ABSTRACT

Prymnesium parvum Effects on Green Algae, Cladocerans and Fish in Field and Laboratory Studies

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Prymnesium parvum, commonly named "golden algae," has severely impacted fisheries in Texas, USA and other parts of the world. This study explored the environmental conditions during fish kills in Texas inland waters known to trigger *P. parvum* bloom formation and ecological impacts on a fish model, *Pimephales promelas*, a model green algae competitor, *Pseudokirchinella subcapitata* and a model cladoceran predator, *Daphnia magna*. *Prymnesium parvum* reduced *D. magna* survival and reproduction in laboratory and field studies, providing the first evidence of *P. parvum* impacts on cladocera. Nutrient treatment of N:P = 20 in the field decreased aquatic toxicity of *P. parvum* to cladocerans and fish. Whereas a potential relationship was observed between lower *P. parvum* related toxicity to fish, lower bacteria densities and fewer observations of *P. parvum* swarming behavior in laboratory studies, future studies are needed to understand the relationships among environmental conditions, grazing pressure, mixotrophy and toxin production.

Prymnesium parvum Effects on Green Algae, Cladocerans and Fish in Field and Laboratory Studies

by

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A Thesis

Approved by the Department of Environmental Science

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Susan P. Bratton, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

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Accepted by the Graduate School
August 2008

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ACKNOWLEDGMENTS

I wish to thank the many institutions and individuals whose support and assistance made this research possible. The Department of Environmental Science at Baylor University funded tuition. Texas Parks and Wildlife Department and the Glasscock Fund for Excellence in Environmental Science funded the research. Dr. Bryan Brooks, Dr. James Grover of the University of Texas at Arlington, Dr. Daniel Roelke of Texas A & M University that assisted greatly with guidance and instruction on the continuation of these studies. Dr. Jason Baker, Mrs. Reagan Errera and Mr. Rateesh Nair assisted in *P. parvum* culturing and microscopy. Special appreciation to Mr. Ted Valenti, Dr. Jacob Stanley, and Dr. Alejandro Ramirez for their guidance and insights. Mieke Lahousse and Barry Fulton assisted with laboratory work and toxicity testing. Ms. Sara Seagraves performed nutrient analysis. Facilities and equipment for toxicity testing were provided by Dr. Bryan Brooks. Mrs. Laura Davalos-Lind assisted on *P. kirchinella* culturing and testing, as well of bacteria counts methods. Thank you to my committee members, Dr. Susan Bratton and Dr. Robert Doyle for their guidance, ideas and insight, especially to my thesis advisor Dr. Bryan Brooks, for his patience and leadership. Special thanks to my family, my parents, and particularly my husband, Justin Boeck for his invariable support.

CHAPTER ONE

Introduction

The occurrence of harmful algal blooms (HABs) at the global scale has become a frequent phenomenon along marine coasts and in freshwater systems. The progressive expansion of HABs has been associated with anthropogenic activities and natural causes. The main focus has been on HAB species that exert detrimental effects on human health, natural resources, particularly fisheries, and marine and freshwater ecosystems (GEOHAB, 2001). *Prymnesium parvum (*Carter, 1937*)*, commonly named "golden algae," has impacted negatively diverse marine and freshwater systems around the world since the late 1930s (Shilo et al., 1953; McLaughlin, 1958; Roelke et al., 2007). Blooms of *P. parvum* in Texas were first documented in 1985 (Texas Parks and Wildlife, 2003), and in recent years increasing loss of local revenues has resulted from an increased frequency and magnitude of fish kills apparently resulting from *P. parvum* blooms. An understanding of environmental conditions leading to bloom formation of this particular harmful alga has become an imperative for the mitigation of future blooms (Baker et al., 2007; Grover et al. 2007; Roelke et al. 2007).

Prymnesium parvum is a unicellular haptophyte with two flagella for locomotion and an additional anterior appendage referred to as haptonema (Lee, 1980), which provides a convenient feature for classification. A unique trophic characteristic of *P. parvum* is that it is a mixotrophic organism: it can ingest bacteria, other algae and even cellular organelles of predators in addition to producing energy through photosynthesis (Tillman, 1998; Skovgaard, et al., 2003, Baker et al., 2007). Further, Uronen et al. (2005)

suggest that algae that form toxic blooms may possess common adaptations such as mixotrophic tendency, and production of toxins to reduce competition or serve as antipredatory defense mechanisms.

Prymnesium parvum is known to have several life history strategies that enable it to gain competitive advantages in phytoplankton communities, and finally increase in numbers to form blooms (Uronen et al. 2005). Several studies suggest that the biological activity of *P. parvum* toxins is not due to a single compound, but to a set of toxins with varying biological activity (See Table 1) (Shilo, 1981; Fistarol et al., 2003; Barreiro et al., 2005; Uronen et al., 2005). These compounds have been reported to change the permeability of the cell membranes, cause necrosis and peel the epithelial tissues of the gills and digestive system, causing lethality to aquatic breathing organisms (Granéli, et al., 1999, Shilo, 1967; Meldahl et al., 1994; Hallegraeff, 1993). Shilo (1981) suggested that *P. parvum* toxins were acidic polar phosphoproteolipids, which target biological membranes, particularly gill breathing organisms such as fish and benthic invertebrates (Moestrup, 1992). More recent investigations have characterized two toxins, prymnesin-1 (PRM1) and prymesin-2 (PRM2) (Igarishi et al. 1998), as an unbrached single chain of 90 carbons (Sasaki et al., 2006) and complex structures of polycyclic ether and polyhydroxyl groups, comparable with other HABs toxins chemical structures like brevetoxins, maitotoxin and cigatoxin (Mariussen, et al., 2005). Prymnesins are believed to be responsible for the major hemolytic and neurotoxic effects attributed to the phytoflagellates (Bergman et al., 1964; Igarashi, 1998). However, production of other toxins by *P. parvum*, which would be consistent with other HABs, has not been reported in the literature.

Studies have been conducted to evaluate *P. parvum* toxicity under various environmental conditions, including salinity, temperature, nutrient conditions, grazing pressure and growth competition (See Table 1). Since Shilo (1953) identified *P. parvum* as a toxin producer, various studies have employed a wide variety of models and responses to identify environmental factors that may trigger toxin production by the algae. The vast majority of these previous studies, however, have focused on estuarine and marine conditions and not inland waters. For examples, salinities ranging from 5 parts per thousand to the levels of seawater have strongly suggested salinity effects on *P. parvum* growth and toxin production. Although light cycle has been reported to influence the "inactivation" (light) or "activation" the toxin(s) (dark) (Parnas, Reich and Bergmann, 1962; Reich and Parnas, 1962; Dafni, et al., 1971), it is quite possible that allelochemicals produced by these organisms are simply transformed by photolysis. Increased irradiance in natural systems plays an important role on the formation of *P. parvum* blooms but not necessary toxin production (Dafni, et al., 1971; Reich and Parnas, 1962; Rahat and Jahn, 1965). Another important factor influencing the magnitude of *P. parvum* related impacts is site-specific pH. Shilo (1953) was the first to explore the importance of a pH-toxicity relationship using fish and amphibian models, which represents one of the first attempts to utilize live organism to assess adverse effects of *P. parvum*. Several studies had demonstrated the influence of pH levels and the presence of $Ca^{2+,}$ Mg^{2+} and Na^{2+} to increase mortality to fish (Ulitzur and Shilo, 1966; Shilo and Sarig, 1989). A specific cationic complex with the *P. parvum* toxin is suggested to occur at higher pH (7-9), which may make the toxin less ionized and more nonpolar to be more reactive with biological membranes (e.g., fish gills).

Table 1. Literature review of previous *Prymnesium parvum* experiments associated with adverse effects under various conditions

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured				
							Salinity (psu)	Temp. (^oC)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Errera et al. 2008	no	*Pimephales promelas*	~5 X 10^5	Acute Fish Mortality	Field/ Lab	LC_{50}	11, 13, 22	~ 2.0	8.4, 8.7	f/2 media + BSE*	Natural Photoperiod
Baker et al. 2007	no	*Pimephales promelas*	3.4-104.9	Acute Fish Mortality	Lab	LC_{50}	.5, 7.5, 17.8, 28, 35	5, 11, 20, 29, 35	NS [1]	f/2 media + 93µM HCO_3	14, 122, 217, 336, 420/ 12: 12
Grover et al. 2007	no	*Pimephales promelas*	500 cell.ml^{-1}	Acute Fish Mortality	Lab	LC_{50}	4	10, 20, 30	6-9	f/2 media + 93µM HCO_{3+} BSE*	150/ 12:12
Roelke et al. 2007	yes	*Daphnia magna* and *Pimephales promelas*	~5 X 10^5	Chronic *D. magna* Toxicity (fecundity) and Acute Fish Mortality	Field/ Lab	*D. magna* fecundity and LC_{50}	11, 13, 22	~ 2.0	8.4, 8.7	f/2 media + BSE*	Natural Photoperiod
Olli and Trunov 2007	no	*Rhodomonas salina* and *Pavlova lutheri*	140	Self toxicity	Lab	Growth rate	6	18	8. 4	f/2 media	100/ 12:12

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured: Salinity (psu)	Temp. (°C)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Sopanen et al. 2006	no	*Copepod Eurytemora affinits* and *Acartia bifilos*	2, 5, 10, 50 and 100	Copepod grazing effect	Lab	1. Grazing effects on *R salina* and *P. parvum* 2. Egestion 3. Egg production 4. Hemolytic Activity	6	16	NS	80:1, 16:4, and 58:3. 6	24 h inc.
Barreiro et al. 2005	yes	*Rhodomonas salina* and *Brachionus plicatilis*	2, 50 and 100 cells ml^{-1}	Rotifer growth and mortality rate and grazing experiments	Lab	1. Grazing effect on *R. salina* 2. Hemolytic activity	6	16	NS	80:1, 4:1,	14:10

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured: Salinity (psu)	Temp. (°C)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Uronen, et al. 2005	no	Horse Blood cells Cryptomonad *Rhodomonas salina*	5	1. Hemolytic activity 2. Allelopathic experiments on *R. salina*	Lab	Allelopathic effects on *R. salina*	seawater	16	NS	58: 3. 6 16:4 80:1	330 / 14: 10
Hägstrom and Granéli 2004	no	Horse Blood cells		Hemolytic	Lab	Hemolysis/ HE_{50}	26	17 ± 1	NS	58: 3. 6	120/ 16:8
Granéli and Johanson 2003	yes	10 nauplii/ 10 ml	3. 0 and 2. 0	1. *Artemia salina*	Lab	Dilution series/24 h LC_{50}	7	25	8	58: 3. 6 58:0. 9	24 h darkness
		5-10 ciliate/ 10 ml	18. 4 and 11. 5	2. Allelopathic growth with phytoplankton species in batch cultures	Lab	72 hr growth rate	7	20	NS	4:1 16:1 64:1	100 16 : 8

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured: Salinity (psu)	Temp. (^oC)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Fistarol et al. 2003	no	Species of dinoflagellates nanoflagellates cyanobacteria, chrysophyceans diatoms and ciliates Horse blood cells	3. 5 X 10^8	1. Allelopathic 2. Hemolytic	Lab	HE_{50}	7. 1	11	7-8. 4	116: 7. 2	80 / 19 : 5
Rosseta and McManus 2003	yes	5 scallops	NS	1. Acute Toxicity	Lab	Dilution series/% mortality	NS	20	NS	NS	100 / 12 :12
			4. 8 X 10^4	2. Acute Scallops bioassay	Lab	Toxicity/ mortality	NS	20	NS	f/2	100/ 12:12
				3. Food experiment of growth	Lab	growth rate	Seawater	20	NS	NS	100 / 12:12
Skovgaard and Hansen 2003	no	Dinoflagellate *Heterocapsa rotundata*	1. 6	Immobilization and lysis	Lab	Cell density *P. parvum* dilution series/feeding frequencies	30	25	>8. 5	NS	90-100 / 16 :-8 h

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured				
							Salinity (psu)	Temp. (°C)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Skovgaard et al. 2003	no	Prey *Rhodomonas baltica*	2-5 X 10^4	Feeding experiment	Lab	*P. parvum* feeding activity on prey under nutrient limitation	10	15	NS	3. 2:1 6:1 80: 1	90-100 / 16 :-8 h
Tillman 2003	no	*Oxyrrhis marina*	16	1. Grazing experiment on prey *O. marina* 2. Mortality of prey	Lab	*P. parvum* EC_{50} and grazing effects on *O. marina*	10	15	NS	f/10 media	30
Johansson and Granéli 1999	no	NS	12. 8, 8. 8, and 42. 0	Hemolytic	Lab	Hemolysis / HE_{50}	9%	16		32: 2. 0 32:0. 2	200 / 16 :-8
Lindholm et al. 1999	no	*Perca, Alburnus* and *Rutilus* species 20 Artemia larvae in 300	10 000- 40 000 cells m^{-1}	1. Fish Mortality 2. *Artemia salina*	Field/ Lab	Fish and *A. salina* mortality	4-5 2. 5%	20-25	~ 8	On the field: TN:TP	Natural light : dark cycle Continuous illumination

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured				
							Salinity (psu)	Temp. (°C)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Igarashi et al. 1999	yes	Mouse blood	NS	1. Hemolytic	Lab	Hemolysis/ HC_{50}	NS	25	6-9	NS	NS
		5 fish/20 ml	NS	2. Fish Mortality	Lab	Effect of pH and Ca^{2+}/ 24 h LC_{50}	NS	17	7-8	NS	NS
		Mice	NS	3. Intra peritoneal injection	Lab	Diluted toxin/ 24 h LD_{99}	NS	NS	NS	NS	NS
Guo et al. 1996	no	carp	NS	Waterborne exposure	Field/ fishponds	Fish poisoning symptoms	8	20-24	7.2-9.3	Limitation of phosphate Ca2+ and Mg2+	NS
Nejstgaard et al. 1995	no	Copepod *Calanus finmarchicus*	Terms of food conc. (mg C m^{-3})	NS	Lab	Effects on grazing rates	NS	NS	NS	f/2; f/20 dilution media	*25* / 14:14

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured: Salinity (psu)	Temp. (°C)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Meldahl et al. 1994	yes	*Artemia salina*	≈10^8 cell ml^{-1} *P. parvum*	1. *Artemia salina*	Lab	Dilution series/24 h LC_{50}	25	NS	NS	NS	24 h darkness
				2. Hemolytic		Dilution series/EC_{50}	NS	15,20	5. 5	NS	160/ 12:12
				3. Inhibition of neuro-Trans-mitter							
Padilla 1977	no		NS	Hemolytic	Lab	Hemolytic activity as a pH response	Sea-water	25	5. 5	NS	NS
Kim and Padilla 1977	no	NS	≈10^9 cell ml^{-1} *P. parvum*	Hemolytic	lab	Hemolysis/HD_{50}	NS	25	90	NS	NS
Binford et al. 1973	no	Bovine blood cells	NS	Hemolytic	Lab	Binding parameter of hemolysin	NS	37	4. 6-7. 0	NS	NS
Dafni, Ulitzur and Shilo 1972	no	Blood cells	2. 4 x 10^{6}-11. 6 x 10^6 $cell.ml^{-1}$	Hemolytic	Lab	Hemolytic activity as light and PO_4 response	NS	26-28	8	S_{60} media	NS

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured: Salinity (psu)	Temp. (^oC)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Martin, Padilla and Brown 1971	no	NS	NS	Hemolytic	Lab	Hemolytic activity by effect of primaquine treatment/ HD_{50}	NS	25	NS	NS	NS
Padilla 1970	no	Rat erythrocytes	1. 33-2. 95 x 10^8 $cell.ml^{-1}$	Hemolytic	Lab	Hemolytic	3. 3 %-10. 0 % ASW	25	NS	NS	40 W fluorescent light
Ulitzur and Shilo 1970	no	*Pseudomonas fluorescens* *Micrococcus lysodeikticus* *Bacillus subtilis*	0. 03-3 µg/10^9 cells	Effect on bacteria	Lab	Toxin effect on bacteria compared with activities of detergents	18	18 ,35	NS	NS	NS
Shilo 1967	no	5 minnows/40 ml	NS	*P. parvum* Growth	Lab	Toxin effect under specific conditions effect	NS	27-37	NS	NS	NS
Ulitzur and Shilo 1966	no	5 *Gambussia* minnows/ 8 ITU^2	NS	Fish mortality	Lab	Fish mortality by *P. parvum* ITU	NS	29	9	NS	NS

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured				
							Salinity (psu)	Temp. (°C)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Rahat, and Jahn 1965	no	*Gambussia* minnows/ dilution series	1. 46 x 10^6- 3. 74 x 10^6	Fish mortality	Lab	Fish mortality on "dark" cultures	NS	21-23	8. 2- 8. 4	Basal media componen ts	Dark
Ultizur and Shilo 1964	no	3 minnows/ dilution series on ITU[2]	NS	1. Intraperiton eal injection	Lab	Fish mortality	NS	NS	NS	NS	NS
			NS	2. Fish mortality	Lab	Fish mortality	NS	26-28	7-9	NS	Continuous fluorescent light
Bergman, Parnas and Reich 1963	no	10 *Gambussia* minnows/ 10mg/ml toxin	NS	1. Subcutaneo us Injection	Lab	LD_{50}	NS	10-30	7-9	NS	NS
Bergman, Reich and Parnas 1961	no	3 *Gambussia* minnow/ 18 ml dilution series	NS	Fish mortality	Lab	LD_{50}	5 %	20	8. 0- 8. 2	NS	40 W fluorescent lamp/ 12:12

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured: Salinity (psu)	Temp. (^oC)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Yariv and S. Hestrin 1961	yes	Bovine erythrocytes. 5 *Gambussia* fish/ 20 ml	NS	Hemolytic	Lab	Hemolytic activity Fish mortality	5, 8	NS	NS	NS	NS
McLaughlin J. J. A. 1958	yes	6-30 *Lebistes* and *Gambussia* species	NS	Fish mortality	Lab	Fish mortality activity under different pH and effect of ammonia	NaCl [0. 3- 6. 0%]	≈ 34	6-8	K_3PO_4	NS
Shilo and Shilo 1953	no	2 *Gambussia* minnows/20 ml 5 tadpoles/ 5 ml	NS	Control by Ammonium sulfate in fish ponds/ toxicity tadpoles	Lab/ Meso cosms	Lytic activity of ammonium sulfate on *P. parvum*	NS	2-30	6. 5- 9. 5	NS	Natural Photoperiod

Table 1. —Continued

Author (s) and Date	Multiple Models	Organism (s)/ Cells	*P. parvum* Cell Density (Cell. ml^{-1} X 10^3)	Bioassay	Lab/ Field Study	Endpoints	Water Quality Conditions Measured				
							Salinity (psu)	Temp. (°C)	pH	N:P NO_3^{3-}: PO_4^3 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Shilo and Aschner 1953	yes	5 X 10	NS	1. Toxicity to minnow and tadpoles	Lab	Minimum lethal dose in minnows or tail curvature on tadpoles	Sea-water diluted 1:10	15-25	7. 5-8. 5	NS	NS
				2. *P. parvum* growth		Comparison of *P. parvum* growth and toxicity under specific conditions		22	7-9	Various culture media Nut. added: egg yolk, fish peptone Antibiotic	Diffuse day light Fluorescent light laboratory interior

1. N.S.: Not Shown
*BSE: Barley Straw Extract

Furthermore, a study by Ulitzur and Shilo (1964) observed the increase of pH in the media caused higher mortality to fish up to four times, even in the absence of divalent cation treatments. Fish survival appeared to increase at lower pH (6-5. 5) assuming ionization of the hydrogen cation. However, the majority of previous studies in Table 1 employed non-standardized in vivo bioassays to assess biological effects of *P. parvum* or in vitro hemolytic bioassays, which have limited ecological relevance unless the in vitro model response is related to adverse effects at the individual and population levels.

Numerous studies examining the aquatic impacts of *P. parvum* suggest that its impacts to aquatic life varies largely among environmental and biological conditions such as salinity, light:dark cycles, cell density, nutrient stoichiometry and temperature (See Table 1). Bioassays to determine lethal and sublethal effects of *P. parvum* on aquatic organisms suggest either positive or negative effects on coexisting algae and grazers population dynamics (Uronen et al., 2005). *Prymnesium parvum* allelochemicals can impact co-existing algal competitors (Fistarol, et al., 2003, Granéli and Johanson, 2003b, Legrand et al., 2003) and these allelochemicals appear to immobilize its prey (Skovgaard and Hansen, 2003). Other studies suggest that the toxins may sublethally reduce the growth of grazers such as species of ciliates, copepods and rotifers (Nejstgaard et al. 1995, Nejstgaard and Solberg, 1996); however, lethality has not been observed as often for grazers of *P. parvum* as has been reported for fish. Recent studies by Sopanen et al. (2006) showed the allelopathic effects of the *P. parvum* on competition for food sources within the grazer communities. The effect of copepods species grazing on *Rhodomonas salina* (high quality food) treatments and *P. parvum* (toxic food) treatments had a significant effect on fecundity, and grazing rates when *P. parvum* cells were present.

Grazers showed inactivity in *P. parvum* treatments, which suggest the negative effect was most likely, caused by the toxins rather that starvation (Sopanen et al., 2006). However, controversial results from a variety of earlier studies may suggest that the responses to *P. parvum* toxins can be species specific (Sopanen et al., 2006), because some organisms appear to be more sensitive to the toxins at lower concentrations (Nejstgaard, 1995). For example, the copepod *Calanus finmarchicus* was used to quantify effects on the grazing rate on *P. parvum* and other algae (Nejstgaard, 1995) (See Table 2). *Calanus finmarchicus* survived to the toxin but generally low feeding rates were shown (Nejstgaard et al., 1995). Both studies suggested that the copepod avoided feeding upon *P. parvum*; however, feeding rates were significantly lower when experimental cultures were nutrient limited (Sopanen, 2006; See Table 1), which may have increased *P. parvum* toxicity. Nevertheless whether *P. parvum* evolved allelopathy as a life history strategy to reduce predation remains undefined.

The variability of toxicity between laboratory and field conditions makes the study of the ecotoxicology of any contaminant and particularly *P. parvum* a challenging proposition. It appears that non-optimal conditions of nutrient limitation may influence the ambient toxicity of *P. parvum*, causing cellular stress followed by excretion of the toxins into the water (Johanson and Granéli, 1999b). Previous studies in our laboratory studied the relationship between environmental conditions (temperature, salinity, light) and *P. parvum* toxicity (Baker et al., 2007). As previously mentioned, because standards for *P. parvum* toxins are not available, quantitation of the toxins in water is not possible.

In the Baker et al. (2007) study, I used standardized toxicity test responses and toxicological benchmark concentrations (e.g. , LC_{50} values) as an indirect measurement

of bioavailable toxin concentrations. The Baker et al. (2007) study identified that non-optimal growth conditions caused higher fish mortality and lower LC_{50} values per unit cell than conditions more optimal for growth. In fact, Baker et al. (2007) suggested that *P. parvum* produced and released higher levels of bioavailable allelochemicals when cells are stressed by non-optimal environmental conditions. More recently, I tested the toxicity to *Daphnia magna* of semicontinuous *P. parvum* laboratory cultures grown at different temperatures, and nutrient-enriched conditions (low nitrogen, and low phosphorus) using a chronic 10 d *D. magna* reproduction study to assess whether sublethal effects may be linked to these conditions. *Daphnia magna* fecundity significantly decreased under low temperatures, and low phosphorus conditions (Figure 1) (unpublished data). However, the relationship between non-optimal environmental conditions, production and modes/mechanisms of action of *P. parvum* toxins remain inconclusive.

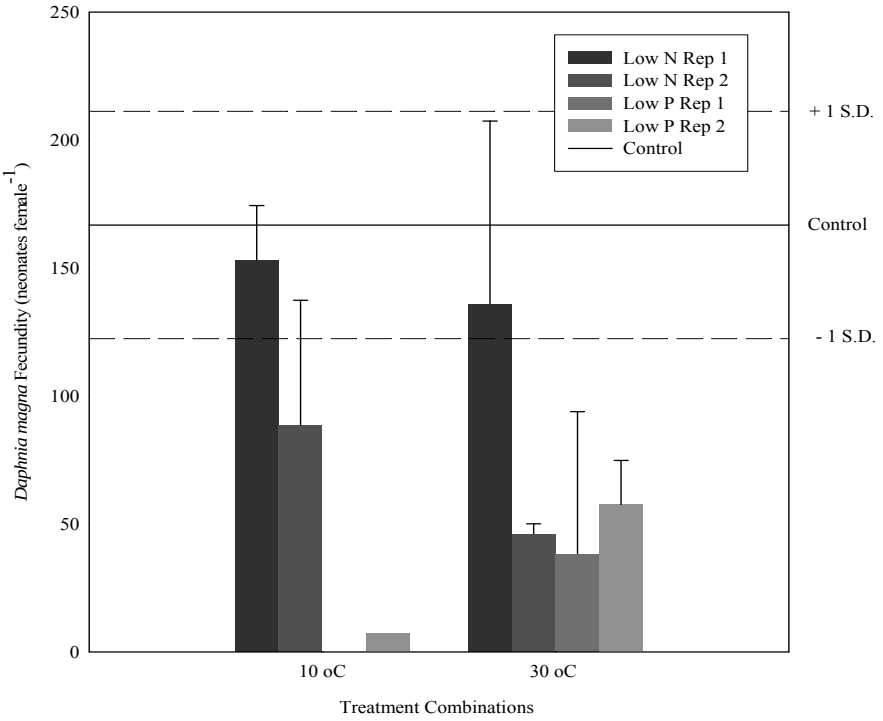


Figure 1. *Daphnia magna* fecundity responses following a 21d exposure to either N or P limited conditions and two temperature combinations at salinity of 4 practical salinity units (psu) under controlled laboratory conditions often associated with bloom conditions in Texas reservoirs (unpublished data).

Standardized bioassays with organisms representing different components of an aquatic food web (e.g., algal competitors, zooplankton grazers, fish) have particular relevance for understanding *P. parvum* impacts to aquatic life because: 1. standards for measuring concentrations of toxins are not available at this time; 2. standardized models are routinely used to develop numeric water quality criteria, assess wastewater effluent safety, and examine the quality of ambient aquatic conditions because standardized responses of model organisms integrate physical, biological and chemical constituents; 3. responses from studies employing standardized assays are generally more repeatable due to higher quality control of experimental conditions; and 4. information obtained from these standardized model organisms can be readily transferable to other laboratories

because of the robustness of standardized experimental designs and reliability of data. Unfortunately very few studies have used standardized models to examine *P. parvum* impacts to aquatic life, particularly in inland or less saline waters. In fact, my research (Baker et al. 2007; Grover et al. 2007; Roelke et al. 2007; Errera et al. 2008) represents the only published studies with standardized *in vivo* models (Table 1), and the vast majority of the current published information on *P. parvum* impacts in inland waters.

As noted in Table 1 above, a number of different types of effects have been associated with *P. parvum* blooms. Several other studies discussed below have provided information regarding the potential mechanisms and modes of action for *P. parvum* toxins. Some of these effects including hemolysis, calcium channel antagonism, and fish lethality were studied by Igarashi et al. (1994). Hemolytic activity by *P. parvum* allelochemicals presumes that the molecule has a direct action on membrane surfaces, resulting in either polarization or charge distribution within the molecule (See Table 2). Fish mortality has been related to increasing Ca^{2+} and pH levels that can cause a possible intracellular Ca^{2+} influx, suggesting that Ca^{2+} ATPase may have an active role as the main transporter of Ca^{2+} into the membrane by a cation pore formation of the *P. parvum* alellochemicals and higher pH conditions causes higher intracellular levels lowering the pump activity, thus leading to cell death (Ulitzer et al., 1964; Igarashi 1998). Extracts containing toxins from *P. parvum* can induce high ion fluxes across cell membranes, resulting in lysis at high concentrations (Meldahl and Fonnum, 1995). Damage to secondary lamella of fish gills can generally increase permeability, produce lesions and is often fatal (Ulitzur and Shilo, 1966). In addition, tadpoles appear to be relatively sensitive to the allelochemicals, but such sensitivity apparently decreases following

metamorphosis to adult air breathers (Ulitzer and Shilo, 1966).

More recent studies identified that extracts from *P. parvum* block uptake and stimulate Ca^{2+}-dependant release of glutamate in rat synaptosomes (see Table 2) (Mariussen et al. , 2005). Because structures of prymnesins and their glutamate and GABA release mechanisms appear similar to maitotoxin, a toxin produced by a marine dinoflagelate (*Gambierdiscus toxicus*) associated with red tide (Murata and Yasumoto, 2000), further characterization of such potential targeting of *P. parvum* allelochemicals on the central nervous system (CNS) of fish is required. It is unknown, for example, if prymnesins can cross the blood-brain barrier due to their large molecular size to even interact with the CNS.

Study Objectives and Hypotheses

It is clear that an advanced understanding to *P. parvum* effects on a freshwater competitor species and grazer species, particularly in inland waters, require additional study. The main objective of this research project was to understand *P. parvum* effects on green algae competitors (*Pseudokirchinella subcapitata*, formerly known as *Selenastrum capricornutum*) and predators (the cladoceran *Daphnia magna*), which represent critical components of fisheries in Texas reservoirs. Further, I investigated these effects under environmental conditions (nutrient stoichiometry, temperature) previously shown to influence *P. parvum* toxicity.

Table 2. Potential mechanisms and modes of action *Prymnesium parvum* toxins under specific conditions

Author (s) and Date	Organisms or System	Endpoints	Experimental conditions	Synopsis
Maurissen et al. 2005	Rat Brain Synap tosomes	Recovery hemolytic (EC_{50}) activity and transmitter (glutamate) release and uptake from rat brain synaptosomes	Toxin production promoted under phosphate limitation growth conditions (Meldahl et al., 1994)	*Prymnesium* toxin-induced inhibition of glutamate uptake as a result of spontaneous Ca^{2+}-dependent and Ca^{2+}-release and inhibition of uptake . Effects are compared to maitotoxin. Hemolytic activity measured
Igarashi et al. 1999	Mouse Blood Fish	Hemolytic Activity Fish Mortality	High pH conditions (pH=8) reduces the Ca^{2+} cationic activity in pump leading to death of cell.	Modes of action of the hemolytic and fish toxicity activities of prymnesins influenced by pH and Ca^{2+} concentration
Meldahl et al. 1994	Erythro-cytes, synap-tosomes neuron transmi-tters and synaptic vesicles	Toxic effect towards hemolytic cells, synaptosomes and synaptic vesicles	*P. parvum* toxin extracts tested on 3 different test for toxicity activity comparison	Mechanism of action on synaptosomal membranes by inhibition of uptake of glutamate and GABA. *Artemia salina* and hemolytic assays are compared to sensitivity of synaptosomal membranes
Ulitzur and Shilo 1966	*Gambussia* minnows	Effect of *Prymnesium* toxin and cofactor on gill tissue	*Gambussia* minnows pretreated on cofactors to change gill permeability increasing sensitivity to external toxicants	*Prymnesium* toxin-cofactor active complex as a primary effectors on gill tissues to determine the sensitivity to other toxicants
Bergmann et al. 1963	*Gambussia* minnows	Effect on prymnesyn toxin by peritoneal injection affected by Ca^{2+} ions	Peritoneal and subcutaneous injection of prymnesium toxin and Ca^{2+} alone to compare toxicity effects	Determination of toxicity by peritoneal injection on fish affected by Ca^{2+} ions and the comparison by other routes of application.
Yariv and Hestrin. 1961	Bovine Erythrocytes *Gambussia* fish	Hemolytic activity : Fish mortality ratio	Toxin solubility patterns to determine the lipid toxic material by cation and anion salts	Ability of prymnesyn to enhance the susceptibility of fish to lethal action

Based on previous population growth studies and related aquatic impacts under non-optimal setting and environmental conditions typical of Texas inland waters (Figure 1), I developed the following hypotheses that may identify relevant physiological and ecological responses of *P. parvum* effects on potential phytoplankton competitors and zooplankton grazers and the role of the nutrient-saturated conditions on its toxicity. The research hypotheses for this thesis are:

I. *Prymnesium parvum* toxicity responses are greater in nutrient-limited conditions.

II. *Prymnesium parvum* effects on grazers are different between dietary/waterborne and waterborne-only exposure routes.

III. *Prymnesium parvum* effects on competitors by waterborne routes of exposure may be greater in non-optimal conditions for growth.

A potential significance of the results of this research will be to assist development of a predictive understanding of environmental and ecological conditions that lead to *P. parvum* toxic bloom formation. Further, an advanced understanding of nutrient influences on *P. parvum* toxic bloom formation will support efforts to manage reservoir fisheries. These experiments will provide novel information for *P. parvum* impacts and the potential routes of exposures under various environmental conditions.

CHAPTER TWO

Methods

I. *Limnocorral Study*

An enclosed experiment was performed in collaboration with researchers at Texas A & M University at Lake Possum Kingdom, Texas (USA) during the early spring of 2005 (Roelke et al., 2007). Eight experimental enclosures conducted in triplicate were nearly fill with lake water and received nutrient addition to levels of f/2 media (Roelke et al. 2007; Guillard and Ryther, 1962). Initial nutrient concentration of N: P was 800 µM and 40µM, respectively. Some enclosures received additions of *P. parvum* to a population density of approximately 5 X 10^5 cell.ml^{-1}. Barley Straw Extract (BSE) was added as mitigation for bloom control at 50-fold manufacture's recommendation (Ecological Laboratories, City, State, Country); this concentration was selected based on preliminary laboratory studies. Water samples from each experimental unit were collected and transported on study Day 0, Day 14, and Day 28 to Baylor University's Ecotoxicology and Aquatic Research Laboratory (Waco, TX, USA) during the 28 d experiment. Ambient toxicity for each enclosure was evaluated using US Environmental Protection Agency toxicity testing standard methods (USEPA, 1994, 2002). *Daphnia magna* 10 d static renewal chronic-toxicity bioassays and 48 h acute-toxicity assay *P. promelas* to determine adverse effects and LC_{50} values, respectively. The significant difference in responses across the experimental treatments was calculated using a General Linear Model (GLM) using statistical program SPSS.

LC_{50} values for *P. promelas* toxicity tests were estimated as percentage of ambient sample using Probit or Trimmed Spearman Karber (TSK) techniques, as appropriate. Low values of LC_{50} imply high toxicity, while estimated $LC_{50} > 50\%$ implies that an undiluted ambient sample was toxic, but that toxicity was reduced by a relatively small dilution with RHW (Roelke et al., 2007). The findings from this limnocorral experiments, particularly organismal responses to the nutrient-enrichment conditions supported the design of a laboratory study described below.

II. *Laboratory Study*

Experimental Design

This experiment explored different environmental conditions typical of Texas inland waters, based on previous studies and literature research. One temperature ($15^{o}C$) and 2 nominal levels of nutrient stoichiometry (N:P= 4:1, 24.4:1,) were cross classified such that each replicated treatment combination was represented as an individual experimental culture of *P. parvum*. A series of samples of the experimental cultures were taken during growth phase until they reached stationary phase (no growth) (Figure 2) following previously reported techniques (Baker, et al. 2007). Samples were analyzed for *P. parvum* motile and non–motile cells (temporary encystment) and bacteria counts, chlorophyll *a*, and nutrient analyses including orthophosphate-phosphorus (PO_4-P), nitrite-nitrogen plus nitrate-nitrogen (NO_2-N, NO_3-), ammonia-nitrogen (NH_3-N), total nitrogen (TN), and total phosphorus (TP). Once cultures reached stationary phase, aliquots were taken for the following analyses: 1) a 96 hr growth inhibition study with a model green algae (*P. subcapitata*) to examine effects on a competitors; 2) a 10 d static-

renewal chronic study with a model cladoceran (*D. magna*). These test procedures were based on standard US EPA methods (USEPA, 1994, 2002). *Pseudokirchinella subcapitata* growth was measured by cell count using a standard hemocytometer and/or Sedgewick Rafter settling chamber and *in vivo* fluorescence using a Trilogy Field Fluorometer Model 10-AU (Turner Designs, Sunnyvale, CA, USA). Aliquots that were used for *P. subcapitata* and *D. magna* studies were manipulated in two ways: 1) *P. parvum* cells were removed from the media by filtration in order to expose organisms to allelochemicals found in water only; 2) no removal of cells to observe adverse affects *via* dietary exposure (*D. magna*) or growth inhibition (*P. subcapitata*).

Cultures

Prymnesium parvum

Cultures of a Texas strain of *P. parvum* (UTEX LB 2797) were grown in a defined media of artificial sea water as described by Kester, et al. (1967) (Table 3) modified by Grover, et al. (unpublished) at 5. 8 psu, light irradiance of 140 μE m^{-2} d^{-1}, and a temperature of 20 degrees Celsius, which resembles the close to optimal growing conditions for stock cultures (James Grover, personal communication). For this study, cultures were diluted to two practical salinity units (psu) salinity media, which represented a typical salinity of Texas inland reservoirs where *P. parvum* blooms occur (e.g., Lake Possum Kingdom). This media was treated with different nutrient-enrichment levels of nitrogen, phosphorus, trace metals, and vitamins as described by MacLachlan (1973) (Table 4). These cultures were maintained in an incubator at temperature of 15 degrees Celsius on a 12:12 photoperiod with an irradiance of approximately 140 μE m^{-2} s^{-1}. This photoperiod was used because blooms typically begin in Texas at approximately

this photoperiod in fall and early winter in temperate climate temperatures that typically range about 15 degrees Celsius (Baker et al., 2007). Twenty liter glass carboys were filled with a working volume of 18,000 ml for each treatment replicate. Each carboy was inoculated with approximately 2,500 cell ml^{-1} of *P. parvum* stock culture, following procedures of previous studies by Grover et al. (2007). Carboys were mixed, and rotated daily.

Table 3. Composition of Artificial Seawater (ASW) at 32 psu (Berges, et. al., 2001)

Solute	Mass Stock Solution g/L	Final Concentration in media (L)
Salt Solution I. Anhydrous salts		
NaCl	21.19	363 mM
$NaSO_4$	3.55	25.0 mM
KCl	0.599	8.04 mM
$NaHCO_3$	0.74	2.07 mM
KBr	0.086	725 µM
H_3BO_3	0.0230	372 µM
Na F	0.0028	65.7 µM
Salt Solution II. Hydrated Salts		
$MgCl_2$. $6H_20$	9.592	41.2 mM
$CaCl_2$. $2H_20$	1.344	9.14 mM
$SrCl_2$. $6H_20$	0.0218	82 µM
TOTAL	36.5826	

*18. 2 MΩ /cm nanopure is used as solvent.
32 psu ASW is diluted down to a working volume of 18 000 ml

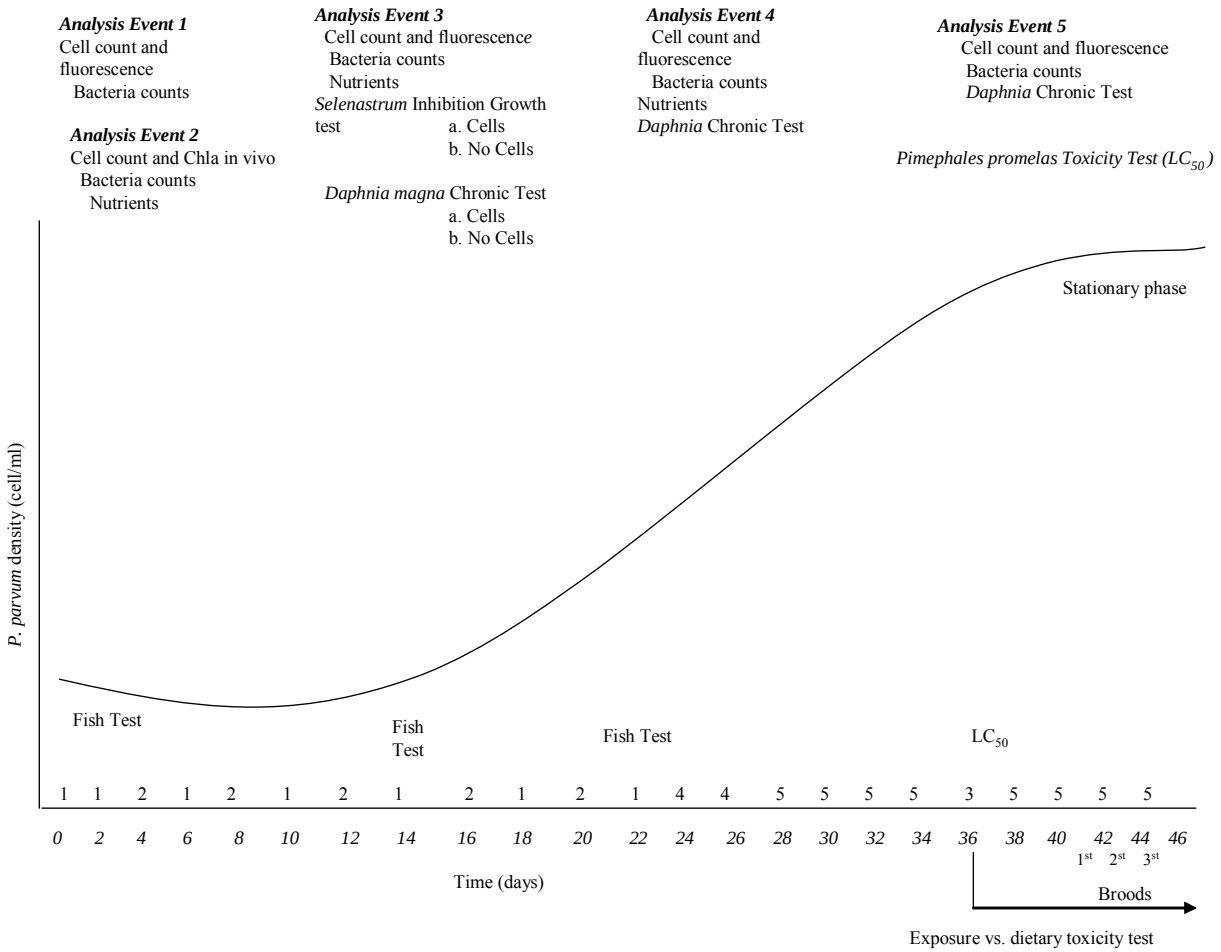


Figure 2. Conceptual diagram of the timeline of sampling and analyses performed during the test period.

Pseudokirchinella subcapitata and Daphnia magna

Less than 24 hours old *D. magna* and *P. subcapitata* were selected for all experiments from stock cultures maintained at Baylor University's Ecotoxicology and Aquatic Research Laboratory (Waco, TX, USA). Algae and Daphnids were mass cultured as previously described (EPA, 1996; Hemming et al., 2002). *P. subcapitata* was grown from strain cultures (UTEX 1648) under fluorescence light at room temperature. Cell density was determined as described below. *Daphnia magna* were fed a 12 mL algae-Cerophyll® suspension once daily (Dzialowski et al. 2006). Culture water was renewed every other day by static renewal to maintain water quality (EPA 1996; OECD 1998). Adults were observed daily and neonates were counted every other day. These records of fecundity were measured to monitor of the overall health of the culture.

Quality Control and Quality Assurance

Preliminary toxicity tests were performed using sodium chloride as a reference toxicant to determine *P. subcapitata* growth responses and *D. magna* reproduction at different salinities (Figure 3 a., Figure 3 b.; USEPA, 2002). Subsequently, salinity of two psu was selected for this study since the concentration had no significant negative effect on *P. subcapitata* growth or *D. magna* reproduction and it corresponded to approximate salinities of Texas Reservoirs experiencing *P. parvum* blooms (e.g., Lakes Possum Kingdom, Granbury, Whitney).

Table 4. f/2 Media Components (Guillard and Ryther, 1962).

Solute	Mass Stock solution (mg/L)	Volume of Stock Solution (ml) in 18 L working volume	Final Concentration in Media (μM/L)
$NaNO_3$	96.11	18	880
KH_2PO_4. H_2O	3.645	18	36
Trace Metals[1]	n. a.	18	n. a.
Vitamin B12	.25	9	0.55
Biotin	.15	9	0.5
Thiamine	1	18	50

Trace Metals Stock Solution	Mass Stock Solution mg/500 ml	Concentration of Stock Solution	Final Concentration in f/2 media (μM/L))
$CoSO_4$. $7H_20$	14	50 μM	0.04
$ZnSO_4$. $7H_20$	23	80 μM	0.08
$CuCl_2$. $2H_20$	6.8	40 μM	0.05
$MnSO_4$. H_20	152	0. 9 μM	0.9
$NaMoO_4$. $2H_20$	7.3	30 μM	0.03
$Fe(NH_4)_2(SO_4)_2$	4,590	11. 7 mM	11.7
Na_2EDTA*	4,350	11. 7 mM	11.7

* NaEDTA is consider part of the Trace Metal Stock Solution

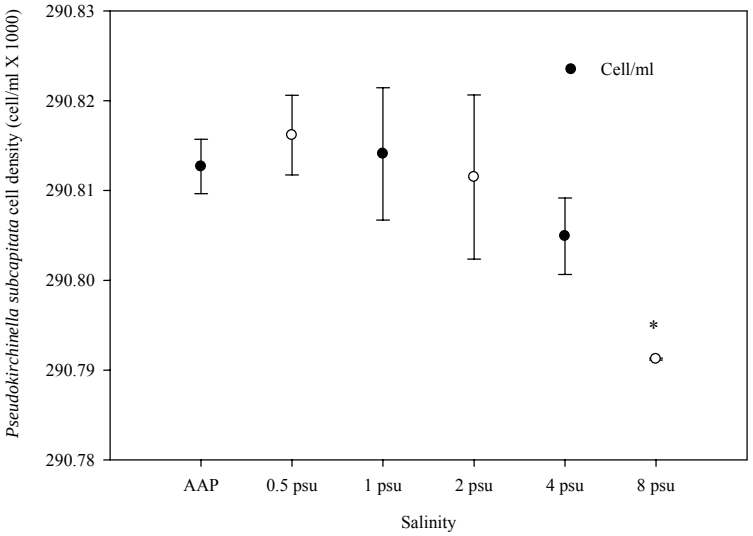
Sampling and Analyses

Experimental cultures were sampled for cell and bacteria counts and chl *a* every two days, and nutrient analyses every four days until cultures reached stationary growth phase (day 36) (See Appendix A)(Baker et al., 2007). At stationary growth phase, experimental cultures were sampled, and aliquots were divided for cell removal and no removal manipulation. These samples were used to perform the following experiments: 1) a 48 h fish acute toxicity test using the fathead minnow (*Pimephales promelas*); 2) a 96 h growth study with the green alga *P. subcapitata*; 3) and a 10-day static renewal chronic-toxicity *D. magna* study. Each of these test procedures were based on USEPA testing standard methods (USEPA, 1994, 2002). Fish 48 hr acute toxicity tests were performed at the time of inoculation (Day=0), mid-point (Day= 14), and at the end of the test (Day=36) to provide a surrogate measurement for the levels of bioavailable toxin production.

Cell Counts

Cell counts were calculated using a manual microscope counting method. *Prymnesium parvum* and *P. subcapitata* cell density (cell.ml^{-1}) were determined using a Bright-line hemacytometer counting chamber by Hausser Scientific Model 1475 for the dense cultures, and Sedgewick Rafter counting cell by Wildlife Supply Company Model # 1801-A10 for the diluted samples (Guillard, 1978). The expanded count for *P. parvum* cells were standardized by the chamber depth and the total surface area. Microscopy was performed using a Zeiss brightfield compound microscope. Cell counts were estimated by in vivo fluorescence, due to the simplicity and sensitivity of the technique,

using a Trilogy Fluorometer Model from Turner designs (USEPA, 2002). Standard curves of fluorescence vs cell density were determined to normalize cell measurements.



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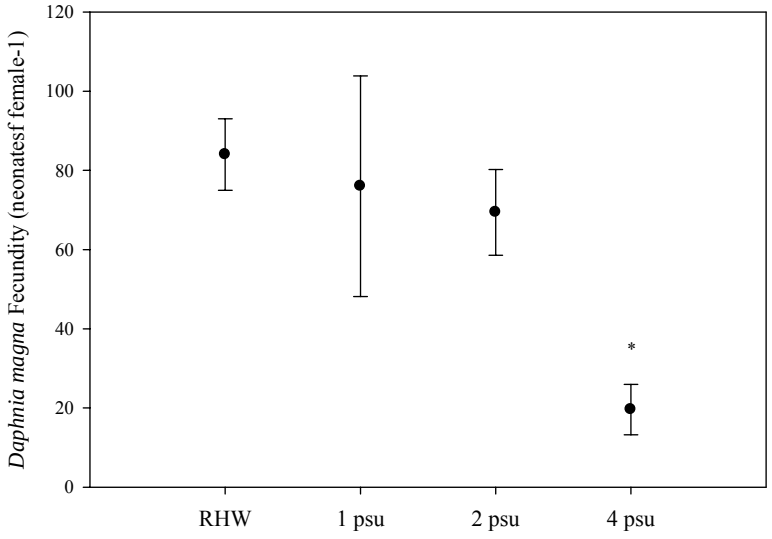


b.

Figure 3. a. *Pseudokirchinella* subcapitata growth response (mean ±S. D cell.ml^{-1}) as a 72 h Dose-Response for NaCl Reference Toxicant. b. *Daphnia magna* fecundity (mean ±S. D.) during a 21d exposure to for NaCl (* represents $p < .05$).

Bacteria

Bacteria aliquots were sampled and preserved with 4% formalin. Bacteria cell density was determined by direct-count method using an orange acridine fluorescent dye and the epifluorescent microscope (Hobbie, 1977). Epifluorescence filter cartridges were set in a Nikon Eclipse Microscope E600 utilizing a 100X fluorometric objective and 15x ocular. Cell counts were performed in 20 random Whipple grid fields by direct analysis or Photo Analysis. Photo analysis was performed by capturing 15-20 images on a known area of the computer monitor, and adjusting a high contrast black background to remove hot pixels.

Nutrient Analysis

The standard operation procedures for the determination of orthophosphate-phosphorus (PO_4-P), nitrite-nitrogen plus nitrate-nitrogen (NO_2-N, NO_3-N), ammonia-nitrogen (NH_3-N), total nitrogen (TN), total phosphorus (TP) using standard colorimetric techniques (EPA 365.3, 353.2, 365.1 and 353.2) are described under the Center for Reservoir and Aquatic Systems Research (CRASR) methods. Samples were analyzed using a Lachat Quickchem 8500 Flow Injection Autoanalyzer. Methods were designed by CRASR personnel to meet USEPA guidelines for approved analytical measurements (USEPA, 1993; USGS, 1993), and have been included in multiple Quality Assurance Project Plans approved by USEPA.

Water Chemistry

Reconstituted Hard Water (RHW) was prepared according to USEPA Whole Effluent Toxicity Testing methods (USEPA, 2002) as a control and for use as dilution media. During this study, water chemistry was measured in our control and highest

treatment samples. Dissolved Oxygen (DO), temperature, pH, salinity and conductivity measurement was recorded on a regular basis. DO and temperature were recorded using YSI Model 55 hand held system; pH measurements were taken using ORION Model 720A pH meter. Salinity and conductivity parameters were measured using YSI Model 30 hand held system. Alkalinity and hardness were measured on freshly prepared RHW by amperiometric and colorimetric titration, respectively (American Public Health Association et al, 1995).

Toxicity Experiments

Samples for *P. subcapitata* and *D. magna* toxicity testing were divided for two types of testing: 1) cells were separated from samples and organisms were exposed to cell free sample to characterize responses waterborne exposure to both organisms; 2) samples not manipulated, which allowed for characterization of adverse affects via dietary and waterborne exposure routes in *D. magna* or growth inhibition through competition with *P. subcapitata*. Cell free samples were filtered onto 47-mm Whatman glass-fiber filters with mesh size 1.2 μm (Whatman Inc., Clifton, NJ) under low pressure (approximately 4 mmHg) to avoid disruption of the cells.

Pseudokirchneriella subcapitata Growth Inhibition Tests

As previously introduced, *P. subcapitata* were exposed to *P. parvum* experimental treatments with or without *P. parvum* for 96 h. Five concentrations using a 0.5 dilution series were performed with four replicates per concentration level. The Algal Assay Procedure (AAP) media control was used as control and for dilution media. The *P. subcapitata* growth conditions were adapted to 15 degrees Celsius when exposed to competitor species *P. parvum* in order to maintain its physiology to avoid added stressing

the organism and potential inducing release of toxins from intercellular stores. No attempt was made to assess the effect of reduced temperature on *P. subcapitata* in this study. Growth conditions of samples without *P. parvum* cells or exposed to its allelochemicals were maintained at 25 degrees Celsius as determined by EPA inhibition growth methods. The response of the green algal population to cell free samples were measured as changes in cell density (cells per mL) and chlorophyll absorbance measurements using the techniques previously identified. For the competition experiment with *P. parvum* and *P. subcapitata*, only cell counts were used to enumerate both organisms. Fluorescence and direct cell counts of growth data from each nutrient treatment level were statistically analyzed by ANOVA with Dunnett's Test to determine significant responses ($p < 0.05$).

Daphnia magna Toxicity Tests

Similar to the green algae studies, less than 24-h *Daphnia* were exposed to *P. parvum* experimental treatments with or without *P. parvum* for 10 days (USEPA, 1994). Three concentrations using a 0.5 dilution series were performed using five replicates per concentration level. Static renewal was performed every other day, and the test was generally following USEPA protocols (1994). *Daphnia magna* responses include survival, and total neonate production (Meyer et al., 1986). These responses allows for linkages between the response of the individual *D. magna* and its population dynamics (Forbes et al., 1998), providing a robust endpoint for potential ecological impact caused by *P. parvum* allelochemicals (Forbes et al., 1998).

Pimephales promelas Acute Toxicity Tests

A series of 48 h acute toxicity tests were performed through the *P. parvum* growth period to assess bioavailability of allelochemicals of the experimental cultures to fish on study days 0, 14, 21 and 28. Juvenile (< 14 day-old) fathead minnows were exposed to samples from *P. parvum* experimental treatments with and without cells for 48 h. Five concentrations using a 0.5 dilution series were performed using four replicates per concentration level for static toxicity tests. Fish mortality responses and subsequently derived LC_{50} values were used as indirect measurements of bioavailability toxin concentrations. LC_{50} values for *P. promelas* toxicity tests were estimated as percentage of ambient sample using Probit or Trimmed Spearman Karber (TSK) techniques, as described on the enclosure studies. The test procedures were based on standard US EPA toxicity testing methods (USEPA, 2002).

Data Analysis

Toxicity data was analyzed using analysis of variance (ANOVA) parametric with a Dunnet's test to identify differences between samples and control media and RHW-only controls ($p < 0.05$). The individual responses were analyzed using Dunnett's t-test to identify treatments that werere significantly different from the mean of the control, and a linear regression or a nonlinear regression analysis when suitable, to determine the effect of *P. parvum* to these different organisms. The data was manipulated to Logarithmic transformation for the "best fit" on the linear models.

Experimental cultures that had lag or no growth were eliminated from this analysis. All statistics were performed using TOXSTAT (Version 3.4, WEST, Inc. and

Gully, 1994), PROBIT (Version 1.5, USEPA) and Sigma Plot (Version 10.0, Systat Software, Inc, 2006).

Resources and Environment Institutional Animal Care and Use Committee (IACUC)

According to the University Animal Care and Use Committee at Baylor University, it is required by law to submit a review of the use of live vertebrate animals in research. The application for approval to use *Pimephales promelas* in our experimental toxicity testing, protocol No. 04-03, was approved to be in compliance to assure the appropriate use, care and treatment of the fish according to the Public Health Service Policy on the Human Care and Use Laboratory regulations of the Animal Welfare Act (Public Law 99-158) (Baylor University, 2006)

CHAPTER THREE

Results

I. *Limnocorral Studies*

The limnocorral experiment performed in Lake Possum Kingdom coincided with a *P. parvum* bloom that was identified by State of Texas personnel as responsible for a substantial fish kill. During this study, *P. parvum* abundance was greater and ambient toxicity to fish and cladocerans was reduced in the enclosures that received nutrient addition at levels of N: P= 20 (N= 880 µM, P= 40µM; Roelke et al., 2007). Specifically, *D. magna* reproduction and mortality were significantly greater and lower ($p < 0.05$), respectively, in limnocorrals receiving nutrient treatments (Figure 4) relative to limnocorrals not receiving nutrient amendment. Fecundity in laboratory control organisms was excellent (mean = 36.6, ±2.41) and all general water quality parameters and test conditions for these studies conformed to USEPA recommendations. My observations in Figure 4 provide the first evidence suggesting that cladocerans are sublethally impacted by *P. parvum*.

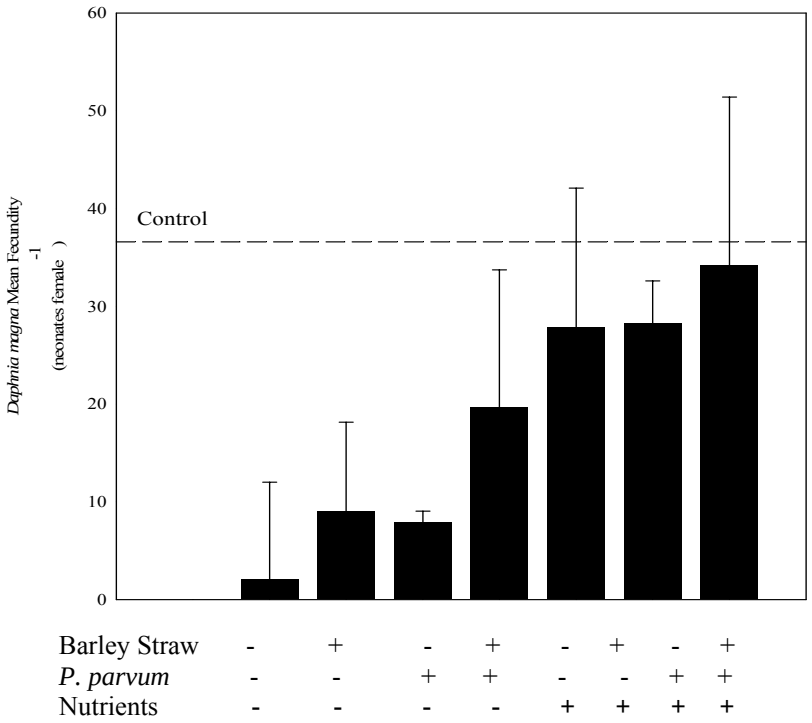
A similar decrease in toxicity with nutrient treatment was observed for fish survival. A Fish No Observed Effect Concentration (NOEC) value on Day 0 was 9.8% (dilution of ambient water), indicating that ambient conditions in the impoundment experiencing a *P. parvum* bloom were appreciable toxic to fish. Fish NOECs among the limnocorrals ranged between 4.8 and 8. 8% on Day 14 (data not shown). By Day 28 toxicity to fish were reduced in limnocorrals that received nutrient additions (Figure 5). . The addition of BSE had no significant ($p < 0.05$) effect on *P. parvum* bloom

proliferation, suggesting limited utility of this treatment to mitigate bloom formation and associated fish kills (Roelke et al. 2007).

II. *Laboratory Studies*

Prymnesium parvum Growth

In total, 9 experimental cultures were exposed to consistent light and temperature for a growth period of 36 days under nutrient-deficient treatments. Nutrient analyses during the growth period exhibited a dramatically different N:P ratios across the nutrient treatments relative to the initial nominal N:P ratios of 24.4 and 4 for f/2 and low nitrogen treatments, respectively. Subsequently, the initial nominal molar ratio treatments were verified to be treatment levels ranging from low to high N:P molar ratios during the growth period (11.43, 12.69, 14.90, 15.94; Table A. 2), though all four treatment ratios were relatively similar compared to previous observations of N:P on *P. parvum* toxicity. Concentrations of NO_3^-, and NH_4^- were fairly constant during the 36 day study; all measurements of total nitrogen were over the analytical detection limit of 10000 μg NOx-N/L (USEPA, 1993; USGS, 1993). Concentrations of PO_4^- had a small decline over the study period. Figure A.3 a-d shows the complete nutrient analysis data during the *P. parvum* laboratory study. The experimental treatments that reached exponential growth were the two intermediate molar ratios of 12.69 and 14.90, reaching higher densities of 2.4 and 3.3 X 10^4 cell.ml^{-1}, respectively. Conversely, the lower and upper molar ratio treatments of 11.43 and 15.94 had lower cell densities of 8 and 3.3 X10^3 cell.ml^{-1}, respectively. A calibration curve ($R^2 = 0.992$) for fluorescence and direct cell count *for P. parvum* is provided in Appendix B.1.



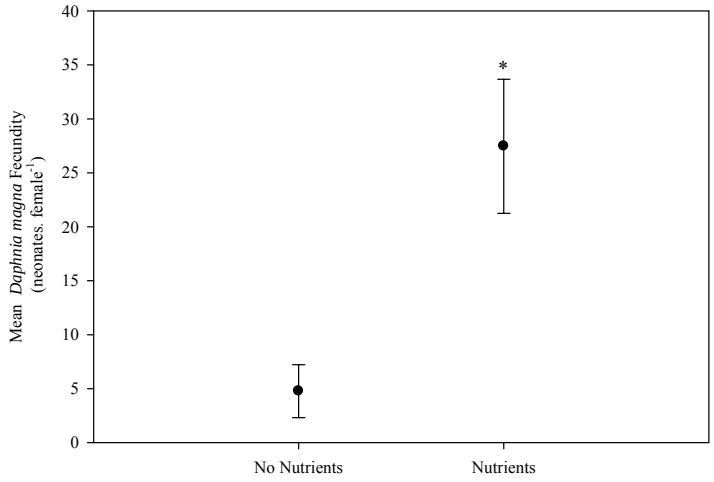


Figure 4 a. *Daphnia magna* mean fecundity responses following a 10 d laboratory exposure to nutrient, *Prymnesium parvum* and barley straw extract treated enclosures (N=3, ±SD) and b. responses to limnocorrals receiving or not receiving nutrients (N=12, ±SE) following a 28 d study at Lake Possum Kingdom, Texas, USA. (* represents $p < 0.05$).

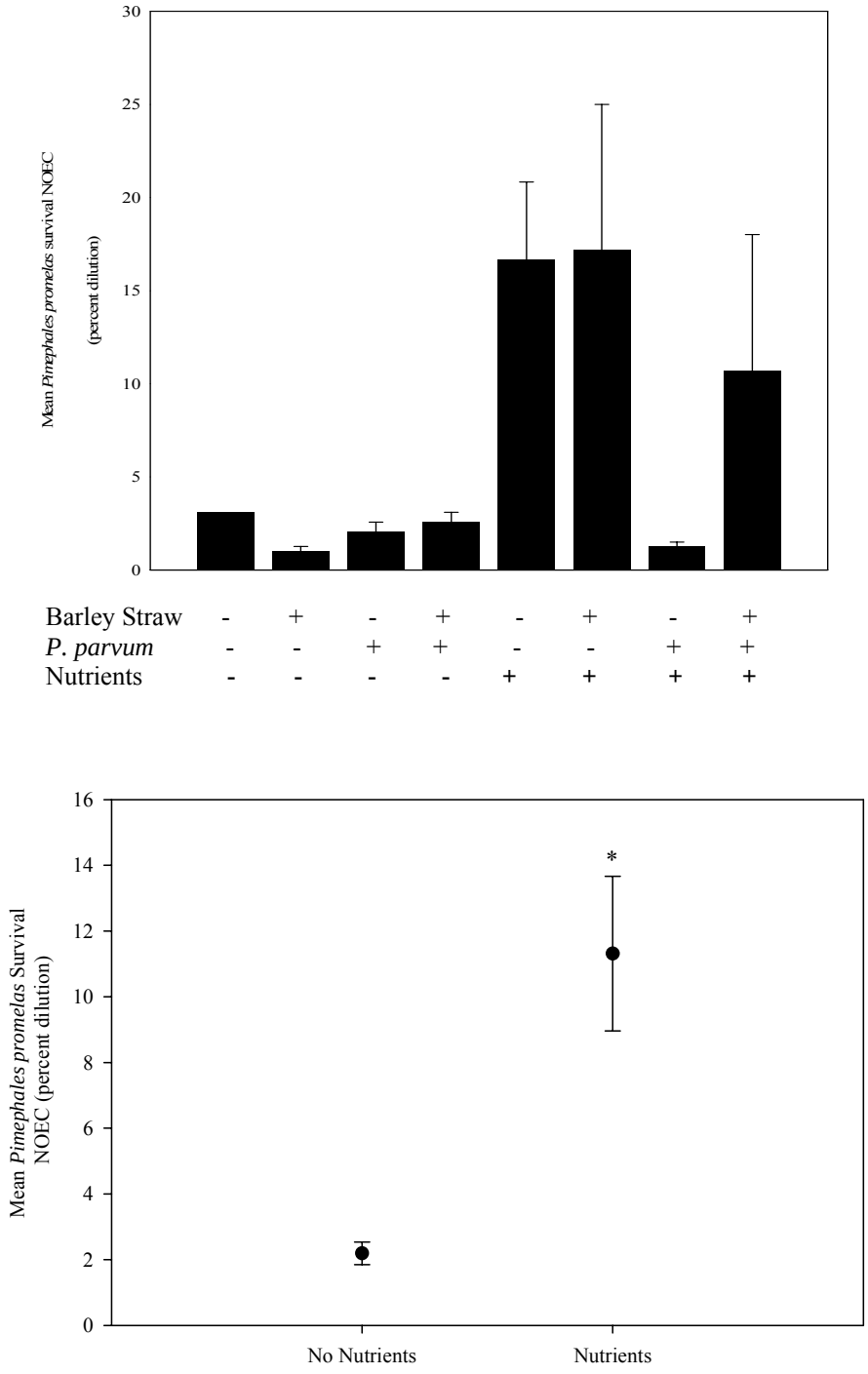


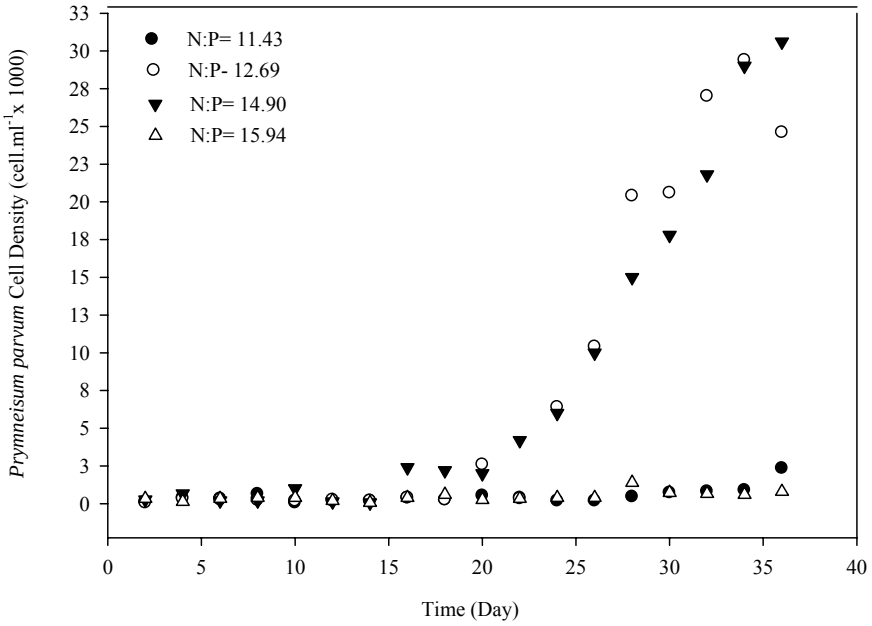
Figure 5. a. *Pimephales promelas* mean survival No Observed Effect Concentrations (percent dilution of ambient samples) following a 48 h laboratory exposure to samples from nutrient, *Prymnesium parvum* and barley straw extract treated enclosures (N=3, ±SD) and b. responses to limnocorrals receiving or not receiving nutrients (N=12, ±SE) following a 28 d study at Lake Possum Kingdom, Texas, USA. (* represents $p < 0.05$).

Figures 6.b displays the percentage of motile cells from the total *P. parvum* cell density. The increment of motile cell density over the growth period may or may not be related to nutrient depletion, since nutrient analyses showed a consistent concentration (except for PO_4^-) at the end of the growth period.

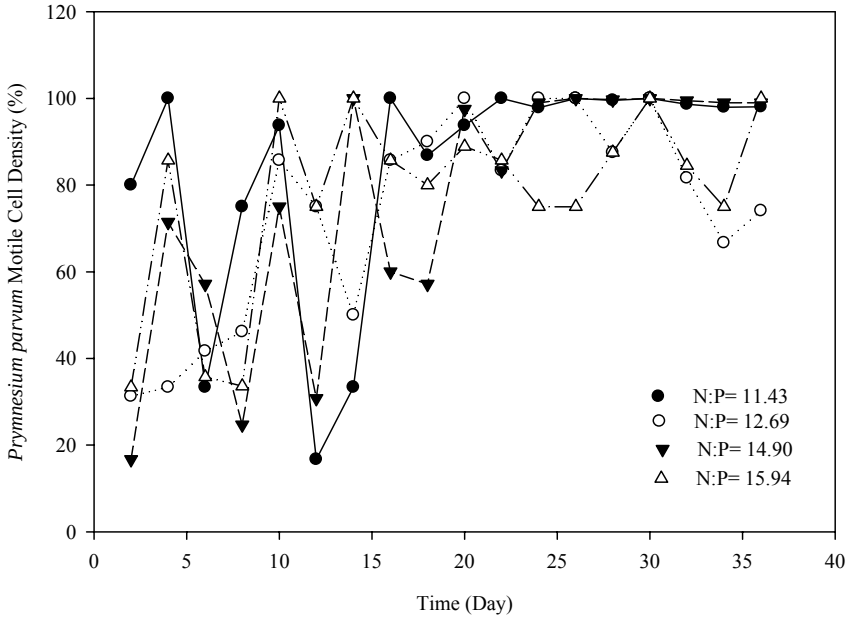
Biological Responses

Pseudokirchinella subcapitata Growth

The adverse effects of *P. parvum* to *P. subcapitata* cell density were evaluated following exposure to each treatment level containing either *P. parvum* cells or cell free filtrate. *P. subcapitata* initial cell density inoculated was approximately 1.0×10^4 $cell.ml^{-1}$, and *P. parvum* at the start of the competition study for molar ratio treatments N:P= 11.43,12.69,14.90, 15.94 was 2.3×10^3, 2.4×10^4,8×10^2, $3.0 \times 10^4 cell.ml^{-1}$, respectively. Growth of *P. subcapitata* in samples with *P. parvum* at the two lowest N:P ratios of 11.43 and were significantly stimulated relative to the f/2 media control at 15°C, which had a markedly higher N:P ratio of 24.4. Higher N:P treatment levels of 14.90 and 15.94 exhibit a growth of 5.7×10^4 and 5.3×10^4 $cell.ml^{-1}$as opposed to *P. parvum* growth that exhibit a cell density of 2.3×10^3 and $1.9 \times 10^4 cell.ml^{-1,}$ respectively. Growth responses of *P. subcapitata* as a function of *P. parvum* filtrates (no cell) samples with higher nutrient ratios (N:P= 14.90, 15.94) at 25°C were significantly different from their control. *Pseudokirchinella subcapitata* final densities (96 h) were recorded as N:P= 11.43, 12.69, 14.90, 15.94 as follows 3.8×10^4, 1.1×10^4, 4.4×10^4, 4.3×10^4. Growth in all nutrient treatment levels was significantly inhibited by cell free filtrate (Figure 7).



a.



b.

Figure 6. a. *Prymnesium parvum* cell density (cells.ml^{-1}) following 36 d of growth at 15^{o}C. b. *Prymnesium parvum* % motile cell density portion during the growth period.

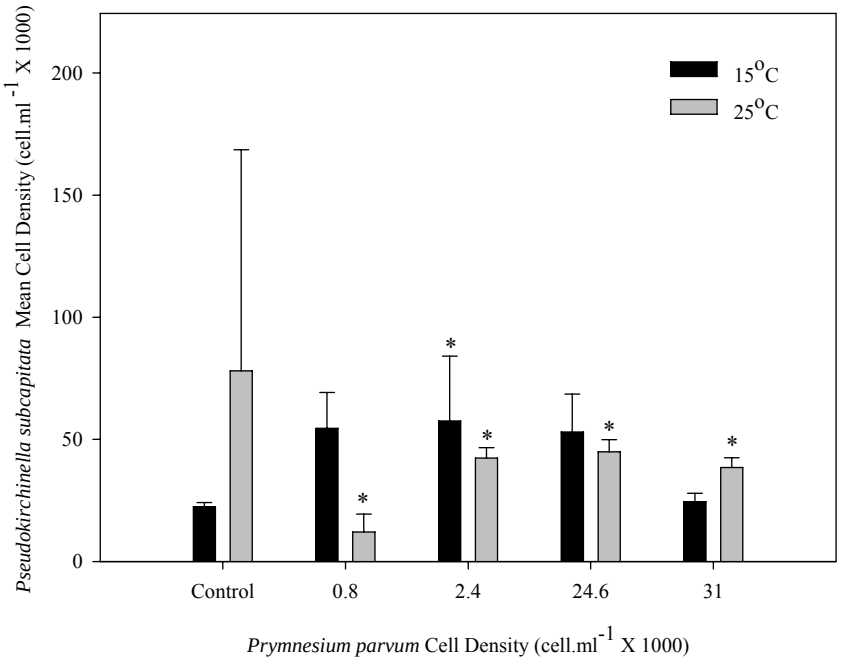
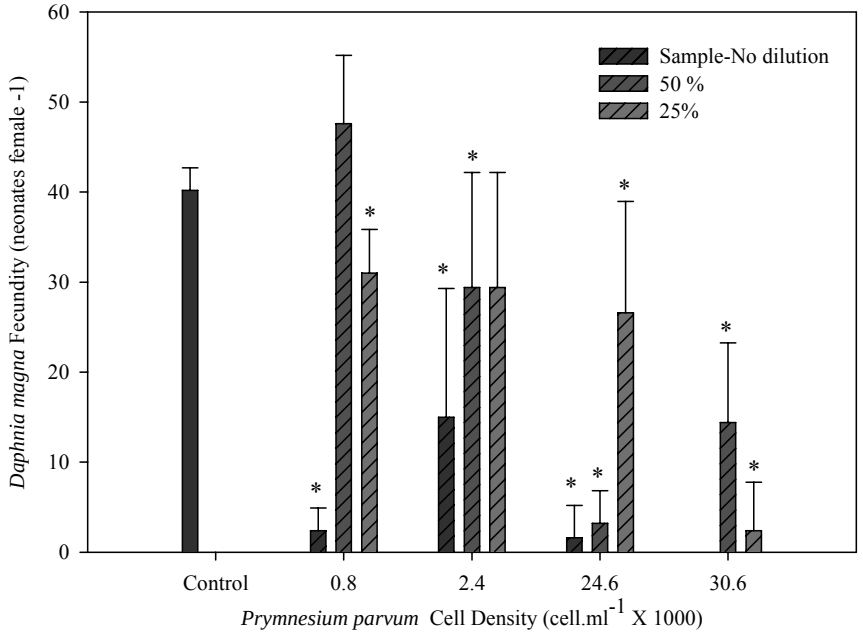


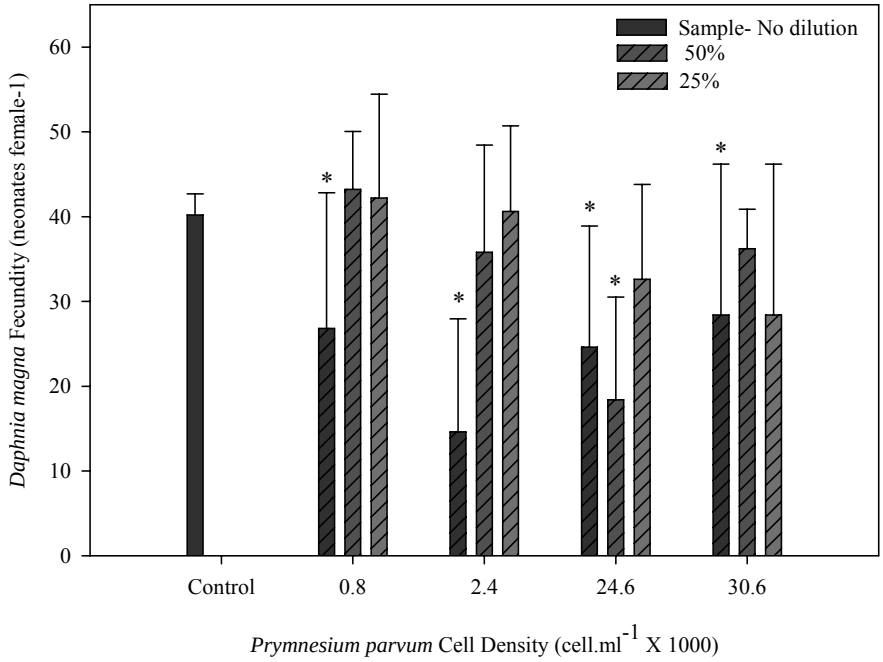
Figure 7. *Pseudokirchinella subcapitata* 96 h growth inhibition bioassay (mean ± S. D. cell density) performed at 15°C with *Prymnesium parvum* present or at 25°C with filtrate without *Prymnesium parvum* (* represents $p < 0.05$).

Daphnia magna Reproduction

A 10 day static renewal reproduction test was performed to determined adverse effects on *D. magna* to samples with *P. parvum* cells and allelochemicals. *D. magna* reproduction was significantly different ($p < 0.05$) from the control (mean = 40.2, ± 2.5) in all samples along a nutrient gradient (N:P = 11.43, 12.69, 14.90, 15.94; Figure 8. a and Figure 8. b). The lowest and highest N:P cultures were less toxic to *D. magna* reproduction than the 12.69 and 14.90 cultures. Specifically for samples with *P. parvum* cells, NOEC and LOEC values were 29.4, and 15 neonates.female^{-1} for N:P: 11:43, and 47.6 and 31 neonates.female^{-1} for N:P 15.94. Cell free filtrate exhibited a NOEC and LOEC value of 35.8 and 14.6 neonates.female^{-1} for N:P 11.43 and 43.2 and 26.8 neonates.female^{-1} for N:P 15.94, respectively.



a.



b.

Figure 8 a. *Daphnia magna* fecundity (mean ± S. D. neonates female^{-1}) exposed to samples with *Prymnesium parvum* cells for a 10 day period. b. *Daphnia magna* fecundity (mean ± S. D. neonates female^{-1}) exposed to samples with no *P. parvum cells* for 10 day period (* represents $p < 0.05$*).* Mean *D. magna* fecundity in laboratory controls was 40.2 (± 2.49) neonates female^{-1}.

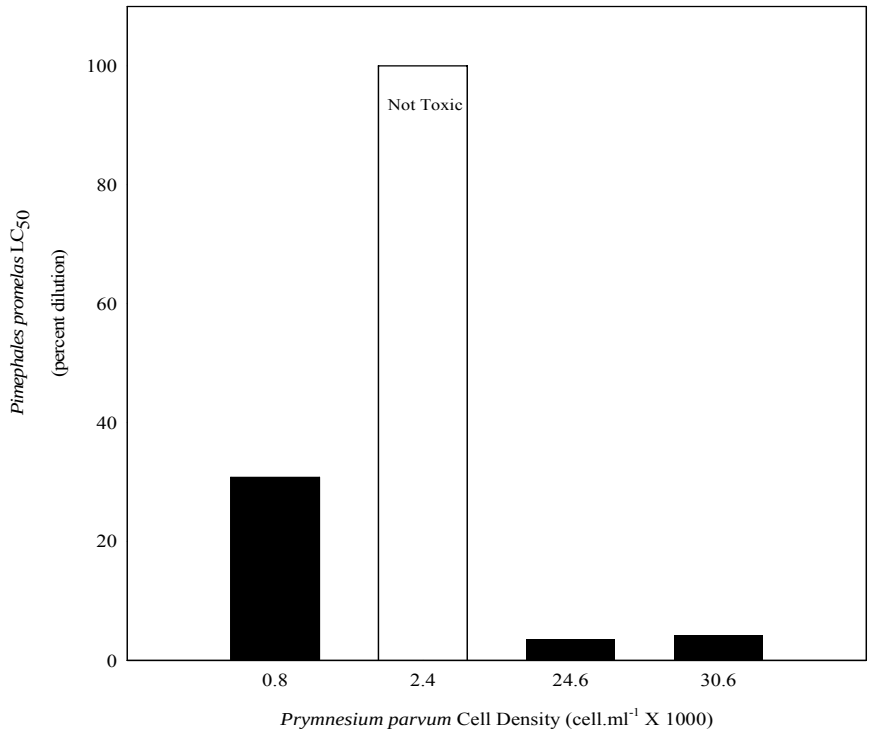
Pimephales promelas Survival

Fish survival was most severely affected on Day 28 for samples with *P. parvum* cells at N:P levels of 12.69, 14.90 and 15.94, and the 12.69 N:P level of cell free filtrate. Figure 9 a and Figure 9 b depicts *P. promelas* survival as a function of presence or absence of *P. parvum* cells, respectively. The LC_{50} values for the 12.69, 14.90, 15.94 N:P treatment levels were 3.65, 4.2 and 30.8% respectively. Whereas only the N:P level of 11.43 was not toxicity when *P. parvum* was present (Figure 9a), responses to cell free filtrate demonstrated reduced or no toxicity, except for one sample N:P= 12.69 that expressed a LC_{50} value of less than 50% (Figure 9b).

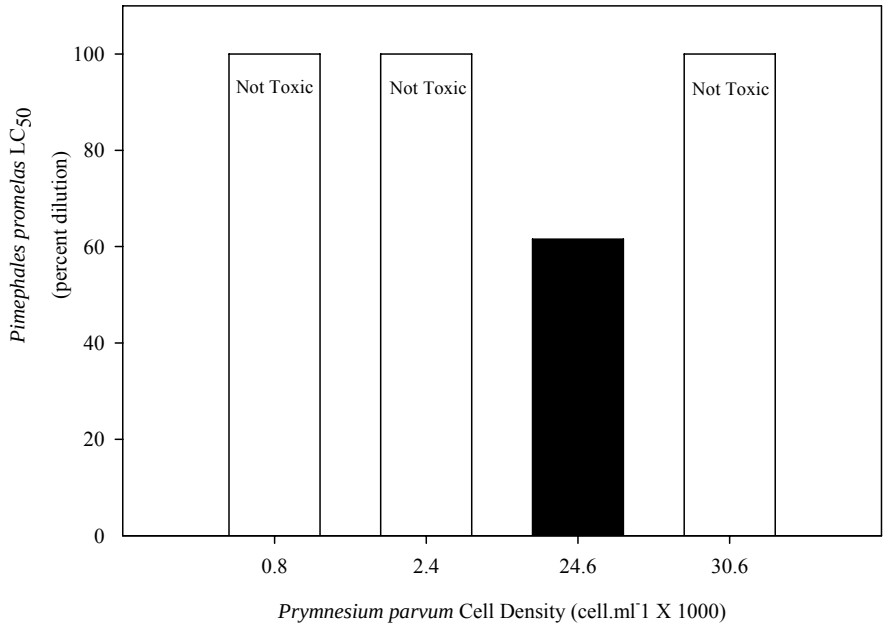
Lethal and sublethal toxicity to the three model organisms (*P. subcapitata*, *D. magna*, *P. promelas*) for undiluted samples with *P. parvum* present (non-filtered samples) is displayed in Figure 10, 11 and 12. Only the lowest N:P treatment level of 11. 43 stimulated growth of *P. subcapitata* compared to the other three N:P levels (Figure 10). Although *D. magna* reproduction was sublethally affected by *P. parvum*, the N:P treatment level of 14.90 caused 100% mortality (Figure 11). Generally, a weaker relationship was observed between *P. parvum* cell density and *P. subcapitata* growth (R^2 = 0.467, p = 0.317) than for *D. magna* reproduction (R^2 = 0.29, p = 0.1462) and *P. promelas* survival (R^2 = 0.961, p = 0.0198; Figure 12).

Prymnesium parvum Behavior

At the end of the growth period, *P. parvum* clusters or "swarming" behavior was observed. Subsequently, bacterial density and *P. parvum* swarming behavior may provide an indicator of increased toxin production and it could be linked to adverse effects on *P. promelas* (Figure 13).



a.



b.

Figure 9. a. *Pimephales promelas* fish mortality expressed as LC_{50} determined on samples with *Prymnesium parvum* cells at the end of growth period. b. *Pimephales promelas* fish mortality expressed as LC_{50} determined on *P. parvum* cell free filtrate at the end of growth period.

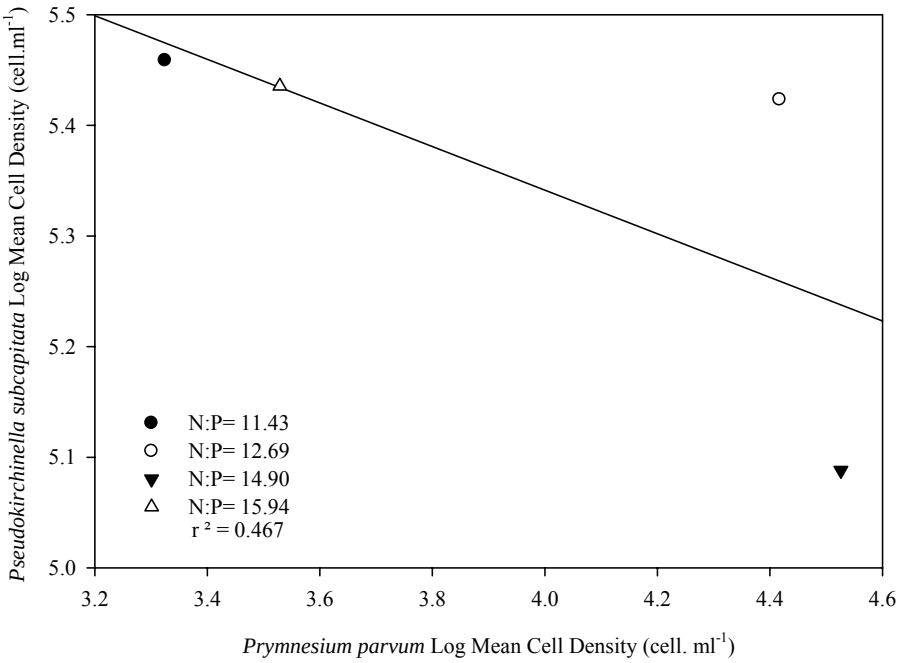


Figure 10. *Pseudokirchinella subcapitata* Inhibition Growth (Log mean ± S. D. cell density) on undiluted sample (higher concentration) as a function of *Prymnesium parvum* cell density (F= 1.755, *p*= 0.317).

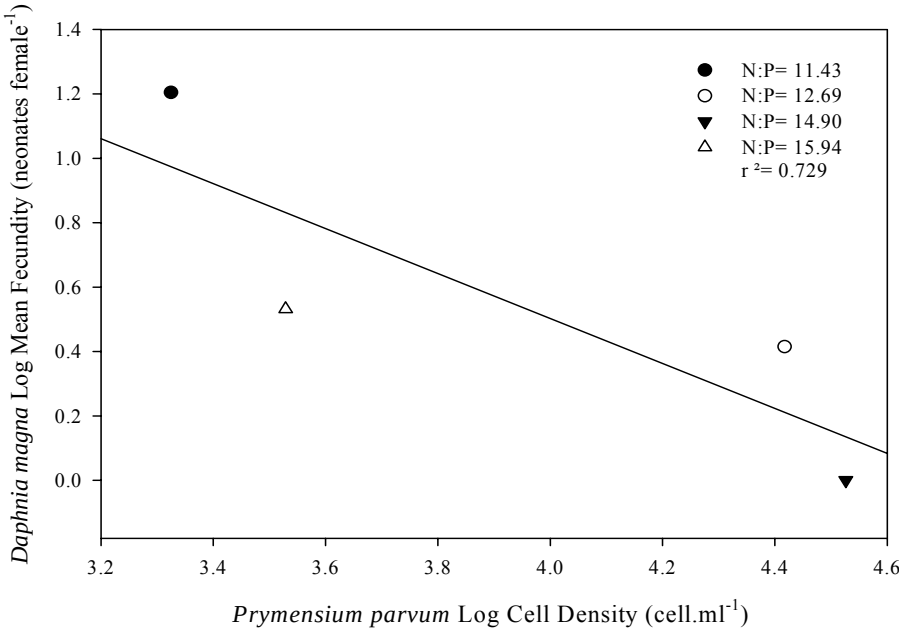


Figure 11. *Daphnia magna* Fecundity (log mean ± S. D. neonates female^{-1}) on undiluted sample (higher concentration) as a function of *Prymnesium parvum* cell density (F= 5.381, *p*= 0.1462).

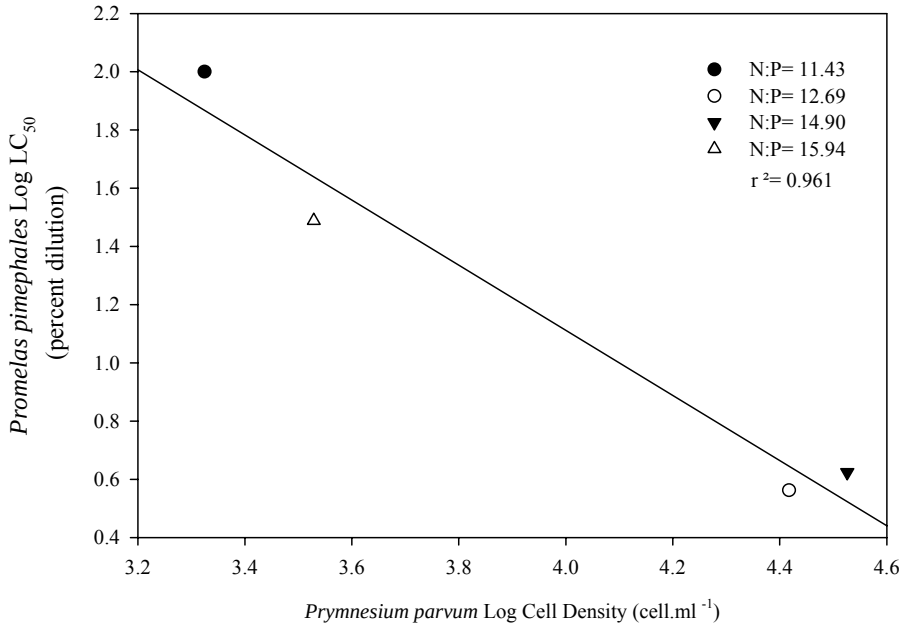


Figure 12. *Pimephales promelas* fish mortality expressed as Logarithm transformed LC_{50} determined on samples with *Prymnesium parvum* at the end of growth period (F = 49.012, $p = 0.0198$).

At the highest N:P ratio of 15. 94 the bacterial density was lower than other treatment levels. Interestingly, the most toxic treatment level (N:P = 12. 69) to *P. promelas* survival possessed higher bacteria density and higher *P. parvum* clustering behavior than the other treatment levels (Figures 13, 14) . Conversely, treatment levels with lower toxicity to *P. promelas* (N:P= 11.43, 14.90) included relatively lower bacteria densities and observations of *P. parvum* swarming behavior. The highest bacteria level was observed at an N:P of 14. 90, which possessed one of the highest acute toxicities to fish; however, no clusters were observed at this treatment level. No relationships between *P. parvum* swarming behavior and bacteria cell density (R^2= 0.0199, p= 0.8589) or fish mortality (R^2= 0.0068, p= 0.9176) were observed.

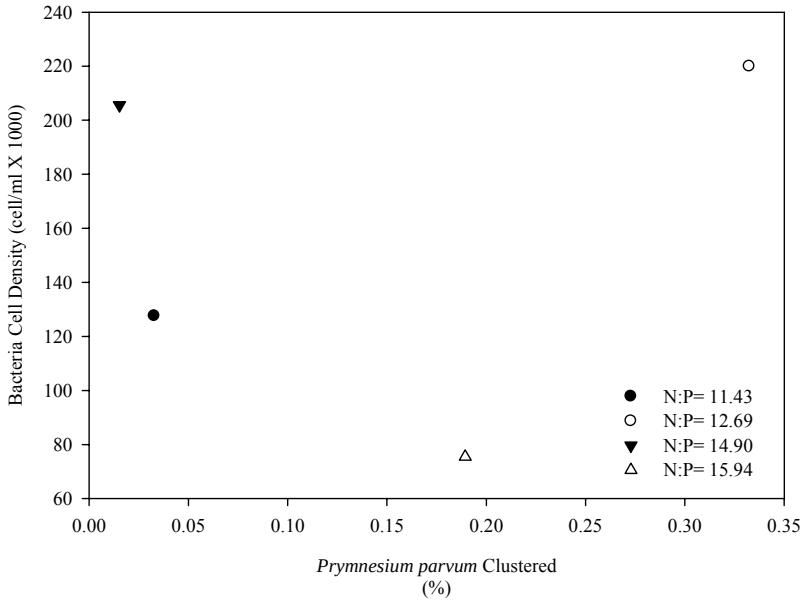


Figure 13. *Prymensium parvum* swarming behavior as a function of bacteria cell density (cell.ml^{-1}) at the end of the growth period (F = 0.0406, p = 0.8589).

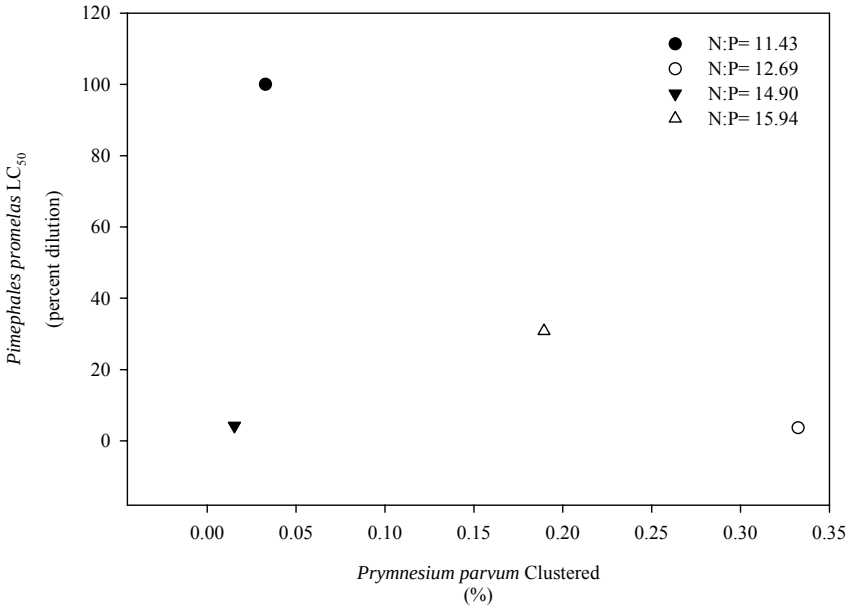


Figure 14. *Prymensium parvum* swarming behavior as a function of *Pimephales promelas* fish mortality expressed as LC_{50} determined on samples with *Prymnesium parvum* at the end of growth period (F = 0.0137, p = 0.9176).

CHAPTER FOUR

Discussion

The main objective of this thesis was to explore the potential effects of *P. parvum* on green algae competitors and zooplankton grazers in freshwater ecosystems. I first tested for these effects during a limnocorral experiment in a reservoir experiencing a *P. parvum* bloom and subsequently performed laboratory experiments to identify responses of algae, zooplankton and fish to *P. parvum*. Such couplings of laboratory and field responses are routinely employed in a weight of evidence approach to define stressor effects on aquatic systems (Brooks et al. 2004). *Prymnesium parvum* population densities elevated to bloom conditions in Lake Possum Kingdom during the spring of 2005 such that fish kills observed in the impoundment were attributed to *P. parvum*, indicating that ambient toxicity to fish and potentially other aquatic life (e.g., grazers, competitors) was likely prior to initiating the enclosure experiment. Although the indirect estimation of bioavailable toxins released by *P. parvum* in the water column under different laboratory and field conditions has been measured in various studies previously (Shilo and Aschner, 1953, McLaughlin, 1958 , Ulitzur and Shilo, 1966, Igarahsi et al., 1999), research to which I recently contributed represents the first implementation of standardized aquatic model organisms to understand *P. parvum* impacts to freshwater or marine ecosystems (Baker et al., 2007, Grover et al., 2007, Roelke et al., 2007, Errera et al., 2008).

In this study, I further employed these standardized models to identify toxicological benchmark concentrations (TBCs; e.g., LC_{50} , NOEC values) of field and

laboratory treatments. These TBCs may serve as useful biosensors for providing useful surrogate measures of bioavailable toxin levels to aquatic life because analytical standards are not available for the known *P. parvum* allelochemicals (prymnesins), though other compounds may be produced by the organism. Thus, quantitation of toxins produced by *P. parvum* is not analytically possible at the present time. Using fish as a model organism, for example, can provide more ecologically relevant information on the potential harm from *P. parvum* blooms than in vitro hemolytic activity, which has been historically employed in marine and estuarine studies, since fish-kills are the primary ecologically and economically important impact associated with this harmful algal species.

During the limnocorral study, fish mortality and cladoceran lethal and sublethal responses (reproduction) were more pronounced at the beginning of the study (Day 0), when a fish LC_{50} value of 9.8% was observed and *D. magna* could not reproduce, than at the end of this experiment. Although fish kills have been associated with *P. parvum* blooms for over 50 years, this study provided the first observation of *P. parvum* impacts on cladocerans. The different trophic impacts and interactions with *P. parvum* are yet to be fully understood in inland waters; however, studies of the adverse effects of a marine strain of *P. parvum* (Fistarol et al, 2003, Tillmann, 2003) have identified adverse effects on other grazers, including copepods, ciliates and rotifers, when *P. parvum* was present in samples or in cell free filtrate, which should have contained allelochemicals released to the water column. Specific effects observed in these studies included reduced growth, fecundity, and survival of the predacious grazers.

Effects of nutrient addition at molar N:P ratio of 20 were apparent after 14 d on *P. parvum* population growth, resulting in a dramatic increase in fish survival and

cladoceran reproduction by day 28 (Figure 4, 5), but the addition of BSE had no effect. Moreover, laboratory studies performed by Grover et al. (2007) examined the effect of repeated doses of ammonium and BSE as potential preventative treatment techniques; however, BSE were also ineffective at reducing *P. parvum* abundance and ambient toxicity to fish. These findings agree with the enclosure experiment that BSE had no effect reducing ambient toxicity to fish (mortality) and cladocerans (mortality and fecundity) even when applied at levels 50 fold higher than the recommended dosage by the manufacturer. In treatments not receiving nutrient additions, *D. magna* did not reproduce by Day 28 to the levels of limnocorrals receiving nutrient treatment (Figure 5). My laboratory cladoceran responses are consistent with in-lake conditions and ambient zooplankton community structure, which indicated that cladocerans were not present during the toxic *P. parvum* bloom in Spring 2005 (Roelke et al 2007).

At the end of the 4 week study (Day 28), a nutrient addition main effect was detected for *D. magna* reproduction and fish survival, indicating a significant decrease in ambient sublethal toxicity to cladocerans and fish (Figures 4b, 5b; Roelke et al., 2007). In addition to providing the first observation of cladoceran responses to *P. parvum* bloom formation, this observation subsequently also provides the first evidence of amelioration of ambient toxicity to cladocerans by nutrient amendments. However, my experimental findings agree with previous laboratory culture studies and suggest that nutrients strongly influence the ability of *P. parvum* to form blooms and its production of allopathic compounds (Roelke et al 2007).

When *P. parvum* experience a nutrient-limited environment, allelochemical production may increase such that the availability of nutrients is a controlling factor for algal growth and biomass when *P. parvum* gains a selective advantage over other

competitor species (Granéli et al, 2003). Toxin production could also be a result of excess of organic carbon storage, and lipid and carbohydrate production in excess to build up material for cell division functions (Granéli, 2003). Alternatively, production of allelochemicals may be a direct response to nutrient limiting conditions (Johansson and Granéli, 1999), and when nutrients are available, toxin production may decrease as algal biomass increases (Roelke et al, 2007). This influence is complex, however, because nutrient enrichment makes higher population of *P. parvum* densities possible, while also reducing the production of toxins due to more optimum conditions for growth.

My results are consistent with other laboratory studies for marine and estuarine systems where *P. parvum* allelopathy and grazer inhibition was reduced under nutrient-sufficient conditions (Johansson and Granéli, 1999, Granéli and Johansson, 2003, Barreiro et al, 2005, Uronen et al, 2005). The extent to which nutrient stoichiometry changes are linked to harmful algal blooms and toxin productions remains unknown (Granéli, 2006). *Prymnesium parvum* toxin production is enhanced when under both P- and N-limitation at significantly higher molar ratios differences (N:P= 4:1, 64:1), and some differences have been observed between marine and inland freshwater systems. Most adverse effects observed on marine systems (e.g. inhibition growth, grazer avoidance; Granéli and Johansson, 2003) were more pronounced at nitrogen deficient conditions as opposed to freshwater studies (Baker et al., 2007, Grover et al, 2007) in which phosphorus-limited conditions were more toxic to cladocerans and fish. However, my observations on toxicity when a necessary N:P ratio around 16 or lower, suggest that toxicity is linked to stress in general, not only by P or N-limitation but potentially to the presence of competitors or potential grazers, and it is strongly related to cell density.

Granéli (2006) has shown that *P. parvum* is able to avoid grazing specifically by excretion of toxins into the water. My results support this allelopathy mechanism for the Texas strain of *P. parvum*, because *D. magna* survival and/or reproduction was reduced by all nonoptimal N:P treatment levels when *P. parvum* cells population were observed. In fact, these previous studies suggest that toxin production is a response to physiological stress caused by nutrient limitation. My observation of the strong suppression of fish and cladoceran impacts by nutrient addition suggests that targeted and time-limited nutrient manipulations might be used to mitigate the effects of *P. parvum* blooms (Roelke et al., 2007).

During the spring experiments in Lake Possum Kingdom, Texas, enclosures with no addition of nutrients showed a slight change on phytoplankton succession of other dominant species, including chlorophytes, such that *P. parvum* population density was dominant (Roelke et al, 2007). In those enclosures receiving nutrient treatment, *P. parvum* population densities increased rapidly by day 14, but the phytoplankton community structure shifted to a dominance of chlorophytes by the end of the study period, which paralleled the previously discussed decline in ambient toxicity to fish and cladocerans. Following the observations from this limnocorral study my laboratory study further examined the potential impact of *P. parvum* on a model chlorophyte competitor.

Most blooms in Texas have occurred during the cool months (fall to early-spring) suggesting that temperature is an important factor. Also, light intensity and salinity varies with season and location signifying an important niche aspect in the growth of *P. parvum* (Baker et al., 2007). The lower range of physical conditions in Texas inland waters are not optimal for growth or abundance of *P. parvum* at the time when blooms occur. The selection of light, salinity and temperature parameters in my study were based

findings from previous laboratory culture experiments (Baker, et al., 2007, Grover, et al., 2007). Specifically, my laboratory study used temperature and salinity conditions (e.g., 15^{o}C and 2 psu) associated with bloom conditions for the Texas strain of *P. parvum*. *Prymnesium parvum* growth had a lag for the first 16 to 20 day, showing a slow growth and low or no toxicity to juvenile fish on the dates tested (Days 0, 14, 21). This could have resulted from low inoculated cell density and the lower temperature that may have affected growth and uptake of nutrients. Baker et al (2007) described that growth rate of the Texas strain is relatively insensitive to a wide range of salinity (e.g., 1 – 22 psu), but it grows optimally at salinities greater than inland waters where *P. parvum* generally blooms (e.g., salinities 2-4; TPWD, 2003).

Furthermore, the intensity of light affecting *P. parvum* algal growth in relation to temperature is not well defined; however, the response of *P. parvum* to light intensity may change on a case to case basis, but an increase and saturation of algal growth and photosynthesis on light intensity ranging from 100 to 200 μmol. m^{-2}. s^{-1} was reported by Baker et al. (2007). Light intensity and photoperiod potentially play an important role on *P. parvum* growth and production of the allopathic compounds. It has been found that excessive illumination inhibits the growth of *P. parvum* (Padan et al., 1967); however, light intensity in all the experimental units in this study was regulated and the experimental units were rotated to ensure uniform light exposure. Moreover, several authors agree with the theory that released toxins are photo-sensitive, and in prolonged periods of illumination, toxicity disappears. Cultures grown in the dark with lesser cells could be more toxic. It is believe that cellular metabolic activity is continued during the dark period, but light is needed to replenish energy and uptake of nutrients. If the cell

exhausts its reserve during prolonged dark cycles, it would not be advantageous since the cell cannot divide (Chisholm and Brand, 1981).

The effects of nutrient and salinity on the ecology of marine and freshwater phytoplankton are based on species-specific physiological differences (Cleave et al., 1981). Fundamentally, the ecology of marine and freshwater phytoplankton includes similarities. However, the differences of their physiological ecology can be influenced by the availability of resources, nutrient-limited uptake, growth and resource competition. Generally, marine algae are often nitrogen limited compared to freshwater algae, which are more limited by phosphorous (Hecky and Kilham, 1988). In addition, salinity is the predominant factor influencing the adaptation to osmotic and ionic properties in both marine and freshwater organisms (Wetzel, 2001). Salinity affects the productivity of algae osmotically because osmotic pressure inhibits photosynthesis in some freshwater algae (Cleave et al., 1981). In this study, I also evaluated the effects of increased salinity on *P. subcapitata* growth by comparing the effects of the modified media (f/2 media; Guillard and Ryther, 1962) to the standard algal assay procedure media (AAP; US EPA 1994, 2002). No significant impact was observed, which is consistent with other studies that have reported no significant growth inhibition on *P. subcapitata* at salinities below 9 parts per thousands (Specht, 1975, Cleave et al., 1981). Thus, the green algae responses observed in my study were not related to salinity.

The availability of nutrients and the ability to compete for the limiting resource is considered the main factor controlling algal growth and biomass (Granéli and Johansson, 2003). Granéli and Johansson (2003) describes that in marine environments there is evidence that elevated extracellular release of organic compounds occurs under conditions of nutrient limitation, especially along shorelines where these circumstances

may occur. *Prymnesium parvum* related-toxicity as a response as a nutrient-deficient stress has been well demonstrated in marine and brackish waters (Johansson and Granéli, 1999; Skovgaard, et al. 2003; Granéli and Johanson, 2003;Hägstrom and Granéli, 2004; Uronen, et al., 2005; Sopanen et al., 2006) and more recently in inland waters by research to which I have contributed (Baker et al., 2007; Roelke et al., 2007; Errera et al., 2008). Toxin production has been identified as a mechanism specifically used to control the growth of competing algae and potential grazers when growth is limited (Uronen et al, 2007). In this study I observed negative effects on a model competitor species, *P. subcapitata* but only when *P. parvum* was removed and green algae were exposed to cell free filtrate, which presumably contained allelochemicals. Conversely, the presence of *P. parvum* at 15 degrees Celsius had a significant stimulatory effect on the growth of *P. subcapitata* at the lowest N:P level and a stimulatory though not significant effect at other N:P levels. For example, initial densities for *P. subcapitata* inoculated at approximately $1.0 \text{ X } 10^4$ cell ml^{-1} increased almost 3 times higher than *P. parvum* growth, specifically at higher N:P treatments (14.90, 15.94).

The particular characteristics of *P. parvum* population growth and production of allelochemicals in inland water conditions under non-optimal growth conditions described in this study are in general agreement with previous research in marine systems. *Prymnesium parvum* production of allelochemicals may serve as a mechanism to control grazers and as a mixotrophic trait to immobilize potential prey organisms (e.g., bacteria) (Skovgaard and Hansen, 2003). Tillman (1998) studied in more detail the phagotrophic characteristics of *Prymnesium pattelliferum*, and the potential for toxin production to either kill or allow potential prey organisms to be encountered before ingesting. Similar observations were detected on this study with *P. parvum*. For the first 20 d of growth, the

percentage of encysted *P. parvum* cells was greater than motile (30% or more); it is possible that encysted cells do not actively take up nutrients from or release toxins to the media. Interestingly, at late exponential growth phases aggregations of motile cells (feeding swarms) were observed and experimental cultures with the highest percentage of motile and active cells exerted sublethal and lethal toxicity to *D. magna* and fish, respectively. This suggests that mixotrophic behavior may be linked *P. parvum* cellular stress release, thus increasing its toxin production and release (Legrand et al., 2001). My results showed higher toxicity on treatment levels with higher bacterial density, and higher *P. parvum* clustering behavior (N:P 12.69) compared to cultures with lower bacteria and swarming clusters that were not as toxic to fish. But the lack of relationship of these observations should be further investigated since one of the treatment levels with higher toxicity (N:P 14.90) had no clusters but higher bacterial densities. Bacterial populations in the media may be related the nutritional status and toxin production of *P. parvum* either by mixotrophic predation or bacterial competition with *P. parvum* for nutrients. *Prymensium parvum* cells may display such swarming behavior when nutrient conditions are non-optimal, subsequently shifting to mixotrophic strategies such as ingestion of bacteria as another source of nutrients (Fistarol et al, 2003; Granéli, 2003; Tillman, 2003, Uronen et al, 2007).

Conclusions and Recommendations

Baker et al. (2007) predicted that *P. parvum* should experience high growth and abundance during warmer temperatures (~30^{o}C), but the bloom occurrence in Texas inland winter conditions during cooler winter temperatures indicate that other factors are important for *P. parvum* bloom formation. Subsequently, ecological factors such as

zooplankton grazing, species competition and nutrient limitation are likely important for bloom initiation and termination events. The findings of my laboratory study provides the first attempt to define sublethal and lethal effects on the freshwater competitor *P. subcapitata* and the grazer *D. magna* under previously studied nutrient conditions and non-optimal temperature and salinity levels that increase toxicity to juvenile fish in marine systems. However, my study initially suffered from limited replication of N:P treatments due to the large culture conditions required to perform the types of ecotoxicity studies in this thesis. In addition, N:P treatment levels were analytically verified to be different from my nominal concentrations, and those N:P levels were very similar. The variability between laboratory and field conditions makes any study in ecology and ecotoxicology challenging, but cladoceran and green algae responses in my laboratory studies were generally consistent with observations by Roelke et al. (2007) of in-lake phytoplankton and zooplankton community structure. My results support that under non-optimal conditions *P. parvum* growth is limited and the production and adverse of allelochemicals can be significant, but nutrient amendments may be useful to ameliorated bloom formation. Further, my limnocorral and laboratory *in vivo* results provide more ecologically realistic information than previous studies with *in vitro* hemolysis models because algal competitors, zooplankton grazers and fish are important parts of freshwater reservoir food webs. Future research is need in several areas to advance an understanding of *P. parvum* effects on aquatic systems. These include:

1. field studies are needed to understand the interactions among of seasonal, spatial and ecological factors that influence bloom formation and termination in impoundments

2. analyticals standards of *P. parvum* toxins are needed to perform toxicology to understand the mechanisms of toxicity of these toxins to aquatic life, terrestrial wildlife (shorebirds) and mammals
3. analyticals standards of *P. parvum* toxins are needed to perform studies to determine how light, pH and ionic composition influences the fate of these toxins in reservoirs experiencing blooms
4. the potential relationships among bacterial abundance, nutrient limitation, mixotrophy and toxicity requires additional study.

APPENDICES

APPENDIX A

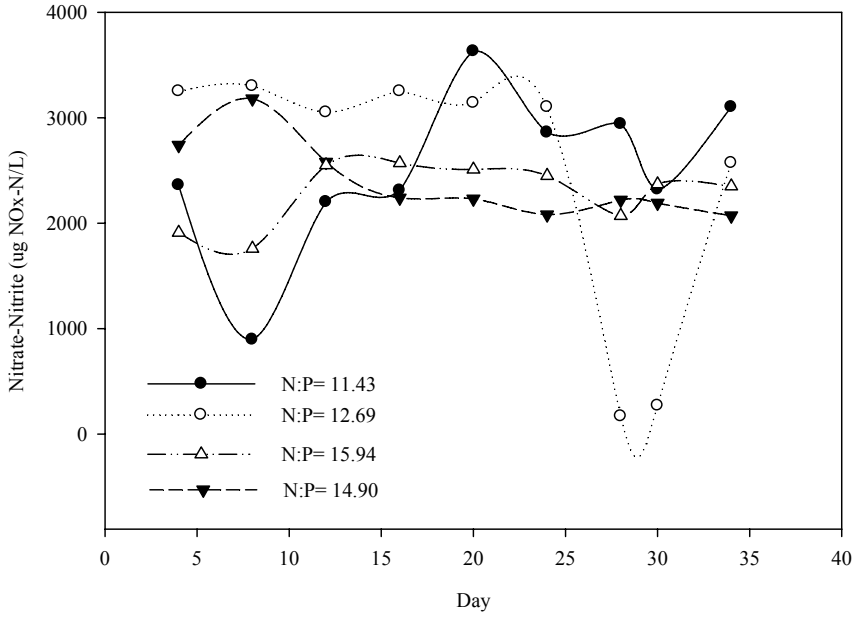
Table A.1. Water chemistry results on experimental cultures at the end of *Prymnesium parvum* growth period.

Sample	pH	DO (mg/l)	Conductivity (μS)	Salinity (ppt)	Temperature (ºC)
N:P= 11.43	6.64	8.44	3679	1.90	21 5
N:P= 1269	6.80	7.47	3685	1.90	21.5
N:P= 14.90	6.80	8.7	3811	2.00	21.5
N:P= 15.94	6.80	7. 94	3782	2.00	21.6

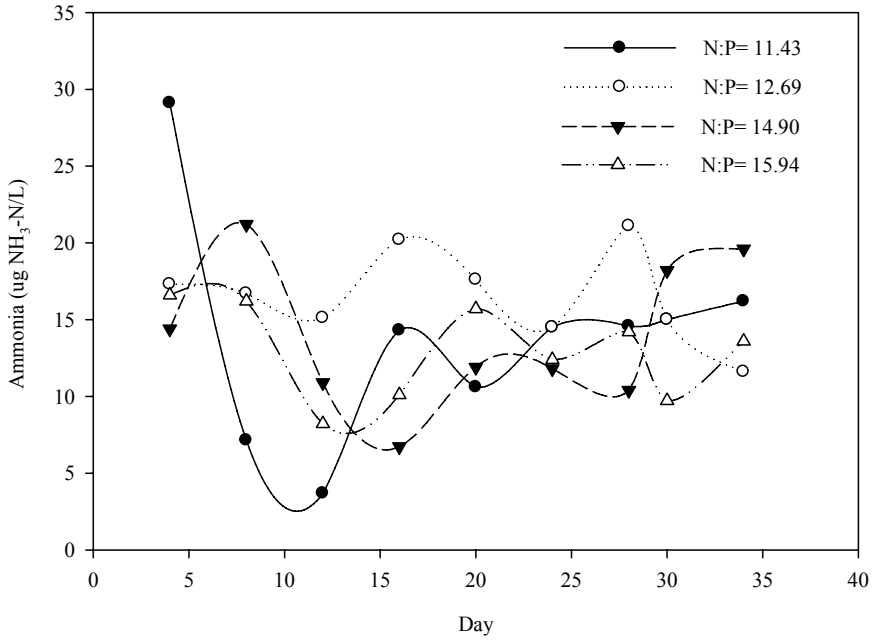
Table A. 2 Nominal and Observed nutrient stoichometry at the beginning and the end of *Prymnesium parvum* growth period

	N:P 11.43			N:P= 12.69			N:P= 14.90			N:P= 15.94		
Day	[Nominal] N:P(μM))	[Observed] DIN:SRP* (μM)	Molar Ratio	[Nominal] N:P(μM))	[Observed] DIN:SRP (μM)	Molar Ratio	[Nominal] N:P(μM))	[Observed] DIN:SRP (μM)	Molar ratio	[Nominal] N:P(μM))	[Observed] DIN:SRP (μM)	Molar Ratio
4	144:36:00	137. 52: 16. 69	8. 24	144:36:00	196. 60: 16. 50	11. 92	880:36:00	170. 53: 12. 95	13. 17	880:36:00	233. 21: 8. 56	27. 26
8	144:36:00	126. 78: 15. 79	8. 03	144:36:00	228. 49: 13. 69	16. 69	880:36:00	135. 77: 14. 04	9. 67	880:36:00	236. 74: 15. 76	15. 03
12	144:36:00	182. 60: 15. 30	11. 93	144:36:00	184. 93: 13. 92	13. 29	880:36:00	157. 29: 11. 69	13. 46	880:36:00	218. 78: 14. 88	14. 7
16	144:36:00	184. 16: 14. 72	12. 51	144:36:00	160. 37: 13. 46	11. 91	880:36:00	165. 90: 14. 27	11. 63	880:36:00	233. 42: 15. 08	15. 48
20	144:36:00	180. 28: 11. 59	15. 55	144:36:00	160. 02: 14. 11	11. 34	880:36:00	259. 86: 14. 79	17. 57	880:36:00	225. 38: 14. 11	15. 97
24	144:36:00	175. 76: 14. 75	11. 91	144:36:00	149. 31: 13. 69	10. 91	880:36:00	205. 17: 11. 24	18. 26	880:36:00	222. 31: 14. 30	15. 54
28	144:36:00	148. 77: 14. 56	10. 22	144:36:00	159. 20: 13. 11	12. 15	880:36:00	210. 89: 11. 27	18. 72	880:36:00	122. 13: 13. 11	9. 32
32	144:36:00	169. 86: 13. 53	12. 56	144:36:00	157. 62: 12. 14	12. 98	880:36:00	166. 67: 11. 98	13. 91	880:36:00	193. 79: 12. 53	15. 47
36	144:36:00	168. 71: 14. 17	11. 9	144:36:00	149. 15: 11. 49	12. 98	880:36:00	222. 43: 12. 59	17. 67	880:36:00	184. 27: 12. 53	14. 71
Mean			11. 43			12. 69			14. 90			15. 94

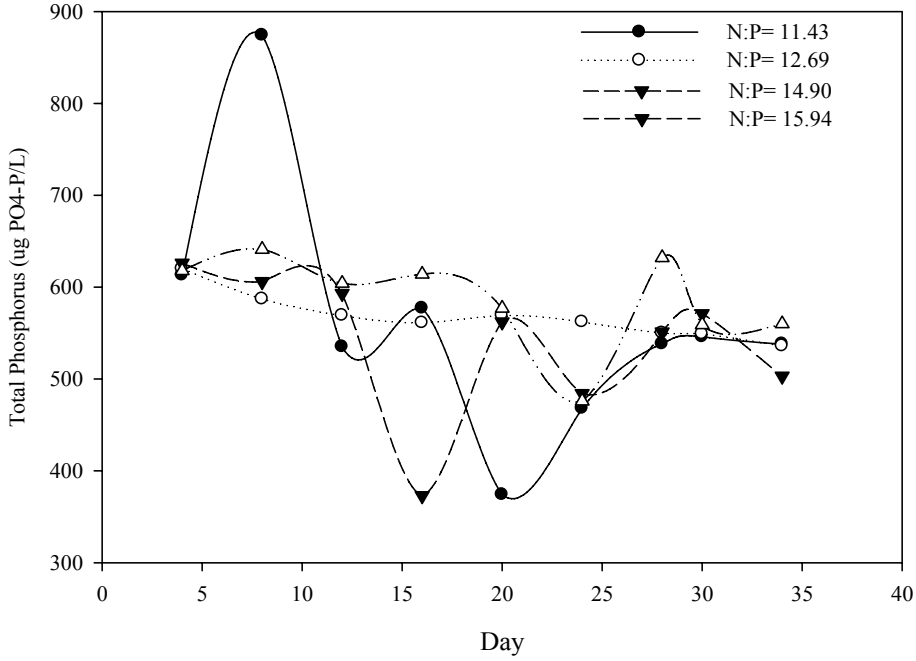
*DIN: dissolved inorganic nitrogen (NO_3, NO_2 and NH_4) : SR(soluble reactive phosphorous or orthophosphate) ratio.


Nitrate-Nitrite (ug NOx-N/L)
4000
3000
2000
1000
0
N:P= 11.43
N:P= 12.69
N:P= 15.94
N:P= 14.90
0
5
10
15
20
25
30
35
40
Day

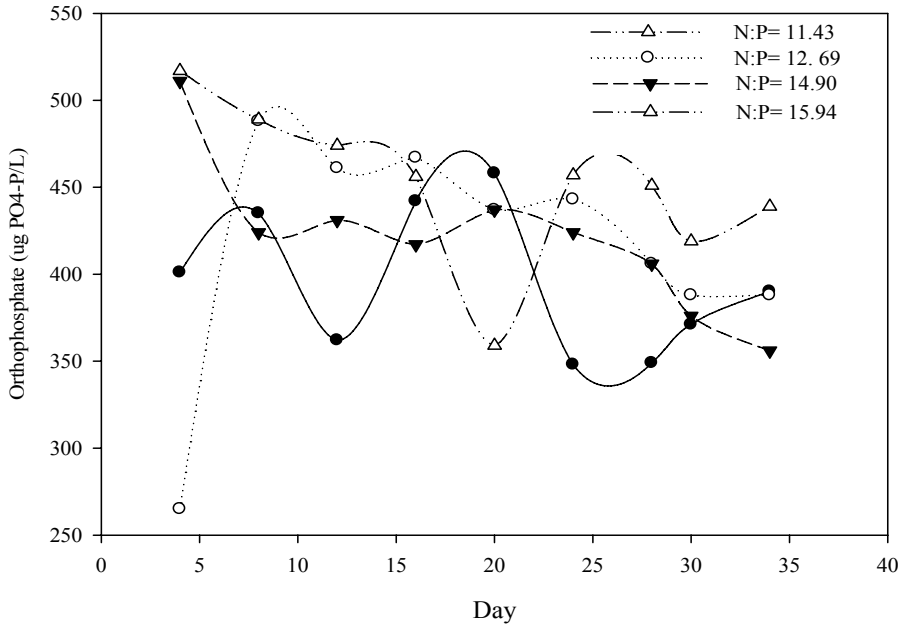
a.


Ammonia (ug NH3-N/L)
35
30
25
20
15
10
5
0
N:P= 11.43
N:P= 12.69
N:P= 14.90
N:P= 15.94
0
5
10
15
20
25
30
35
40
Day

b.



c.



d.

Figure A.3 a-d. Nutrient analytical chemistry for experimental cultures during *P. parvum* growth period (Total Nitrogen data not available).

APPENDIX B

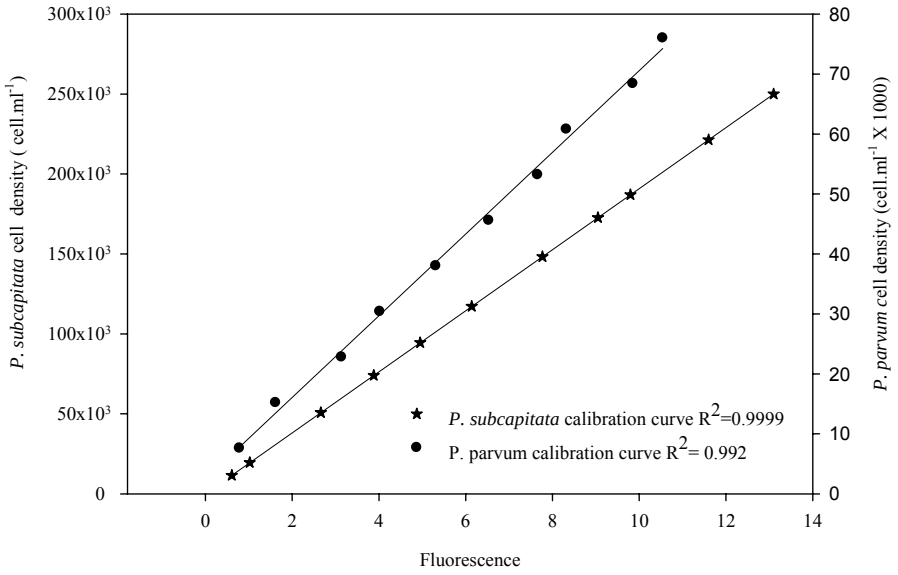


Figure B.1 *Pseudokirchinella subcapitata* and *Prymensium parvum* calibration curves

APPENDIX C

Table C.1 Bacteria cell density (cell.ml^{-1}) at the start and the end *P. parvum* growth phase as opposed to *P. parvum* cell density (cell.ml^{-1}), and the number of observations on *P. parvum* clustered cells.

Treatment N:P Molar Ratio	Bacteria Cell density (cell.ml^{-1})		Mean DIN:SRP	*P. parvum* clustered or "swarm" observations (% of clustered cell in sample)
	Start	End		
11. 43	2. 20 X 10^4	1. 28 X 10^5	163. 83: 14. 57	0. 1894
12. 69	2. 39 X 10^4	2. 20 X 10^5	171. 74: 13. 57	0. 0153
14. 90	2. 15X 10^4	2. 06X 10^5	192. 77: 12. 76	0. 0328
15. 94	2. 26 X 10^4	7. 55 X 10^4	207. 78: 13. 43	0. 3324

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