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MITIGATION OF MALATHION'S ACUTE TOXICITY BY FOUR SUBMERSED MACROPHYTE SPECIES

WILLIAM R. BROGAN III* and RICK A. RELYEA
Department of Biological Sciences, University of Pittsburgh, Pennsylvania, USA

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Abstract: Some submersed macrophyte species rapidly sorb some insecticides from the water, potentially reducing exposure for aquatic species. The rates at which macrophytes remove insecticides, however, can differ widely among plant species. Furthermore, few studies have examined how much macrophytes actually influence insecticide toxicity to sensitive animals. The authors quantified the ability of several macrophyte species to mitigate insecticide toxicity by comparing the survival of the aquatic herbivore, *Daphnia magna*, following exposure to a factorial combination of 3 malathion concentrations (0 μg/L, 3 μg/L, and 24 μg/L) and 7 macrophyte treatments (no macrophytes, 4 different macrophyte monocultures, and 2 inert substrates: plastic plants and polypropylene rope). The authors also quantified the rate that different macrophytes reduced malathion's toxicity by exposing *D. magna* to water samples collected from each treatment after 2 h, 8 h, and 48 h of exposure. The results revealed that whereas 3 μg/L and 24 μg/L of malathion decimated *D. magna* in the no-macrophyte, plastic plant, and rope treatments, all 4 macrophyte species strongly mitigated these effects. When the authors compared the rate at which malathion's toxicity decreased, they found that all macrophytes negated malathion's toxicity within 2 h, whereas it took more than 8 h in the absence of macrophytes or in the presence of inert substrates. These results demonstrate that numerous macrophyte species can equally and strongly mitigate insecticide toxicity, whereas inert substrates cannot. *Environ Toxicol Chem* 2013;32:1535–1543. © 2013 SETAC

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INTRODUCTION

Using insecticides is a primary strategy for controlling pest damage to economically valuable lands and human health. An unintended byproduct of insecticide use, however, is the exposure of non-target species. For example, insecticides commonly enter surface waters via runoff, spray drift, and irrigation effluent, leading to exposure of aquatic communities that can cause shifts in species composition [1,2] and diversity [3]. Thus, preventing adverse environmental impacts of insecticides is an important goal, and advancing our understanding of the factors that might mitigate these effects is imperative.

In recent decades, research has explored the efficacy of agricultural best management practices for mitigating and remediating the environmental impacts of insecticides in aquatic ecosystems [4–7]. The primary focus of such work has been to evaluate the efficacy of using various species of emergent vegetation in constructed wetlands and vegetated drainage ditches to reduce the transport of insecticides in runoff from sprayed fields into aquatic ecosystems of economic or ecological importance. This research has demonstrated that best management practices can reduce the environmental transport of some insecticides into adjacent aquatic ecosystems effectively. Still, ecologically relevant concentrations of insecticides are detected frequently in surface waters of aquatic ecosystems located near agricultural lands [8]. In addition, recent surveys have suggested that surface waters located in urban areas can possess similar insecticide loadings as those in agricultural settings [9]. Yet, despite the frequent exposure of non-target aquatic habitats to insecticides, a paucity of information currently exists on the ecological factors contained within these environments that might also mitigate insecticide effects.

Submersed macrophytes are a ubiquitous component of aquatic ecosystems that can achieve high standing biomass and may be able to mitigate insecticide toxicity to aquatic taxa. For example, macrophytes can remove many insecticides from the water column via sorption, potentially reducing the risk of exposure for aquatic animals [10,11]. In fact, evidence suggests that for highly hydrophobic compounds (i.e., log octanol–water partition coefficient [K_{OW}] > 6.0) such as organochlorine (e.g., DDT) and pyrethroid (e.g., lambda cyhalothrin) insecticides, some submersed macrophyte species can sorb 80% or more of the compounds within 1 d [12,13]. Sorption of less hydrophobic compounds by macrophytes, however, is much slower. For example, Gao et al. [14] reported that more than 70% of the malathion ($\log K_{OW} = 2.3$) concentration applied remained after 1 d in the presence of submerged macrophytes, and only 20% of the total concentration applied was extractable from plants after 8 d. Nevertheless, Gao et al. [14] still found that malathion dissipated from the water column faster in the presence of macrophytes than in the absence of plants or in the presence of autoclaved (dead) macrophytes. Thus, a necessary next step is to examine whether and to what extent macrophytes can actually influence the toxicity of relatively hydrophilic insecticides such as malathion.

The degree to which macrophytes actually influence the toxic effects of insecticides in aquatic communities is poorly understood. Recently, Brogan and Relyea [15] examined the mitigating influence of submersed macrophytes on malathion's toxicity to the aquatic zooplankter, *Daphnia magna*. The authors discovered that malathion was up to 9 times less toxic in the presence of realistic densities of the common macrophyte *Elodea canadensis* than in the absence of macrophytes (median lethal concentration in absence of macrophytes [$LC50_{no\text{-}macrophytes}$] = 2.8 μg/L). Furthermore, they found that *E. canadensis*

* Address correspondence to wbrogan23@gmail.com.
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dramatically increased the rate at which malathion's toxicity decreased in the water column relative to environments containing no plants. In fact, at the highest macrophyte densities tested, the toxicity of water that had been dosed with approximately 30 μg/L of malathion to *D. magna* returned to control levels (nontoxic) within 2 h after the insecticide had been applied. Although the study by Brogan and Relyea [15] demonstrated that *E. canadensis* can mitigate malathion's effects on sensitive aquatic species, it was not designed to elucidate the mechanism driving this effect. One way to begin narrowing down a mechanism is to test the mitigating effects of other macrophyte species that vary in their influence on the persistence of insecticides in the water column.

Some evidence suggests that macrophyte species may differ in the rates at which they remove insecticides from the water column. Such evidence could lead to important differences in mitigating insecticides between macrophyte species. For example, Gao et al. [14] showed that nearly 50% of the malathion concentration applied to the test systems was extractable from tissues of the submersed macrophyte *Myriophyllum aquaticum* 8 d after application, whereas less than 25% was bound by *E. canadensis*. In addition, Crum et al. [10] demonstrated that the submersed macrophytes *Chara globularis* and *Elodea nuttallii* as well as the floating macrophyte *Lemna gibba* differed in the rate at which they sorbed different insecticides (i.e., chlorpyrifos, coumaphos, and diazinon) from the water by as much as 630%, although the relative sorption rates of the macrophyte species depended strongly on the insecticide. Currently, the mechanisms driving species-specific differences in macrophyte effects on aqueous insecticide concentrations are poorly understood but likely include differences in organic matter content [10] and the molecular machinery involved in binding, transporting, and degrading pesticide molecules [14]. Before attempting to elucidate the mechanisms driving species-level differences in insecticide uptake, however, the critical next step is to determine whether these differences are even biologically relevant by asking whether plant species differ in the degree to which they affect insecticide toxicity to sensitive species.

The goal of the present study was to determine whether and to what extent several globally abundant macrophyte species differ in their ability to mitigate malathion's toxicity to aquatic taxa. Malathion is an organophosphate insecticide that kills animals by irreversibly binding and inhibiting the function of acetylcholinesterase enzymes. It is considered highly toxic to aquatic insects and many other invertebrates. Recent market reports identify malathion as one of the most commonly applied organophosphate insecticides in the United States, 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 [16]. However, despite its toxicity and popularity, ecotoxicological experiments examining malathion's effects on aquatic taxa under semi-natural and natural conditions are relatively rare.

The present study tested whether macrophyte species differ in the degree to which they mitigate the toxicity of multiple malathion concentrations to animals, as well as in the rate at which they reduce the toxicity of water that has been exposed to malathion. We also examined the mitigating effects of 2 different inert substrates to determine whether insecticide mitigation occurs merely as a result of the added surface area the presence of plants provides. The null hypotheses we tested were 1) all macrophyte species will reduce malathion's toxicity by the same amount relative to environments containing no macrophytes, 2) all macrophyte species will reduce the toxicity of water treated with malathion at equal rates, and 3) environments containing inert substrates will not mitigate malathion's toxicity relative to environments containing no macrophytes.

MATERIALS AND METHODS

Experimental design

To examine the abilities of different submersed macrophyte species to mitigate the toxic effects of insecticides, we conducted an experiment at the University of Pittsburgh's Pymatuning Laboratory of Ecology in Pennsylvania, USA in July 2011. We compared the survival of the cladoceran *D. magna* exposed to a complete factorial cross of 3 nominal concentrations of the insecticide malathion (0 μg/L, 2.5 μg/L, 25 μg/L) in each of 7 macrophyte treatments (no macrophytes, plastic plants, polypropylene rope, *E. canadensis*, *M. spicatum*, *Ceratophyllum demersum*, and *Vallisneria americana*). Each of the 21 treatment combinations was replicated 4 times for a total of 84 experimental units.

We chose the 4 submersed macrophyte species for this experiment because they are all locally abundant throughout northwestern Pennsylvania and they represent both highly dissected (*M. spicatum* and *C. demersum*) and broadleaf (*E. canadensis* and *V. americana*) growth forms. Although no literature currently leads us to predict differences in insecticide uptake or mitigation ability among macrophyte growth forms, plants with highly dissected leaves possess higher surface area per unit mass. This may increase sorption rates if sorption is the underlying mechanism of mitigation. All macrophyte species were collected from field sites during 15 June to 17 June 2011 (Table 1). Although the Geneva Marsh and Crystal Lake sites (PA, USA) have had no direct exposure to insecticides in the past 5 yr (J. Bish, Pennsylvania Game Commission, Hartstown, PA, USA personal communication), it is possible that incidental insecticide exposure has occurred in the Pymatuning or Conneaut Lakes (PA, USA) as a result of their proximity to agriculture. After collection, all macrophytes were washed under running tap water to remove attached invertebrates and epiphytic algae. Each species was then planted in a separate 1200 L cattle tank containing well water and terrestrial topsoil as a rooting substrate and nutrient source. Mesh lids designed to block 60% of solar irradiance were placed over each cattle tank to reduce water temperature and to prevent colonization by invertebrates. We kept the macrophytes in the cattle tanks until they were harvested for the experiment on 20 June 2011.

The malathion concentrations that we selected for the present study span the range of likely exposure scenarios for species inhabiting surface waters in the US. Though malathion application data for urban and industrial sectors in the US are sparse, the US Environmental Protection Agency (USEPA) has recently calculated the estimated environmental concentrations for this insecticide in California surface waters based on input from agricultural sources [17]. Models generated using data, including typical application amounts, frequencies (every 2–14 d), and expected drift patterns for more than 50 terrestrial crops, reveal surface water estimated environmental concentrations for malathion range between 0 μg/L and 36 μg/L (mean = 9 μg/L). In addition, malathion's use in insect-pest eradication programs can produce average surface water concentrations of 50 μg/L after spraying events [18]. If we assume these data are representative of exposure scenarios in other states where similar data are currently unavailable, the concentrations we chose are well within realistic exposure scenarios.

Table 1. Collection sites of four submersed macrophyte species tested for their ability to mitigate malathion's toxicity to *Daphnia magna*

Collection site	GPS coordinates	Species collected
Geneva Marsh	41°35′19.12″N, 80°14′40.61″W	*Elodea canadensis* *Ceratophyllum demersum*
Pymatuning Lake	41°37′18.11″N, 80°32′9.94″W	*C. demersum* *Myriophyllum spicatum*
Crystal Lake	41°33′13.6″N, 80°22′9.26″W	*M. spicatum*
Conneaut Lake	41°36′13.88″N, 80°17′58.36″W	*Vallisneria americana*

We used *D. magna* as the test species in the present experiment in part because of its widespread use in toxicological testing. However, daphnids are also considered critical herbivores in aquatic food webs, because they provide a key link between primary producers, planktivorous predators, and water quality [19,20]. The *D. magna* used in the present experiment were drawn from a mixture of 18 genetically distinct clones originating from Katholieke Universiteit Leuven, Belgium. We used a mixture of genetically distinct lineages to increase the genetic variability among the animals selected. Furthermore, by using laboratory-reared clones, we ensured that the test animals had not been exposed to contaminants for dozens of generations prior to the present study. The *D. magna* populations were housed in 500 mL glass jars containing 300 mL of UV-filtered well water, and the populations were culled during water changes that occurred every 2 wk. We added 1 mL of concentrated *Scenedesmus spp*. algae grown in high-phosphorus COMBO medium to each jar every other day. Although *D. magna* neonates (i.e., <24 h old) are typically used for toxicological testing [21,22], coordinating reproduction to achieve the large number of *D. magna* needed for the present study (∼3500 animals) prevented our use of neonates. Instead, we used intermediate-sized individuals (∼instars 3–6) that had not yet started to produce eggs.

Toxicity test setup

We performed the experiment in outdoor 0.95-L glass jars containing well water and loamy sediment. On 20 July, we removed all coarse organic debris from loamy terrestrial topsoil (collected on site) and added 100 g of soil to each jar. We then added 700 mL of UV-filtered well water, which had been allowed to sit in an open container for 48 h, to each jar. We allowed the jars to sit overnight to allow the suspended sediment to settle. The following day, we selected shoots of each macrophyte species from culture pools along with inert substrates to include in the experiment. For *E. canadensis* and *C. demersum*, which form minimal or no root structures, we cut each shoot 15 cm below the shoot apex and added approximately 5.7 g fresh weight to each appropriate jar. For *M. spicatum* and *V. americana*, which form more extensive root systems, we clipped the shoots 15 cm above the sediment. In addition, we clipped the roots and any stolons down to 1 cm. We then weighed out 5.7 g fresh weight of each macrophyte species and added the macrophytes to randomly assigned jars.

We ensured that the basal end of each macrophyte made contact with the sediment by combining all shoots destined for each jar into a single bouquet. We then gently screwed a stainless steel hexagonal nut around the base of each bouquet to anchor it to the sediment of each jar. We also attached a nut to the artificial plants (plastic and rope), and placed a stainless steel nut in each jar containing no plants. After the experiment, the macrophytes were removed, dried at 65 °C for 24 h, and then weighed to determine dry weight biomass densities. The mean (± standard error) dry weight for each species inside of each jar was as follows: *E. canadensis* = 0.54 ± 0.03 g, *M. spicatum* = 0.55 ± 0.01 g, *C. demersum* = 0.49 ± 0.01 g, and *V. americana* = 0.54 ± 0.05 g. Because observed dry biomass densities for submersed macrophytes typically range from 0.05 g/L to 0.8 g/L [23], the densities used in the present experiment fall well within this range.

A major goal of the present study was to observe how macrophytes influence the toxicity of malathion under abiotic conditions that macrophytes and *D. magna* would experience in nature. Thus, after adding macrophytes, we moved all jars outside and placed them inside glass aquaria that were positioned on their sides in 300 L pools positioned on wooden tables. We randomly assigned each jar to an aquarium and placed 7 jars into each of 12 aquaria dispersed throughout 4 pools (Figure 1). Using this design allowed us to expose the test systems to natural fluctuations in temperature and light, while preventing rain from entering the testing chambers and diluting the insecticide concentrations. After the jars were in place, we added approximately 100 L of cool well water to each pool (approximately one-half of the height of a jar) to buffer against unnaturally rapid temperature fluctuations. We quantified the abiotic environment in each jar by recording pH, temperature (digital pH meter, Oakton Instruments), and dissolved oxygen (digital oxygen meter; WTW) 1 h before applying malathion, as well as pH and dissolved oxygen 48 h after applying malathion.

We allowed the macrophytes to acclimate to the testing conditions for 4 d prior to applying insecticides. During this time, we visually inspected the plants and observed no changes in coloration or decay of leaves or shoots.

Malathion application

On 26 July 2011, we applied technical grade (99.1%) malathion (Chem Service) to each jar. To achieve nominal concentrations of 0 μg/L, 2.5 μg/L, and 25.0 μg/L, we added 0 mL, 0.457 mL, and 4.573 mL, respectively, of stock solution (0.123 mg/mL malathion dissolved in EtOH carrier) to 1.5 L of UV-filtered water to create our working solutions. We used a separate container for each working solution. After mixing each for approximately 30 s, we added 50 mL into each appropriate jar to bring the total volume in each jar to 750 mL. During dosing, we slowly poured control and treated water into each jar

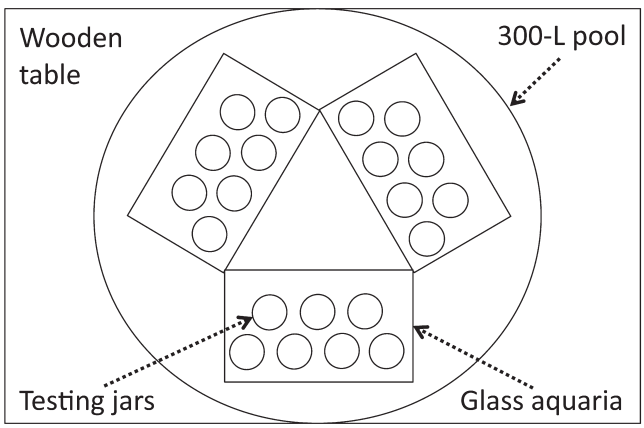

Figure 1. Experimental setup consisting of glass jars positioned inside sideways-oriented aquaria. All aquaria were placed in plastic 300-L pools filled with approximately 100 L of well water. See *Methods* for complete details.

to ensure thorough mixing inside each container without disturbing the sediment. We began applying malathion, starting with insecticide-free controls, at 10:00 h, and worked up to the 25 μg/L treatments, finishing at 11:00 h. Although we did not perform an ethanol control in the present experiment, other experiments have demonstrated that ethanol concentrations (0.5 mL ethanol/L water) twice as high as those used in the present study (0.203 mL ethanol/L water) had no adverse effects on *D. magna* survival [24].

To determine the actual malathion concentrations achieved for each treatment, we applied 50 mL of each working solution (using the same solution described previously) to 2 separate glass jars containing 700 mL UV-filtered water using identical application techniques as we used for the experimental containers. We then took 450 mL of water from each of these jars and transferred it to 2 separate 500 mL, pre-cleaned amber glass jars (VWR International). We stored the jars in a 3 °C refrigerator until analysis. All samples were sent to an independent laboratory for analysis using gas chromatography–mass spectronomy (University of Georgia Agricultural and Environmental Services Laboratory, Athens, GA, USA) within 1 wk of being collected. The actual malathion concentrations corresponding to the nominal concentrations of 0 μg/L, 2.5 μg/L, and 25 μg/L were 0 μg/L, 3.3 μg/L, and 23.7 μg/L (hereafter referred to as 3 μg/L and 24 μg/L), respectively.

Determining macrophyte effects on the reduction of malathion's toxicity

After applying each insecticide treatment, we added 10 *D. magna* individuals to each jar in the same order that the jars were dosed. Thus, *D. magna* were added to each jar approximately 20 min after it had been treated with the appropriate malathion concentration. After all *D. magna* were added, and each day thereafter, we added 1 mL of high-phosphorus *Scenedesmus* algae raised in COMBO medium [25] to each jar to serve as food. After 48 h, we removed the macrophytes from each jar, gently shaking them in a separate container of well water to ensure that no *D. magna* had been removed while removing the macrophytes. We then quantified the number of surviving *D. magna* individuals in each jar using a protocol modified slightly from the Organization for Economic Co-operation and Development standardized testing guidelines [26]. Specifically, we applied a gentle burst of water over immobile individuals with a transfer pipette. We considered an individual to have survived if it began to swim vertically in the water column within 3 applications of this stimulus. Thus, whereas most non-survivors were clearly dead (no movement and faded color), any individuals still twitching but unable to swim in response to the stimulus were also considered dead.

Comparing macrophyte effects on the rate at which malathion's toxicity is reduced

In addition to comparing the amount that different macrophyte species reduced malathion's toxicity, we also compared the rate that different macrophyte species reduced the toxicity of water treated with malathion. To do this, we used a glass pipette to remove 25 mL of water from each jar at 2 h, 8 h, 24 h, and 48 h following insecticide treatment applications to test the toxicity of this water to *D. magna*. We removed the water samples in the same order that the jars had been dosed so that the duration between insecticide application and water collection was equal for each jar. We then transferred the water collected from each jar into a 50 mL glass vial and immediately added 10 *D. magna* to each vial. The vials were brought indoors, where we quantified *D. magna* survival (using the criteria described previously) 48 h after they had been added to each vial. During this 48-h exposure period, we fed the *D. magna* in each vial 0.25 mL of high-phosphorus *Scenedesmus* algae daily. Thus, survival data for this phase of the experiment represented the number of surviving individuals 48 h after being exposed to water collected from each jar at each timepoint that we extracted the water from the original jars.

When selecting *D. magna* individuals to be exposed to water collected from the outdoor jars 24 h after malathion application, we tried pouring the animals through a metal sieve, which appeared to affect the animals' survival. Although we saw no evidence that the animals included in this group were unhealthy as we were adding them to the testing vials, 48-h survival in the controls for this group was 58%, whereas animals in the groups exposed to control water collected after 2 h, 8 h, and 48 h always exhibited >90% survival. We also observed higher within-treatment variation in *D. magna* survival in the animals tested at 24 h. Therefore, we decided to omit the data for the 24-h timepoint from our analyses.

Statistical analysis

To compare the amount that each macrophyte treatment mitigated malathion's toxicity, we compared the effects of different malathion concentrations on *D. magna* survival across macrophyte treatments. To do so, we first performed analysis of variance (ANOVA) on *D. magna* survival 48-h following malathion exposure. The full-factorial model included macrophyte treatment, malathion concentration, and their interaction as sources of variation. Due to unequal variances, we rank-transformed the survival data before analysis. When significant effects of the treatment interaction were detected, we used Games-Howell multiple comparison tests to examine the effects of increasing malathion concentrations on ranked *D. magna* survival within each macrophyte treatment.

To compare the rate of malathion removal from the water column in the presence of the different macrophyte species, the inert-substrate controls, and the no-macrophyte treatment, we used Dunnett's test. Specifically, we measured 48-h *D. magna* survival after exposure to water collected at 2 h, 8 h, and 48 h following insecticide application. We also compared ranked survival of animals exposed to water treated with 3 μg/L and 24 μg/L of malathion to survival in the controls at each timepoint. This allowed us to compare the time that it took the toxicity of the water to return to control levels within each macrophyte treatment.

We determined the effect of the different macrophyte treatments on aqueous pH, dissolved oxygen, and temperature 1 h before applying malathion using a multivariate analysis of variance (MANOVA). We also quantified pH and dissolved oxygen 48 h after applying malathion. We again analyzed the data using a MANOVA, but we included malathion concentration and the macrophyte-by-malathion concentration interaction in the model to account for any effects of these sources of variation. Where appropriate, we used ANOVAs to examine treatment effects on each response variable and Tukey's multiple comparisons tests to determine differences between treatments.

RESULTS

Effects of macrophyte treatments on reduction of malathion's toxicity

In the outdoor jars, the 48 h survival of *D. magna* was affected by macrophyte treatment ($F_{6,63} = 3.6$, $p = 0.004$),

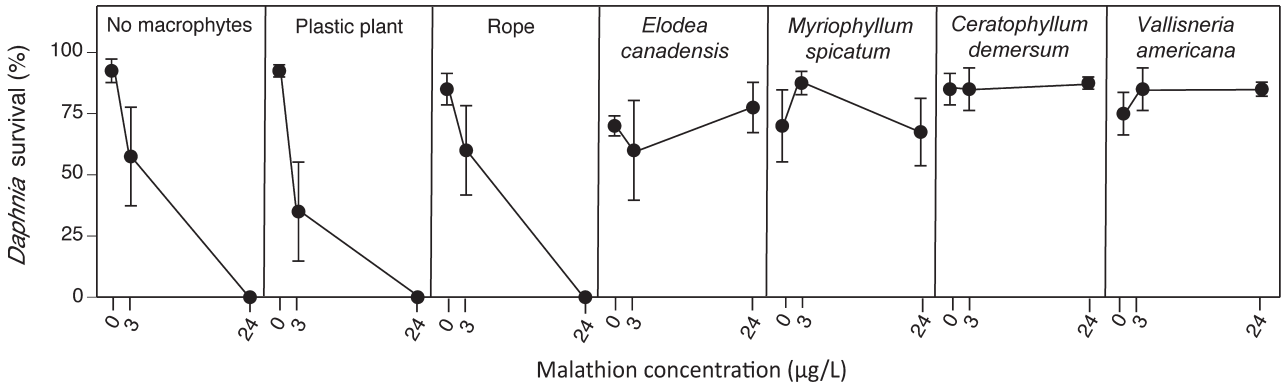

Figure 2. *Daphnia magna* 48-h survival following exposure to 3 malathion concentrations in the presence of each of 7 macrophyte treatments. Data are means ± 1 standard error.

malathion concentration ($F_{2,63} = 10.8$, $p < 0.001$), and their interaction ($F_{12,63} = 3.8$, $p < 0.001$). Due to the significant macrophyte-by-malathion treatment interaction, we compared the ability of each species to mitigate malathion's effects by comparing *D. magna* survival at each malathion concentration. As malathion concentrations increased, we observed significant negative effects on *D. magna* survival in the no-macrophyte, plastic plant, and rope treatments ($F_{2,9} > 8.4$, $p < 0.01$) but no effect of malathion concentration on *D. magna* survival in the presence of *E. canadensis, M. spicatum, C. dermersum*, or *V. americana* (Figure 2; $F_{2,9} < 0.6$, $p > 0.59$). Responses to malathion were similar in the no-macrophyte, plastic plant, and rope treatments, where 24 µg/L of malathion caused significant decreases in *D. magna* survival relative to the 0 and 3 µg/L treatments ($p < 0.02$); the latter 2 treatments did not differ ($p > 0.094$). We also examined *D. magna* survival across the 7 macrophyte treatments in the 0 µg/L malathion treatments and found no significant differences ($F_{6,21} = 1.435$, $p = 0.248$). This demonstrates that although some of the macrophytes were collected from sites that might have encountered incidental prior exposure to pesticides, the plants themselves had no significant negative impact on *D. magna* survival.

Effects of macrophyte treatments on the rate at which malathion's toxicity is reduced

We discovered that the rate at which malathion's toxicity decreased in the water column was substantially faster in the presence of any of the 4 live macrophyte species than in the no-macrophyte or inert-substrate treatments. For example, in the no-macrophyte, plastic plant, and rope treatments, Dunnett's test revealed significantly reduced *D. magna* survival following exposure to water collected 2 and 8 h following applications of 3 and 24 µg/L of malathion (Figure 3; $p < 0.012$). Furthermore, in the no-macrophyte and plastic-plant treatments, water treated with 24 µg/L of malathion was still toxic to *D. magna* 48 h after the insecticide had been applied ($p < 0.034$). In treatments containing any of the 4 living macrophyte species however, water receiving 3 µg/L or 24 µg/L of malathion was nontoxic to *D. magna* within 2 h following applications of the insecticide ($p > 0.149$). Though survival of *D. magna* exposed to water collected just 2 h after applications of 24 µg/L of malathion in the presence of *V. americana* was only approximately 40%, Dunnett's test revealed no difference from survival in the controls ($p = 0.110$). In addition, in the presence of *C. demersum*, survival of *D. magna* following exposure to water collected 48 h after

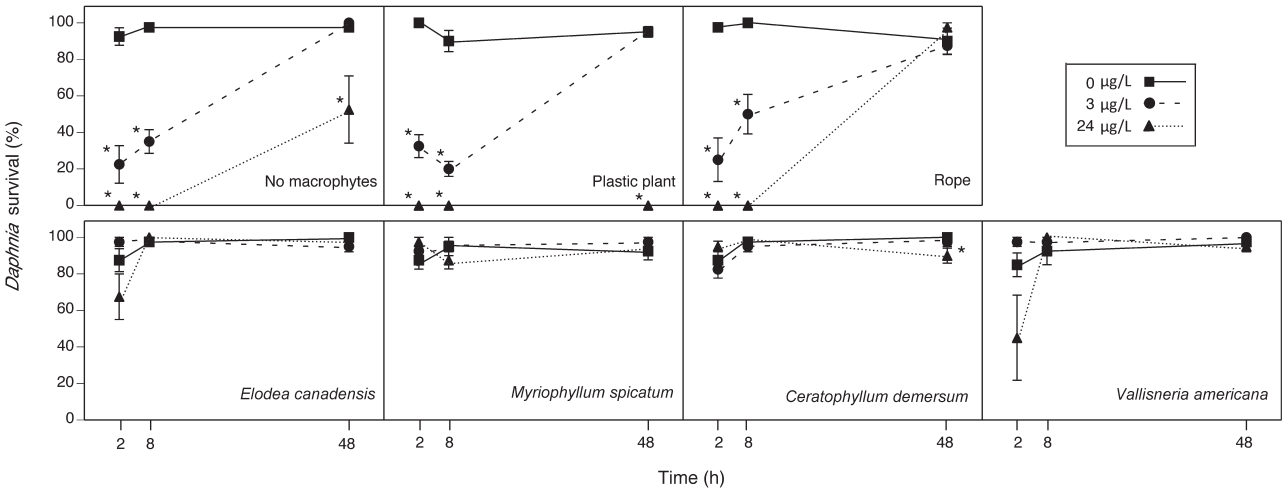

Figure 3. The effects of macrophyte treatment on the toxicity of water collected 2 h, 8 h, or 48 h after malathion applications of 0 µg/L, 3 µg/L, or 24 µg/L. For treatments that had received insecticides, water toxicity was assessed at each sampling time and within each macrophyte treatment by comparing *Daphnia magna* 48-h survival to the controls. Asterisks indicate insecticide treatments in which *D. magna* survival was significantly lower than in insecticide-free controls. Data are means ± 1 standard error.

malathion applications reduced *D. magna* survival by a small (<10%) but statistically significant amount compared with controls ($p = 0.023$). This was the case even though no differences in survival were observed following exposure to water collected at 2 h and 8 h.

Effects of macrophyte treatments on the abiotic environment

Both before and after malathion applications, we found significant multivariate effects of macrophyte treatment on pH and dissolved oxygen (Wilk's λ, $F_{12,124} > 66.3$, $p < 0.001$). The multivariate effect of macrophytes were driven by univariate effects of pH ($F_{6,63} > 224.1$, $p < 0.001$) and dissolved oxygen ($F_{6,77} > 6.5$, $p < 0.001$). Compared to the no-macrophyte treatment, pH samples collected at either timepoint did not differ in the rope treatment ($p = 0.651$); were 5% to 7% lower in the plastic-plant treatment ($p < 0.001$); and were 11% to 27% higher in treatments containing any of the 4 macrophyte species (Figure 4; $p < 0.001$). At the first sample time (1 h prior to dosing), dissolved oxygen levels in the no-macrophyte treatment did not differ from the plastic plant, rope, *E. canadensis*, *C. demersum*, or *V. americana* treatments ($p > 0.196$). However, dissolved oxygen in the presence of *M. spicatum* was at least 8% higher than all other macrophyte treatments and was 25% higher than the no-macrophyte treatment ($p < 0.003$). In the sample collected 48 h after dosing, dissolved oxygen levels in the no-macrophyte treatment did not differ from the rope or *V. americana* treatments ($p > 0.99$). They were, however, 27% higher in the presence of plastic plants ($p < 0.001$) and 13% to 26% lower than in the presence of *E. canadensis, C. demersum*, and *M. spicatum* ($p < 0.001$). The average temperature in the jars prior to adding malathion was 30.5 °C (range = 27.3–32.8 °C) and was not influenced by any treatments ($F_{6,77} = 1.163$, $p = 0.335$).

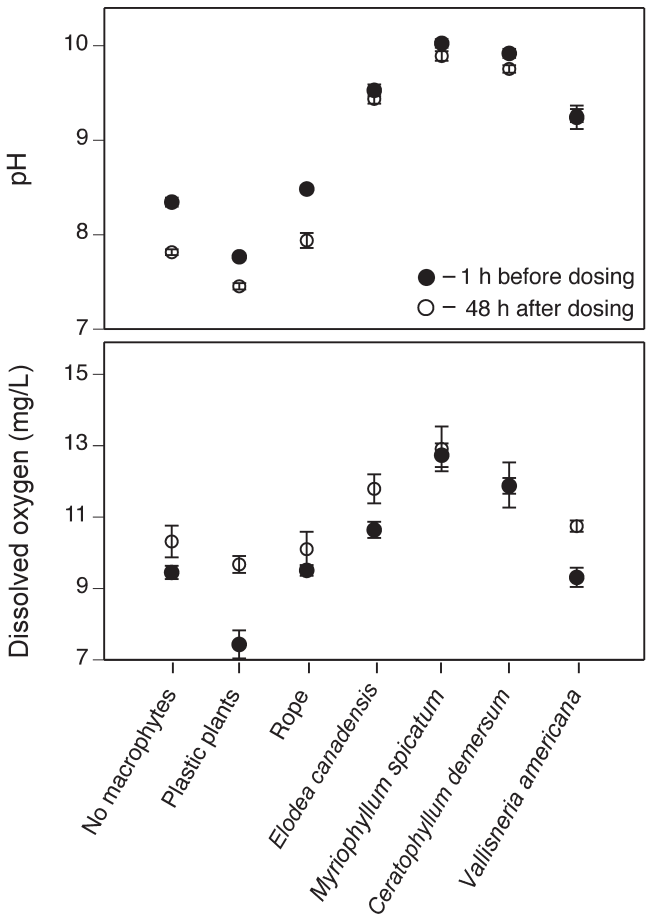

Figure 4. The effects of macrophyte treatment on pH and dissolved oxygen in 1-L jars 1 h before and 48 h after malathion application. Data are means ± 1 standard error.

The multivariate effect of malathion concentration (Wilk's λ, $F_{4,124} = 25.4$, $p < 0.001$) was also driven by significant univariate effects of pH ($F_{2,63} = 12.2$, $p < 0.001$) and dissolved oxygen ($F_{2,63} = 27.2$, $p < 0.001$). Whereas pH did not differ between treatments exposed to 0 μg/L and 3 μg/L of malathion ($p = 0.713$), concentrations of 24 μg/L increased pH levels by approximately 3% compared with the controls ($p < 0.001$). Malathion's effect on dissolved oxygen occurred because water exposed to 0 μg/L of malathion had approximately 7% greater dissolved oxygen levels than water dosed with 3 μg/L ($p < 0.001$), and nearly 13% higher dissolved oxygen than 24 μg/L malathion treatments ($p = 0.025$).

DISCUSSION

In the present study, we tested the amount and rate at which 4 species of submersed macrophytes (*E. canadensis, M. spicatum*, *C. demersum*, and *V. americana*) and 2 inert substrates (plastic plants and polypropylene rope) mitigated the toxic effects of a common insecticide (malathion) on the aquatic herbivore *D. magna*. We discovered that all 4 macrophyte species strongly and equally mitigated the toxicity of malathion to *D. magna*, whereas the inert substrates had no mitigating effect. For example, exposure to 24 μg/L of malathion left no *D. magna* survivors in the absence of macrophytes or in either inert substrate treatment. This same concentration had no effect, however, on *D. magna* survival in the presence of *E. canadensis*, *M. spicatum*, *C. demersum*, or *V. americana* relative to insecticide-free controls. As a result, the data support our first hypothesis that all 4 macrophyte species can mitigate the amount of malathion's toxicity to a similar degree.

The present experiment demonstrated that the presence of each macrophyte species tested prevented any *D. magna* mortality from occurring even after exposure to malathion concentrations that were more than 13 times higher than typical LC50 values for *D. magna* (e.g., LC50 = 1.8 μg/L) [27]. In contrast, the effects of malathion on *D. magna* survival we observed in the no-macrophyte, plastic-plant, and rope treatments of the present experiment were similar to documented effects reported in studies employing more traditional toxicological experimental designs [28]. Although creating environments conducive to maintaining healthy submersed macrophyte populations in the present study made it impractical for us to adhere strictly to traditional toxicity testing guidelines using *D. magna* (e.g., USEPA, Organization for Educational Technology and Curriculum, American Society for Testing and Materials, etc.), the similarities between our results and others employing standardized protocols provides external validity that our testing methodology did not strongly influence malathion's toxicity to this species.

Whereas the present study demonstrates strong mitigating effects of submersed macrophytes on zooplankton exposed to insecticides, other studies comparing insecticide toxicity to zooplankton in the presence and absence of macrophytes have found mixed evidence of mitigation. For example, the results from the present study are highly consistent with previous work demonstrating that different densities of *E. canadensis* can strongly mitigate the effects of malathion on *D. magna* in a biomass-dependent manner [15]. Brock et al. [29] also found

some evidence of insecticide mitigation by macrophytes when they contrasted the effects of 35 μg/L of another organophosphate insecticide, chlorpyrifos, between macrophyte-dominated and phytoplankton-dominated communities using indoor 850 L experimental units. They observed that cladocerans were eliminated within hours in the phytoplankton-dominated system, while it took approximately 2 wks for comparable mortality to occur in macrophyte-dominated systems. While the findings of Brock et al. [29] suggest that macrophytes might have had a mitigating effect on zooplankton assemblages, the results of the present study must be interpreted with caution, because the responses of the macrophyte- and phytoplankton-dominated systems that were exposed to insecticides were examined in separate years (1988 and 1989, respectively); thus, they might have had different community compositions prior to dosing.

In another study, Roessink et al. [30] compared the effects of a range of concentrations (10–250 ng/L) of the pyrethroid insecticide lambda-cyhalothrin in outdoor 500-L macrophyte- and phytoplankton-dominated mesocosms. Although they did not observe any clear mitigating effects of submersed macrophytes on zooplankton assemblages, they did observe stronger indirect effects of the insecticide in phytoplankton-dominated microcosms than in macrophyte-dominated microcosms. However, the phytoplankton- and macrophyte-dominated systems used in their study differed in numerous confounding factors, including initial species composition and nutrient environment. Thus, it is impossible to determine the influence that insecticides had relative to the effects of different ecological interactions in phytoplankton- versus macrophyte-dominated systems in studies by Brock et al. [29] and Roessink et al. [30]. Clearly, more studies designed to examine the influence that macrophytes might have on the ecological effects of different insecticides in more complex communities are needed.

Another important discovery in the present study was that all 4 macrophyte species expedited the rate at which malathion's toxicity decreased in the water column relative to treatments containing no macrophytes, plastic plants, or rope. For example, in the no-macrophyte, plastic-plant, and rope treatments, water treated with either 3 μg/L or 24 μg/L of malathion was still significantly toxic to *D. magna* 8 h after the insecticide had been applied. In fact, in the no-macrophyte and plastic plant treatment, 24 μg/L of malathion was still toxic to *D. magna* 48 h after the application. It is unclear why the toxicity of the water treated with 24 μg/L of malathion was not toxic to *D. magna* after 48 h in the presence of rope, given that the rope treatment showed nearly identical patterns to the no-macrophyte and plastic-plant treatments in all other endpoints measured. Regardless, the difference between live macrophyte and control treatments was clear. In the presence of each of the 4 macrophyte species, water collected just 2 h following the application of any of the tested malathion concentrations was no longer toxic to *D. magna*. This evidence supports our second hypothesis that macrophyte species will reduce the toxicity of water treated with malathion at equal rates and more quickly than in the absence of macrophytes.

Finally, we also found support for our third hypothesis that insecticide mitigation by macrophytes is not merely an artifact of the added surface area that results from the presence of plants. We demonstrated this by showing that 2 types of inert substrates—which approximated the morphology of submersed macrophytes (plastic plants) or possessed very high surface area (rope)—did not cause any decrease in malathion's toxicity to *D. magna* or in the rate at which malathion was removed from the water column relative to treatments containing no macrophytes. These results are consistent with previous studies showing that autoclaved (dead) macrophytes with no living epiflora removed negligible amounts ($< 10\%$) of malathion over a period of 8 d, whereas living, submersed plants removed approximately 80% of malathion over this same interval [14]. Taken together, all of the evidence from the present study suggests that aquatic plants must be alive to mitigate malathion's toxicity.

Of course, living macrophytes host a diverse epiphytic floral community. While our rinsing procedure appeared to remove nearly all epiphytic algae from the macrophytes we used in our experiment, it is possible that the epiphytic bacterial and algal communities may have contributed to the mitigation of malathion's effects that we observed in the present study. However, bacteria collected from natural waters degrade malathion relatively slowly, compared with the rates that we indirectly observed in our experiment (half-life $\approx$ 32 h with 5.0×10^8 colony forming units) [31]. In fact, Mohamed et al. [32] selected a bacterial strain (*Bacillus thuringiensis*) specifically for its ability to degrade malathion in wastewater treatment, yet it took approximately 3 d for 7.87×10^{11} colony forming units per milliliter to reduce aqueous malathion concentrations by half. Given the relatively slow degradation rates of malathion by bacteria and the likely low biomass of algae present on the plants in the present study, we attribute the mitigation we observed primarily to the effects of macrophytes.

Several mechanisms can help explain the faster reduction of malathion's toxicity by living macrophytes relative to the no-macrophyte and inert substrate treatments. One possibility is that macrophytes could be rapidly sorbing malathion onto their tissues and thus reducing the toxicity of the water column to *D. magna*. Gao et al. [14] investigated the rates at which malathion concentrations decreased in a liquid culture medium containing either 2 submersed macrophytes (*E. canadensis*, *Myriophyllum aquaticum*) or the floating macrophyte *Lemna minor*. They found that after 48 h, measured malathion concentrations in the water column had decreased by only 40% and 15% in the presence of *M. aquaticum* and *E. canadensis*, respectively. Relating this to the present study, these results suggest that if the live macrophytes we tested were sorbing malathion at similar rates to those Gao et al. [14] observed, the malathion concentration 48 h following applications of 24 μg/L should still have been approximately 14 μg/L, which remains enough to cause substantial *D. magna* mortality. Yet, in the presence of live macrophytes, we observed high *D. magna* survival following malathion applications of 24 μg/L, where the animals were exposed for the entire 48 h duration of the experiment and when they were exposed to water collected just 2 h after dosing. Based on this evidence, it is unlikely that sorption was the sole mechanism by which the living macrophytes mitigated malathion's toxicity to *D. magna*.

Another possible mechanism to explain the ability of macrophytes to mitigate insecticide effects is the increase in water pH associated with the presence of live plants. During photosynthesis, macrophytes remove and retain dissolved carbon dioxide while adding oxygen to the water, both of which increase aqueous pH [33]. Although the size of our testing containers required us to prune the macrophyte roots and shoots in this experiment, the high pH and dissolved oxygen concentrations we observed in the presence, but not in the absence, of the live plants demonstrated healthy photosynthetic activity. Macrophyte effects on pH are potentially very important because malathion's half-life decreases rapidly with

increases in pH [34]. For example, in water with pH = 8 (i.e., the no-macrophyte, plastic-plant, and rope treatments of our experiment), malathion's half-life is approximately 8 h at the temperatures recorded in the present study (~30 °C). However, with each unit increase in pH (i.e., from pH = 8 to pH = 9), malathion's half-life is predicted to decrease by an order of magnitude. Thus, in water with pH greater than 9 (i.e., the live macrophyte treatments), malathion's half-life would likely be substantially less than 1 h and might be on the order of only a few minutes. Of course, it is possible that macrophytes mitigated malathion's effects via a combination of sorption and through their effects on water pH. The present experiment was not designed to tease apart these mechanisms, but an important next step will be to determine the relative importance of macrophyte sorption versus macrophyte effects on pH in mitigating insecticides such as malathion.

CONCLUSIONS

In the present study, we discovered that 4 different macrophyte species exhibited equal mitigating effects on malathion's toxicity to the sensitive aquatic species *D. magna*. Furthermore, we demonstrated that mitigation does not occur in the presence of 2 separate inert substrates. These results advance our current understanding of the influence that submersed macrophytes have on the toxicity of insecticides that until recently have been extrapolated largely from studies examining the sorption of insecticides from the water column by macrophytes. Whereas the mechanisms underlying the mitigating effects that we observed remain unclear, the literature suggests that sorption by macrophytes and the effects of macrophytes on water pH may be playing a critical role. The results of the present study suggest that incorporating submersed macrophytes into agricultural best management practices, which almost exclusively employ emergent macrophytes, could provide a highly effective alternative to reducing the insecticide loads contained in runoff. Furthermore, our results indicate that management strategies seeking to remove submersed macrophytes to improve the aesthetic quality or recreational functionality of water bodies (e.g., lakes, reservoirs, golf courses, etc.) could unintentionally decrease the resistance and resilience of these aquatic environments to common contaminants.

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