ABSTRACT

Prymnesium parvum in Inland Waters—Comparative Toxicity, Microcystin Allelopathy, and Toxin Photodegradation

Susan Virginia James, M.S.

Mentor: Bryan W. Brooks, Ph.D.

The harmful algal species *Prymnesium parvum*, a toxin-producing mixotrophic haptophyte, has caused massive fish kills in slightly saline inland Texas water bodies. In the first study, standardized aquatic test models from multiple trophic levels were simultaneously employed to compare sensitivities to *P. parvum* toxins. Fish mortality was the most sensitive endpoint, though adverse reproduction effects in two invertebrate species were observed; a green algal species was not adversely affected. A second study employed a probabilistic risk assessment approach and examined potential allelopathy of the cyanotoxin microcystin-LR to *P. parvum*. A concentration of 4,392.8 $\mu g\ l^{-1}$ significantly inhibited *P. parvum* growth over a portion of the study, which corresponded to a 9% probability of detecting this concentration in the environment. Finally, the effect of sunlight on toxicity of *P. parvum* cell-free filtrate was assessed. Exposure to eight hours of full or ~50% sunlight eliminated toxicity to *Pimephales promelas*, suggesting toxin photodegradation.

Prymnesium parvum in Inland Waters—Comparative Toxicity, Microcystin Allelopathy, and Toxin Photodegradation

by

Susan Virginia James, B.S.

A Thesis

Approved by the Department of Environmental Science

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Susan P. Bratton, Ph.D., Chairperson

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Approved by the Thesis Committee

_____
Bryan W. Brooks, Ph.D., Chairperson

_____
Joe C. Yelderman, Jr., Ph.D.

_____
Owen T. Lind, Ph.D.

Accepted by the Graduate School
August 2009

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J. Larry Lyon, Ph.D., Dean

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ABBREVIATIONS

ASW	Artificial Seawater
ED	Exposure Distribution
ELISA	Enzyme Linked Immunosorbent Assay
HAB	Harmful Algal Bloom
LC_{50}	Median Lethal Concentration
LOAEL	Lowest Observable Adverse Effect Level
LOEC	Lowest Observable Effect Concentration
LT_{50}	Median Lethal Time
MC-LR	Microcystin-LR
NOAEL	No Observable Adverse Effect Level
NOEC	No Observable Effect Concentration
RHW	Reconstituted Hard Water
SSD	Species Sensitivity Distribution
US EPA	United States Environmental Protection Agency

ACKNOWLEDGMENTS

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DEDICATION

This work is dedicated to those who truly know me.

“The only true wisdom is in knowing you know nothing.”—Socrates

CHAPTER ONE

Introduction and Overview

Introduction

An increasing global problem that marine, estuarine, and freshwater ecosystems must face is the greater frequency and severity of harmful algal blooms (HABs) (Lopez et al. 2008). Anthropogenic sources into receiving aquatic systems, such as sewage, detergents, agricultural run-off, and industrial run-off, have caused more water bodies to be impaired by eutrophication. The loading of nutrients into aquatic systems as been associated with increases in the occurrence of HABs over the past decades (Heisler et al. 2008, Dawson et al. 1998). Since activities that cause the excessive nutrient inputs are unlikely to subside in the near future, algal blooms will likely continue to occur frequently over widely distributed locations. Dense algal blooms in water bodies can be aesthetically unpleasing and distress local economies by reducing tourism. This is perhaps a superficial consequence of HABs, but it is important to local communities that rely on income from water related recreation. More importantly from an ecological perspective, the presence of algae in high numbers can be detrimental to ecosystems by altering both the physical and chemical environment. Altered parameters that can negatively affect aquatic life include increased dissolved organic material and light attenuation, as well as decreases in dissolved oxygen following algae decay. However, the primary reason that algal blooms are unfavorable is that several of the algal species produce toxins that can adversely affect aquatic organisms and/or human health (Lopez et

al. 2008). Although all algal species are primary producers and thus generally considered a critical component of aquatic ecosystems, when their biomass exceeds a certain amount and their toxin production exceeds a certain threshold, there can be devastating effects. HAB species are found in both freshwater and marine environments throughout the world.

The fact that harmful algal blooms are not confined to one or even a few geographic locations on varying spatial scales makes it difficult to establish management strategies between and within watersheds. In America, almost every coastal state has reported problems associated with harmful algal blooms (Lopez et al. 2008). Examples of coastal blooming species (with an associated toxin) are *Pyrodinium* spp. (saxitoxins), *Karenia brevis* (brevetoxins), *Gambierdiscus toxicus* (ciguatoxins), and *Pseudo-nitzschia* spp. (domoic acid) (Van Dolah et al. 2001). These toxins are associated with human, fish and shellfish poisonings, that result in symptoms ranging from nausea, vomiting, and cramps, to blurred vision, hypertension, seizures, tachycardia, paralysis, and death (Van Dolah et al. 2001). In addition to being a marine/coastal problem, HAB species also cause adverse effects in freshwater systems. Cyanobacteria, or blue-green algae, are widely documented as being a problem common to freshwater systems. At least 35 states have reported problems with cyanobacteria, including diverse locations such as New York, Oregon, Iowa, Nebraska, and Florida (Lopez et al. 2008). One unique HAB species of particular interest to my research, which originated in a marine environment and migrated into inland Texas water bodies, is *Prymnesium parvum*. Since its first identification in Texas, *P. parvum*, has since been confirmed in New Mexico, Colorado, Wyoming, North Carolina, South Carolina, Georgia, Arkansas, Alabama, and Oklahoma

and has been suspected in Florida and Nebraska (Lopez et al. 2008, Baker et al. 2007). This thesis focuses on *P. parvum* because it has become an invasive species of concern to Texas, causing widespread damage to ecosystems.

P. parvum is a eurythermal, euryhaline, flagellated HAB species that produces the toxins prymnesin-1 and prymnesin-2 (Igarashi et al.1999). These toxins aid in the mixotrophic life strategy for obtaining nutrients, and consequently are attributed to massive fish mortalities in the environment (Baker et al. 2007). In addition to photosynthesizing like all other algae, *P. parvum* employs its toxins to immobilize and lyse single-celled prey that it then phagocytizes (Skovgaard and Hansen 2003). The purpose of my research is to better understand the effects that *P. parvum* toxins have in the environment as well as what in the environment affect *P. parvum* and its toxins. An advanced understanding of this organism and its associated toxins will support efforts of fisheries management and mitigation.

Overview

The following three chapters detail the research I conducted to further understand *P. parvum* and its impacts in inland waters. Chapter Two focuses on the comparative toxicity of *P. parvum* cell-free filtrates to several aquatic model organisms. By performing simultaneous ecotoxicity experiments with a common culture, relative sensitivities to the toxins were assessed. This is critical because purified prymnesins are not available. Standard research protocols and model organisms were utilized, unlike in much of the past studies (Brooks et al. in press), in order to ensure quality research and repeatablilty. Model organisms selected from multiple trophic levels tested included a

fish species, *Pimephales promelas*, two invertebrate species, *Brachionus calyciflorus* and *Daphnia magna*, and a competitor algal species *Pseudokirchneriella subcapitata*.

In Chapter Three I performed a probabilistic assessment of the cyanobacterial toxin microcystin-LR (MC-LR), because allelopathy from cyanobacteria may reduce *P. parvum* growth (Roelke et al. in press). Data used in the probabilistic distributions came from over 200 peer-reviewed studies. This assessment provided information on the probability that MC-LR will occur in freshwater systems and adversely affect aquatic organisms, which subsequently was used to select treatment levels of a study with MC-LR and *P. parvum*. *P. parvum* growth was observed over 27 days to establish thresholds of MC-LR toxicity.

The fourth chapter includes a novel pilot study of photolytic degradation of *P. parvum* toxins. Cell-free filtrates of *P. parvum* were exposed to two intensities of natural sunlight (full sunlight, ~50% sunlight) for eight hours and dark treatment levels at ambient temperature and 4^{o}C. Because quantitation of prymnesins is not possible at this time, toxicity tests were conducted with *P. promelas* as a surrogate for bioavailable toxin concentrations. Fish mortality and the onset of ecotoxicological responses to the filtrates containing extracellular *P. parvum* toxins were examined to estimate photolysis rates.

CHAPTER TWO

Comparative Toxicity of *Prymnesium parvum* Toxins to Freshwater Organisms

Introduction

Prymnesium parvum

Prymnesium parvum, commonly known as golden algae due to its golden pigment, is a eurythermal, euryhaline, flagellated haptophyte that employs a mixotrophic life strategy (Legrand et al. 2001). Mixotrophy describes when energy is obtained both photosynthetically and via phagotrophy (Burkholder et al. 2008). The ability to regulate its position in the water, obtain nutrients in multiple manners, and live in a wide range of temperatures and salinities likely give *P. parvum* a competitive advantage and enable it to thrive under diverse environmental conditions. The reason it is of concern as a harmful algal species is that *P. parvum* produces toxins called prymnesins (Igarashi et al. 1999). These prymnesins have been found to severely affect ecosystems, especially at the higher trophic level of the fish. *P. parvum* is responsible for massive fish kills in Texas reservoirs that have caused millions of dollars in economic loss (Baker et al. 2007). This ability to proliferate in diverse locations and greatly impact ecosystems, yet not appear to affect human health has caused *P. parvum* to be an interesting species of research. Much investigated has been conducted on factors affecting this species growth and toxicity, as well as what effects it has on other species. For example, temperature, salinity, and light have been found to influence *P. promelas* growth, and it is more toxic under nutrient

limited conditions (Larsen and Bryant 1998, Baker et al. 2007, Granéli and Johansson 2003a, b, Skovgaard et al. 2003).

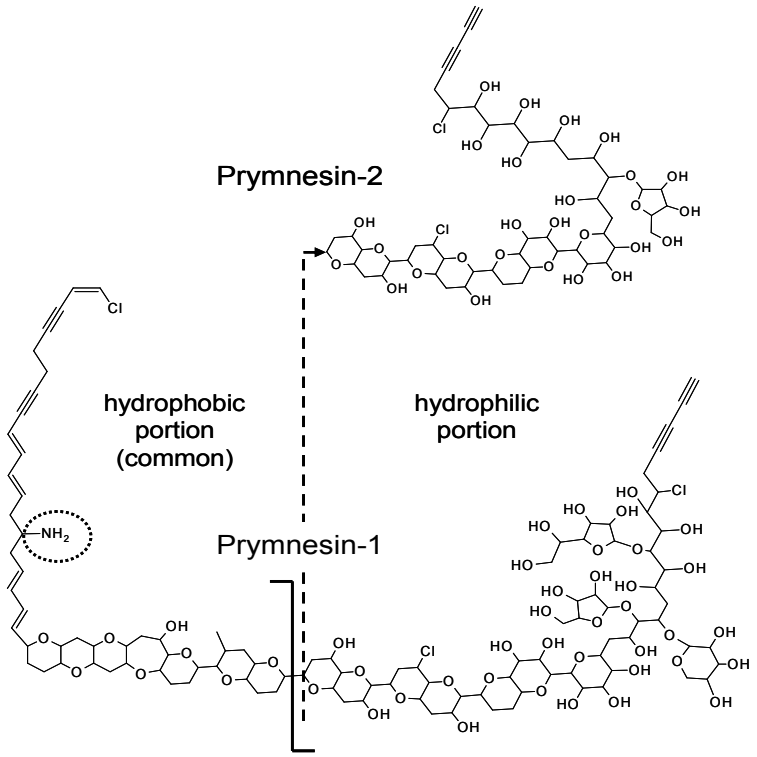

Figure 1. Structure of prymnesins-1 and -2 (modified from Valenti et al. accepted).

Prymnesins

The structures of prymnesin -1 and -2 (Figure 1) were elucidated a decade ago and found to be very large glycosides with hydrophilic and hydrophobic properties (Igarashi et al. 1998, Igarashi et al. 1999). They consist of large chains of 90 carbon atoms and trans-1, 6-dioxadecaline units with conjugated double/triple bonds at each terminal end (Igarashi et al. 1999). These compounds are amphiphilic, with uneven distributions of sugars and hydroxyl groups, three chlorine atoms, and one nitrogen atom. The amine present on the hydrophobic end, which is common to both prymnesins, suggests that these compounds might be weak ionizable bases with pKa values > 8

(Valenti et al. accepted). This results in variable toxicity depending on the pH of the environmental matrix.

Prymnesins are toxic to gill-breathing species such as fish, arthropods, mollusks, and the gill-breathing stage of amphibians (Paster 1973). The toxins target the permeability mechanism of the gill, which disrupts ionoregulation (Yariv and Hestrin 1961). Experiments have shown that on a cellular level, prymnesins have lytic action on blood cells, hepatocytes, and Hela cells (Bergmann et al. 1963, Shilo et al. 1960). However, no data suggests that prymnesins are toxic to humans, although further study is warranted, particularly for chronic effects (Paster 1973).

The biggest difficulty in researching prymnesins and their effects on other organisms is that neither purified standards nor analytical techniques to measure concentrations in water samples have been developed. Consequently, accurate quantitation in surface waters is not possible at this time. In addition, experiments cannot be performed utilizing discrete, verified concentrations of toxins in order to compare effects. This markedly increases uncertainty when attempting to integrate the results of prior published studies to estimate ecological risks. One method of study has involved culturing *P. parvum* and studying either the effect of whole cultures or cell-free filtrates on other organisms (Baker et al. 2007, Roelke et al. 2007, Brooks et al. in press). However, because toxins are produced in different amounts under different culture conditions (Baker et al. 2007, Granéli and Johansson 2003a, b), relative species sensitivities to prymnesins cannot be accurately determined. To overcome this limitation, my study tested the toxicity of *P. parvum* cell-free filtrates concurrently to different organisms from different trophic levels. I specifically selected *Pimephales promelas*

survival to quantify adverse impacts on a juvenile fish, survival and reproduction of *Daphnia magna* and *Brachionus calyciflorus* models to assess effects on predators of *P. parvum*, and model growth of the green algae model *Pseudokirchneriella subcapitata* to examine competitor responses. Test cultures were grown under laboratory conditions meant to simulate natural conditions when *P. parvum* has been reported to bloom in Texas inland, slightly saline waters. This is particularly important because little research has been done to study *P. parvum* in freshwater systems with salinities ≤4 psu. For example, of the 95 toxicity assays described in 44 peer-reviewed journal articles reviewed in Brooks et al. (in press), only about half of the assays reported salinity (in ppt or psu). Of these assays with reported salinities, only 23% of them utilized a salinity ≤4 psu; a majority of those papers was from the same research group that is concerned with *P. parvum* in inland Texas water bodies. In addition, all bioassays were completed based upon standard methods, which has not been the case for much of the prior research (Brooks et al. in press). Using standard methods helps to ensure quality control and repeatability of experiments and should be used whenever possible.

Materials and Methods

Prymnesium parvum Stock Cultures

Approximately 9 x10^3 cells ml^{-1} of *P. parvum* from a non-axenic previously cultured toxic batch grown from a culture obtained from The University of Texas at Austin Culture Collection of Algae (strain UTEX LB 2797) was used to inoculate a defined media of artificial seawater (ASW; Berges et al. 2001;, Appendix A) diluted to 2.4 psu with Nanopure® (Thermo Fisher Scientific, Waltham, MA, USA) water and

enriched with f/8 levels of nutrients and f/2 levels of trace metals and vitamins in glass carboys in triplicate (Guillard 1975; appendices A, B). Nutrient limitation in f/8 media was previously shown by our group to cause greater toxicity associated with *P. parvum* (Brooks et al. in press). Algae were grown in a VWR Model 2015 incubator (West Chester, PA, USA) at 20±1^{o}C on a 12:12 light:dark cycle until late stationary phase. The temperature, salinity, and photoperiod were selected to represent environmental conditions observed when *P. parvum* blooms have occurred in inland Texas water bodies (Brooks et al. in press). Toxicity of cultures was preliminarily assessed using *P. promelas* on day 15 following the standard bioassay described below in order to verify that all cultures were indeed producing toxin(s). On day 36, when the cultures were in late stationary phase, cells were separated from culture media via filtration (Whatman GF/C; VWR International, West Chester, PA, USA) in order to prepare cell-free filtrates for assessing effects of extracellular toxins (Barreiro et al. 2005). Cell-free filtrate (instead of whole cultures) was used to: 1) observe effects from extracellular toxins; 2) not confound results with toxin exposure via ingestion of cells; and 3) prohibit the chance of further toxin production of *P. parvum* cells during exposures. *P. parvum* filtrate composites from the triplicate cultures was collected and stored in foil covered glass containers at 4±1˚C in the dark for less than 24 hrs prior to initiation of toxicity assays. Average cell count before harvesting, as determined using a hemocytometer, was approximately 2.98x10^{5} cells ml^{-1}. Cell-free filtrate used for these toxicity experiments was serially diluted using a 0.4 dilution factor to various percentages of initial *P. parvum* filtrate with 2.4 psu ASW, which was also used as controls.

Toxicity Bioassays

Pimephales promelas. Acute mortality for *P. promelas* to *P. parvum* cell-free filtrates was assessed generally according to US EPA method 2000.0 (US EPA 2002). Less than 96-hour old juveniles (all hatched within 24 hours) obtained from Environmental Consulting & Testing (Superior, WI, USA) were allowed to feed on *Artemia* nauplii at least two hours before initiation of the study. Using 250 ml of test solution in 600-ml beakers, four replicates with ten fish each were loaded in each of seven treatments plus an ASW control. Beakers were placed in an incubator (Norlake®; Hudson, WI, USA) maintained at 25±1˚C on a 16:8 light:dark cycle. Mortality was assessed at 24 and 48 hours after test initiation.

Brachionus calyciflorus. Rotifer cysts obtained from Florida Aqua Farms, Inc. (Dade City, FL) were allowed 24 hours to hatch in reconstituted hard water (RHW). Neonates were allowed to feed on Roti-rich™ supplemented with lab-cultured algae, *Pseudokirchneriella subcapitata*, before initiation of the test. Acute reproduction responses were assessed according to standard method 8420 (APHA et al. 1998). Six neonates were transferred into five replicate borosilicate glass test tubes of five concentrations plus an ASW control, each supplemented with an algae and Roti-rich™ food mixture. Test tubes were loaded onto a Glas-Col Model 099A RD4512 rotary wheel (VWR International, West Chester, PA, USA) to maintain the food mixture in suspension. The rotary wheel was placed in a VWR Model 2015 incubator (West Chester, PA, USA) and kept in the dark at 25±1˚C for 48 hrs. After 48 hrs the total

number of rotifers per tube was determined under a stereomicroscope and growth rate (r) was calculated with the equation

$$r = (\ln N_d - \ln N_0)/t, \quad (1)$$

where N_d is number of organisms at 48 hrs, N_0 is number of organisms at initiation (6 rotifers), and t is duration of test (2 days).

Daphnia magna. Prior to experimentation, *D. magna* were cultured as previously described by Hemming et al. (2002), and fed a Cerophyl®-*P. subcapitata* mixture daily (Knight and Waller 1992). Effects of *P. parvum* cell-free filtrate on *D. magna* reproduction was assessed over a 10 day study generally according to US EPA methods (US EPA 1994) with modifications previously reported by our laboratory (Dzialowski et al. 2006, Stanley et al. 2007, Roelke et al. 2007). Briefly, one <24-hour-old neonate was transferred into 25 mls of test solution for ten replicates at five test concentrations, plus an ASW control. Organisms were then placed in the incubator (Norlake®; Hudson, WI, USA) at 25±1˚C on a 16:8 light:dark cycle. Each daphnid was fed daily with 600 µl of *P. subcapitata* and Cerophyl® mixture. The number of neonates per female was determined during static renewals every other day for ten days when adults were transferred to fresh exposure water.

Pseudokirchneriella subcapitata. The effect of *P. parvum* cell-free filtrate on *P. subcapitata* growth was evaluated generally according to US EPA test method 1003.0 (US EPA 2002). *P. subcapitata* was cultured for a week in Algal Assay Procedure (AAP) medium (Appendix C), harvested by centrifugation (15 minutes at 10,000 rpm at 4˚C), and resuspended in RHW. Culture tubes (25x200 mm) with 50 mls volume of cell-

free filtrate for five replicates at five concentrations pre-dosed with AAP nutrients were inoculated to a cell density of approximately10^4 cells ml^{-1} of *P. subcapitata.* Culture tubes in slanted test tube racks were grown at 24±1˚C for 96 hours under constant illumination in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) and swirled twice daily. Cell density was estimated by *in vivo* chlorophyll *a* fluorescence with a Turner Design Model 10-AU-005-CE fluorometer (Sunnyvale, CA, USA) at 0, 24, 48, 72, and 96 hours. At the conclusion of the study, samples were preserved in Lugol's solution (Wetzel and Likens 2001) and cell densities were enumerated microscopically.

Statistical Analyses

Data from experimental treatment levels were converted to densities of *P. parvum* (cells ml^{-1}) from associated percentages of initial *P. parvum* filtrate to determine ecotoxicological benchmark values, such as No Observable Adverse Effect Levels (NOAELs) and the concentrations required to cause mortality in fifty percent of organisms (LC_{50}s), on a per cell basis. For *D. magna*, *B. calyciflorus*, and *P. subcapitata* studies, NOAELs and LOAELs of sublethal responses to *P. parvum* filtrate were determined using a one-way ANOVA with a Dunnett's test (SAS v.9.1., Cary, NC, USA). Statistically significant differences from controls were determined at $\alpha \leq 0.05$. Fisher's Exact Test was used to identify treatment level differences from control for *D. magna* survival (US EPA 2002). An LC_{50} value for *P. promelas* was estimated using the Trimmed Spearman Karber method (Hamilton et al. 1977).

R*esults*

Prymnesium parvum Stock Cultures

Each of three *P. parvum* cultures experienced exponential growth through day 15 and reached late stationary growth phase by day 36 when cells were harvested (Figure 2). As noted below, cells were highly toxic to fish at the end of exponential growth. Cell density in the three carboys were similar and the average cell density in composited cultures on day 36 was 2.98 x 10^5 cells ml^{-1}.

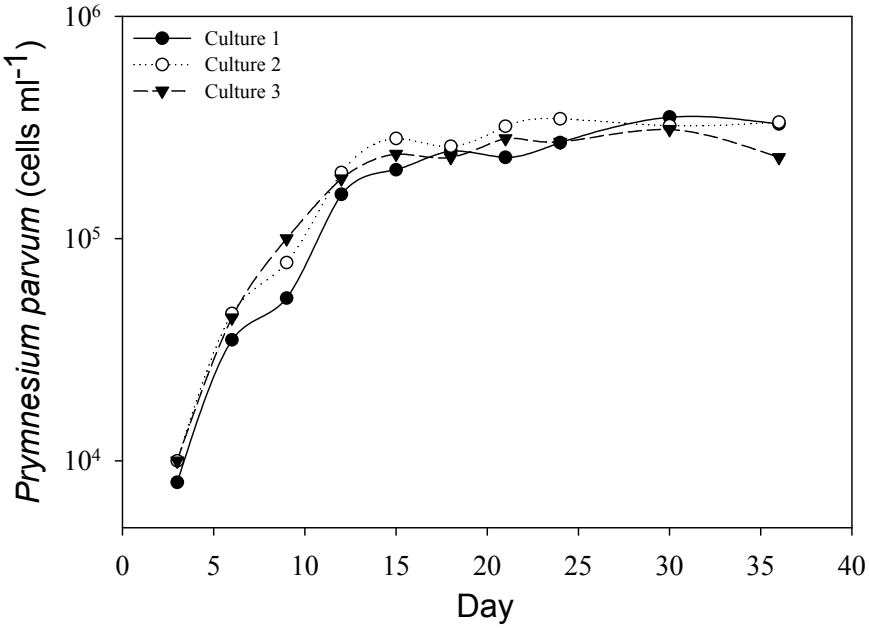

Figure 2. *Prymnesium parvum* cell concentrations (cells ml^{-1}) in three replicate carboys with f/8 nutrients grown over a 36 study period at 2.4 psu and 20°C with a 12:12 light:dark cycle.

Toxicity Bioassays

Survival of juvenile *P. promelas* (48 hr) following exposure to *P. parvum* filtrates showed that laboratory cultures examined in this study were highly toxic (Figure 3). An

LC_{50} for *P. promelas* was estimated at 2.18 x 10^4 cells ml^{-1} (Table 1). Conversely, growth of *P. subcapitata* was not inhibited by any level of *P. parvum* filtrate; rather, growth was significantly stimulated ($p < 0.05$) at the three highest treatment levels (Figure 4). At 96 hours, the green algae density of 1.56 x 10^6 cells ml^{-1} (±2.3 x 10^5 cells ml^{-1}) in the highest treatment level was markedly greater than that of the 2.4 psu ASW control treatment, which had a density of 3.34 x 10^5 cells ml^{-1} (±0.85 x 10^5 cells ml^{-1}). Accordingly, the NOAEL of *P. parvum* filtrates to *P. subcapitata* growth under these experimental conditions was at least 2.98 x 10^5 cells mL^{-1} (Table 1).

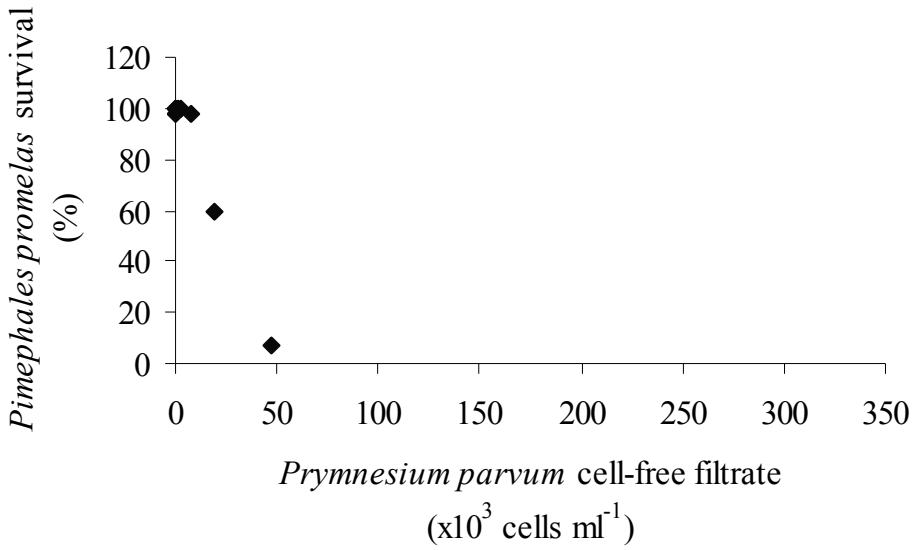

Figure 3. Mean survivorship (n = 4) of *Pimephales promelas* exposed to *Prymnesium parvum* cell-free filtrate.

Survival and reproduction of *D. magna* and *B. calyciflorus* were less sensitive than fish mortality, but were significantly adversely affected by *P. parvum* filtrates ($p < 0.05$). *D. magna* control survival was 100% and mean reproduction was 41.9 ±6.3 neonates $female^{-1}$ (N=10). NOAEL and LOAEL values for *D. magna* survival

Table 1. Ecotoxicological benchmark values for model aquatic organisms exposed to *Prymnesium parvum* cell-free filtrates

Organism	Endpoint		Benchmark Value (cells ml^{-1})
Pimephales promelas	48 hr LC_{50}	Survival	2.18×10^4
Brachionus calyciflorus	48 hr NOAEL	Survival	4.77×10^4
		Population Growth Rate	1.91×10^4
Daphnia magna	10 d NOAEL	Survival	1.19×10^4
		Reproduction	4.77×10^4
Pseudokirchneriella subcapitata	96 hr NOAEL	Growth	2.98×10^5

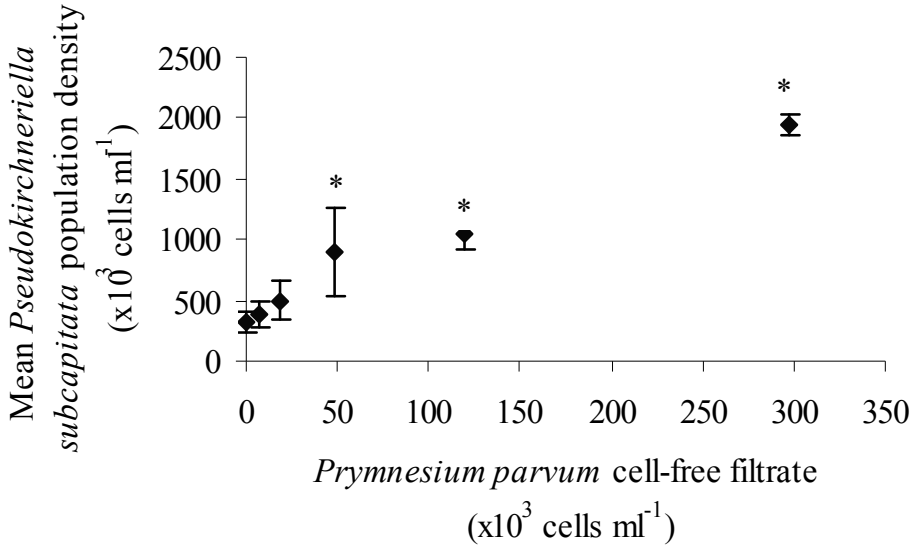

Figure 4. Mean 96-hr growth of *Pseudokirchneriella subcapitata* (cells ml^{-1} ± SD) exposed to *Prymnesium parvum* cell-free filtrate. *$p<0.05$

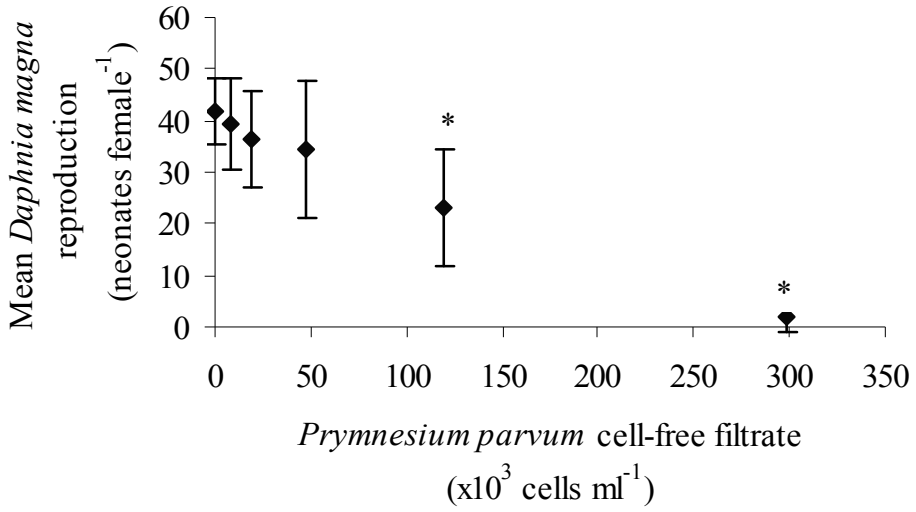

Figure 5. Mean 10 day *Daphnia magna* reproduction (±SD) exposed to *Prymnesium parvum* cell-free filtrates. *p<0.05

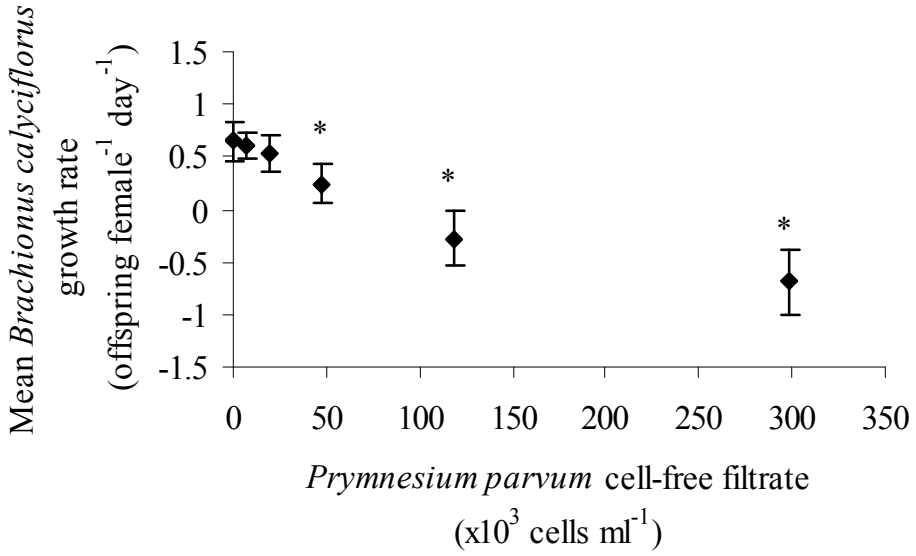

Figure 6. Mean 48-hr *Brachionus calyciflorus* growth rate (±SD) exposed to *Prymnesium parvum* cell-free filtrates. *p<0.05

were determined at 1.19 x 10^5 cells ml^{-1} and 2.98 x 10^5 cells ml^{-1}, respectively (Table 1). *D. magna* reproduction was significantly reduced at 1.19 x 10^5 cells ml^{-1} (LOAEL), but was not affected at 4.77 x 10^4 cells ml^{-1} (NOAEL) (Table 1; Figure 5). Rotifer survival was adversely affected at the two highest treatment levels, resulting in a NOAEL of 4.77 x 10^4 cells ml^{-1} and a LOAEL of 1.19 x 10^5 cells ml^{-1} (Table 1). NOAEL and LOAEL values for *B. calyciflorus* population growth (Figure 6) were more sensitive than survival, as determined at 1.91 x 10^4 cells ml^{-1} and 4.77 x 10^4 cells ml^{-1}, respectively (Table 1).

Discussion

In this study, four standardized *in vivo* bioassays were utilized to examine the comparative toxicity of cell-free *P. parvum* filtrates containing excreted toxins. Standardized models are routinely used to assess wastewater effluent safety and examine the quality of ambient aquatic conditions because they are generally more repeatable and transferable among research laboratories due to higher quality control and consistency of experimental conditions (US EPA 2002). Such techniques have particular relevance for understanding *P. parvum* impacts to aquatic life because standards for measuring concentrations of toxins are not available at this time (Schug et al. in press).

Further, these standardized laboratory responses can be used to predict field responses to contaminants and support development of predictive models for *P. parvum* (Baker et al. 2007; Baker et al. 2009; Grover et al. in press). Unfortunately, most of the previous *in vivo* studies reviewed did not use standardized aquatic toxicological methods (Brooks et al. in press). In addition, comparisons of relative sensitivities to *P. parvum* toxins in previous published studies are challenging because nutrient limitation, light, temperature, salinity and pH of study conditions influences toxin production and/or

potency, and corresponding toxicological responses. Thus, an understanding of the comparative sensitivities to *P. parvum* toxins remained indefinable prior to this study, particularly when projecting such differential responses to inland waters with salinities ≤4 psu.

Juvenile *P. promelas* survival was distinctly more sensitive to *P. parvum* filtrates than the other model bioassays evaluated in this study. This effect is confirmed by the observation of massive fish kills occur in the environment; however, the results of my study also suggest that that lower trophic levels are also being adversely affected during fish kills. In addition to adverse affects on fish, *P. parvum* filtrate significantly impacted reproduction of *D. magna* and *B. calyciflorus*. Previous studies demonstrated that *P. parvum* toxins adversely affect predators of *P. parvum* such as copepods, ciliates and dinoflagelates under more saline conditions than investigated in my study (Fistarol et al. 2003; Granéli and Johansson 2003a; Tillmann 2003; Barreiro et al. 2005; Sopanen et al. 2006). A recent experiment by Roelke et al. (2007) in Lake Possum Kingdom, Texas, USA demonstrated sublethal effects of *P. parvum* on *D. magna* reproduction. These observations corresponded to an absence of cladocerans in the zooplankton community and a decrease in biovolumes of copepod nauplii and total rotifers when a *P. parvum* bloom occurred (Roelke et al. 2007). In my laboratory study, negative effects were documented of *P. parvum* toxins on survival and reproduction of *D. magna* and survival and population growth of the freshwater rotifer *B. calyciflorus* at salinities comparable to Lake Possum Kingdom (e.g., 2.4 psu). Similarly, Barreiro et al. (2005) reported weak effects of *P. parvum* filtrates on population growth of *B. plicatilis* in a study performed

with seawater at 6 psu, but observed more pronounced reductions in population growth when this marine *Brachionus* species ingested *P. parvum*.

Interestingly, a recent experiment in Lake Whitney, Texas, USA by Schwierzke et al. (in press) reported a marked shift in the zooplankton community during a *P. parvum* bloom that reduced *P. promelas* survival and *D. magna* reproduction to levels similar to those reported here. In the Schwierzke et al. (in press) study, the zooplankton community became dominated by a freshwater rotifer (*Notholca* sp.) under highly toxic conditions to fish and cladocerans. Lake Whitney is located downstream of Lake Possum Kingdom and Lake Granbury (Grover et al. in press), forming a three impoundment cascade of the Brazos River in central Texas; each of these reservoirs have experienced severe fish kills associated with *P. parvum*. It is recognized that zooplankton can develop tolerance and even resistance to aquatic contaminants through various mechanisms (Clubbs and Brooks 2007), including induction of cytochrome P450 monooxygenases, which serve as important detoxification mechanisms for toxicants and toxins. Such observations between marine and freshwater species of rotifers indicate differential sensitivities among rotifer genera and may suggest development of resistance to *P. parvum* toxins. It may be that *Notholca* sp. in these cascading reservoirs of the Brazos River are developing resistance to *P. parvum* toxins, but evidence to date of decreased susceptibility to *P. parvum* has only been observed in the most downstream impoundment.

P. parvum has been repeatedly reported to adversely affect algal competitors (Fistarol et al. 2003; Granéli and Johansson 2003 a, b; Legrand et al. 2003; Skovgaard et al. 2003; Uronen et al. 2005). In this study, growth of the model freshwater green algae *P. subcapitata* significantly increased in the presence of increasing levels of *P. parvum*

filtrates. The fact that *P. subcapitata* was not adversely affected is counterintuitive to what one might expect based on the previous literature and because all treatment levels contained the same concentrations of nutrient media to avoid differential nutrient limitation across treatment levels (US EPA 2002), although photoinduced breakdown of the contaminants may have increased available dissolved organic carbon (DOC). It would be beneficial for *P. parvum* if its toxins inhibited the growth of species that compete for the same resources. This was not observed to be the case in this study. However, similar to invertebrates and vertebrates, aquatic algae and plants can develop tolerance to contaminants by inducing detoxification pathways such as glutathione (Brain and Cedergreen 2009).

Toxic activity associated with *P. parvum* has been demonstrated to be "inactivated" by light (Parnas et al. 1962; Reich and Parnas 1962), though photolytic degradation of the molecules are a more likely explanation (Manahan 2000), particularly when exposure to light occurs in the 400 to 510 nm and ultraviolet ranges (Rahat and Jahn 1965). Granéli and Johansson (2003) reported that *P. parvum* cells grown under nutrient-deficient conditions produced allelopathic substances to competitors: within 0-36 hrs of their study, growth rate was substantially lower for three species of marine algae exposed to *P. parvum* filtrates grown under various nutrient conditions compared to the control. However, between 36-72 hrs growth rates were occasionally higher for experiments completed with filtrates from nutrient-deficient cultures (Granéli and Johansson 2003). In addition to *P. subcapitata* developing tolerance or being less sensitive to *P. parvum* toxins, photodegradation of the toxins could have occurred during the 96 hr *P. subcapitata* growth period in this experiment, particularly since US EPA

(2002) protocols with continuous illumination were followed. Subsequently, photodegradation products from the toxins and algal exudates (Vasconcelos et al. 2002) could have increased DOC, which would have been mineralized by bacteria in these non-axenic cultures, increasing the levels of CO_2 for uptake, and potentially stimulating the growth response observed. However, DOC and CO_2 were not quantified at any treatment level.

Here I assessed the relative sensitivities of common *in vivo* standardized bioassays to *P. parvum* filtrates following culture at experimental conditions similar to *P. parvum* bloom conditions in Texas inland waters (Roelke et al. 2007) and those non-optimal for growth, which results in more toxin production per cell (Baker et al. 2007). Additional studies are required to understand how comparative sensitivities of inland fish, zooplankton, and phytoplankton communities influence competition, predation and associated bloom dynamics of *P. parvum*. These future efforts will support predictive modeling of bloom formation and termination (Grover et al. in press) and further will be supported by robust analytical methods to quantify toxins in water and tissue matrices (Schug et al. in press).

CHAPTER THREE

Probabilistic Ecological Assessment of Microcystin-LR: Application in a Case Study of Allelopathy to *Prymnesium parvum*

Introduction

Microcystins

Microcystins are cyclic heptapeptide algal toxins produced by a number of cyanobacterial species, including members of *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc* and *Oscillatoria* (Chorus and Bartram 1999). Approximately 80 microcystin congeners have been identified, such as microcystin-LR, -RR, -LA, and -YR. They all share a common structure of 3-Amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-diene acid (ADDA) and five amino acids connected by peptide bonds. The different variants are formed mainly by variations of the α-amino acids found at positions 2 and 4 (Antoniou 2008). Microcystin-LR (MC-LR), for example, has leucine (L) and arginine (R) as the two amino acids (Figure 7). This particular variant, which is the toxin examined in this study, is the most commonly found microcystin in natural waters (Wiegand and Pflugmacher 2005.). Additional congeners are formed by alteration of other constituent amino acids by way of substitutions, methyl esterification, or demethylation; these alterations can markedly affect toxicity (Kotak and Zurawell 2007). Generally, microcystins with non-polar amino acids at positions 2 and 4 are more toxic than those with polar amino acid because of the increased likelihood of partitioning to lipid tissues (Sivonen and Jones 1999). For example, the non-polar leucine on MC-LR

contributes to its high degree of toxicity (Kotak and Zurawell 2007). However, despite having non-polar properties, MC-LR, like all microcystins, is soluble in water (de Maagd et al. 1999). While this limits passive uptake into tissues, other transport mechanisms allow the toxin to enter organisms and elicit toxicity.

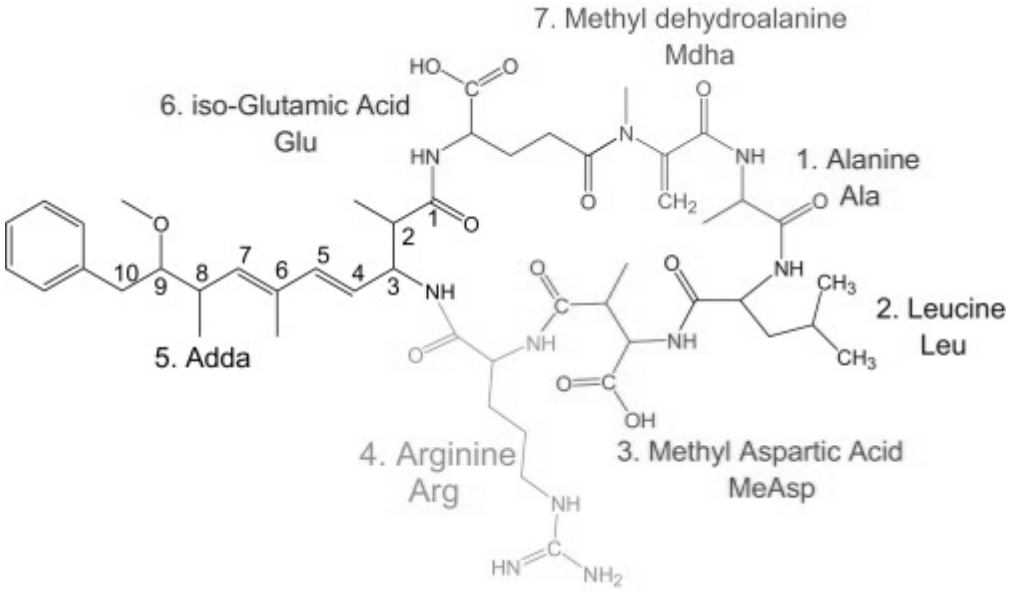

Figure 7. Structure of microcystin-LR (modified from Antoniou 2008).

Microcystin-LR

In humans, microcystin-LR is transported into liver cells by membrane-bound proteins responsible for bile acid transport. Once in hepatocytes, MC-LR acts as a protein phosphatase inhibitor of PP1 and PP2A, which results in hyperphosphorylated cytosolic and cytoskeletal proteins and ultimately necrosis that expresses as hepatoenteritis (MacKintosh et al. 1990). The most well known human mortality event attributed to microcystin was from water taken from the Tabocas Resrvoir in Caruaru, Pernambuco state, Brazil in 1996 (Pouria 1998). All 126 patients of a hemodialysis clinic

using water from this reservoir developed signs and symptoms of acute neurotoxicity and subacute hepatotoxicity; sixty of these patients died. Laboratory testing of patients's liver samples and serum detected microcystin-LR, -RR, and -AR. (Pouria 1998). Other mammals that have been documented to be poisoned by microcystins include sheep, horses, and livestock (Carmichael 1992, Codd 1995). For instance, three adult cattle and calves in Vernal, Utah, were determined to have died from ingesting microcystin-containing waters in 2004 (Williams 2007).

The majority of experimental toxicity information regarding MC-LR is also mammalian, as is necessary for establishing guideline values to protect human health. There are no Federal regulations for any HAB species or their associated toxins in the United States, but microcystin-LR is also the only algal toxin that has a provisional guideline value (of 1 $\mu g\ l^{-1}$) established for drinking water by the World Health Organization (WHO, Lopez et al. 2008). Health Canada established a drinking water guideline of 1.5 $\mu g\ l^{-1}$ of MC-LR. (Kotak and Zurawell 2007). There is also toxicity data on the effects of microcystin-LR to aquatic organisms, but more information is needed for organisms at various trophic levels to fully characterize its effects on aquatic ecosystems (Murphy 2003).

Aquatic life can be affected by microcystins indirectly by ingestion and metabolism of intracellular or extracellular toxins or directly through absorption of extracellular toxins. Of all microcystins, MC-LR is the variant with the most experimentally observed toxicity to aquatic species. Studies have shown MC-LR to affect algae, macrophytes, zooplankton, and fish (Pflugmacher 2002, Mitrovic et al. 2005, Demott et al. 1993, Reinikainen et al. 2002, Oberemm et al. 1997) Additionally,

MC-LR can bioaccumulate, which is expected based on the estimated octanol-water partitioning coefficient of 2.16 (Ward and Codd 1999). Grazers appear to be the most likely organisms to bioaccumulate and transfer microcystins to higher trophic levels (Kotak et al. 1996, Ferrao-Filho 2002).

Microcystin-LR is a relatively stable toxin. For example, Metcalf and Codd (2000) used boiling and microwaving and observed no MC-LR degradation. In addition, purified toxins are relatively stable under irradiation by sunlight alone, with a half-life of approximately ten days (Tsuji et al.1995). When MC-LR without pigment was irradiated with UV at 238 nm, its concentration was halved within one hour. Higher intensities of light also increase the degradation rate; total MC-LR elimination was observed at ten minutes at an intensity of 2550 uW/cm^2 (Tsuji et al. 1995). In addition, Jones and Orr (1994) suggested that degradation by specific bacteria may be integral in natural biodegradation of microcystin.

Algal species must compete for essential resources in the environment. This can be facilitated by being more adept at acquiring nutrients or by way of excreting allelochemicals. Roelke et al. (in press) proposed a possible allelopathic response from cyanotoxins to another harmful algal species, *Prymnesium parvum*. *P. parvum* has been observed to bloom in some Texas reservoirs (e.g., Lake Whitney, TX) and not others (e.g., Lake Waco, TX), although they are present in Lake Waco. Lake Waco has a high density of cyanobacteria, including species that produce the cyanotoxin microcystin-LR. In the Roelke et al. (in press) mesocosm study, a dose-dependent reduction of *P. parvum* cell density was observed when organisms were exposed to varying proportions of Lake Waco and Lake Whitney water: the higher the proportion of Lake Waco water, the lower

the growth of *P. parvum*. Roelke et al (in press) concluded that salinity, nutrients, and anthropogenic contaminants were not responsible for such responses. However, Roelke et al (in press) could not rule out toxins/ allelochemicals produced by cyanobacteria or bacteria. Because MC-LR had been measured in Lake Waco in the past and species that produce MC-LR were present at the time of the Roelke et al (in press) study, they proposed that allelochemicals produced by cyanobacteria may have caused *P. parvum* growth inhibition. The purpose of my study detailed in this chapter examined whether *P. parvum* growth responses may have been caused by MC-LR.

In this study, I examined the effect of MC-LR on growth of the harmful algal species, *Prymnesium parvum*. Before choosing experimental treatment levels, probabilistic distributions of exposure and effects were developed to design the study to be environmentally relevant and to compare my results to previously reported observations. An exposure distribution (ED), a probability distribution of reported concentrations of a compound measured in the environment, was developed following an extensive literature review. The probability of observing a concentration at or below a selected value can be interpolated from the log-normal probability of exposure. A species sensitivity distribution (SSD), which is a tool frequently applied in ecological risk assessment (Solomon et al. 2000), was also developed from values published in the literature found to elicit effects on aquatic organisms. An SSD is a distribution of species that elicit a response to the same compound, and the probability of observing an adverse effect at or below a selected exposure concentration can be interpolated from the log-normal probability distribution of effects (Solomon et al. 2000). The treatment levels utilized in this experiment were selected based on the ED, but the SSDs allowed for a

prediction, based on where other primary producers were located in the distribution, at what concentrations adverse effects of MC-LR may be observed for *P. parvum*.

Materials and Methods

Probabilistic Exposure and Effects Distributions

An extensive literature review was conducted to find reported environmental levels in surface waters for microcystins (Appendix D, table 1.) in order to develop a probabilistic exposure distribution. The literature values were not limited to MC-LR because many detection methods (e.g., enzyme linked immunosorbent assay or ELISA) are not variant specific, and thus neither was the available data. However, because the -LR variant is the most common form, it is likely that a majority of the unspecific measurements included this particular variant. The ED was created by ranking the data in increasing order and calculating a percent rank for each concentration using a Weibull formula:

$$j = (i * 100) / (n + 1), \quad (2)$$

where j is the percent rank, i is the rank assigned to the measured microcystin value after placing the values in increasing order (1 to n), and n is the number of measurements (Solomon et al. 2000). The concentrations were plotted on a log scale against their respective percent rank, which was on a probability scale. A regression line was added to develop a line of best fit for the percent rank and concentration data (Solomon et al. 2000). The equation of this line was then used to calculate the 10, 25, 50, 75, and 90th centile values, which were selected to serve as nominal treatment levels in the study.

These centile values represent the probability of detecting a microcystin concentration at or below a corresponding concentration in a freshwater system.

Nominal treatment levels were also selected by evaluating the peer-reviewed literature for MC-LR concentrations that elicit effects on various aquatic organisms (Appendix D, tables 2, 3.). These values were used to develop an SSD (SigmaPlot, v10.0, San Jose, CA, USA). The process was similar to development of the exposure distribution, except the concentrations were experimentally derived ecotoxicological benchmark values or thresholds of adverse response (Solomon et al. 2000). Because of the data availability, two SSDs were developed: one using median lethal concentration (LC_{50}) values and another using No Observable Effect Concentration (NOEC) values. The first SSD utilized values from assays employing lethality as an endpoint and the second SSD utilized values from assays using sublethal endpoints. After determining where algae and macrophytes were located on the distribution, nominal treatment levels initially selected from the ED were refined to encompass these responses.

Prymnesium parvum Stock Culture

A stock culture of *P. parvum* was initiated from a strain obtained from The University of Texas at Austin Culture Collection of Algae (strain UTEX LB 2797) that was originally isolated from the Colorodo River in Texas. The culture was maintained in a 20-L glass carboy filled with 10 L of 2.4 psu ASW (Berges et al. 2001) enriched with f/2 levels of vitamins, trace metals, and nutrients (Guillard 1975; appendices A, B). The carboy was maintained in an incubator (VWR Model 2015, West Chester, PA, USA) at 20˚C at 12:12 light:dark cycle at approximately 100 µmol photons/m^2/s, which generally followed approaches detailed in Chapter Two. The culture was swirled daily and allowed

to grow until stationary phase, at which time these organisms were used to inoculate the experimental units at each treatment level. Cell density of the stock culture at the time of inoculation was approximately 2 x 10^5 cells ml^{-1}.

Experimental Design

Experimental units consisted of five replicate culture tubes (25x200 mm) each containing 50 mls of test solution at one of six different nominal concentrations of MC-LR (0, 0.0623, 0.807, 13.9, 239, and 3090 µg l^{-1}). The highest test solution concentration was made by mixing approximately 1 mg of purified (≥95%) MC-LR (Alexis Biochemicals/Enzo Life Sciences, Inc., Farmingdale, NY, USA) into 308 mls of f/2 medium. The other four concentrations were prepared from a concentrated stock solution by diluting approximately 1 mg MC-LR into two liters of f/2 medium; f/2 medium was used as a diluent for all concentrations. A final set of five culture tubes consisted of the 500 µg l^{-1} stock solution with no algal addition, hereafter referred to as blanks, were used to examine MC-LR degradation at the end of the study period. After all test solutions were made, each culture tube (except for blanks) was inoculated with approximately 10,000 cells ml^{-1} of *P. parvum*.

After inserting foam plugs, all flasks were placed on slanted test tube racks in an incubator (VWR model 2015, West Chester, PA, USA) maintained at 20˚C on a 12:12 L:D cycle for 27 days. Culture tubes were swirled once in the morning and vortexed in the afternoon before taking a fluorescence reading, after which their positions were rotated within the incubator. *In vivo* fluorescence, determined with a Turner fluorometer model 10AU (Turner Designs, Sunnyvale, California, USA), was used to measure

chlorophyll *a*, and values were converted to cell density using a standard curve of cell counts determined microscopically using a hemocytometer (US EPA 2002).

Analytical Verification of MC-LR Concentrations

Nominal concentrations of each culture tube were verified with ELISA that used competitive inhibition to determine the concentrations, with a range of detection of 0.1 to 5 $\mu g\ l^{-1}$ (Abraxis, Steamwhistle, PA, USA). Final MC-LR concentrations on day 27 were estimated with ELISA for all treatment levels to estimate the amount of toxin remaining at the end of the study. Any concentration that would or were expected to fall beyond the method limit of detection was diluted to the detection range of the ELISA.

Statistical Analyses

Final cell densities on day 27 were enumerated by both fluorescence and hemocytometer to verify the standard curve. Daily growth of each treatment was compared to the controls by a one way analysis of variance (ANOVA) and a Dunnett's test (JMP v6.1, SAS Institute, Cary, NC, USA) when a significant difference ($\alpha = 0.5$) was detected. Specific growth rates (μ day $^{-1}$) were calculated using change in cell densities according the formula (Levasseur et al. 1993):

$$\mu = [\ln(Nj) - \ln(Ni)] / \text{time (d)}, \quad (3)$$

where *Ni* equals the cell density at time i and *Nj* is the cell density at time j. Exponential growth rate for days 2 through 7 were calculated for each replicate at each treatment level by plotting the natural log of the cell density against time. The exponential growth rate was then determined as the slope of the line of best fit. Mean exponential growth rate values were then determined for each treatment level, analyzed by ANOVA, and used to

compare findings of this study to a 7-day microcosm study conducted by Roelke et al. (in press).

Results

Exposure and Effects Distributions

The ED (Figure 8) included 211 concentrations of microcystin reported in the peer-reviewed literature (Appendix D, table 3). Values were available from water bodies in the United States, but data also came from Canada, Finland, Australia, and Japan. The highest reported value used to make the distribution was 12,176 µg l^{-1} microcystin from the Copco Reservoir in California, USA (Jacoby and Kann 2007); 121 values were under 1 µg l^{-1}. Regression analysis of the exposure distribution resulted in the equation $y = -0.6235x + 0.54588$ (r^2=0.94). The calculated 10, 25, 50, 75, and 90th centile values were 0.0623, 0.807, 13.9, 239, and 3090 µg l^{-1} of microcystin, respectively.

The mortality SSD of MC-LR included 12 LC_{50} values (Figure 9) reported from seven publications (Appendix D, table 2). Based on the available data, a mayfly (*Hexagenia* sp.) hatchling nymph was the most sensitive (96 hr LC_{50} = 49 µg l^{-1}, ~9th centile; Smith et al. 2008) and *Daphnia pulicaria* was the least sensitive species (48 hr LC_{50} = 21,400 µg l^{-1}, ~84th centile; Demott et al. 1991) to MC-LR. Another cladoceran, *Daphnia hyalina*, had a a 48 hr LC_{50} value midway between these two of 11,600 µg l^{-1} MC-LR, which corresponds to approximately the 78th centile (DeMott et al. 1991). An HC_5 value, which corresponds to a prediction that 95% of aquatic organisms would experience acute mortality following exposure at or above this concentration, was identified as 23.5 µg l^{-1} MC-LR.

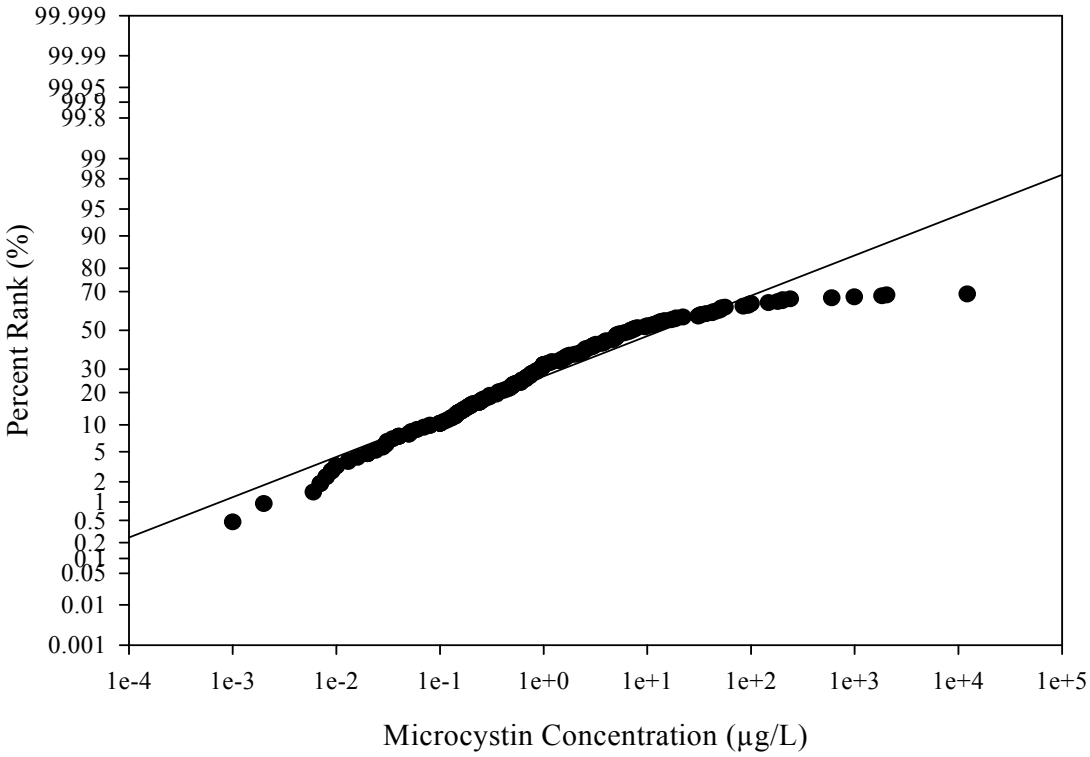

Figure 8. Probabilistic exposure distribution of microcystin in freshwater ecosystems based on 211 concentration values from the peer-reviewed literature ($r^2 = 0.94$).

A second SSD (Figure 10) utilized NOEC values for various responses, including 15 values from six publications (Appendix D, table 3). The most sensitive species was *Ceratophyllum demersum*, with a six-week oxygen production inhibition NOEC at 0.1 µg l^{-1} MC-LR (Pflugmacher 2002). The least sensitive species in the distribution were a macrophyte (*Wolffia arrhiza*) and a filamentous alga (*Chladophora fracta*); both had a growth NOEC of 10,000 µg l^{-1} MC-LR (Mitrovic et al. 2005). An HC_5 value, which corresponds to a prediction that 95% of aquatic organisms would not experience adverse effects following exposure at or above this concentration, was identified as 0.056 µg/L MC-LR.

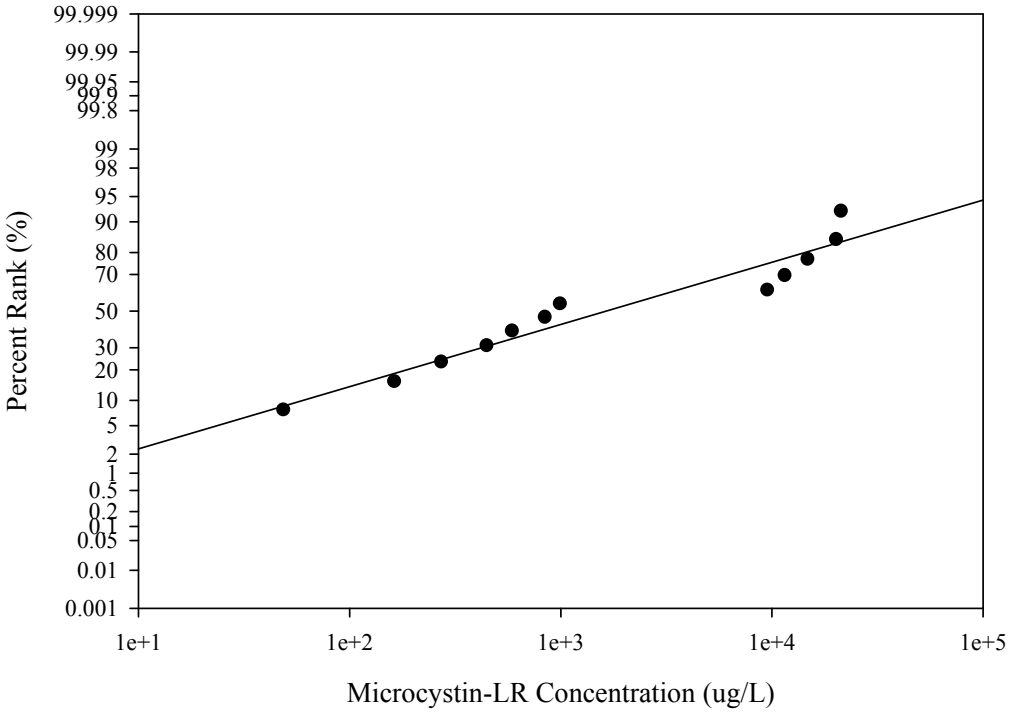

Figure 9. Species sensitivity distribution for microcystin-LR mortality effects on aquatic organisms using LC_{50} values (n = 12, r^2 = 0.93).

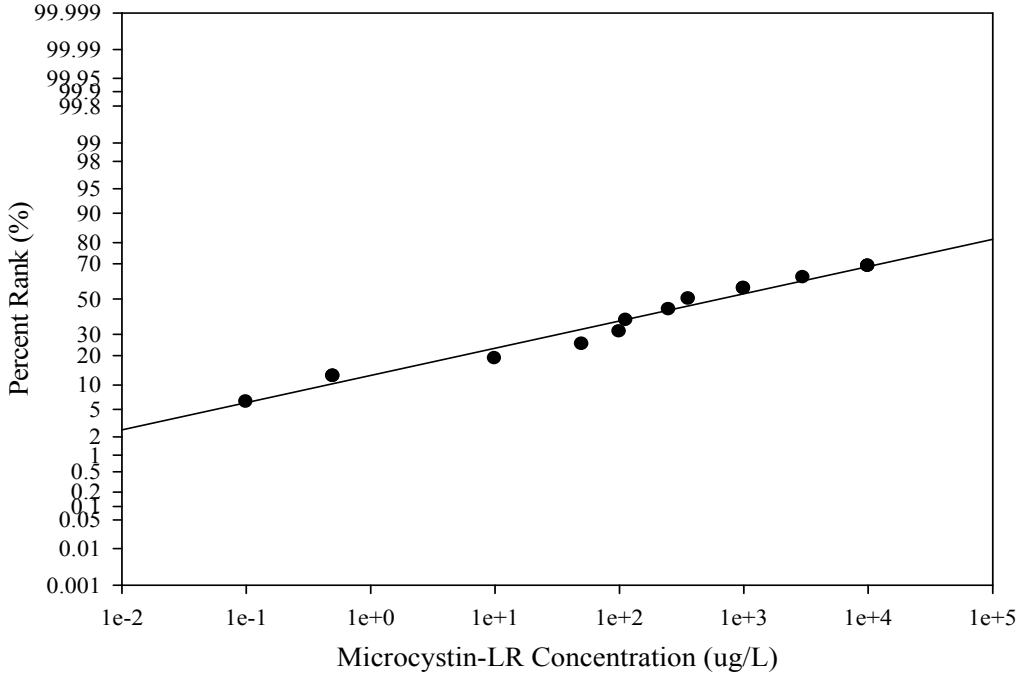

Figure 10. Species sensitivity distribution for microcystin-LR effects on aquatic organisms using No Observable Effects Concentration values (n = 15, r^2 = 0.98).

Coupling information from EDs and the acute and chronic SSD provides robust predictions of the probability of observing adverse aquatic effects to stressors (Solomon et al. 2000). Based on the probabilistic assessment of MC-LR in this study, 95% of aquatic organisms are predicted to experience acute mortality following exposure to MC-LR above 23.5 μg l^{-1} approximately 55% of the time (Table 2). However, the HC_5 value of 0.056 μg l^{-1} MC-LR for sublethal responses is predicted to be exceeded greater than 90% of the time in freshwater ecosystems experiencing cyanobacterial blooms from MC producing species (Table 2).

Table 2. Calculated centile values for microcystin-LR (MC-LR) probabilistic distributions of environmental exposure and lethal and sublethal effects. SSD = species sensitivity distribution.

	MC-LR concentration (μg l^{-1})		
	Exposure SSD	Effect SSD	
Centile		NOEC	LC_{50}
1%	0.0008	0.0012	4.06
5%	0.01	0.056	23.5
10%	0.062	0.443	60.0
15%	0.175	1.78	113
20%	0.399	5.38	187
25%	0.807	13.9	287
30%	1.52	32.6	423
35%	2.73	71.8	605
40%	4.77	152	850
45%	8.17	313	1,182
50%	13.9	640	1,633
55%	23.6	1,305	2,258
60%	40.4	2,695	3,138
65%	70.5	5,700	4,410
70%	127	12,555	6,312
75%	239	29,435	9,293
80%	483	76,021	14,297
85%	1,099	229,746	23,623
90%	3,090	923,833	44,436
95%	14,303	7,266,482	113,351
99%	253,433	347,989,468	656,627

Analytical Verification of MC-LR Concentrations

Measured MC-LR concentrations were 102 to 154% of the nominal concentrations; therefore, the measured values were used for all statistical analyses (Table 2). Measured initial concentrations approximated the 12th, 25th, 52nd, 76th, 91st centiles; all values were approximately zero to two centiles higher than those chosen for nominal concentrations based on the ED. A second ELISA was performed to measure the final concentrations (day 27) of MC-LR remaining in the culture tubes (Table 3). Degradation was observed in all but the second lowest concentration (0.8 µg l^{-1}); however, this minor inconsistency may be explained by measurement at the lower range of the ELISA limit of detection. Otherwise, there was 53 to 84 % MC-LR remaining in the experimental units at the termination of the experiment.

Table 3. Nominal microcystin-LR concentrations and mean measured values (n = 5) for *Prymnesium parvum* treatment levels on study days 0 and 27.

Day 0 Nominal Concentration (µg l^{-1})	Day 0 Measured Concentration (µg l^{-1})	Measured Percent of Nominal (%)	Day 27 Measured Concentration (µg l^{-1})	Day 27 Measured Percent of Day 0 (%)
0	0	0	0	0
0.0623	0.09	140	0.05	57
0.807	0.8	102	1.1	128
13.9	16.6	119	14.0	84
239.0	278.6	117	211.5	76
3090.0	4392.8	142	2589.4	59
500.0	768.7	154	410.3	53

Prymnesium parvum Growth

From day 17 until the end of the study on day 27 (except day 24), the lowest MC-LR treatment level (0.09 µg l^{-1}) significantly increased *P. parvum* cell density ($p < 0.05$),

which is evident in Figure 12. However, on day 6-8, 13-14, and 22-23 (approximately one quarter of the duration of the test), the highest MC-LR treatment level (4392.8 µg l^{-1}) significantly decreased *P. parvum* cell density relative to controls ($p < 0.05$). There was a significant ($p < 0.05$) reduction of cell density by the 16.6 µg l^{-1} treatment on day 13 only. All other treatments were not significantly different from the controls throughout the study period ($p > 0.05$). Specific growth rate (Figure 11) was similar in all treatments for most of the test duration. Specific growth rate was the highest in the first five days, decreased until around 15 days, then leveled off until day 27. Analysis of exponential growth rate (Table 4) from days two to seven showed that there was no significant difference of exponential growth rate among the treatment levels ($p>0.05$).

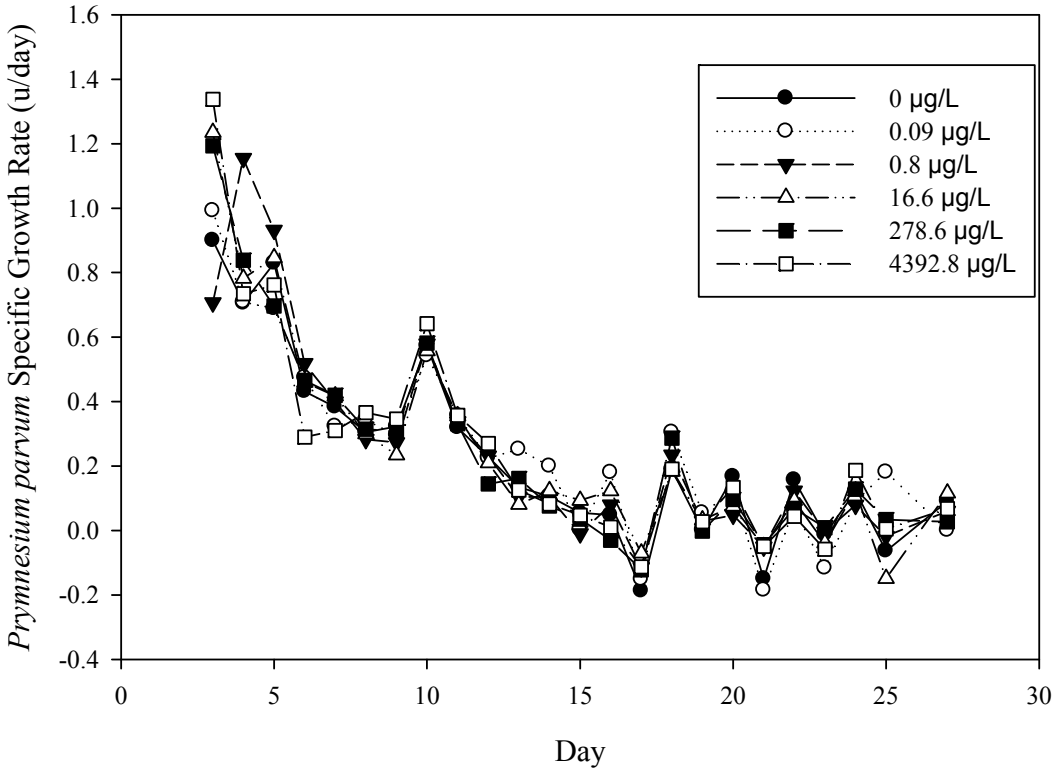

Figure 11. Specific growth rate of *P. parvum* during a 27 d exposure to five microcystin-LR concentrations and a control.

Table 4. Mean exponential growth rate and standard deviation of *Prymnesium parvum* exposed to six microcystin-LR concentrations.

MC-LR Concentration ($\mu g\ l^{-1}$)	Exponential Growth Rate (divisions day^{-1})	Standard Deviation ($\mu g\ l^{-1}$)
0	0.700	0.099
0.09	0.678	0.109
0.8	0.945	0.265
16.6	0.939	0.403
278.6	0.903	0.417
4392.8	0.868	0.416

Discussion

Microcystin-LR has been detected in water bodies throughout the world. When cyanobacteria that produce microcystin are present in an aquatic ecosystem, concentrations are reported to range from ≤1 to ≥12,176 $\mu g\ l^{-1}$ microcystin (Appendix D, table 1). The microcystin-LR concentrations used for the exposure distribution in this study encompassed a wide range of environmentally relevant values. More than 100 values were at or below the recommended WHO provisional drinking water guideline value of 1 $\mu g\ l^{-1}$; however, 90 values were reported above this threshold value in unfinished drinking water supplies.

Exposure and effects distributions are useful tools employed for probabilistic hazard and risk assessment (Solomon et al. 2000). In fact, these approaches are well accepted by the scientific and regulatory communities (Posthuma et al. 2001); for example, SSDs are used retrospectively in probabilistic hazard assessment to support development of numeric ambient water quality criteria in the US (Stephan 2006) and

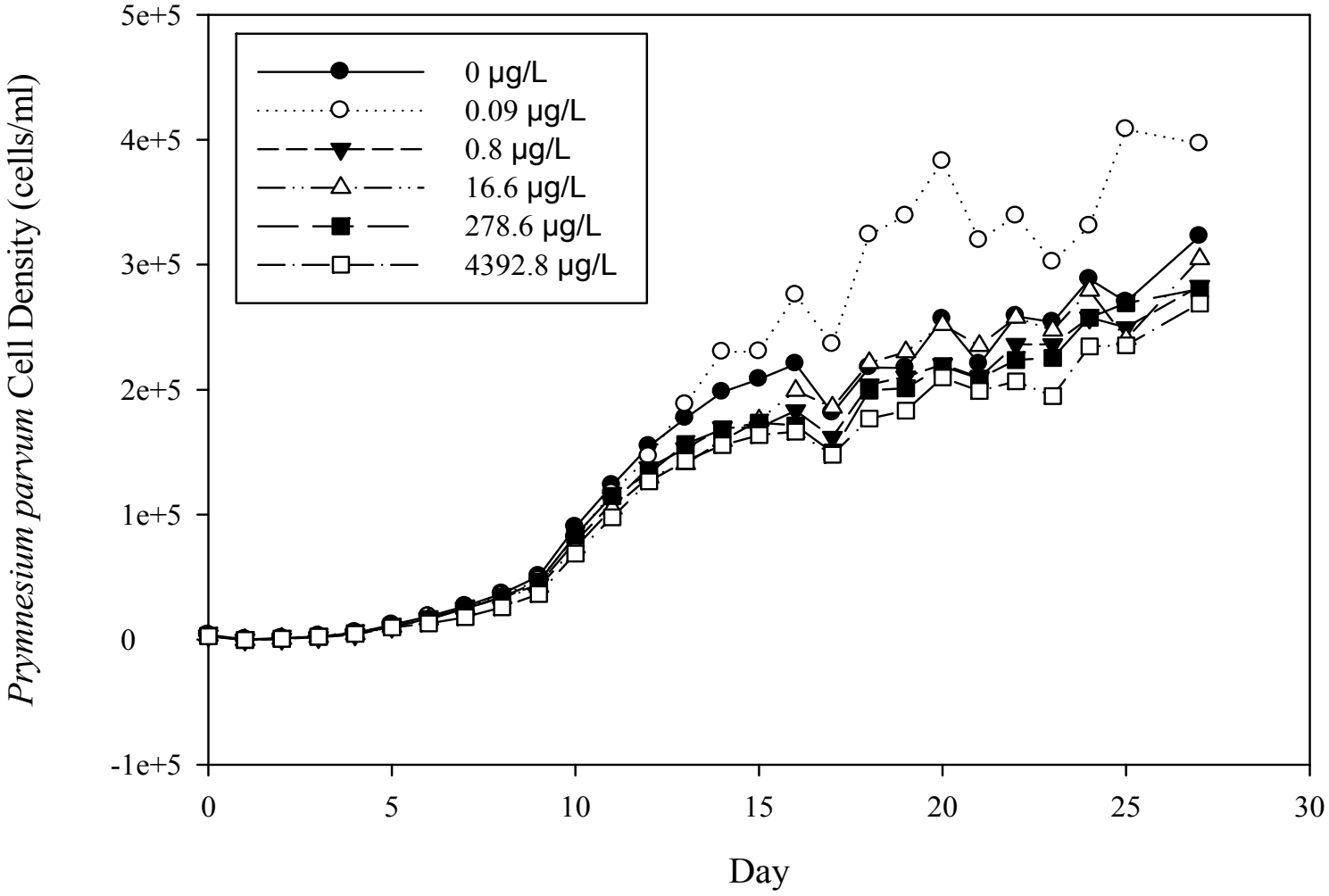

Figure 12. Mean (n=5) *Prymnesium parvum* cell densities (cells ml^{-1}) from day zero to day 27 for five microcystin-LR concentrations and a control.

prospectively examine environmental safety of chemicals in the European Union (Williams et al. 2009). Understanding the amount of risk associated with an environmental contaminant, including biotoxins, supports sound science-based management decisions in aquatic ecosystems. Because multiple species from different trophic levels are incorporated into SSDs, it allows for a prediction of ecological thresholds at which a contaminant may adversely affect an ecosystem. For example, according to the MC-LR SSDs, 1% of freshwater organisms are predicted to be lethally affected at or below a concentration of 4.06 $\mu g\ l^{-1}$ MC-LR and sublethally affected at or below 0.0012 $\mu g\ l^{-1}$. These concentrations or greater are predicted to be encountered in freshwaters 38% and 1% of time, respectively, according to the ED (Table 2). Further, 10% of species are predicted to be lethally affected at or below 60.0 $\mu g\ l^{-1}$ and sublethally affected at or below 0.443 $\mu g\ l^{-1}$, which corresponds to 64% and 21% of the time, respectively (Table 2). These numbers indicate that sublethal endpoints, such as plant oxygen production and growth or invertebrate hatching delay, will be affected at much lower concentrations than mortality and subsequently should be expected to occur more frequently.

An important limitation to these probabilistic predictions is that I included only detection data from the peer-reviewed literature (Appendix D, table 1). It may be reasonable to presume that analytical measurements were performed in these studies when cyanobacterial blooms were occurring or believed to be possible in the freshwater systems examined. Thus, the outcome of the probabilistic assessment presented here is likely applicable to ecosystems experiencing cyanobacterial blooms with MC-producing organisms. However, this novel assessment provides useful information to support

environmental management decisions relevant to fisheries and unfinished drinking water in such inland water bodies.

Roelke et al. (in press) identified that *P. parvum* growth was inhibited in a dose-dependent manner by increasing concentrations of Lake Waco water, which contained high densities of cyanobacteria. Because inhibition was observed when the cyanobacterial cells were filtered, Roelke et al (in press) suggested that dissolved compounds from cyanobacteria were likely responsible for growth inhibition. Further, two cyanobacterial species, *Microcystis* sp. and *Anabaena* sp., present in Lake Waco produce MC-LR (Lopez et al. 2008). Although cell densities of cyanobacteria were determined by Roelke et al. (in press), toxin levels such as MC-LR were not examined. In my study, the highest treatment level of MC-LR (4,392.8 $\mu g\ l^{-1}$) was identified as the LOEC approximately 25% of the time and 278.6 $\mu g\ l^{-1}$ as the NOEC for *P. parvum* growth inhibition. The LOEC and NOEC values for *P. parvum* growth corresponded to the 91st and 76th centiles of the MC-LR ED, respectively (Figure 8). The NOEC corresponds to the 44th centile in the sublethal SSD. However, the concentration that caused this inhibitory effect is at the 91st centile of environmental concentrations of microcystins, indicating that only 9% of the time should MC-LR be observed at levels observed to reduce *P. parvum* growth in this study. Therefore, based on the probabilistic assessment efforts in this thesis, there is a low probability that MC-LR was solely responsible for *P. parvum* growth inhibition observed by Lake Waco water in the Roelke et al. (in press) study. The observed growth inhibition in my study is not a pronounced indication of adverse effect, but it is possible that if there is even slight inhibition it can allow other species to out-compete *P. parvum* for the same resources, or allow a grazer

not susceptible to the toxin to impact population density. In fact, Jonsson et al. (2009) concluded that algal toxins are not allelopathic mechanisms to suppress competitors. After analysis of 21 allelopathy studies, predator deterrence was identified as a more likely explanation for the role of algal toxins, whereas allelopathy of competitors was suggested to be a non-adaptive side effect (Jonsson et al. 2009).

Another point to take into consideration when comparing the results of my study to those of the Roelke et al. (in press) study is difference in the environmental conditions. Specifically, the Roelke et al (in press) study was performed in the field where October temperatures varied, whereas my study was performed at a constant 20^{o}C in a laboratory incubator. In addition, Lake Waco water was phosphorus limited; and my cultures were not under any nutrient limitation. When phosphorus is limited, algae can upregulate the enzyme alkaline phosphatase in order to hydrolyze organic phosphorus compounds (Hoppe 2003). MC-LR acts as a protein phosphatase inhibitor (Chorus and Bartram 1999). Therefore, if *P. parvum* were phosphorus limited in the Roelke et al. (in press) study, it may have been that the combination of nutrient stress and inability to utilize phosphatases to compensate for the nutrient stress caused the observed *P. parvum* growth inhibition. Roelke et al. (in press) used additional treatments that were nutrient enriched and observed inconsistent results. The treatments exhibited *P. parvum* growth inhibition and variable phytoplankton growth, but there was also stimulation of zooplankton, specifically rotifers, which could have affected *P. parvum* biomass.

My study cannot completely eliminate MC-LR as an inhibitor of *P. parvum* in the Roelke et al (in press) study particularly because MC-LR was not measured in Lake Waco waters; however, my findings suggests that other dissolved chemicals may

contribute to an explanation of the observed inhibition of *P. parvum* growth. For example, other compounds produced by cyanobacteria include other cyanotoxins, methylisoborneol, and geosmin (Chorus and Bartram 1999). Additionally, an allelochemical produced by a different organism, such as bacteria, could have caused the observed inhibition.

Studies have shown there to be adverse effects from MC-LR to different species of plants and algae. For instance, Pflugmacher (2002) found growth inhibition as low as 1.0 $\mu g\ l^{-1}$ on the submerged macrophyte *Ceratophyllum demersum* over six weeks and oxygen inhibition to occur at MC-LR concentrations as low as 0.05 $\mu g\ l^{-1}$; complete death was observed at 5,000 $\mu g\ l^{-1}$ after 24 hours. In contrast, Mitrovic et al. (2005) found that even at a concentration as high as 10,000 $\mu g\ l^{-1}$, growth and peroxidase activity of the filamentous alga *Chlodophora fracta* and growth of the aquatic macrophyte *Wolffia arrhiza* were not adversely affected over a five day study. In my study, population growth of *P. parvum* was not adversely affected up to 278.6 $\mu g\ l^{-1}$, and significantly ($p < 0.05$) inhibited by concentrations of 4,392.8 $\mu g\ l^{-1}$ for 25% of the study period. Adverse effects to *P. parvum* could be detected at about half of the NOEC value for the alga *C. fracta* and the macrophyte *W. arrhiza* and several orders of magnitude more than the submerged macrophyte *C. demersum*. This demonstrates the differences in sensitivities that different species of algae and plants have to MC-LR. However, cell density was the main endpoint measured in my study, so it is possible that a more sensitive effect, such as oxygen production, was inhibited by treatment levels employed in this study.

Another sublethal endpoint not measured in this experiment that could have been affected by MC-LR is *P. parvum* motility. Kearns and Hunter (2001) performed an

experiment with MC-LR on a motile green alga *Chlamydomonas reinhadtii* and found that 10 μg l^{-1} MC-LR was able to paralyze the green algae and cause them to sink more rapidly than the untreated cells. This paralysis affects settling, even though the cells themselves are still viable. In an aquatic system, this settling could create a competitor-reduced zone in a lake water column that might allow for the proliferation of cyanobacteria. It also may possibly lead to higher rates of grazer predation because lack of motility would decrease the ability to avoid predators. Because *P. parvum* cells must be fixed before enumerating them, neither my study nor the study conducted by Roelke et al. (in press) commented on the motility of the *P. parvum* cells. Higher grazing of immotile cells could have resulted in lower *P. parvum* population growth in the Roelke et al. (in press) study. Conversely, because my study was conducted in the absence of grazers and thus I could not account for grazer x MC-LR interactions, which may have influenced observed growth inhibition observed by Roelke et al (in press).

In addition to seeing no adverse effects on growth of *P. parvum* at concentrations under 4,392.8 μg l^{-1}, there was actually significant stimulation of growth at the lowest concentration, 0.09 μg l^{-1} MC-LR. One possible explanation is that *P. parvum* was able to detoxify the toxin. Such detoxification pathways were proposed by Ou et al. (2005) for another mixotrophic alga, the chrysomonad *Poteriochromonas* sp. Like *P. parvum* growth was at a concentration of 0.09 μg l^{-1}, growth of *Poteriochromonas* was found to be stimulated in the presence of MC-LR at higher concentrations ranging from 100 to 4,000 μg l^{-1}. Because the growth rate of *Poterioochromonas* was four to five times higher than the control, the authors concluded that the toxins served as growth stimulus. However, they also observed that toxin-treated cells showed low cellular viability,

suggesting that growth enhancement by microcystins was an abnormal process. In addition, glutathione, malondialdehyde, and superoxide dismutase (SOD) content were measured and found to be upregulated within eight hours of exposure to MC-LR (500 µg l^{-1}). A high level of SOD activity indicated that SOD was involved in decreasing oxidative stress caused by MC-LR (Ou et al. 2005). Antioxidant activity was not measured in my study, but could be a valuable endpoint in future studies to understand how *P. parvum* protects itself from MC-LR induced damage. In addition, *Poterioochromonas* was able to detoxify MC-LR at concentrations of 1,050, 2,500, and 4,000 µg l^{-1} (Ou et al. 2005). In my study, 16-47% MC-LR degradation was observed in experimental units over a 27-day study period. It is possible that *P. parvum* absorbed and detoxified MC-LR, though not as efficiently as *Poterioochromonas*, which exhibited high degrees of detoxification within a few hours of exposure. Degradation of exposure levels in my study could also have resulted from bacterial biotransformation (Jones and Orr 1994).

This area of algal toxin research has many future possibilities. For example, further studies to assess alternative toxicological endpoints, such as oxygen or alkaline phosphatase production and motility inhibition, from MC-LR exposure to *P. parvum* would help to better understand sublethal effects that it potentially has to *P. parvum*. In addition, using other toxins (e.g., saxitoxins) or congeners (e.g., MC-RR) that can be produced by cyanobacterial species that produce MC-LR could help identify other potential causes of growth inhibition seen in Roelke et al. (in press). In addition, increasing the robustness of my probabilistic effects distributions could be achieved by performing more standardized toxicological bioassays and incorporating the findings into

the SSDs presented here. This would be a valuable tool for aquatic ecosystems managers facing cyanobacterial blooms.

CHAPTER FOUR

Sunlight Photodegradation of Toxins Produced by *Prymnesium parvum*: A Pilot Study

Introduction

The harmful algal species *Prymnesium parvum* employs a mixotrophic feeding strategy (Skovgaard et al. 2003), which includes acquiring energy through photosynthesis and heterotrophically by way of phagotrophy. To facilitate this life history strategy, *P. parvum* produces and excretes toxins called prymnesins (prymnesin-1 and prymnesin-2, Figure 1) to the water column (Igarashi et al. 1998). These toxins have been observed to immobilize and lyse open single-celled prey that *P. parvum* cells then engulf (Skovgaard and Hansen 2003).

Studies have shown that under stressful environmental conditions, toxicity of *P. parvum* can be increased (Graneli and Johansson 2003a, 2003b; Uronen et al. 2005; Baker et al. 2007). Although there are no methods to detect or measure prymnesins at this time, increased toxicity has been assessed with in vitro hemolytic assays and with model aquatic organisms (see Brooks et al. in press for a review). For example, nutrient limited media has been demonstrated to result in higher toxicity to ciliates and other phytoplankton species than nutrient replete cultures (Graneli and Johansson 2003a, b). Additionally, the effect of temperature, salinity, and light on the growth and toxicity of *P. parvum* have been evaluated. Larsen and Bryant (1998) used the invertebrate organism *Artemia* sp. as a test organism for three European strains of *P. parvum* and Baker et al. (2007) used the model fish species *Pimephales promelas* for a Texas strain of *P. parvum*.

Although there are differences among strains and differences in sensitivities of the test organisms, in general, *P. parvum* is growth inhibited below 10˚C and above 30˚C. However, toxicity was not affected by temperature in the Larsen and Bryant study (1998), though toxicity to fish was maximal at low temperatures in the Baker et al. (2007) study. In addition, Baker et al (2007) found maximum toxicity to *P. promelas* at extremely low and high values of salinity (Baker et al 2007); however, Larsen and Bryant (1998) did not report a relationship between salinity and toxicity to *Artemia*. Further, when Larsen and Bryant (1998) exposed light to cultures of different strains of *P. parvum*, they observed increased growth rate with increasing light, with saturation at about 200 μmol photons/ m^2/s, but toxicity to *Artemia* was not affected. Similarly, Baker et al. (2007) observed that optimal growth was reached at 200 μmol photons/m^2/s and toxicity to fish was not affected by light intensity. Thus, although light intensity may not strongly influence site-specific toxin production by *P. parvum* and associated ambient aquatic toxicity, relative to other physical and chemical factors, light intensity may influence the stability of toxins in aquatic ecosystems. Rahat and Jahn (1965) found that *P. parvum* growth was possible in the dark when glycerol was added to the culture medium. Results of their experiments showed that cultures grown in the dark were more toxic than those grown with alternating dark and light periods, indicating that light is not a requirement for toxin synthesis. They also concluded that any toxicity assays performed from cultures grown in alternating light-dark periods only reveal the net results of production and "inactivation" of the toxin (Rahat and Jahn 1965). Another study also demonstrated that constant illumination with a fluorescent lamp reduced toxicity and "inactivated" the toxins (Reich and Parnas 1962). However, it is possible

that the cultures in this study did not produce toxins while under constant illumination. Toxicity was observed when they alternated light and dark periods (Reich and Parnas 1962). While such studies manipulating the light environment of whole cultures were important for identifying factors that affect the physiological status and resulting toxicity of *P. parvum* cells, they did not allow for evaluation of which factors affect the environmental fate of toxins once released into the water column.

Only one published study performed light experiments with toxins separated from the cells (Parnas et al. 1962). Parnas et al. (1962) removed cells by centrifugation and the filtrate was exposed to artificial light and one wavelength of ultraviolet light. Toxicity tests were then performed with *Gambusia affinis* fish to evaluate the differences in toxicity of the different light treatments. They found that "photoinactivation" of the toxin occurred and that it was not affected by the presence of cells, cell pigments, oxygen, or glutathione. Whereas Parnas et al (1962) used the term "photoinactivation" to describe this observation, it is likely that photolysis of the prymnesins occurred, which reduced the concentration of bioavailable toxins, reduced exposure to fish, and thus reduced the magnitude of toxicity.

Photolysis is a chemical process by which molecules are broken down through the absorption of light (Newman and Unger 2003). Certain chemical groupings and classes, such as compounds with conjugated double bonds and aromatic rings, are more susceptible to be degraded because their internal energy states correspond to the incoming light energy (Newman and Unger 2003). There are two forms of photolysis, direct and indirect. Direct photolysis occurs when photons directly interact with and break molecular bonds. Indirect photolysis occurs when photons are absorbed by another

compound, such as humic acid, which then forms a reactive species, such as a hydroxyl radical, which in turn facilitates the degradation of the contaminant (Newman and Unger 2003).

The purpose of the pilot experiment presented in this chapter was to investigate the possible photolytic effect of natural sunlight on *P. parvum* cell-free filtrates from cultures grown under conditions comparable to when blooms occur in Texas inland water bodies. Filtrates were exposed to two different sunlight intensities or no sunlight and toxicity tests were performed and results compared among treatment levels. It was important to isolate excreted toxins from *P. parvum* because this eliminates any confounding results due to physiological status of the organism or toxin production when cells remain in whole culture. This represents a first attempt to evaluate the effect of natural light, rather than artificial laboratory light, on extracellular prymnesins.

Materials and Methods

Prymnesium parvum Culture

P. parvum cells originally obtained from The University of Texas at Austin Culture Collection of Algae (strain UTEX LB 2797) were cultured in an incubator (VWR Model 2015, West Chester, PA, USA) at 20˚±1˚C in 2.4 psu artificial sea water (ASW; Berges et al 2001, Appendix A) in f/8 media (Guillard 1975; Appendix B) on a 12:12 light:dark cycle. After 39 days, the cells were filtered out of the media using glass fiber filters (GF/C, Whattmann), and the filtrate collected in a foil-covered Erlenmeyer flask. The flask was stored under refrigeration (5±1 ˚C) in the dark for less than 24 hours before the sunlight study was initiated.

Sunlight Exposure

Three light treatment levels were selected including full sunlight, partial sunlight, and no sunlight. The partial sunlight treatment was made by placing a shade cloth affixed to a frame over the top of the beakers, which were selected as experimental units for incubation of cell-free filtrate. The dark treatment level was held under a cardboard box beside the light treatments. To perform the sunlight exposures, 600-ml beakers filled with 500 mls of cell-free filtrate or ASW controls were placed in their respective light treatment level. The study was conducted at the Baylor Experimental Aquatic Research (BEAR) facility at the Lake Waco Wetlands, Waco, Texas for eight hrs beginning approximately one hour after sunrise. Light intensity was quantified with two readings approximately every 20 minutes for the full and partial sunlight treatment levels using a light meter (Table 5). At the end of the eight hr period, all beakers were covered with Parafilm, placed in the dark in an ice-filled cooler, and returned to the lab where they were stored overnight under refrigeration (4±1˚C) in the dark. Less than 24 hours later, toxicity assays with the model test organism *Pimephales promelas* were initiated with samples from the light exposure study. In addition, toxicity was assessed for filtrate that was stored under refrigeration in the dark after harvesting the cells, which hereafter is called “lab dark control”.

Laboratory Toxicity Bioassays

Acute toxicity of the filtrates was assessed generally according to US EPA method 2000.0 (US EPA 2002) using *Pimephales promelas* as a model test organism. Less than 48-hour old juveniles (all hatched within 24 hours) obtained from Environmental Consulting & Testing (Superior, Wisconsin, USA) were allowed to feed

on *Artemia* nauplii at least two hours before initiation of the test. The *P. parvum* cell-free filtrate for each treatment was diluted with ASW to dilutions of 100, 50, 25, 12.5, 6.25, and 3.13% filtrate. All solutions were adjusted to pH of 8.5, including the ASW diluent, before mixing the dilutions and filling the test chambers. The test chambers consisted of three replicate 100-ml beakers filled with 80 mls of test solution at each concentration for each of the four treatments. Beakers of light-exposed ASW and reconstituted hard water (RHW; APHA et al 1995) not exposed to ambient sunlight served as controls. The beakers were loaded with five fish each and placed into an incubator (Norlake®; Hudson, Wisconsin, USA) at 25±1˚C on a 16:8 L:D cycle for 48 hrs. Modification to the EPA test method was made by using a time-to-death study design in which mortality was assessed at several time points, rather than only at 24 and 48 hrs. Time points used were 1, 2, 3, 4, 5, 6, 9, 12, 18, 21, 24, and 48 hrs. The number of dead fish was recorded at every time point, but not removed until 24 hours according to standard methods (US EPA 2002).

Statistical Analyses

Median lethal concentration (LC_{50}) values were calculated for all filtrates using an appropriate method of analysis based on attributes of the data (e.g., Spearmann-Karber or Probit; US EPA 2002). Median lethal time to death (LT_{50}) values were determined using the Trimmed Spearmann-Karber method.

Results

Experimental Conditions

During the sunlight study period mean ambient light intensity (±SD) for the full and partial natural sunlight treatment levels was 11,165 (±6,640) lux and 5,750 (±3,989)

lux, respectively (Table 5). Thus, the partial sunlight treatment level was approximately 50% of mean full sunlight during the 8 hr study period.

Laboratory Toxicity Bioassays

Cell-free filtrates of *P. parvum* exposed to either full sunlight or ~50% sunlight did not produce significant *P. promelas* mortality during the 48-hr acute study. In fact, there was not a single mortality observed at any dilution of these light treatments (Figure 13); all ASW and RHW controls also had 100% survival. However, the cell-free filtrate maintained in the dark adjacent to the natural sunlight treatment levels was highly toxic to fish with a 24 and a 48-hour LC_{50} value of 19.50% filtrate (95% confidence intervals: 14.99-25.38%). Such a magnitude of toxicity to fish was similar to that of the cell–free filtrate stored in the dark under refrigeration (dark lab control) in the laboratory during the natural sunlight study period: an acute 24 and 48 LC_{50} value of 14.03% filtrate (10.29-19.13%) was determined. Results from the time-to-death study showed that the 100% treatment levels of both laboratory and field dark treatments all organisms were dead within three hrs. In the 25% filtrate treatment level for both dark treatments, 50% mortality (LT_{50}) was calculated at 2.3 hrs (95% confidence interval 1.37–3.84 hrs) (Figure 14).

Discussion

In this experiment, the two *P. parvum* cell-free filtrates maintained in the dark demonstrated to be acutely toxic to fish while the two filtrates exposed to sunlight produced no mortality over a 48 hr period. The complete disappearance of toxicity of filtrates after sunlight exposure provides strong evidence that photolysis of the toxins

Table 5. Light intensity for full sunlight and partial sunlight treatments during *P. parvum* cell-free filtrate exposure period.

	Full Sunlight	Partial Sunlight
Time	Mean Light Intensity (lux)	Mean Light Intensity (lux)
9:00 AM	5,760	3,165
9:20 AM	4,350	1,950
9:40 AM	5,565	3,200
10:00 AM	8,405	5,235
10:22 AM	6,680	3,445
10:43 AM	32300	21,750
11:00 AM	13,095	6,865
11:20 AM	8,735	5,210
11:42 AM	9,620	4,770
12:12 PM	23,000	8,590
12:28 PM	9,115	4,625
12:50 PM	4,680	2,145
1:00 PM	9,500	4,890
1:20 PM	4,645	2,380
1:41 PM	14,180	7,290
2:14 PM	8,270	3,980
2:30 PM	9,455	4,865
2:50 PM	11,600	5,940
3:20 PM	13,250	6,200
3:40 PM	21,650	9,605
4:02 PM	11,395	5,375
4:21 PM	12,985	6,415
4:43 PM	8,560	4,350

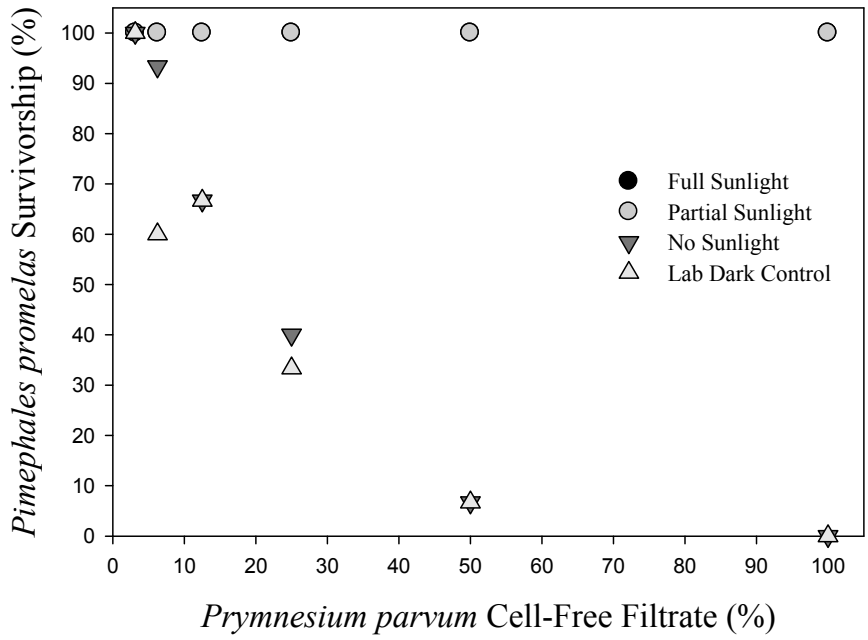

Figure 13. *Pimephales promelas* 24 hour survivorship following exposure to *Prymnesium parvum* cell-free filtrates treated with different natural sunlight intensities and a laboratory dark control.

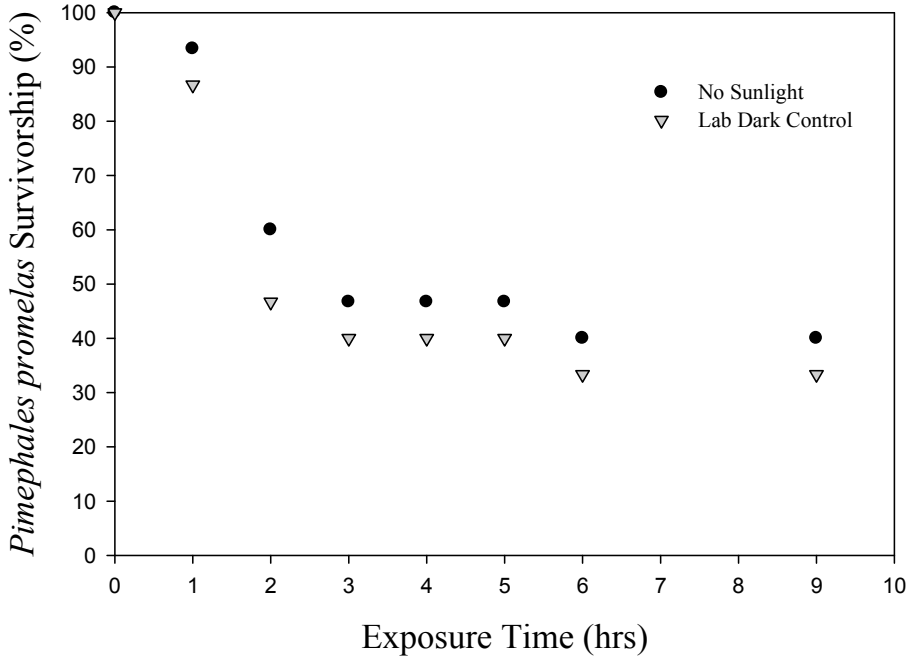

Figure 14. *Pimephales promelas* survivorship for the first ten hours of the 48 hr acute study when exposed 25% filtrate of the no sunlight treatment level and a laboratory dark control.

occurred. The findings in this study are consistent with those of Parnas et al. (1962) who reported that when a *P. parvum* supernatant was exposed to light from a tungsten lamp for just 1.5 hrs toxicity to *Gambussia* sp. was removed, and such toxicity elimination was not influenced by the addition of cell pigments, oxygen, or glutathione. Parnas et al (1962) concluded that a photochemical process was involved in "inactivation" of toxins present in the *P. parvum* cell-free filtrate. However, Parnas et al (1962) could not suggest how the photons may interact with the bonds of the prymnesins because the structure was not elucidated until thirty years after the study was published (Igarashi et al. 1998).

Prymnesins are very large molecules with 90 carbon atoms and trans-1, 6-dioxadecaline units with conjugated double/triple bonds at each terminal end (Igarashi et al. 1999). These compounds are amphiphilic, with uneven distributions of sugars and hydroxyl groups, three chlorine atoms, and one nitrogen atom (Valenti et al. in review). It is not possible to identify at this time how the prymnesin molecules break apart during photolysis, especially because they are such large molecules with many bonds that could be subject to being broken by photons. However, one likely susceptible chromophore (or, a region that can absorb ultraviolet and visible light) (Connell 2005) is the series of single and double bonded carbons that is found on the hydrophilic portion of the prymnesins. It appears reasonable that this region absorbs photons and causes degradation of the parent toxins. In addition, indirect photolysis reactions may have occurred. Decaying algae, other algal exudates and bacterial activity likely increased the dissolved organic matter in the stock culture, which could not be removed when the cells were harvested and cell-free filtrate prepared for light experiments. Dissolved organic matter is photosensitive and can release reactive species that can facilitate degradation of

environmental contaminants (Newman and Unger 2003), and potentially contributed to prymnesin degradation in this study. Thus, it is not possible, based on the findings from this study, to determine whether direct and indirect photolysis was primarily responsible for photodegradation of *P. parvum* toxins. However, this question deserves additional study because the factors influencing toxin photodegradation rates, and thus the magnitude and duration of prymnesin exposures, ultimately resulting in ambient aquatic toxicity, can provide important information for environmental management of fish kills resulting from *P. parvum* bloom formation.

Not only were the dark treatments highly toxic to fish, but the onset of toxicity was rapid, with complete mortality in the 100% filtrate within three hours and 50% death in the 25% filtrates at approximately 2.3 hrs. Toxins produced by *P. parvum* result in a rapid onset of toxic effects because the toxins exert toxicity at the level of the gill, which is a vulnerable surface directly exposed to bioavailable chemicals in aquatic systems (Terao et al.1996). Because fish toxicity serves as a useful surrogate for the concentration of bioavailable toxins in a sample, the magnitude of toxic response may provide a biosensor for toxin concentrations. In this study, toxin concentrations appeared to be reduced to very low levels because fish toxicity was eliminated by full and ~50% sunlight exposure. In addition, LC_{50} values were very similar in the dark treatment in the field (LC_{50} = 19.5%) outside and the dark lab refrigerated control (LC_{50} = 14.03%), highlighted by overlapping 95% confidence intervals. Such comparable toxicity to fish suggests that temperature, which was 4^{o}C in the lab and approximately 26^{o}C in the field, did not influence toxin stability in this study. Further, the magnitude of survivorship responses did not continue to appreciably decrease with exposure duration in the time-to-

death study after the first 3 hrs of the exposure period (Figure 13). This observation is not consistent with aquatic toxicological response thresholds, which generally decrease in concentration with increasing exposure duration (Rand 1995). Subsequently, this observation suggests that the magnitude of exposure to bioavailable toxins changed over this 3 hr period, potentially because toxins were degraded by incubator lights during the fish bioassay study.

The observed loss in toxicity to fish in this study when exposed to sunlight suggests that nighttime toxin release of toxins by *P. parvum* cells may be important for the environmental management of inland and coastal waters experiencing fish kills. Producing such large compounds is energetically costly, so it would be a more advantageous life history strategy to release the toxins when light exposure would be minimal; however, the relationship between toxin synthesis and release by *P. parvum* and diurnal light regimes are not understood. The information collected from this may support future fisheries management and mitigation measures for harmful *P. parvum* blooms. Understanding direct and indirect photolytic degradation pathways for prymnesins can also improve the design of laboratory studies. For example, light minimization when handling samples and photoperiod consideration in toxicological experimental designs are needed.

This study represents a pilot effort to understand the effects of sunlight on the environmental fate and exposure of toxins produced by *P. parvum*. Additional studies evaluating the effect of experimental conditions should be performed, specifically examining a parallel toxicity test in complete darkness in addition to a test with standard conditions. In addition, cell-free filtrates were only exposed to full or partial sunlight for

a period of eight hrs; thus, it would be useful to examine additional sunlight intensities and exposure durations. For example, a future study could utilize multiple time points, such as 30 minutes, and 1, 2, 4, and 8 hrs to examine the duration of exposure to sunlight necessary to significantly reduce ambient toxicity. The results from these additional studies could advance the knowledge gained from this study, could have implications in the way ambient toxicity is assessed in the future, and possibly allow for a surrogate estimate of an aggregate photolysis half-life of toxins produced by *P. parvum*.

APPENDICES

APPENDIX A

Culture Media

Table A.1. F/2 and f/8 media for culturing *Prymnesium parvum*. Modified from Guillard 1975.

	f/2 Medium	f/8 Medium
Nutrients		
$NaNO_3$	75 mg	18.75 mg
$NaH_2PO_4 \cdot H_2O$	5 mg	1.25 mg
Trace Metals		
Na_2 EDTA	4.36 mg	4.36 mg
$FeCl_3 \cdot 6H_2O$	3.15 mg	3.15 mg
$CuSO_4 \cdot 5H_2O$	0.01 mg	0.01 mg
$ZnSO_4 \cdot 7H_2O$	0.022 mg	0.022 mg
$CoCl_2 \cdot 4H_2O$	0.01 mg	0.01 mg
$MnCl_2 \cdot 2H_2O$	0.18 mg	0.18 mg
$Na_2MoO_4 \cdot 2H_2O$	0.006 mg	0.006 mg
Vitamins		
Thiamin HCl	0.1 mg	0.1 mg
Biotin	0.5 µg	0.5 ug
B_{12}	0.5 µg	0.5 ug
Seawater	to one liter	to one liter

Add nutrients and trace metals to seawater and autoclave. After it cools, add 0.2 µm syringe filtered vitamins, then inoculate with *P. parvum* and place in incubator.

APPENDIX B

Artificial Seawater

Table B.1. Recipe for artificial seawater used to culture *Prymnesium parvum*. Modified from Berges et al. 2001.

Salt Solution I—Anhydrous Salts

NaCl	21.19 g
Na_2SO_4	3.55 g
KCl	0.599 g
$NaHCO_3$	0.174 g
KBr	0.0863 g
H_3BO_3	0.0230 g
NaF	0.0028 g
Nanopure water	1/3 L

Salt Solution II—Hydrated Salts

$MgCl_2 \cdot 6H_2O$	9.592 g
$CaCl_2 \cdot 2H_2O$	1.344 g
$SrCl_2 \cdot 6H_2O$	0.0218 g
Nanopure water	2/3 L

Autoclave salt solutions separately and combine after they cool. Dilute to a salinity of 2.4 psu using Nanopure water.

APPENDIX C

Algal Assay Procedure (AAP) Medium

Table C.1. Algal Assay Procedure medium for culturing *Pseudokirchneriella subcapitata.* Modified from US EPA 2002.

Stock Solution	Compound	Amount Dissolved in 500 ml Nanopure water
1. *Macronutrients*		
A.	$MgCl_2 \cdot 6H_20$	6.08 g
	$CaCl_2 \cdot 2H_20$	2.20 g
	$NaNO_3$	12.75 g
B.	$MgSO_4$	7.35 g
C.	K_2HPO_4	0.522 g
D.	$NaHCO_3$	7.50 g
2. *Micronutrients*		
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg
	$CuCl_2 \cdot 2H_20$	0.006 mg
	$Na_2EDTA \cdot 2H_20$	150.0 mg
	Na_2SeO_4	1.196 mg

Add 1 ml of each stock solution per 1 L of media.

APPENDIX D

Literature used in creating exposure and effects distributions in Chapter Three.

Table D.1. Measured environmental microcystin exposure values used for the exposure distribution.

Reference	[MC] µg l^{-1}	Location	Reference	[MC] µg l^{-1}	Location
McDermott		Wisconsin, US	Graham and	0.8	Missouri/ Iowa, US
1995	200	Suamico River	Jones 2007	7	Missouri/ Iowa, US
	180	Green Bay			
	1.7	Green Bay	Fristachi et al.	0.007	US/ Canada
	1.2	Quarry Park Lake	2007; Carmichael	0.053	US/ Canada
	0.7	Long Lake #1	2001	0.013	US/ Canada
	19	Long Lake #2		0.007	US/ Canada
	0.3	Becker Lake		0.04	US/ Canada
	3.8	Round Lake		147.1	US/ Canada
	2.4	Boot Lake		4.7	US/ Canada
	22	Beaver Dam Lake #2		0.03	US/ Canada
	8	Beaver Dam access stream			
	2	Long Lake	Kotak and		Canada
	6.8	Kettle Moraine Lake	Zurawell 2007	2.48	Little Beaver Lake
	0.5	Green Lake		11.2	Little Beaver Lake
	17	Green Lake Dam		1.5	Nakamun Lake
	50	Lake Weyauwega		1	Driedmeat Lake
	12.4	Mirror Lake			
	7.4	Shadow Lake	Murphy et al.		Canada
			2003	0.17	Hamilton Harbor, Lake Ontario

Table D.1 continued

Reference	[MC] µg l^{-1}	Location	Reference	[MC] µg l^{-1}	Location
McDermott	11.2	White Lake	Murphy et al.	0.19	Hamilton Harbor, Lake Ontario
1995	5	Hartman Lake	2003	0.01	Hamilton Harbor, Lake Ontario
	1	Fox River-Omro Park		0.01	Hamilton Harbor, Lake Ontario
	52	Lake Butte de Mortes		0.02	Hamilton Harbor, Lake Ontario
	3	Wolf River		0.06	Hamilton Harbor, Lake Ontario
	51	Lake Winnebago #1		33.1	Hamilton Harbor, Lake Ontario
	56	Lake Winnebago #2		0.25	Hamilton Harbor, Lake Ontario
				5.03	Hamilton Harbor, Lake Ontario
Jacoby and		Pacific Northwest, US		0.96	Hamilton Harbor, Lake Ontario
Kann 2007	12176	Copco Reservoir, CA		0.47	Hamilton Harbor, Lake Ontario
	2032	Iron Gate Reservoir, CA		238.8	Hamilton Harbor, Lake Ontario
	46.73	Klamath River, CA		202.2	Hamilton Harbor, Lake Ontario
	0.7	Tenmile Lakes, OR		0.98	Hamilton Harbor, Lake Ontario
	1.61	Tenmile Lakes, OR		0.24	Hamilton Harbor, Lake Ontario
	0.19	Crane Prarie, OR		0.024	Lake Erie
	4.92	Crane Prarie, OR		0.035	Lake Erie
	0.68	Lava Lake, OR		0.142	Lake Erie
	0.009	Paulina Lake, OR		0.302	Lake Erie
	84	Paulina Lake, OR		0.016	Lake Erie
	2.9	Wickiup Reservior, OR		0.009	Lake Erie
	2.54	Diamond Lake, OR		0.008	Lake Erie
	0.36	Suttle Lake, OR		0.001	Lake Erie
	0.51	Suttle Lake, OR		0.006	Lake Erie
	0.19	Lake Selmac, OR		0.002	Lake Erie
	13.5	Lake Selmac, OR		0.371	Lake Erie
	0.03	Odell Lake, OR		0.407	Lake Erie
	5.01	Odell Lake, OR		0.2	Lake Erie
	10	Odell Lake, OR		0.028	Lake Erie
	600	Upper Klamath (Agency) Lake , OR		0.031	Lake Erie
	14.6	Owyhee Reservoir, OR		0.009	Lake Erie
	18.5	Vancouver Lake, WA		0.008	Lake Erie

Table D.1 continued.

Reference	[MC] µg l^{-1}	Location	Reference	[MC] µg l^{-1}	Location
Jacoby and	0.91	Vancouver Lake, WA	Murphy et al.	0.009	Lake Erie
Kann 2007	7	Vancouver Lake, WA	2003		
	9.5	Vancouver Lake, WA			
	0.25	Lake Garrett (Hicks), WA	Young et al.		Aland, Finland
	0.85	Lake Garrett (Hicks), WA	2006	0.070	Hogbolstad
	0.26	Lake Garrett (Hicks), WA		1.400	Godby trask
	0.75	Lake Garrett (Hicks), WA		12.100	Prasttrasket
	0.74	Lake Garrett (Hicks), WA		0.130	Basttjarnan
	0.68	Lake Garrett (Hicks), WA		0.260	Brantsbole trask
	0.86	Lake Garrett (Hicks), WA		6.400	Nato Hemviken
	1	Green Lake, WA		0.200	Gloskars trask
	32	Green Lake, WA		0.250	Gloskars trask
	3	Green Lake, WA		0.760	Brantsbole trask
	100	Green Lake, WA		0.620	Brantsbole trask
	0.17	Green Lake, WA		0.670	Brantsbole trask
	2.2	Green Lake, WA		1.000	Stromma trask
	1	Lake Sammamish, WA		0.700	Kaldersfjarden
	43	Lake Sammamish, WA		2.300	Lembote Bytrask
	0.13	Lake Sammamish, WA		6.000	Lembote Bytrask
	0.17	Lake Sammamish, WA		5.400	Vargata trask
	0.16	Lake Sammamish, WA		4.600	Vargata trask
	0.1	Lake Sammamish, WA		5.100	Godby trask
	0.14	Lake Union, WA		0.100	Norra Langsjon, Saltvik
	0.15	Lake Union, WA		0.940	Overby insjo
	0.12	Lake Union, WA		0.240	Sodra Slemmern
	0.14	Lake Union, WA		0.610	Hogskar
	0.18	Lake Washington, WA		0.160	Lillfjarden
	0.62	Lake Washington, WA		1.640	Katthavet
	0.68	Lake Washington, WA		3.100	Prasttrasket
	1.15	Lake Washington, WA		0.440	Hagn trask
	4	Kitsap Lake, WA		42.300	Vargata trask

Table D.1 continued.

Reference	[MC] µg l^{-1}	Location	Reference	[MC] µg l^{-1}	Location
Jacoby and	13	Steilacoom Lake, WA	Young et al.	36.800	Vargata trask
Kann 2007	0.8	Waughop Lake, WA	2006	7.540	Prasttrasket
	0.13	Moran State Park Lake, WA		3.100	Brantsbole trask
	0.5	Lake Cassidy, WA		0.210	Brantsbole trask
	3	Lake Cassidy, WA			
	0.5	Ketchum Lake, WA	Jones and		Temora, Australia
	3	Ketchum Lake, WA	Orr 1994	990	Lake Centenary
	2.73	Lake Lawrence, WA		1830	Lake Centenary
	0.99	McIntosh Lake, WA			
	0.54	McIntosh Lake, WA	Harada and		Japan
	0.51	McIntosh Lake, WA	Tsuji 1998	0.02	Lake Sagami
	1.52	McIntosh Lake, WA		0.04	Lake Sagami
	0.54	McIntosh Lake, WA		0.05	Lake Sagami
	0.48	McIntosh Lake, WA		0.6	Lake Sagami
				0.04	Lake Tsukui
Touchette et		North Carolina, US		0.08	Lake Tsukui
al. 2007	0.3	Kerr Scott		3.18	Lake Tsukui
	0.13	Tuckertown		0.14	Lake Tsukui
	0.11	Oak Hollow		52	Lake Tsukui
	0.2	Jordan		3.9	Lake Tsukui
	0.24	Falls		2.5	Lake Tsukui
	0.15	Narrows		0.05	Lake Tsukui
	0.28	Lake Rhodhiss		1.78	Lake Tsukui
	0.15	Michie		0.24	Lake Tsukui
	0.1	High Rock		0.04	Lake Tsukui
	0.35	Tillery		1.07	Lake Tsukui
	0.14	High Point Lake		93.8	Lake Tsukui
				0.08	Lake Tsukui
Williams et al.		Florida, US		0.02	Lake Tsukui
2007	0.05	Harris Chain of Lakes			
	0.5	Harris Chain of Lakes			

Table D.1 continued.

Reference	[MC] µg l^{-1}	Location	Reference	[MC] µg l^{-1}	Location
Williams et al.	3.6	Harris Chain of Lakes			
2007	0.01	St. Johns River			
	0.6	St. Johns River			
	31	St. Johns River			
	0.1	Lake Okeechobee			
	0.1	Lake Okeechobee			
	95	Lake Okeechobee			

Table D.2. Values used for median lethal concentration species sensitivity distribution.

Reference	Organism	Statistic	Endpoint	Concentration ($\mu g\ l^{-1}$)
Smith et al. 2008	*Hexagenia* spp. hatchling nymph	96 hr LC_{50}	mortality	49
Liu et al. 2002	*Misgurnus mizolepis* larvae--stage 34	7d LC_{50}	mortality	164.3
Reinikainen et al. 2002	*Eurytemora affinis*	48 hr LC_{50}	mortality	274
DeMott et al. 1991	*Diaptomus birgei*	48 hr LC_{50}	mortality	450
Liu et al. 2002	*Misgurnus mizolepis*juvenile	7d LC_{50}	mortality	593.3
Metcalf et al. 2002	*Artemia salina*	72 hr LC_{50}	mortality	850
DeMott et al. 1991	*Diaptomus birgei*	48 hr LC_{50}	mortality	1,000
DeMott et al. 1991	*Daphnia pulex*	48 hr LC_{50}	mortality	9,600
DeMott et al. 1991	*Daphnia hyalina*	48 hr LC_{50}	mortality	11,600
Kiviranta et al. 1992	*Aedes aegypti*	48 hr LC_{50}	mortality	14,900
Chen et al. 2005	*Daphnia magna*	48 hr LC_{50}	mortality	20,300
DeMott et al. 1991	*Daphnia pulicaria*	48 hr LC_{50}	mortality	21,400

Table D.3. No observable effect concentration values used in the sublethal species sensitivity distribution.

Reference	Organism	Statistic	Endpoint	Microcystin Concentration (ug l^{-1})
Pflugmacher 2002	*Ceratophyllum demersum*	6 week NOEC	oxygen production inhibition	0.10
Pflugmacher 2002	*Ceratophyllum demersum*	7 week NOEC	growth inhibition	0.5
Oberemm et al. 1997	*Danio rerio* larvae	end of embryonic stage NOEC	decreased survival and growth:weight	0.5
Smith et al. 2008	*Hexagenia* spp. egg	16 day NOEC	hatching delay	10
Reinikainen et al. 2002	*Eurytemora affinis*--adult males + females	5 day NOEC	survival time	50
Smith et al. 2008	*Hexagenia* spp. egg	16 day NOEC	overall hatching success (# eggs & percentage hatched)	100
Chen et al. 2005	*Daphnia magna*	21 day NOEC	reproduction decreased per alive female	113
Reinikainen et al. 2002	*Eurytemora affinis* adult females	7 day NOEC	survival time	250
Chen et al. 2005	*Daphnia magna*	21 day NOEC	survival rate decreased	360
Reinikainen et al. 2002	*Eurytemora affinis* egg	4 day NOEC	cumulative hatching frequency and time	1,000
Reinikainen et al. 2002	*Eurytemora affinis* nauplii	4 day NOEC	cumulative # dead nauplii increase	1,000
Mitrovic et al. 2005	*Lemna minor*	5 day NOEC	growth--weight and frond number decrease; root length decrease; peroxidase increase	3,000
Smith et al. 2008	*Hexagenia* spp. pre-emergence (large) nymph	7 day NOEC	mortality	10,000
Mitrovic et al. 2005	*Chladophora fracta*	5 day NOEC	growth--weight decrease; peroxidase increase	10,000
Mitrovic et al. 2005	*Wolffia arrhiza*	5 day NOEC	growth--frond number decrease	10,000

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