoverman, 2006; Relyea & Diecks, 2008). Instead, the increase in periphyton in the single high malathion treatment was likely caused by the death of tadpoles, which would reduce periphyton consumption. Additionally, the high concentration of malathion may have favoured an increase in periphyton by decreasing tadpole foraging. Decreased foraging in response to AchE inhibition has been reported previously for amphibians

(Bridges, 1999; Weis *et al.*, 2001). For carbaryl, only the high single treatment caused a delayed reduction in periphyton. While a number of studies have observed pesticide-induced trophic cascades that affect zooplankton and phytoplankton (e.g. Boone *et al.*, 2005; Boone & Bridges-Britton, 2006), few studies have examined direct and indirect effects of pesticides on phytoplankton and periphyton (Mills & Semlitsch, 2004; Relyea & Diecks, 2008). This is an important oversight as these algal groups serve different functions in creating pond structure, cycling nutrients and as a resource for primary consumers.

The impact of pesticides on amphibians depends upon how toxins directly alter amphibian health and how they indirectly affect amphibian resources. Malathion reduced the survival of leopard frog tadpoles in all treatments even though the concentrations of malathion that tadpoles were exposed to (3, 35 and 384 ppb) were one to three orders of magnitude lower than LC50 values determined in laboratory environments (2400 ppb; Relyea, 2004). The U.S. EPA typically estimates the level of concern for pesticides at 5 or 10% of the LC50 value (Jones *et al.*, 2004). Based on this reasoning, we would expect little or no tadpole mortality below 120–240 ppb (i.e. 5–10% of the leopard frog LC50), which is not consistent with our results. Carbaryl also decreased tadpole survival at concentrations lower than 10% of the predicted LC50 value for leopard frogs (LC50 = 2200 ppb; Relyea, 2003). In this case, mortality resulted from slower development and occurred because tadpoles could not metamorphose before their 'ponds' dried and mortality was not observed at the highest carbaryl concentration.

When resources are limiting tadpole growth, decreased tadpole survival should reduce competition. This can result in increased growth and development of the survivors. Conversely, when a trophic cascade lowers periphyton abundance, increased competition should reduce growth and development. Malathion exposure not only altered the survival of the leopard frogs, but also affected their development and growth. Leopard frogs in the weekly low treatment had reduced survival, growth and development. In contrast, leopard frogs exposed to single medium or single high applications also had lower survival, but increased growth and unaffected development. Since this mortality occurred early in the experiment (personal observation), the reduced density of leopard

frogs (and possibly reduced foraging activity) allowed a short-term increase in periphyton that was subsequently consumed by the remaining survivors and resulted in increased mass at metamorphosis. It is unclear why tadpoles in the weekly low treatment did not experience a similar increase in growth since they also experienced a small decline in survival; one possible explanation is that tadpole mortality occurred later in this treatment, leaving less time for survivors to benefit from reduced densities.

Among the carbaryl treatments, survival, development and growth were only affected by the weekly low and single medium treatments, where they were all reduced. Since food (periphyton) was unaltered in these treatments, this trend was likely due to reduced resources after day 21 or impaired growth and development as a result of pesticide toxicity. The latter explanation is more probable for the weekly low carbaryl treatment as there is little evidence for a trophic cascade at any point. Unlike the malathion treatments, tadpoles in these treatments did not experience reduced competition for periphyton as a result of tadpole mortality; most of the mortality occurred at the end of the larval period. Larger mass at metamorphosis is associated with higher juvenile survival, improved mating success, earlier time to reproduction and production of higher quality eggs (Howard & Kluge, 1985; Smith, 1987; Altwegg & Reyer, 2003). Earlier time to metamorphosis is associated with early reproductive success, increased survival and increased juvenile growth rate (Smith, 1987; Altwegg & Reyer, 2003).

Amphibian life history was unaffected in the single high carbaryl treatment even though a trophic cascade beginning with a decrease in cladocerans led to a large decrease in tadpole resources (periphyton). This result is unexpected since tadpoles exposed to 10% less carbaryl had lower survival, development and growth; however, similar patterns of decreasing effects of carbaryl on amphibian life history with increasing concentrations have been found in some laboratory and mesocosm studies. For example, mesocosm studies of southern leopard frogs (*Rana sphenoccephala* Cope) show increasing survival and mass with increasing concentrations of carbaryl (3.5 and 7.0 ppm: Boone & James, 2003; 2 and 5 ppm: Mills & Semlitsch, 2004). In contrast, mesocosm studies of effects of carbaryl on American toads (*Bufo americanus*) and gray treefrogs (*Hyla versicolor* LeConte)

across this same gradient (3.5 and 7.0 ppm) show declining survival and increasing mass (Boone & Semlitsch, 2001), while overwintered green frogs (*Rana clamitans* Latreille) in the same experiment were not affected by exposure to carbaryl. Boone & Semlitsch (2002) attribute these differences to the point in ontogeny in which organisms are exposed as well as the length of the larval period, with individuals with shorter larval periods being more negatively affected by exposure; however, there are limited data to investigate this hypothesis.

Collectively, these results suggest that both insecticides can cause comparable trophic cascades that affect zooplankton and phytoplankton abundances; however, their effects on amphibians diverge especially with exposure to higher concentrations of insecticides. While nearly all treatments caused trophic cascades initiated by decreases in zooplankton, exposure to a single application of more than 10% of the leopard frog LC50 value of carbaryl or malathion produced very different effects on amphibians and periphyton. Malathion had early-acting direct lethal and sublethal effects on amphibians, while carbaryl had delayed sublethal effects on amphibians or no effects at all. Malathion caused reduced foraging and early death of tadpoles, while carbaryl exposure reduced growth, development and, because of slower development, survival. Community responses to toxins may be similar for many contaminants when the main effect is driven through one causal pathway and the time frame of the effects is similar. When multiple direct and indirect trait- and density-mediated effects of pesticides on communities interact, the community response depends on the strength and timing of these interactions and is less generalisable (e.g. Relyea & Hoverman, 2006). To develop predictive models for the effects of contaminants on communities, we need to incorporate sublethal (as well as lethal) effects of contaminants into community ecology-based models and focus on the timing of these effects. Traditional toxicity tests, which focus on mortality and not behavioural and developmental effects, provide insufficient information for the development of such models.

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