ABSTRACT

Influence of pH and Total Dissolved Solids on Harmful Algal Blooms of *Prymnesium parvum*

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Harmful blooms of *Prymnesium parvum* in inland waters continue to expand, in part, due to anthropogenic influences and climate change. This thesis examines influences of pH and total dissolved solids (TDS) on *P. parvum* blooms, growth, and toxicity to aquatic life. Chapter 1 evaluated pH effects on *P. parvum* bloom development and toxicity by manipulating *in situ* limnocorrals during 21 d pre-bloom and bloom development experiments. Though neutral pH levels preempted *P. parvum* bloom development, higher pH resulted in bloom formation and ambient toxicity. Chapter 2 examined whether TDS with major ionic composition associated with hydraulic fracturing influenced *P. parvum* growth and toxicity across nutrient conditions. Though no growth was observed at 130 mg/L, higher treatment levels of TDS stimulated *P. parvum* growth and increased acute toxicity to fish. Such novel observations provide pH and TDS thresholds for potentially supporting management of *P. parvum* growth and acute toxicity in inland waters.

Influence of pH and Total Dissolved Solids on Harmful Algal Blooms of *Prymnesium parvum*

by

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A Thesis

Approved by the Department of Environmental Science

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ABBREVIATIONS

ASW	Artificial Seawater
CHL-A	Chlorophyll-*a*
CI	Confidence Interval
HAB	Harmful Algal Bloom
HF	Hydraulic Fracturing
LC_{50}	Median Lethal Concentration
LT_{50}	Median Lethal Time
N	Normal
NTU	Nephelometric Turbidity Units
pK_a	Logarithmic Acid Dissociation Constant
PSU	Partial Salinity Units
RHW	Reconstituted Hard Water
RM ANOVA	Repeated Measures Analysis of Variance
SPSS	Statistical Package for the Social Sciences
TDS	Total Dissolved Solids
US EPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant
YSI	Yellow Springs Instruments

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DEDICATION

To my grandparents

Kenneth and Sylvia Finlan

and

John and Jean Prosser

CHAPTER ONE

Low pH Preempts Bloom Development of a Toxic Haptophyte

Introduction

Harmful algal blooms (HABs) of *Prymnesium parvum* are global phenomena occurring in marine, estuarine and inland ecosystems (Edvardsen and Paasche 1998; Lundholm and Moestrup 2006; Moestrup 1994). *Prymnesium parvum*, commonly called "golden algae" and the "Texas tide," is a mixotrophic flagellated haptophyte known to produce toxins that may severely impact aquatic organisms (Brooks et al. 2010). There is some debate whether *P. parvum* is invasive to inland systems (Lutz-Carrillo et al. 2010), or whether it has been present for many years, only recently becoming an environmental issue due to anthropogenic influences and climatological changes that favor *P. parvum* growth (Roelke et al. 2012). Either way, salinization (Roelke et al. 2010a; Roelke et al. 2010b) and eutrophication (Hallegraeff 1993; Roelke et al. 2007; Roelke et al. 2010b) have created conditions that allow for the proliferation of *P. parvum* blooms in inland waters. In particular, some Texas inland reservoirs have experienced devastating *P. parvum* blooms and conservative estimates suggest that over 30 million fish (Sager et al. 2008; Southard et al. 2010) have been killed since 1985 when blooms were first observed in the Pecos River (James and De La Cruz 1989). *Prymnesium parvum* is now present in at least 5 major river basins and has affected over 33 reservoirs in Texas (Southard et al. 2010). A recent *P. parvum* bloom in Dunkard Creek, Pennsylvania/West Virginia in 2009 highlights the expansion of this organism even further northward (Brooks et al. 2011a).

To date, *P. parvum* is present in at least 18 states in the United States (Roelke et al. 2011; Sager et al. 2008).

Of the toxins produced by *P. parvum*, prymnesin-1 and prymnesin-2 were initially characterized (Igarashi et al. 1998; Igarashi et al. 1999) and thus have historically been held responsible for toxicity to aquatic organisms. However, toxicity may not solely result from exposure to prymnesins, but from other products such as fatty acids (Henrikson et al. 2010), fatty acid amides (Bertin et al. 2012a, 2012b), or toxins yet to be characterized (Schug et al. 2010). Whereas the adaptive functions of these toxins remain unclear, the resultant fish kills, thought to be caused by the disruption of ion regulation in gills, are severe (Edvardsen and Imai 2006; Ulitzur and Shilo 1966; Yariv and Hestrin 1961). Though less understood, the effects of these toxins on other organisms within aquatic ecosystems may have equally severe impacts (Brooks et al. 2010). In particular, *P. parvum* can affect zooplankton grazers by reducing fecundity (Brooks et al. 2010; Granéli and Johansson 2003a; Larsen et al. 1998; Roelke et al. 2007) and altering predator-prey dynamics (Barreiro et al. 2005; Olli and Trunov 2007; Skovgaard and Hansen 2003). Furthermore, differential sensitivities among species to toxins produced by *P. parvum* can cause shifts in planktonic community structure (Brooks et al. 2010; Fistarol et al. 2003; Schwierzke et al. 2010), with longer term consequences yet unknown.

A number of factors apparently influence the magnitude of toxins produced by *P. parvum* in inland waters, as evidenced by inconsistent correlation between population density of *P. parvum* and observed toxicity in laboratory and field studies (Baker et al. 2007; Shilo and Aschner 1953). Physical system conditions that influence *P. parvum*

bloom dynamics include salinity, temperature, light, and river inflows (Baker et al. 2007; Baker et al. 2009; Roelke et al. 2011), while chemical conditions include inorganic nutrient concentrations (Granéli and Johansson 2003a; Grover et al. 2007; Johansson and Granéli 1999; Roelke et al. 2007). The interplay among these processes leads to counter-intuitive observations. For example, optimum conditions for growth result in lowest toxicity to fish for the Texas strain of *P. parvum* (Baker et al. 2007; Baker et al. 2009), which differ markedly from late winter/early spring conditions of Texas impoundments (Roelke et al. 2011) when bloom associated fish kills typically occur (Southard et al. 2010).

Another chemical variable shown to affect the magnitude of *P. parvum* associated toxicity in marine and inland waters is pH. Over 50 years ago, Shilo and colleagues (Shilo and Aschner 1953; Ulitzur and Shilo 1964) demonstrated that *P. parvum* was not ichthyotoxic at pH 7 and below, but became consistently more toxic to fish at higher pH levels in studies at salinities typical of marine / estuarine ecosystems. Similar relationships between pH and toxicity were recently extended to inland waters by our research group. Valenti et al. (2010) identified that the potency of *P. parvum* toxins increased at higher pH by conducting bioassays with *Pimephales promelas* and *Daphnia magna* as model aquatic organisms. Such relationships were consistently observed in laboratory studies with monocultures of *P. parvum* at various levels of nutrient limitation and in field studies of samples collected during extensive fish kills attributed to *P. parvum* (Valenti et al. 2010).

Interactions between pH and *P. parvum* may represent an important determinant of site-specific blooms, because natural, anthropogenic, and climatological factors

influence pH dynamics in surface waters and thus the ambient toxicity of a wide range of organic and inorganic stressors (Brooks et al. 2011b; Valenti et al. 2011). Though influences of pH on *P. parvum* bloom development are unknown, it may be reasonable for pH to affect bloom formation if toxin(s) potency provides advantages for competition and predation. The primary objective of the present study was to evaluate whether pH influences *P. parvum* bloom development and toxicity. To this end, we manipulated pH levels (7, 7.5, 8.5) of *in situ* enclosures during pre-bloom and bloom development conditions in Lake Granbury, TX, USA, an impoundment consistently impacted by *P. parvum* HABs, and followed the resulting plankton dynamics.

Materials and Methods

Experimental Design

We selected Lake Granbury (Granbury, TX, USA) for our in-lake experiments. It was chosen because of its history of experiencing devastating *P. parvum* blooms during late winter/early spring months. Lake Granbury is an impoundment of the Brazos River in central Texas with a surface area of 34 km^2, a volume of 188 x 10^6 m^3, and a mean width, depth, and length of 0.6 km, 5 m, and 45 km, respectively. In-lake experiments that employed large enclosures were conducted in a shallow cove of Lake Granbury, chosen for its sufficient depth and seclusion from heavy boat traffic. Enclosures were translucent and impermeable closed-bottomed cylinders with dimensions of 1 m diameter and 2 m depth (total volume: 1.57 m^3) (Aquatic Research Instruments, Hope, ID, USA). Floating frames anchored in the cove supported the enclosures. Each enclosure was filled with ambient site water and attached to the frames with a portion of the enclosure above the water line. This prevented surrounding cove water from entering the enclosures

through wave action after experiment initiation. Enclosures were left uncovered to allow for air exchange throughout the study.

Two experiments of 21d duration were conducted to assess the effect of pH on *P. parvum* and plankton community dynamics, and ambient toxicity. The first experiment was conducted in February 2010 during a pre-bloom period. The second experiment was conducted in March 2010 during a period of bloom development. Both experiments evaluated the effects of two pH treatment levels, pH 7 and 7.5. For each experiment, unmanipulated ambient controls were also included (pH ~8.5). Three replicate enclosures were used for each pH level.

pH Manipulation

Following initial titration studies, the pH was adjusted to the designated treatment level by slowly adding 0.1 N sulfuric acid to each enclosure. During this process, water inside each experimental unit was gently stirred and pH was monitored to ensure a uniform pH at various depths. Throughout each experiment, pH was monitored twice weekly in each enclosure using a calibrated Hydrolab Quanta (Hach Company, Loveland, CO, USA) or YSI 600 XLM multiprobe (Yellow Springs Instruments, Yellow Springs, OH, USA). If needed, pH was readjusted to its designated treatment level following the aforementioned procedure. In addition to twice weekly discrete pH measurements in each enclosure, YSI 6600 XLM multiprobes were suspended in the middle of one randomly selected enclosure for each treatment level and set to continuously monitor pH at 15 min intervals throughout the entire study.

Water Quality Monitoring

Water quality parameters (temperature, dissolved oxygen, salinity, specific conductivity, and turbidity) of each enclosure and the cove were measured at days 0, 7, 14, and 21 (Table A1). Water quality measurements were taken semi-weekly at a uniform depth of 0.5 m. In addition, Secchi depth of each enclosure and the cove were monitored (Table A1). Ammonia (NH_4) concentrations were measured from 100 mL samples collected at a depth of 0.5 m and analyzed using a Flow Solutions IV Autoanalyzer (OI Analytical, College Station, TX, USA) (Armstrong and Sterns 1967). Ammonia levels were assessed relative to the US EPA's current ambient water quality criteria for ammonia (*Aquatic life ambient water quality criteria for ammonia-freshwater* 2009; Harwood and Kuhn 1970) and were all found to be well below thresholds likely to cause adverse effects to aquatic organisms (data not shown).

Zooplankton and Phytoplankton Biomass.

Prymnesium parvum cell densities were estimated from a 100 mL sample collected from a depth of 0.5 m and fixed with 5% v/v glutaraldehyde (Sigma-Aldrich, Grade II, 25%). A 1 mL sub-sample was taken from each sample and settled for at least 24 hours using Utermöhl algal settling chambers (Utermöhl 1958). Inverted, phase-contrast light microscopy was used to enumerate *P. parvum* cell densities in each sample (Leica Microsystems, Inc., Buffalo Grove, IL, USA). Cells were counted in a minimum of 20 random fields of view at 400x magnification. If less than 200 *P. parvum* cells were observed, a subsequent 20 random fields of view were then counted (Hayden et al. 2012).

Zooplankton were enumerated and total zooplankton biomass was estimated from a 50 mL concentrated water sample collected using a 12 L Schindler plankton trap from a

depth of 0.5 m and fixed with formalin (2%). Sub-samples of approximately 10 mL were removed from each field collected sample and settled for at least 24 hours using Utermöhl algal settling chambers (Utermöhl 1958). Zooplankton biovolume was determined by using the same microscopy and enumeration techniques as were used when determining *P. parvum* cell density, with additional measurements taken of appropriate dimensions of each individual that enabled geometric estimation of organism biovolume (Hayden et al. 2012).

Total phytoplankton biomass was determined from triplicate 50 mL samples collected at a depth of 0.5 m. The samples were filtered (Whatman GF/F, 25 mm), extracted (90% acetone), and the chlorophyll-*a* fluorescence was measured using a 10-AU fluorometer (Turner Designs, Inc., Sunnyvale, CA; (Strickland and Parsons 1968). Total phytoplankton biomass was then estimated based on the chlorophyll-*a* fluorescence values.

Acute Toxicity to Fish

On days 7, 14, and 21, ambient acute toxicity of the cove and each enclosure was assessed from 4 L samples collected from a depth of 0.5 m. To limit pH drift during holding time, samples were collected such that there was no air-space in the sample container. After collection, samples were stored in the dark at 4˚C and used to initiate water toxicity bioassays within 24 hours. 48 hr toxicity bioassays were performed to estimate acute toxicity to larval (< 48 hrs) *P. promelas* (fathead minnows). Bioassays were conducted based on standardized methods (*Methods for measuring the acute toxicity of effluents receiving waters to freshwater and marine organisms* 2002). Larval *P. promelas* were fed *Artemia* nauplii to satiation 2 hours prior to test initiation.

Bioassays were conducted at 25^{o}C in darkness to avoid amelioration of *P. parvum* toxicity by light (James et al. 2011). The initial pH of each sample was determined using a standard bench-top pH meter calibrated before each use (Accumet combination pH probe, 720A Orion pH meter). Standard 100 mL beakers were used as test chambers for the bioassays. Mortality was monitored at 24 hr and 48 hr time periods. Samples were diluted with reconstituted hard water (RHW), which also served as a control, to define dose response relationships (*Methods for measuring the acute toxicity of effluents receiving waters to freshwater and marine organisms* 2002). All bioassays were performed in a climate controlled environmental chamber (ISO 9001, Nor-Lake, Inc., Hudson, WI).

Sublethal Toxicity to Cladocerans

On day 21 of both the pre-bloom and bloom development experiments, 10 d toxicity assays were performed to define the sublethal effects of *P. parvum* to the model cladoceran, *D. magna*. Bioassays were conducted according to US EPA (USEPA, 1994) standard methods with minor modifications (*10-day chronic toxicity test using daphnia magna or daphnia pulex. Environmental response team, compendium of ert standard operating protocols #2028* 1994; Dzialowski et al. 2006). Briefly, test chambers were filled with 25 mL of sample with 5 replicates per enclosure. Here again, RHW served as a control for cladoceran bioassays. One < 24 h old *D. magna* was introduced in each experimental unit and then fed daily 600 μL of a mixture of *Pseudokirchneriella subcapitata* (UTEX, Austin, TX, USA) and cereal grass media (ScholAR Chemistry, West Henrietta, NY, USA; (Hemming et al. 2002; Knight and Waller 1992).

pH Toxicity Identification

A pH adjustment study was performed on day 21 of the bloom development experiment following standard methods of pH adjustment for toxicity identification evaluations (TIE; (*Methods for aquatic toxicity identification evaluations: Phase i toxicity characterization procedures* 1991). TIEs are used to identify causative toxicants / toxins in freshwater and marine water bodies when ambient toxicity (e.g., fish kills) is observed. pH adjustment TIEs are employed to determine if the potency of substances causing toxicity in surface waters are influenced by pH.

Enclosure samples were collected and stored as described above. Three 500 mL aliquots were taken from each enclosure sample; one was adjusted to pH 7, one to pH 8.5, and one was left at ambient pH of the various enclosure treatment levels (i.e., 7, 7.5 or ambient (~8.5)). The pH adjustments were attained using either 0.1 N HCl (Fisher Scientific, ACS Grade) or 0.1 N NaOH (VWR International) and pH was determined using a standard bench-top pH meter (USEPA 1991). pH manipulation studies were performed in 100 ml glass beakers filled with 80 ml of test solution, and mortality to fish observed over 48 hr. To ensure that pH adjustments were not responsible for any observed mortality, RHW controls were subjected to the same pH adjustments.

Statistical Analysis

Repeated-measures ANOVAs were used to examine influences of experimental design on *P. parvum* cell densities, chl-*a* levels, and zooplankton biomass (SPSS 19.0). One-way ANOVAs were used to examine *D. magna* reproduction responses to the treatment levels, because sublethal *D. magna* bioassays were only performed at T21 for both experiments (SPSS 19.0). LC_{50} (% of ambient sample) values were estimated for *P.*

promelas 48 hr acute toxicity bioassays using Probit or Trimmed Spearman Karber statistical programs, as appropriate (US EPA 2002). One-way ANOVA with Tukey HSD post-hoc tests was used to examine influences of pH adjustment during the TIE study on LC_{50} values of samples modified to pH 7 or 8.5 from ambient pH (SigmaPlot 11.0).

Results

Pre-Bloom and Bloom Development Conditions in Lake Granbury

Prymnesium parvum cell densities in the cove during our first experiment were 0.21×10^6, 0.76×10^6, 0.67×10^6, and 0.42×10^6 cells/L at T0, T7, T14, and T21, respectively (Hayden et al., 2012). No acute toxicity to *P. promelas* was observed in the cove at any point during the first experiment. Because cell densities did not exceed a designated bloom density level of 10×10^6 cells/L and ambient toxicity was not observed, we refer to this period as pre-bloom (Hayden et al. 2012). During our second experiment, *P. parvum* cell densities in the cove were 1.1×10^6, 2.6×10^6, 0.46×10^6, and 0.08×10^6 cells/L at T0, T7, T14, and T21, respectively. Although cell densities did not exceed a designated bloom density threshold of 10×10^6 cells/L, acute toxicity to *P. promelas* was observed by T14 with a 48 hr LC_{50} value of 77.5% of ambient cove water. Consequently, we refer to this period as bloom development (Hayden et al. 2012).

Prymnesium parvum *Cell Densities*

In both experiments, observed pH values in enclosures, based on discrete (Table 1) and continuous (Table 2) measures, were consistent with targeted pH treatment levels. During the pre-bloom experiment, *P. parvum* cell density exceeded the bloom threshold in the untreated control enclosures (pH ~8.5), but only on day 21 (Figure 1A). In fact, *P.*

parvum growth was significantly ($p<0.05$) inhibited by both pH treatment levels such that cell densities did not exceed bloom thresholds (Figure 1A). In the bloom development experiment, *P. parvum* cell growth also exceeded the bloom threshold concentration (Figure 1b) as early as study day 7 with *P. parvum* cell density continuing to increase throughout the 21 d study period (Figure 1B). Similar to the pre-bloom experiment, *P. parvum* growth was also significantly ($p < 0.05$) inhibited at the pH 7 and pH 7.5 treatment levels such that population densities never reached the bloom threshold (Figure 1B).

Table 1. Median (± SE), maximum, and minimum pH values for ambient, low, and intermediate pH treatment levels monitored semi-weekly throughout the pre-bloom and bloom development periods.

Period	Treatment	Median pH (±SE)	Maximum pH	Minimum pH
Pre-bloom	Ambient pH	8.4 ± 0.05	8.8	8.2
	Low pH	7.0 ± 0.01	7.4	6.7
	Intermediate pH	7.4 ± 0.04	7.9	7.1
Bloom development	Ambient pH	8.6 ± 0.01	8.7	8.5
	Low pH	7.1 ± 0.04	7.6	6.5
	Intermediate pH	7.9 ± 0.03	8.8	7.2

Table 2. Median (± SD), maximum, and minimum pH values for ambient, low, and intermediate pH treatment levels monitored continuously at 15 min intervals throughout the pre-blooms and bloom development periods.

Period	Treatment	Median pH (±SD)	Maximum pH	Minimum pH	N
Pre-bloom	Ambient pH	8.5 ± 0.23	9.34	8.00	610
	Low pH	6.9 ± 0.26	7.53	6.65	606
	Intermediate pH	7.5 ± 0.09	7.68	7.43	330
Bloom development	Low pH	6.7 ± 0.29	7.32	6.27	1000
	Intermediate pH	7.5 ± 0.24	7.98	7.46	1000

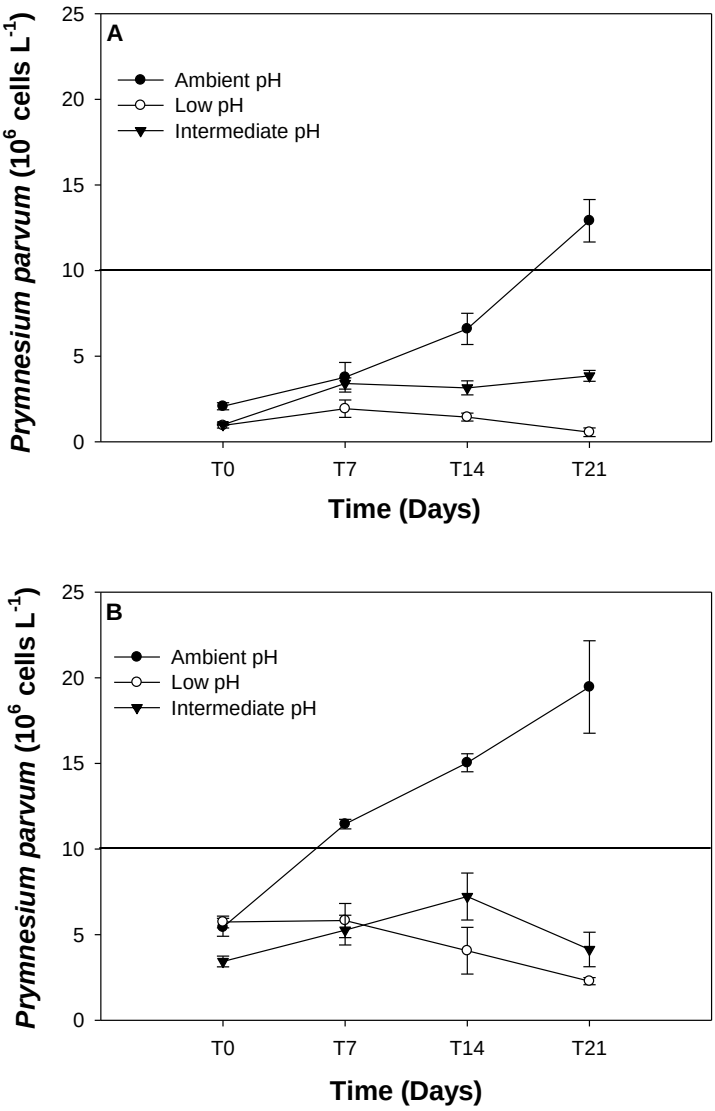

Figure 1. Mean *Prymnesium parvum* cell density (cells/L) (N=3; ±SD) through time (days) in low (7), intermediate (7.5), and high (ambient, ~8.5) pH treatment enclosures in experiments conducted during (A) pre-bloom and (B) bloom development periods. In both experiments, low and intermediate pH levels significantly reduced ($p<0.05$) *P. parvum* cell density compared to the higher ambient pH treatment level. The horizontal line indicates a *P. parvum* harmful bloom threshold of 10 x 10^6 cells/L.

Phytoplankton and Zooplankton Biomass

With the exception of *P. parvum* growth observations described above, pH manipulations did not affect other plankton. Chl-*a* levels were not significantly different ($p > 0.05$) among the pH 7, pH 7.5 and ambient treatment levels under pre-bloom or bloom development conditions (Figure 2A, B). Similarly, zooplankton biomass was not significantly affected by pH treatments ($p > 0.05$; Figure 3A, B).

Acute Toxicity to Fish

In both experiments, acute toxicity of *P. parvum* to larval *P. promelas* was completely ameliorated by the pH 7 treatment level. Throughout the pre-bloom experiment, acute toxicity to fish was not observed at any treatment level until day 21 when the ambient enclosures became acutely toxic to *P. promelas*. On day 21, the mean 48 hr LC_{50} value for the ambient treatment level was 17%, whereas both the pH 7 and 7.5 treatment levels remained free from acute toxicity to fish (Figure 4A). In the bloom development experiment, acute toxicity to fish was observed in the ambient and pH 7.5 treatment levels as early as day 7 ($p < 0.05$); however, the pH 7 manipulated enclosures remained free from acute toxicity ($p > 0.05$) to fish throughout the 21 d study (Figure 4B).

SubLethal Toxicity to Cladocerans

At T21, *D. magna* fecundity was not significantly ($p > 0.05$) different among the pH treatment levels (7, 7.5) and the ambient (8.5) enclosures for the pre-bloom (Figure 5A) or bloom development (Figure 5B) experiments. For example, during the pre-bloom study, *D. magna* exposed to samples from the ambient pH (8.5) enclosures produced

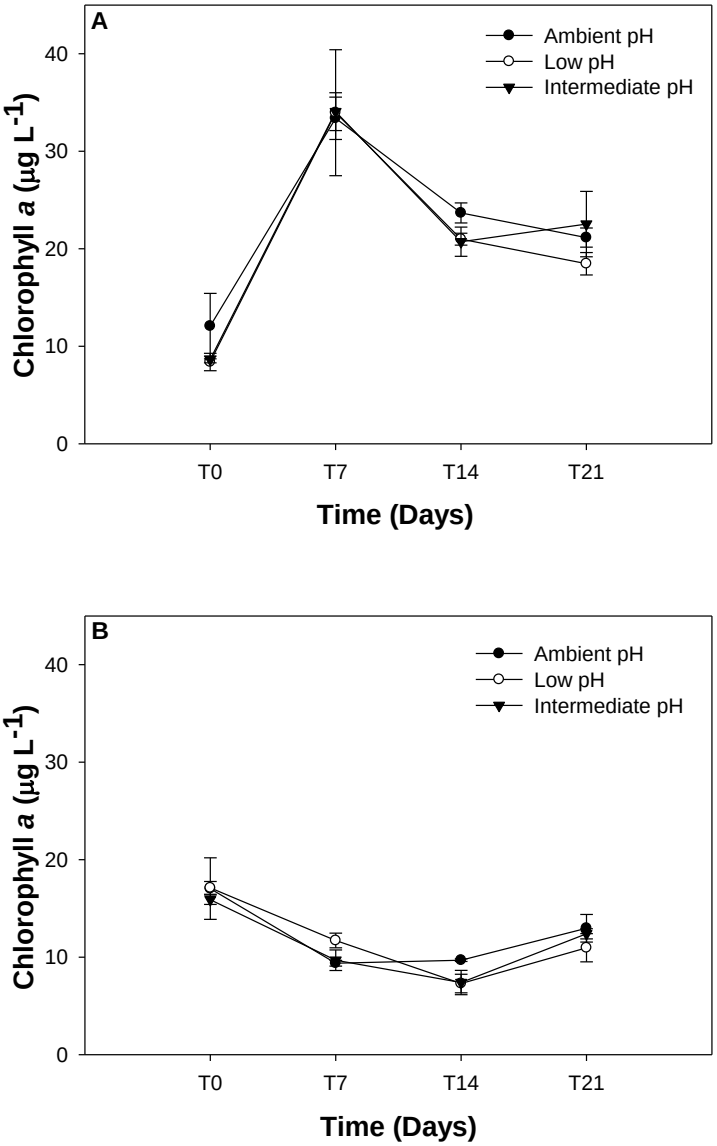

Figure 2. Mean Chlorophyll *a* (µg/L) (N=3; ±SD) through time (days) in low (7), intermediate (7.5), and high (ambient, ~8.5) pH treatment enclosures in experiments conducted during (A) pre-bloom and (B) bloom development periods. In both experiments, total phytoplankton biomass was not significantly ($p>0.05$) affected by low or intermediate pH treatment levels compared to higher ambient pH enclosures.

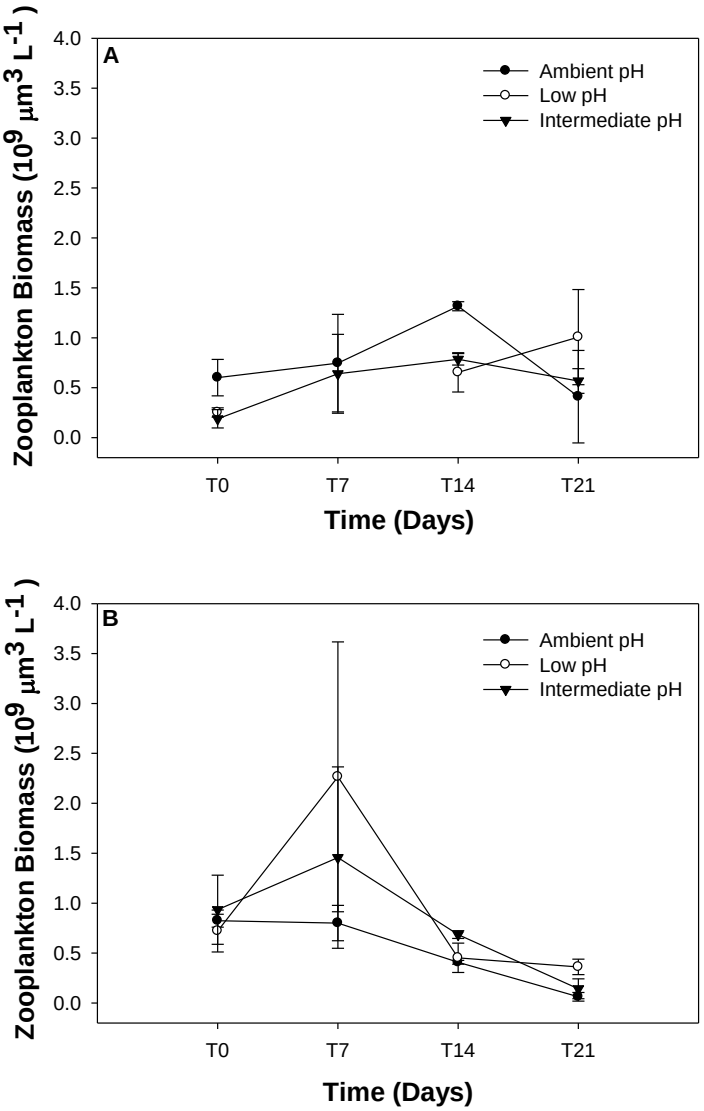

Figure 3. Mean total zooplankton biomass (µm^3/L) (N=3; ±SD) through time (days) in low (7), intermediate (7.5), and high (ambient, ~8.5) pH treatment enclosures in experiments conducted during (A) pre-bloom and (B) bloom development periods. In both experiments, total zooplankton biomass was not significantly ($p>0.05$) affected by low or intermediate pH treatment levels compared to higher ambient pH enclosures.

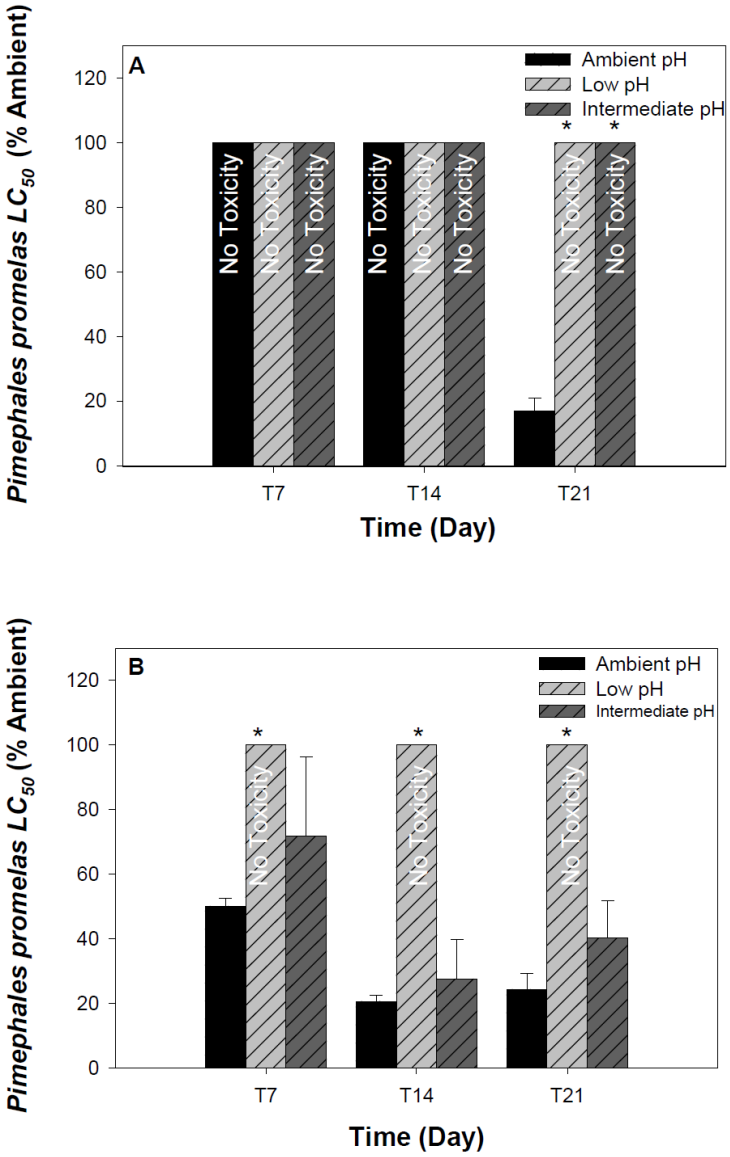

Figure 4. Mean *Pimephales promelas* 48 h LC_{50} (% Ambient) (N=3; ±SD) through time (days) in low (7), intermediate (7.5), and high (ambient, ~8.5) pH treatment enclosures in experiments conducted during (A) pre-bloom and (B) bloom development periods. * denotes pH treatment level with significantly lower ($p<0.05$) toxicity than ambient enclosures.

more neonates (54 mean neonates/female) than those exposed to the pH 7 (47 mean neonates/female) and 7.5 (33 mean neonates/female) treatment levels. For comparison, RHW laboratory control organisms produced 51 (±14.1) mean neonates/female, which indicates enclosures were not sublethally toxic to *D. magna* during the first study. However, *D. magna* exposed to the pH 7 treatment level under bloom development conditions produced twice as many neonates (e.g., 35 mean neonates/female) compared to the pH 7.5 (17 mean neonates /female) and ambient pH 8.5 (17 mean neonates/female) treatment levels. Such values for the pH 7 enclosures were consistent with reproduction of RHW laboratory controls (e.g., 37 (±7.7) mean neonates/female).

pH Toxicity Identification

The pH adjustment study confirmed that pH influenced the potency of toxins associated with blooms of *P. parvum*. Specifically, acute toxicity (LC_{50}) to *P. promelas* was completely ameliorated ($p < 0.05$) when acutely toxic enclosure samples from the pH 7.5 and ambient pH (8.5) treatment levels were experimentally reduced to pH 7 in the laboratory. Conversely, samples from pH 7 enclosures remained free of acute toxicity to fish even when pH was increased to 8.5, suggesting that *P. parvum* toxins were not present at acutely toxic levels in these enclosures (Figure 6).

Discussion

Though a number of chemical, physical and biological factors influence the development of *P. parvum* blooms in inland waters, pH was previously shown to influence the potency of *P. parvum* (Shilo and Aschner 1953; Ulitzur and Shilo 1964; Valenti et al. 2010). Here we report for the first time that pH appears to govern *P. parvum*

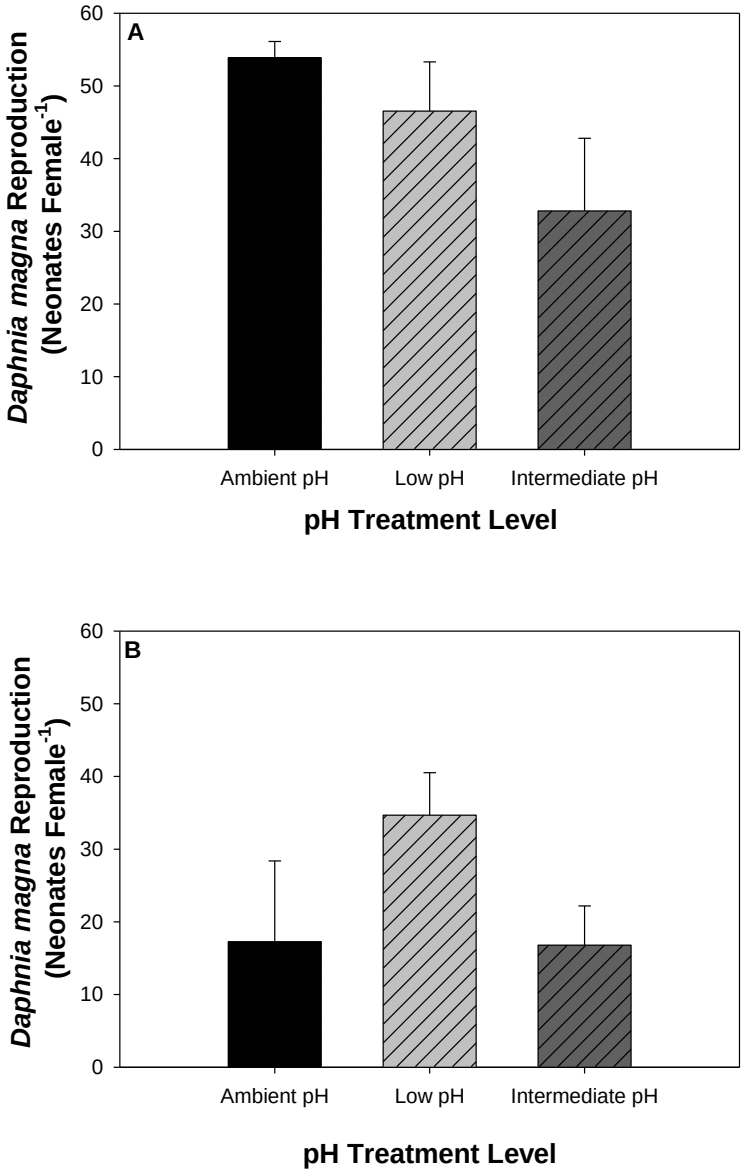

Figure 5. Mean *Daphnia magna* reproduction (neonates/female) (±SE) through time (days) in low (7), intermediate (7.5), and high (ambient, ~8.5) pH treatment enclosures in experiments conducted during (a) pre-bloom and (b) bloom development periods. Number of neonates produced was not significantly affected by pH treatment level in either experiment ($p>0.05$).

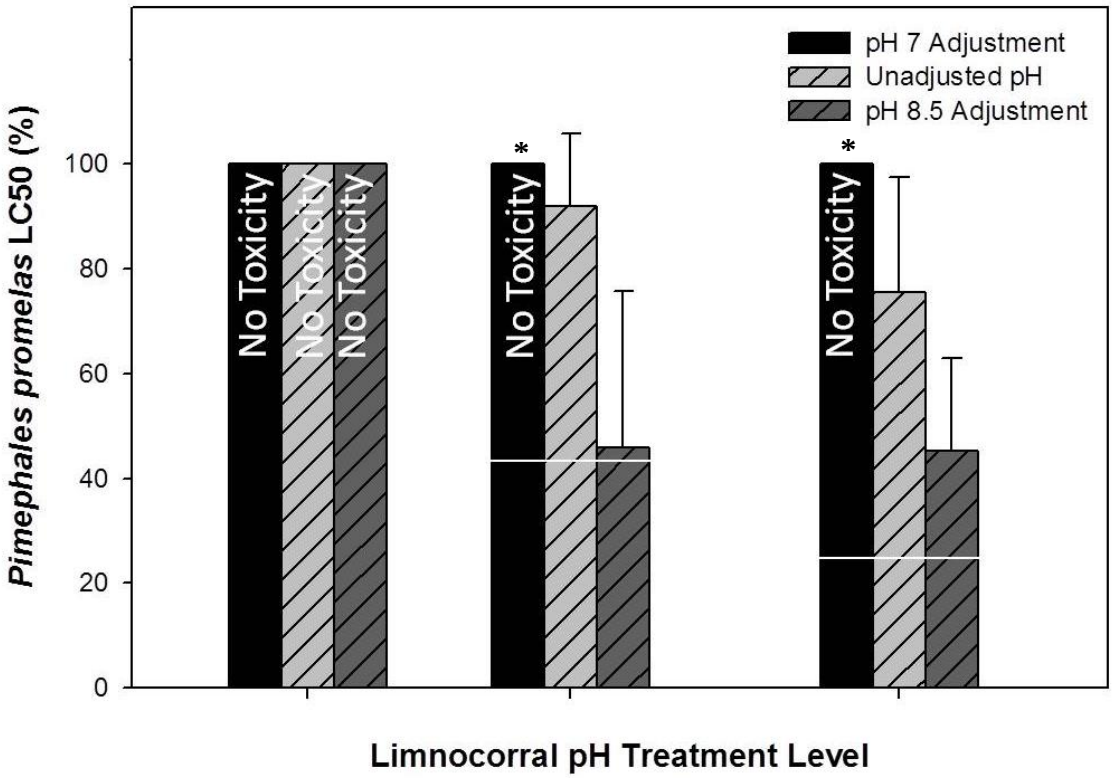

Figure 6. Mean *Pimephales promelas* 48 h LC_{50} values (% ambient) (N = 3; ±SD) in pH 7, 7.5, and ambient (~8.5) treatment levels from the bloom development study following a toxicity identification evaluation (TIE) pH adjustment to pH 7, 8.5 or unadjusted. * indicates pH treatment levels with significantly lower ($p < 0.05$) toxicity than the ambient treatment level. White reference lines indicate mean LC_{50} value of pH treatment level enclosure samples before initiation of TIE pH adjustment.

bloom formation and associated toxicity to aquatic life in inland waters. In the present studies examining pre-bloom and bloom development conditions, neutral pH levels preempted *P. parvum* bloom development, resulting in no ambient toxicity. However, higher pH (~8.5) resulted in bloom formation and ambient toxicity during both pre-bloom and bloom development periods. During both experiments, reducing pH to 7 and 7.5, which did not adversely affect total phytoplankton and zooplankton biomass, significantly suppressed *P. parvum* growth such that densities in these treatment levels

never exceeded the bloom threshold for *P. parvum*. However, pH 8.5 resulted in exponential *P. parvum* growth that surpassed the bloom threshold in both pre-bloom and bloom development experiments.

Adverse effects of *P. parvum* have been documented for a number of aquatic organisms (Brooks et al. 2010), including allelopathic interactions affecting growth of prey and competitor phytoplankton (Barreiro et al. 2005; Olli and Trunov 2007) and zooplankton grazers (Granéli and Johansson 2003a; Larsen et al. 1998; Uronen et al. 2007). *Prymnesium parvum* blooms can alter the structure of plankton communities (Fistarol et al. 2003; Schwierzke et al. 2010) and the function of the microbial loop by increasing dissolved organic carbon available to the bacterial community (Uronen et al. 2007). As a result, *P. parvum* blooms may have lasting repercussions on food webs of inland water bodies. In the present study, *D. magna* reproduction was less sensitive to *P. parvum* than *P. promelas* mortality responses. Such observations are consistent with previous reports in inland waters (Roelke et al. 2007). For example, Brooks et al. (2010) identified that acute fish mortality was more sensitive than chronic, sublethal responses of rotifers and cladocerans to *P. parvum*. Similarly, Valenti et al. (2010) demonstrated that cladoceran reproduction was less sensitive than fish mortality.

Observations in the present study are consistent with previous work identifying that low pH reduces *P. parvum* related toxicity to aquatic organisms across a range of salinities (McLaughlin 1958; Shilo and Aschner 1953; Ulitzur and Shilo 1964; Valenti et al. 2010). Early studies of pH and *P. parvum* were conducted under high salinity conditions associated with marine or estuarine systems (Shilo and Aschner 1953; Ulitzur and Shilo 1964) rather than the low salinities of Texas reservoirs. Valenti et al. (2010)

used aspects of Toxicity Identification Evaluation Methods (*Methods for aquatic toxicity identification evaluations: Phase i toxicity characterization procedures* 1991) to examine the influence of pH on aquatic toxicity of samples collected from Texas reservoirs experiencing fish kills attributed to *P. parvum*. In addition, Valenti et al. (2010) performed laboratory experiments with *P. parvum* cultures to define pH influences on aquatic toxicity. In both cases, the magnitude of *P. parvum* associated toxicity to fish and cladocerans was enhanced as pH increased to 8.5 and ameliorated when pH decreased to 6.5 (Valenti et al. 2010). In an effort to interpret such observations, Valenti et al. (2010) hypothesized that toxins produced by *P. parvum* behave like weak bases and become more nonionized, bioavailable, and toxic with increasing pH. Observations in the present study are also consistent with more recent, groundbreaking findings from Bertin et al. (2012a, b) who provided a novel report of a new class of toxins, fatty acid amides, produced by *P. parvum* (Bertin et al. 2012a), and demonstrated that increasing pH increased toxicity of two of these fatty acid amides, oleamide and linoleamide, to rainbow trout gill cells (Bertin et al. 2012b). Clearly, additional toxicological research is warranted on impacts of fatty acid amides associated with *P. parvum* blooms in inland waters.

Bertin et al. (2012b) further proposed that increased ion pairing of divalent cations to fatty acid amides occurs at elevated pH, which can result in increased bioavailability. Here again, observations from the pH adjustment portion of the present study are consistent with findings from previous reports (Valenti et al. 2010; Bertin et al. 2012b). Toxicological observations *in vivo* by Valenti et al. (2010) and *in vitro* by Bertin et al. (2012b) using pH adjustment approaches similar to those employed in the current

study consistently observed increased toxicity at elevated pH. Henrikson et al. (2010) reported that various fatty acids are produced by *P. parvum*, and further speculated that these molecules were responsible for aquatic toxicity associated with *P. parvum* blooms in inland waters. Because acute toxicity to fish in the present study was completely ameliorated in enclosure samples from the pH 7.5 and ambient pH (8.5) treatment levels following reduction to pH 7, it is unlikely that fatty acids, which are more nonionized, bioavailable, and toxic at lower pH, were responsible for pH dependent toxicological observations. Thus, it remains clear that pH may influence the potency of toxins released by *P. parvum* in inland waters (Valenti et al. 2010). Such observations are important because spatiotemporal pH dynamics in inland waters influences site-specific toxicity and risks of naturally occurring and anthropogenic contaminants (Brooks et al. 2011b; Valenti et al. 2011).

Based on observations in the present study, it appears likely that site-specific pH can influence the development of *P. parvum* blooms by altering toxin(s) potency. In Texas, *P. parvum* blooms are not observed in impoundments with pH levels of circumneutral pH. For example, though *P. parvum* is present in surface waters of east Texas, fish kills attributed to *P. parvum* have not been reported (Southard et al., 2010). In fact, surface water pH levels in many parts of east Texas where *P. parvum* is found, but does not bloom, are generally low due to a wet climate, increased vegetation and lower soil pH levels. Conversely, pH of impoundments in central and west Texas, where *P. parvum* blooms are observed, generally exceed pH 8 due to a dry climate, less vegetation, and limestone bedrock (Valenti et al. 2010). Further, it may be possible that greater toxicity exists during the day when pH is generally higher due to photosynthesis,

and lower toxicity observed at night when pH decreases due to continued respiration in the absence of photosynthesis (Valenti et al. 2011). Relationships among factors influencing spatiotemporal surface water pH dynamics, particularly in response to anthropogenic activities and climatic changes (Brooks et al. 2011b) and bioaccumulation and toxicity of toxins produced by *P. parvum* require future study.

From an evolutionary perspective, producing and releasing exotoxins provide important competitive advantages to *P. parvum* because they can serve as deterrents to grazers, increase heterotrophic feeding efficiency, and suppress growth of phytoplankton competitors (Skovgaard and Hansen 2003; Tillmann 1998, 2003). Thus, it has been largely believed that these allelopathic interactions function as a mechanism of *P. parvum* bloom formation and that blooms might not occur without this strategy (Turner et al. 1998). In contrast, Jonsson et al. (2009) suggested that toxin production is not a mechanism of *P. parvum* bloom formation since cell-cell interactions necessary for allelopathy to occur may be too few and far between under the low cell densities associated with pre-bloom conditions. Jonsson et al. (2009) does concede, however, that allelopathy may serve to prolong a bloom once formed by suppressing the growth of other algal species and by immobilizing potential grazers. Based on experimental conditions in this study, *P. parvum* bloom formation appeared dependent on pH enhancement of toxin potency. Future studies are clearly necessary to define the role of pH on toxin activities during development of *P. parvum* blooms.

Preemptive environmental management strategies for *P. parvum* blooms are challenging, and a number of approaches have been examined (Grover et al. 2007; Hagström et al. 2010; Kurten et al. 2010). Though McLaughlin (1958) suggested

reducing pH as a bloom management and mitigation strategy for *P. parvum* in brackish systems over 60 years ago, pH adjustment has not yet been formally explored as a *P. parvum* bloom mitigation approach in inland waters. Whereas lowering the pH of entire lakes or reservoirs would clearly be impractical and unwise, it may be possible to adjust the pH of targeted regions of affected systems in an effort to provide refuge for fish and other aquatic organisms from devastating *P. parvum* blooms. Such considerations are not trivial; impacts of acidification due to atmospheric deposition on lentic systems have received much study. For example, acidification of surface waters can reduce species abundance and richness (Havens and Heath 1989) and favor the proliferation of smaller zooplankton species (Havens and Hanazato 1993). Moderately adjusting surface waters of enclosures in Lake Granbury to neutral pH, however, did not significantly affect total phytoplankton or zooplankton biomass under either pre-bloom or bloom development conditions. Because *P. parvum* blooms appear to originate in coves that experience lower inflows than the main stems of reservoirs (Grover et al. 2011; Roelke et al. 2010b), coves may represent more reasonable management units to create refuge habitats and potentially preempt bloom formation.

CHAPTER TWO

Influence of Total Dissolved Solids on *Prymnesium parvum* Growth and Toxicity

Introduction

In recent years, the range of *Prymnesium parvum* harmful algal blooms (HABs) has expanded past coastal marine and estuarine ecosystems and now include inland waters as well (Moestrup 1994, Edvardsen and Paasche 1998, Lundholm and Moestrup 2006). In addition to the increased range of distribution of these HABs, an increase in bloom frequency, intensity, and duration (Hallegraeff 1993) has been observed over the past several decades. The influences of anthropogenic practices on water quality and climate patterns appear to be pronounced enough, in some cases, to produce conditions favoring *P. parvum* growth and toxin production (Brooks et al. 2011, Roelke et al. 2012) and; therefore, may be responsible for the worsening global impact of *P. parvum*. Since first linked to fish kills in the Pecos River of Texas in 1985, the range of *P. parvum* HABs has now expanded to include at least 18 states within the U.S. (Sager et al. 2008, Roelke et al. 2011). In particular, it may be possible that natural resource extraction activities contribute to *P. parvum* expansion in inland waters of the U.S.

Relationships among factors influencing *P. parvum* HAB development are complex (Prosser et. al 2012). Site-specific nutrient availability (Grover et al. 2007, Roelke et al. 2007, Johansson and Granéli 1999, Granéli and Johansson 2003), pH (Shilo and Aschner 1953, Ulitzur and Shilo 1964, Valenti et al. 2010, Prosser et al. 2012), temperature (Baker et al. 2007, Grover et al. 2007, Baker et al. 2009), sunlight intensity (Parnas et al. 1962, Reich and Parnas 1962, Rahat and Jahn 1965, James et al. 2011), and

inflow events (Roelke et al. 2010b, Roelke et al. 2011, Schwierzke-wade et al. 2011) influence *P. parvum* HAB dynamics in inland waters. In addition, site-specific salinity thresholds are necessary for *P. parvum* HABs to occur (Baker et al. 2007, Baker et al. 2009, Roelke et al. 2011). Further, because *P. parvum* is a euryhaline species, salinity appears to be a critical factor in determining the expansion of these HABs further inland. Further, it has been suggested that ionic composition of surface waters can increase the bioavailability of *P. parvum* toxins resulting in higher toxicity (Bertin et al. 2012a; Manning and La Claire 2010; Ulitzur and Shilo 1966). Whereas the role of salinity in influencing *P. parvum* HAB dynamics is relatively well understood, the effect of site-specific ionic constituents on *P. parvum* growth and toxicity is largely unknown.

The most recent expansion of *P. parvum* HABs in the U. S. was documented during a 2009 fish kill in Dunkard Creek, located along the Pennsylvania/West Virginia border (Renner 2009). This HAB was responsible for widespread mortalities including at least 18 different fish species and 14 species of freshwater mussels (Renner 2009; Wozniak 2011). The Dunkard Creek area and others along the Appalachian Mountain Range are rich in Marcellus Shale stores making these areas prime for natural resource extraction. An emerging concern for *P. parvum* is that wastes from mining practices can increase TDS levels in aquatic systems, thus impairing surface waters when *P. parvum* blooms occur (Brooks et al. 2011a). However, it remains critical to develop an understanding of the site-specific influences of ionic constituents contributing to increased TDS levels on *P. parvum* HABs (Brooks et al. 2011a). It may also be possible that increased TDS levels resulting from disposal of produced waters during hydraulic

fracturing may contribute to *P. parvum* growth and toxicity if not properly managed (Brooks et al. 2011a).

Natural gas in the Marcellus Shale region is found mainly in the pores of shale and is extracted via either naturally or artificially created fractures in the shale (Kargbo et al. 2010). Seismic and volcanic activities naturally form fractures along fault gradients that allow for easy access to underground gas reservoirs. In areas such as the Marcellus Shale Region (240,000 km^2) located along the Appalachian Mountain Range, large stores of natural gas are present yet inaccessible due to limited seismic and volcanic activities (Kargbo et al. 2010). As a result, an extraction technique known as hydraulic fracturing is often used to mine this valuable resource. Wells are first drilled to the shale layer (~1.6 km deep in the Marcellus Shale Region) and lined with cement (Kargbo et al. 2010). Next, highly pressurized fluid is used to create and enlarge fractures in the shale by injecting highly pressurized fluid into the well. Fracking fluid consists of a water and sand mixture that may be supplemented with chemicals such as surfactants, acids, biocides, and diesel fuel required to maintain the fractures in the shale throughout the mining process (EPA 2004; Kargbo et al. 2010). Colborn et al. (2010) compiled a list of 632 chemicals often used in natural gas mining; long-term impacts of these additives on human and ecosystem health are widely unknown. Once used, waste fluid contains initial chemical additives and increased concentrations of ionic constituents (e.g., Na^+, Cl^-), heavy metals, and radionuclides leached from the bedrock (Kargbo et al. 2010; Soeder and Kappel 2009). It has been suggested that hydraulic fracturing procedures may use up to 3 million gallons (Harper 2008) of fracking fluid per gas well, 80% of which may be unrecoverable (Karbgo et al. 2010). When recovered, the wastewater, with typical TDS

levels between 6000 and 225000 mg/L (Wozniak 2011), is disposed of by either evaporation or is processed by wastewater treatment plants (WWTP) (Karbgo et al 2010). In other regions of the US, produced waters are disposed by deep well injection. However, fracturing wastewater that has been treated by WWTPs still may contaminate surface waters because conventional WWTPs are typically not equipped for TDS removal and, as a result, may release effluent with TDS levels above the limit of 500 mg/L set by USEPA for drinking water (Karbgo et al 2010, Brooks et al 2011a, Wozniak 2011), subsequently impacting aquatic ecosystems and contaminating drinking water sources (Osborn et al. 2011). Other concerns of hydraulic fracturing include the potential destruction or contamination of underground aquifers (Karbgo et al 2010) from the unrecovered waste, the triggering of earthquakes (Karbgo et al 2010), further stress on water supplies in drought prone areas (Soeder and Kappel 2009, Karbgo et al 2010, Wozniak 2011), hazards of poorly understood chemicals in produced waters to human health (Colborn et al. 2011), and decreased biodiversity as organisms less sensitive to high TDS levels may be favored (Wozniak 2011).

In addition to direct TDS effects on ecological integrity, indirect effects of TDS modified instream conditions of Dunkard Creek, PA/WV by increasing TDS levels (e.g., Na^+, Cl^-, Renner 2009), which, in addition to other conditions (e.g., low instream flows (Brooks et al. 2011a)), influenced development of a *P. parvum* HAB. Such observations are important because natural resource extraction efforts are projected to increase in the US, yet the potential influences of these activities on *P. parvum* bloom expansion and toxicity in inland waters remain undefined. The purpose of this study was to determine whether TDS, composed of concentrations and ratios of major ionic constituents

associated with hydraulic fracturing activities in the Marcellus Shale Region, influence *P. parvum* growth and toxicity.

Methods and Materials

Prymnesium parvum *Cultures*

A starter culture of *P. parvum* was obtained from UTEX and was inoculated in sterilized artificial seawater (ASW) media with a salinity of 2.4 psu (Table B1.; Appendix B). Trace metals, F2 nutrients, and 0.2 µm filtered vitamin stock solutions were added to the culture at a concentration of 1 mL stock per 1 L ASW (Baker et al. 2007, Baker et al. 2009, Brooks et al. 2010, Valenti et al. 2010, James et al. 2011; Table B2.; Appendix B). The culture was maintained at 20 ˚C with a 12:12 hr light/dark cycle (Model 2015, VWR International, Cornelius, OR), and hand swirled daily. Every two days, a 5 mL sample of the *P. parvum* culture was preserved using Lugol's solution and the cell density was enumerated via a hemocytometer and light microscopy. Experiments were initiated once the culture reached a density of 10×10^6 cells/L, a level previously identified as the HAB threshold in inland waters of Texas (Roelke et al 2011).

Experimental Design

An experiment was conducted to address the primary objective of this study. Artificial seawater was used as the base for all TDS treatment levels to ensure the presence of trace elements necessary for *P. parvum* survival. Environmentally relevant concentrations of NaCl and $CaCl_2$ were added to ASW to create TDS treatment levels (130, 1000, 4000, 8000 mg/L) (Table B3.; Appendix B). Stoichiometry of dominant ions associated with these TDS treatment levels were chosen based on U.S. EPA data for

produced waters from HF during Marcellus Shale exploration (L Reynolds, personal communication). Artificial seawater of 2.4 psu, previously studied extensively by our lab (Brooks et al. 2010, Valenti et al. 2010, James et al. 2011), served as a control (Table B1.; Appendix B). Each treatment level contained trace metals and 0.2 µm filtered vitamin solutions at a concentration of 1 mL/L (Table B2.; Appendix B). Each solution was mixed thoroughly and a YSI Conductivity meter (Model 30, Yellow Springs Instruments, Yellow Springs, OH, USA) was used to measure the conductivity (µS) and salinity (psu) of each treatment level (Table 3).

Table 3. Salinity (psu) and conductivity (µS) of HF and control (2400 mg/L) stock solutions prior to inoculation of *Prymnesium parvum.*

Nutrient Regime	TDS (mg/L)	Salinity (psu)	Conductivity (µS)
F2	130	0.1	292.5
	1000	1.1	2072
	2400	2.4	-
	4000	4.2	7290
	8000	8.6	14140
F4	130	0.1	279.9
	1000	1.1	2080
	2400	2.4	-
	4000	4.3	7390
	8000	8.5	13970
F8	130	0.1	277.4
	1000	1.1	2078
	2400	2.4	-
	4000	4.3	7360
	8000	8.5	14060

Three different nutrient concentrations (F2, F4, F8 nutrients; Guillard 1975) were employed to evaluate effects of TDS treatment levels on *P. parvum* growth and toxicity across a nutrient gradient. For each nutrient solution, 2.4 psu ASW was also included as

a control. Three replicate 1 L flasks were used as experimental units for each treatment level. When the cell density of the *P. parvum* stock culture surpassed a HAB threshold of 10×10^6 cells/L (Roelke et al. 2011), experimental units were inoculated to an initial density of 100 cells/mL. Prior to inoculation, a 48 hr acute toxicity bioassay was performed on the *P. parvum* stock culture to ensure that each experimental unit was inoculated with a culture of *P. parvum* producing toxicity to juvenile *Pimephales promelas* (US EPA 2002). Bioassays conducted with the *P. parvum* starter culture consisted of both whole culture and cell-free samples. Cell-free filtrate was obtained by gravitational filtration of each sample through a 1.2 μm pore glass microfiber filter as previously described (Valenti et al. 2010).

The experiment was performed in a growth chamber at 20 °C with a 12:12 hr light/dark cycle (Model ISO 9001, Nor-Lake, Inc., Hudson, WI). Each flask was swirled daily and their position in the growth chamber was randomized every other day. Every fourth day, *P. parvum* cell densities were enumerated microscopically from each experimental unit following preservation with Lugol's solution (Wetzel and Likens 1991). The study was concluded once *P. parvum* growth reached stationary growth phase. At study termination, a 48 hr acute toxicity bioassay with juvenile *P. promelas* (US EPA 2002) was conducted to determine whether treatment levels resulted in acute toxicity to fish. These bioassays were conducted with whole cultures in darkness to preserve *P. parvum* toxicity because previous studies by our research team demonstrated that light can ameliorate *P. parvum* acute toxicity to fish (James et al. 2011). Mortality was assessed at 0.5, 1, 2, 4, 8, 12, 24, and 48 hr time periods to identify the onset of acute toxicity.

Nutrient Analysis

At study initiation, dissolved N and P concentrations were analyzed for each TDS and control treatment solution according to standard methods using a flow-injection auto-analyzer (Lachat QuikChem 8500 and Series 520 XYZ Autosampler; APHA (Clesceri et al. 1998). Dissolved N and P concentrations (µg/L) for each stock solution are reported in Table B4. (Appendix B).

Statistical Analysis

Prymnesium parvum specific growth rates were calculated for experimental units using the equation

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

where r is the growth rate (µ/day), N_d is the number of organisms at the beginning of the steady growth state, N_0 is the number of organisms at study initiation, and t is the time (days) to reach steady state growth. The beginning of steady state was determined as the time at which the maximum *P. parvum* density was reached and followed by a general decline.

LC_{50} (%) values were estimated for all *P. promelas* acute toxicity bioassays at each time mortality was (0.5, 1, 2, 4, 8, 12, 24, 48 hrs) observed using Probit, Spearman Karber or graphical analysis by linear regression, depending on data characterisitics. LT_{50} (hr) values were also estimated for each experimental replicate based on mortality observations over 48 hrs. *Prymnesium parvum* cell densities, growth rates, and 48 hr LC_{50} and LT_{50} values were then compared using Analysis of Variance (One-Way ANOVA for normally distributed data; Kruskal-Wallace One-Way ANOVA on Ranks for non-

normally distributed data) and Dunnett's post-hoc test to examine whether TDS treatment levels significantly affected ($\alpha \leq 0.05$) response variables across the nutrient gradient (SigmaPlot 11.0).

Results

Prymnesium parvum *Growth*

F2 Nutrients. Prymnesium parvum growth was stimulated in a dose dependent manner by TDS treatment levels under an F2 nutrient regime. Because no growth was observed in the 130 mg/L treatment, it was not included in statistical analyses. Mean cell densities for each TDS treatment level, control, 1000, 4000, and 8000 mg/L, were observed at 1.5×10^5, 1.3×10^3, 6×10^5, and 12×10^5 cells/mL, respectively, on day 54 (Figure 7). *Prymnesium parvum* cell densities were significantly different at T54 among the treatment levels ($P<0.05$). Post-hoc analysis revealed that *P. parvum* cell densities in controls (2.4 psu) were only significantly lower than those in the 8000 mg/L treatment level ($P<0.05$; Figure 7).

F4 Nutrients. Similar to the results of the F2 nutrient regime, *P. parvum* growth was stimulated in a dose dependent manner by TDS treatment levels under an F4 nutrient regime. Also similar to F2 observations, no growth was observed in the 130 mg/L treatment level. Mean cell densities of each TDS treatment level, control, 1000, 4000 and 8000 mg/L, were observed at 8.2×10^5, 2.4×10^3, 1.5×10^5, and 9.2×10^5 cells/mL, respectively, on day 54 (Figure 8). *Prymnesium parvum* cell densities at T54 were not significantly different among the treatment levels ($P=0.064$).

F8 Nutrients. Consistent with observations of F2 and F4 nutrient conditions, *P. parvum* growth was similarly stimulated in a dose dependent manner under an F8 nutrient

regime, except again for no growth at the 130 mg/L TDS treatment level. Mean cell densities of each treatment level, control, 1000, 4000 and 8000 mg/L, were detected at 1.8 x 10^5, 5.4 x 10^3, 5.8 x 10^5, and 5.1 x 10^5 cells/mL, respectively, on day 54 (Figure 9). *Prymnesium parvum* cell densities at T54 were significantly different ($P<0.016$) among the treatment levels; however, post-hoc analysis showed that *P. parvum* cell densities in the control treatments were not significantly different than any treatment levels ($P>0.05$).

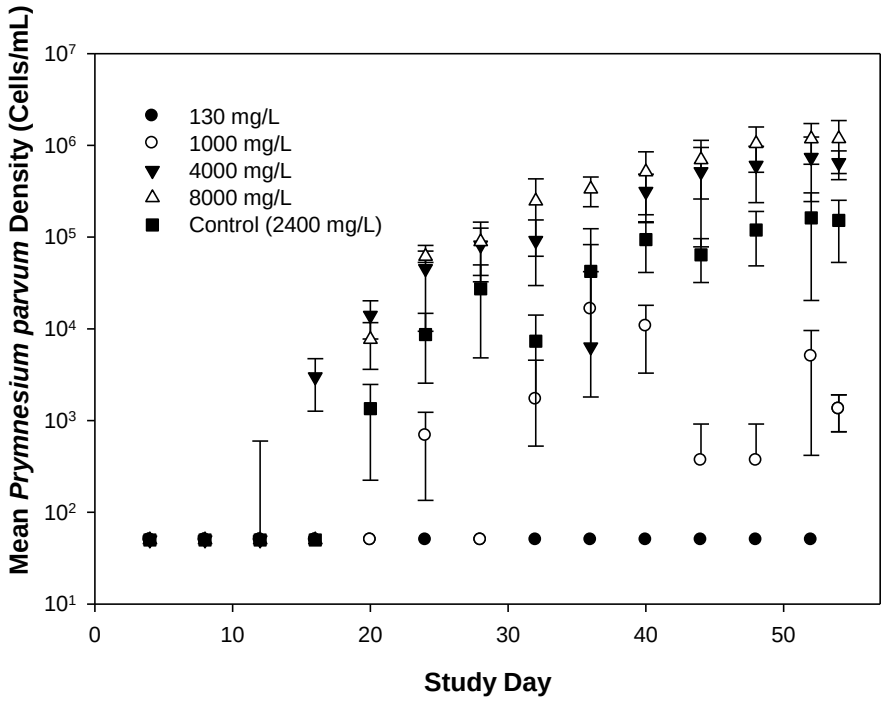

Figure 7. Mean *Prymnesium parvum* cell density (cells/mL) (±SD, n=3) over a 54 day study in 130, 1000, 4000, and 8000 mg/L Total Dissolved Solids treatment levels and controls (2400 mg/L) with F2 nutrients.

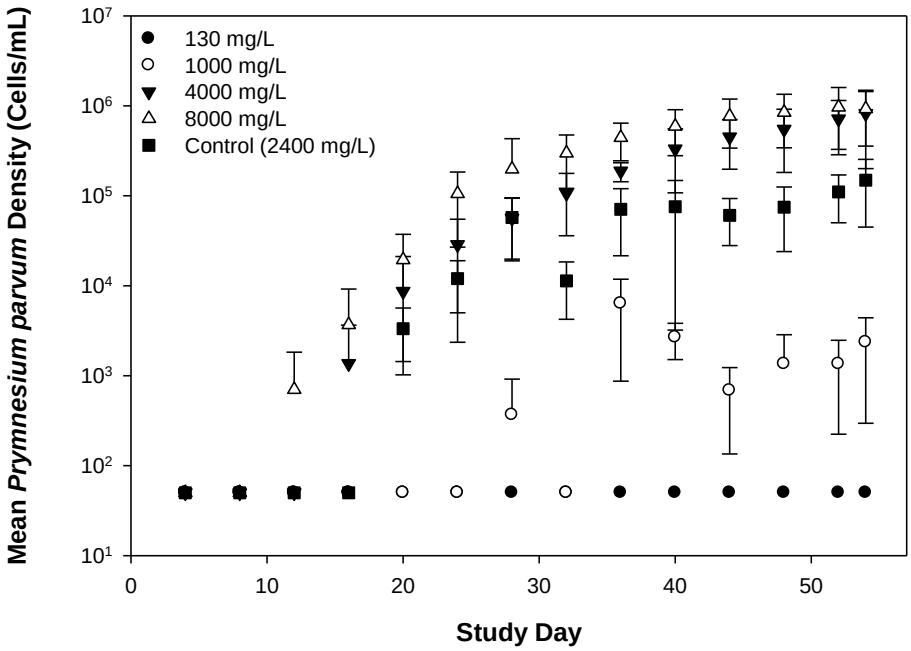

Figure 8. Mean *Prymnesium parvum* cell density (cells/mL) (±SD, n=3) over a 54 day study in 130, 1000, 4000, and 8000 mg/L Total Dissolved Solids treatment levels and controls (2400 mg/L) with F4 nutrients.

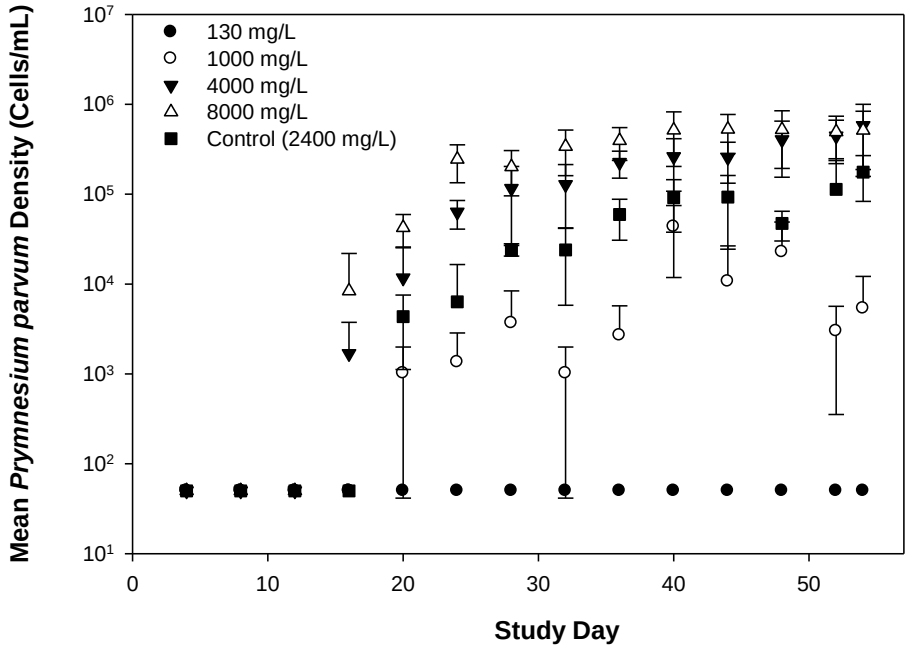

Figure 9. Mean *Prymnesium parvum* cell density (cells/mL) (±SD, n=3) over a 54 day study in 130, 1000, 4000, and 8000 mg/L Total Dissolved Solids treatment levels and controls (2400 mg/L) with F8 nutrients.

Specific Growth Rates

In addition to cell densities, growth rate responses to TDS treatment levels across the nutrient gradient were examined. Consistent with observations of cell densities, *P. parvum* growth rates through time were stimulated in a dose dependent manner by TDS treatment levels for each nutrient gradient (Appendix Table B5, Figure 10). For the F2 and F8 nutrient regimes, a significant difference was not observed in *P. parvum* growth rates between the treatment levels ($P>0.05$; Figures 10A, C). In the F4 nutrient regime, *P. parvum* growth rates in controls were significantly greater than those of the 1000 mg/L treatment level ($P<0.05$), but not significantly different from the 4000 and 8000 mg/L treatment levels ($P>0.05$; Figure 10B).

Acute Toxicity to Fish

At T_0 the 48 hr LC_{50} value of the *P. parvum* whole-cell starter culture (3.2×10^4 cells/mL) to juvenile *P. promelas* was estimated to be 2.1×10^4 cells/mL of the original culture. The cell-free fraction of the starter culture; however, was not acutely toxic to *P. promelas*, which suggests the *P. parvum* was not releasing appreciable amounts of toxins in the culture media when the study was initiated.

Mean 48 hr *P. promelas* LC_{50} values for the control, 1000, 4000 8000 mg/L treatment levels are listed in Table 4. As noted above, no growth was observed at the 130 mg/L treatment level throughout the study; thus, no toxicity was observed at this TDS treatment level for each of the three nutrient conditions. For F2 nutrients, *P. promelas* LC_{50} values of controls were significantly greater than those of the 1000 mg/L treatment level ($P<0.05$), but not significantly different from the 4000 and 8000 mg/L treatment levels ($P>0.05$). For the F4 nutrient regime, control 48 hr *P. promelas* LC_{50} values were

significantly greater than those of the 1000 mg/L treatment level ($P<0.05$), but not significantly different from the 4000 and 8000 mg/L treatment levels ($P>0.05$). For F8 nutrients, control 48 hr LC_{50} values were significantly greater than those of the 4000 and 8000 mg/L treatments ($P<0.05$). However, no toxicity was observed in the 1000 mg/L treatment level with F8 nutrients. Mean LC_{50} values calculated for each time-point are reported in Table B6 (Appendix B).

Table 4. Mean *Pimephales promelas* 48 hr LC_{50} values (10^4 cells/mL) (± SD; n=3) for Total Dissolved Solids treatment level and control (2400 mg/L) treatment levels under F2, F4, and F8 nutrient regimes.

Nutrient Regime	Treatment (mg/L)	Mean 48 hr LC_{50} (10^4 cells/mL ±SD)
F2	1000	0.08 ± 0.04
	2400	10.15 ± 8.00
	4000	<0.80 ± 0.28
	8000	<1.45 ± 0.84
F4	1000	0.18 ± 0.16
	2400	10.23 ± 8.20
	4000	<1.01 ± 0.76
	8000	<1.14 ± 0.84
F8	1000	Not Toxic
	2400	8.79 ± 1.42
	4000	<0.71 ± 0.52
	8000	<0.63 ± 0.40

Time-to-Acute Toxicity (LT_{50})

As noted above, time-to-mortality was observed during the 48 hr *P. promelas* acute toxicity bioassays and was assessed at 0.5, 1, 2, 4, 8, 12, 24, and 48 hr time-points. Mean LT_{50} values for each TDS treatment under F2, F4, and F8 nutrient regimes are provided

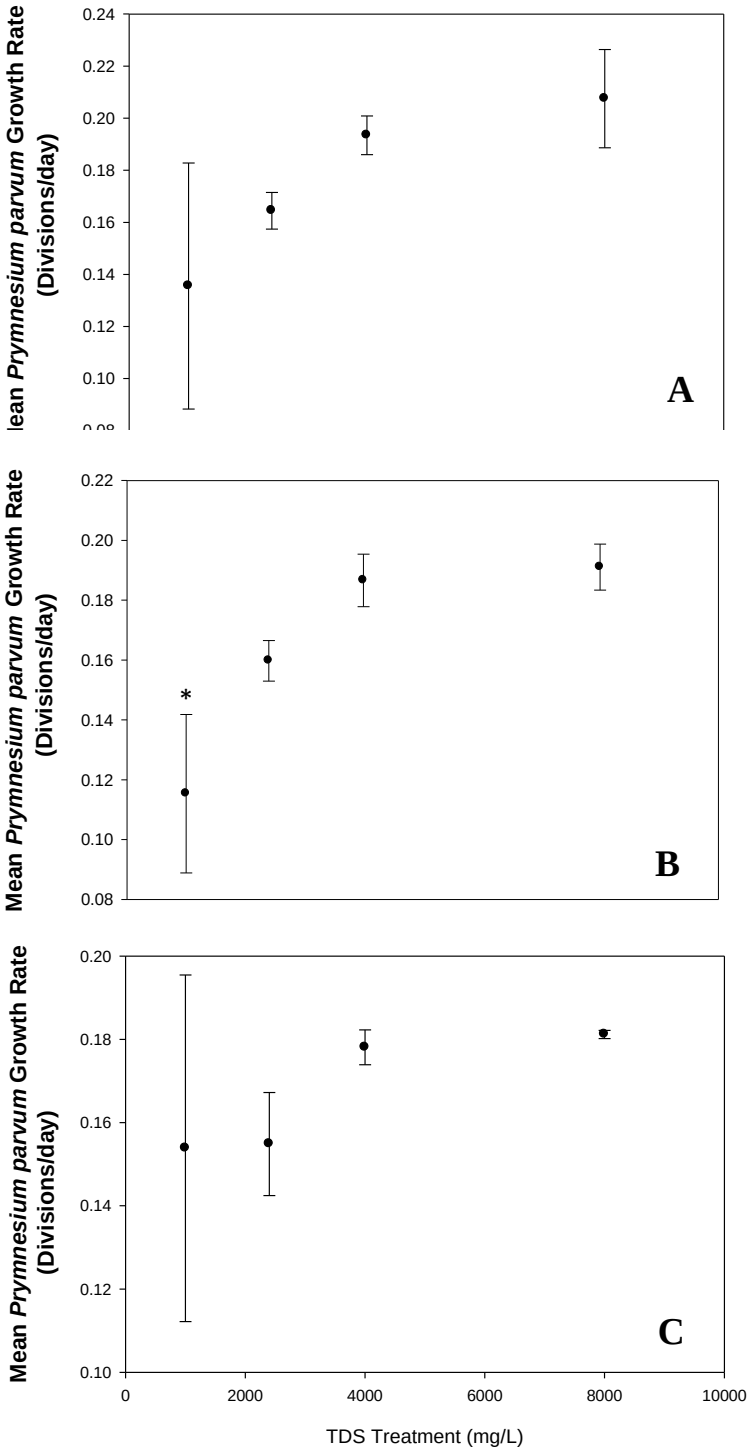

Figure 10. Mean *Prymnesium parvum* specific growth rate (μ/day) (±SD, n=3) in Total Dissolved Solids treatment levels (1000, 4000, 8000 mg/L) and controls (2400 mg/L) across F2 (A), F4 (B), and F8 (C) nutrient regimes. * indicates treatment significantly different from control.

in Table 5. LT_{50} values among the treatment levels were significantly different from controls (P=0.020) under the F4 nutrient regime, and altered by the F2 and F8 nutrient regimes, though not significantly (P=0.058 and 0.055, respectively). Under the F4 nutrient regime control LT_{50} values were significantly greater than those of the 1000 mg/L treatment level (P<0.05), but not significantly different from the 4000 and 8000 mg/L treatment levels (P>0.05).

Table 5. Mean *Pimephales promelas* LT_{50} (hr) (±SD, n=3) for the 100% concentration of hydraulic fracturing TDS (1000, 4000, and 8000 mg/L) and control (2400 mg/L) treatments under F2, F4, and F8 nutrient regimes.

	Mean LT_{50} (hr)		
Treatment (mg/L)	F2	F4	F8
1000	0.48 ± 0.30	22.38 ± 23.56	Not Toxic
2400	17.02 ± 26.83	4.78 ± 3.02	2.27 ± 2.76
4000	0.25	0.25	0.25
8000	0.25	0.25	0.25

Discussion

The recent expansion of *P. parvum* HABs to include Dunkard Creek PA/WV following a TDS pulse in the system has sparked much interest to better understand the impact of TDS on *P. parvum* HAB dynamics in inland waters. Some impoundments in Texas have relatively stable salinity levels with inputs mainly shaped by geological formations. Roelke et al. (2011) analyzed historical salinity data for three impoundments of the Brazos River in central Texas and identified salinity thresholds for *P. parvum* HAB occurrence. In Lakes Granbury and Whitney blooms occur only when salinity levels are above 0.5 psu, whereas HABs occur in Lake Possum Kingdom when salinity levels are

above 1.5 psu (Roelke et al. 2011). The development of such salinity threshold levels for inland waters is useful for environmental risk management; however, in areas such as Dunkard Creek where waters may receive TDS inputs from multiple sources, the development of similar threshold levels require additional attention. Dunkard Creek is located in the Appalachian Mountain Range, which is home to large shale and coal stores and, as a result, widespread natural resource extraction operations occur. These practices produce waste with high TDS levels, which may contaminate inland waters. In particular, this study set out to explore the effects of ionic constituents associated with hydraulic fracturing wastewaters at environmentally relevant TDS concentrations on both *P. parvum* growth and toxicity to determine whether a TDS threshold for *P. parvum* HABs exists.

The results of this study are concurrent with the findings of Baker et al. (2009) in which it was observed that at 20 °C *P. parvum* growth and growth rate increased in a dose dependent manner at inland salinity ranges under both nutrient sufficient and deficient conditions. As specific growth rates at 0.5 psu were markedly decreased when compared to those at 4 psu, a lower threshold limit for *P. parvum* growth was suggested by Baker and colleagues (2009) to be between 0.5-1 psu. The findings of this study support this suggested threshold as no growth was observed at 0.13 psu (130 mg/L); however, bloom densities were observed at 1 psu (1000 mg/L). Baker et al (2009) further reported that specific growth rates between 0.1-0.3 have resulted in a *P. parvum* HABs in inland Texas reservoirs. In fact, specific growth rates in this study were between 0.1-0.3 mean μ/day at all TDS treatment levels and *P. parvum* densities in the 1000, 4000, and 8000 mg/L treatments did exceed a bloom threshold of 10×10^6 cells/L at some point

throughout the study under all nutrient regimes, with 1000 mg/L F4 nutrient treatment as the only exception. Based on these observations, ionic constituents associated with produced waters from hydraulic fracturing may, indeed, stimulate *P. parvum* blooms in the Marcellus Shale Region of the Appalachian Mountain Range to a similar extent that is observed in Texas inland reservoirs.

In addition to TDS levels (Baker et al. 2007 and 2009), reduced nutrient availability (Granéli and Johansson 2003a, 2003b; Grover et al. 2007; Valenti et al. 2010) has also been shown to influence *P. parvum* HAB dynamics and, as a result, this study assessed *P. parvum* toxicity across nutrient regimes and at varying TDS levels corresponding to the lower salinity known to produce toxicity to fish. Acute toxicity to fish in the control treatments (2400 mg/L TDS (2.4 psu) ASW) in this study were comparable to results of Valenti et al. (2010), which showed that cultures grown in 2.4 psu ASW media under nutrient rich conditions (F2) were less toxic than those grown under nutrient limited conditions (F8). Both 4000 and 8000 mg/L hydraulic fracturing treatments were highly toxic with LC_{50} values beyond the lowest tested concentration level and with LT_{50} values of just 0.25 hrs after test initiation for all nutrient regimes. Time-to-mortality observations; however, show that both 4000 and 8000 mg/L treatments were slightly less toxic under F2 conditions than those under F8 conditions. However, the opposite was observed in the 1000 mg/L treatments in which toxicity was greatest under F2 nutrient conditions. Although unexpected, these findings may be explained by the often inconsistent correlation between *P. parvum* density and observed toxicity seen in both laboratory and field studies (Baker et al. 2007, Shilo and Aschner 1953). Even though the 1000 mg/L treatments under F2 and F4 nutrient conditions exhibited similar

LC_{50} values as the 4000 and 8000 mg/L treatments, the LT_{50} values show that the time to observed mortality in the 1000 mg/L treatments were consistently greater than the 4000 and 8000 mg/L treatments. Ulitzur and Shilo (1966) observed that *P. parvum* icthyotoxicity was greatest when ionic co-factors were present and suggested that these co-factors complete the structures of *P. parvum* toxins, thus increasing bioavailability and toxicity. If the amount of co-factors present increases with increasing salinity, it may then be reasonable to expect inland waters with higher salinities to experience more devastating HABs than those at a lower salinities. In fact, Brooks et al. (2011a) reported that while toxic HABs have been observed in Texas reservoirs at salinities of 1-2 psu, HABs observed in reservoirs with salinities of 2-3 psu tend to exhibit a higher toxicity on a system wide scale (Brooks et al 2011a). Based on the observations of the present study, it may be that while acute toxicity to fish was similar at these different salinities, the time to observed mortality (LT_{50} values) may be greater at lower salinity levels thus resulting in potentially lower toxicity in lower salinity waters.

Past studies also suggest that the types, rather than just the amount, of ionic co-factors present may also affect *P. parvum* HAB dynamics. For example, Yariv and Hestrin (1961) found that co-factors consisting of monovalent cations (Na^+ and K^+) promoted a lesser degree of icthyotoxicity, whereas divalent cations (Ca^{2+} and Mg^{2+}) promote greater toxicity to fish. Similarly, Ulitzur and Shilo (1964) found that toxicity was inhibited when NaCl was an important co-factor; however, when $CaCl_2$ was added as co-factor toxicity was increased. As noted above, it has been suggested that these co-factors complete the structures of Prymnesins-1 and -2 (Ulitzur and Shilo 1966, Manning and LaClaire 2010), allowing them to become more bioavailable and more toxic. A

recent study by Bertin et al. (2012b), however, proposed that fatty acid amides may actually be responsible for *P. parvum* toxicity and further suggested that increased pairing of these fatty acid amides to divalent cations may also result in increased bioavailability. The hydraulic fracturing solutions used in this study consisted of mainly NaCl and $CaCl_2$; *P. parvum* cultured under these conditions reached HAB densities and exhibited toxicity to fish in a fashion consisted with previous reports.

Based on the conditions of the present study, a TDS threshold for *P. parvum* bloom development and toxicity was identified between 130-1000 mg/L. Such observations may support *P. parvum* HAB management in watersheds in which salinization results from anthropogenic influences. Bloom management in areas at high risk of *P. parvum* HAB occurrence appears possible by coupling laboratory and field studies to develop predictive models of site-specific factors and conditions that govern development of *P. parvum* HABs (Brooks et al 2011a, Grover et al 2012).

APPENDICES

APPENDIX A

Water Quality Parameters for Chapter 1 Experiments

Table A1. Mean temperature, specific conductance, salinity, dissolved oxygen, turbidity, and Secchi depth during our two in-lake experiments. Shown are the means and standard deviations for conditions in the coves and each of the treatment levels which were low pH (7), intermediate pH (7.5) and ambient pH (~8.5).

Period	Treatment	Mean Temperature (˚C)	Mean Specific Conductivity (µS/cm)	Mean Salinity (PSU)	Mean Dissolved Oxygen (mg/L)	Mean Turbidity (NTU)	Mean Secchi Depth (cm)
Pre-bloom	Cove	8.6 ± 1.80	0.76 ± 0.11	0.36 ± 0.05	10.2 ± 2.70	20.0 ± 3.70	46.0 ± 5.00
	Ambient pH	8.0 ± 0.05	0.89 ± 0.00	0.43 ± 0.00	11.9 ± 0.04	15.0 ± 0.33	57.7 ± 1.70
	Low pH	8.1 ± 0.13	0.91 ± 0.00	0.44 ± 0.00	12.1 ± 0.05	14.6 ± 1.10	63.0 ± 0.87
	Intermediate pH	8.0 ± 0.04	0.89 ± 0.01	0.43 ± 0.00	11.9 ± 0.17	14.2 ± 0.70	58.3 ± 0.15
Bloom Development	Cove	17.4 ± 1.70	1.4 ± 0.04	0.72 ± 0.02	9.1 ± 1.10	3.0 ± 2.30	93.8 ± 27.7
	Ambient pH	17.5 ± 0.04	1.4 ± 0.00	0.71 ± 0.00	9.5 ± 0.11	4.1 ± 0.68	93.7 ± 3.40
	Low pH	17.5 ± 0.05	1.4 ± 0.00	0.71 ± 0.00	9.5 ± 0.10	4.5 ± 0.69	90.8 ± 2.50
	Intermediate pH	17.5 ± 0.02	1.4 ± 0.00	0.70 ± 0.00	9.6 ± 0.11	3.9 ± 0.31	94.9 ± 3.20

APPENDIX B

Media Recipes and Bioassay Results for Chapter 2 Experiments

Table B1. Recipe for concentrated (~30 psu) artificial seawater (ASW) media used to culture *P. parvum*. Dilute solution to target salinity (2.4 psu) using Nanopure and autoclave and cool before use.

Stock Solution	Chemical	Amount (g)	Nanopure (L)
Anhydrous	NaCl	63.57	1
	Na_2SO_4	10.65	
	KCl	1.80	
	$NaHCO_3$	0.52	
	KBr	0.26	
	H_3BO_3	0.07	
	NaF	0.01	
Hydrous	$MgCl_2 \cdot 6H_2O$	28.78	2
	$CaCl_2 \cdot 2H_2O$	4.03	
	$SrCl_2 \cdot 6H_2O$	0.07	

Table B2. Recipes for F2, F4, and F8 nutrient, trace metal, and vitamin stock solutions for culturing *P. parvum*. Dose for all stocks is 1 mL stock/1L media. * indicates stock to be filtered using 0.45µm pore filter prior to inoculation. All stocks are autoclaved and cooled before use. Modified from Guillard 1975.

Stock Solution	Nutrient Regime	Chemical	Amount	Nanopure (mL)
Nutrients	F2	$NaNO_3$	7.50 g	100
		$NaH_2PO_4 \cdot H_2O$	0.50 g	100
	F4	$NaNO_3$	3.75 g	100
		$NaH_2PO_4 \cdot H_2O$	0.25 g	100
	F8	$NaNO_3$	1.88 g	100
		$NaH_2PO_4 \cdot H_2O$	0.13 g	100
Trace Metals	F2, F4, F8	Na_2 EDTA	2.18 g	400
		$FeCl_3 \cdot 6H_2O$	1.58 g	
		$CuSO_4 \cdot 5H_2O$	3.4 mg	
		$ZnSO_4 \cdot 7H_2O$	11.5 mg	
		$CoCl_2 \cdot 4H_2O$	7.1 mg	
		$MnCl_2 \cdot 2H_2O$	76.0 mg	
		$Na_2MoO_4 \cdot 2H_2O$	3.7 mg	
Vitamins*	F2, F4, F8	Thiamin HCl	100 mg	500
		Biotin	0.53 mg	
		B_{12}	0.28 mg	

Table B3. Amounts of salts added to 2.5 L of Nanopure water to achieve the HF stock solutions. * denotes the salts associated with HF while the remaining salts serve as the ASW base.

Target TDS (mg)	NaCl* (mg)	$CaCl_2$* (mg)	KBr (mg)	$NaHCO_3$ (mg)	NaF (mg)	$MgCl_2$ (mg)	$SrCl_2$ (mg)	H_3BO_3 (mg)	KCl (mg)	Na_2SO_4 (mg)
130	245.34	100.28	0.42	0.84	0.01	46.43	0.11	0.11	2.90	17.19
1000	1887.22	771.36	3.21	6.48	0.10	357.18	0.81	0.86	22.31	132.19
4000	7548.88	3085.43	12.85	25.92	0.42	1428.72	3.25	3.43	89.22	528.77
8000	15097.75	6170.86	25.71	51.83	0.83	2857.45	6.49	6.85	178.44	1057.54

Table B4. Analytically measured Dissolved N and P concentrations of hydraulic fracturing and control (2400 mg/L) stock solutions for Experiments 1-3.

TDS Treatment (mg/L)	Nutrient Regime	Dissolved P (µg/L)	Dissolved N (µg/L)
130	F2	1120	8660
	F4	450	4620
	F8	339	3080
1000	F2	768	6470
	F4	322	2500
	F8	145	1550
2400	F2	502	6170
	F4	196	2910
	F8	126	1940
4000	F2	276	3320
	F4	126	3700
	F8	18.8	2690
8000	F2	54.9	5680
	F4	-	-
	F8	41.2	2190

Table B5. Mean *P. parvum* specific growth rates (μ/day; ±SD**)** from T0 to time to steady-state growth for Total Dissolved Solids treatment levels and controls (2400) across F2, F4, and F8 nutrient regimes.

Treatment Level (mg/L)	Nutrient Regime	Mean growth rate (divisions/day) (±SD)
1000	F2	0.14 ± 0.05
2400		0.16 ± 0.01
4000		0.19 ± 0.01
8000		0.21 ± 0.02
1000	F4	0.12 ± 0.03
2400		0.16 ± 0.01
4000		0.19 ± 0.01
8000		0.19 ± 0.01
1000	F8	0.15 ± 0.04
2400		0.15 ± 0.01
4000		0.18 ± 0.00
8000		0.18 ± 0.00

Table B6. Mean LC_{50} values (%) (± SD; n=3) for *P. promelas* at 0.5, 1, 2, 4, 8, 12, 24, and 48 hr time-points for all TDS level and control (2400) treatment levels under F2, F4, and F8 nutrient regimes.

		Mean LC_{50} (%) (±SD)							
Nutrient Regime	Treatment (mg/L)	0.5-hr	1-hr	2-hr	4-hr	8-hr	12-hr	24-hr	48-hr
F2	1000	-	64.51 ± 7.49	63.75 ± 8.06	61.47 ± 4.12	59.02 ± 7.25	59.02 ± 7.25	57.40 ± 5.04	56.64 ± 4.25
	2400	Not Toxic	Not Toxic	78.14 ± 19.81	72.73 ± 23.62	66.87 ± 30.01	66.87 ± 30.01	66.87 ± 30.01	66.87 ± 30.01
	4000	3.55 ± 1.03	2.69 ± 0.48	-	1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00
	8000	4.44 ± 0.65	1.98 ± 0.36	1.44 ± 0.36	1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00
F4	1000	Not Toxic	Not Toxic	91.02 ± 15.55	91.02 ± 15.55	91.02 ± 15.55	91.02 ± 15.55	91.02 ± 15.55	85.44 ± 13.60
	2400	Not Toxic	Not Toxic	87.12 ± 22.30	83.91 ± 27.87	69.35 ± 16.09	69.35 ± 16.09	69.35 ± 16.09	63.67 ± 10.89
	4000	3.31 ± 0.93	2.05 ± 0.07	1.31 ± 0.14	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00
	8000	1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00
F8	1000	Not Toxic	Not Toxic	Not Toxic	Not Toxic	Not Toxic	Not Toxic	Not Toxic	Not Toxic
	2400	91.02 ± 15.55	86.37 ± 23.62	71.03 ± 25.54	67.63 ± 29.74	60.61 ± 23.04	59.86 ± 23.04	59.86 ± 23.04	57.40 ± 23.55
	4000	1.61 ± 0.66	1.38 ± 0.26	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00
	8000	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00	<1.23 ± 0.00

APPENDIX C

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