SAFE AND SUBMERGED: HOW ECOLOGICAL EFFECTS OF AQUATIC PLANTS MITIGATE INSECTICIDE IMPACTS IN FRESHWATER COMMUNITIES

by

William Robert Brogan III

Bachelor of Science, Ithaca College, 2007

Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

KENNETH P. DIETRICH SCHOOL OF ARTS AND SCIENCES

This dissertation was presented

by

William Robert Brogan III

It was defended on

February 27, 2014

and approved by

Dr. Walt Carson, Associate Professor, Dept. of Biological Sciences, University of Pittsburgh

Dr. Susan Kalisz, Professor, Dept. of Biological Sciences, University of Pittsburgh

Dr. Brian Traw, Assistant Professor, Dept. of Biological Sciences, University of Pittsburgh

Dr. Katia Engelhardt, Research Associate Professor, University of Maryland Center for Environmental Sciences, Appalachian Laboratory

Dissertation adviser: Dr. Rick Relyea, Professor, Dept. of Biological Sciences, University of Pittsburgh

SAFE AND SUBMERGED: HOW ECOLOGICAL EFFECTS OF AQUATIC PLANTS MITIGATE INSECTICIDE IMPACTS IN FRESHWATER COMMUNITIES

William R. Brogan III, PhD

University of Pittsburgh, 2014

A major goal in ecology and toxicology is to better predict the environmental impacts of anthropogenic contaminants. A key step towards accomplishing this goal is to understand how ecological interactions can influence both the direct and indirect impacts of contaminants in nature. While many of the factors that exacerbate contaminant impacts have been well studied, ecological factors that can mitigate these effects are relatively poorly understood. In this dissertation, I examine the mitigating influence that submerged plants, a common feature of aquatic ecosystems, have on the impacts of the widely used insecticide malathion in freshwater communities. In chapters one and two, I test the degree to which different realistic submerged plant densities and different plant species, respectively, influence malathion's toxicity to the ecologically important zooplankton species, *Daphnia magna*. I show that each increase in plant density reduced both the amount and duration of malathion's toxicity, and that the ability to mitigate malathion's toxicity is a generalizable phenomenon across submerged plant species. In chapter three, I demonstrate that the mechanism traditionally thought to play the largest role in mitigating insecticide toxicity, sorption to plant tissues, plays virtually no role in the mitigation of malathion. Instead, I present the first evidence that increased water pH caused by plant photosynthesis is the primary mechanism driving the mitigating effects of plants on this insecticide. Finally, in chapter four I test whether plants can mitigate malathion's direct and indirect effects at larger spatial scales and in more ecologically complex communities. I show

that in the absence of plants, realistic malathion exposures decimate sensitive cladoceran zooplankton, initiating trophic cascades that result in sustained phytoplankton blooms. However, in the presence of submerged plants, even at low densities, malathion had no effect on community structure. My research provides the first evidence that submerged plants are capable of mitigating the toxicity of a widely used insecticide at multiple spatial scales and levels of biological organization. My findings can help improve toxicological models designed to predict insecticide effects in aquatic environments and mitigation strategies (e.g., best management practices) for reducing the environmental impacts of insecticides.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

"Not everything that counts can be counted, and not everything that can be counted counts."

- Albert Einstein, PhD

The research that I present in this dissertation is the culmination of many years of hard work. However, it also much more than that. This dissertation is a tangible result of the profound impact that my friends, family, colleagues, and critics have all had on me over the years. There are many people who have helped to shape me not only as a scientist, but as a person. Because it is not possible for me to sufficiently thank everyone who has influenced and supported me over the years, I ask that if you are not mentioned below, please know that I do not forget you. I deeply appreciate the role that you have played in my life and my knowing you has made me the man that I am today. Thank you.

First, I would like to thank my advisor, co-author, and mentor Rick Relyea. Rick perfectly embodies what it is to be a mentor. Sure, he has all of the obvious characteristics: knowledge, experience, and leadership that he has used to teach me how to do interesting, rigorous science. However, it is Rick's qualities that have nothing to do with science that I have found most influential on my professional and personal development. For example, Rick epitomizes what it is to be passionate about work and life. In meetings where he and I would

discuss ongoing studies during the field season, Rick was always downright eager to come to look at the experiment and put his hands into the water to decode what the biological communities were trying to tell us, often repeating "just another day in paradise!" I hope to maintain the same level of passion for my work that I observe in Rick every day. But even Rick's passion for his work pales in comparison to the one trait that I admire about him the most: his calmness. Even in my most volatile moments, when I felt the suffocating panic of an experiment failing or a grant deadline that I was certain I would never meet, Rick always had a way to make me understand that it just wasn't as big a deal as I was making it and that I could handle the challenge. The calmness that Rick displays and transfers to others is something that I deeply hope to master as he has. I believe that this ability is rooted deeply in a mentality that, no matter what, things are going to be okay. Rick has provided me with a model of what a successful mentality looks like and how it translates so effortlessly to happiness in life. That is why Rick's mentorship has extended way beyond my development as a scientist. I am a better person and a better man for having met you, Rick, so thank you.

I also owe an immeasurable debt of gratitude to my lab mates. I will forever have a special kinship with Dr. Jess Hua and Heather Shaffery. Without you by my side as my friends, colleagues, teammates, and occasionally my therapists (perhaps too often), I am certain that I would have given up before the end of my first year. To my senior lab mates Drs. Aaron Stoler, Maya Groner, Rickey Cothran and John Hammond, I thank each of you for always graciously offering your friendship and assistance with every conceivable aspect of my work. You have been some of my strongest supporters and greatest critics. Without a doubt, you have each immensely influenced what it means to me to be a scientist and colleague. To my junior lab mates RJ Bendis and Devin Jones, and the host of undergraduates who I have had the privilege

of working with over the years, I have truly enjoyed watching you develop and I just hope that I have been half as influential to you as my senior colleagues were to me.

There are also many people outside of my lab that I wish to thank. My academic committee Drs. Walt Carson, Susan Kalisz, Brian Traw, and Katia Engelhardt, whose wisdom and guidance have turned my nebulous ideas into the research program contained in these pages. In fact, I must credit Walt Carson with the origin of my entire dissertation, as he was the first to ask me whether aquatic plants might influence the effects of anthropogenic contaminants, a question that has served as the major theme for my entire dissertation and will propel me into my career. I also thank my colleagues George Meindl, Matt Koski, Kate Lecroy, Eric Griffin, Mike Chips, Nathan Brouwer, Alison Hale, Tarek Elnaccash, Marnin Wolfe, and Steven Tonsor for their friendship and input over the years. Finally, I thank the entire departmental administrative staff who have made navigating graduate student so pleasant and easy.

Finally, I dedicate my dissertation to those who have been the greatest source of support and strength for me in my life. To my parents, Barbara and Bob, your unconditional support during even my most tumultuous times has often been the only thing that has gotten me through. I know that I have not always made it easy, but I truly appreciate the sacrifices you have made on my behalf so that I could achieve this accomplishment. To my big brother, Justis, you have been a major source of guidance for me through the years. You have always been there for me and I will forever look up to you and be there for you. Lastly, to my fiancé, Erin, you are the reason that I have the courage to pursue my dreams. I know that no matter what happens, we will get through it by always being there to love each other, make each other laugh, and support one another. I could not have done this without you.

1.0 INTRODUCTION

Over the past century, ecologists and toxicologists have strived to better predict the impacts of environmental perturbations on biological communities. Ecologists have historically employed a deductive approach; developing and refining theoretical models of species interactions to predict biological effects of natural perturbations (Paine 1969, Bender et al. 1984, Novak et al. 2011). In contrast, toxicologists have used a more inductive approach; collecting extensive data on species sensitivities to anthropogenic contaminants, such as pesticides, and then using models to extrapolate the effects to complex communities (Cairns 1986, Newman 2010). While each field has greatly expanded our understanding of environmental perturbation impacts, there is growing appreciation that integrating these disciplines (i.e. ecotoxicology) can further advance our ability to predict pesticide effects in nature (deNoyelles et al. 1994, Fleeger et al. 2003, Rohr et al. 2006, Relyea and Hoverman 2006). This is becoming an increasingly important goal as exposure of non-target ecosystems like aquatic habitats to pesticides is projected to increase for the foreseeable future (Laurence et al. 2001).

One way that incorporating ecological theory into toxicology can improve our ability to predict pesticide effects in nature is by considering the influence of ecological interactions on direct (i.e. lethal and sublethal) pesticide effects to sensitive species. Traditionally, toxicologists have assessed direct pesticide effects by performing single-species tests under standardized laboratory conditions designed to eliminate any sources of environmental variation (Newman

2010). While this approach is necessary for comparing the relative toxicity of large numbers (i.e. > 1,000) of pesticide active ingredients, accumulating evidence suggests that in nature, ecological interaction modifiers (*sensu* Wootton 1994, 2002) can dramatically alter pesticide direct effects to sensitive species (Fig. 1.1). For example, in aquatic communities, insecticide toxicity to sensitive species can increase several-fold in the presence of predators (Hanazato and Dodson 1995, Hanazato 2001, Relyea and Mills 2001, Relyea 2003), competitors (Hanazato 2001, Mills and Semlitsch 2004), and pathogens (Kieseker 2002, Coors and De Meester 2008). Despite the clear influence that ecological interaction modifiers can have on insecticide direct effects, the primary focus to date has been on understanding factors that exacerbate insecticide toxicity, while factors that might mitigate toxicity have received comparatively little attention.

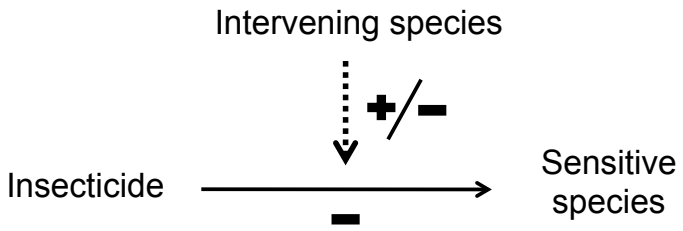

Figure 1.1. Diagram illustrating the positive or negative indirect effect (dashed arrow) of an interaction modifier on insecticide toxicity (solid arrow) to a sensitive species. Adapted from Wootton 1994.

Ecological interactions can also cause indirect contaminant effects in nature that traditional toxicity tests are not designed to predict. For example, it is becoming well established that at environmentally realistic concentrations, insecticides can initiate trophic cascades in aquatic communities (Fig. 1.2; Hanazato & Yasuno 1987, Fairchild et al.1992, Fleeger et al. 2003). Insecticides typically decimate cladoceran zooplankton, a key consumer of phytoplankton

(Larsson and Dodson 1993). This allows phytoplankton to bloom, which shades the water column and can reduce periphyton biomass. As a result, insecticides can indirectly affect the growth and survival of periphyton grazers at concentrations that traditional toxicological tests predict should be harmless (Relyea and Diecks 2008, Relyea and Hoverman 2008). Although indirect effects such as trophic cascades can exacerbate insecticide effects in aquatic communities, there is a paucity of literature examining factors that may dampen the magnitude of these cascading effects, despite the key implications for basic and applied science.

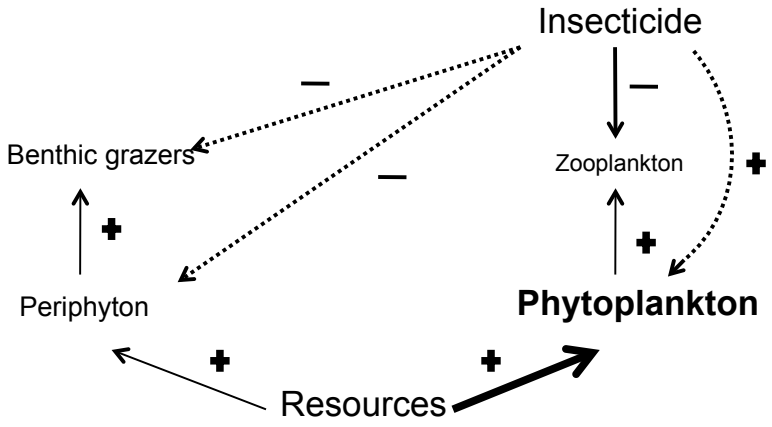

Figure 1.2. Observed direct (solid lines) and indirect (dashed lines) effects of insecticides in simplified aquatic communities containing zooplankton, phytoplankton, periphyton, and periphyton grazers. Adapted from Relyea and Diecks 2008.

For my dissertation, I address these gaps in our understanding by examining the ability of submerged aquatic plants to mitigate direct and indirect insecticide effects in aquatic communities. Since the pioneering work of Brock et al. (1992), the influence of submerged plants on insecticide fate and effects has been an issue of interest to ecotoxicologists. Primarily,

researchers have examined the rate at which insecticides sorb (i.e. bind) to plant tissues from the water column and use these rates to extrapolate the degree to which plants might mitigate insecticide toxicity (Karen et al. 1999, Crum et al. 1999, Gao et al. 2000a,b, Hand et al. 2001, de Carvalho et al. 2007, Thomas and Hand 2011). However, very few studies to date have actually quantified how much submerged plants influence the ecological effects of insecticides and the few studies that do are confounded by comparing treatments across years (Brock et al. 1992) or because researchers performed simultaneous manipulations of submerged plants and other contaminants such as nutrients (Roessink et al. 2005). My dissertation contains the first studies designed specifically to isolate and examine the influence of submerged plants on the ecological effects of insecticides.

I chose to examine the mitigating influence of submerged plants on the toxicity of the widely used organophosphate insecticide malathion. Since its introduction in the 1940's, malathion has been one of the most frequently applied insecticides in the United States, with at least 5.0×10^6 lbs applied annually over the past decade (Kiely et al. 2004, Grube et al. 2011). Malathion is a common insecticide used in insect pest eradication programs and during such events, surface water concentrations of the insecticide can exceed 780 μg/L (Newhardt 2006). However, during more common agricultural applications, expected environmental malathion concentrations in surface waters, taking aerial drift and application frequencies into account, range from 0-36 μg/L (Odenkirchen and Wente 2007). Thus, in my dissertation, I examine the influence of submerged plants on the ecological effects of malathion concentrations that span this latter expected range.

In chapter two, I examine the degree to which the cosmopolitan submerged plant *Elodea canadensis* influences malathion's direct toxicity to the key aquatic herbivore, *Daphnia magna.* I

hypothesize that *E. canadensis* will reduce malathion's toxicity (across five concentrations ranging from 0 – 30 μg/L) to *D. magna*, relative to environments containing no plants, and that the magnitude of these mitigating effects will increase with plant density (density range: 0 - 1,102 g dry weight/m^3). I also compared the rate at which each *E. canadensis* density detoxifies malathion by exposing *D. magna* to water samples collected at several time points over a 48 h period following malathion applications. I discovered that *E. canadensis* reduced malathion's toxicity in a density-dependent manner, with the highest plant densities making malathion up to nine times less toxic. I also discovered that malathion detoxification rate increased with plant density. For example, water treated with 30 μg/L of malathion was still lethal to *D. magna* after 48 h in the absence of *E. canadensis* while water treated with the same concentration was no longer toxic after just 2 h in the presence of high plant densities. This paper is co-authored with Rick Relyea and is published in *Environmental Toxicology and Chemistry* (Brogan and Relyea 2013a).

While my second chapter demonstrates that one plant species (*E. canadensis*) is able to mitigate malathion's toxicity to *D. magna*, chapter three considers how generalizable this ability is across different submerged plant species. Further, I test whether this ability is driven by traits of the living plants themselves or if mitigation instead occurs simply as a result of the added substrate (i.e. for sorption) provided by the addition of plants. Because no studies examining what plant traits may influence insecticide mitigation ability exist, I selected four common submerged plant species (*E. canadensis*, *Myriophyllum spicatum, Ceratophyllum demersum, and Vallisneria americana*) that differ widely in morphology and life histories (Nichols and Shaw 1986, Blindow 1992, Barrat-Segretain et al. 2002) and compared the magnitude to which and rate at which they mitigated malathion's toxicity to *D. magna,* relative to treatments containing

no plants. I also performed two inert substrate treatments containing polypropylene rope and plastic plants, respectively, to control for any mitigating effects of simply adding mass to each container. I discovered that each plant species reduced malathion's toxicity by an equal magnitude and at the same rate, while inert substrates had no mitigating effects. My findings demonstrate not only that the ability to mitigate malathion's toxicity is generalizable across plant species, but also that these mitigating effects are driven by traits of living plants, not merely their mass. This study was conducted with Rick Relyea and is published in *Environmental Toxicology and Chemistry* (Brogan and Relyea 2013b).

Having provided the first unequivocal evidence that submerged plants can mitigate an insecticide's toxicity to animals, in chapter four I examine the mechanism driving this effect. We introduce the current paradigm employed by toxicological models (e.g., AQUATOX, Park et al. 2008) that plants mitigate insecticide effects via sorption, the rate of which is predicted using an insecticide's octanol-water partition coefficient (i.e. K_{ow}). However, while insecticides possessing high log K_{ow} values (log $K_{ow} > 4$), such as DDT and many pyrethroid insecticides, sorb rapidly to submerged plant tissues (Gao et al. 2000a, Hand et al. 2001, Liestra et al. 2003, Carvalho et al. 2007), malathion has a relatively low log K_{ow} value (log $K_{ow} = 2.75$) and binds slowly to plants (Gao et al. 2000b). Because my earlier research shows that submerged plants do in fact mitigate malathion's toxicity, I test an alternative hypothesis that submerged plants actually detoxify malathion by increasing water pH via photosynthesis, which causes malathion to break down rapidly via alkaline hydrolysis (Wolfe et al. 1977, Seaman and Reidl 1978). To tease apart the effects of increased water pH from other functions of plants (e.g., sorption), we compared the toxicity of several malathion concentrations (range: 0 – 36 μg/L) across four treatments where I independently manipulated the presence of plants (plants present or absent)

and water pH (low pH or high pH) using either chemical additions or by manipulating the shading environment of plants. I found that chemically increasing water pH reduced malathion's toxicity by the same amount as adding unshaded (i.e. photosynthetic) plants. Further, I discovered that sorption played virtually no role in mitigation, as malathion was equally toxic to *D. magna* in water containing shaded (i.e. non-photosynthetic) plants and in the absence of plants (at low pH). This discovery demonstrates that a previously unexamined mechanism (pH-mediated mitigation) may play a major role in buffering aquatic communities from many insecticides. This study is co-authored by Rick Relyea and is in press at *Chemosphere*.

While chapters 2-4 demonstrate the ability of submerged plants to mitigate malathion's direct effects on sensitive species in microcosms, chapter five addresses the degree to which this ability scales up to more spatially and ecologically complex aquatic communities under several environmentally relevant insecticide-exposure scenarios (control, single "pulse" exposure, or repeated "press" exposures). I test the hypotheses that, 1. The magnitude of malathion's direct and indirect effects will increase with the number of insecticide exposure events, and 2. Submerged plants will mitigate these effects more as plant density increases. Overall, the data supported hypothesis 1; in the absence of plants, repeated malathion applications caused dramatic declines in cladoceran abundance followed by phytoplankton blooms that were not observed following single or control exposures. With respect to hypothesis 2, we found that submerged plants mitigated malathion's toxicity to cladocerans and prevented phytoplankton blooms, but mitigation did not increase with plant density because even the lowest plant densities strongly mitigated malathion's effects. Although these results suggest that plants may buffer communities from realistic malathion exposure events, I also discovered that plants had negative effects on the growth and abundance of some benthic algal and animal species, suggesting that

there may be costs associated with living in dense plant beds for some taxa. This study was conducted with Rick Relyea and is currently in review at *Freshwater Biology*.

In the final chapter I synthesize my work, discussing the relevance of my research to natural systems and important remaining questions that need to be addressed. I also consider potential applications of my findings to current pesticide mitigation strategies such as agricultural best management practices. Finally, I place my discoveries in the context of broader ecological theory, discussing how models designed to predict the ecological impacts of perturbations could be improved by incorporating interactions documented in my work and other studies.

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

2.1 INTRODUCTION

Insecticides are important tools for improving human health and the productivity of forestry and agriculture. However, projected increases in insecticide usage for the foreseeable future will likely lead to greater exposure for natural ecosystems (Laurence 2001). Insecticides pose a significant threat to aquatic habitats as they can exacerbate declines in already threatened taxa (Davidson 2004, but see Bradford et al. 2011) and decrease biodiversity (Relyea 2005, Geiger et al. 2010). Thus, a major contemporary challenge for ecologists and toxicologists is to better understand the factors that influence the environmental effects of insecticides in aquatic habitats.

Traditional toxicological models designed to predict the impacts of insecticides in aquatic communities are derived from results of laboratory tests that determine concentrations at which some effect occurs (e.g., LC50 = the concentration of an insecticide that kills 50% of a population; Newman 2010). To directly compare the relative toxicity of a large number of insecticides, agencies responsible for registering and regulating pesticides across the globe (e.g., United States Environmental Protection Agency, Organisation for Economic Co-operation and Development, ASTM International, etc.) 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 that the environmental conditions are not only important in determining the outcome of toxicity tests, but also that they incorporate the reality of what organisms experience in nature (Hanazato and Dodson 1995, Relyea and Hoverman 2006, Relyea 2010).

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 (Zaga et al. 1998, Edginton et al. 2004), predatory stress (Hanazato and Dodson 1995, Hanazato 2001, Relyea and Mills 2001, Relyea 2004), and competitive stress can all make insecticides more lethal to animals (Boone and Semlitsch 2001, Boon and James 2003). 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 (Karen et al. 1998, Crum et al. 1999). 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., DDT) and pyrethroid (e.g., lambda-cyhalothrin) insecticides (Gao et al. 2000a, Hand et al. 2001). For less lipophilic compounds—such as the commonly applied organophosphate insecticides chlorpyrifos (Log $K_{ow} = 4.81$) and malathion (Log $K_{ow} = 2.75$), the amount of insecticides removed from the water column by macrophytes typically ranges from 0 - 50% in a 24-h period (Van Donk et al. 1995, Karen et al. 1998, Gao et al. 2000b).

Though it is clear that some submersed macrophytes possess the ability to reduce the aqueous concentrations of some insecticides, there is very limited evidence for the ability of submersed macrophytes to mitigate the effects of insecticides on sensitive aquatic taxa. In one study comparing the ecological effects of the organophosphate insecticide chlorpyrifos (35 µg/L) between macrophyte-dominated and phytoplankton-dominated artificial test systems (~ 0.85 m^3), Brock et al. (1992) found that cladocerans were eliminated within hours in the phytoplankton-dominated system whereas it took several weeks for die-offs to occur in the macrophyte-dominated system. In addition, Roessink et al. (2005) 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). Though these studies did find 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 directly test the extent to which macrophytes alone influence the ecological impacts of insecticides. For example, Brock et al. (1992) 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. Additionally, Roessink et al. (2005)

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 as these animals serve as key drivers of aquatic community dynamics (Sarnelle 2005) and water quality (Lathrop et al. 1999). Specifically, we addressed two hypotheses: 1) As submersed macrophyte density increases, malathion's toxicity to *Daphnia magna* will decrease, and 2) As submersed macrophyte density increases, malathion's toxicity in the water column will decrease at a faster rate.

2.1.1 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 x 10^6 kg of active ingredient applied annually in the agricultural sector and another 1.8 to 3.6 x 10^6 kg applied annually in the home, garden, industrial and governmental sectors of the United States alone (Grube et al. 2011). Recently, the United States Environmental Protection Agency (USEPA) 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 (Odenkirchen and Wente 2007). Based on these values for more than 50 terrestrial crops, the EEC for malathion in water is 9 ± 27 µg/L (mean ± 95% CI). Further, aerial applications of malathion used to control insect pests can produce even higher concentrations in surface waters. For example, in the 1990's, the spraying of malathion for Mediterranean fruit fly control resulted in average surface water concentrations of approximately 50 µg/L (Ando et al. 1996).

2.2 METHODS

2.2.1 Experimental design

We conducted the experiment at the University of Pittsburgh's Pymatuning Laboratory of Ecology in Pennsylvania, USA. To investigate the effect of submersed macrophytes on insecticide toxicity, we examined the survival of the cladoceran zooplankter, *Daphnia 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 weight (DW) /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 DW/m^3 to more than 800 g DW/m^3; Duarte and Kalff 1990). On 15 June, 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 5 years (*pers. comm.* Jerry Bish, PA Game Commission). 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 colonization by any invertebrates and to reduce water evaporation. *Elodea* was kept in these conditions for 23 d before being used in the experiment.

The malathion concentrations that we chose for this experiment span the range of concentrations estimated or observed to be present in surface waters following typical agricultural and pest control practices (Ando et al. 1996, Odenkirchen and Wente 2007). 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 (Odenkirchen and Wente 2007). However, such worst-case scenarios are likely rare occurrences for a majority of freshwater habitats and so we elected to use concentrations that would more commonly occur in nature.

2.2.2 Test species

In winter 2010, we obtained 18 genetically distinct *Daphnia magna* (hereafter called *Daphnia*) clones originating from Katholieke Universiteit Leuven, Belgium. Using these lab-reared clones for our experiment instead of 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 our study. Further, using these clones ensured that there was genetic variability among

the *Daphnia* populations used in our 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 wks. *Daphnia* were fed 1 mL of concentrated *Scenedesmus spp.* algae that had been grown in a high-phosphorus COMBO medium (Kast-Hutchinson et al. 2001). Because of the logistical issues associated with coordinating the reproduction of these animals to achieve the very large number of *Daphnia* used in this experiment (7,200 total), we did not use < 24 h-old neonates to test malathion's toxicity. Instead, we used intermediate sized individuals (~ instars 3-6) that had not yet produced eggs.

2.2.3 Toxicity test setup

On 8 July, 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 above), 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 ± 148.4 g DW/m^3 (mean ± SD).

Although we performed this experiment in test systems 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 of 300-L pools that were 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. Once the jars were in place, we added ~ 10 cm of cold well water to each pool until it rose to approximately one half of the height of the test systems. Placing the pools on flat tables ensured that the water level outside of each test system 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 of 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.

2.2.4 Malathion applications

On 12 July, we applied the appropriate concentration of technical grade (99.1%) malathion (Chem Service Inc.) to each test system. We elected to use technical-grade malathion instead of 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 make our working solutions. This large batch of working solution provided a sufficient volume for dosing each appropriate test system plus two additional jars for malathion concentration

analysis. Though 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* (Kast-Hutchinson et al. 2001). 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) for analysis using GC/MS within 1 wk 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 encountered by the *Daphnia* in our study would be even higher than reported but this would not affect the overall conclusions.

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

Once 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.

2.2.6 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 to *Daphnia*, we also compared *the rate* at which different *Elodea* densities 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* 48 h after they had been added to each vial using the criteria described above. Thus, the response data for this experiment were the number of surviving *Daphnia* after 48 h of exposure to water collected from each jar at each time point.

2.2.7 Measuring *Elodea's* effects on water pH, DO, 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.

2.2.8 Statistical analysis

To determine the effect of *Elodea* density on the survival of *Daphnia* exposed to malathion, we compared *Daphnia* $LC50_{48\text{-h}}$ 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 (Finney 1971). 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. (2003) have demonstrated that 84% confidence intervals approximate an α

= 0.05. In one of the *Elodea* treatments (889 g DW/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% CI = 26 to 4356 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. While the utility of Dunnett's test in toxicological testing is controversial (Delignette-Muller et al. 2011), we emphasize that we used this approach simply as a tool for comparing 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 malathion addition 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 ANOVAs to examine treatment effects on each response variable. We used Tukey's multiple comparisons tests to determine differences between treatments.

2.3 RESULTS

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

As *Elodea* density increased, malathion's lethality to *Daphnia* decreased (Fig. 2.1). One way to quantify this is by estimating the $LC50_{48\text{-}h}$ values for malathion within each *Elodea* treatment. The $LC50_{48\text{-}h}$ value for *Daphnia* in the absence of *Elodea* (2.8 µg/L) was significantly lower than the LC50 values of all treatments containing *Elodea* (Table 2.1). Moreover, with each increase in *Elodea* density, we observed a significant increase in the estimated LC50 value for *Daphnia* exposed to malathion.

Table 2.1. $LC50_{48\text{-}h}$ values and 84% confidence intervals calculated for *Daphnia magna* exposed to malathion in the presence of different densities of the submersed macrophyte, *Elodea canadensis*.

***Elodea* density (g DW/m^3)**	***Daphnia* LC50 value (µg/L)**	**Lower 84% CI**	**Upper 84% CI**
0	2.8[a]	2.1	3.1
344	5.5[b]	4.8	6.3
612	14.0[c]	11.5	17.2
889	-*	-	-
1,102	25.2[d]	19.5	36.6

[a-d] Superscripts indicate significant differences between groups based on the overlap of 84% CI's.

* LC50 estimates for 889 g DW/m^3 were not reliable because the highest *Daphnia* mortality only approached 50%.

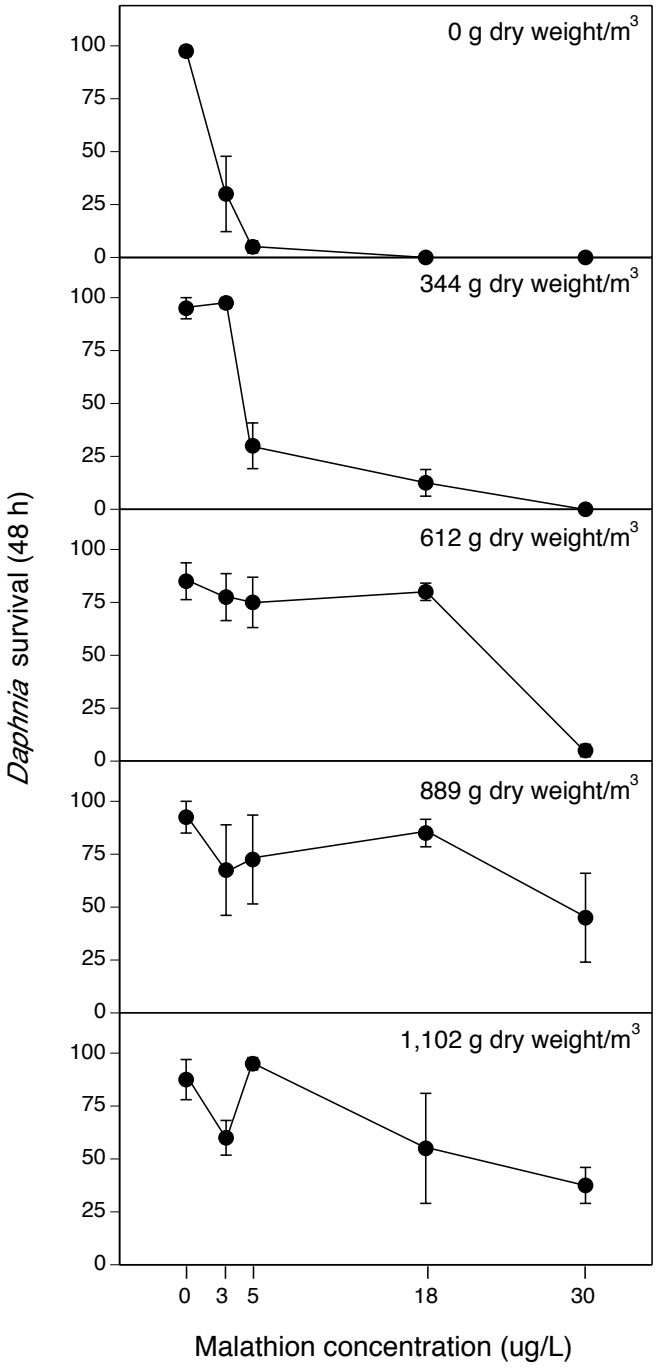

Figure 2.1. Survival data for *Daphnia magna* (n = 10) exposed to a factorial combination of malathion concentrations (0, 3, 5, 18, 30 µg/L) and *Elodea* densities (0, 344, 612, 889, 1102 g DW/m^3). Data are means ± 1 SE.

2.3.2 *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 ($p \geq 0.081$). However, in jars receiving applications of 5, 18 and 30 µg/L of malathion, water detoxification rates increased with macrophyte density. For example, with 0 g DW/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. 2.2; $p \leq 0.011$). With 344 g DW/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 DW/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 DW/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 that we observed occurred with 1,102 g DW/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 that 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 ($p > 0.066$).

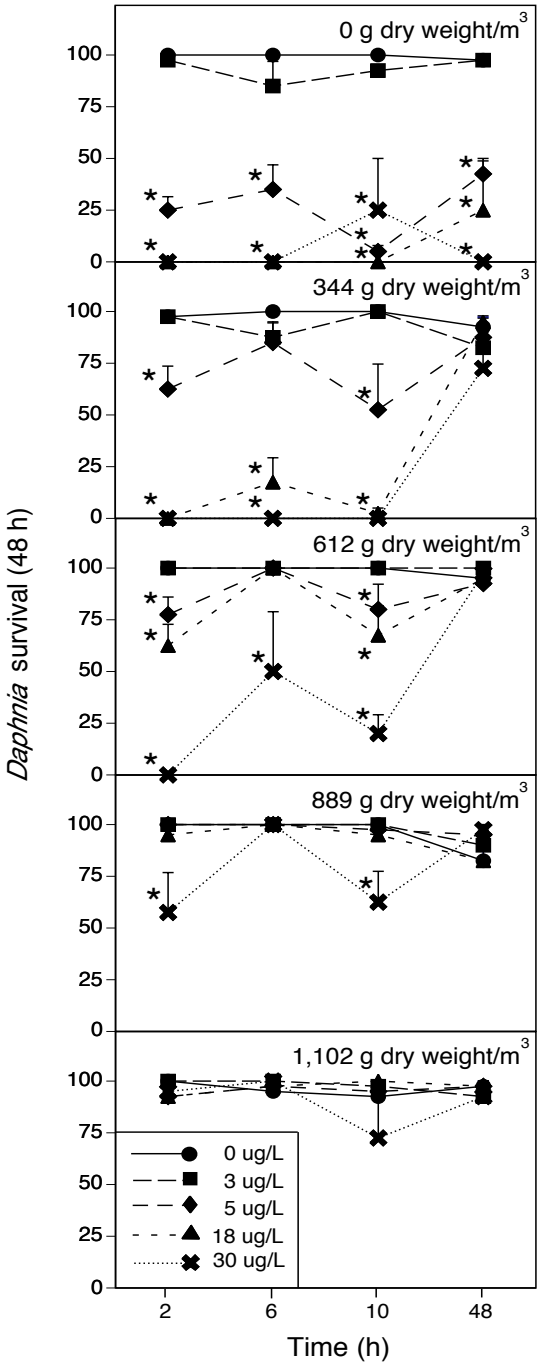

Figure 2.2. The influence of *Elodea* density on the toxicity of water collected 2, 6, 10, or 48 h after malathion applications of 0, 3, 5, 18, and 30 µg/L. We quantified water toxicity by examining *Daphnia* survival 48 h after exposure to each respective water sample. Asterisks indicate treatments 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 plus 1 SE.

2.3.3 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 *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. 2.3; all $p < 0.029$). Dissolved oxygen also increased with each increase in *Elodea* density (Fig. 2.3; 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. 2.3; $p < 0.001$) 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 ($p > 0.463$). Though we detected significant multivariate effects of malathion concentration on the abiotic environment 48 h after malathion applications, the range of pH (9.2 to 9.4) and DO values (12.3 to 16.8 mg/L) that we observed across malathion treatments were unlikely to have resulted in significant biological effects on *Daphnia* or *Elodea* so they will not be discussed further.

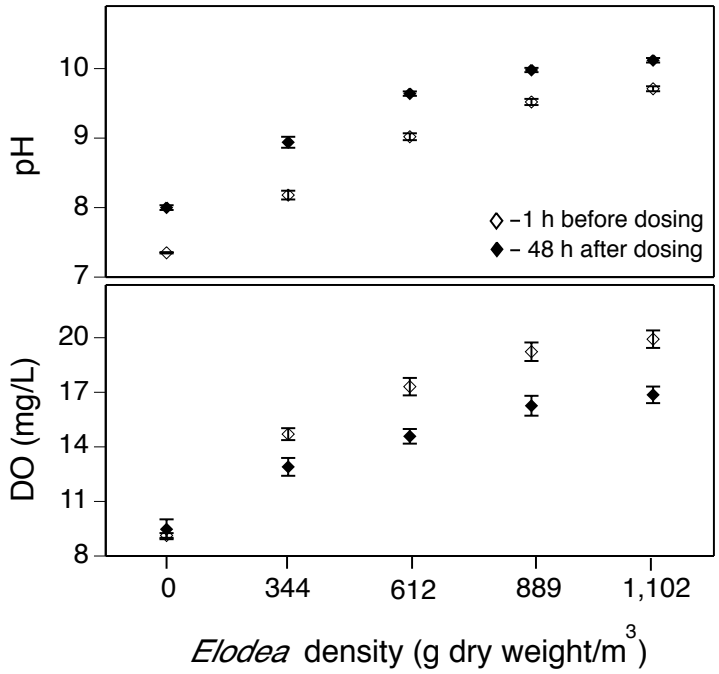

Figure 2.3. The influence of *Elodea* density on the toxicity of water collected 2, 6, 10, or 48 h after malathion applications of 0, 3, 5, 18, and 30 µg/L. We quantified water toxicity by examining *Daphnia* survival 48 h after exposure to each respective water sample. Asterisks indicate treatments 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 plus 1 SE.

2.4 DISCUSSION

While previous studies have reported mitigating effects of emergent vegetation, contained within agricultural constructed wetlands and drainage ditches, on the toxicity of insecticides to aquatic taxa (Lizotte et al. 2011), 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 *Elodea canadensis* substantially reduced the lethality of the popular insecticide malathion to the keystone herbivore, *Daphnia magna* (Sarnelle 2005), and also increased the rate at which water treated with malathion was detoxified.

By generating $LC50_{48\text{-}h}$ 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*. Further, these data demonstrate that this mitigating effect increases with *Elodea* density. In fact, we found that the $LC50_{48\text{-}h}$ estimates for *Daphnia* significantly increased with each increase in *Elodea* density. For example, comparing the 0 g DW/m^3 *Elodea* treatment to the 344, 612, and 1,102 g DW/m^3 *Elodea* density treatments, we observed approximately 2, 5, and 9-fold increases in the $LC50_{48\text{-}h}$ estimates for *Daphnia*.

The estimated $LC50_{48\text{-}h}$ 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, http: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 *Daphnia magna* (USEPA, 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.

Our experiment also revealed that the rate at which *Elodea* reduces the toxicity of water following the application of malathion increases with increasing *Elodea* density. For example, in jars containing 0 g DW/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. However, in jars containing 1,102 g DW/m^3 of

Elodea, 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 DW/m^3 of *Elodea* in response to this malathion concentration, we expected to observe at least a partial reduction in *Daphnia* survival following exposure 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 first water extraction that took place at 2 h. Though 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. Additionally, 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. (2000b) examined the rate at which malathion concentrations decreased in culture medium in the presence of two submersed macrophyte species (*Myriophyllum aquaticum* and *Elodea Canadensis*; Gao et al. 2000b). However, the macrophyte densities (100,000 g fresh weight/m^3) used in that study were ten times higher than even the maximum *Elodea* density used in our study (~10,000 g fresh weight/m^3). Thus, one would expect that the authors would have observed higher malathion dissipation rates compared to our study. Interestingly, the opposite appears to have occurred. For example, whereas Gao et al. (2000b) documented less than a 50% reduction in aqueous malathion concentration over 48 h (nominal concentration applied = 1,000 μg/L), our data suggest much higher dissipation rates as 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 our experiment. For example, *Elodea* could be sorbing malathion onto its surfaces and thus reducing water toxicity to *D. magna*. However, though 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 (Gao et al. 2000a, Hand et al. 2001), malathion is relatively hydrophilic (Log K_{ow} = 2.75) and it remains unclear how much macrophytes will sorb this insecticide. In the aforementioned experiment by Gao et al. (2000b), the authors found no evidence that malathion was taken up by macrophytes during the first 48 h following exposure (Gao et al. 2000b). 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 can not determine how much of malathion's disappearance from the water column was due to sorption versus other breakdown processes.

Another mechanism that might contribute substantiantially to malathion's disappearance from the water column is the rise in pH associated with each increase in *Elodea* density in our study (Fig. 2.3). Increases in aqueous pH are known to affect the persistence of many insecticides (Chapman and Cole 1982). For example, Wolfe et al. (1977) demonstrated that each unit increase in pH (e.g., pH 8 to pH 9) decreases malathion's half-life by approximately one order of magnitude (Wolfe et al. 1977). 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 DW/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.

2.5 CONCLUSIONS

The field of ecotoxicology is beginning to fully explore the influence of ecological interactions 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, *Elodea canadensis*, can dramatically reduce the toxicity of the insecticide malathion to *Daphnia 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. Additionally, 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 which 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 like 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.

2.6 ACKNOWLEDGEMENTS

We thank Zach Zbinden, Jess Hua, and Rickey Cothran for their help with the experiments. Our thanks to Luc De Meester's lab for providing us with the *Daphnia magna* clones used in this experiment. Finally, we thank the staff at the Pymatuning Laboratory of Ecology for all of their help. This research was funded by a G. Murray McKinley grant to WRB and a National Science Foundation grant to RAR.

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

3.1 INTRODUCTION

The use of insecticides is a primary strategy for controlling pest damage to economically valuable lands and human health. However, an unintended byproduct of insecticide usage is the exposure of non-target species. For example, insecticides commonly enter surface waters via runoff, spray drift, and irrigation effluent, leading to exposure for aquatic communities that can cause shifts in species composition (Brock et al. 2000, Relyea 2005) and diversity (Geiger et al. 2010). 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 (BMPs) for mitigating and remediating the environmental impacts of insecticides in aquatic ecosystems (Schulz 2004, Moore et al. 2006, Reichenberger et al. 2007, Werner et al. 2010). The primary focus of this work has been on evaluating 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. While this research has demonstrated that BMPs can be effective at reducing the environmental transport of some insecticides into adjacent aquatic ecosystems,

ecologically relevant concentrations of insecticides are still frequently detected in surface waters of aquatic ecosystems located near agricultural lands (Gilliom 2007). Additionally, recent surveys suggest that surface waters located in urban areas can possess similar insecticide loadings as those in agricultural settings (Hoffman et al. 2009). Yet, despite the frequent exposure of non-target aquatic habitats to insecticides, there is currently a paucity of information on the ecological factors contained within these environments that might also mitigate insecticide effects.

Submersed macrophytes are a globally 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 (Crum et al. 1999, Thomas and Hand 2011). 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 (Gao et al. 2000a, Hand et al. 2001). However, sorption of less hydrophobic compounds by macrophytes is much slower. For example, Gao et al. 2000b reported that over 70% of the malathion ($\log K_{ow} = 2.75$) concentration applied remained after 1 d in the presence of submerged macrophytes and that only 20% of the total concentration applied was extractable from plants after 8 d. Nevertheless, this study 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 like malathion.

The degree to which macrophytes actually influence the toxic effects of insecticides in aquatic communities is poorly understood. Recently, Brogan and Relyea (2013a) examined the mitigating influence of submersed macrophytes on malathion's toxicity to the aquatic zooplankter, *Daphnia magna*. They discovered that malathion was up to nine times less toxic in the presence of realistic densities of the common macrophyte *Elodea canadensis* than in the absence of macrophytes ($LC50_{no\text{-}macrophytes}$ = 2.8 μg/L). Further, they found that *E. canadensis* 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 ~ 30 μg/L of malathion to *D. magna* returned to control levels (non-toxic) within just 2 h after the insecticide had been applied. Although the study by Brogan and Relyea (2013a) 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, which could lead to important differences in insecticide mitigation between macrophyte species. For example, Gao et al. (2000b) 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 *Elodea canadensis*. Additionally, Crum et al. (1999) demonstrated that the submersed macrophytes *Chara globularis* and *Elodea nuttallii*, and 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%, though 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 (Crum et al. 1999) and the molecular machinery involved in binding, transporting, and degrading pesticide molecules (Gao et al. 2000b). However, before attempting to elucidate the mechanisms driving species-level differences in insecticide uptake, 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 our 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. The most 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 x 10^6 kg of active ingredient applied annually in the agricultural sector and another 1.8 to 3.6 x 10^6 kg applied annually in the home, garden, industrial and governmental sectors (Grube et al. 2011). However, despite its toxicity and popularity, ecotoxicological experiments examining malathion's effects on aquatic taxa under semi-natural and natural conditions are relatively rare.

We 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 two different inert substrates to determine whether insecticide mitigation occurs merely as a result of the added surface area provided by the presence of plants. 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.

3.2 METHODS

3.2.1 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, United States in July 2011. We compared the survival of the cladoceran *Daphnia magna* exposed to a complete factorial cross of three nominal concentrations of the insecticide malathion (0, 2.5, 25 μg/L) in each of seven macrophyte treatments (no macrophytes, plastic plants, polypropylene rope, *Elodea canadensis*, *Myriophyllum spicatum*, *Ceratophyllum demersum*, or *Vallisneria americana*). Each of the 21 treatment combinations was replicated four times for a total of 84 experimental units.

We chose the four 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.

While 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, which may increase sorption rates if sorption is the underlying mechanism of mitigation. All macrophyte species were collected from field sites during 15 to 17 June (Table 3.1). While the Geneva Marsh and Crystal Lake sites have had no direct exposure to insecticides in the past 5 y (*personal communication*, Jerry Bish, Pennsylvania Game Commission), it is possible that incidental insecticide exposure has occurred in Pymatuning or Conneaut lakes 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 and each species was planted in a separate 1,200 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.

Table 3.1. 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	*Ceratophyllum demersum*
		Myriophyllum spicatum
Crystal Lake	41°33'13.6"N, 80°22'9.26"W	*Myriophyllum spicatum*
Conneaut Lake	41°36'13.88"N, 80°17'58.36"W	*Vallisneria americana*

The malathion concentrations that we selected for this study span the range of likely exposure scenarios for species inhabiting surface waters in the U.S. Though malathion application data for urban and industrial sectors in the United States are sparse, the U.S. Environmental Protection Agency has recently calculated the estimated environmental concentrations (EEC) for this insecticide in California surface waters based on inputs from agricultural sources (Odenkirchen and Wente 2007). Models generated using data including typical application amounts, frequencies (every 2 to 14 d), and expected drift patterns for more than 50 terrestrial crops reveal surface water EECs for malathion to range between 0 to 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 (Ando et al. 1996). If we assume these data are representative of exposure scenarios in other states where similar data are

currently unavailable, the concentrations that we chose are well within realistic exposure scenarios.

We used *Daphnia magna* as the test species in this experiment in part because of its widespread use in toxicological testing. However, daphnids are also considered to be critical herbivores in aquatic food webs because they provide a key link between primary producers, planktivorous predators, and water quality (Lathrop et al. 1999, Sarnelle 2005). The *D. magna* used in the 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 used in our study. Further, by using laboratory-reared clones for our experiment, we ensured that the test animals had not been exposed to contaminants for dozens of generations prior to our 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 wks. 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 (ASTM 2004, ASTM 2007), coordinating reproduction to achieve the large number of *D. magna* needed for this experiment (~ 3,500 animals) prevented our use of neonates. Instead, we used intermediate sized individuals (~ instars 3 to 6) that had not yet begun producing eggs.

3.2.2 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 letting the suspended sediment settle. The following day, we selected shoots of each macrophyte species from culture pools along with inert substrates for inclusion 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. For *M. spicatum* and *V. americana*, which form more extensive root systems, we clipped the shoots 15 cm above the sediment. Additionally, we clipped the roots and any stolons down to 1 cm. We weighed out 5.7 g fresh weight of each macrophyte species and added the macrophytes to their randomly assigned jars.

We ensured that the basal end of each macrophyte contacted 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 (± SE) 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. As observed dry biomass densities for submersed macrophytes typically range from 0.05 to 0.8 g/L (Duarte and Kalff 1990) the densities used in our 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 of 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 seven jars into each of twelve aquaria dispersed throughout four pools (Fig. 3.1). 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 insecticide concentrations. Once 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 (Oakton digital pH meter), and dissolved oxygen (DO; Oakton Instruments; WTW digital oxygen meter) 1 h before applying malathion as well as pH and DO 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.

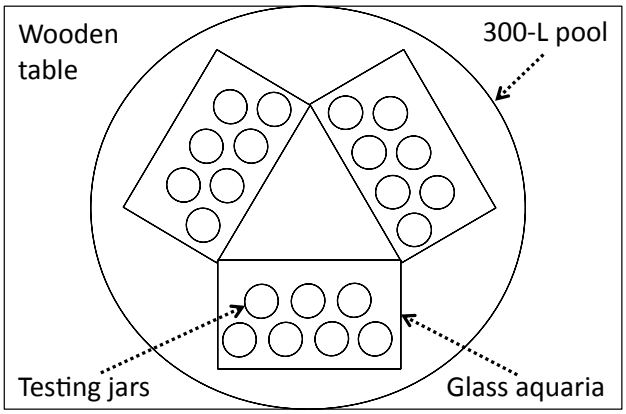

Figure 3.1. Depiction of the experimental setup consisting of glass jars positioned inside of sideways-oriented aquaria. All aquaria were placed in plastic 300-L pools filled with approximately 100 L of well water. See methods section for complete details.

3.2.3 Malathion application

On 26 July, we applied technical grade (99.1%) malathion (Chem Service Inc.) to each jar. To achieve nominal concentrations of 0, 2.5, and 25.0 μg/L, we added 0, 0.457, 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 make our working solutions. We used a separate container for 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 in each jar to 750 mL. During dosing, we slowly poured control and treated water into each jar to ensure thorough mixing inside of 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 this experiment, other experiments have demonstrated that ethanol concentrations (0.5 mL ethanol/L water) twice as high as those used in our study (0.203 mL ethanol/L water) had no adverse effects on *D. magna* survival (Kast-Hutchinson et al. 2001).

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 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 two separate 500-mL pre-cleaned amber glass jars (VWR). We stored the jars in a 3°C refrigerator until analysis. All samples were sent to an independent laboratory for analysis using GC/MS (University of Georgia Agricultural and Environmental Services Laboratory) within 1 wk of being collected. The actual malathion concentrations corresponding to the nominal

concentrations of 0, 2.5, and 25 μg/L were 0, 3.3, and 23.7 μg/L (hereafter referred to as 3, and 24 μg/L).

3.2.4 Determining macrophyte effects on the amount that malathion's toxicity is reduced

After applying each insecticide treatment, we added 10 *Daphnia* individuals to each jar in the same order that the jars were dosed. Thus, *Daphnia* were added to each jar approximately 20 min after it had been treated with the appropriate malathion concentration. Once all *D. magna* were added, and each day thereafter, we added 1 mL of high-phosphorus *Scenedesmus* algae raised in COMBO medium (Kilham et al. 1998) to each jar to serve as food for *D. magna*. 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 slightly modified from OECD standardized testing guidelines (OECD 1984). 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 three applications of this stimulus. Thus, while most non-survivors were clearly dead (no movement and faded color), any individuals that were still twitching but unable to swim in response to the stimulus were also considered dead.

3.2.5 Comparing macrophyte effects on the rate at whch 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, 8, 24, 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 treatment 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 ten *D. magna* to each vial. The vials were brought indoors where we quantified *D. magna* survival (using the criteria described above) 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 following exposure to water collected from each jar at each time point 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 applications, we tried pouring the animals through a metal sieve, which appeared to affect the survival of these animals. 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, 8, 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 time point group from our analyses.

3.2.6 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 this, we first performed an 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 first rank-transformed the survival data before analysis. When significant effects of the treatment interaction were detected, we used Games-Howell multiple comparisons 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, 8, and 48 h following insecticide application and compared ranked survival of animals exposed to water treated with 3 and 24 μg/L of malathion to survival in the controls at each time point. This allowed us to compare the time that it took for 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, DO, and temperature 1-h before applying malathion using a multivariate analysis of variance (MANOVA). We also quantified pH and DO 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.

3.3 RESULTS

3.3.1 Effects of macrophyte treatments on the amount that malathion's toxicity is reduced

In the outdoor jars, the 48-h survival of *D. magna* was affected by macrophyte treatment ($F_{6,63}$ = 3.6, p = 0.004), 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* (Fig. 3.2; $F_{2,9}$ < 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 two treatments did not differ (p > 0.094). We also examined *D. magna* survival across the seven 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 even though some of the macrophytes were collected from sites that may have encountered incidental prior exposure to pesticides, the plants themselves had no significant negative impact on *D. magna* survival.

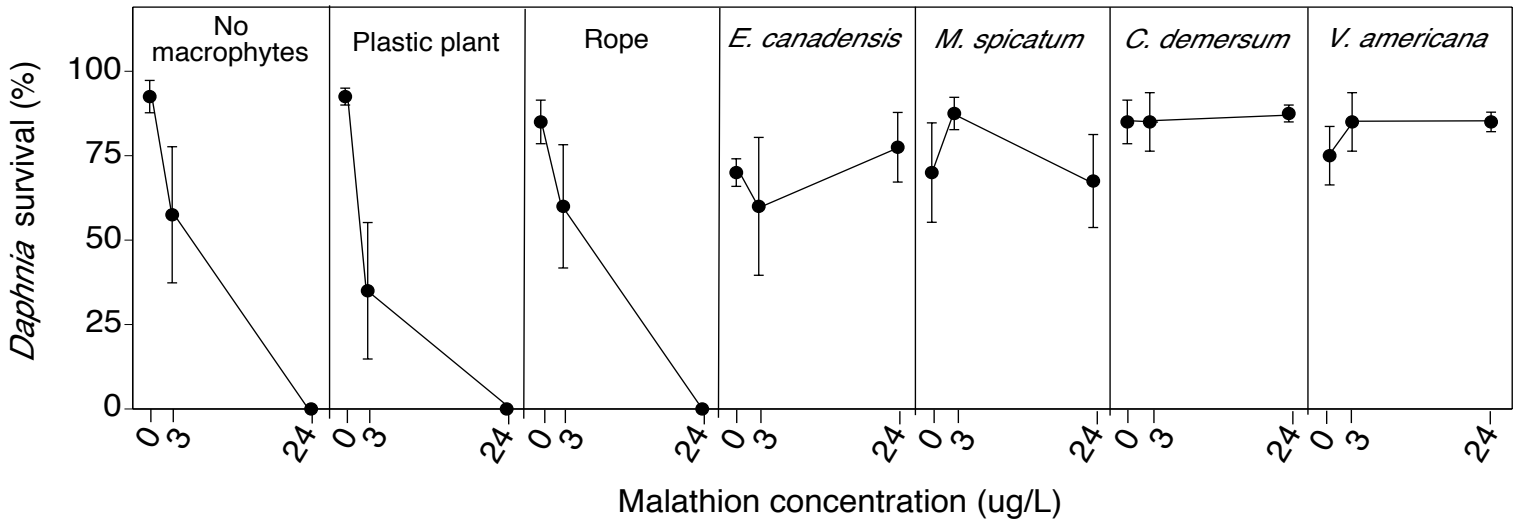

Figure 3.2. *Daphnia magna* 48-h survival following exposure to three malathion concentrations in the presence of each of seven macrophyte treatments. Data are means ± 1 SE.

3.3.2 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 four live macrophyte species than in 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 (Fig. 3.3; $p < 0.012$). Further, 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 ($p < 0.034$). However, in treatments containing any of the four living macrophyte species, water receiving 3 or 24 µg/L of malathion was non-toxic 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 ~40%, Dunnett's test revealed no difference from survival in the controls ($p = 0.110$). Additionally, in the presence of *C. demersum*, survival of *D. magna* following exposure to water collected 48 h after malathion applications reduced *D. magna* survival by a small (< 10%) but statistically significant amount compared to controls ($p = 0.023$) even though no differences in survival were observed following exposure to water collected at 2 and 8 h.

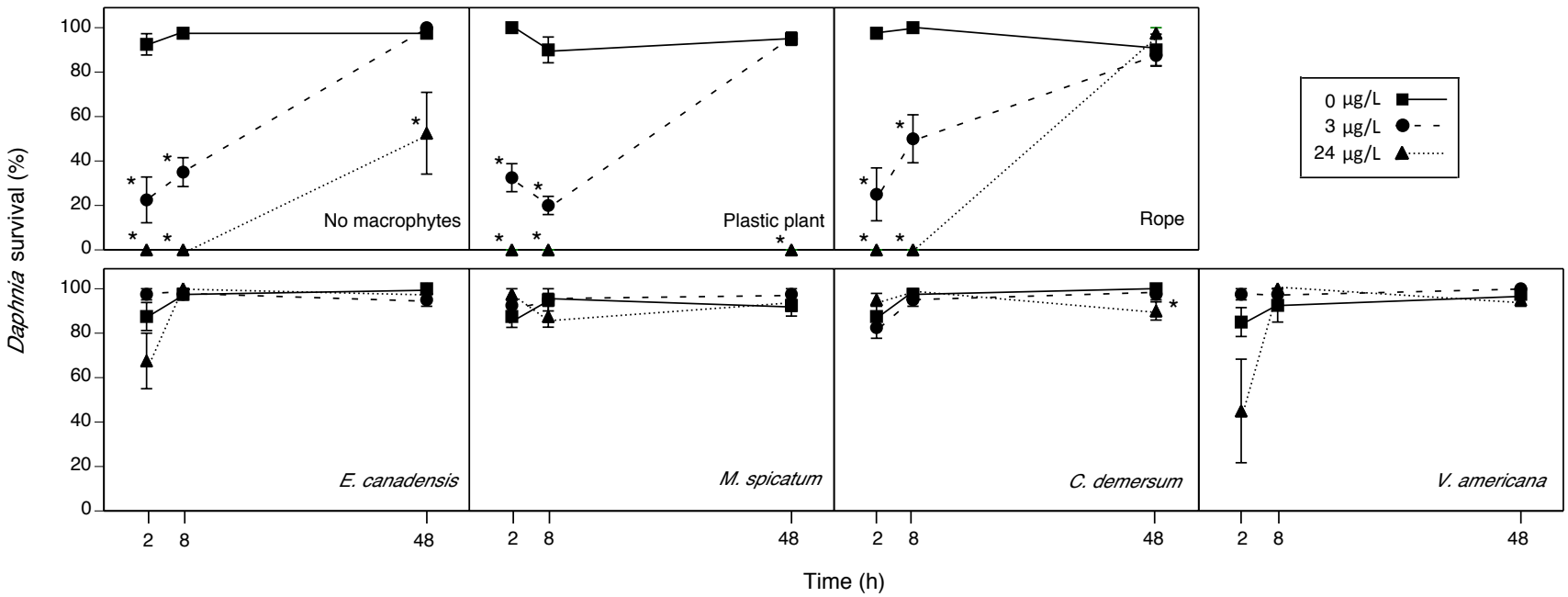

Figure 3.3. The effects of macrophyte treatment on the toxicity of water collected 2, 8, or 48 h after malathion applications of 0, 3, or 24 µg/L. For treatments that had received insecticides, water toxicity was assessed at each sampling time and within each macrophyte treatment by comparing *D. magna* 48-h survival to the controls. Asterisks indicate insecticide treatments where *D. magna* survival was significantly lower than in insecticide-free controls. Data are means ± 1 SE.

3.3.3 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 DO ($F_{6,77} > 6.5$, $p < 0.001$). Compared to the no-macrophyte treatment, pH samples collected at either time point 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 four macrophyte species (Fig. 3.4; $p < 0.001$). At the first sample time (1 h prior to dosing), DO 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, DO in the presence of *M. spicatum* was at least 8% higher than all other macrophyte treatments and was 25% higher than in the no-macrophyte treatment ($p < 0.003$). In the sample collected 48 h after dosing, DO levels in the no-macrophyte treatment did not differ from the rope or *V. americana* treatments ($p > 0.99$) but were 27% higher than 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 to 32.8°C) and was not influenced by any treatments ($F_{6,77} = 1.163$, $p = 0.335$).

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 DO ($F_{2,63} = 27.2$, $p < 0.001$). While pH did not differ between treatments exposed to 0 and 3 µg/L of malathion ($p = 0.713$), concentrations of 24 µg/L increased pH levels by ~3% compared with controls ($p < 0.001$). Malathion's effect on DO occurred because water exposed to 0 µg/L of

malathion had approximately 7% greater DO levels than water dosed with 3 µg/L ($p < 0.001$), and nearly 13% higher DO than 24 µg/L malathion treatments ($p = 0.025$).

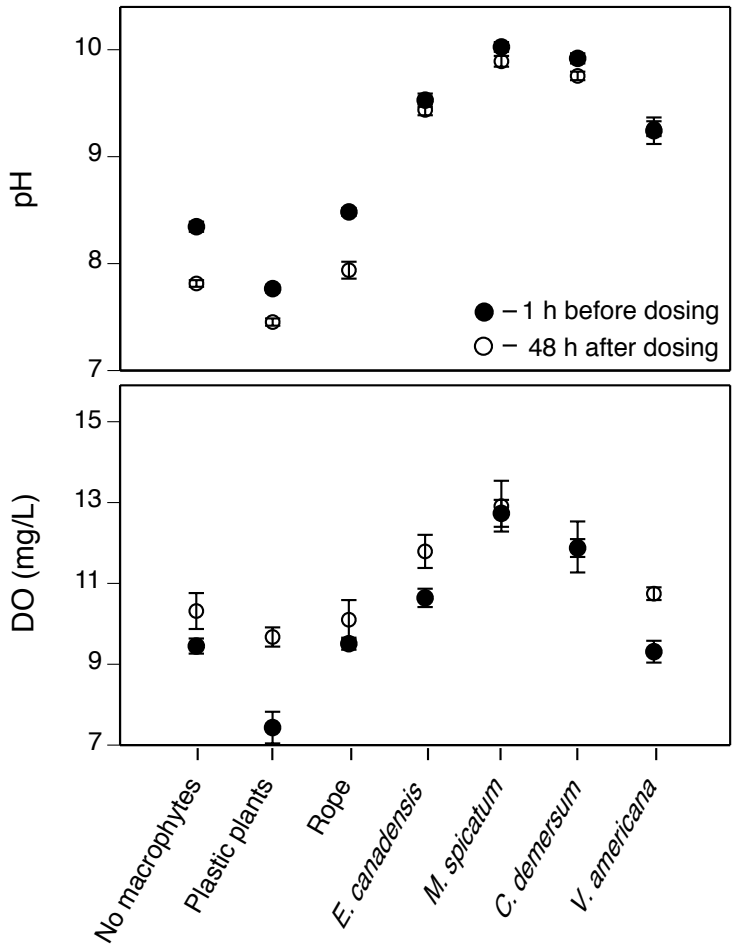

Figure 3.4. The effects of macrophyte treatment on pH and dissolved oxygen in 1-L jars 1 h before and 48 h after malathion application. Data are means ± 1 SE.

3.4 DISCUSSION

We tested the amount and rate at which four species of submersed macrophytes (*E. canadensis, M. spicatum*, *C. demersum*, and *V. americana*) and two inert substrates (plastic plants and polypropylene rope) can mitigate the toxic effects of a common insecticide (malathion) on the aquatic herbivore *D. magna*. We discovered that all four macrophyte species strongly and equally mitigated the toxicity of malathion to *D. magna*, whereas the inert substrates had no mitigating effect. For example, while 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 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 four macrophyte species can mitigate the amount of malathion's toxicity to a similar degree.

Our experiment demonstrated that the presence of each macrophyte species tested prevented any *D. magna* mortality from occurring even following 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; Kikuchi et al. 2000). In contrast, the effects of malathion on *D. magna* survival that we observed in the no-macrophyte, plastic-plant, and rope treatments of our experiment are similar to documented effects reported in studies employing more traditional toxicological experimental designs [Kegley et al. 2010; http:www.pesticideinfo.org]. Although creating environments conducive to the maintenance of healthy submersed macrophyte populations in our study made it impractical for us to strictly adhere to traditional toxicity testing guidelines using *D. magna* (e.g., USEPA, OETC, ASTM, 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.

While our 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 this experiment 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 (Brogan and Relyea 2013a). Brock et al. (1992) 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 ~ 2 wks for comparable mortality to occur in macrophyte-dominated systems. While the findings of Brock et al. (1992) suggest that macrophytes may have had a mitigating effect on zooplankton assemblages, the results of this study must be interpreted with caution as the responses of the macrophyte- and phytoplankton-dominated systems that were exposed to insecticides were examined in separate years (1988 and 1989, respectively) and, thus, may have had different community compositions prior to dosing.

In another study, Roessink et al. (2005) compared the effects of a range of concentrations (10 to 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 as well as 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 the studies by Brock et al. (1992) and Roessink et al. (2005). Clearly, more studies designed to examine the influence that macrophytes may have on the ecological effects of different insecticides in more complex communities are needed.

Another important discovery in the present study was that all four 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 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 four 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 resulting from the presence of the plants. We demonstrated this by showing that two 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 that 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 (Gao et al. 2000b). Taken together, all of the evidence from our study suggests that aquatic plants must be alive to mitigate malathion's toxicity.

Of course, living macrophytes host a diverse epiphytic floral community and, while our rinsing procedure appeared to remove nearly all epiphytic algae from the macrophytes 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 our study. However, bacteria collected from natural waters degrade malathion relatively slowly, compared with the rates that we indirectly observed in our experiment (half-life ≈ 32 h with 5.0×10^8 colony forming units; Paris et al. 1981). In fact, Mohamed et al. (2010) even 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/mL 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 our study, we attribute the mitigation that we observed primarily to the effects of macrophytes.

There are several mechanisms that 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. (2000b) investigated the rates at which malathion concentrations decreased in a liquid culture medium containing either of two 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 our study, these results suggest that if the live macrophytes we tested were sorbing malathion at similar rates to what Gao et al. (2000b) observed, the malathion concentration 48 h following applications of 24 µg/L should still have been approximately 14 µg/L, which is still 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 whole 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 (Halstead and Tash 1982). 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 that we observed in the presence, but not

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 (Wolfe et al. 1997). For example, in water with pH = 8 (i.e. the no-macrophyte, plastic plant and rope treatment of our experiment), malathion's half-life is approximately 8 h at the temperatures recorded in our study (~30°C). However, with each unit increase in pH (i.e. 8 to 9), malathion's half-life is predicted to decrease by an order of magnitude. Thus, in water with pH > 9 (i.e. the live macrophyte treatments), malathion's half-life would likely be substantially less than 1 h and may 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. Unfortunately, the present experiment was not designed to tease apart these mechanisms, but an important next step would be to determine the relative importance of macrophyte sorption versus macrophyte effects on pH in mitigating insecticides like malathion.

3.5 CONCLUSIONS

In this study, we discovered that four different macrophyte species exhibited equal mitigating effects on malathion's toxicity to the sensitive aquatic species *D. magna.* Further, we demonstrated that mitigation does not occur in the presence of two 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 largely been extrapolated from studies examining the sorption of insecticides from the water column by macrophytes. While 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 current 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. Further, 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.

3.6 ACKNOWLEDGEMENTS

We thank Zach Zbinden and Jess Hua for their assistance with the experiments. Our thanks to Rickey Cothran for experimental ideas that improved the present study and the Pymatuning Laboratory of Ecology staff for their support during this project. This research was funded by a G. Murray McKinley grant to WRB and a National Science Foundation grant to RAR.

4.0 A NEW MECHANISM OF MACROPHYTE MITIGATION: HOW SUBMERGED PLANTS REDUCE MALATHION'S ACUTE TOXICITY TO AQUATIC ANIMALS

4.1 INTRODUCTION

A major contemporary challenge in ecotoxicology is to identify and understand the factors that can mitigate the effects of contaminants in aquatic communities. In the past 15 years, aquatic plants have emerged as one factor that can have a strong influence on the transport, fate, and ecological effects of many contaminants (Cooper et al. 2004, Reichenberger et al. 2007, Moore et al. 2011). However, the degree to which plants mitigate contaminant effects in aquatic ecosystems is highly variable and there is a critical need for research that examines the mechanisms driving contaminant mitigation.

The current approach used in models designed to predict the influence of aquatic plants on the fate and effects of insecticides in surface waters (e.g., AQUATOX, CATS, etc.; Park et al. 2008) is based primarily on the octanol-water partition coefficient (K_{ow}) of an insecticide. For highly hydrophobic insecticides (i.e. log K_{ow} > 5) such as DDT and pyrethroids, plants can remove nearly all of a compound from the water column within a few hours (Gao et al. 2000b, Hand et al. 2001, Leistra et al. 2003). For less hydrophobic insecticides (log K_{ow} < 3), however, plants remove the insecticides from the water column much more slowly. As a result, these chemicals can be detected in the water for several days after application (Crum et al. 1999, Gao

et al. 2000b). Under the current sorption paradigm, one is left to conclude that plants should have weak mitigating effects on insecticides with low log K_{ow} values (e.g., Gao et al. 2000b). While this is a logical extrapolation, new evidence suggests that this is not always the case.

Recent studies demonstrate that aquatic plants can strongly mitigate the toxicity of the widely used organophosphate insecticide malathion to the sensitive aquatic zooplankter *Daphnia magna*, despite malathion's relatively low octanol-water partition coefficient (log K_{ow} = 2.75). In one study, Brogan and Relyea (2013a) tested the lethality of malathion to *Daphnia* across a range of densities of the submerged plant *Elodea canadensis* and demonstrated that with each increase in plant density, malathion's toxicity to *Daphnia* decreased. Further, by removing samples of water at several time points following malathion applications and then exposing *Daphnia* to them, the researchers discovered that in the absence of *Elodea*, water treated with as little as 5 μg/L of malathion was still toxic to *Daphnia* after 48 h, whereas *Elodea* detoxified water treated with up to 30 μg/L of malathion within 2 to 6 h. In a subsequent study, Brogan and Relyea (2013b) found that four species of submerged plants all strongly and equally mitigated malathion's toxicity to *Daphnia* relative to systems containing no plants or plastic-plant controls. These studies demonstrated clear mitigating effects of submerged plants on malathion's toxicity, but they were not designed to elucidate the mechanism by which the plants were able to mitigate an insecticide that current toxicological models suggest should be weakly mitigated.

One alternative mechanism that could mitigate malathion's toxicity is the increase in pH caused by plants that can in turn cause the breakdown of malathion. During photosynthesis, submerged plants take up dissolved carbon dioxide from the water; this initiates the conversion of carbonic acid into CO_2 and increases water pH by shifting the bicarbonate buffer system towards the more alkaline molecules bicarbonate and carbonate (Wetzel 2001). This increase in

pH may be important because malathion is rapidly hydrolyzed under alkaline aquatic conditions. For example, malathion's half-life in water is approximately 3 d at a pH of 7, but it is 19 h at a pH of 8 and 2.4 h at a pH of 10 (no temperature data provided; Seaman and Riedl 1986). Wolfe et al. (1977) reported similar half-lives at 30°C, although at pH 10 malathion's half-life was substantially less than 1 h. While these studies suggest that alkaline hydrolysis could potentially play an important role in the detoxification of malathion in water, no studies to date have examined whether alteration of pH is a mechanism whereby plants can mitigate the lethal effects of insecticides on animals. This is an important step as some of malathion's breakdown products resulting from alkaline hydrolysis (e.g., malaoxon, diethyl fumurate) can be more toxic to aquatic animals than malathion itself (Bender 1969, Aker et al. 2008).

We addressed this important gap in our knowledge by exploring whether plant-mediated and chemical-mediated changes in water pH can alter malathion's toxicity to *Daphnia*. Our approach allows us to tease apart the independent influence of pH on malathion's toxicity from the effects of other potential interactions between plants and malathion, such as sorption. Based on our hypothesis that aquatic plants mitigate the effects of insecticides on sensitive animal taxa by a mechanism of increasing water pH, we made the following predictions: (1) adding aquatic plants in full sunlight to allow photosynthesis will mitigate malathion's effect on *Daphnia,* (2) chemically increasing water pH by the same amount as a photosynthesizing plant will mitigate malathion's effect on *Daphnia* to a similar degree, and (3) adding aquatic plants in complete shade to prevent photosynthesis but to allow other plant-insecticide interactions such as sorption will not mitigate malathion's effect on *Daphnia*.

4.2 METHODS

4.2.1 Experimental design

We tested these predictions using an experiment conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology in July 2012. We exposed *Daphnia magna* to five nominal malathion concentrations (0, 1, 5, 10, 50 μg/L) crossed in a factorial manner with four aquatic environments (plants in full sunlight, plants in complete shade, chemical additions to maintain low pH without plants, and chemical additions to maintain high pH without plants). The 20 treatments were replicated four times for a total of 80 experimental units.

4.2.2 Insecticide selection

While a major reason that we performed the present study using malathion was to test hypotheses arising from discoveries in previous studies (Brogan and Relyea 2013a,b), we also selected this insecticide because it's widespread usage and potential for contamination of surface waters. With approximately 10-14 million kg applied annually (Kiely et al. 2004, National Pesticide Use Database, www.ncfap.org/database/national.php), malathion is one of the most common active ingredients applied in the U.S. (Grube et al. 2011). The nominal concentrations in the present study span a range of estimated environmental concentrations for malathion exposure in aquatic environments (0-36 μg/L), accounting for application frequencies and estimated drift (Odenkirchen and Wente 2007).

4.2.3 Species collection and husbandry

For our malathion toxicity assays, we used a mixture of four genetically distinct *Daphnia magna* (hereafter *Daphnia*) clones originating from Katholieke Universiteit Leuven, Belgium that we had reared in the lab since winter 2010 using methods described in Brogan and Relyea (2013a,b). Approximately 1 month prior to the start of our experiment, we stopped culling the populations and pooled individuals from all *Daphnia* families together in a 15-L container so that we could generate enough gravid females to use < 24-h old neonates for our 48-h survival test. No ephippia were observed in our *Daphnia* cultures at any point within 2 months prior to our experiment.

We collected and cultured *Elodea canadensis* (hereafter *Elodea*) from three artificial ponds located in northwestern Pennsylvania, USA (41°35'19.12"N, 80°14'40.61"W) on 15 June. More detailed descriptions of the ponds and culturing methods for *Elodea* are described in Brogan and Relyea (2013a,b). We cultured the plants for 25 d before adding them to the experiment.

4.2.4 Experimental setup

The experimental units were 0.95-L glass jars containing 700 mL of UV-filtered well water. On 9 July, we set up all 80 jars and added *Elodea* shoots to the appropriate treatments. To set up the treatments containing plants, we harvested the top 12 cm of *Elodea* shoots in our culturing tanks and rubbed each shoot under running well water to remove all visible periphyton and invertebrates. We then blotted each plant shoot dry with paper towels and added 5.7 g of *Elodea* (fresh weight) to all appropriate jars. This produced an average dry weight biomass ~ 800 g

DW/m^3 in our experimental jars; this represents a high, but realistic, submerged plant biomass for freshwater ecosystems (Duarte and Kalff 1990, Hopson and Zimba 1993).

Because the goal of this experiment was to distinguish the mitigating influence of *Elodea's* natural effects on water via changes in pH versus other mechanisms such as sorption, we performed the experiment outdoors where plants would be exposed to natural daily fluctuations in light and temperature. To do this, we placed the experimental testing jars in sideways-oriented aquaria, which kept out rainwater, that were set in 300-L wading pools positioned on wooden tables. After placing the jars and aquaria in the wading pools, we filled each pool with approximately 100 L of cool well water. This water served as a buffer to prevent the temperature of the water inside each jar from fluctuating widely throughout the day and night. While our experimental setup deviates from standardized protocols used in traditional toxicological tests (e.g., United States Environmental Protection Agency 1996), this was necessary for us to examine the impact that submerged plants have on insecticide toxicity under semi-realistic environmental conditions.

After placing the jars outdoors, we created the shaded and unshaded-plant treatments for jars containing *Elodea*. Unshaded plants were simply kept uncovered and exposed to natural sunlight levels in the glass aquaria. These jars were randomly assigned to aquaria throughout our experimental array. However, the jars assigned to the shaded-plant treatment were all placed together in two sideways-oriented aquaria. The aquaria were covered on all sides with bed sheets and wrapped in aluminum foil to prevent any sunlight from reaching the jars contained within. In this way, we were able to allow the plants to interact with the water in each jar while preventing the plant from photosynthesizing and causing an increase in water pH. We allowed the plants to acclimate to these testing conditions for 4 d before chemically manipulating pH in the jars

lacking plants and subsequently dosing the experiment with malathion.

We recognize that randomly placing all of the shaded-plant treatment jars into two shaded aquaria does not satisfy the conditions of a complete randomized experimental design. However, we used this approach because individually shading each shaded-plant treatment jar (e.g., by wrapping each jar in aluminum foil or a similar material) would have made applying malathion and sampling each jar logistically very difficult to accomplish in an appropriate time frame. Further, the testing conditions to which these and all other jars were exposed were virtually identical as the pools were all on tables right next to each other outside. Thus, compared to the impacts of the treatment manipulations, we do not expect that the physical location of the jars had a significant influence on the response of *Daphnia* to the malathion treatments.

At 0900 h on 13 July, we began manipulating water pH in the jars lacking plants. To create low-pH treatments, we added 1M hydrochloric acid (HCl) while monitoring pH using a calibrated digital pH meter (Oakton Instruments) until the pH stabilized within 0.1 pH units of 7.5. After creating all low-pH treatments, the pH meter was rinsed thoroughly and recalibrated. We then created high-pH treatments by adding 1M sodium carbonate (i.e. soda, Na_2CO_3) while monitoring pH until it stabilized within 0.1 pH units of 9.5. These jars were then randomly assigned to aquaria in our experimental array. The pH targets of 7.5 and 9.5 are within the range of natural pH values in lentic systems and were designed to represent aquatic habitats containing little or no vegetation and habitats characteristic of high submerged plant densities, respectively (e.g., Ondok et al. 1984, Frodge et al. 1990).

4.2.5 Sampling abiotic variables

After chemically manipulating water pH in jars containing no plants, we recorded the aqueous pH of all jars from 1100 h to 1300 h (hereafter referred to as "-1 h" relative to malathion treatment applications). To monitor changes in pH over time, we also recorded pH 24 and 48 h after applying malathion. In addition, we measured the dissolved oxygen concentration (DO) and temperature of each jar using a calibrated water quality probe (YSI Inc.) both before (i.e. -1 h) and 48 h after applying malathion (see Appendix A for statistical procedure and results for DO and temperature). To prevent significant amounts of sunlight from reaching plants located in the shaded-plant treatments during sampling of abiotic variables, we removed one shaded jar at a time from the covered aquarium and placed it in an opaque box to record pH, DO and temperature.

4.2.6 Malathion applications

On 13 July, we applied technical grade malathion (99.1% purity; Chem Service Inc.) to each appropriate jar. We used technical grade malathion instead of commercial formulations containing inert ingredients because the goal of this study was to specifically understand how water chemistry affects the toxicity of the active ingredient. To achieve nominal concentrations of 0, 1, 5, 10 and 50 μg/L, we added 0, 0.006, 0.03, 0.06, or 0.3 mL, respectively, of malathion stock solution (0.123 mg malathion/mL ethanol) to clean, 100-mL glass jars containing 50 mL of UV-filtered water to make our working solutions. We mixed the malathion working solutions thoroughly and poured the contents into a corresponding experimental jar to bring the total volume of each test system to 750 mL. This approach ensured adequate mixing of the insecticide

throughout the water column in each jar. We did not perform a control for the ethanol carrier in this experiment because other studies have demonstrated that there should be no adverse effects of ethanol on *Daphnia* at higher concentrations (0.5 mL ethanol/L water) than those used in our study (0.4 mL ethanol/L water; Kast-Hutchinson et al. 2001). In addition, we have included ethanol controls in other studies employing similar experimental designs in which 1 mL ethanol/L water had no adverse effects on *Daphnia* survival (Brogan III, WR and Relyea, RA *unpublished data*).

At 1400 h, we began applying one malathion concentration treatment at a time, starting with 0 μg/L and working up in concentration. To quantify the actual concentration of malathion achieved for each treatment, we applied 50 mL of working solution to each of two additional glass jars containing 700 mL of UV-filtered water. We then poured 450 mL of this water into 500-mL pre-cleaned amber glass jars and stored the jars in a 3°C refrigerator until analysis. On 16 July (3 d after dosing), we sent the jars in coolers of ice to an independent laboratory (University of Georgia Agricultural and Environmental Services Laboratory) for extraction and analysis using GC/MS. The actual malathion concentrations corresponding to the nominal concentrations of 0, 1, 5, 10, and 50 μg/L were 0, 0.75, 3.6, 6.3, and 35.6 μg/L (hereafter referred to as 0, 1, 4, 6, and 36 μg/L). The discrepancy between the actual and nominal malathion concentrations is likely the result of some breakdown of the insecticide in the 4 d between the water collection and extraction of the insecticide from the water samples. Thus, our reported malathion concentrations may differ from the actual concentrations that the *Daphnia* experienced in the jars, so they should be interpreted as approximate values representing distinct treatments as opposed to exact concentrations for inclusion in formal toxicity assessments for regulatory purposes.

4.2.7 *D. magna* 48-h survival assays

After applying malathion to all jars of each malathion treatment, we added 10 *Daphnia* to each jar in the same order in which we applied the insecticide before moving on the next malathion treatment. Thus, *Daphnia* were added to each jar within 5 min of malathion's application. After 48 h (i.e. at 1400 h on 15 July), we terminated the experiment. Starting with the 0 µg/L malathion treatment and working up in concentration, we recorded the final abiotic data from each jar and then brought the jars indoors for processing. We quantified the number of surviving *Daphnia* in each jar using a transfer pipet to gently blow water over each *Daphnia* individual. We counted an individual as alive if it responded during three applications of the stimulus by swimming vertically in the water column. For jars containing *Elodea*, we first removed the plants and carefully transferred them to containers of clean water. We inspected these containers as well as the original jars and recorded the number of surviving *Daphnia*.

4.2.8 Statistical analysis

To examine the effects of our treatments on pH over the course of the experiment, we performed univariate ANOVAs on pH data from before malathion was applied (-1 h), as well as 24 and 48 h after malathion applications. Because no malathion treatments had been applied prior to dosing, we performed a one-way ANOVA on pH at -1 h, with plant-pH treatments as a fixed factor. However, pH sampled 24 and 48 h after malathion applications were analyzed using a full factorial two-way ANOVA model with malathion and plant-pH treatments as fixed factors. When appropriate, we performed Tukey's multiple comparisons tests to compare the effects of

each treatment on our response variables. All abiotic data met the assumptions of general linear models.

To determine the effects of plant-pH treatments on malathion's toxicity to *Daphnia,* we performed a two-way univariate ANOVA on rank-transformed, 48-h survival. Where significant interactions occurred, we used Student-Newman-Keuls test to compare *Daphnia* survival among treatments. In these analyses, we excluded one experimental unit (treatment: shaded-plant) because we discovered a larval damselfly had gotten into the jar and caused very low *Daphnia* survival (20%) compared to the other replicates of this treatment, which had high survival (90%).

4.3 RESULTS

4.3.1 Treatment effects on pH before malathion applications

Univariate ANOVAs revealed significant effects of plant-pH treatment on water pH 1 h before malathion applications ($F_{3,76} = 854.5$, $p < 0.001$). In comparing the plant-pH treatments at -1 h, Tukey's tests revealed water pH in the no-plant/low-pH treatment did not differ from the shaded-plant treatment (Fig. 4.1; $p = 0.217$). However, the pH in the no-plant/high-pH treatment was slightly (~ 4%) lower than the unshaded-plant treatment ($p < 0.001$). More importantly, the no-plant/high-pH and unshaded-plant treatments had much higher pH levels than the other two treatments ($p < 0.001$).

4.3.2 Treatment effects on pH after malathion applications

After applying malathion, we again sampled pH at 24 and 48 h to track any treatment-specific changes in pH and to determine whether adding malathion influenced water pH. At 24 and 48 h after malathion applications, univariate ANOVAs revealed significant effects of plant-pH treatment on water pH ($F_{3,60} > 195.2$, $p < 0.001$) but no effect of malathion ($F_{4,60} < 1.8$, $p > 0.131$) or the malathion by plant-pH interaction ($F_{12,60} < 1.3$, $p > 0.256$). In both samples, pH levels in the treatments were (from lowest to highest): shaded-plant < no-plant/low pH< no-plant/high pH< unshaded-plants. While all differences between treatments were significant (Fig. 4.1, $p < 0.01$), the difference in pH between the two low-pH treatments or between the two high-pH treatments were relatively small compared to the difference in pH between the low- and high-pH treatments.

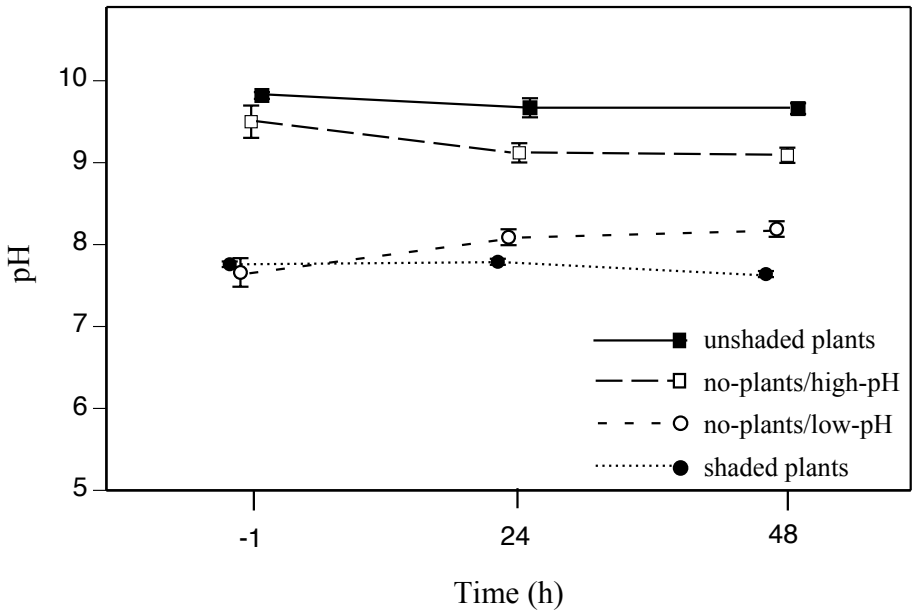

Figure 4.1. The effects of plant and chemical pH treatments on water pH in experimental jars over time. Data are means ± 1 SE.

4.3.3 Treatment effects on malathion's toxicity to *D. magna*

The primary goal of the experiment was to examine the influence of water pH and plant presence on malathion's toxicity to *Daphnia*. We discovered that *Daphnia* 48-h survival was affected by plant-pH treatment ($F_{3,59} = 14.7$, $p < 0.001$), malathion concentration ($F_{4,59} = 28.0$, $p < 0.001$), and their interaction ($F_{12,59} = 2.4$, $p = 0.013$). Because of this interaction, we compared *Daphnia* survival across plant-pH treatments within each malathion concentration. In the presence of 0 and 1 μg/L of malathion, *Daphnia* 48-h survival was equally high across all of the plant-pH treatments (Fig. 4.2, $p > 0.799$). However, in the presence of intermediate malathion concentrations (4 and 6 μg/L), *Daphnia* survival was 62% to 78% higher in the no-plant/high-pH and unshaded-plant treatments, respectively, than in no-plant/low-pH or shaded-plant treatments

($p < 0.007$). Further, in the presence of both 4 and 6 µg/L of malathion, *Daphnia* survival did not differ between no-plant/high-pH and unshaded-plant treatments ($p > 0.786$), nor between no-plant/low-pH and shaded-plant treatments ($p = 1.0$), respectively. At malathion concentrations of 36 µg/L we did not observe differences in *Daphnia* 48-h survival between any plant-pH treatments ($p > 0.117$); fewer than 50% of *Daphnia* survived in each of the plant-pH treatments. However, while the higher variance in *Daphnia* survival at 36 µg/L precluded statistical significance, it is worth noting that the trend of higher *Daphnia* survival in the no-plant/high-pH and unshaded-plant treatments, compared to the two low-pH treatments, was still consistent.

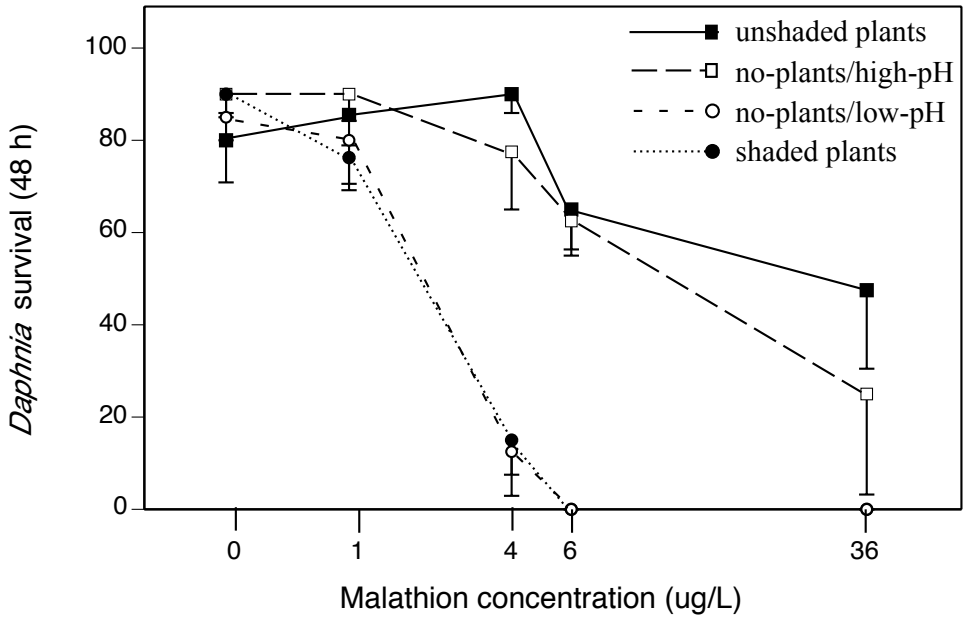

Figure 4.2. *Daphnia magna* 48-h survival following exposure to five malathion concentrations in the presence of four plant and chemical pH treatments. Data are means ± 1 SE. Note that the *x* axis is using a log scale (i.e. log [concentration +1]).

4.4 DISCUSSION

In this experiment, we examined the primary mechanism driving the mitigating effects of submerged plants on the toxicity of the widely used insecticide malathion to sensitive animals. By separately manipulating pH levels and the presence of plants, we were able to compare the magnitude of the mitigating effects of plant-elevated pH alone versus other plant-insecticide interactions such as sorption. Overall, we were able to achieve very similar pH values within 0.1 standard units in the no-plant/low-pH and shaded-plant treatments and within 0.32 units in the no-plant/high-pH and unshaded-plant treatments, respectively, prior to applying malathion. The difficulty in precisely matching the pH levels in the latter treatments occurred because of our uncertainty in predicting how much the pH in the unshaded-plant treatments would increase due to photosynthesis throughout the day.

Despite this challenge, we found strong support for our hypothesis that malathion's toxicity to *Daphnia* would be mitigated by increases in pH. We discovered that relative to treatments where pH was kept low via the addition of HCl or by blocking plant photosynthesis via shading, simply increasing water pH using Na_2CO_3 reduced malathion's toxicity to *Daphnia* to the same degree as when photosynthetically active submerged plants were present. We also found support for our hypothesis that shaded plants would not mitigate malathion's toxicity. Our results suggests that the mitigating effect of submerged plants on malathion's toxicity could be predicted entirely based on the pH of the water and that sorption played no role in mitigation, a finding that is consistent with malathion's low binding affinity to organic substrates (log K_{ow} = 2.75), and its demonstrated weak sorption to plants (Gao et al. 2000b).

While we did not measure specific degradation pathways in our study, alkaline hydrolysis is the most likely mechanism driving malathion's detoxification in high pH environments (Wolfe

et al. 1977, Seaman and Riedl 1986). It is possible that other mechanisms, such as photolysis, played a role in malathion's detoxification but in the only study to our knowledge that has examined malathion's breakdown via direct sunlight in natural waters, it took 16 h to degrade only 50% of the insecticide (Wolfe et al. 1977). Compared with malathion breakdown rates caused by alkaline hydrolysis (half-life < 2.5 h; Wolfe et al. 1977, Seaman and Riedl 1986), processes such as photolysis appear to be of relatively low importance. Further, a review of pesticide degradation pathways by Burrows et al. (2002) suggests that, in general, the direct photodegradation of most insecticides is a relatively minor breakdown pathway in aquatic environments.

Our study shows that we do not need to invoke the mechanism of sorption to explain the mitigating effects of aquatic plants on all insecticides in surface water. Instead, we found that for insecticides like malathion, water chemistry can play a primary role in mitigating toxicity. This finding challenges current models that primarily use an insecticide's K_{ow} value to predict the influence of aquatic plants on its fate and environmental effects (AQUATOX, CATS; Park et al. 2008). While most of these models do include abiotic water parameters like pH, no models to our knowledge incorporate the influence of abiotic conditions on the removal of contaminants from the water by processes such as hydrolysis. Thus, these models may be missing an important insecticide degradation pathway in aquatic ecosystems.

In the United States, surface water pH values exceeding 8 are common (based on average daily pH values at ~ 17:30 h from United States Geological Survey real-time surface water quality monitoring data; http://waterwatch.usgs.gov/wqwatch), suggesting that pH-mediated insecticide mitigation may be a widespread phenomenon. While freshwater ecosystems containing high densities of submerged plants often contain high pH (Ondok et al. 1984, Barko

and Godshalk 1988, Frodge et al. 1990), highly eutrophied waters dominated by phytoplankton (or periphyton) can also posses high pH (e.g., Talling 1976, Toivonen and Huttunen 1995). This is important as many eutrophied aquatic environments receiving high nutrient inputs from runoff may also experience exposure to high insecticide concentrations. However, it is also important to note that well-buffered waters possessing high alkalinity (i.e. containing high concentrations of bicarbonates and carbonates) can be resistant to fluctuations in pH, even during periods of intense photosynthesis (Wurts and Durborow 1992, Wetzel 2001). Our findings suggest that malathion's toxicity should be mitigated in high pH environments (compared to neutral or acidic waters), regardless of the mechanism by which high pH is achieved. A critical next step will be testing this prediction while controlling for confounding factors that may also alter malathion's toxicity or the effects of pH on malathion's toxicity.

Our results could also improve current agricultural best management practices (BMPs). BMPs typically contain high densities of emergent plants for sorbing insecticides and other contaminants from the water (Cooper et al. 2004, Kröger et al. 2009). These treatment systems are capable of reducing concentrations of many insecticides, but especially those with relatively high log K_{ow} values (Moore et al. 2011). However, as emergent plants perform gas exchange with the air and not the water, aqueous pH levels in these mitigation systems are likely to remain relatively low (i.e. pH < 8, Wetzel 2001). Our results suggest that for insecticides like malathion, which posses low K_{ow} values but are hydrolysable under alkaline conditions, a more effective mitigation strategy may be to construct sections of BMPs to either contain submerged vegetation or at least elevated pH levels. Examining the efficacy of implementing such changes to current mitigation systems is an area in need of further research.

In addition to malathion, waters containing high pH may mitigate other widely used

insecticides that are known to undergo relatively rapid alkaline hydrolysis (e.g., carbaryl and carbofuran; Wolfe et al. 1978, Chapman and Cole 1982). However, this is not necessarily a mechanism that will be generalizable for all pesticides. For example, many of the increasingly used pyrethroid insecticides have breakdown rates that differ very little across a wide range of pH levels (National Pesticide Information Center; http://npic.orst.edu/ingred/aifact.html). Even other insecticides within the same chemical class as malathion (e.g., chlorpyrifos) can persist for weeks under alkaline conditions (Christensen et al. 2009) and their toxicity appears to be largely unaffected by the presence of submerged plants (Brock et al. 1992). Further, some insecticides, such as the organophosphate compound diazinon, actually persist longer in water under alkaline conditions and break down faster under acidic conditions (Harper et al. 2009). For insecticides like diazinon, it is reasonable to predict that submerged plants (and high pH in general) may have the opposite effect on toxicity to invertebrate taxa than we observed for malathion. Clearly, more research is needed to understand how ecological complexity can influence the toxicity of insecticides so that we can better predict and mitigate their effects moving forward.

Although our results suggest that managing aquatic ecosystems for high pH (by promoting submerged plants or via other methods) may be an appropriate strategy for minimizing impacts of some contaminants in certain cases, it is important to consider the full biological and economic consequences of management strategies before employing them. In general, most aquatic animals appear to be tolerant to alkaline conditions but high pH levels can be toxic to some species. For example, high pH can inhibit normal sodium channel function and result in toxic elevations in blood ammonia levels in several fish species (Wright et al. 1989, Ip et al. 2001, Scott et al. 2005). Further, high water pH can increase the toxicity of some metal contaminants to aquatic animals (e.g., copper, zinc, and cadmium; Cusimano et al. 1986).

Clearly, before any management strategy is chosen, the potential biological impacts of increasing pH need to be carefully considered. Further, the economic costs (i.e. recreational, aesthetic, etc.) of managing for high pH need to be evaluated by land managers and stakeholders before selecting a strategy. Given the potentially widespread applications of our research, future studies evaluating the costs and benefits of different management approaches are needed.

4.5 ACKNOWLEDGEMENTS

We would like to thank Kelly DeRolf and Dr. Andrew Turner for their assistance and input. This research was funded by a NSF Doctoral Dissertation Improvement Grant (DEB 12-09084), a Society for Freshwater Science General Endowment award, and a G. Murray McKinley grant to WRB and a NSF grant to RAR (DEB 11-19430).

5.0 SUBMERGED MACROPHYTES MITIGATE DIRECT AND INDIRECT INSECTICIDE EFFECTS IN FRESHWATER COMMUNITIES

5.1 INTRODUCTION

A contemporary challenge facing ecologists and ecotoxicologists is to elucidate factors that can mitigate anthropogenic contaminant impacts in aquatic ecosystems. Traditionally, the ecological effects of contaminants, such as insecticides, are based on laboratory toxicity studies using a small number of test species and then extrapolated to entire communities (Newman 2010). However, such tests are designed to eliminate sources of environmental variation (e.g., Organisation for Economic Co-operation and Development 1984, ASTM International 2007), but accumulating evidence suggests this can lead to discrepancies between predicted and actual insecticide effects in nature (Fleeger et al. 2003, Relyea and Hoverman 2006, Forbes et al. 2008).

One common cause of discrepancy between predicted and actual insecticide effects is the influence of ecological interactions. For example, in freshwater communities, natural stressors such as competition and predation can exacerbate direct insecticide toxicity (Hanazato 2001, Boone and Semlitsch 2001, Kieseker 2002, Boone et al. 2007). Further, insecticides can affect the growth and survival of relatively resistant species at concentrations that traditional toxicity tests predict should have no effect through indirect trophic interactions. For example, low concentrations of many insecticides decimate cladoceran zooplankton, initiating trophic cascades

that cause phytoplankton blooms. As the phytoplankton bloom, they shade the benthos, reduce periphyton growth, and adversely affect benthic grazer growth and survival (Mills and Semlitsch 2004, Relyea and Diecks 2008, Relyea and Hoverman 2008). While a preponderance of studies in aquatic ecosystems have targeted factors that exacerbate insecticide effects, comparatively few studies have identified factors that might mitigate these effects.

Growing evidence suggests that submerged macrophytes can mitigate insecticide effects in aquatic ecosystems. For example, Brogan and Relyea (2013a) demonstrated that realistic densities of a cosmopolitan submerged macrophyte, Canadian waterweed (*Elodea canadensis*), caused up to nine-fold reductions in the toxicity of the insecticide malathion to the *cladoceran Daphnia magna* in small (0.95-L) outdoor jars. Moreover, each increase in macrophyte density caused greater mitigation. We also understand the mechanism of this mitigation; as submerged macrophytes photosynthesize, they increase water pH by taking up CO_2, which decreases carbonic acid concentration and shifts the bicarbonate buffer system towards the more alkaline bicarbonate and carbonate (Wetzel 2001). This higher pH causes malathion to degrade more rapidly via alkaline hydrolysis (Wolfe et al. 1977, Brogan and Relyea 2014). However, as the mitigating effects of submerged macrophytes on insecticide toxicity have thus far only been documented at the microcosm scale, a critical next step is to examine whether these effects occur in more spatially and ecologically complex communities.

In addition to mitigating insecticide direct effects, submerged macrophytes may also dampen the indirect cascading effects of insecticides in freshwater communities. For example, macrophytes can suppress phytoplankton growth via allelopathy (Hilt and Gross 2008) and aqueous nutrient competition (Sand-Jensen and Borum 1991, van Donk and van de Bund 2002). Thus, even if zooplankton decline following insecticide exposure, submerged plants may still

prevent phytoplankton blooms. While predicting the impacts of macrophytes on phytoplankton is relatively straightforward, their effects on periphyton and grazers (e.g., snails and larval amphibians) are still poorly understood. For example, to our knowledge, no studies have examined the impact of submerged macrophytes on the growth or survival of larval amphibians. Thus, there is a need for studies examining the influence that macrophytes have on grazers, particularly in the presence of perturbations like insecticides.

When examining the influence of macrophytes on insecticide effects in aquatic communities, there is also a need to consider different insecticide-exposure scenarios. For example, depending on weather patterns and application frequencies, insecticide exposure in aquatic communities can occur either as single "pulse" or repeated "press" events (*sensu* Bender and Case 1984, Yodzis 1988, Paine et al. 1998). However, studies examining community responses to different insecticide exposure scenarios have received little attention. In the few studies investigating the ecological effects of pulse and press insecticide perturbations, press exposures have ecological effects that are longer lasting and many times larger in magnitude than pulse perturbations (Hanazato and Yasuno 1990, Boone et al. 2001, Relyea and Diecks 2008). While submerged macrophytes can mitigate pulse insecticide applications in microcosm studies (Brogan and Relyea 2013a,b), their ability to mitigate the effects of recurring press exposures has never been examined. Clearly, considering different exposure regimes is critical for understanding the factors influencing realistic insecticide impacts in freshwater communities.

To address these gaps in our understanding, we examined the mitigating role of a range of natural macrophyte densities in freshwater communities containing phytoplankton, periphyton and 22 species of animals (zooplankton, snails, and larval amphibians) during several realistic insecticide-exposure scenarios. We used the organophosphate insecticide malathion (Diethyl 2-

dimethoxyphosphorothioylsulfanylbutanedioate) because it is one of the most commonly used active ingredients in the U.S. (Grube et al. 2011), with 10-14 million kg applied annually (Kiely et al. 2004, National Pesticide Use Database, www.ncfap.org/database/national.php). Despite malathion's popularity, few studies have examined its ecological effects in non-target communities. We hypothesized that the magnitude of malathion's direct and indirect effects would increase with the number of insecticide exposure events (control < pulse < press) and that these effects would decrease with increasing submerged macrophyte density.

5.2 METHODS

5.2.1 Experimental design

The experiment was conducted at University of Pittsburgh's Pymatuning Laboratory of Ecology. We used a completely randomized, factorial design crossing four macrophyte densities (0, 10, 50, and 100 *E. canadensis* shoots initially planted) with three malathion exposure scenarios (no insecticide, a single application, and repeated applications every three wks). The 12 treatment combinations were replicated four times for a total of 48 experimental units.

5.2.2 Experimental setup

The experimental units were outdoor 1,200-L mesocosms containing 850 L of well water. On 2 May, we added 95 L of sediment to each mesocosm and on 28 to 31 May, we manually planted the appropriate number of macrophyte shoots in each mesocosm, simulating planting in the 0-

macrophyte treatment. The macrophytes were collected and mixed from three local wetlands. Prior to adding them to the mesocosms, we placed the collected plants in 200-L wading pools containing well water and sediment for 2 wks to allow any attached invertebrate eggs to hatch.

On 21 May, we established microbial, algal and zooplankton communities in each mesocosm. To do this, we used a zooplankton tow to collect water from the same ponds where we had collected the macrophytes, combined the water samples, and removed all predatory invertebrates. We then added 200-mL aliquots of this water to each mesocosm. On 26 May, we added five unglazed, vertically oriented clay tiles (10 x 10 cm) to the north side of each mesocosm to serve as periphyton samplers.

Next, we collected and added larval amphibians to our mesocosms. From 28 to 29 May we collected 30 pairs of breeding gray treefrogs (*Hyla versicolor*) and placed them into individual containers to oviposit. We then mixed the resulting eggs and moved them to 200-L wading pools containing aged well water. Once hatched, we fed the tadpoles *ad libitum* until reaching an appropriate handling size (~10 mg). On 16 June (defined as day 0 of the experiment), we added 20 gray treefrog tadpoles to each mesocosm. The densities of 10 tadpoles/species/m^2 are well within natural densities (Morin 1983, R.A. Relyea, E.E. Werner, D.K. Skelly, K.L. Yurewicz, *unpublished data*). We also set aside 20 tadpoles for staging (all tadpoles were at Gosner stage 25; Gosner 1960) and weighing (mass ± 1 SE: 11.4 ± 0.6 mg). In addition, we assessed 24-hr survival of 20 tadpoles following handling (survival was 100%).

To represent grazer communities commonly found in wetlands, we also added freshwater snails to the mesocosms. On 5 May, we collected pond snails (*Physa acuta* and *P. gyrina*, which can only be differentiated by dissecting their internal genitalia) and rams horn snails (*Helisoma trivolvis*) from local ponds. To prevent adult snail endoparasites from being introduced to the

mesocosms, the snails used in the experiment were hatched from eggs of the snails collected from local ponds and were cultured in clean well water in 200-L wading pools. On 17 June (day 1), we sorted all hatched pond snails into small (< 10 mg), medium (10 to 20 mg), and large (> 20 mg) size classes. We added five pond snails from each size class to each mesocosm. On 24 June (day 8), we sorted rams horn snails into small (< 100 mg) and large (> 100 mg) size classes (range = 17 to 211 mg) and added 4 small and 3 large rams horn snails to each mesocosm. While these snail densities are considerably lower than what can occur in wetlands in western Pennsylvania, (A.M. Turner, *unpublished data*), we added the maximum number possible to each mesocosm given the lower-than-expected number of hatchlings produced during culturing. We also assessed the 24-h survival of pond snails and rams horn snail following handling; we found 100% survival for each taxa.

5.2.3 Insecticide applications

Once all animals were added, we did not disturb the mesocosms for 10 d. On 3 July (day 19), we applied the insecticide treatments using technical grade malathion (99.1% active ingredient; Chem Service Inc., West Chester, Pennsylvania, USA). Our original target concentration was 18 μg/L, which is well within the US Environmental Protection Agency's estimated environmental concentration (EEC) for surface waters (0 to 36 μg/L; Odenkirchen and Wente 2007). However, 3 d after applying malathion, we assessed zooplankton abundance in 0-macrophyte treatments and found that the insecticide reduced cladoceran abundance, but not significantly (see Results). Given that one of our goals was to determine if the macrophyte could mitigate the toxic effects of malathion on zooplankton, we decided to double the nominal concentration to 36 μg/L (which

is still within the range of the EPA's EEC values) and applied this concentration to the appropriate mesocosms on 17 July (day 37).

To achieve nominal concentrations of 36 μg/L in our tanks, we dissolved 0.88 mL of technical grade malathion (specific gravity = 1.23 g/mL) in 25 mL of ethanol to make a stock solution of (0.042 g/mL). We then added 0.71 mL of this stock solution to each appropriate mesocosm (average volume = 850 L). We elected not to apply an ethanol control because the concentrations we used have had no effect on any taxa in similar, previous experiments (Relyea and Diecks 2008, Relyea and Hoverman 2008, Relyea 2009). After applying malathion, we gently mixed the water in the mesocosms to simulate mixing that would occur during a runoff event. We also mixed control mesocosms to standardize disturbance. Whereas 36 μg/L of malathion was applied only on day 37 in the pulse treatment, we repeated this application procedure on days 55 and 73 for the press treatment. Given malathion's rapid breakdown rate in water ($t_{1/2}$ = 48 h at pH 8; Wang et al. 1991), each application in the press treatment represented a new exposure to our nominal malathion concentration.

We collected water samples within 1 hr of application. Because of the high costs of insecticide analysis, we pooled water collected from the center of the water column of each mesocosm within a given insecticide treatment into 500 mL pre-cleaned amber glass jars. Because pulse and press treatments had received identical malathion applications to this point, we pooled water from these treatments to compare with the control. Immediately after collection, we stored all water samples in a refrigerator kept just above freezing (3°C) until analysis.

The water samples were sent for analysis to an independent testing laboratory (Mississippi State Chemical Laboratory, Mississippi State, Mississippi, USA) on 17 August (38 d after application). The actual concentration for the nominal 36 μg/L sample was reported to be

below the lower detection limit (0.1 μg/L). Further, the control sample revealed a trace amount of the insecticide present (0.156 ug/L). Though actual malathion concentrations in mesocosm experiments are often substantially lower (~ 40% lower) than nominal concentrations (e.g., Relyea and Diecks 2008, Relyea and Hoverman 2008, Relyea 2009, Distel and Boone 2010), our concentrations were lower than typical. One explanation for this discrepancy is that, despite being stored in the dark at 3°C, the malathion broke down during the 38+ d that elapsed between water sample collection and extraction into an organic solvent by the testing laboratory.

While we recognize that our protocols do not meet standards for formal toxicological assessments, our experiment was not designed to determine the impact of a specific malathion concentration on aquatic communities. Instead, our experiment was designed to examine the extent to which submerged macrophytes could mitigate the direct and indirect effects of malathion in freshwater communities. Based on the biological responses that we observed in our study, our malathion applications were sufficiently high to achieve this goal.

5.2.4 Response variables

Throughout the experiment we sampled abiotic variables (aqueous pH, dissolved oxygen (DO), light decay rate, temperature) and biotic variables including the density of each major zooplankton group (cladocerans, copepods, rotifers), phytoplankton abundance (measured as chlorophyll *a*), and periphyton mass several times throughout the experiment using approaches described in Relyea and Diecks (2008) and explained in detail in Appendix B. Although each round of sampling took at least 3 d to complete, we hereafter identify samples by the day that sampling began (i.e. days 26, 47, 68, and 100).

To determine the influence of our treatments on periphyton grazers, we measured snail

abundance and amphibian survival and growth (see Appendix B). We assessed pond snail and rams horn snail abundance on day 68. We also quantified gray treefrog survival, time to metamorphosis, and mass at metamorphosis. The first gray treefrog metamorph emerged on day 30, just 13 d after the initial 18 μg/L malathion application (thus, no indirect effects of malathion were expected on gray treefrogs); the final metamorph emerged on day 94.

Finally, we quantified macrophyte density just before taking down the experiment (day 320). To do this, we placed a stovepipe sampler (*r* x *h*, 0.008 x 0.031 m) in the middle of each mesocosm to standardize the area sampled. We then removed all macrophytes from within the stovepipe, rinsed the macrophytes to remove attached algae, and dried the plants for 24 hrs at 60°C. We then weighed the plants to determine their dry mass.

5.2.5 Statistical analysis

We used general linear models (GLM) to analyze the data from this experiment. To analyze the effects of initial macrophyte density and insecticide treatment on final macrophyte density, we performed a two-way ANOVA. To analyze the effects of the treatments on abiotic response variables over time, we performed a two-way repeated-measures multivariate analysis of variance (rm-MANOVA) on pH, DO, temperature and light decay. To analyze treatment effects on biotic response variables over time, we performed a two-way rm-MANOVA on cladoceran, copepod, and rotifer density, phytoplankton abundance (chlorophyll *a*), and periphyton biomass. When we found significant multivariate effects, we explored the univariate effects on each response variable using two-way rm-ANOVAs. When significant univariate time-by-treatment interactions were detected, we examined treatment effects within each time point using two-way ANOVAs. Where appropriate, we used Tukey's test for post-hoc comparisons. This hierarchical

approach allowed us to control overall experiment-wise error when performing multiple rm-ANOVAs and subsequent ANOVAs. When necessary, we log (+1) transformed our data to meet the assumptions of GLM.

To analyze the effects of the macrophyte and insecticide treatments on snails, which were only measured at a single time point, we performed a two-way MANOVA on log-transformed *Physa spp.* and *H. trivolvis* snail abundance. We performed a separate two-way MANOVA on gray treefrog survival (arcsine-transformed), time to metamorphosis, and mass at metamorphosis. We examined all significant multivariate treatment main effects and interactions using subsequent two-way ANOVAs and Tukey's mean comparison tests.

5.3 RESULTS

5.3.1 Macrophyte density and abiotic variables

In general, we found strong effects of initial macrophyte density on final macrophyte density and on the abiotic environment in our mesocosms. By the end of the experiment, the 10- and 50-macrophyte treatments no longer differed in density, but both contained about 50% less biomass than the 100-macrophyte treatment. In regard to the abiotic effects, the addition of macrophytes generally had no effect on temperature, increased DO, and maintained lower light decay rates relative to mesocosms containing no macrophyes (see Appendix C for full results and figures for macrophyte density and abiotic variables). Because pH is the primary mechanism by which plants mitigate malathion's toxicity (Brogan and Relyea 2014), we discuss only the results for pH further here.

We observed a significant time-by-macrophyte interaction on pH ($F_{9,108} = 8.4$, $p < 0.001$). At each sample date, macrophyte treatment had a significant effect on pH ($F_{3,36} = 13.5$, $p < 0.001$). At day 26, pH in the 10-macrophyte treatment was 9% greater than the 0-macrophyte treatment (Fig. 5.1, $p = 0.001$), but 9% less than the 50- and 100-macrophyte treatments (all $p < 0.001$), which did not differ from each other (all $p > 0.9$). On each subsequent sample date, pH in the 10-, 50- and 100-macrophyte treatments was at least 10% higher than the 0-macrophyte treatment (all $p < 0.002$) and did not differ from each other (all $p > 0.078$).

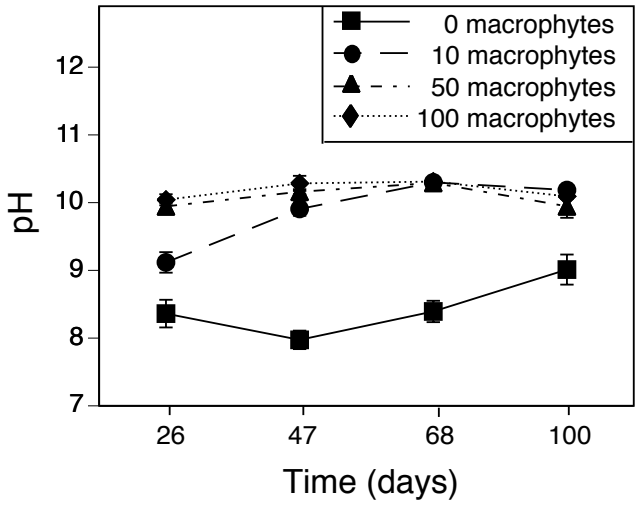

Figure 5.1. The effect of macrophyte density on pH over time. Data are means ± 1 SE.

5.3.2 Biotic variables

The rm-MANOVA on cladoceran, copepod and rotifer densities, phytoplankton abundance, and periphyton biomass showed significant effects of macrophytes, insecticides, time, and all

interactions (Table 5.1). As a result, we separately examined the time and treatment effects on each biotic response variable using two-way rm-ANOVAs (Table 5.2).

Table 5.1. Results of a repeated-measures MANOVA showing the effects of time, macrophyte density, malathion treatment, and their interactions on all biotic response variables. Bold p-values are significant at $\alpha = 0.05$.

Multivariate test (Wilks' lambda)	**df**	***F*-value**	***p*-value**
Macrophyte (M)	15, 89	5.4	**< 0.001**
Insectiide (I)	10, 64	3.3	**0.002**
M x I	30, 130	2.1	**0.002**
Time (T)	15, 288	15.3	**< 0.001**
T x M	45, 468	2.8	**<0.001**
T x I	30, 418	1.8	**0.006**
T x M x I	90, 509	1.6	**0.001**

Table 5.2. Results of repeated measures ANOVAs showing the effects of time, macrophyte density, malathion treatment, and their interactions on each biotic response variable. Bold p-values are significant at $\alpha = 0.05$.

		Cladocerans	**Copepods**	**Rotifers**	**Phytoplankton**	**Periphyton**
Univariate tests	**df**	***p***	***p***	***p***	***p***	***p***
Macrophyte (M)	3,36	**0.002**	0.147	**< 0.001**	**< 0.001**	0.207
Insecticide (I)	2,36	**< 0.001**	0.431	0.146	0.094	0.446
M x I	6,36	**< 0.001**	0.506	0.332	**0.028**	0.860
Time (T)	3,108	**<0.001**	**< 0.001**	**< 0.001**	**0.026**	**< 0.001**
T x M	9,108	0.626	**0.008**	**< 0.001**	**0.006**	**< 0.001**
T x I	6,108	0.091	0.668	0.078	**0.005**	0.089
T x M x I	18,108	**0.001**	**0.009**	0.396	0.262	0.485

5.3.3 Cladocerans

Cladoceran density was influenced by macrophytes, insecticides, the macrophyte-by-insecticide interaction, and the three-way interaction with time (Table 5.2). On day 26 (i.e. after applying 18 μg/L of malathion), cladocerans were marginally affected by insecticide treatment ($F_{2,36}$ = 3.0, p = 0.061) but not macrophytes ($F_{3,36}$ = 0.7, p = 0.534) or the macrophyte-by-insecticide interaction ($F_{6,36}$ = 1.2, p = 0.339). Because there appeared to be a pattern of different cladoceran responses to insecticide treatment within different macrophyte treatments (Fig. 5.2), we conducted Tukey's mean comparisons within each macrophyte treatment but found that pulse and press treatments never differed from the controls (all $p > 0.08$). As noted in the methods, the lack of a malathion treatment effect in the absence of macrophytes led to our decision to increase the malathion concentration from 18 to 36 μg/L.

On sample days 47, 68, and 100 (i.e. after malathion applications of 36 μg/L), we found that the effect of insecticide treatment on cladoceran density depended on macrophyte density (all $F_{6,36} \geq 3.2$, $p \leq 0.012$). In the 0-macrophyte treatment, the pulse insecticide exposure caused a marginally significant decline (76%) in cladocerans, relative to controls, on day 47 (p = 0.053). However, cladocerans returned to control levels by day 68 and remained equal to controls through day 100 (all $p \geq 0.302$). In the press treatment, cladoceran densities were less than 3% of control densities on days 47, 68, and 100 (Fig. 5.2, all $P \leq 0.009$). However, in treatments that contained 10, 50, or 100 macrophytes, cladoceran density in the pulse and press treatments never differed from the controls on any sample date (all $p \geq 0.173$).

5.3.4 Copepods

Copepod density was affected by time, the time-by-macrophyte interaction and the three-way interaction with time (Table 5.2). Two-way ANOVAs revealed that on days 26 and 68, copepod density was affected by macrophytes ($F_{3,36} = 3.2$, $p = 0.036$), but not insecticides ($F_{2,36} = 0.2$, $p = 0.84$) or their interaction ($F_{6,36} = 1.2$, $p = 0.315$). On day 26, the macrophyte effect was driven by a 13-fold higher copepod density in the 10-macrophyte treatment than the 100-macrophyte treatment (Fig. 5.2, $p = 0.035$). On day 68, however, the macrophyte effect was driven by a 2-fold higher copepod density in the 0-macrophyte treatment compared to the 100-macrophyte treatment ($p = 0.031$). In between these two dates (day 47), there were no effects of macrophytes ($F_{3,36} = 2.7$, $p = 0.062$), insecticides ($F_{2,36} = 1.3$, $p = 0.291$), or their interaction ($F_{6,36} = 1.0$, $p = 0.446$).

On day 100, we observed a significant macrophyte-by-insecticide interaction ($F_{6,36} = 2.9$, $p = 0.021$) driven by an effect of insecticides on copepod density in the 10-macrophyte treatment ($F_{2,36} = 8.7$, $p = 0.008$) but not in the other macrophyte treatments (Fig. 5.2, all $F_{2,36} \leq 2.1$, $p \geq 0.179$). With 10 macrophytes, we observed 12 to 15 times higher copepod densities in the press and pulse insecticide treatments compared to the controls ($p \leq 0.016$); the press and pulse treatments did not differ from each other ($p = 0.993$).

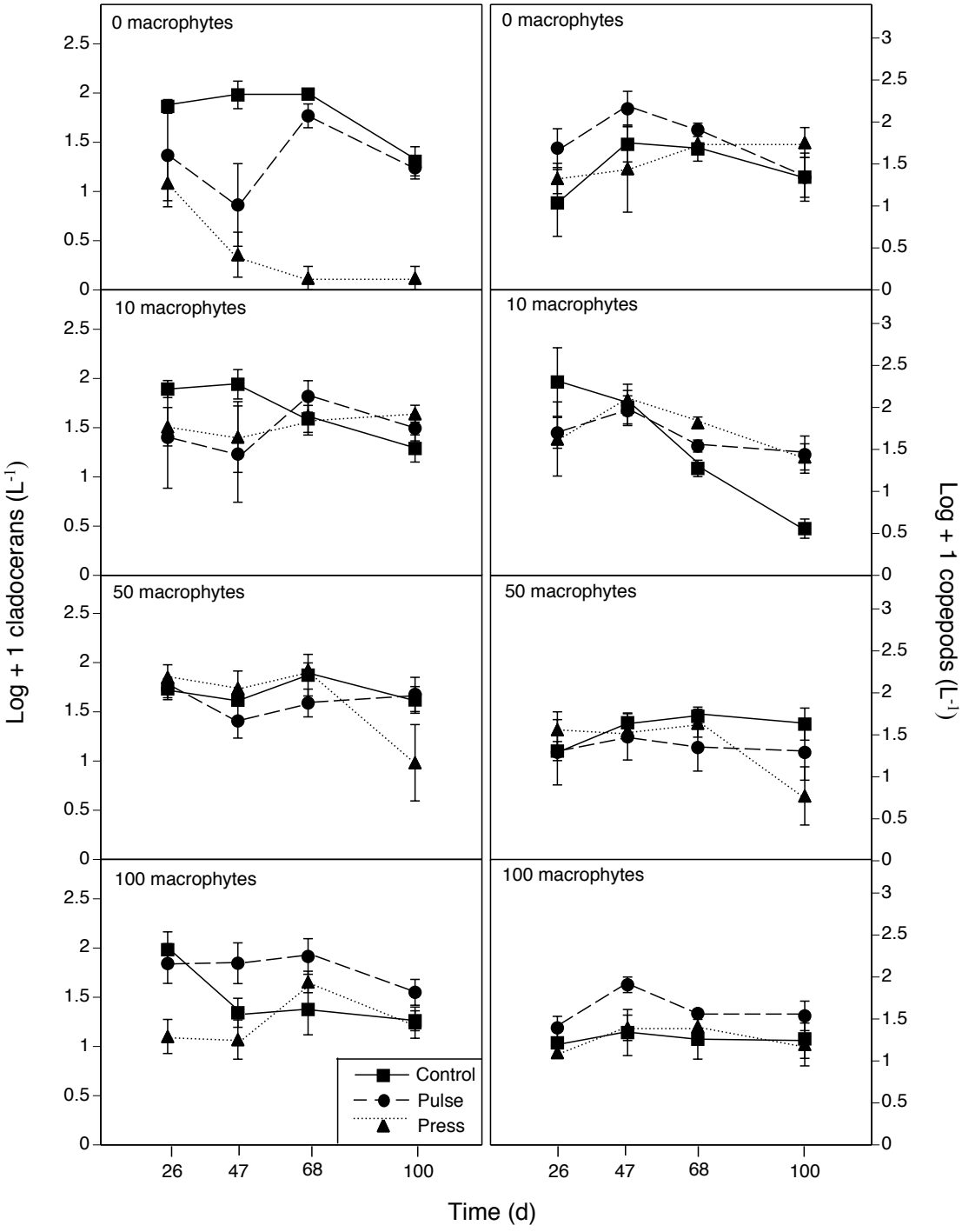

Figure 5.2. The effects of different malathion exposure scenarios in the presence of four macrophyte densities on cladoceran density (left) and copepod density (right) over time. Data are means ± 1 SE.

5.3.5 Rotifers

Rotifer density was affected by macrophytes, time, and the time-by-macrophyte interaction; however, the insecticide had no effect (Table 5.2). Significant univariate effects of macrophyte treatment on rotifer density occurred on each sample day (all $F_{3,36} \geq 7.1$, $p < 0.001$). On day 26, the 50- and 100-macrophyte treatments had five times higher rotifer densities than the 0-macrophyte treatment (Fig. 5.3, all $p \leq 0.004$); the 10- and 0-macrophyte treatments did not differ ($p = 0.993$). On all subsequent sampling dates, rotifer densities in the 10-, 50- and 100-macrophyte treatments were 2 to 13 times higher than in the 0-macrophyte treatment (all $p \leq 0.05$).

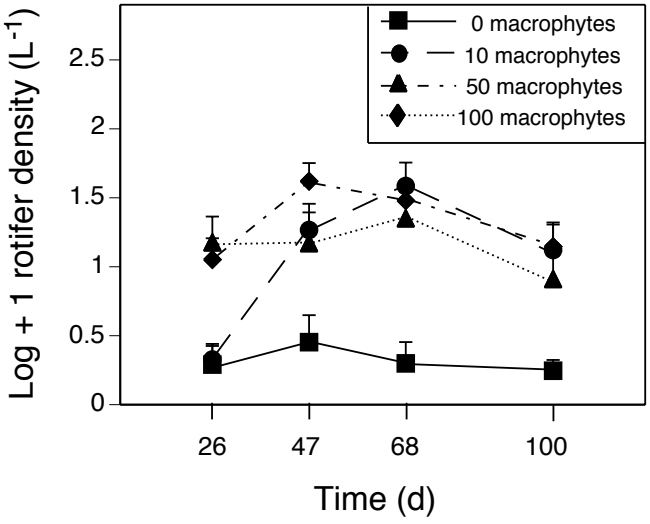

Figure 5.3. Effects of macrophyte density on rotifer density over time. Data are means ± 1 SE.

5.3.6 Phytoplankton

Phytoplankton abundance was affected by macrophytes, the macrophyte-by-insecticide interaction, time, and several interactions with time (Table 5.2). As a result, we performed two-way ANOVAs on phytoplankton abundance within each sample day. On days 26, 47, and 68, we observed effects of macrophyte treatment (all $F_{3,36} \geq 5.0$, $p \leq 0.006$), but not insecticides ($F_{2,36} \leq 2.0$, $p \geq 0.148$) or their interaction ($F_{6,36} \leq 1.4$, $P \geq 0.224$). On day 26, phytoplankton abundance in the 0- and 10-macrophyte treatments was over five and two times higher, respectively, than in the 100-macrophyte treatment (Fig. 5.4, all $p \leq 0.049$); abundance in the 50-macrophyte treatment was intermediate (all $p \geq 0.126$). On day 47, phytoplankton abundance in the 0-macrophyte treatment was more than three times higher than the 10-, 50-, and 100-macrophyte treatments (all $P \leq 0.01$), which did not differ from one another (all $p \geq 0.874$). On day 68, phytoplankton abundance in the 0-macrophyte treatment was over five times higher than in the 10- and 50-macrophyte treatments (all $p \leq 0.004$); the 100-macrophyte treatment did not differ from any of the other treatments (all $p \geq 0.141$).

On day 100, we found an effect of insecticides on phytoplankton abundance, but the effect depended on macrophyte treatment ($F_{6,36} = 6.3$, $p < 0.001$). This interaction occurred because insecticides had a significant effect on phytoplankton when macrophytes were absent ($F_{2,36} = 20.5$, $p < 0.001$) but no effect when macrophytes were present at any density (Fig. 5.4, all $F_{2,36} \leq 2.0$, $p \geq 0.185$). In the 0-macrophyte treatment, the insecticide effect was caused by a nearly 12-fold increase in phytoplankton abundance (i.e. a phytoplankton bloom) in the press insecticide treatment compared to the control and pulse treatments (all $p \leq 0.003$), which did not differ from one another ($p = 0.457$).

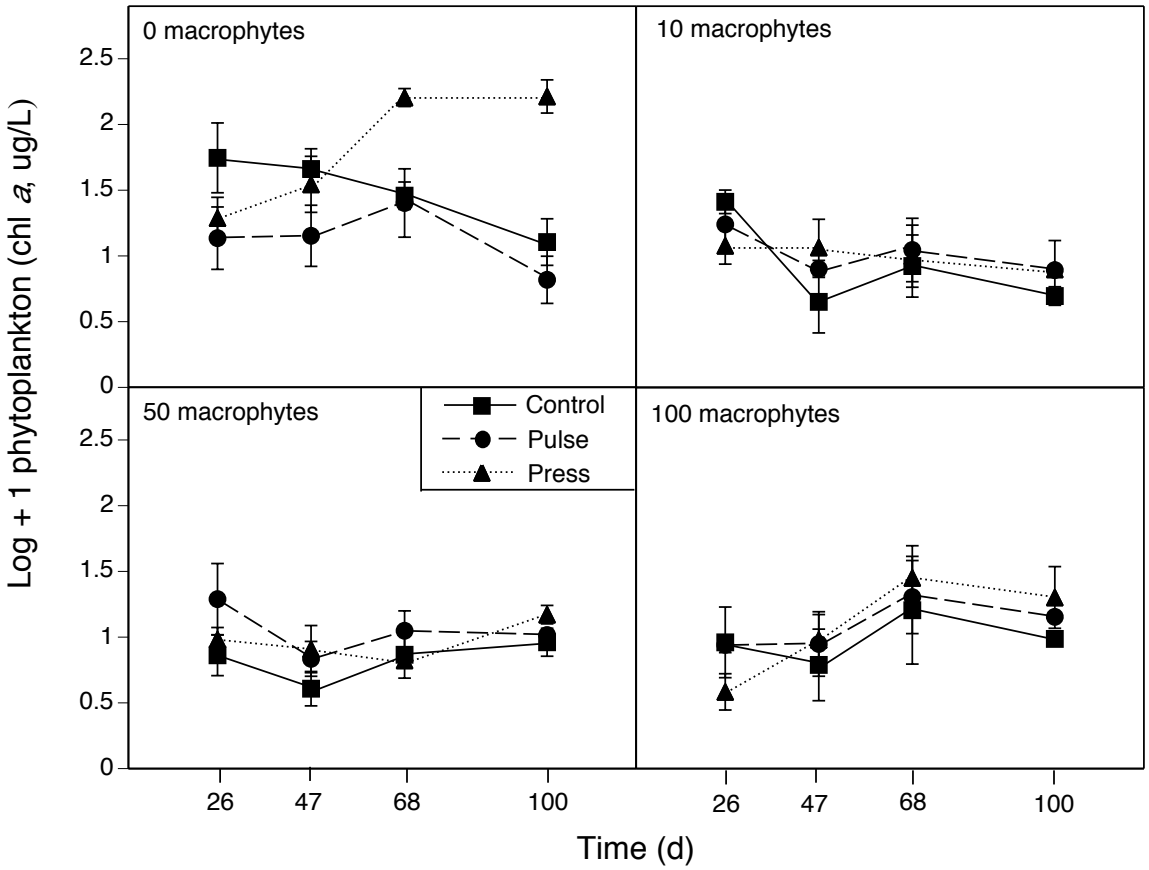

Figure 5.4. Phytoplankton abundance (measured as chlorophyll *a*) over time in mesocosms treated with different macrophyte densities and different malathion application regimes. Data are means ± 1 SE.

5.3.7 Periphyton

Periphyton biomass was affected by time and the time-by-macrophyte interaction, but not by insecticides (see Table 5.2). We detected significant effects of macrophytes on periphyton biomass on days 26, 47, and 100 (all $F_{3,36} \geq 3.1$, $p \leq 0.04$), but not on day 68 ($F_{3,36} = 0.9$, $p = 0.434$). On day 26, Tukey's test revealed a trend of higher periphyton biomass in the 0- and 10-macrophyte treatments than in the 100-macrophyte treatment (Fig. 5.5, all $p \leq 0.09$), though no

treatments differed from the 50-macrophyte treatment (all $p \geq 0.36$). On day 47, we again observed a trend of higher periphyton biomass in the 10-macrophyte treatment than in the 50- and 100-macrophyte treatments (all $p \leq 0.059$), though biomass in the 0-macrophyte treatment did not differ from any of these treatments (all $p \geq 0.29$). Finally, on day 100, periphyton abundance in the 50-macrophyte treatment was three times greater than in the 0-macrophyte treatment ($p = 0.004$); the 10- and 100-macrophyte treatments did not differ from the 0- or 50-macrophyte treatments (all $p \geq 0.265$).

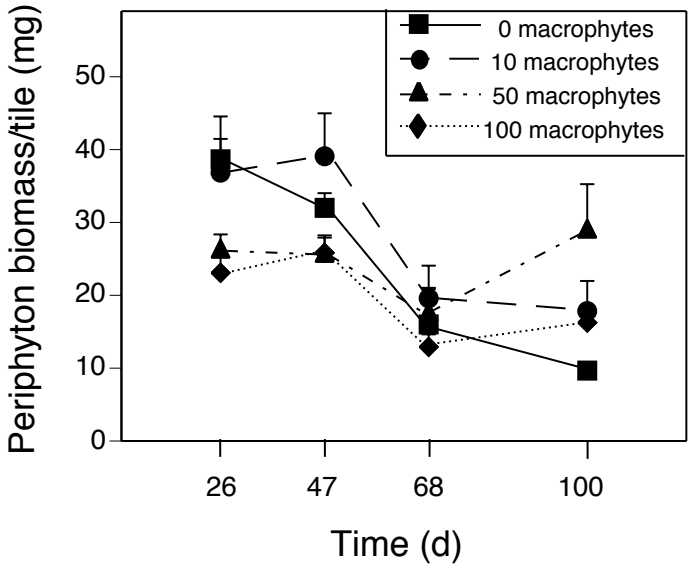

Figure 5.5. Effects of macrophyte density on periphyton abundance measured as biomass on clay tiles over time. Data are means ± 1 SE.

5.3.8 Snails

The two-way MANOVA on snail abundance, which was assessed on day 68, revealed effects of macrophytes (Wilks' $F_{6,70} = 3.3$, $p = 0.007$), insecticides (Wilks' $F_{4,70} = 3.2$, $p = 0.018$) and their interaction (Wilks' λ, $F_{12,70} = 1.9$, $p = 0.05$). For rams horn snails, abundance was not affected by macrophytes ($F_{3,36} = 1.0$, $p = 0.419$), but was affected by insecticides ($F_{2,36} = 4.8$, $p = 0.014$) and the interaction ($F_{6,36} = 2.5$, $p = 0.042$). In the 0-macrophyte treatment, we observed a 10-fold decrease in abundance in the malathion-press treatment compared to the control, although this effect was marginally significant (Fig. 5.6A, $p = 0.064$); abundance in the pulse treatment did not differ from either the press or control treatments (all $p \geq 0.116$). In the 10-macrophyte treatment, insecticides had no effect on rams horn snail abundance (all $F_{2,9} \leq 4.0$, $p \geq 0.05$). In the 50- and 100-macrophyte treatments, insecticides had significant effects (all $F_{2,36} \geq 4.6$, $p \leq 0.043$). Abundance was 75% lower in pulse than in press treatments ($p = 0.039$); controls did not differ from the pulse and press treatments (all $p \geq 0.127$).

Pond snail abundance was significantly affected by macrophytes ($F_{3,36} = 7.5$, $p < 0.001$), marginally significantly affected by insecticides ($F_{2,36} = 3.0$, $p = 0.067$), and not affected by their interaction ($F_{6,36} = 1.7$, $p = 0.14$). The macrophyte effect was caused by a 2.5-fold higher abundance in the 0- and 10-macrophyte treatments than in the 50- and 100-macrophyte treatments (Fig. 5.6B, all $p \leq 0.008$). The insecticide effect occurred because pond snail abundance in the press malathion treatment was more than twice as high as in the pulse treatment ($p = 0.05$), though neither treatment differed from the control (all $p \geq 0.387$).

5.3.9 Amphibians

The MANOVA on gray treefrog life history traits revealed an effect of macrophytes (Wilks' $F_{9,82}$ = 2.29, p = 0.028) but no effect of insecticides (Wilks' $F_{6,68}$ = 1.0, p = 0.405) or their interaction (Wilk's' $F_{18,96}$ = 0.7, p = 0.811). Subsequent ANOVAs revealed that survival was high across all treatments (mean ± 1 SE; 86 ± 2%) and unaffected by macrophytes ($F_{3,36}$ = 0.6, p = 0.629). However, macrophyte treatment affected mass at metamorphosis ($F_{3,36}$ = 6.6, p = 0.001) and time to metamorphosis ($F_{3,36}$ = 5.6, p = 0.003). Compared to the 0-macrophyte treatment, time to metamorphosis did not differ in the 10-macrophyte treatment (p = 0.621) but took 5 d longer in the 50- and 100-macrophyte treatments (Fig. 5.6C, all $p \leq 0.02$). For mass at metamorphosis, sgray treefrog raised with 0-macrophytes were similar in mass to those raised with 10 macrophytes (all $p > 0.348$), but mass in the 50- and 100-macrophyte treatments was approximately 25% lower (Fig. 5.6D, all $p \leq 0.007$).

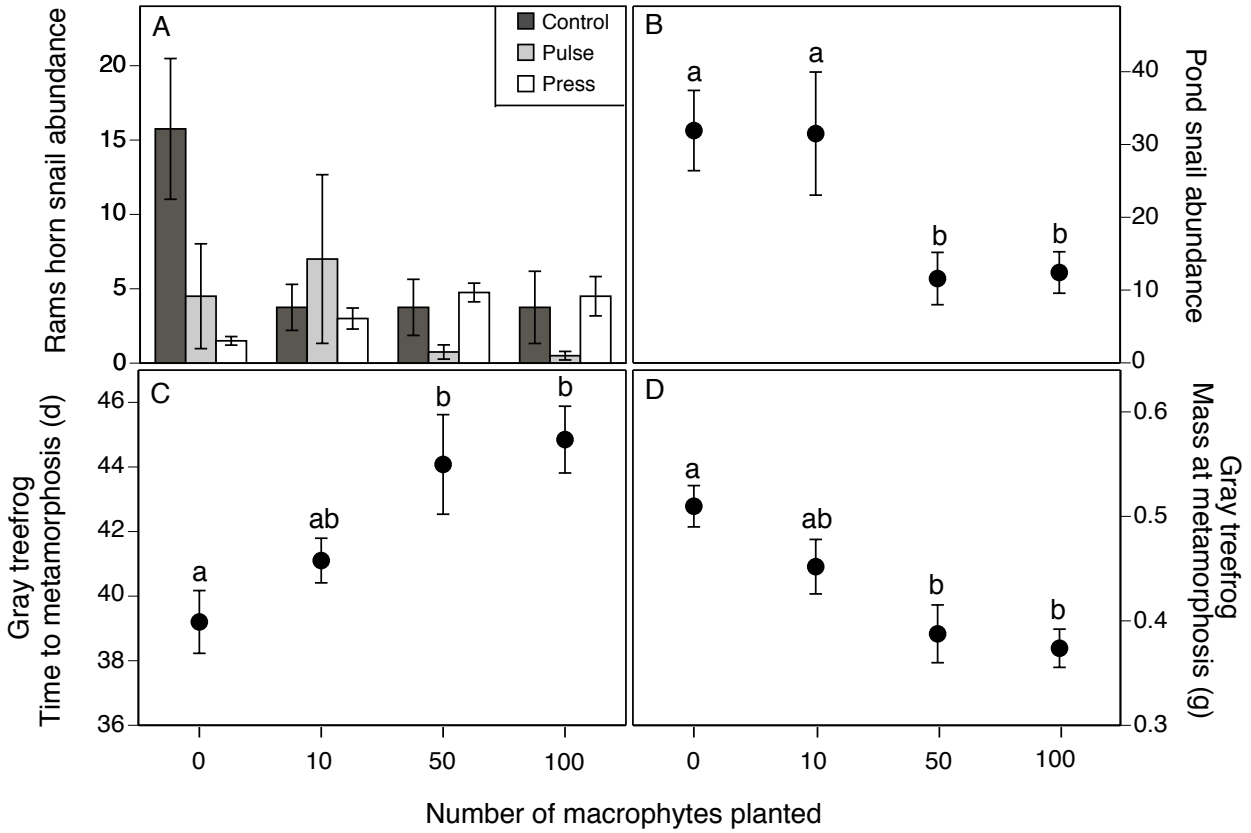

Figure 5.6. The impacts of A) insecticide treatments on rams horn snail abundance on day 68 within each macrophyte treatment, and macrophyte treatment effects on B) pond snail abundance on day 68, and gray treefrog C) mass at metamorphosis and D) time to metamorphosis. For panels B-D, different lowercase letters represent significant macrophyte-treatment differences ($\alpha = 0.05$). All data are means ± 1 SE.

5.4 DISCUSSION

We tested the general hypothesis that the submerged macrophyte *Elodea canadensis* would mitigate the direct and indirect effects of several realistic insecticide exposure scenarios in aquatic communities. Overall, we found that whenever the macrophyte was present, malathion's direct effects were strongly mitigated. This mitigating effect occurred regardless of whether the insecticide was applied as a single application or repeated applications. By buffering cladocerans from malathion's direct lethal effects, macrophytes also dampened the insecticide's cascading effects on the rest of the community. Further, we discovered that medium to high macrophyte densities suppressed the biomass of periphyton, resulting in reduced snail abundance and tadpole growth compared to treatments with low or no macrophytes.

An important prediction in our experiment was that malathion would decimate sensitive cladocerans in the absence of macrophytes but this effect would be mitigated in the presence of macrophytes in a density-dependent manner. Indeed, we discovered that without macrophytes, both pulse and press malathion applications of 36 μg/L reduced cladoceran densities relative to insecticide-free controls. Although cladocerans recovered to control levels within 3 wks after the pulse exposure, the press exposures maintained low cladoceran densities for the duration of the experiment. This result is consistent with reported cladoceran sensitivities to malathion in laboratory experiments (Lethal concentration required to kill 50% of animals; $LC50_{48h} < 5$ μg/L, Kegley et al. 2010; PAN Pesticide Database, http://www.pesticideinfo.org). Other studies conducted in mesocosms have demonstrated similarly toxic effects of comparable malathion concentrations on cladoceran populations (Relyea & Diecks 2008, Relyea & Hoverman 2008).

In contrast to malathion's high toxicity in the absence of macrophytes, when the macrophytes were present the pulse and press malathion treatments had no effect on cladocerans.

Using microcosm experiments, Brogan and Relyea (2013a) found that a similar range of *E. canadensis* densities (i.e. range = 177 to 747 g dry weight/m^3) made malathion up to six times less lethal to the cladoceran *Daphnia magna* than when macrophytes were absent. Further, we have demonstrated that the mitigating effects of submerged macrophytes on malathion's toxicity are primarily driven by the elevated water pH caused by plant photosynthesis (Brogan and Relyea 2014). While wetlands often range from pH 5 to 8 (Mitsch and Gosselink 1986), pH levels of 9 and above are not uncommon in dense macrophyte beds (Raspopov et al. 2002, Nurminen 2003), particularly in the canopy near the surface (Carter et al. 1988, Frodge et al. 1990). It should also be noted that high pH levels also occur during algal blooms (phytoplankton and/or periphtyon; e.g., Kufel et al. 2004). Thus, malathion's toxic effects would also likely be reduced under these conditions. Nevertheless, our discovery that realistic macrophyte densities (Duarte & Kalff 1990) mitigate insecticide toxicity under the more realistic conditions in the present study suggests that this ability may translate to the field, though testing this conclusion is an important next step.

Compared with cladocerans, copepods and rotifers are relatively resistant to malathion (Kegley et al. 2010), so the lack of malathion's direct effects on these taxa was not surprising. Previous work has demonstrated that cladoceran declines following malathion exposure in the absence of macrophytes can result in the competitive release of copepods and rotifers (Hanazato 1998, Relyea and Diecks 2008, Relyea and Hoverman 2008). However, we found no evidence of this indirect effect in our study. In fact, the only case where we observed significantly higher copepod densities following malathion applications (in both the pulse and press treatments) was on day 100 in the 10-macrophyte treatment, where malathion treatment had no effect on cladoceran density at any earlier sample dates.

Although malathion had only minor effects on copepods and rotifers, we observed effects of macrophyte density on these taxa. Copepods were generally less abundant in the 100-macrophyte treatment than in the 0- and 10-macrophyte treatments throughout the experiment, though this relationship depended on the sample date. To our knowledge, no studies have examined mechanisms by which submerged macrophytes might suppress copepod populations. In contrast, rotifers were generally more abundant whenever macrophytes were present. This is likely the result of macrophytes providing rotifers with an important refuge from predators, such as cyclopoid copepods (Duggan et al. 2001).

We also predicted that cladoceran declines following malathion exposure would initiate phytoplankton blooms. When we examined phytoplankton abundance in the malathion pulse treatment, we found no effects of macrophytes, likely because the insecticide caused only ephemeral (< 3 wk) cladoceran declines. In the press treatment, however, consistently low cladoceran densities occurred when macrophytes were absent and these caused phytoplankton blooms that developed by day 68 and persisted through day 100. However, these blooms did not occur whenever the macrophyte was present due to the mitigating effects of the macrophytes on cladocerans. Thus, we found support for our hypothesis that macrophytes do not only mitigate the direct effects of malathion on cladocerans, they also mitigate the subsequent indirect effects on phytoplankton.

The cascading effects that insecticides have in macrophyte-free aquatic communities are becoming well established. For example, Relyea and Diecks (2008) documented phytoplankton blooms in outdoor mesocosms following press, but not pulse, treatments of low malathion concentrations (10 μg/L) because cladocerans were kept at low abundance for several weeks. Other studies have documented phytoplankton blooms following pulse insecticide applications,

but only where concentrations were high enough to apparently cause local extinctions of cladoceran populations (Hanazato and Yasuno 1987, Fairchild et al.1992, Relyea and Hoverman 2008). While phytoplankton blooms are observed following exposure to many different insecticides, the primary mechanism is typically due to dramatic declines in the abundance of cladocerans due to direct insecticide toxicity (*reviewed in* Fleeger et al. 2003). Thus, the present study, which investigates the ecological factors capable of partially or completely mitigating such cascades, has clear conservation and management implications for developing better strategies to protect contaminated freshwater ecosystems.

Despite the sustained phytoplankton blooms in the 0-macrophyte, press malathion treatment in this study, we did not find support for our prediction that the phytoplankton blooms would reduce periphyton mass via competition for light and nutrients. Instead, we found no effects of malathion on periphyton, regardless of macrophyte treatment. However, we would expect this result if, across malathion treatments, grazing pressure was consistently above a threshold level necessary to prevent periphyton mass from increasing beyond a minimum mass. Under such conditions, one would expect that, instead of creating differences in periphyton mass, the phytoplankton blooms would actually manifest as differences in the abundance of grazers in different malathion treatments, possibly driven by changes in periphyton quality or production (Vadeboncouer et al. 2001).

In contrast to periphyton, the abundance of rams horn snails was affected by our malathion treatments and the effect depended on macrophyte density. Without macrophytes, the press malathion treatment tended to decrease rams horn snails abundance relative to controls. Given that rams horn snails (and gastropods in general) exhibit low sensitivity to malathion ($LC50_{48h}$ = 500,000 μg/L; Tchounwou et al. 1991) it is unlikely that the insecticide had any

direct effects on the snails. Instead, the adverse effects of malathion-induced phytoplankton blooms on periphyton may have manifested as reduced snail abundance. However, it is important to note that rams horn snails were the only grazer affected by malathion in the absence of macrophytes and the reasons for this are unclear.

With high macrophyte densities, rams horn snail abundance in pulse malathion treatments was lower than in press and control treatments. Because no phytoplankton blooms or effects on periphyton abundance were observed in the pulse malathion treatment at these macrophyte densities, the mechanism driving these effects is uncertain. Unfortunately, ecotoxicological studies including snails are rare and the impacts of pesticides on snail population dynamics is likely driven by a complex set of factors that our experiment was not designed to differentiate.

While a major focus of the present study was on the influence of macrophytes on malathion's community-level effects, we also discovered important and novel effects of the macrophyte on community structure. For example, during the first two sampling dates (days 26 and 47), periphyton biomass was generally higher in tanks with 0 or 10 macrophytes than 50 or 100 macrophytes. This pattern makes sense as macrophytes and periphyton overlap spatially and compete for light and nutrients in the benthos (Sand-Jensen et al. 1988). Because periphyton is a primary food source for many grazer species, we predicted that such competitive interactions would have important implications for the growth and abundance of tadpoles and snails (Carpenter and Lodge 1986, Sand-Jensen and Borum 1991).

The abundance of pond snails was closely related to periphyton biomass early in the experiment, with the highest abundances occurring with 0 and 10 macrophytes and lower abundances with 50 or 100 macrophytes. Though the total primary producer biomass likely increased as macrophyte density increased (due to the large biomass provided by the plants)

freshwater snails primarily graze algae and are not known to be important herbivores on living macrophyte tissues (Lodge 1985, 1991). Thus, macrophytes likely had an inhibitory effect on *Physa spp.* abundance, mediated through their competitive interactions with periphyton.

Increased macrophyte density also had adverse effects on amphibians, causing gray treefrogs to emerge later and at a smaller mass. As in the case of pond snails, this is likely a result of increased competition for resources driven by the negative effects of higher macrophyte densities on periphyton biomass. An additional possibility is that periphyton quality decreased as macrophyte density increased, but the few experiments addressing this question have found no effect of macrophytes on periphyton quality (Jones et al. 1999, 2000). Regardless of the mechanism, the reduced growth and prolonged larval developmental period experienced by gray treefrogs has important implications because anurans that metamorphose later and at smaller masses experience reduced survival to reproduction and recruitment (Smith 1987, Altwegg and Reyer 2003). More studies examining how different habitats (e.g., macrophyte-free versus macrophyte-dominated) and exposure to anthropogenic contaminants might interact to influence the survival and life-history traits of amphibians are needed as these taxa continue to decline worldwide (Collins and Storfer 2003, Stuart et al. 2004, Blaustein et al. 2010).

5.5 CONCLUSIONS

From a management perspective, our work suggests that promoting healthy submerged macrophyte populations may be an effective strategy for buffering aquatic communities from common contaminants. Not only can submerged plants potentially detoxify contaminants that enter non-target aquatic habitats, but they may also help improve best management practices

(BMPs) designed to prevent non-target habitats from being exposed to contaminants in the first place. For example, constructed wetlands and vegetated drainage ditches used in agricultural BMPs currently rely exclusively on emergent macrophytes and sediment to bind pesticides in runoff and increase their retention time so that less of the compounds pass through (Moore et al. 2011). While this approach is successful for some contaminants (particularly compounds with high binding affinities for organic substrates), this strategy is unlikely to remediate pesticides, such as malathion, that do not rapidly bind to these substrates. Our research suggests that new strategies incorporating submerged macrophytes should be examined as a potential complementary approach to buffering surface waters from contaminants. However, we also found that submerged macrophytes may have some adverse impacts on some species' life-history traits, so it is important to consider this tradeoff in developing a management strategy.

5.6 ACKNOWLEDGEMENTS

We thank Drs. Rickey Cothran, Aaron Stoler, Jess Hua, and John Hammond, as well as Dean Matthews, Kate Henderson, and Heather Shaffery for their assistance. Funding for this research was provided by a National Science Foundation grant to RAR and an LK Darbaker award, Sigma Xi grant-in-aid-of-research, and Society for Wetland Scientists award to WRB.

6.0 CONCLUSIONS

Understanding the factors that influence responses of aquatic communities to natural and anthropogenic perturbations is an increasingly important goal as degrading water quality has become a major threat to freshwater, estuarine, and marine ecosystem integrity (Smith et al. 1999, Kemp et al. 2005). My dissertation explores the ability of globally abundant submerged plants to mitigate the ecological effects of the widely used insecticide malathion in aquatic communities. Because this issue has received very little previous attention, I performed experiments on simplified communities at the microcosm and mesocosm scale in order to address a number of fundamental questions including uncovering the mechanism by which plants mitigate malathion's toxicity. While this approach allowed me to provide the first evidence that submerged plants can strongly mitigate an insecticide's effects at multiple spatial scales, a critical next step is to examine how these results scale up to natural aquatic communities that are exposed to malathion and other perturbations.

Several lines of evidence suggest that malathion's ecological effects might be mitigated in a wide variety of natural aquatic communities. My dissertation research indicates that the magnitude of malathion's ecological effects may be predictable using a single, easily measured water quality variable, pH. Using this finding to generate straightforward and testable predictions, future research should quantify the magnitude of malathion's effects across water bodies differing in pH. Although aquatic ecosystems containing high densities of submerged

plants can achieve pH levels high enough to rapidly detoxify malathion (Ondok et al. 1984; Barko and Godshalk 1988, Frodge et al. 1990), similar pH levels can also be achieved by algal blooms in eutrophied systems (Talling 1976, Toivonen and Huttunen 1995). This is an important consideration as eutrophication is a widespread water quality issue (Carpenter et al.1998) and co-exposure to nutrients and pesticides is likely common in aquatic ecosystems. However, while predicting malathion's effects in homogenous pH environments (i.e. in a thoroughly mixed shallow lake) is straightforward, considering the effects of habitat heterogeneity within water bodies is also important.

In wetlands, lakes, and rivers, patches of dense plant beds, algal production and relatively oligotrophic zones can create a mosaic environment where insecticides may have variable effects depending on local-scale conditions and species distributions. For example, primary productivity in deep lakes is typically dominated by phytoplankton in the pelagic zone and submerged plants and periphyton in the littoral zone. As these habitats can possess very different water chemistries (Wetzel 2001), predicting insecticide effects in these different habitats will require fine-scale consideration of individual site characteristics as well as the route by which exposure occurs (e.g., groundwater leaching, surface runoff, or drift/direct overspray). Habitat heterogeneity is also present in rivers, where pockets of dense submerged plant growth can alter water flow and chemistry on a very local scale (Sand-Jensen and Mebus 1996). For example, Beketov and Leiss (2008) demonstrated that during exposure to the insecticide thiacloprid in streams, submerged plant beds created relatively buffered zones of reduced toxicity surrounded by unvegetated regions that experienced high macroinvertebrate mortality. Understanding how populations and communities will respond to insecticide perturbations in heterogeneous aquatic habitats will require consideration of metacommunity theory, including population source-sink dynamics

(Leibold MA et al. 2004, Mouquet et al. 2006) and migratory patterns of aquatic species (Werner et al. 1983, Schindler et al. 1996, Hanazato 2001, Burks et al. 2001, 2006)

While submerged plants should clearly be considered when predicting insecticide impacts in aquatic environments, they may also have applications for preventing exposure in the first place. Currently, agricultural best management practices (BMPs) such as constructed wetlands and vegetated drainage ditches primarily rely on sorption by emergent plants for removing insecticides in agricultural runoff (Cooper et al. 2004, Kröger et al. 2009). While emergent plants are effective for removing hydrophobic compounds (Moore et al. 2011), these plants may be ineffective at mitigating the impacts of a large number of insecticides that do not bind rapidly to plant tissues (e.g., malathion). Further, because emergent plants perform gas exchange with the air, they do not increase water pH like submerged plants do (Wetzel 2001). As chapter four of my dissertation shows, increasing water pH can cause rapid insecticide breakdown via alkaline hydrolysis, which can mitigate the toxicity not only of malathion (Brogan and Relyea *In press*), but also of several other insecticides that BMPs are currently unable to remediate (e.g., carbaryl, carbofuran; Brogan and Relyea *in prep*). Based on my dissertation research, incorporating submerged plants into BMPs may help reduce exposure of non-target aquatic habitats to a much larger number of contaminants. However, an essential next step is to perform field-scale studies that factor in ecological and economic considerations to determine whether submerged plants can effectively be incorporated into BMPs.

Submerged plants are also a key factor in determining the structure and function of aquatic communities in contexts other than during insecticide exposure. For example, submerged plants inhibit phytoplankton growth via interference competition (i.e. allelopathy; Hilt and Gross 2008, Hu and Hong 2008) and exploitation competition (i.e. nutrient competition; van Donk and

van de Bund 2002). Submerged plants can also suppress phytoplankton via apparent competition. Field experiments examining fish-zooplankton-phytoplankton interactions show that once a critical density of submerged plants is reached, cladoceran zooplankton are able to use plants as refuge from fish predation and, as a result, maintain top-down pressure on phytoplankton, which otherwise bloom when plant densities are low (Schriver et al. 1995). In general, submerged plants are considered a primary factor in maintaining clear-water stable states and preventing shifts to phytoplankton-dominated stables states in aquatic ecosystems (Scheffer et al. 1993). Given their critical ecological role and ability to buffer aquatic communities from perturbations including insecticide exposure, eutrophication and increases piscivorous fish abundance, we recommend that conserving submerged plants should be a priority of aquatic water management, though strategies should incorporate both ecological and economic (i.e. recreation, aesthetics) considerations (van Nes et al. 2002).

Finally, a central goal of my dissertation has been to provide insights to help ecologists develop better models for predicting the impacts of perturbations in ecological communities. Currently, models designed to predict the effects of top-down and bottom-up forcing (i.e. trophic cascades) on community structure primarily consider direct and indirect resource-consumer interactions (Terborgh and Estes 2010). However, the impacts of altering these interactions can be highly dependent upon the presence and strength of other ecological interactions. For example, factors such as prey (including plant) defenses (Agrawal 1998) and refugia (Schriver et al. 1995, Borer et al. 2005), as well as intraguild predation (Finke and Denno 2004, Schmidtz 2007) can all dampen the magnitude of top-down trophic cascades. As my dissertation shows, factors that mitigate perturbation direct effects can also dramatically dampen the magnitude of top-down trophic cascades. The critical next steps will be identifying other ecological

interactions that can dampen top-down and bottom-up trophic cascades, and to include parameters accounting for these factors into ecological models to better predict perturbation impacts in biological communities.

APPENDIX A

CHAPTER 4: DISSOLVED OXYGEN AND TEMPERATURE

A.1 STATISTICAL ANALYSIS

A.1.1 Statistical analysis of dissolved oxygen (DO) and temperature

We performed a multivariate analysis of variance to examine the effects of our treatments on water DO and temperature both before applying malathion and 48 h afterwards. We analyzed data collected before malathion applications using a one-way MANOVA on DO and temperature, with plant-pH treatment as a fixed factor. For samples collected 48 h after malathion applications, we analyzed DO and temperature using a full factorial two-way MANOVA model with malathion and plant-pH treatments as fixed factors. When appropriate, we performed Tukey's multiple comparisons tests to compare the effects of each treatment on our response variables. All abiotic data met the assumptions of general linear models.

A.1.2 Treatment effects on DO and temperature

We recorded dissolved oxygen and temperature data before applying malathion (-1 h) and 48 h after applying the insecticide. At -1 h, we observed a significant multivariate effect of plant-pH treatment on water DO and temperature (Wilk's λ, $F_{6,150} = 24.7$, $p < 0.001$). This multivariate effect was driven by significant univariate effects on DO ($F_{3,76} = 121.7$, $p < 0.001$) but not on temperature ($F_{3,76} = 0.7$, $p = 0.567$). Compared with the shaded-plant treatment, DO levels were 102% and 110% higher in the no-plant/low-pH and no-plant/high-pH treatments, respectively (Fig. A.1, $p < 0.001$), which did not differ from each other ($p = 0.976$). Further, dissolved oxygen in the unshaded-plant treatment was 226% higher than in shaded-plant treatment ($p < 0.001$) and over 50% higher than no-plant/low-pH and no-plant/high-pH treatments ($p < 0.001$). The water temperature at -1 h averaged across all plant-pH treatments was 24.3°C ± 0.1°C (mean ± SE).

At 48 h after applying malathion, we also observed significant multivariate effects of plant-pH treatment (Wilk's λ, $F_{6,118} = 98.0$, $p < 0.001$), malathion concentration (Wilk's λ, $F_{8,118} = 22.6$, $p < 0.001$), and the malathion by plant-pH interaction (Wilk's λ, $F_{24,118} = 2.7$, $p < 0.001$) on DO and temperature. While the plant-pH treatment effect was driven by univariate effects of DO ($F_{3,60} = 575.8$, $p < 0.001$) and temperature ($F_{3,60} = 13.1$, $p < 0.001$), only temperature was affected by malathion concentration ($F_{4,60} = 73.4$, $p < 0.001$) and the interaction ($F_{12,60} = 5.2$, $p < 0.001$). Tukey's test revealed that the effect of plant-pH treatment on DO occurred because, compared with the shaded-plant treatment, DO levels in the no-plant/low-pH and no-plant/high-pH treatments were 283% to 288% higher (Fig. A.1, $p < 0.001$); these latter two treatments did not differ from each other ($p = 0.977$). Further, DO in the unshaded-plant treatment was approximately 600% higher than in the shaded-plant treatment ($p < 0.001$) and over 65% higher than no-plant/low-pH and no-plant/high-pH treatments ($p < 0.001$).

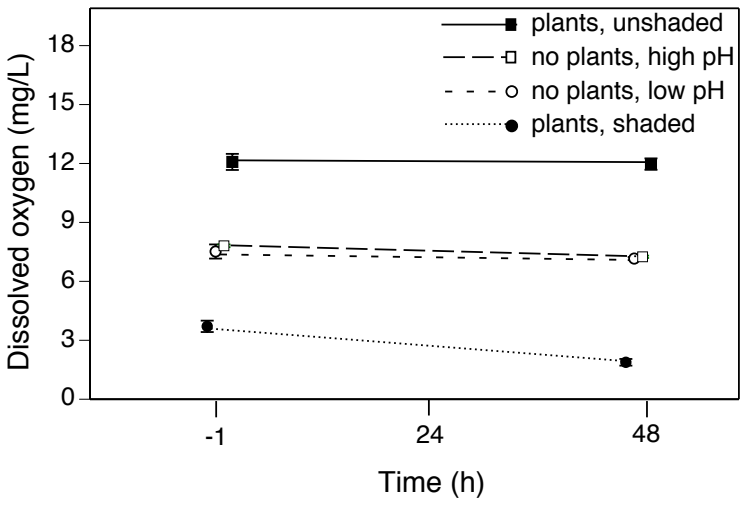

Figure A.1. The effects of plant and chemical pH treatments on water dissolved oxygen concentration in experimental jars over time. Data are means ± 1 SE.

The effect of the malathion-by-plant-pH interaction on temperature in the 48-h sample occurred because in the presence of the higher malathion concentrations (i.e. 4, 6, 36 µg/L), water temperature in jars exposed to sunlight (i.e. no-plant/low-pH, no-plant/high-pH, unshaded-plant treatments) was 0.9°C to 1.4°C higher than water in the shaded-plant treatment jars (Fig. A.2, $p < 0.023$). However, in the 0 and 1 µg/L malathion treatments, water temperature did not differ between plant-pH treatments (all $F_{3,12} \leq 2.0$, $p \geq 0.170$). Because we sampled abiotic variables in order from lowest malathion concentration to highest beginning at 1100 h, a likely explanation for this interaction is that the water in the lower malathion concentration jars (0 and 1 µg/L) was not exposed to high outside temperatures for as long as the jars containing higher malathion concentrations. As the shaded jars were not exposed to direct sunlight, it makes sense that the water temperature in these jars remained relatively low while the temperature in the other plant-pH treatments increased as the afternoon progressed.

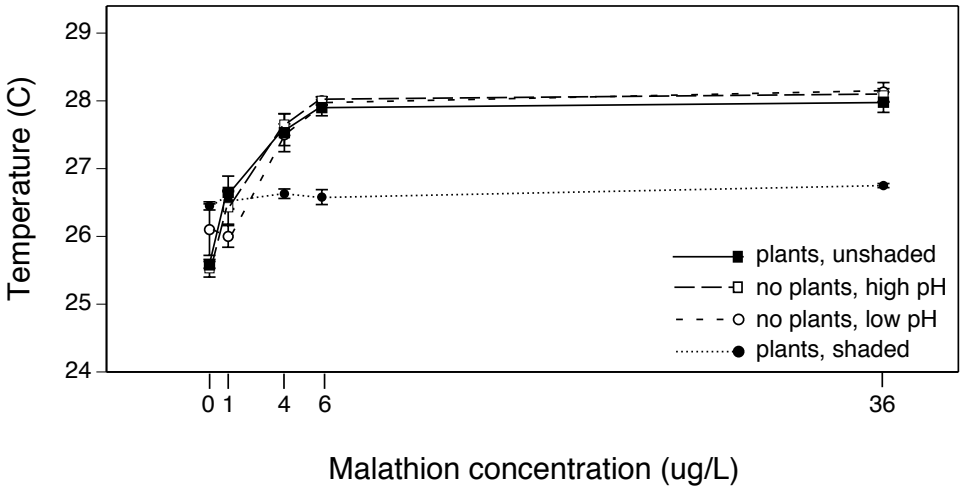

Figure A.2. The influence of plant and chemical pH treatments and malathion concentration on water temperature in samples collected 48 h after malathion applications. Data are means ± 1 SE.

APPENDIX B

CHAPTER 5: ADDITIONAL SAMPLING DETAILS FOR RESPONSE VARIABLES

B.1 ABIOTIC VARIABLES

B.1.1 Sampling abiotic variables

We quantified water temperature and dissolved oxygen using a calibrated digital water meter (WTW, Woburn, Massachusetts, USA) and pH using a calibrated Oakton pH 5 Acorn series sensor (Oakton Instruments, Vernon Hills, Illinois, USA). We quantified the shading effect of phytoplankton on periphyton by measuring the rate of light decay with depth. Using an underwater quantum sensor (LI-COR, Lincoln, Nebraska, USA), we measured photosynthetically active radiation at 10 and 20 cm below the water surface by positioning the light meter so no macrophyte shoots were shading the sensor. To calculate light decay rate (K), we used the formula:

$$K = \frac{\ln(L_{10} / L_{20})}{d}$$

where L_{10} equals the light intensity at 10 cm, L_{20} equals the light intensity at 20 cm, and d is the difference in depth between those two measurements.

B.2 BIOTIC VARIABLES

B.2.1 Sampling zooplankton

Using methods from Relyea & Diecks (2008), we collected zooplankton and identified them to species. We ultimately grouped them into cladocerans, copepods, and rotifers because species within each of these groups exhibit very similar responses to malathion treatments (Relyea & Diecks 2008, Relyea & Hoverman 2008, Hua & Relyea 2012). We collected zooplankton samples using a 0.2-L tube sampler that was plunged approximately 0.25 m deep at five locations in each mesocosm and the water was filtered through a 62-µm Nitex screen. Zooplankton samples were preserved in 30% ethanol for enumeration and identification to species. We identified a total of 18 zooplankton species in the experiment but we ultimately grouped them as cladocerans, copepods, and rotifers because species within each group exhibited very similar responses to our treatments; similar results have been found in past experiments (Relyea and Diecks 2008, Relyea and Hoverman 2008, Hua and Relyea 2012).

B.2.2 Sampling phytoplankton

We also quantified phytoplankton following the protocols of Relyea & Diecks (2008). To sample phytoplankton, we plunged plastic cups approximately 5 cm under the water surface to collect 500 ml of water from each mesocosm. The water was vacuum-filtered through a Fisherbrand GF/C filter (4.25 cm diameter). Samples were wrapped in foil and frozen until chlorophyll *a* analysis. We analyzed all chlorophyll *a* samples within 30 d of collection using methods modified from Arar and Collins (1997). We used a mortar and pestle to grind the filters in 90%

acetone and steeped the samples in the dark for 24 hrs at 3˚C. We then centrifuged the samples for 30 sec at 12,000 rpm and determined the concentration of chlorophyll *a* using a flurometer (TD-700, Turner Designs Inc., Sunnyvale, California, USA).

B.2.3 Sampling periphyton

We measured periphyton by removing a single clay tile from each mesocosm. We scrubbed and rinsed the periphyton from a standardized area of each tile (10 x 5 cm) and then we collected and vacuum-filtered the algae water onto a pre-weighed Fisherbrand GF/C filter (7.0 cm diameter) that had been dried for 24 h at 60˚C. After filtration, we dried the filters for another 24 hrs and re-weighed them to determine periphyton biomass.

B.2.4 Sampling snail abundance

We sampled pond snail and rams-horn snail abundance on day 68 by sinking five plastic cups (350 ml) with rocks to the bottom of each mesocosm so that each cup faced upwards. We placed a single pellet of alfalfa into each cup to attract the snails. After 24 hrs, we removed the cups from each tank and rinsed the contents through a 2-mm sieve. We sorted the snails by species and then counted the number of snails caught by the sieve.

B.2.5 Gray treefrog metamorph collection and processing

We collected gray treefrogs as they metamorphosed to compare survival and larval development. After the first metamorph emerged (day 30), we checked mesocosms daily for metamorphs.

Once collected, we held the metamorphs in the lab in separate containers (one container/mesocosm) until tail resorption (Gosner stage 46; Gosner 1960). Once tail resorption was complete, we euthanized the metamorphs in 2% MS-222 (tricaine methane sulfonate) and preserved them in glass jars containing 10% buffered formalin, allowing us to subsequently assess metamoprh mass at metamorphosis in addition to survival and time to metamorphosis.

We collected gray treefrogs as they metamorphosed to compare survival and larval development. After the first metamorph emerged (day 30), we checked mesocosms daily for metamorphs. Once collected, we held the metamorphs in the lab in separate containers (one container/mesocosm) until tail resorption (Gosner stage 46; Gosner 1960). Once tail resorption was complete, we euthanized the metamorphs in 2% MS-222 (tricaine methane sulfonate) and preserved them in glass jars containing 10% buffered formalin, allowing us to subsequently assess metamoprh mass at metamorphosis in addition to survival and time to metamorphosis.

APPENDIX C

CHAPTER 5: RESULTS FOR MACROPHYTE BIOMASS AND ABIOTIC VARIABLES

C.1 MACROPHYTE BIOMASS

C.1.1 Treatment effects on macrophyte biomass

Over the course of the experiment, *E. canadensis* density increased in all mesocosms containing macrophytes. When we quanitified macrophyte biomass on the last day of the experiment (i.e. day 320), we detected significant effects of macrophyte treatment ($F_{2,27} = 5.8$, $p = 0.006$) and insecticides ($F_{2,27} = 3.7$, $p = 0.029$), but not the interaction ($F_{4,27} = 0.3$, $p = 0.869$). Tukey's tests revealed that the macrophyte treatment effect was driven by an approximately 50% greater *E. canadensis* biomass in the 100-macrophyte treatment compared to the 10- and 50-macrophyte treatments (Fig. S2aA, $p < 0.02$); the latter two treatments did not differ from each other ($P = 0.998$). The insecticide effect was caused by an approximately 50% greater *E. canadensis* biomass in the press treatment than in the control ($p = 0.03$); the pulse treatment did not differ from the control or press treatments (Fig. S2aB; all $p > 0.339$).

This increase in *E. canadensis* density in press treatments could be a result of the repeated inputs of phosphorus provided by each addition of the organophosphate insecticide,

malathion. However, the ability of microorganisms to remineralize nutrients contained in insecticide molecules has received little attention to draw definitive conclusions (but see Omar 1998). A second possibility is that with each malathion application, a new source of nutrients was available in the form of dead cladocerans, where the decomposition of the carcasses could recycle nutrients and facilitate macrophyte growth. However, our study was not designed to elucidate the mechanism driving this pattern.

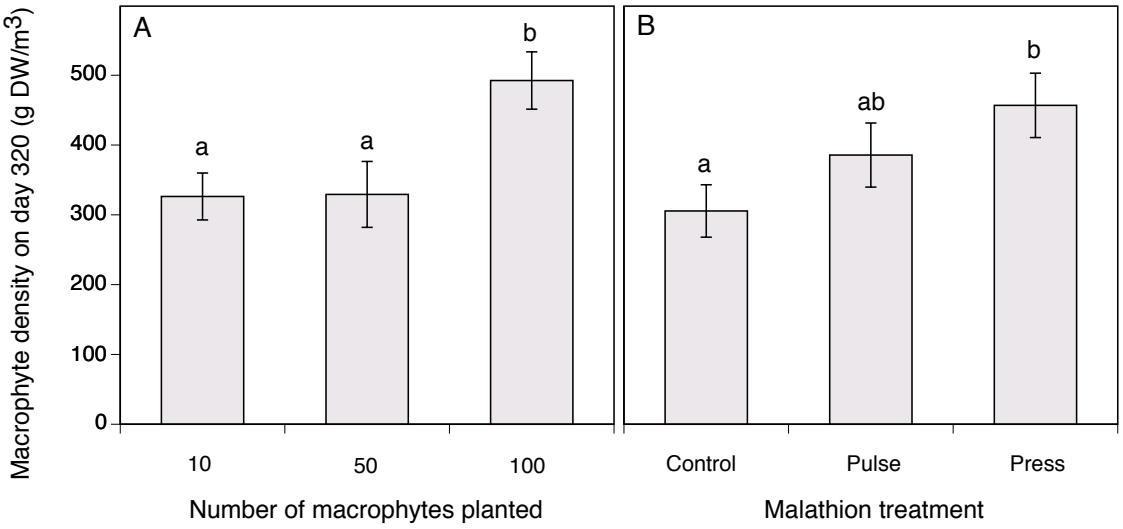

Figure C.1. The effect of A) number of macrophyte shoots planted and B) insecticide treatment on final *E. canadensis* biomass as measured on day 320. Different lower case letters show significant differences ($\alpha = 0.05$). Data are means ± 1 SE and exclude treatments containing no macrophytes.

C.2 ABIOTIC VARIABLES

C.2.1 Treatment effects on abiotic variables

The rm-MANOVA on temperature, pH, dissolved oxygen, and light decay revealed multivariate effects of macrophyte treatment, the macrophyte-by-insecticide interaction, time, and the time-by-macrophyte interaction (Table C.1). Because of the significant multivariate time-by-macrophyte interaction, we examined the univariate time-by-macrophyte interaction effects on each response variable (pH results discussed in main text). Where appropriate, we subsequently examined the univariate macrophyte treatment effects within each sample date.

Average daytime water temperatures were (mean ± 1 SE) 20.8 ± 0.07 °C, 20.6 ± 0.06 °C, 22.6 ± 0.08 °C, and 19.6 ± 0.08 °C on days 26, 47, 68, and 100, respectively. However, we did not observe a time-by-macrophyte interaction ($F_{9,108}$ = 0.3, p = 0.99) or a macrophyte-by-insecticide interaction ($F_{6,36}$ = 1.1, p = 0.38) on water temperature.

Dissolved oxygen was significantly influenced by the time-by-macrophyte interaction ($F_{9,108}$ = 2.7, p = 0.009). We found significant macrophyte treatment effects on dissolved oxygen concentrations at each sample date (all $F_{3,36}$ > 9.1, p < 0.001). Tukey's mean comparisons tests revealed that on all sample dates, dissolved oxygen did not differ among the 10-, 50- and 100-macrophyte treatments (all $p \geq 0.4$), but was at least 30% greater in these treatments than in the 0-macrophyte treatment (Fig. C.4, all $p \leq 0.002$;).

Light decay rate was also influenced by the time-by-macrophyte interaction ($F_{9,108}$ = 4.2, p < 0.001). While there was no effect of macrophyte treatment on light decay on day 26 (Fig. C.3, $F_{3,48}$ = 0.4, p = 0.751), each subsequent sample date revealed a significant macrophyte effect (all $F_{3,48}$ > 4.9, p < 0.006). Tukey's mean comparisons test revealed that at day 47, the light

decay rate in the no-macrophyte treatment was 70% higher than in the 100-macrophyte treatment ($p = 0.006$), but the 10- and 50-macrophyte treatments did not significantly differ from the 0- or 100-macrophyte treatments (all $p \geq 0.07$). On days 68 and 100, light decay rate in the 0-macrophyte treatment was at least 44% greater than in the 10-, 50-, and 100-macrophyte treatments (all $p < 0.001$), which did not differ from each other (all $p \geq 0.73$).

Table C.1. Results of repeated measures MANOVA on water temperature, pH, dissolved oxygen and light decay in mesocosms treated with a factorial combination of four macrophyte densities and three insecticide (malathion) application regimes. Bold p-values are significant at $p < 0.05$.

Source (Wilk's lambda)	df	*F*-value	*p*-value
Macrophyte	12, 88	14.5	**< 0.001**
Insecticide	8, 66	1.3	0.265
Macrophyte x insecticide	24, 116	1.7	**0.037**
Time	12, 278	54.3	**< 0.001**
Time x macrophyte	36, 395	3.1	**0.001**
Time x insecticide	24, 368	1.5	0.057
Time x macrophyte x insecticide	72, 415	1.3	0.06

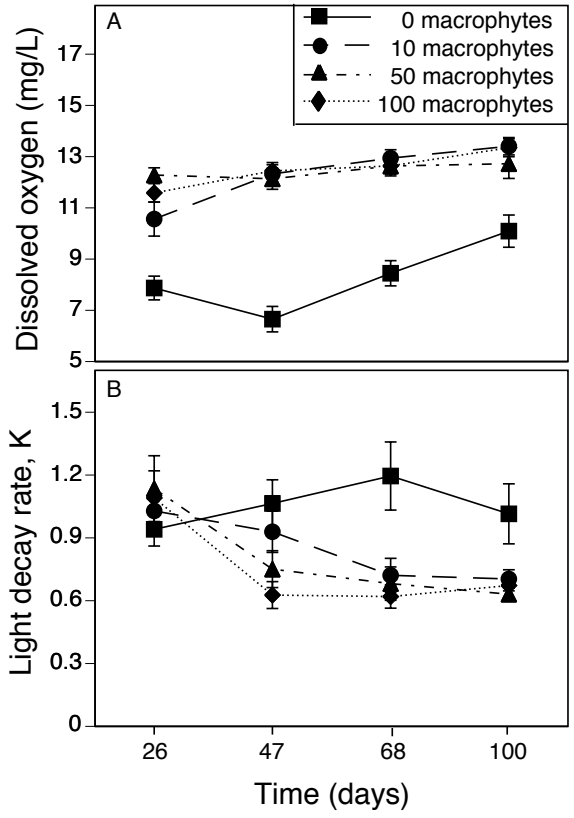

Figure C.2. The effect of macrophyte density on (A) dissolved oxygen and (B) light decay over time (means ± SE).

BIBLIOGRAPHY

Aker WG, Hu X, Wang P, Hwang HM. 2008. Comparing the relative toxicity of malathion and malaoxon in blue catfish *Ictaralurus furcatus*. *Environmental Toxicology* 23:548-554.

Altwegg R, Reyer HU. 2003. Patterns of natural selection on size at metamorphosis in water frogs. *Evolution* 57:872-882.

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. California Environmental Protection Agency EH95-02.

Arar EJ, Collins GB. 1997. *In vitro* determination of chlorophyll *a* and pheophytin *a* in marine and freshwater algae by fluorescence. Method 445.0. National Exposure Research Laboratory, USEPA, Cincinnati, Ohio, USA.

ASTM Standard E1193. 2004. Standard guide for conducting *Daphnia magna* Life-Cycle Toxicity Tests. ASTM International. West Conshohocken, PA, USA.

ASTM Standard E729. 2007. Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. ASTM International. West Conshohocken, PA, USA.

Barko JW, Godshalk GL, Carter V, Rybicki NB.1988. Effects of submersed aquatic macrophytes on physical and chemical properties of surrounding water. Technical Report A-88-11, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

Barrat-Segretain MH, Elger A, Sagnes P, Puijalon S. 2002. Comparison of three life-history traits of invasive *Elodea Canadensis* Michx and *Elodea nuttallii* (Planch.) H. St. John. *Aquatic Botany* 74:299-313.

Bender EA, Case TJ, Gilpin ME. 1984. Perturbation experiments in community ecology: Theory and practice. *Ecology* 65:1-13.

Bender ME, 1969. The toxicity of the hydrolysis and breakdown products of malathion to the fathead minnow (*Pimephales promelas*, Rafinesque). *Water Research* 3:571-582.

Blaustein AR, Han BA, Relyea, RA, Johnson PTJ, Buck JC, Gervasi SS, Kats LB. 2010. The

complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Annals of the New York Academy of Sciences*, **1223**, 108-119.

Blindow I. 1992. Long- and short-term dynamics of submerged macrophytes in two shallow eutrophic lakes. *Freshwater Biology* 28:15-27.

Boone MD, James SM. 2003. Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. *Ecological Applications* 13:829-841.

Boone MD, Semlitsch RD. 2001. Interactions of an insecticide with larval density and predation in experimental amphibian communities. *Conservation Biology* 15:228-238.

Boone MD, Bridges CM, Rothermel BB. 2001. Growth and development of larval green frogs (Rana clamitans) exposed to multiple doses of an insecticides. *Oecologia* 129:518-524.

Boone MD, Semlitsch RD, Little EE, Doyle MC. 2007. Multiple stressors in amphibian communities: Effects of chemical contamination, bullfrogs, and fish. *Ecological Applications* 17:291-301.

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*. *Environmental Toxicology and Chemistry* 30:682-691.

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. *Archives of Environmental Contamination and Toxicology* 23:69-84.

Brock TCM, Van Wijngaarden RPA, Van Geest G. 2000. Ecological risks of pesticides in freshwater ecosystems. Part 2: Insecticides. *Alterra-Report 089,* Wageningen, The Netherlands.

Brogan III WR, Relyea RA. 2013a. Mitigating with macrophytes: Submersed plants reduce the toxicity of pesticide-contaminated water to zooplankton *Environmental Science and Technology* 32:699-706.

Brogan III WR, Relyea RA. 2013b. Mitigating of malathion's acute toxicity using four submersed macrophyte species. *Environmental Science and Technology* 32:1535-1543.

Brogan III WR, Relyea RA. 2014. A new mechanism of macrophyte mitigation: How submerged plants reduce malathion's acute toxicity to aquatic animals. *Chemosphere* _:__-__.

Burrows HD, Canle L M, Santaballa JA, Steenken S. 2002. Reaction pathways and mechanisms of photodegradation of pesticides. *Journal of Photochemistry and Photobiology B: Biol*ogy 67:71-108.

Cairns J Jr. 1986. Community Toxicity Testing: A symposium. ASTM STP 920. Ann Arbor, MI, USA.

Carpenter SR, Lodge DM. 1986. Effects of submersed macrophytes on ecosystem processes. *Aquatic Botany* 26:341-370.

Carter V, Barko JW, Godshalk GL, Rybicki NB. 1988. Effects of submersed macrophytes on water quality in the tidal Potomac River, Maryland. *Journal of Freshwater Ecology* 4:493-501.

Chapman RA, Cole CM. 1982. Observations on the influence of water and soil pH on the persistence of insecticides. *Journal of Environmental Science and Health Part B: Pesticide and Food Contamination* 17:487-504.

Christensen K, Harper B, Luukinen B, Buhl K, Stone D. 2009. Chlorpyrifos Technical Fact Sheet. National Pesticide Information Center, Oregon State University Extension Services. http://npic.orst.edu/factsheets/chlorptech.pdf.

Collins JP, Storfer A. 2003. Global amphibian declines: sorting the hypotheses. *Diversity and Distributions* 9:89-98.

Cooper CM, Moore MT, Bennett ER, Smith Jr. S, Farris JL, Milam CD, Shields Jr. FD. 2004. Innovative uses of vegetated drainage ditches for reducing agricultural runoff. *Water Science and Technology* 49:117-123.

Coors A, De Meester L. 2008. Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. *Journal of Applied Ecology* 45:1820-1828.

Crum SJH, van Kammen-Polman AMM, Liestra M. 1999. Sorption of nine pesticides to three aquatic macrophytes. *Archives of Environmental Contamination and Toxicology* 37:310-316.

Cusimano RF, Brakke DF, Chapman GA. 1986. Effects of pH on the toxicities of cadmium, copper, and zinc to steelhead trout (*Salmo gairdneri*). *Canadian Journal of Fisheries and Aquatic Science* 43:1497-1503.

Davidson C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. *Ecological Applications* 14:1892-1902.

De Carvalho RF, Bromilow RH, Greenwood R. 2007. Uptake of pesticides from water by curly waterweed *Lagarosiphon major* and lesser duckweed *Lemna minor*. *Pest Management Science* 63:789-797.

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. *Environmental Toxicology and Chemistry* 30:2888-2891.

deNoyelles F, Dewer SL, Huggins DG, Kettle WD. 1994. Aquatic mesocosms in ecological effects testing: detecting direct and indirect effects of pesticides. *In* RL Graney, JH Kennedy, JH Rodgers Jr. eds. Aquatic mesocosm studies in ecological risk assessment. Pp:577-603. Lewis, Boca Raton, Florida, USA.

Distel CA, Boone MD. 2010. Effects of aquatic exposure to the insecticide carbaryl are species-specific across life stages and mediated by heterospecific competitors in anurans. *Functional Ecology* 24:1342-1352.

Duarte CM, Kalff J. 1990. Biomass density and the relationship between submerged macrophyte biomass and plant growth form. *Hydrobiologia* 196:17-23.

Duggan IC, Green JD, Thompson K., Shiel RJ. 2001. The influence of macrophytes on the spatial distribution of littoral rotifers. *Freshwater Biology* 46:777-786.

Edginton, AN, PM Sheridan, GR Stephensen, DG Thompson, HJ Boermans. 2004. Comparative effects of pH and vision herbicide on two life stages of four anuran amphibian species. *Environmental Toxicology and Chemistry* 23:815-822.

Fairchild JF, La Point TW, Zajicek JL, Nelson MK, Dwyer J, Lovely PA. 1992. Population-, community- and ecosystem-level responses of aquatic mesocosms to pulsed doses of a pyrethroid insecticide. *Environmental Toxicology and Chemistry* 11:115–129.

Finney DJ. 1971. Probit Analysis. Cambridge University Press, New York, NY.

Fleeger JW, Carman KR, Nisbet RM. 2003. Indirect effects of contaminants in aquatic ecosystems. *Science of the Total Environment* 317:207-233.

Forbes VE, Calow P, Sibly RM. 2008. The extrapolation problem and how population modeling can help. *Environmental Toxicology and Chemistry* 27:1987-1994.

Frodge JD, Thomas GL, Pauley GB. 1990. Effects of canopy formation by floating and submerged macrophytes on the water quality of two shallow lakes. *Aquatic Botany* 38:231-248.

Gao J, Garrison AW, Hoehamer C, Mazur CS, Wolfe NL. 2000a. Uptake and phytotransformation of *o,p'-DDT* and *p,p'-DDT* by axenically cultivated aquatic plants. *Journal of Agriculture and Food Chemistry* 48:6121-6127.

Gao J, Garrison AW, Hoehamer C, Mazur CS, Wolfe NL. 2000b. Uptake and phytotransformation of organosphosphate pesticides by axenically cultivated aquatic plants. *Journal of Agriculture and Food Chemistry* 48:6114-6120.

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 and Applied Ecology* 11:97-105.

Gilliom RJ. 2007. Pesticides in US streams and groundwater. *Environmental Science and Technology* 41:3408-3414.

Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183-190.

Grube A, Donaldson D, Kiely T, Wu L. 2011. Pesticide industry sales and usage: 2006 and 2007 market estimates. USEPA. Washington, DC, USA.

Halstead BG, Tash JC. 1982. Unusual diel pHs in water as related to aquatic vegetation. *Hydrobiologia* 96:217-224.

Hanazato T, Dodson SI. 1995. Synergistic effects of low oxygen concentration, predator kairomone, and a pesticide on the cladoceran *Daphnia pulex*. *Limnology and Oceanography* 40:700-709.

Hanazato T, Yasuno M. 1987. Effects of a carbamate insecticide carbaryl on the summer phytoplankton and zooplankton communities in ponds. *Environmental Pollution* 48:145-159.

Hanazato T. 2001. Pesticide effects on freshwater zooplankton: an ecological perspective. - *Environmental Pollution* 112:1-10.

Hanazato T. 1998. Response of a zooplankton community to insecticide application in experimental ponds: a review and the implications of the effects of chemicals on the structure and functioning of freshwater communities. *Environmental Pollution* 101:361-373.

Hanazato T, Yasuno M. 1990. Influence of time of application of an insecticide on recovery patterns of a zooplankton community in experimental ponds. *Archives of Environmental Contamination and Toxicology* 19:77-83.

Hand LH, Kuet SF, Lane MCG, Maund SJ, Warinton JS, Hill IR. 2001. Influences of aquatic plants on the fate of the pyrethroid insecticides *lambda*-cyhalothrin in aquatic environments. *Environmental Toxicology and Chemistry* 20:1740-1745.

Harper B, Luukinen B, Gervais JA, Buhl K, Stone D. 2009. Diazinon Technical Fact Sheet. National Pesticide Information Center, Oregon State University Extension Services.

http://npic.orst.edu/factsheets/diazinontech.html.

Hilt S, Gross EM. 2008. Can allelopathically active submerged macrophyte stabilize clear-water states in shallow lakes. *Basic and Applied Ecology* 9:422-432.

Hoffman RS, Capel PD, Larson SJ. 2009. Comparison of pesticides in eight U.S. urban streams. *Environmental Toxicology and Chemistry* 19:2249-2258.

Hopson MS, Zimba PV. 1993. Temporal variation in the biomass of submersed macrophytes in Lake Okeechobee, Florida. *Journal of Aquatic Plant Management* 31:76-81.

Hua J, Relyea RA. 2012. East Coast versus West Coast: Effects of an insecticide in communities containing different amphibian assemblages. *Freshwater Science* 21:787-799.

Ip YK, Chew SF, Randall DJ. 2001. Ammonia toxicity, tolerance and excretion. *Fish Physiology* 20:109-148.

Jones JI, Moss B, Eaton JW, Young JO. 2000. Do submerged aquatic plants influence periphyton community composition for the benefit of invertebrate mutualists? *Freshwater Biology* 43:591-604.

Jones JI, Young JO, Haynes GM, Moss B, Eaton JW, Hardwick KJ. 1999. Do submerged aquatic plants influence their periphyton to enhance the growth and reproduction of invertebrate mutualists? *Oecologia* 120:463-474.

Karen DJ, Joab BM, Wallin JM, Johnson KA. 1998. Partitioning of chlorpyrifos between water and an aquatic macrophyte (*Egeria densa*). *Chemosphere* 37:1579-1586.

Kast-Hutchinson K, CV Rider, GA LeBlanc. 2001. The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environmental Toxicology and Chemistry* 20:502-509.

Kegley SE, Hill BR, Orme S, Choi AH. 2010. Pesticide Action Network Pesticide Database. Pesticide Action Network North America. San Francisco, CA, USA.

Kiely T, Donaldson D, Grube A. 2004. Pesticide industry sales and usage: 2000 and 2001 market estimates. U.S. Environmental Protection Agency, Washington DC.

Kieseker JM. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? *Proceedings of the National Academy of the Sciences* 99:9900-9904.

Kikuchi M, Sasaki Y, Wakabayashi M. 2000. Screening of organophosphate insecticide pollution in water by using *Daphnia magna. Ecotoxicology and Environmental Safefty* 47:239–245.

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.

Knäbel A, Stehle S, Schäfer RB, Schulz R. 2012. Regulatory FOCUS surface water models fail to predict insecticide concentrations in the field. *Environmental Science and Technol*ogy 46:8397-8404.

Kröger R, Moore MT, Locke MA, Cullum RF, Steinriede Jr. RW, Testa III S, Bryan CT, Cooper CM. 2009. Evaluating the influence of wetland vegetation on chemical residence time in Mississippi delta drainage ditches. *Agriculture and Water Management* 96:1175-1179.

Kufel L, Kufel L, Kroólikowska J. 2004. The effect of lake water characteristics on decomposition of aquatic macrophytes. *Polish Journal of* Ecology 52:261-273.

Larsson P, Dodson SI. 1993. Chemical communication in planktonic animals. *Archives of Hydrobiologia* 129:129-155.

Lathrop RC, Carpenter SR, Robertson DM. 1999. Summer water clarity responses to phosphorus, *Daphnia* grazing, and internal mixing in Lake Mendota. *Limnology and Oceanography* 44:137-146.

Laurence WF. 2001. Future shock: Forecasting a grim fate for the Earth. *Trends in Ecology and Evolution* 16:531-533.

Leistra M, Zweers AJ, Warinton JS, Crum SJH, Hand LH, Beltman WHJ, Maund SJ. 2003. Fate of the insecticide lambda-cyhalothrin in ditch enclosures differing in vegetation density. *Pest Management Science* 60:75-84.

Lizotte Jr. RE, 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 and Soil Pollution* 220:69-79.

Lodge DM. 1985. Macrophyte-gastropod associations: observations and experiments on macrophyte choice by gastropods. *Freshwater Biology* 15:695-708.

Lodge DM. 1991. Herbivory on freshwater macrophytes. *Aquatic Botany* 41:195-224.

Mills NE, Semlitsch RD. 2004. Competition and predation mediate the indirect effects of an insecticide on southern leopard frogs. *Ecological Applications* 14:1041-1054.

Mitsch WJ, Gosselink JG. 1986. Wetlands. Van Nostrand Reinhold, New York, NY, USA.

Mohamed ZK, Ahmed MA, Fetyan NA, Elnagdy SM. 2010. Isolation and molecular characterization of malathion-degrading bacterial strains from waste water in Egypt. *Journal of Advanced Research* 1:145-149.

Moore MT, Bennett ER, Cooper CM, Smith Jr. S, Farris JL, Douillard KG, Schulz R. 2006. Influence of vegetation in mitigation of methyl parathion runoff. *Environmental Pollution* 142:288-294.

Moore MT, Kröger R, Farris JL, Locke MA, Bennett ER, Denton DL, Cooper DM. 2011. From vegetated ditches to rice fields: thinking outside the box for pesticide mitigation. *In* Pesticide Mitigation Strategies for Surface Water Quality. Goh KS, Bret BL, Potter T, Gan J. (eds.) pp. 29-37.

Morin PJ. 1983. Predation, competition, and the composition of larval anuran guilds. *Ecological Monographs* 54:119-138.

Newhardt K. 2006. Environmental fate of malathion. California Environmental Protection Agency, Department of Pesticide Regulation, California, USA.

Newman MC (ed). 2010. Fundamentals of Ecotoxicology 3rd Edition. CRC Press, NY.

Nichols SA, Shaw BH. 1986. Ecological life histories of the three aquatic nuisance plants, *Myriophyllum spicatum, Potamogeton crispus* and *Elodea canadensis*. *Hydrobiologia* 131:3-21.

Novak M, Wootton JT, Doak DF, Emmerson M, Estes JA, Tinker MT. 2011. Predicting community responses to perturbations in the face of imperfect knowledge and network complexity. *Ecology* 92:836-846.

Nurmenin L. 2003. Macrophyte species composition reflecting water quality changes in adjacent water bodies of lake Hiidenvesi, SW Finland. *Annales Botanici Fennici* 40:199-208.

Odenkirchen E, Wente SP. 2007. Risks of malathion use to federally listed California red-legged frog (*Rana aurora draytonii*). US Environmental Protection Agency Environmental Fate and Effects Division, Washington, DC, USA.

Omar SA. 1998. Availability of phosphorus and sulfur of insecticide origin by fungi. *Biodegradation* 9:327-336.

Ondok JP, Pokorny J, Kvet J. 1984. Model of diurnal changes in oxygen, carbon dioxide and bicarbonate concentrations in a stand of *Elodea canadensis* Michx. *Aquatic Botany* 19:293-305.

Organisation for Economic Co-operation and Development Guideline 202. 1984. *Daphnia* sp., acute immobilization test and reproduction test. Part I: 24 h EC50 acute immobilization test. OECD. Paris, France.

Paine RT. 1969. A note on trophic complexity and community stability. *The American Naturalist* 103:91-93.

Paine RT, Tegner MJ, Johnson EA. 1998. Compounded perturbations yield ecological surprises. *Ecosystems* 1:535-545.

Paris DF, Steen WC, Baughman GL, Barnett Jr JT. 1981. Second-order model to predict microbial degradation of organic compounds in natural waters. *Applied and Environmental Microbiology* 41:603-609.

Park RA, Clough JS, Wellman MC. 2008. AQUATOX: Modeling environmental fate and ecological effects in aquatic ecosystems. *Ecological Modelling* 213:1-15.

Payton ME, Greenstone MH, Shenker N. 2003. Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical signficance? *Journal of Insect Science* 34:1-6.

Raspopov IM, Adamec LA, Husák S. 2002. Influence of aquatic macrophytes on the littoral zone habitats of the lake ladoga, NW Russia. *Preslia* 74:315-321.

Reichenberger S, Bach M, Skitschak A, Frede HG. 2007. Mitigation strategies to reduce pesticide inputs into ground- and surface water and their effectiveness; a review. *Science of the Total Environment* 384:1-35.

Relyea RA, Hoverman JT. 2006. Assessing the ecology in ecotoxicology: A review and synthesis in freshwater systems. *Ecology Letters* 9:1157-1171.

Relyea RA, Hoverman JT. 2008 Interactive effects of predators and a pesticide on aquatic communities. *Oikos* 117:1647-1658.

Relyea RA, Mills N. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proceedings of the National Academy of the Sciences USA* 98:2491-2496.

Relyea RA. 2003. Predator cues and pesticides: A double dose of danger for amphibians. *Ecological Applications* 13:1515-1521.

Relyea RA. 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. *Environmental Toxicology and Chemistry* 23:1080-1084.

Relyea RA. 2005. The impact of insecticide and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications* 15:618-627.

Relyea RA. 2009. A cocktail of contaminants: How mixtures of pesticides at low concentrations affect aquatic communities. *Oecologia* 159:363-376.

Relyea RA. 2010. Multiple stressors and indirect food web effects of contaminants on herptofauna. Pages 475-486 in D. Sparling, ed. Ecotoxicology of Amphibians and Reptiles, 2nd edition.

Relyea RA, Hoverman JT. 2008. Interactive effects of predators and a pesticide on aquatic communities. *Oikos* 117:1647-1658.

Relyea RA, Diecks N. 2008. An unforeseen chain of events: lethal effects of pesticides at sublethal concentrations. *Ecological Applications* 18:1728–1742.

Roessink I, Arts GHP, Belgers JD, Bransen F, Maund SJ, Brock TC. 2005. Effects of lambda-cyhalothrin in two ditch microcosm systems of different trophic status. *Environmental Toxicology and Chemistry* 24:1684-1696.

Rohr JR**,** Kerby, J, Sih A. 2006. Community ecology theory as a framework for predicting contaminant effects. *Trends in Ecology and Evolution*. 21:606-613.

Sand-Jensen J, Borum J. 1991. Interactions among phytoplankton, periphyton, and macrophytes in temperature freshwaters and estuaries. *Aquatic Botany* 41:137-175.

Sand-Jensen J, Møller J, Olesen BH. 1988. Biomass regulation of microbenthic algae in a Danish lowland stream. *Oikos* 53:332-340.

Sarnelle O. 2005. *Daphnia* as keystone predators: effects on phytoplankton diversity and grazing resistance. *Journal of Plankton Research* 27:1229-1238.

Schulz R. 2004. Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution: A review. *Journal of Environmental Quality* 33:419-448.

Scott DM, Lucas MC, Wilson RW. 2005. The effect of high pH on ion balance, nitrogen excretion and behaviour of freshwater fish from an eutrophied lake: A laboratory and field study. *Aquatic Toxicology* 73:31-43.

Seaman AJ, Riedl H. 1986. Preventing decomposition of agricultural chemicals by alkaline hydrolysis in the spray tank. *New York Food Life Science Bulletin* 118:1-7.

Smith DC. 1987. Adult recruitment in chorus frogs: Effects of size and date at metamorphosis. *Ecology* 68:344-350.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306:1783-1786.

Talling JF. 1976. The depletion of carbon dioxide from lake water by phytoplankton. *Journal of Ecology* 64:79-121.

Tchounwou PB, Englande AJ, Malek EA. 1991. Toxicity evaluation of bayluscide and malathion to three developmental stages of freshwater snails. *Archives of Environmental Contamination and Toxicology* 21:351-358.

Thomas KA, Hand LH. 2011. Assessing the potential for algae and macrophytes to degrade crop protection products in aquatic ecosystems. *Environmental Toxicology and Chemistry* 30:622-631.

Toivonen H, Huttunen P. 1995. Aquatic macrophytes and ecological gradients in 57 small lakes in southern Finland. *Aquatic Botany* 51:197-221.

United States Environmental Protection Agency OPPTS 850.1300. 1996. Ecological Effects Test Guidelines: Daphnid chronic toxicity tests. USEPA Prevention, Pesticides and Toxic Substances, Washington D.C., USA.

Vadeboncouer Y, Lodge DM, Carpenter SR. 2001. Whole lake fertilization effects on distribution of primary production between benthic and pelagic habitats. *Ecology* 82:1065-1077.

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. *Archives of Hydrobiology* 133:417-439.

Van Donk E, van de Bund WJ. 2002. Impact of submerged macrophytes including charophytes on phyto- and zooplankton communities: allelopathy versus other mechanisms. *Aquatic Botany* 72:261-274.

Wang T. 1991. Assimilation of malathion in the Indian River estuary, Florida. *Bulletin of Environmental Contamination and Toxicology* 47:238–243.

Werner I, Deanovic LA, Miller J, Denton DL, Crane D, Mekebri A, Moore MT, Wrysinki J. 2010. Use of vegetated agricultural drainage ditches to decrease toxicity of irrigation runoff from tomato and alfalfa fields in California, USA. *Environmental Toxicology and Chemistry* 29:2859-2868.

Wetzel D. 2001. Limnology: Lake and River Ecosystems. Third Edition. Academic Press.

Wolfe NL, Zepp RG, Gordon JA, Baughman GL, Cline DM. 1977. Kinetics of chemical degradation of malathion in water. *Environmental Science and Technology* 11:88-93.

Wolfe NL, Zepp RG, Paris DF. 1978. Carbaryl, propham and chlorpropham: A comparison of the rates of hydrolysis and photolysis with the rate of biolysis. *Water Research* 12:565-571.

Wootton JT. 1994. The nature and consequences of indirect effects in ecological communities. *Annual Review of Ecology and Systematics* 25:443-466.

Wootton JT. 2002. Indirect effects in complex ecosystems: Recent progress and future challenges. *Journal of Sea Research* 48:157-172.

Wright PA, Randall DJ, Perry SF. 1989. Fish gill water boundary layer – a site of linkage between carbon dioxide and ammonia excretion. *Journal of Comparative Physiology B* 158:627-635.

Yodzis P. 1988. The indeterminacy of ecological interactions as perceived through pertubation experiments. *Ecology* 69:508-515.

Zaga A, Little EE, Raben CF, Ellersieck MR. 1998. Photoenhanced toxicity of a carbamate insecticide to early life stage anuran amphibians. *Environmental Toxicology and Chemistry* 17:2543-2553.