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MITIGATING WITH MACROPHYTES: SUBMERSED PLANTS REDUCE THE TOXICITY OF PESTICIDE-CONTAMINATED WATER TO ZOOPLANKTON

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Abstract—In ecotoxicology, appreciation is growing for the influence that ecological interactions have on the toxicity of contaminants, such as insecticides, to sensitive species. Most previous studies, however, have focused on factors that exacerbate insecticide effects on species, while factors that may mitigate these effects have been relatively ignored. In aquatic habitats, a small number of studies have shown that submersed macrophytes can remove some insecticides from the water column via sorption. Although examining sorption dynamics is important for understanding the environmental fate of insecticides, whether and to what extent macrophytes actually mitigate insecticide effects on aquatic species remains unknown. In the present study, the authors examined how much and how quickly several realistic densities of the macrophyte *Elodea canadensis* decreased the toxicity of the insecticide malathion to *Daphnia magna*, a keystone aquatic herbivore. To do this, the authors quantified *Daphnia* survival in outdoor test systems (0.95 L) exposed to a factorial combination of five *Elodea* densities crossed with five malathion concentrations. The authors discovered that malathion's lethality to *Daphnia* decreased with increasing *Elodea* density. Furthermore, the rate at which *Elodea* reduced malathion's toxicity in the water column increased with macrophyte density. These results provide strong evidence that submersed macrophytes can mitigate the ecological impacts of a popular insecticide and further support that ecological interactions can strongly influence contaminant environmental effects. Environ. Toxicol. Chem. 2013;32:699–706. © 2012 SETAC

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INTRODUCTION

Insecticides are important tools for improving human health and the productivity of forestry and agriculture. Projected increases in insecticide use for the foreseeable future, however, will likely lead to greater exposure for natural ecosystems [1]. Insecticides pose a significant threat to aquatic habitats, because they can exacerbate declines in already threatened taxa [2,3] and decrease biodiversity [4,5]. Thus, a major contemporary challenge for ecologists and toxicologists is to understand the factors that influence the environmental effects of insecticides in aquatic habitats better.

Traditional toxicological models designed to predict the impacts of insecticides in aquatic communities are derived from the results of laboratory tests that determine concentrations at which some effect occurs (e.g., median lethal concentration [LC50] equals the concentration of an insecticide that kills 50% of a population) [6]. To compare the relative toxicity of a large number of insecticides directly, agencies responsible for registering and regulating pesticides worldwide (e.g., the U.S. Environmental Protection Agency [U.S. EPA], the Organisation for Economic Co-operation and Development [OECD], the American Society for Testing and Materials International [ASTM]) have established standardized testing guidelines designed to provide unambiguous cause and effect relationships by examining species in isolation of most biotic and abiotic environmental variation. However, there is a growing recognition not only that the environmental conditions are important in determining the outcome of toxicity tests, but also that they incorporate the reality of what organisms experience in nature [7–9].

To date, research that has incorporated natural environmental conditions has primarily focused on identifying factors that increase the toxicity or ecological impacts of insecticides. For example, variation in the abiotic environment [10,11], predatory stress [12–15], and competitive stress can all make insecticides more lethal to animals [16–17]. In contrast, studies examining the ecological factors that might mitigate insecticide effects are rare, despite the clear conservation and societal implications.

Submersed macrophytes possess traits that may allow them to at least partially mitigate the direct effects of insecticides on sensitive aquatic taxa. For example, macrophytes can sorb insecticides, potentially reducing the duration and intensity of exposure experienced by aquatic taxa [18,19]. In fact, submersed macrophytes can sorb up to 90% of insecticides from the water column within 24 h, but such high sorption rates only occur for highly lipophilic compounds (i.e., Log octanol-water partition coefficient, $K_{OW} > 6.0$), such as organochlorine (e.g., dichlorodiphenyltrichloroethane [DDT]) and pyrethroid (e.g., lambda-cyhalothrin) insecticides [20,21]. For less lipophilic compounds—such as the commonly applied organophosphate insecticides chlorpyrifos ($\text{Log}\, K_{OW} = 4.81$) and malathion ($\text{Log}\, K_{OW} = 2.3$)—the amount of insecticides removed from the water column by macrophytes typically ranges from 0 to 50% in a 24-h period [18,22,23].

Although it is clear that some submersed macrophytes have the ability to reduce the aqueous concentrations of some insecticides, very limited evidence exists regarding the ability of submersed macrophytes to mitigate the effects of insecticides on sensitive aquatic taxa. In one study, which compared the ecological effects of the organophosphate insecticide chlorpyrifos

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(35 μg/L) between macrophyte-dominated and phytoplankton-dominated artificial test systems (~0.85 m^3), Brock et al. [24] found that cladocerans were eliminated within hours in the phytoplankton-dominated system. In contrast, it took several weeks for die-offs to occur in the macrophyte-dominated system. In addition, Roessink et al. [25] examined the effects of five concentrations of the pyrethroid insecticide lambda-cyhalothrin (ranging from 10–250 ng/L) in macrophyte-dominated and phytoplankton-dominated ditch test systems (~0.5 m^3). In macrophyte-dominated systems, the authors estimated the no observable effect concentration (NOEC) of lambda-cyhalothrin on *Chaoborus obscuripes* to be at least 10 ng/L, whereas the NOEC was less than 10 ng/L in phytoplankton-dominated systems (no lower concentrations were tested). Although these studies found differences in the indirect effects of insecticide exposure on community structure and function between phytoplankton- and macrophyte-dominated systems, the influence of insecticide exposure versus idiosyncratic differences in ecological interactions on the community responses is unclear.

While these studies compared the effects of insecticides in macrophyte-dominated versus phytoplankton-dominated environments, they were not designed to test the extent to which macrophytes alone influence the ecological impacts of insecticides directly. For example, Brock et al. [24] compared the effects of chlorpyrifos on aquatic communities inhabiting macrophyte-dominated systems in 1988 with the effects of chlorpyrifos on similar (but not identical) communities inhabiting open-water systems in 1989. In addition, Roessink et al. [25] examined the response of macrophyte- and phytoplankton-dominated communities that differed in nutrient environment and species composition. To understand the influence that submersed macrophytes have on the biological effects of insecticides in aquatic communities, we need experiments that are designed specifically to address whether the manipulation of macrophytes in a system can alter insecticide effects on sensitive species.

We addressed this challenge by conducting an outdoor experiment that manipulated macrophyte density and insecticide concentration to determine whether, and to what extent, macrophytes could mitigate the lethality of the popular insecticide malathion to *Daphnia magna*. Studies elucidating the impacts of environmental stressors on *Daphnia* population dynamics are imperative because these animals serve as key drivers of aquatic community dynamics [26] and water quality [27]. Specifically, we addressed two hypotheses. First, as submersed macrophyte density increases, malathion's toxicity to *D. magna* will decrease; and second, as submersed macrophyte density increases, malathion's toxicity in the water column will decrease at a faster rate.

Insecticide background

Malathion is an organophosphate insecticide that inhibits acetylcholineesterase function in the nervous system. Malathion is commonly used for both agricultural and residential pest control throughout the world, with approximately 9.1 to 11.3 × 10^6 kg of active ingredient applied annually in the agricultural sector and another 1.8 to 3.6 × 10^6 kg applied annually in the home, garden, industrial, and governmental sectors of the United States alone [28]. Recently, the U.S. EPA determined the estimated environmental concentration (EEC) for malathion in California surface waters based on application frequencies (every 2 to 14 d), rates, and expected drift [29]. Based on these values for more than 50 terrestrial crops, the EEC for malathion in water was found to be 9 ± 27 μg/L (mean ± 95% confidence interval). Furthermore, aerial applications of malathion used to control insect pests can produce even higher concentrations in surface waters. For example, in the 1990s, the spraying of malathion for Mediterranean fruit fly control resulted in average surface water concentrations of approximately 50 μg/L [30].

MATERIALS AND METHODS

Experimental design

We conducted the experiment at the University of Pittsburgh's Pymatuning Laboratory of Ecology, Pennsylvania, USA. To investigate the effect of submersed macrophytes on insecticide toxicity, we examined the survival of the cladoceran zooplankter, *D. magna*, when exposed to a range of concentrations of the organophosphate insecticide, malathion, in the presence of different densities of the macrophyte *Elodea canadensis* (hereafter called *Elodea*). We used a complete factorial design, crossing five *Elodea* densities (0, 344, 612, 889, and 1,102 g dry wt/m^3) with five nominal malathion concentrations (0, 2.5, 10, 25, and 50 μg/L) for a total of 25 treatment combinations. Each treatment was replicated four times for a total of 100 experimental units.

Elodea canadensis is a globally widespread, submersed macrophyte that lives at a wide range of densities (i.e., from less than 50 g dry wt/m^3 to more than 800 g dry wt/m^3) [31]. On June 15, 2011, we collected *Elodea* from three separate shallow ponds in northwestern Pennsylvania. None of these ponds have been treated with any chemicals (nutrients, pesticides, etc.) within the past five years (J. Bish, Pennsylvania Game Commission, Pennsylvania, USA, personal communication). Once collected, we mixed and cultured the macrophytes in 300-L culture pools containing 50 L of loamy sediment. We placed a 40% shade cloth over the top to prevent any invertebrates from colonizing and to reduce water evaporation. The macrophytes were kept in these conditions for 23 d before being used in the experiment.

The malathion concentrations we chose for this experiment spanned the range of concentrations estimated or observed to be present in surface waters following typical agricultural and pest control practices [29,30]. Assuming the California data are representative of exposure scenarios in other regions where similar data are unavailable, these concentrations likely represent realistic exposure scenarios for aquatic taxa. Direct malathion application to surface waters for mosquito control (EEC = 539 μg/L) and for protecting aquatic crops (EEC = 1,404–1,797 μg/L) can produce dramatically higher exposure scenarios [29]. Such worst case scenarios, however, are likely rare occurrences for a majority of freshwater habitats; therefore, we elected to use concentrations that would occur more commonly in nature.

Test species

In winter 2010, we obtained 18 genetically distinct *D. magna* (hereafter called *Daphnia*) clones originating from Katholieke Universiteit Leuven, Belgium. Using these lab-reared clones for our experiment rather than animals collected directly from nature allowed us to ensure that the lineages had not been exposed to any environmental contaminants for dozens of generations prior to the present study. Furthermore, using these clones ensured genetic variability among the *Daphnia* populations used in the present study. We housed the *Daphnia* in 500-ml glass jars containing 300 mL of UV-filtered well water. We culled the *Daphnia* populations and performed water

changes every two weeks. *Daphnia* were fed 1 ml of concentrated *Scenedesmus spp.* algae that had been grown in a high-phosphorus COMBO medium [32]. Because of the logistical issues associated with coordinating the reproduction of these animals to achieve the very large number of *Daphnia* used in the present experiment (7,200 total), we did not use less than 24-h-old neonates to test malathion's toxicity. Instead, we used intermediate sized individuals (~instars 3–6) that had not yet produced eggs.

Toxicity test setupp

On July 8, 2011, we set up our aquatic test systems, which were 0.95-L glass jars. To do this, we removed all coarse organic debris from loamy terrestrial topsoil (collected on site) and added 100 g of this soil to each jar to serve as a nutrient source and rooting substrate for *Elodea*. We then added 700 ml of aged, UV-filtered well water to each jar. We let the jars sit overnight to allow the suspended sediment to settle. The following day, we haphazardly selected *Elodea* shoots from the culture pools, cut each shoot 15 cm below the apex, and added the appropriate number of shoots to each jar. To span the range of *Elodea* densities commonly observed in nature (see *Experimental Design*), we added 0, 3, 6, 9, or 12 *Elodea* shoots to each jar, which created density treatments of 0, 344 ± 60.7, 612 ± 62.8, 889 ± 101.7, and $1{,}102 \pm 148.4$ g dry weight/m^3 (mean $\pm$ standard deviation).

Although we performed the present experiment in jars designed to maximize our control over the abiotic and biotic environment inside each jar, we also wanted to expose the macrophytes and zooplankton to environmental conditions that were somewhat representative of what they would experience in nature. To achieve this, we moved the jars outside and placed them in glass aquaria positioned on their sides inside 300-L pools located on wooden tables. We randomly assigned each jar to an aquarium and placed ten jars into each of the twelve aquaria in the pools. This setup allowed us to expose the jars to natural temperature and light fluctuations while preventing rain from entering and diluting the water. After the jars were in place, we added approximately 10 cm of cold well water to each pool until it rose to approximately one half of the height of the jars. Placing the pools on flat tables ensured that the water level outside of each jar was equal. We drained each pool twice daily (at 11:00 h and 15:00 h) and added new, cool well water to help buffer the water inside the jars from reaching unnatural temperature extremes. To allow *Elodea* to acclimate to the jars conditions, we let the jars sit outside for 3 d prior to applying insecticides. During this time, we visually inspected the plants and determined that they were healthy, as evidenced by new foliar growth and production of roots extending into the sediment.

Malathion application

On July 12, 2011, we applied the appropriate concentration of technical grade (99.1%) malathion (Chem Service) to each test system. We elected to use technical-grade malathion rather than commercial formulations (typically containing ~50% malathion) because little information exists about the degree to which aquatic organisms are actually exposed to the inert ingredients comprising the other 50% of commercial formulations of malathion. To achieve nominal concentrations of 0, 2.5, 10, 25, and 50 μg/L, we added 0, 0.366, 1.463, 3.660, and 7.320 ml, respectively, of stock solution (0.123 mg malathion/ml ethanol) to 1.2 L of UV-filtered water to create our working solutions. This large batch of working solution provided a sufficient volume for dosing each appropriate test system, plus provided two additional jars for malathion concentration analysis. Although we did not perform a control for the ethanol carrier in this experiment, other experiments have documented no adverse effects of ethanol at concentrations (0.5 ml ethanol/L water) higher than those used in our study (0.41 ml ethanol/L water) on *Daphnia* [33]. We used a separate container to make each working solution. After mixing each working solution for approximately 30 s, we added 50 ml into each appropriate jar to bring the total volume of each test system to 750 ml. We applied the malathion stock solution to each test system in a circular motion that ensured thorough mixing and even distribution inside of each container. We began applying malathion at 12:00 h and finished at 14:00 h.

To determine the actual malathion concentrations achieved for each treatment, we applied 50 ml of each working solution (same solution as above) to two separate glass jars containing 700 ml of UV-filtered water, using identical application techniques as we used for the experimental containers. We then took 450 ml of this water and transferred it to 500-ml pre-cleaned amber glass jars and stored the jars in a 3°C refrigerator until analysis. All samples were sent to an independent laboratory (University of Georgia Agricultural and Environmental Services Laboratory, Athens, Georgia, USA) for analysis using gas chromatography–mass spectrometry within one week of being collected. The actual malathion concentrations corresponding to the nominal concentrations of 0, 2.5, 10, 25, and 50 μg/L were 0, 3.2, 4.7, 17.7, and 29.6 μg/L (hereafter referred to as 0, 3, 5, 18, and 30 μg/L). Because water samples collected during dosing were not analyzed for one week, it is possible that some malathion breakdown occurred during this time, resulting in the discrepancy between our nominal and actual malathion concentrations. If breakdown did occur, then the true malathion concentrations the *Daphnia* in the present study encountered would be even higher than reported. This, however, would not affect the overall conclusions.

Determining the effect of Elodea *density on malathion's toxicity*

After the insecticide was applied, we added 10 *Daphnia* to each jar. Because the malathion application took 2 h, *Daphnia* were added to each test system 2 h after it had received its malathion application (i.e., *Daphnia* were added in same order that malathion was applied). Each day we fed the *Daphnia* in the jars by adding 0.5 ml of the algae solution that was being fed to the *Daphnia* cultures. After 48 h, we removed the *Elodea* from the jars to facilitate *Daphnia* survival counts and gently shook the shoots in a separate container of water to ensure that no *Daphnia* had been removed from the jars during *Elodea* removal. We then counted the number of surviving *Daphnia* in each jar by applying a gentle burst of water over the individuals with a transfer pipette. We considered an individual to have survived if it began to swim vertically in the water column within three applications of this stimulus. Any individuals that were twitching but unable to swim were considered dead.

Determining Elodea*'s effect on the rate of decrease in malathion's toxicity*

In addition to comparing the amount that different *Elodea* densities reduced malathion's toxicity, we also compared the rate at which they caused malathion's toxicity to decrease in the water column. To accomplish this, we removed small amounts of water from the jars over time and tested the toxicity of this sampled water against new groups of *Daphnia*. We used a glass

pipette to remove 25 ml of water from the middle of the water column of each jar at 2, 6, 10, and 48 h after we had applied malathion. Again, this step was done in the same order that the jars had been dosed so that the duration between insecticide application and water collection was equal for each test system. We then transferred the water from each jar to a separate 50-ml glass vial and immediately added 10 *Daphnia* to each vial. We transferred the vials indoors, where they were kept at 20°C under a 12:12-h light:dark cycle. We fed *Daphnia* 0.25 ml of *Scenedesmus spp.* algae daily. After 48 h, we quantified the number of surviving *Daphnia* after they had been added to each vial using the criteria described above. Thus, the response data for the present experiment were the number of surviving *Daphnia* after 48 h of exposure to water collected from each jar at each time point.

Measuring Elodea*'s effects on water pH, dissolved oxygen, and temperature*

We documented the effects of *Elodea* on water pH (using a calibrated digital pH meter; Oakton Instruments), dissolved oxygen (DO) and temperature (using a calibrated digital oxygen meter; WTW), 1 h before applying malathion to the experiment. In addition, we documented water pH and DO in each test system 48 h after applying malathion.

Statistical analysis

To determine the effect of *Elodea* density on the survival of *Daphnia* exposed to malathion, we compared *Daphnia* $LC50_{48\,h}$ values between each macrophyte density treatment. To estimate these values for each *Elodea* density treatment, we used probit analyses to fit sigmoid-shaped curves to the *Daphnia* survival data. If necessary, data were smoothed to ensure equal or decreasing survival with increasing malathion concentration and adjusted for mortality in the controls using Abbott's formula [34]. To compare the effects of different *Elodea* densities on the *Daphnia* LC50 values, we examined the overlap between the 84% confidence intervals. Payton et al. [35] have demonstrated that 84% confidence intervals approximate an $\alpha = 0.05$. In one of the *Elodea* treatments (889 g dry wt/m^3), the highest mortality levels only approached 50%. As a result, this distribution of mortality values produced LC50 estimates that were not reliable (LC50 = 64 μg/L, 84% confidence interval = 26 – 4,356 μg/L).

To determine whether *Elodea* densities differed in the rate at which they reduced malathion's toxicity in the water column, we compared the amount of time it took for the toxicity of water treated with each concentration of malathion to return to control levels in each *Elodea* density treatment. To do this, we used Dunnett's tests to compare *Daphnia* survival 48 h after exposure to control water versus water treated with each respective malathion concentration collected at each sampling time point within each *Elodea* density treatment. Due to unequal variances, we first rank transformed the survival data. Although the utility of Dunnett's test in toxicological testing is controversial [36], we emphasize that we used this approach simply as a tool to compare the rates at which different *Elodea* densities detoxified the water. This is in contrast to the more conventional uses of Dunnett's tests, such as trying to determine acceptable and unacceptable contaminant loads in the environment.

Finally, we evaluated the effects of *Elodea* density on aqueous pH, DO, and temperature immediately prior to adding malathion using a multivariate analysis of variance (MANOVA). We also examined the effect of *Elodea* density, malathion treatment, and the interaction on pH and DO 48 h following the application of malathion. Where appropriate, we used univariate analysis of variances (ANOVAs) to examine treatment effects on each response variable. We used Tukey's multiple comparisons tests to determine differences between treatments.

RESULTS

Influence of Elodea *density on malathion's lethality to* Daphnia

As *Elodea* density increased, malathion's lethality to *Daphnia* decreased (Fig. 1). One way to quantify this is by estimating the $LC50_{48\,h}$ values for malathion within each *Elodea* treatment. The $LC50_{48\,h}$ value for *Daphnia* in the absence of *Elodea* (2.8 μg/L) was significantly lower than the LC50 values of

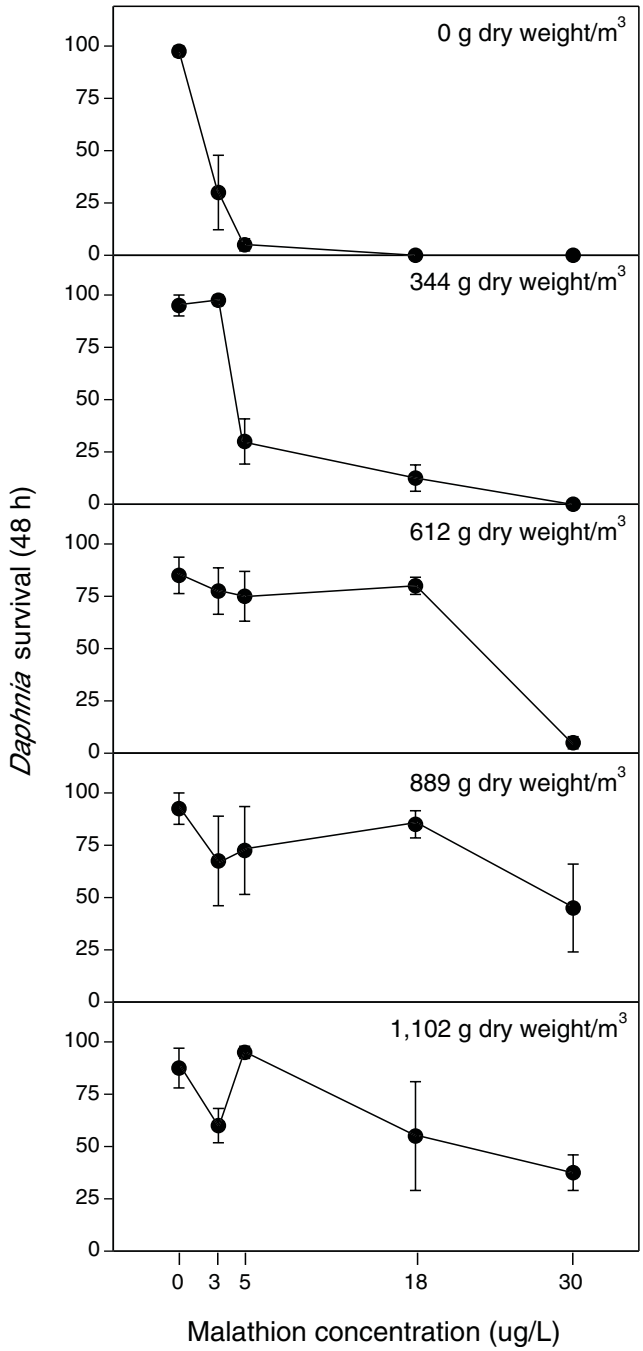

Fig. 1. Survival data for *Daphnia magna* ($n = 10$) exposed to a factorial combination of malathion concentrations (0, 3, 5, 18, 30 μg/L) and *Elodea canadensis* densities (0, 344, 612, 889, 1,102 g dry wt/m^3). Data are means ± 1 standard error.

all treatments containing *Elodea* (Table 1). Moreover, with each increase in *Elodea* density, we observed a significant increase in the estimated LC50 value for *Daphnia* exposed to malathion.

Elodea*'s effect on the rate of decrease in malathion's toxicity*

In general, we observed that the toxicity of a given malathion concentration in the water column decreased at a faster rate, relative to insecticide-free controls, with each increase in *Elodea* density. The exception was in all jars receiving 3 μg/L of malathion, in which *Daphnia* survival never differed from insecticide-free controls ($p \geq 0.081$). In jars receiving applications of 5, 18, and 30 μg/L of malathion, however, water detoxification rates increased with macrophyte density. For example, with 0 g dry weight/m^3 of *Elodea*, water collected from jars at 2, 6, 10, and 48 h following the application of 5, 18, and 30 μg/L of malathion always caused greater than 50% *Daphnia* mortality (Fig. 2; $p \leq 0.011$). With 344 g dry weight/m^3 of *Elodea*, it took 6, 48, and 48 h for *Daphnia* survival to return to control levels in the 5, 18, and 30 μg/L malathion treatments, respectively ($p > 0.108$). With 612 g dry weight/m^3 of *Elodea*, it took just 6 h for *Daphnia* survival to return to control levels in the 5, 18, and 30 μg/L malathion treatments ($p > 0.561$). With 889 g dry weight/m^3 of *Elodea*, it took only 2 h for *Daphnia* survival to return to control levels in the 5 and 18 μg/L malathion treatments, but took 6 h in the 30 μg/L treatment ($p \geq 0.369$). The strongest mitigative effect we observed occurred with 1,102 g dry weight/m^3 of *Elodea*; under this condition, each water sample collected between 2 and 48 h after the initial malathion application caused no more *Daphnia* mortality than that which occurred in the no-malathion controls ($p \geq 0.054$).

Finally, an interesting phenomenon we observed when examining the rate at which different *Elodea* densities detoxify the water column was the apparent decrease in *Daphnia* survival following exposure to water collected from the jars between 6 and 10 h following malathion application. To examine this pattern further, we performed Wilcoxon signed-ranks tests on *Daphnia* survival following exposure to water collected after 6 h versus 10 h in each malathion and *Elodea* treatment combination. These analyses confirmed that none of the apparent differences between *Daphnia* survival in the samples collected at 6 and 10 h were significant ($p > 0.066$).

Effects of Elodea *and malathion on water pH, DO, and temperature*

When we analyzed pH, DO, and temperature immediately prior to applying malathion, we found multivariate effects of

Table 1. Median lethal concentration ($LC50_{48h}$) values and 84% confidence intervals (CIs) calculated for *Daphnia magna* exposed to malathion in the presence of different densities of the submersed macrophyte, *Elodea canadensis*[a]

Elodea density (g dry wt/m^3)	*Daphnia* LC50 value (μg/L)	Lower 84% CI	Upper 84% CI
0	2.8A	2.1	3.1
344	5.5B	4.8	6.3
612	14.0C	11.5	17.2
889	–[b]	–	–
1,102	25.2D	19.5	36.6

[a] Letters indicate significant differences between groups based on the overlap of 84% CIs.
[b] LC50 estimates for 889 g dry weight/m^3 were not reliable because the highest *Daphnia* mortality only approached 50%.

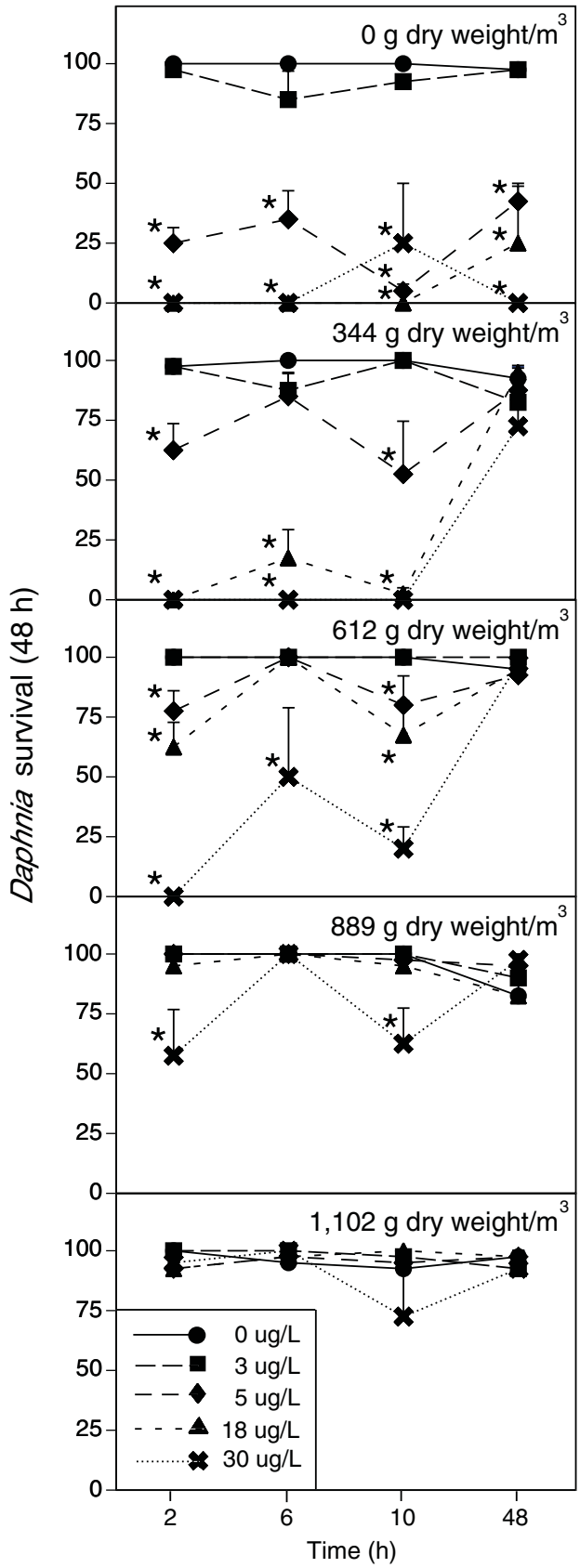

Fig. 2. The influence of *Elodea canadensis* density on the toxicity of water collected 2, 6, 10, or 48 h after malathion applications of 0, 3, 5, 18, and 30 μg/L. We quantified water toxicity by examining *Daphnia magna* survival 48 h after exposure to each respective water sample. Asterisks indicate treatments where *Daphnia* survival was significantly lower than in insecticide-free treatments at a given sampling time and *Elodea* density. For clarity, data are presented as means + 1 standard error.

Elodea density (Wilk's λ, $F_{12,246} = 49.8$, $p < 0.001$). The multivariate effects were driven by univariate effects of pH ($F_{4,95} > 494.3$, $p < 0.001$) and DO ($F_{4,95} > 113.3$, $p < 0.001$). There was no effect of *Elodea* treatment on temperature ($F_{4,95} = 0.8$, $p = 0.513$) as the five *Elodea* densities were all within 1°C of each other (mean ± SE; 29.8 ± 0.1). Tukey's test revealed that pH increased significantly with each increase in *Elodea* density (Fig. 3; all $p < 0.029$). Dissolved oxygen also increased with each increase in *Elodea* density (Fig. 3; all $p < 0.021$), except for the two highest *Elodea* densities, which did not differ ($p > 0.760$).

When we analyzed pH and DO 48 h after applying malathion, we observed significant multivariate effects of *Elodea* density (Wilk's λ, $F_{8,148} = 75.9$, $p < 0.001$), as well as effects of malathion concentration (Wilk's λ, $F_{8,148} = 31.5$, $p < 0.001$), but not the *Elodea*-by-malathion interaction (Wilk's λ, $F_{32,148} = 1.5$, $p = 0.061$). The effects of *Elodea* density were driven by univariate effects of pH ($F_{4,16} > 3.7$, $p < 0.009$) and DO ($F_{4,16} > 65.6$, $p < 0.001$). Tukey's tests revealed that each increase in *Elodea* density caused a corresponding increase in pH (Fig. 3; $p < 0.001$) except for the highest two *Elodea* density treatments, which did not differ ($p = 0.152$). Dissolved oxygen also increased with each increase in *Elodea* density ($p < 0.001$) with the exception of the two highest *Elodea* densities, which did not differ ($p > 0.463$). Although we detected significant multivariate effects of malathion concentration on the abiotic environment 48 h after malathion applications, the range of pH (9.2 − 9.4) and DO values (12.3 − 16.8 mg/L) that we observed across malathion treatments were unlikely to have resulted in significant biological effects on *Daphnia* or *Elodea*; therefore, they will not be discussed further.

DISCUSSION

Although previous studies have reported the mitigating effects of emergent vegetation contained within agricultural constructed wetlands and drainage ditches on the toxicity of insecticides to aquatic taxa [37], the present study appears to be the first experimental demonstration that submersed macrophytes can strongly mitigate the lethal effects of insecticides on an aquatic species. Specifically, we discovered that the common macrophyte *E. canadensis* substantially reduced the lethality of the popular insecticide malathion to the keystone herbivore, *D. magna*, [26] and also increased the rate at which water treated with malathion was detoxified.

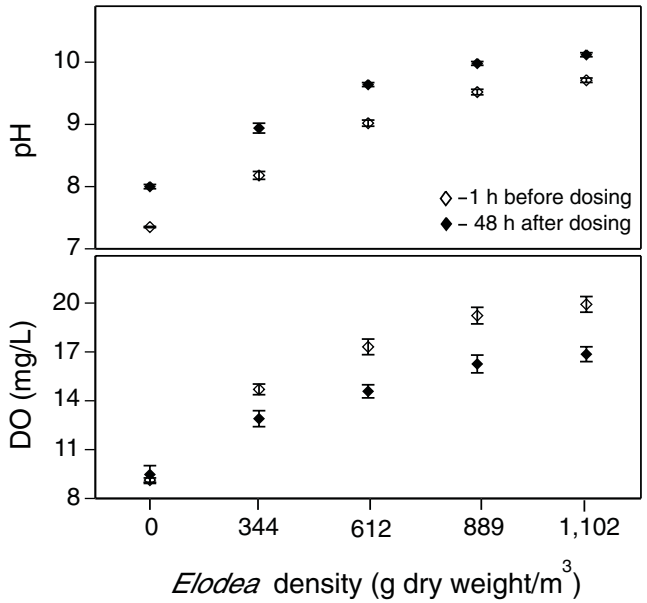

Fig. 3. The effects of *Elodea canadensis* density on pH and dissolved oxygen (DO) before and after malathion applications to outdoor experimental test systems. Data are means ± 1 standard error.

By generating $LC50_{48h}$ estimates for *Daphnia* exposed to malathion in the presence of five different *Elodea* densities, we found strong support for our hypothesis that *Elodea* would reduce malathion's lethality to *Daphnia*. Furthermore, these data demonstrate that this mitigating effect increases with *Elodea* density. In fact, we found that the $LC50_{48h}$ estimates for *Daphnia* significantly increased with each increase in *Elodea* density. For example, comparing the 0 g dry weight/m^3 *Elodea* treatment to the 344, 612, and 1,102 g dry weight/m^3 *Elodea* density treatments, we observed approximately twofold, fivefold, and ninefold increases in the $LC50_{48h}$ estimates for *Daphnia*.

The estimated $LC50_{48h}$ value for *Daphnia* exposed to malathion in the absence of *Elodea* (2.8 μg/L) is consistent with other studies employing more traditional toxicological experimental designs [38] (www.pesticideinfo.org). Thus, while incorporating submersed macrophytes into our experiment made it impractical for our study to adhere to traditional toxicity testing guidelines using *D. magna* (U.S. EPA, OETC, ASTM, etc.), the similarity between our results and others provides external validity that our testing methodology did not strongly influence malathion's toxicity to this species.

The present study also revealed that the rate at which *Elodea* reduced the toxicity of water following the application of malathion increased with increasing *Elodea* density. For example, in jars containing 0 g dry weight/m^3 of *Elodea*, the average survival of *Daphnia* exposed to water extracted from treatments that had initially received 5, 18, or 30 μg/L of malathion was less than 50% even 48 h after malathion had been applied. In jars containing 1,102 g dry weight/m^3 of *Elodea*, however, regardless of the malathion concentration that had been applied, *Daphnia* survival never significantly differed from controls following exposure to water collected from the test systems at any sampling interval after the initial application. Thus, our data also strongly support our second hypothesis that higher *Elodea* densities increase the rate at which malathion's toxicity in the water column is reduced.

Curiously, we did not observe significant lethal effects of water extracted at any time point following the application of 3 μg/L malathion to *Daphnia* in any of the *Elodea* treatments. Given the low survival (less than 50%) of *Daphnia* directly added to the jars containing 0 g dry weight/m^3 of *Elodea* in response to this malathion concentration, we expected to observe at least a partial reduction in *Daphnia* survival after being exposed to water collected from these test systems, particularly at the early extraction time points (e.g., after 2 h). Malathion breakdown during the time interval before the 2 h water extraction is not a likely cause of this difference because the *Daphnia* placed directly into the jars, which experienced substantial mortality, were added simultaneously with the water extraction that took place at 2 h. Although the mechanisms underlying this observation are unclear, it is possible that the *Daphnia* in these jars faced greater exposure as their swimming movements near the benthos could have resuspended sediment particles bound to malathion that the *Daphnia* then ingested. In addition, it is possible that desorption of malathion from the sediments caused an exposure that the

Daphnia in test vials (which contained only water from the jars) would not have encountered. While such mechanisms would be interesting to tease apart, they cannot be separated by our experiment and are thus beyond the scope of the present study.

Though no previous studies have examined the rates at which submersed macrophytes can reduce the toxicity of water to aquatic taxa following insecticide exposure, a small body of research has examined dissipation rates of insecticides in the presence of submersed macrophytes. For example, Gao et al. [23] examined the rate at which malathion concentrations decreased in culture medium in the presence of two submersed macrophyte species (*Myriophyllum aquaticum* and *E. canadensis*). However, the macrophyte densities (100,000 g fresh wt/m^3) used in that study were 10 times higher than even the maximum *Elodea* density used in the present study (~10,000 g fresh wt/m^3). Thus, one would expect that the authors would have observed higher malathion dissipation rates compared to the present study. Interestingly, the opposite appears to have occurred. For example, whereas Gao et al. [23] documented less than a 50% reduction in aqueous malathion concentration over 48 h (nominal concentration applied = 1,000 μg/L), the present study's data suggest much higher dissipation rates, because all of the macrophyte treatments containing *Elodea* made the water completely non-toxic to *Daphnia* within 48 h, even at the highest malathion concentrations tested.

Because so few data are available on the role that submersed macrophytes play in the dissipation of malathion from aquatic environments, it is difficult to draw broad conclusions about the factors that may have influenced malathion's toxicity to *Daphnia* in the present study. For example, *Elodea* could be sorbing malathion onto its surfaces and thus reducing water toxicity to *Daphnia*. However, although many highly-lipophilic insecticides with Log K_{ow} values greater than 6.0 (e.g., pyrethroid and organochlorine insecticides) will bind rapidly to submersed macrophytes [20,21], malathion is relatively hydrophilic (Log $K_{ow} = 2.3$), and it remains unclear how much macrophytes will sorb this insecticide. In the aforementioned experiment by Gao et al. [23], the authors found no evidence that malathion was taken up by macrophytes during the first 48 h following exposure. Though they attribute the dissappearance of malathion from the water column after 48 h to sorption by *Elodea*, the authors only measured malathion's concentration in *Elodea* on day 8 and thus cannot determine how much of malathion's disappearance from the water column was due to sorption versus other breakdown processes.

Another mechanism that might contribute substantially to malathion's disappearance from the water column is the rise in pH associated with each increase in *Elodea* density in the present study (Fig. 3). Increases in aqueous pH are known to affect the persistence of many insecticides [39]. For example, Wolfe et al. [40] demonstrated that each unit increase in pH (e.g., from pH8 to pH9) decreases malathion's half-life by approximately one order of magnitude [40]. Their data suggest that at pH levels similar to those documented in our no-macrophyte treatments (i.e., pH ~8), malathion's half-life in water is slightly less than 10 h at the average daytime water temperatures occurring in our study (~30°C). However, malathion's half-life is expected to decrease to approximately 1 h in the 344 g dry weight/m^3 *Elodea* density treatments (pH = 9) and to substantially less than 1 h in the highest *Elodea* treatments (pH = 10). Though it is unknown how much decreasing the half-life of an insecticide may affect its toxicity, it is possible that reductions in malathion's persistence could be contributing to the lower toxicity of this insecticide that we observed at higher *Elodea* densities. Thus, an important future step is to compare the relative effects of macrophyte sorption versus differences in pH resulting from the presence of macrophytes on insecticide persistence and toxicity. While dissolved oxygen also correlated positively with *Elodea* density, the authors know of no studies indicating that the differences in DO between *Elodea* treatments observed in the present study would cause differences in malathion's persistence or toxicity.

CONCLUSIONS

The field of ecotoxicology is beginning to explore the influence of ecological interactions more fully when examining the effects of toxic contaminants in the environment. Despite major advancements in this area, however, relatively little attention has focused on the ecological factors that can potentially reduce the biological impacts of contaminants in nature. We performed the first experiment to explicitly test the extent to which submersed macrophytes mitigate the direct toxic effects of a common insecticide contaminant. Our results demonstrate that the common waterweed *E. canadensis* can dramatically reduce the toxicity of the insecticide malathion to *D. magna*, an herbivorous zooplankton species that plays a key role in the functioning of many aquatic ecosystems. Moreover, the mitigating effect of *Elodea* increases with increases in its density. In addition, we discovered that *Elodea* can remove malathion quickly from the water column, but that the rate at which this macrophyte does so is also related to the plant's density. These findings suggest that processes that reduce the abundance of submersed macrophytes, such as eutrophication or vegetation eradication programs, may indirectly increase the susceptibility of sensitive aquatic taxa to other contaminants such as insecticides. Future research should focus on the generalizability of contaminant mitigation ability across other species of submersed macrophytes and other insecticides. In addition, an important next step is to examine whether the mitigative influence of submersed macrophytes on free-swimming *Daphnia* also applies to other aquatic species that may spend more time perching on macrophyte shoots or even ingesting macrophytes or their epiphytes directly. Such research will help to fill important gaps in our understanding of the ways that biological components of ecosystems may buffer the environment from increasingly common exposure to contaminants.

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REFERENCES

1. Laurence WF. 2001. Future shock: Forecasting a grim fate for the Earth. *Trends Ecol Evol* 16:531–533.
2. Davidson C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. *Ecol Appl* 14: 1892–1902.
3. Bradford DF, Knapp RA, Sparling DW, Nash MS, Stanley KA, Tallent-Halsell NG, McConnell LL, Simonich SM. 2011. Pesticide distributions and population declines of California, USA, alpine frogs, *Rana muscosa*, and *Rana sierrae*. *Environ Toxicol Chem* 30:682–691.
4. Relyea RA. 2005. The impact of insecticide and herbicides on the biodiversity and productivity of aquatic communities. *Ecol Appl* 15: 618–627.
5. Geiger F, Bengtsson J, Berendse F, Weisser WW, Emmerson M, Morales MB, Ceryngier P, Liira J, Tscharntke T, Winqvist C, Eggers S,

Bommarco R, Pärt T, Bretagnolle V, Plantegenest M, Clement LW, Dennis C, Palmer C, Oñate JJ, Guerrero I, Hawro V, Aavik T, Thies C, Flohre A, Hänke S, Fischer C, Goedhart PW, Inchausti P. 2010. Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic Appl Ecol* 11:97–105.

6. Newman MC. (ed)., 2010. *Fundamentals of Ecotoxicology*, 3rd ed. CRC Press, NY.
7. Chapman PM. 2002. Integrating toxicology and ecology: Putting the "eco" into ecotoxicology. *Mar Pollut Bull* 44:7–15.
8. Relyea RA, Hoverman JT. 2006. Assessing the ecology in ecotoxicology: A review of pesticide effects in freshwater systems. *Ecol Lett* 9:1157–1171.
9. Relyea RA. 2010. Multiple stressors and indirect food web effects of contaminants on herptofauna. In Sparling D, ed, *Ecotoxicology of Amphibians and Reptiles*, 2nd ed. pp. 475–486.
10. Zaga A, Little EE, Raben CF, Ellersieck MR. 1998. Photoenhanced toxicity of a carbamate insecticide to early life stage anuran amphibians. *Environ Toxicol Chem* 17:2543–2553.
11. Edginton AN, Sheridan PM, Stephensen GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and vision herbicide on two life stages of four anuran amphibian species. *Environ Toxicol Chem* 23:815–822.
12. Hanazato T, Dodson SI. 1995. Synergistic effects of low oxygen concentration, predator kairomone, and a pesticide on the cladoceran *Daphnia pulex*. *Limnol Oceanogr* 40:700–709.
13. Hanazato T. 2001. Pesticide effects on freshwater zooplankton: An ecological perspective. *Environ Pollut* 112:1–10.
14. Relyea RA, Mills N. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proc Natl Acad Sci U S A* 98:2491–2496.
15. Relyea RA. 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. *Environ Toxicol Chem* 23:1080–1084.
16. Boone MD, Semlitsch RD. 2001. Interactions of an insecticide with larval density and predation in experimental amphibian communities. *Conserv Biol* 15:228–238.
17. Boone MD, James SM. 2003. Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. *Ecol Appl* 13:829–841.
18. Karen DJ, Joab BM, Wallin JM, Johnson KA. 1998. Partitioning of chlorpyrifos between water and an aquatic macrophyte (*Egeria densa*). *Chemosphere* 37:1579–1586.
19. Crum SJH, van Kammen-Polman AMM, Liestra M. 1999. Sorption of nine pesticides to three aquatic macrophytes. *Arch Environ Contam Toxicol* 37:310–316.
20. Gao J, Garrison AW, Hoehamer C, Mazur CS, Wolfe NL. 2000. Uptake and phytotransformation of *o,p′*-DDT and *p,p′*-DDT by axenically cultivated aquatic plants. *J Agric Food Chem* 48:6121–6127.
21. Hand LH, Kuet SF, Lane MCG, Maund SJ, Warinton JS, Hill IR. Influences of aquatic plants on the fate of the pyrethroid insecticides *Lambda*-cyhalothrin in aquatic environments. *Environ Toxicol Chem* 20:1740–1745.
22. Van Donk E, Prins H, Voogd HM, Crum SJH, Brock TCM. 1995. Effects of nutrient loading and insecticide application on the ecology of *Elodea*-dominated freshwaster test systems I. Responses of plankton and zooplanktivorous insects. *Arch Hydrobiol* 133:417–439.
23. Gao J, Garrison AW, Hoehamer C, Mazur CS, Wolfe NL. 2000. Uptake and phytotransformation of organosphosphate pesticides by axenically cultivated aquatic plants. *J Agric Food Chem* 48:6114–6120.
24. Brock TCM, Crum SJH, van Wijngaarden R, Budde BJ, Tijink J, Zuppelli A, Leeuwangh P. 1992. Fate and effects of the insecticide Dursban 4E in indoor elodea-dominated and macrophyte-free freshwater ecosystems: I. Fate and primary effects of the active ingredient Chlorpyrifos. *Arch Environ Contam Toxicol* 23:69–84.
25. Roessink I, Arts GHP, Belgers JD, Maund SJ, Brock TCM. 2005. Effects of lambda-cyhalothrin in two ditch test system systems of different trophic status. *Environ Toxicol Chem* 24:1684–1696.
26. Sarnelle O. 2005. *Daphnia* as keystone predators: Effects on phytoplankton diversity and grazing resistance. *J Plankton Res* 27: 1229–1238.
27. Lathrop RC, Carpenter SR, Robertson DM. 1999. Summer water clarity responses to phosphorus, *Daphnia* grazing, and internal mixing in Lake Mendota. *Limnol Oceanogr* 44:137–146.
28. Grube A, Donaldson D, Kiely T, Wu L. 2011. Pesticide industry sales and usage: 2006 and 2007 market estimates. U.S. Environmental Protection Agency, Washington, DC.
29. Odenkirchen E, Wente SP. 2007. Risks of malathion use to federally listed California red-legged frog (*Rana aurora draytonii*). U.S. Environmental Protection Agency, Environmental Fate and Effects Division, Washington, DC.
30. Ando C, Gallavan R, Wofford P, Bradley A, Kim D, Lee P, Troiano J. 1996. Environmental monitoring results of the Mediterranean fruit fly eradication program, Riverside County 1994. EH95-02. California Environmental Protection Agency, Sacramento, CA, USA.
31. Duarte CM, Kalff J. 1990. Biomass density and the relationship between submerged macrophyte biomass and plant growth form. *Hydrobiologia* 196:17–23.
32. Kilham SS, Kreeger DA, Lynn SG, Goulden CE, Herrera L. 1998. COMBO: A defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* 377:147–159.
33. Kast-Hutchinson K, Rider CV, LeBlanc GA. 2001. The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environ Toxicol Chem* 20:502–509.
34. Finney DJ. 1971. *Probit Analysis*. Cambridge University Press, New York, NY, USA.
35. Payton ME, Greenstone MH, Shenker N. 2003. Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical signficance? *J Insect Sci* 34:1–6.
36. Delignette-Muller ML, Forfait C, Billoir E, Charles S. 2011. A new perspective on the Dunnett procedure: Filling the gap between NOEC/LOEC and ECx concepts. *Environ Toxicol Chem* 30:2888–2891.
37. Lizotte RE Jr, Moore MT, Locke MA, Kröger R. 2011. Effects of vegetation in mitigating the toxicity of pesticide mixtures in sediments of a wetland mesocosm. *Water Air Soil Pollut* 220:69–79.
38. Kegley SE, Hill BR, Orme S, Choi AH. 2010. *PAN Pesticide Database*. Pesticide Action Network, North America, San Francisco CA, USA.
39. Chapman RA, Cole CM. 1982. Observations on the influence of water and soil pH on the persistence of insecticides. *J Environ Sci Health Part B: Pestic Food Contam Agric Wastes* 17:487–504.
40. Wolfe NL, Zepp RG, Gordon JA, Baughman GL, Cline DM. 1977. Kinetics of chemical degradation of malathion in water. *Environ Sci Technol* 11:88–93.