THE ROLE OF LIFE HISTORY IN THE TOXICITY OF THE TOXIC GOLDEN HAPTOPHYTE *PRYMNESIUM PARVUM*

by

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ABSTRACT

THE ROLE OF LIFE HISTORY IN THE TOXICITY OF THE TOXIC GOLDEN HAPTOPHYTE *PRYMNESIUM PARVUM*

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Prymnesium parvum is a harmful algal species that causes widespread fish kills. It is usually motile, but non-motile stages have been observed that may represent an encysted stage. The relationship of these life history changes to the abundance and acute toxicity to fish of *P. parvum* was studied. As part of research into factors regulating abundance and toxicity of this species, a series of experiments was conducted. The first was a semi-continuous culture experiment examining limiting nutrient (N vs. P), temperature (14 or 20 °C), and dilution rate (at four levels). Samples from the lower temperature generally exhibited greater acute lethal toxicity to fish and toxicity increased with dilution rate. The increase of toxicity with dilution rate was surprising, because increased dilution rate decreases the degree of nutrient limitation and other studies indicate that increased severity of nutrient limitation promotes toxicity. In this study, all cultures contained high proportions of non-motile cells, suggesting a hypothesis that small populations grow larger and deplete nutrients available in the medium, and then release toxin

triggered by a stressed environment of decreased nutrient availability. If the environment remains stressful, encystment is eventually triggered, leading to a reduction of toxin production. With the assumption that toxins degrade, we would expect to see toxicity levels decrease.

To test a hypothesis that seasonal variations trigger formation of non-motile cells, nutrient-limited laboratory batch cultures contrasted summer and winter conditions typical of Texas inland waters. Another experiment addressed variations of salinity and temperature in a full factorial design with two other factors that might affect life history and acute toxicity to fish: silica availability (cell walls of cysts might be silicified), and mixing by aeration. Another experiment compared nutrient-sufficient and nutrient-limited cultures. An additional treatment added glucose as a carbon source for nutrient-limited cultures, to encourage mixotrophy, which is potentially related to toxic activity.

Acute toxicity to fish in simulated summer and winter cultures continuously increased for 20 days and then remained very high for another 22 days. This toxic activity did not differ significantly between summer and winter conditions until days 35 and 42, when winter cultures were more toxic. Non-motile cells remained sparse in both treatments, while motile cells dominated. In the four-factor experiment, acute toxicity to fish was significantly related to several interactions among experimental factors, but no strong relationships to non-motile cells were evident. In the remaining experiment, nutrient-sufficient cultures with highest abundance produced the greatest proportion of non-motile cells. In these cultures, acute toxicity to fish varied over time in relation to the numbers of motile cells and dropped when non-motile cells became dominant. In contrast, nutrient-limited cultures maintained lower total abundance, dominance by motile cells, and higher toxicity to fish. These observations suggest that high population density induces the non-motile phase, rather than stressful conditions of nutrient limitation or seasonal variation, which induce higher toxic activity.

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CHAPTER 1

GENERAL INTRODUCTION

Algal bloom formation is a common occurrence in inland and coastal waters worldwide. In recent years harmful algal blooms have occurred with increased severity and duration, which may be a result of increased nutrient pollution of inland and coastal waters. Negative impacts of algal blooms can include massive fish kills and human health concerns. In order to mitigate these environmentally deleterious occurrences, research focused on understanding the triggers for these harmful algal blooms is imperative.

Golden algae, *Prymnesium parvum*, are a harmful algal species of major interest in the state of Texas and other states in the south central USA (Barkoh and Fries 2010, Southard et al. 2010). The first recorded bloom formation of *P. parvum* occurred in 1985 along the Pecos River (James and de la Cruz 1989). Golden algae produce toxic chemicals which have caused and continue to cause widespread fish kills in lakes and rivers resulting in negative impacts on local economies, including fisheries and tourism markets. Fish kills statewide have created economic losses in the millions of dollars (Southard 2010). Since the first bloom was discovered in 1985, the majority of fish kills have occurred within the past 10 years (Roelke et al. 2011). These large fish-killing blooms in riverine reservoirs occur primarily in winter, following extended periods of low flow, and forecasts of declining river flows due to climate change and increasing human use suggest that bloom frequency could increase in the future (Roelke et al. 2011).

The factors leading to bloom formation of golden algae are still poorly understood. However, prior research indicates that blooms may be triggered by eutrophic conditions and brackish water (Guo et al. 1996). The inland waters of western Texas and many other parts of the American southwest often have sufficient salinity to support growth of *P. parvum*. Many of

these water bodies are eutrophic, and high salinity tends to occur during periods of low river flow that are also associated with blooms of *P. parvum* (Roelke et al. 2011). This species produces a combination of different toxins that are cytotoxic, ichthytoxic, and exhibit hemolytic activity (Brooks et al. 2010, Manning and LeClaire 2010). These toxins are most dangerous to gill breathing organisms, especially fish, due to the exposure of the gills to the toxic waters. The toxins lyse the exposed cells and cause hemorrhaging. One of the major issues of concern is when and under what conditions will these harmful algae bloom and create such toxic conditions. Although fish are especially susceptible to the toxins of *P. parvum*, the functional role of these toxins may have more to do with inhibition of organisms that graze on *P. parvum* (Graneli and Johannson 2003, Rosetta and McManus 2003, Sopanen et al. 2008), immobilization of prey for mixotrophic feeding (Tillman 2003, Nygaard and Tobiessen 1993), or allelopathic effects on competing algae (Fistarol et al. 2003, Fistarol et al. 2005). Efforts to uncover the mechanisms and dynamics of toxic events are necessary to build a predictive understanding of *P. parvum*.

The influence of life history transitions such as encystment and germination on the formation of harmful algal blooms has long been suggested (Anderson 1997) but the details of environmental cues, timing, and relation to population dynamics remain essentially unknown. A bloom in Norway was characterized by an initial domination of non-motile cells which shifted to dominance by motile cells coupled with a rise in toxicity during the bloom event (Johnsen 2010). In 2009, researchers in Greece observed a *P. parvum* bloom in Lake Koronia with flagellated motile cells, as well as, what they described as non-motile coccoid cells (Genitsaris 2009). It is not clear that these non-motile cells are true cysts, since they lack a thickened cell wall, and they might be a form that enhances sinking so that *P. parvum* can feed mixotrophically on microbial prey in the benthos. Germination of presumed cysts present in pond sediments has been noted by fish hatchery personnel in the Texas Parks and Wildlife Department (G. Southard, personal communication).

The observation of non-motile cells within our laboratory as well as in the current literature (Johnsen 2007, Genitsaris 2009), inspired the need to further investigate the role of such cells and their relation to the toxicity of this organism. While it is known that *P. parvum* forms these non-motile stages and cysts, the cues for immobilization, encystment and germination are unknown. The figure below represents current understanding of how changes in life history can affect toxicity during bloom development (Brooks et al., 2011). This hypothesized life history states that a small active population of cells will grow into a larger population coupled with an increase in toxicity. Since *P. parvum* is a mixotrophic species, these cells can use the released toxins to immobilize or lyse their prey. As the prey population is depleted or if prey is not available to begin with, the toxicity is expected to decline followed by immobility or encystment. This last step coincides with the bloom termination, which can occur from other causes such as hydraulic flushing, grazing, or viral infection.

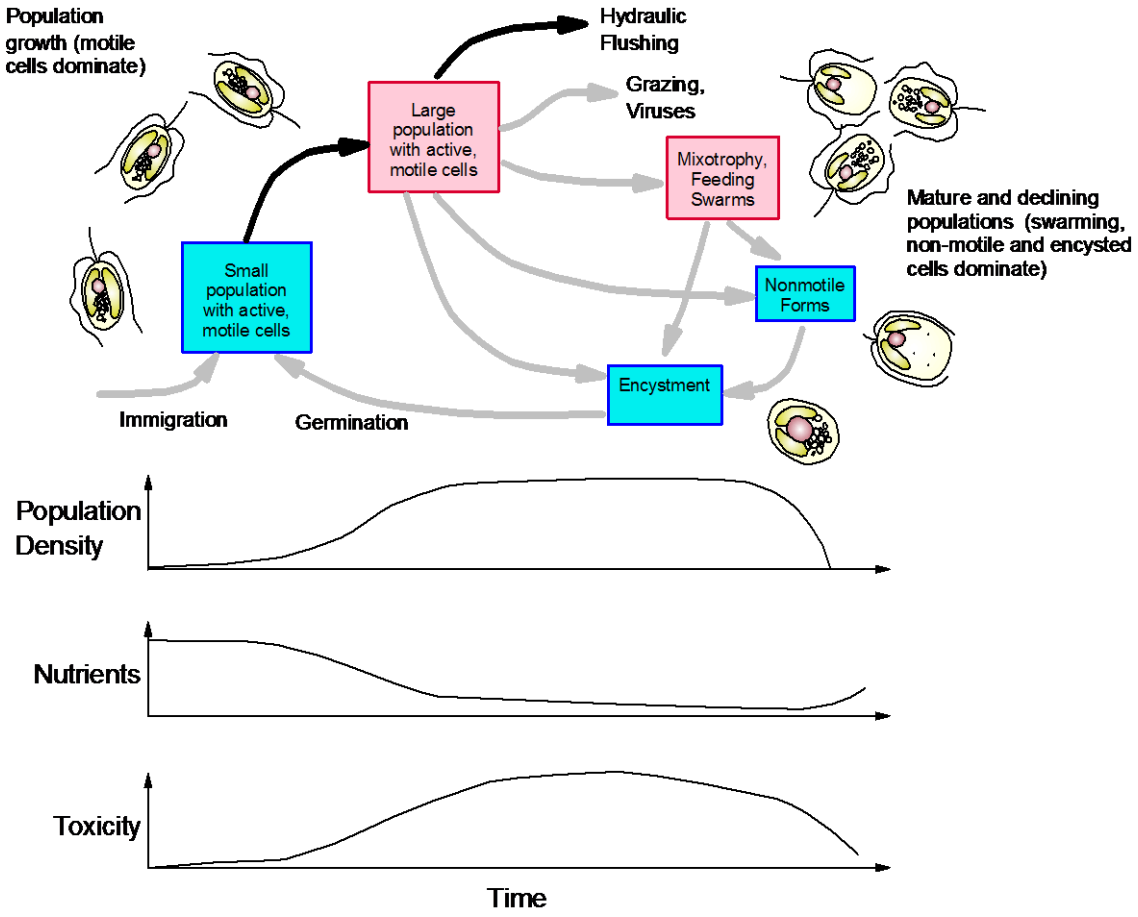

Figure 1.1 Life history and bloom dynamics of *P. parvum*. Red boxes indicate stages known to be toxic. Blue boxes indicate stages known or suspected to be nontoxic. Black arrows show transitions known to be important in dynamics of toxic blooms. Gray arrows show those transitions whose importance to bloom dynamics is less well understood. Graphs show typical dynamics of population density, nutrients and toxic activity during fish-killing blooms. Figure from Brooks et al., 2011.

The strain of *P. parvum* used in the research described here (UTEX LB ZZ181, isolated by J. Glass from the Colorado River) often undergoes life history changes in culture, sometimes producing populations dominated by non-motile cells, and providing a unique opportunity to undertake controlled studies. The experiments undertaken are part of a larger set of investigations of this harmful algal species, conducted by researchers at University of Texas at Arlington, Texas A&M University, and Baylor University. This broader project has been

sponsored by agencies with a strong interest in this species: the Texas Parks & Wildlife Department, the US Fish & Wildlife Service, and others, and encompasses additional laboratory studies, observational and experimental field studies, and mathematical modeling. Observations on these life history changes will allow these processes to be incorporated into the developing understanding of this species' population dynamics. The results should also open new lines of study involving the proximate extracellular signals mediating these life history shifts, and may eventually lead to novel bloom control strategies based on the manipulation of life history events.

For the work described in this dissertation, several laboratory culture experiments were done to examine the role of life history shifts between motile and non-motile stages in the toxicity of *P. parvum*. These experiments examined such shifts within the context of other factors known or suspected to affect the toxic activity of this species, such as growth rate, nutrient limitation, temperature, and salinity. The motivating hypotheses and objectives of each individual experiment are summarized in the following sections. Data from the series of experiments were also combined into a synthetic regression analysis to assess the relative influence on toxic activity of the factors manipulated and observed in the individual experiments.

1.1 Growth and Toxicity of *Prymnesium parvum* in Relation to Growth Rate, Nutrient Limitation, and Temperature in Semi-continuous Cultures

Although many aspects of the initiation and termination of *P. parvum* blooms remain uncertain, temperature, nutrient and light availability are likely to be important. In laboratory studies, *P. parvum* is more toxic under nutrient limitation (nitrogen and phosphorous) (Dafni et al. 1972, Johansson and Graneli 1999) and at suboptimal temperature, light, and salinity conditions (Baker et al. 2007). Similar responses to varying light, temperature, salinity and other parameters have been reported for a variety of algal species producing toxins (Parnas et al. 1962, Dafni et al. 1972, Larsen et al. 1993, Larsen and Bryant 1998).

Previous work has been done to study the effects of nutrient limitation on *P. parvum*. Johansson and Granéli 1999 studied nutrient limitation (N and P) under semi-continuous conditions, and measured toxicity using hemolytic assays. They found that hemolytic activity was higher in the cultures grown under N or P deficiency. Toxicity was enhanced by either N or P limitation, suggesting that toxicity is related to physiological stress rather than to a particular nutrient.

Semi-continuous cultures are used to approximate true continuous, chemostat cultures. In a true chemostat, culture volume is continuously removed from the culture vessel at a constant rate, while being continuously replaced with the same volume of fresh media. With the semi-continuous method, a certain culture volume is removed periodically and also replaced, but discretely in time rather than continuously. Both true chemostats and semi-continuous cultures create defined states of nutrient limitation. The resident population must grow at a rate that balances the dilution rate imposed by the replacement of culture volume, and in this steady state growth, a single nutrient limits the population growth rate (Droop 1974). Quantitatively, higher dilution rates imply higher growth rates, which in turn require higher concentrations of the limiting nutrient both within cells and in the extracellular medium. According to the Droop equation for nutrient kinetics, as the dilution rate is increased there increase in nutrient quota per cell follows (Droop 1973):

$$\mu=\mu'_{max}\left(1-\frac{Q_{min}}{Q}\right)$$

where μ is the population growth rate ($time^{-1}$), Q is the quota of nutrient per cell ($mol\ cell^{-1}$), μ'_{max} is a maximal growth rate parameter ($time^{-1}$), and Q_{min} is the "subsistence" quota ($mol\ cell^{-1}$) at which growth rate goes to zero.

For *P. parvum*, the observation that nutrient limitation makes populations more toxic leads to a prediction that in continuous or semi-continuous cultures, toxicity would decrease with increasing dilution rate. As the growth rate increases, the quota for the limiting nutrient increases, and toxicity should decrease as the degree of nutrient limitation decreases.

Using phosphorus-limited batch cultures sampled at high frequency, Skingel et al. (2010) found that cell quotas for phosphorus ranged up to several hundred fmol cell^{-1}, but quotas decreased over time, and populations developed strong hemolytic activity as the quota dropped below about 5 fmol cell^{-1}, similar to the quotas at which others observed high toxic activity (Johansson and Granéli 1999, Uronen et al. 2005). Population growth rate also declined over time, and the following parameter estimates were obtained for Droop's equation: μ'_{max} at 0.50 d^{-}1 and Q_{min} at 5.0 fmol cell^{-1}. These estimates should be treated cautiously, because errors of estimation were large, and even lower quotas down to 1-2 fmol cell^{-1} were observed in the late stationary of these populations.

In the semi-continuous experiment conducted for this study, it was expected that toxic activity of *P. parvum* would increase for cultures at lower dilution rates characterized by greater nutrient limitation. This expectation was tested with both nitrogen and phosphorus as limiting nutrients, and at two different temperatures to see whether the hypothesized effect varied with these factors.

1.2 Growth and Toxicity of *Prymnesium parvum* in Relation to Simulated Seasonal Conditions

Several observations motivate an investigation of how seasonal changes influence the toxicity of *P. parvum* and its transitions to non-motile or encysted stages. In the south central U.S., fish-killing blooms of this species tend occur during the cooler months of the year between October and April (Southard et al. 2010, Roelke et al. 2011). Greater toxicity to fish has been observed in laboratory cultures grown at low temperatures that are suboptimal for *P. parvum* growth (Baker et al. 2007). Later, Baker et al. (2009) observed an interaction between salinity and temperature, which could weaken the negative effect of low temperature on growth and might explain how blooms of high population density can occur during the cooler months.

Encystment of algae has been viewed as a survival strategy that occurs under suboptimal conditions (Green et al. 1982, Johnsen et al. 2010). Optimal growth for *P. parvum*

has been reported to be between 25 ^{o}C and 30 ^{o}C (Larsen and Bryant 1998, Baker et al. 2007). Therefore, it may be hypothesized that under winter conditions transitions to non-motile or encysted cell stages might be observed. If such an effect occurs, the effect of suboptimal growth conditions on toxicity would be complex: although previous studies suggest that suboptimal conditions can stimulate toxic activity, non-motility or encystment under such conditions could reduce it. These complex effects are likely to be time-dependent, so in this experiment a time-series approach was taken with several sequential observations of population status and toxic activity.

1.3 Factors Affecting Toxic Activity and Non-motile Stages of *Prymnesium parvum*: An Experiment Exploring Salinity, Temperature, Aeration, and Silica Additions

The experiment described in section 1.2 examined the toxic activity of *P. parvum* in cultures simulating the winter and summer conditions found in Texas lakes where blooms have occurred. Non-motile cells proved too scarce in that study, and thus their formation could not be related to the variations in toxic activity observed. Because the experimental design used in that experiment tested only particular combinations of salinity and temperature characteristic of two seasons, the possible influence of interactions of these and other factors on toxic activity and formation of non-motile stages were not fully assessed. Full factorial experimental designs are necessary to assess multiple factors and their interactions, and that was the goal of this next experiment. Temperature, and salinity are known to affect the growth and toxicity of *P. parvum* (Baker 2007, Granéli and Salomon 2010), and they can also be hypothesized to affect the formation of non-motile cells. Two levels of these factors were crossed in the experimental design used here. Two additional factors were also explored, aeration and silica additions. Aeration has been reported to enhance the toxicity of *P. parvum* cultures (Igarashi et al. 1998, Skingel et al. 2010), and thus it has been suggested that the degree of wind mixing might affect toxicity of natural populations (Granéli and Salomon 2010). Populations might also respond to

signals of mixing as a cue for life history transitions such as formation of non-motile stages or cysts. Siliceous scales have been observed on cysts of *P. parvum* (Pienaar et al. 1994), thus silica in the medium might enable building the thickened cell coverings needed for encystment.

1.4 Population Density and Nutrient Limitation of *Prymnesium parvum* in Relation to Toxicity and Formation of Non-motile Stages

Field observations suggest that non-motile stages might be associated with less toxic natural populations, and observations during some of laboratory experiments conducted in this study suggested that non-motile cells occur under lab conditions and are associated with reduced toxicity to fish. The seasonal conditions and factors such as temperature, salinity, aeration, and silica tested in the experiments described in sections 1.2 and 1.3 identified factors influencing toxicity to fish, but did not find strong influences on motile versus non-motile cells.

Nitrogen and phosphorus are inorganic nutrients that play a pivotal role in *P. parvum* growth and toxin production (Fistarol et al. 2003, Granéli and Johansson 2003, Skovgaard and Hansen 2003, Fistarol et al. 2005, Granéli and Salomon 2010). Lake Varsgsundet in Finland experienced a massive fish-kill event in the summer of 1997 (Lindholm et al. 1999). It had been determined that nitrogen and phosphorus had been in high concentrations prior to the bloom event. Through the duration of the bloom nitrogen and phosphorus concentrations declined. Granéli and Salomon (2010) concluded that the decline in these nutrient concentrations stimulated the increase in toxin production. This also gives *P. parvum* a competitive advantage over other phytoplankton. In another example, The Oued Mellah Reservoir in Morocco endured a bloom in 1999 (Sabour et al. 2002). The N:P ratio was high during the bloom. Nitrogen was high in concentration, while phosphorus levels were below the detection limit (Sabour et al. 2002). The depletion of phosphorus was suggested as the factor that led to the increased toxin production (Granéli and Johansson 2003). These examples provide a connection between nutrient availability and bloom formation and toxin production.

While the effects of nutrient depletion on toxin production have been well documented in several experiments, the effects of nutrient dynamics on the motile and non-motile cell phases of *P. parvum* have yet to be fully explored. The hypothesis for last experiment in the series reported here is that nutrient deficiency induces a shift from a population dominated by the motile cell stage to a population dominated by the non-motile cell stage as the severity of the nutrient depletion increases over time. Such a trend of progressive nutrient depletion occurs in batch cultures, so that experimental approach was adopted. Toxic activity would be expected to vary over time under this hypothesis, being high at times when the population was dominated by motile cells and low at times when the population was dominated by non-motile cells. Because the degree of nutrient depletion might affect the hypothesized shift to non-motility, both nutrient-sufficient (high phosphorus) and nutrient-limited (low phosphorus) media were used. Additionally, a low-phosphorus medium with glucose additions was used, to create conditions favoring mixotrophic nutrition (Harman and Grover 2008). Mixotrophy is hypothesized to increase both toxic activity and motility, since both properties might function to increase prey capture rates.

CHAPTER 2

GENERAL METHODS

2.1 *Prymnesium parvum* Culture Strain

The strain of *Prymnesium parvum* used in all experiments is from the UTEX Algal Collection (UTEX LB ZZ181, isolated by J. Glass from the Colorado River). All experimental cultures were inoculated from *P. parvum* stock cultures incubated at 20 ºC with an irradiance of 150 µE m^{-2} s^{-1} and a 12:12 photoperiod. The stock cultures of *P. parvum* were maintained in 5.8 psu artificial sea water (ASW, Kester 1967, as modified by Baker et al. 2007) media with f/2 levels of nitrogen and phosphorous, vitamins, and trace metals (MacLachlan 1973). Experimental cultures either used this artificial seawater medium diluted to various working salinities, or modified media with a salt composition resembling that of Texas waters where blooms of *P. parvum* have occurred (Baker et al. 2009). Some experimental cultures also had nutrient levels modified from those of the stock medium, Modifications of media for the various experimental cultures are detailed in chapter 4, along with any changes in temperature or other growth conditions from the stock culture conditions. None of the cultures were axenic and so samples were taken to measure bacterial populations during each experiment

2.2 Sampling and Analysis

Live samples of 15 mL were withdrawn from the culture flasks with sterile pipets and examined under the microscope prior to adding preservative. The numbers of motile and non-motile cells in these live samples were counted at 400X using a Sedgwick Rafter Slide. This assay was done quickly without a high amount of precision. 20 grids were counted with anywhere from 5 to 60 cells per grid depending on the time of the experiment. Samples of 15

mL were preserved with several drops of Lugol's solution in 20 mL scintillation vials. These counts were done with the Sedgewick Rafter slide at 400X magnification. Aliquots of 10 mL were preserved with 0.5 mL of a 10 % formalin solution for bacterial counts. These samples were stained with acridine orange, filtered onto a polycarbonate filter, and bacteria were enumerated with epifluorescence microscopy (Hobbie et al 1977). Another 10 mL of culture was filtered through a 25mm Whatman glass fiber filter for chlorophyll *a* analysis. The filter was frozen in a scintillation vial with 1 mL magnesium carbonate. For chlorophyll *a* extraction, the vials were filled with 9 mL of a 90 % acetone solution and stored in the dark for about 12 hours. The amount of chlorophyll present was then analyzed using a fluorometer (Welschmeyer 1994). Another 50 mL of cultures was filtered onto a 45 mm glass fiber filters and stored in the freezer for particulate phosphorus analysis (Menzel and Corwin 1965).

2.3 Toxicity Analysis

Samples were tested for acute lethal toxicity to fish using a 48-hour test with juvenile fathead minnows (*Pimephales promelas*; US EPA 1991). Whole culture samples were diluted over a range of seven fractions into reconstituted hard water, with corresponding dilutions of sterile culture media used as controls. Toxicity to fish was represented as LC_{50}, expressed as a percentage dilution of the original culture sample. Fish survival was tested in a 0.5 dilution series from 100% to 6.25% whole culture. This toxicity testing was performed by the personnel in Dr. Bryan Brook's lab at Baylor University. Two replicate chambers with 5 fish per chamber were used for each toxicity analysis. Acute LC_{50} values were estimated using Probit (Finney 1971) or Trimmed Spearman-Karber (Hamilton et al 1977). Each estimated LC_{50} was then multiplied by the cell concentration of *P. parvum* to estimate acute LC_{50} as the concentration of cells that resulted in 50% fish mortality. This calculation was done both for total cell concentrations (motile and non-motile), and motile cell concentrations only. The cell-based LC_{50}

estimates are reported here to compare the toxicity of *P. parvum* with cultures of different cell concentrations, for both total and motile cells.

2.4 Regression Analysis

A multiple regression analysis of data from all experiments in this study was used to conduct a synthetic analysis that assessed the relative influence of several factors on toxic activity of *P. parvum*. Response variables measuring toxic activity were LC_{50} on a percent dilution basis, LC_{50} on a motile cell concentration basis, and LC_{50} on a total cell concentration basis, calculated as explained in section 2.3. Independent variables included: age of culture (d), dilution rate (d^{-1}), total cell density (cells/mL), proportion of motile cells (cells/mL), and phosphorus quotas (fmol/cell). Dilution rate was coded to zero for batch culture experiments, and age was taken as the day that cultures were sampled to measure response and independent variables. These latter two factors differed among experiments due to differences in their designs and sampling schedules (Table 2.1). These factors were included in the overall regression analysis because they address the life history changes that were the focus of this study, are known or suspected to affect toxic activity, and were observed in all of the individual experiments composing this study.

Table 2.1 Dilution rates and days sampled for the experiments conducted in this study

Experiment	Dilution rate (d^{-1})	Days sampled
Semi-continuous (section 4.1)	0.034, 0.060, 0.124, 0.182, 0.357	21, 35
Seasonal (section 4.2)	0 (batch)	7, 14, 21, 28, 35, 42
Factorial (section 4.3)	0 (batch)	28
Nutrient-limitation (section 4.4)	0 (batch)	7, 14, 21, 28, 35, 42

To achieve normal distributions, total cell density, motile cell density, and phosphorus quotas were natural-log transformed. An arcsine square root transformation was used for the proportion of motile cells. The response variables were also natural-log transformed to achieve normality. Residual plots were analyzed to confirm that the model follows the assumptions of the regression analysis. Systat (version 11.0, Systat Software 2008) was used for statistical analyses.

Additional statistical analyses were done to examine further the results of the individual experiments undertaken in this study. These analyses are described in chapter 4, which reports the detailed designs and results of each individual experiment.

CHAPTER 3

GENERAL RESULTS

The results for all experiments were analyzed together using multiple linear regression. The goal of these overall analyses was to examine relationships between acute toxicity to fish and several factors known or suspected to affect such toxicity, including the motility of cells as an indicator of their life-history stage. The response variables included the following measurements of toxic activity: LC_{50} on a percent dilution basis, LC_{50} on a motile cell concentration basis, and LC_{50} on a total cell concentration basis, calculated as explained in section 2.3. Independent variables included: age of culture (d), dilution rate (d^{-1}), total cell density (cells/mL), proportion of motile cells (cells/mL), and phosphorus quotas (fmol/cell). Dilution rate was coded to zero for batch culture experiments, and age was taken as the day that cultures were sampled to measure response and independent variables. For all regression models, both the raw regression coefficients (B), and standardized coefficients (Beta) were obtained. The latter are computed after standardizing response and independent variables by subtracting the mean and dividing by the standard deviation. These standardized coefficients provide a way of identifying influential independent variables that corrects for their different scaling and units of measurement. Non-significant regression terms were retained in regression models for two reasons: to provide statistical control that would improve the estimation of significant effects, and so that all hypothesized effects would be compared on a similar basis.

To visualize the correlations between the variables analyzed by multiple regression, a scatterplot matrix was produced (Fig. 3.1). The abundance of motile cells appears to be relatively high throughout the various days of the experiments. In other words, the age of the culture does not appear to have much of an effect on motile cell abundance. This relationship

also appears to be the same when observing the proportion of motile cells by day. Another important observation concerning the age of the cultures, is that the older the culture corresponds to greater toxicity, or a lower LC_{50}. When observing LC_{50} on a percentage basis, as the number of total and motile cells increase, the toxicity also increases (LC_{50} decreases). When comparing phosphorous quotas to LC_{50} of motile cells, as the P quota rises, there is an increase in toxicity. This relationship is not as clear when looking at LC_{50} on a total cell basis, but the opposite relationship is seen when comparing P quotas to LC_{50} on a percentage basis. In this case, as the P quota increases, the toxicity decreases.

The transformations reported in section 2.4 were made to achieve normality and to reduce skew. These transformations also resulted in achieving a reasonable degree of linearity among the dependent and independent variables. Among the relationships analyzed by multiple regression, the least linear were between measures of toxic activity and dilution rate.

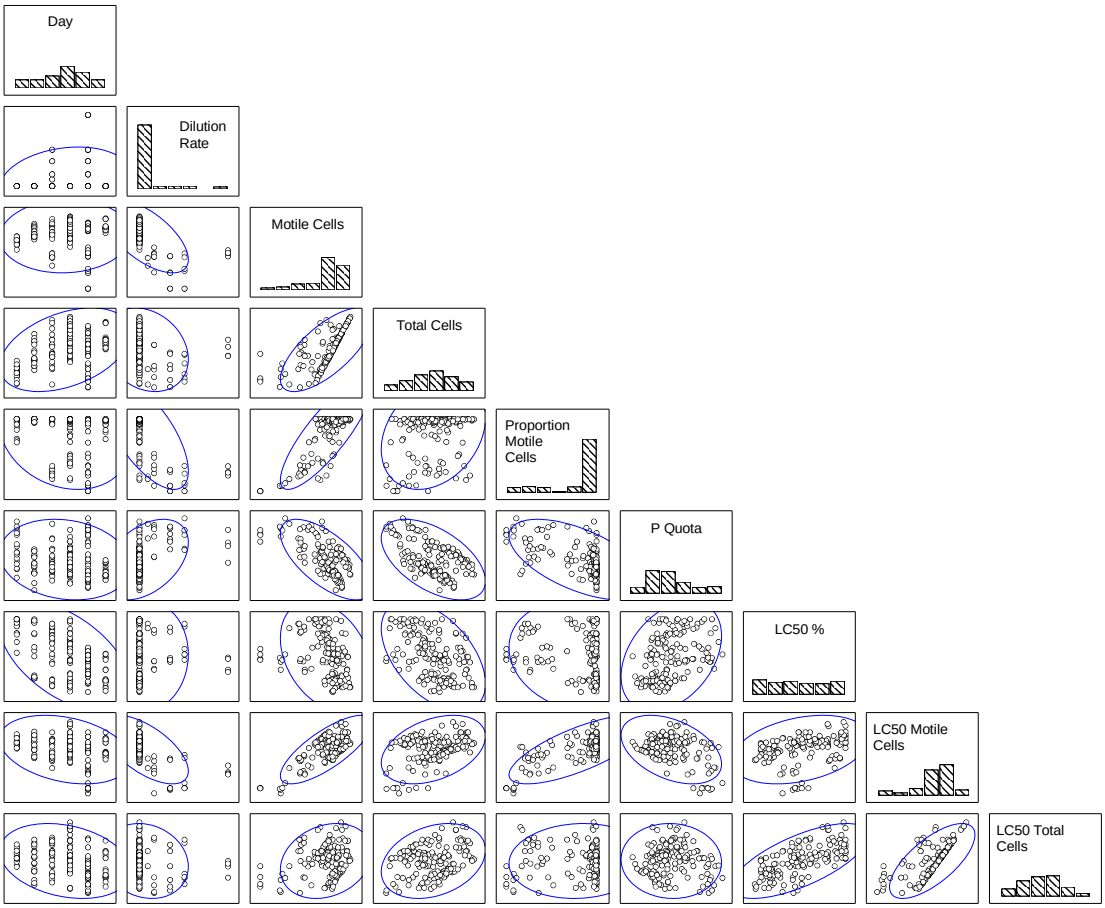

Figure 3.1 Scatterplot matrix of the dependent and independent variables included in the multiple regression analysis.

3.1 LC_{50}%

The response variable used for this model was LC_{50} on a percentage basis. It is a percent dilution of the whole culture sample. No corrections were made for population density (motile or total cell density). The independent variables used were: age of culture (in days), dilution rate (d^{-1}), total cell density (cells/mL), the proportion of motile cells (cells/mL), and the phosphorous quotas (fmol/cell).

The overall regression model was significant ($F = 21.75$, $p < 0.001$) (Table 3.2). The residual plots followed the assumptions of the regression analysis. This model had intermediate goodness of fit among the three models examined (adjusted $R^2 = .404$). Age of culture ($p <$

0.001) and total cell density (p = 0.005) were the significant terms. Both are also strongly significant with p-values < 0.01 (Tables 3.1, 3.3). Age of culture and total cell density also have negative coefficients. So, older cultures and those with higher densities of total cells have greater toxicity. Standardized regression coefficients show that culture age had an effect about twice as strong as that of population density. While the proportion of motile cells did not have a significant p-value, it had a negative coefficient, meaning the higher the proportion of motile cells, the greater the toxicity. This effect was weaker than the significant effects of age and total population density, but was the strongest of the non-significant effects.

Table 3.1 Summary statistics for multiple regression analysis with LC_{50} % as dependent variable

Model	R^2	Adjusted R^2	Std. Error of Estimate
LC_{50} %	0.424	0.404	1.330

Table 3.2 ANOVA for LC_{50} % regression model

	Sums of Squares	df	Mean Squares	F	p-level
Regression	192.4	5	38.479	21.75	< 0.001
Residual	261.8	148	1.77		
Total	454.2				

Table 3.3 Coefficients summary for LC_{50} % regression model

	Beta	Std. Error of Beta	B	Std. Error of B	t	p-level
Intercept			7.043	1.515	4.650	< 0.001
Age of Culture	-0.445	0.074	-0.074	0.012	-5.996	< 0.001
Dilution rate	-0.092	0.085	-2.280	2.112	-1.080	0.282
Total cells	-0.231	0.081	-0.303	0.106	-2.864	0.005
Proportion motile cells	-0.167	0.097	-1.055	0.613	-1.721	0.087
P quota	0.118	0.089	0.171	0.128	1.334	0.184

3.2 LC_{50} of Total Cells

The response variable used for this model was LC_{50} on a total cell basis. This was calculated by multiplying the LC_{50} on a percentage basis of the whole culture by the total cell population density. This calculation presumes that all cells contribute to toxic activity and provides a way to compare toxic activity independent of population density. The independent variables used were: age of culture (in days), dilution rate (d^{-1}), the proportion of motile cells (cells/mL), and the phosphorous quotas (fmol/cell).

The overall model was significant ($F = 3.71$, $p = 0.007$) (Table 3.8). The residual plots followed the assumptions of the regression analysis. This model had the poorest fit among the three models examined (adjusted $R^2 = .066$). Age of culture was the only significant term ($p = 0.001$) (Tables 3.7, 3.9). The age of culture variable had a negative coefficient therefore, the older cultures exhibited the greatest toxicity.

Table 3.4 Summary statistics for multiple regression analysis with LC_{50} of total cells as dependent variable

Model	R^2	Adjusted R^2	Std. Error of Estimate
LC_{50} of Total Cells	0.090	0.066	1.507

Table 3.5 ANOVA for LC_{50} of total cells regression model

	Sums of Squares	df	Mean Squares	F	p-level
Regression	33.7	4	8.42	3.71	0.007
Residual	338.5	149	2.27		
Total	372.1				

Table 3.6 Coefficients summary for LC_{50} of total cells regression model

	Beta	Std. Error of Beta	B	Std. Error of B	t	p-level
Intercept			10.235	1.069	9.574	< 0.001
Age of Culture	-0.277	0.085	-0.042	0.013	-3.246	0.001
Dilution rate	-0.156	0.106	-3.502	2.384	-1.469	0.144
Proportion motile cells	-0.152	0.121	-0.868	0.694	-1.251	0.213
P quota	-0.106	0.103	-0.138	0.135	-1.024	0.308

3.3 LC_{50} of Motile Cells

The response variable used for this model was LC_{50} on a motile cell basis. This was calculated by multiplying the LC_{50} on a percentage basis of the whole culture by the population density of motile cells. This calculation presumes that motile cells are primarily responsible for toxic activity and provides a way to compare toxic activity that is independent of their density alone. The independent variables used were: age of culture (d), dilution rate (d^{-1}), total cell density (cells/mL), the proportion of motile cells (cells/mL), and the phosphorous quotas (fmol/cell).

The overall model was significant ($F = 64.84$, $p < 0.001$) (Table 3.5). The residual plots followed the assumptions of the regression analysis. This model had the best fit among the three models examined (adjusted $R^2 = .676$). Age of culture, dilution rate, total cell density, and proportion of motile cells were the significant factors ($p < 0.005$) (Tables 3.4, 3.6). The age of culture variable had a negative coefficient, meaning that the older the culture, the more toxic it becomes. The dilution rate also had a negative coefficient; the semi-continuous cultures with the higher dilution rates exhibited the greatest toxicity. The proportion of motile cells had a positive coefficient, which indicates that after correcting toxic activity to the density of such cells, increasing dominance of such cells in the culture actually decreases toxic activity by raising LC_{50}. Total cell density also had a positive coefficient, indicating that higher total cell densities

also reduce toxic activity, when this is measured on a motile cell basis. Standardized regression coefficients indicate that of the significant effects in the regression model, the strongest were those of the proportion of motile cells and total cell density.

Table 3.7 Summary statistics for multiple regression analysis with LC_{50} of motile cells as dependent variable

Model	R^2	Adjusted R^2	Std. Error of Estimate
LC_{50} of Motile Cells	0.687	0.676	1.396

Table 3.8 ANOVA for LC_{50} of motile cells regression model

	Sums of Squares	df	Mean Squares	F	p-level
Regression	631.9	5	126.37	64.84	< 0.001
Residual	288.5	148	1.95		
Total	920.3				

Table 3.9 Coefficients summary for LC_{50} of motile cells regression model

	Beta	Std. Error of Beta	B	Std. Error of B	t	p-level
Intercept			-2.969	1.589	-1.868	0.063
Age of Culture	-0.302	0.054	-0.071	0.012	-5.529	< 0.001
Dilution rate	-0.185	0.062	-6.563	2.216	-2.960	0.003
Total cells	0.381	0.059	0.713	0.111	6.417	< 0.001
Proportion motile cells	0.516	0.071	4.649	0.643	7.225	< 0.001
P quota	0.118	0.065	0.243	0.134	1.809	0.072

3.4 Summary of General Analysis

Overall, the model with LC_{50} on a motile cell basis had the best fit based on the adjusted R^2. This suggests that motile cells are the ones responsible for toxic activity and that measuring toxicity in terms of the abundance of motile cells can be very useful. It is important to note that using motile cells as the basis to quantify toxicity alters the relationships of toxic

activity to total cell density and the proportion of motile cells contribute to toxic activity. In this best-fitting model both total cells and proportion of motile cells had positive coefficients. This means that the greater number of total and proportion motile cells actually lead to less toxicity. This is not the case for the other two models (LC_{50} percent and LC_{50} total cells) where both total cells and proportion motile cells had negative coefficients. In all three models, the age of culture was an important factor. It is possible that complex, time-dependent changes in total population density, motility, and toxic activity arise. As these quantities change, a high total population might be very toxic because it is dominated by motile cells, or it might also be dominated by non-motile cells that were recently active and producing toxins. The results in section 4.3.4 demonstrate that dynamic variations in population density, motility and toxic activity took place in some of the experimental cultures. Time-dependent changes might also explain why phosphorus quota had only weak effects in this regression analysis, despite the emphasis that prior literature places on nutritional status as a correlate of toxic activity in *P. parvum*. As the age of the culture progresses, phosphorus and other nutrients are depleted and phosphorus quotas decline, while the toxic activity increases. The strong influence of age on toxic activity might in fact arise in part from an effect of nutrient status. If phosphorus quotas are measured with more error than culture age, age might be a good proxy for nutritional status. There could also be additional processes related to culture age, such as accumulation of toxins or of intercellular signals to produce toxins, which could confound the estimation of nutrient effects on toxicity.

CHAPTER 4

EXPERIMENTS

4.1 Growth and Toxicity of *Prymnesium parvum* in Relation to Growth Rate, Nutrient Limitation, and Temperature in Semi-continuous Cultures

4.1.1 Introduction

The experiment reported here used semi-continuous cultures to approximate continuous culture conditions to explore the relationships between nutrient limitation, growth rate, and toxic activity in *P. parvum*. Two limiting nutrients were used: nitrogen and phosphorous, and two temperatures were used: 14 and 20 ºC. Cell composition was measured to determine nutrient status. Toxic activity was determined as acute toxicity to fish (*P. promelas*), given the strong interest in fish-killing blooms of this species. Several dilution rates were used at each temperature, and it was expected based on chemostat theory that lower dilution rates would be associated with indications of nutrient limitation and high toxic activity, as explained in section 1.1.

4.1.2 Methods

Semi-continuous cultures were used to examine effects of two limiting nutrients (N versus P), two temperatures (14 °C and 20 °C), and four dilution rates (0.034, 0.060, 0.124, 0.182 d^{-1} at 14 ºC, and 0.060, 0.124, 0.182, 0.357 d^{-1} at 20 ºC) on abundance, cell nutrient composition, and toxicity to fish of *P. parvum*. Duplicate cultures of 350 mL in 500 mL Erlenmeyer flasks were maintained at all combinations of these factors. The media used were modifications of artificial seawater designed to closely resemble the ion composition of north Texas lakes (LW medium, Baker et al. 2009). Based on prior work, it was expected that the

20 ºC cultures would reach steady state in 3 weeks and the 14 ^{o}C cultures in 5 weeks. At the end of these time periods, samples were tested for lethal toxicity to fish using a 48-hour test with juvenile fathead minnows (*Pimephales promelas*; US EPA 1991).

Every other day culture dilutions were performed: the required volume (according to the dilution rate) was poured out into a sterile graduated cylinder. The same volume was replaced with sterile medium, dispensed with a sterile graduated cylinder

Table 4.1 Dilution rate (d^{-1}) with corresponding volume of sterile media to replace (mL)

Dilution rate (d^{-1})	Volume to replace (mL)
0.034	20
0.060	35
0.124	70
0.182	100
0.357	180

The live counts discussed in chapter 2 were done on days 14, 17, and 21 for the 3 week cultures and days 28, 31, and 35 for the 5 week cultures. Samples of 15 mL were preserved with several drops of Lugol's solution in 20 mL scintillation vials. These samples were taken on days 0, 7, 14, and then daily until day 21 for the 3 week cultures and on days 0, 7, 21, 28, and then daily until day 35 for the 5 week cultures. These counts were done with the Sedgewick Rafter slide at 400X magnification. Samples from the end of the experiment (day 21 or day 35 for 3 week and 5 week cultures, respectively) were tested for acute lethal toxicity to fish using a 48-hour test with juvenile fathead minnows (*Pimephales promelas*; US EPA 1991).

4.1.3 Results

None of the cultures reached an apparent steady-state as anticipated. By about 2 to 3 weeks (depending on the temperature), the total cell abundance began to fluctuate in most cultures (Fig. 4.1). Densities of motile cells were often >10X lower than total cell density and various trends with time were observed, with no obvious relationship to the experimental treatments.

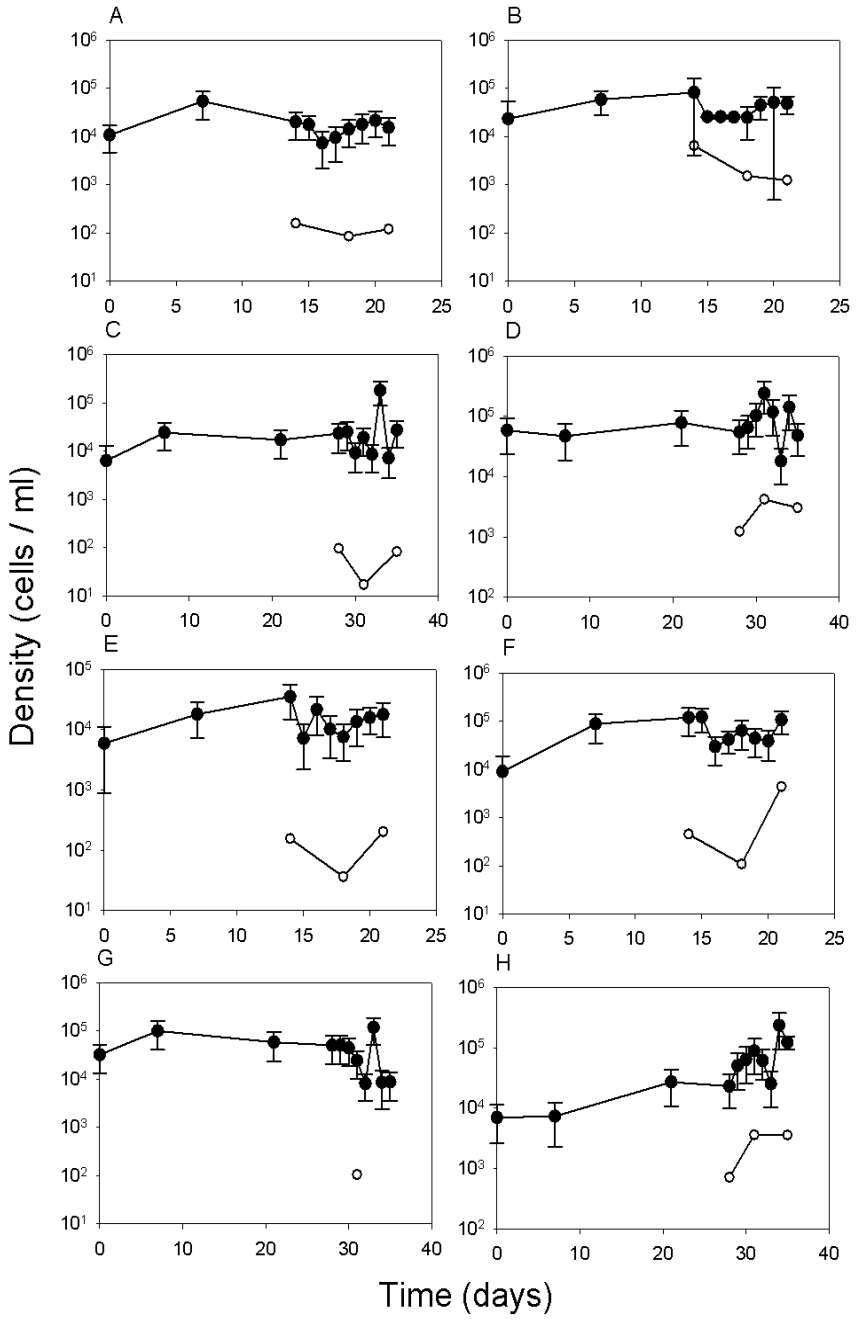

Figure 4.1 Density of preserved (total) cells/mL (black circles) and motile cells/mL (white circles) over the duration of the experiment. A-D represents nitrogen limited cultures. E-H represents phosphorous limited cultures. (A) 0.182 d^{-1} 14 ºC low N, (B) 0.034 d^{-1} 14 ºC low N, (C) 0.357 d^{-1} 20 ºC low N, (D) 0.060 d^{-1} 20 ºC low N, (E) 0.182 d^{-1} 14 ºC low P, (F) 0.034 d^{-1} 14 ºC low P, (G) 0.357 d^{-1} 20 ºC low P, (H) 0.060 d^{-1} 20 ºC low P. Error bars represent standard deviation over two replicates.

Fig 4.2 below shows replicates separately for the last day of the experiment when nutrient composition and toxicity were measured. There was no apparent relationship to dilution rate. The generally decreasing relationship that would be expected based on standard chemostat theory was not observed.

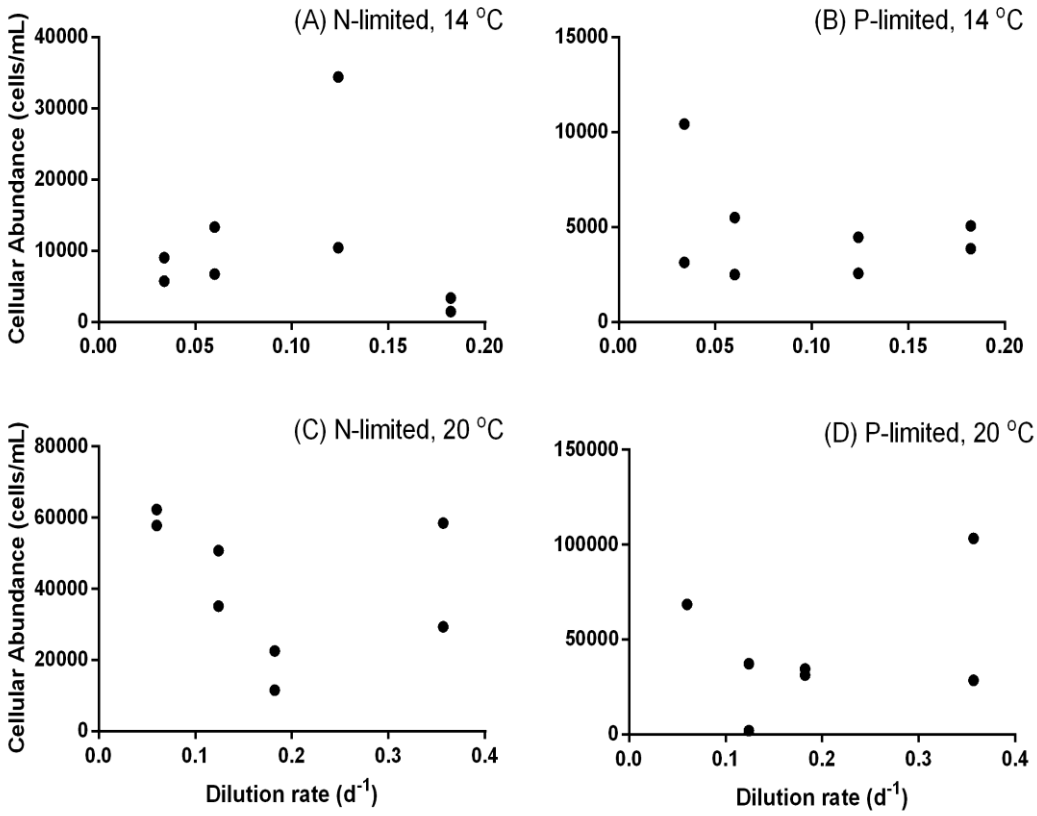

Figure 4.2 Total cellular abundance (cells/mL) at the endpoint of the experiment for each dilution level in the small-volume semi-continuous cultures. Each replicate is shown separately.

The nitrogen and phosphorous quotas from the last day of the experiment show a lot of scatter (Fig. 4.3) and no tendency to follow the increasing relationship expected theoretically from Droop's equation (section 1.1). The highest dilution level represents some of the lowest phosphorous quotas. Generally, the 14 ºC cultures exhibited higher phosphorous quotas.

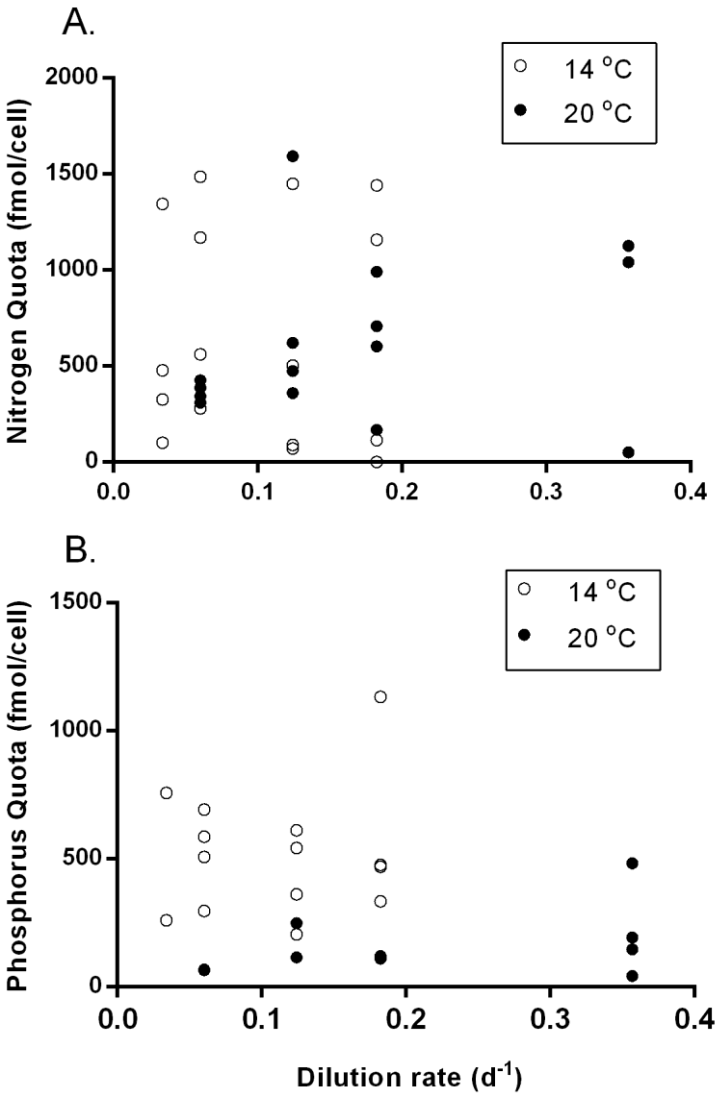

Figure 4.3 Cellular nutrient quotas in fmol/cell for each dilution rate (d^{-1}). (A) nitrogen and (B) phosphorus quotas. The white circles represent 14 ^{o}C cultures and the black circles represent 20 ^{o}C cultures.

Two measures of acute toxicity to fish were considered in this experiment. The LC_{50} value is the percentage dilution of a raw culture sample that kills 50% of fish during the 48 h test and it indicates the toxicity of cultured populations without conveying any information relating to

their sizes: a sample could be highly toxic because a large population of cells expresses low toxic activity, or because a smaller population expresses higher activity. The LC_{50} value expressed as the cells per ml causing 50% mortality of fish conveys information about the relative toxic activity of cells. The latter is obtained by multiplying the abundance (cells / mL) in the undiluted culture samples, by the LC_{50} on a percentage basis (the first toxicity measure). For both measures, lower values of LC_{50} indicate higher toxicity. And for both measures, results contradict the hypothesis that more nutrient-limited cells grown at lower dilution rates should be more toxic. By both measures, either the highest dilution rate was the most toxic to fish, or the lowest dilution rate was the least toxic, or both, and there was a generally decreasing trend of LC_{50} in relation to dilution rate (Figs. 4.4 and 4.5). However, the cultures with higher dilution rates tended to have higher abundance of motile cells.

Samples from the lower temperature of 14 ºC generally exhibited greater toxicity (lower LC_{50}) than those at 20 ºC (ANOVA, $p < 0.001$), which is consistent with other work using the same strain isolated from Texas inland waters (Baker et al. 2007). Toxicity also increased with increased dilution level (ANOVA, $p < 0.001$). There was also a significant interaction of temperature and dilution rate (ANOVA, $p < 0.001$), due to the strong effect of temperature at the two middle dilution rates. When live cell counts were conducted dense samples were characterized by a large number of non-motile, or encysted, cells. The toxicity of cultures was related to the density of motile cells determined 3 days before the toxicity tests were done (Fig. 4.6; correlation $r = -0.85$, $p < 0.01$) for both 14 and 20 ºC.

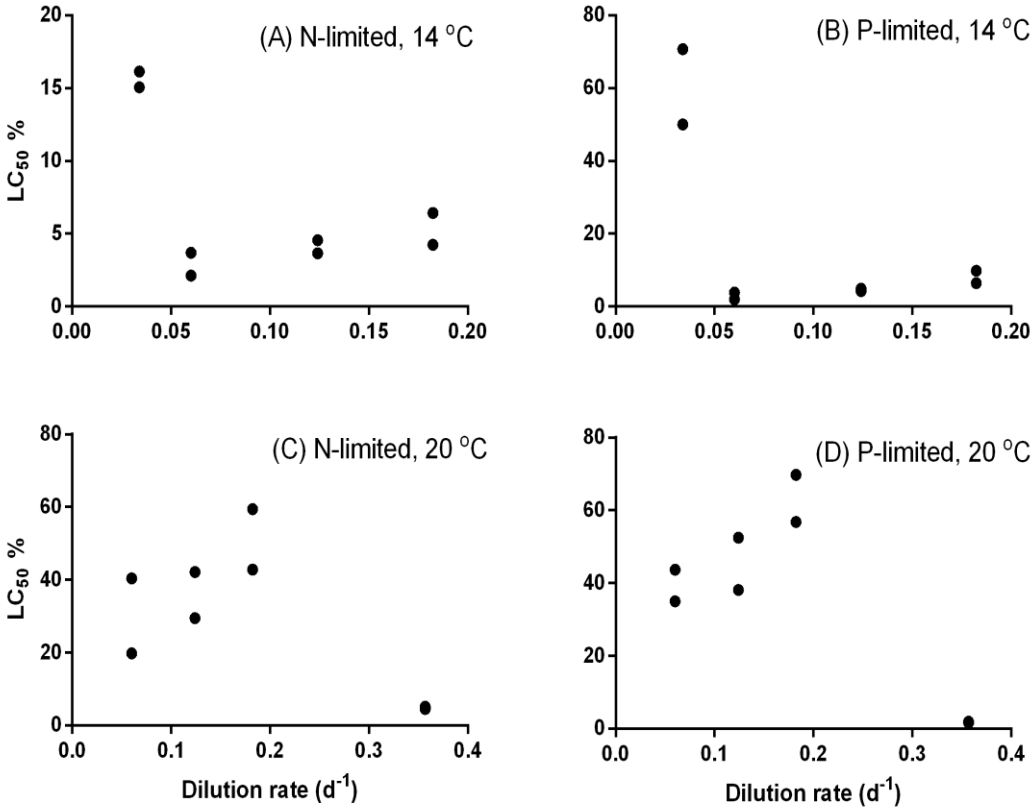

Figure 4.4 Toxicity to fish in relation to dilution rate (d^{-1}) in small-volume semi-continuous cultures after 21 (14 °C) or 35 (20 °C) days of growth. Toxicity is highest when LC_{50} is lowest.

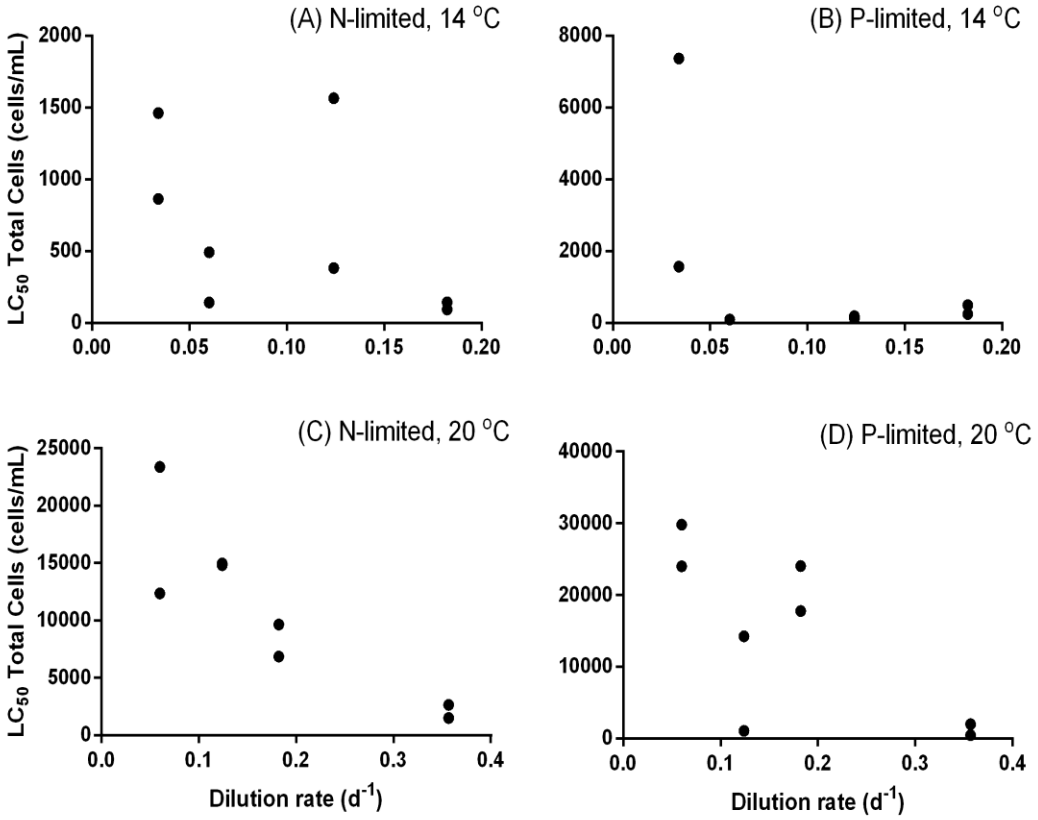

Figure 4.5 Toxicity to fish in relation to dilution rate (d^{-1}) in small-volume semi-continuous cultures after 21 (14 °C) or 35 (20 °C) days of growth. LC_{50} is expressed as total number of cells. Toxicity is highest when LC_{50} is lowest.

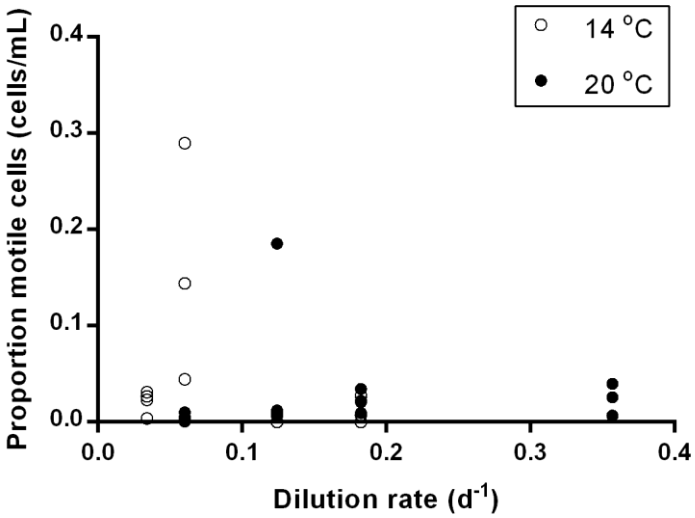

Figure 4.6 Proportion motile cells (cells/mL) 3 days prior to toxicity results. White dots represent 14 ºC cultures and black dots represent 20 ºC cultures.

4.1.4 Discussion

The results of this experiment did not agree with the prior expectations that informed its design. Acute toxicity to fish increased with the dilution rate, rather than decreasing as predicted. The increase of toxicity with increasing dilution rate was surprising, because other studies indicate that increased severity of nutrient limitation promotes toxicity (Dafni et al. 1972, Johansson and Granéli 1999). Increased dilution rate for semi-continuous cultures should decrease the degree of nutrient limitation, leading to reduced toxicity. Another unexpected result was that cell quota of the limiting nutrient was apparently unrelated to dilution rate, rather than increasing with it as expected. Observations during this study suggest that the formation of non-motile or encysted cells could contribute to these unexpected results. If such cells are inactive or not growing and dividing, their cell quotas for the limiting nutrient would not necessarily follow the same relationship as expected for active, growing cells. Quotas were measured based on the total number of cells, and thus they average together cells of different types. In many cases,

this average quota was higher than is expected for nutrient limited cells (Johansson and Granéli 1999, Uronen et al. 2005, Skingel et al. 2010). Based on these prior studies, high quotas would be associated with low toxic activity, and thus high proportions of non-motile, nutrient-rich cells at low dilution rates could explain the lower toxicity to fish observed for such cultures.

A complication of this study is that the semi-continuous culture technique used here might not have produced steady state conditions. The two-day cycle of dilution undoubtedly produced a brief phase of nutrient enrichment followed by a period of starvation. It is possible that such cycles, which would not occur in a truly continuous culture, act to trigger the production of non-motile cells. An additional complication is that toxic activity was assessed at only one point in time, when the experiment was terminated. The relatively large sample volumes required to measure acute toxicity to fish, and the expense of such tests precluded making multiple determinations to determine the dynamics of toxic activity. In a study where a more logistically tractable hemolytic assay was used for frequent determinations of toxic activity, toxicity went through oscillations with apparent periods of 5 – 10 days (Skingel et al. 2010). The Skingel study did not examine live samples for the presence of non-motile cells, but it is possible that oscillations in the formation of such cells occurred, and were related to oscillations of toxicity.

There were different types of non-motile or encysted cells observed in this experiment, with different morphologies. Although some had the thick cell walls expected for true cysts, more resembled the non-motile, coccoid cells reported in the literature (Genitsaris et al. 2009). The association of high proportions of such cells with low toxicity to fish in this experiment supports the interpretations of field population dynamics offered by these authors. They suggested that non-motile forms appear towards the end of toxic blooms, and are related to bloom termination as these non-motile cells reduce toxin production and settle to the sediments.

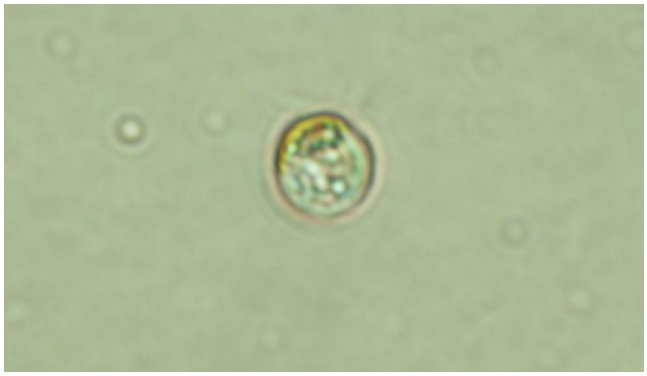

Figure 4.7 Coccoid *Prymnesium parvum* cell observed in culture sample.

These observations and the results found here suggest a working hypothesis that small populations grow and deplete available nutrients, and then release toxins triggered by a stressed environment of low nutrient availability. If the environment remains stressful, non-motility and encystment are eventually triggered, leading to a reduction of toxin production. Because the toxins degrade in sunlight (James et al. 2011), toxicity would then decline. Stressed, toxin-producing cells are suspected to exhibit mixotrophy and ingest bacterial or other microbial prey if available, releasing toxins to enhance the capture of prey. The experiment reported here was intended to examine the hypothesis that toxic activity of *P. parvum* is related to nutrient limitation, in a context of different limiting nutrients and temperatures. Instead of supporting this hypothesis, the results suggested a new hypothesis that toxic activity is influenced by transitions from motile cells actively producing toxin to other cell types, non-motile and encysted. The goal of the additional research described in subsequent sections of this chapter is to build an understanding of the life history of *P. parvum*, particularly its encystment and formation of non-motile cells, and explore how these transitions relate to toxicity.

4.2 Growth and Toxicity of *Prymnesium parvum* in Relation to Simulated Seasonal Conditions

4.2.1 Introduction

In the culture experiments reported here, temperature, salinity, and photoperiod were manipulated to simulate summer and winter environmental conditions likely to occur in reservoirs of the south central U.S. A nutrient-limited medium was used for all cultures because of the expected enhancement of toxicity under nutrient limited conditions (Johansson and Granéli 1999). Toxicity to fish was assayed weekly during the course of a seven week experiment. Based on the hypothesized life cycle of *P. parvum* (Fig. 1.1, chapter 1), it was expected that cultures would first develop dense populations of motile cells and high toxic activity, and then display transitions to non-motile states and reduced toxic activity, with such transitions occurring earlier or to greater extent in simulated winter conditions.

4.2.2 Methods

The summer experimental cultures were grown at 30 ^{o}C with a salinity of 4 psu and a 14:10 photoperiod. In contrast, the winter experimental cultures were grown at 14 ^{o}C with a salinity of 2 psu and a 10:14 photoperiod. Both summer and winter groups were quadruplicated.

The LW medium developed to mimic ion compositions of Texas inland waters (Baker et al. 2009) was used. However, all cultures received limiting supplies of nitrogen and phosphorous, 40 uM and 1.5 uM respectively. These nutrient concentrations are representative of the relatively eutrophic Texas lakes where fish-killing blooms of *P. parvum* have occurred. All cultures were grown at volumes of 4 liters in 5 liter Pyrex carboys to allow sufficient volume for multiple toxicity sampling over the seven week experiment.

Live algal samples were withdrawn from the flasks and examined under the microscope prior to adding preservative. The numbers of motile and non-motile cells were counted using a Sedgwick Rafter Slide. 20 grids were counted and typically there were 5 to 60 cells per grid

depending on the time of the experiment. These were done every two days until day 10 and then weekly from day 14. These samples of about 15 mL were then preserved with several drops of Lugol's solution in 20 mL scintillation vials, for later determinations of total cell abundance based on counts of 300 cells in 10 grids per sample. Samples for determinations of motile cells and total abundance were taken every two days until day 10 and then weekly from day 14. Weekly throughout the experiment, 10 mL of culture was preserved with 0.5 ml of a 10 % formalin solution for bacterial counts. Samples were tested for acute lethal toxicity to fish using a 48-hour test with juvenile fathead minnows (*Pimephales promelas*; US EPA 1991) weekly to day 42. The sampling schedule is provided in Table 4.2.

Table 4.2 Sampling schedule for experiment of growth and toxicity of *Prymnesium parvum* in relation to simulated seasonal conditions

Sample	**Day**
Live and preserved counts	Every two days until day 10. Then weekly from day 14
Particulate phosphate, CHN, bacteria, chlorophyll *a*, acute toxicity to fish	6, 14, 21,28, 35, 42

4.2.3 Results

Total cells were slightly greater in the summer cultures than the winter cultures. At the endpoint, the summer cultures reached about 80,000 cells/mL, while the winter cultures reached about 60,000 cells/mL (Fig 4.8). Only on day 7 was there a significant difference between summer and winter cultures in total cells ($F = 19.99$, $p = 0.004$).

Non-motile cells were relatively scarce in this experiment. The highest number they reached was about 10,000 cells/mL on day 35 of the experiment in both the winter and summer simulated conditions. The number of motile cells greatly outnumbered the non-motile cells

reaching over 80,000 cells/mL in the summer conditions and 60,000 cells/mL in the winter conditions (Fig. 4.8). There were no significant differences in the number of non-motile cells between winter and summer conditions.

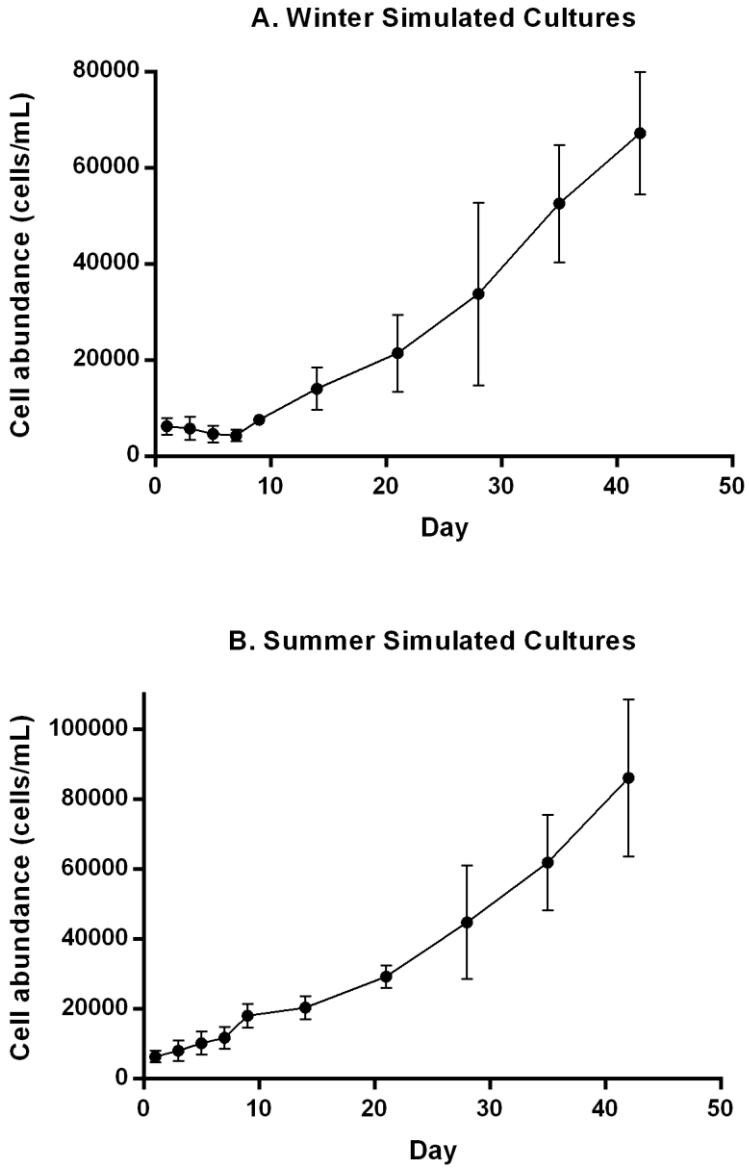

Figure 4.8 Total cell abundance for cultures in: (A) winter simulated conditions, and (B) summer simulated conditions from day 7 to day 42. Error bars represent ± standard deviation.

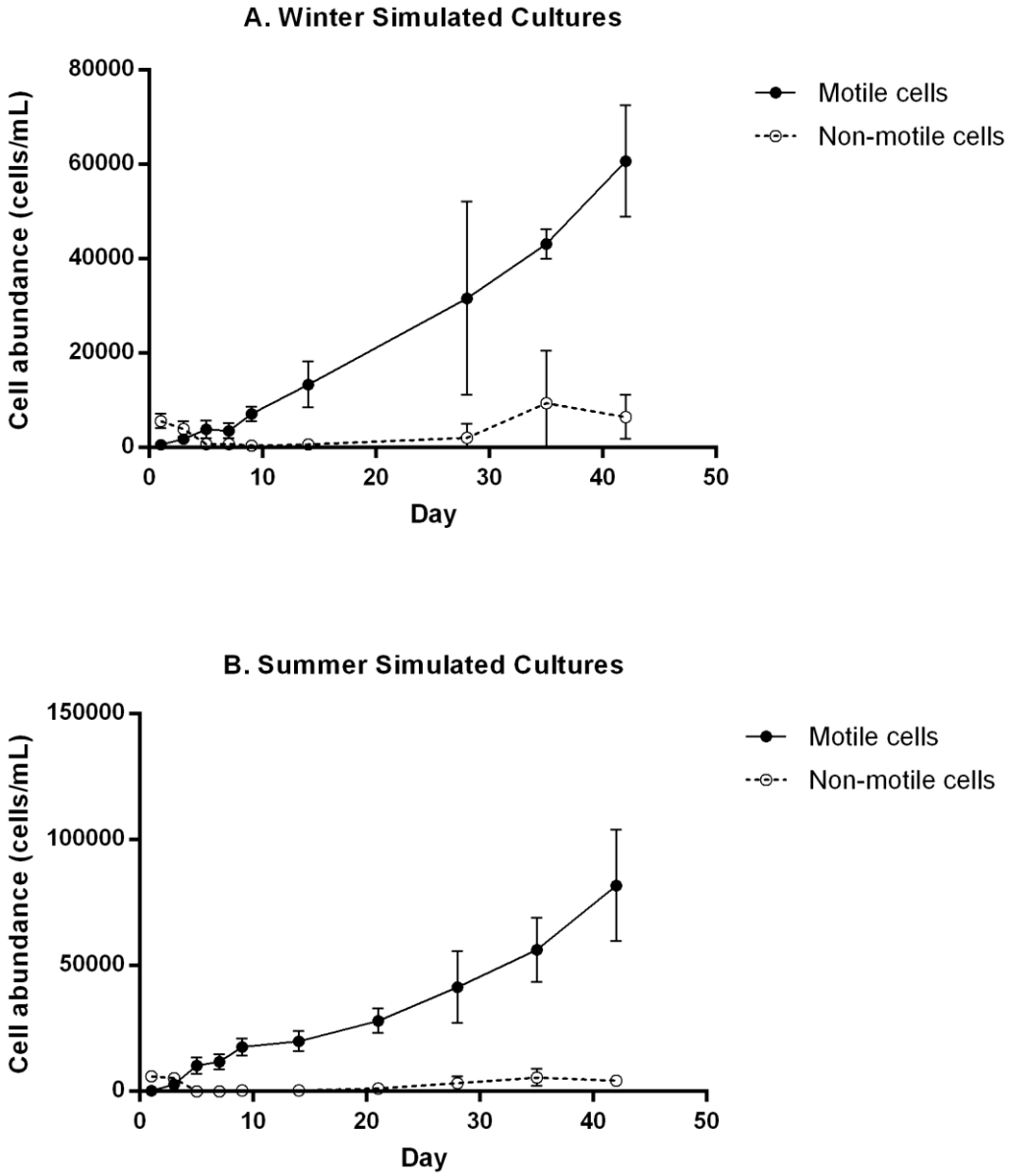

Figure 4.9 Motile and non-motile cell abundance for cultures in: (A) winter simulated conditions, (B) summer simulated conditions from day 7 through day 42. Error bars represent ± standard deviation.

Both culture conditions started out with low toxicity. By day 21 the toxicity increased greatly. When observing toxicity to fish on a LC_{50} cell basis, there was a greater difference

between summer and winter cultures than there was for LC_{50} as percent dilution (Figs. 4.10 and 4.11) (Table 4.3). Both culture conditions then remained toxic with the same differences or lack of differences until the end of the experiment (Table 4.3).

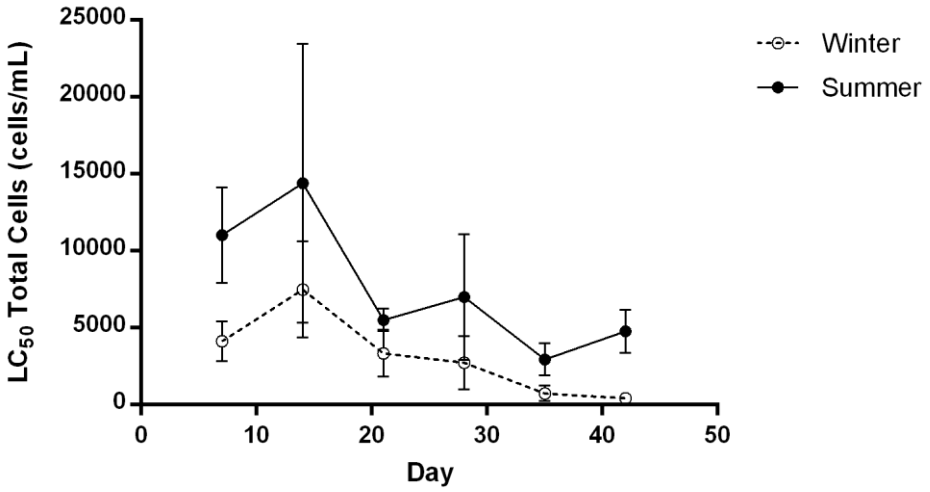

Figure 4.10 LC_{50} on a cell-basis for summer and winter simulated cultures. Black dots represent summer conditions, white dots represent winter conditions. Error bars represent ± standard deviation.

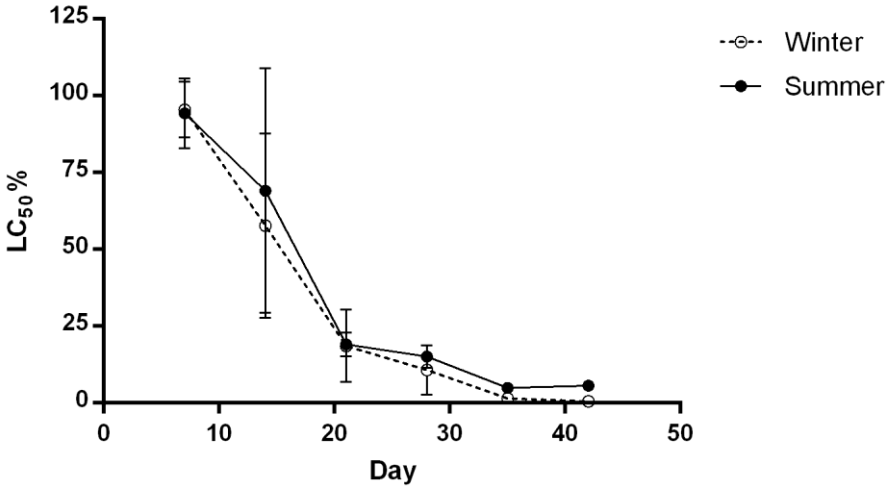

Figure 4.11 LC_{50} % for summer and winter simulated cultures. Black dots represent summer conditions, white dots represent winter conditions. Error bars represent ± standard deviation.

The winter and summer conditions both had cell quotas of less than 100 fmol/cell of phosphorus after day 14. In the winter cultures, by day 21 the cell quota begins to drop which is the same time the toxicity starts to increase (Fig. 4.12). Phosphorus quotas were significantly different between summer and winter cultures on day 7 and day 14 (Table 4.3).

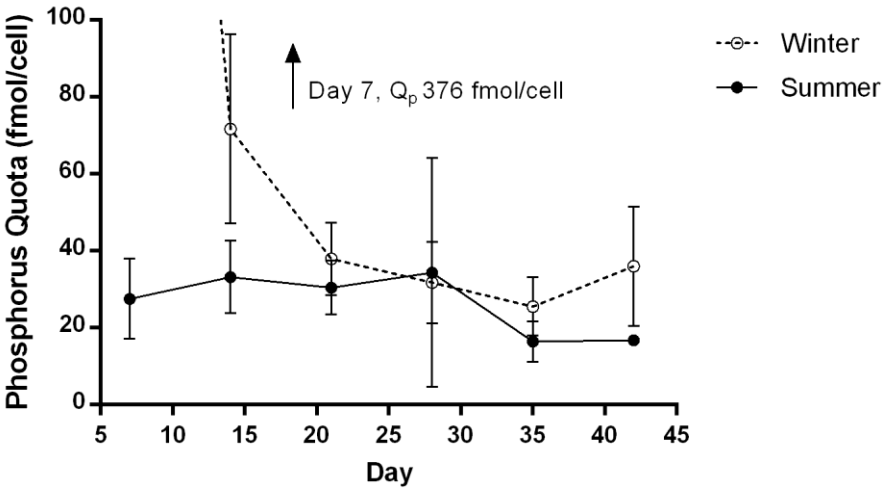

Figure 4.12 Winter and summer phosphorous quotas (fmol/cell). Dark circles represent summer cultures, white circles represent winter cultures. Error bars represent ± standard deviation.

Table 4.3 ANOVAs testing for differences between summer and winter cultures in total cells, non-motile cells, LC_{50}%, LC_{50} of cells, and phosphorus quota. Bold p-values indicated significance at α=0.05

Measure	Day	F-ratio	P-value
Total cells	7	19.99	**0.004**
	14	5.38	0.06
	21	3.32	0.11
	28	0.79	0.40
	35	1.04	0.34
	42	2.15	0.19
Non-motile	7	2.57	0.15
	14	0.12	0.73
	21	0.35	0.57
	28	0.38	0.56
	35	0.43	0.53
	42	0.82	0.39
LC_{50}%	7	0.008	0.93
	14	0.32	0.59
	21	0.06	0.80
	28	1.22	0.31
	35	10.33	**0.018**
	42	111.70	**0**
LC_{50} cells/mL	7	16.94	**0.006**
	14	2.08	0.19
	21	6.56	**0.04**
	28	3.70	0.10
	35	14.14	**0.009**
	42	38.10	**0.0008**
Phosphorus quota	7	14.70	**0.008**
	14	8.56	**0.03**
	21	1.63	0.24
	28	0.03	0.87
	35	3.94	0.09
	42	6.14	0.05

4.2.4 Discussion

In many algae, the production of cysts or other resting stages is a response to deteriorating conditions at the end of the growing season, and the onset of winter (Reynolds 2006). If that is the case for *Prymnesium parvum*, we would expect the stress of the cooler temperature to induce a non-motile or even encysted stage. Non-motile cells were rare in both winter and summer simulated conditions. While high toxicity is related to lower temperatures and salinities (Granéli 2010, Baker 2007) which were the simulated winter conditions, it does not appear that the switch to the non-motile phase is due to the stress of these conditions. Other possible triggers for the non-motile phase should be explored, which could include, nutrient conditions or salinities other than those used here, or population densities at levels other than those seen here.

Cultures from both summer and winter conditions simulated here were very toxic to fish by about week 3 and remained toxic to the end of the experiment. The winter simulated cultures were expected to be toxic based on previous studies with temperature and toxicity of golden algae (Baker 2007). However, once the summer simulated cultures became toxic, they remained very toxic as the number of motile cells increased. Non-motile cells were scarce at this time, suggesting that motile cells are the cell type responsible for the toxicity of the culture. This trend was the same for the winter simulated cultures, even though the motile cell count was slightly less than summer conditions.

As expected, toxicity remained higher in the simulated winter cultures than the summer cultures (Baker et al. 2007). Since motile cells were the dominant cell type in summer and winter cultures, these results do not clarify whether high proportions of non-motile cells lead to lower toxicity.

The results of this study do confirm that reduction of cellular phosphorus quotas below 100 fmol cell^{-1} is associated with the onset of toxic activity (Skingel et al. 2010). In these experiments, phosphorus quotas of both summer and winter cultures fluctuated between about

10 and 30 fmol cell^{-1}, quotas that were associated with high toxic activity in previous studies. However, phosphorus quotas remained above the estimated subsistence quota at which growth ceases (about 5 fmol cell^{-1}) and the lower quotas observed in dense, severely phosphorus-limited populations (about 2 fmol cell^{-1}; Skingel et al. 2010). Both the simulated winter and summer conditions were suboptimal for growth of *P. parvum*, and during this 42-day experiment, populations did not rise to levels at which severe phosphorus depletion occurred. Nevertheless, cellular phosphorus quotas were much lower than the levels characterizing exponentially growing cells at conditions closer to optimal (about 1000 fmol cell^{-1}; Skingel et al. 2010).

This experiment simulated the summer and winter conditions of temperature and salinity characterizing inland waters of Texas where blooms of *P. parvum* have occurred (Southard et al. 2010, Roelke et al. 2011), to address a hypothesis that seasonal conditions might induce formation of non-motile stages and cysts. Large numbers of such stages were not observed. This study did not employ a factorial design and did not independently vary conditions such as salinity and temperature that change seasonally. Although the results did not support seasonality as a trigger for life history changes in *P. parvum*, it is still possible that interactions of these or other factors could affect such changes. To reveal interactions, a full factorial experiment is needed, and is the focus of the next section.

4.3 Factors Affecting Toxic Activity and Non-motile Stages of *Prymnesium parvum*: An Experiment Exploring Salinity, Temperature, Aeration, and Silica Additions

4.3.1 Introduction

The previous experiment sought to explain the effect of seasonal changes. However, only temperature, salinity, and photoperiod were manipulated, and not all possible combinations of these factors were explored. Thus a factorial experiment was needed to assess interactions

between these factors in their influence on toxicity and non-motile cell stages. This section reports such an experiment, adding two additional factors: aeration and the supply of silica.

The effect of aeration on the toxicity of *P. parvum* has been addressed in the literature (Igarashi et al. 1995, Igarashi et al. 1998, Skingel et al. 2010). Strongly aerated cultures have produced greater toxic activity compared to non-aerated cultures (Igarashi et al. 1995). The resulting suggestion is that blooms occurring during windy, turbulent conditions will exhibit increased toxic activity (Granèli and Salomon 2010). While it is still unconfirmed why aeration increases toxin production, it has been suggested that the stress placed upon the cells by the turbulence created by aeration is a factor (Granèli and Salomon 2010). It can thus be hypothesized that the creation of this stressful environment will urge the motile, active cells into the non-motile phase.

The cysts of *P. parvum* have been characterized by the appearance of siliceous scales covering the outer cell wall (Pienaar et al. 1994). In order to permit production of non-motile cells, silica was added to cultures as Na_2SiO_3. The hypothesis is that the cells will utilize the additional silica available to build the thickened cells was unique to the encysted, non-motile form.

In this experiment, two levels of temperature and salinity were crossed with aeration (present vs. absent), and silica addition (present vs. absent) in a full factorial experimental design. Photoperiod was not manipulated, however.

4.3.2 Methods

One liter flasks were used with a working volume of 800 mL LW media. Media, of f/2 concentration, were prepared as described in chapter 2. After autoclaving, cultures requiring the silica addition received 0.8 mL of a silica stock solution (38.08 g Na_2SiO_3 in 1000 mL Millipore water), delivered aseptically using a sterile 0.2 µm Nalgene nylon syringe filter. The bubbling of cultures was achieved with the use of aquarium air pumps and sterile silicone tubing, using

humidified and filtered (0.2 µm) ambient air, at a flow rate of about 10 mL s^{-1} (Skingel et al. 2010).

The four experimental factors were presented at two levels each: (1) temperature: 14 ^{o}C vs 20 ^{o}C; (2) salinity: 2 psu vs 4 psu salinity; (3) aeration: present vs absent; (4) silica: 134 µM silica addition vs. no silica addition. All possible combinations of these factor levels were duplicated, for a total of 32 experimental cultures.

Aliquots of 5 mL were withdrawn from the flasks and examined live under the microscope prior to adding preservative. The numbers of motile and non-motile cells were counted using a Sedgwick Rafter Slide. 20 grids were counted and 5 to 60 cells per grid were counted depending on the densities of the cultures. These live counts were done every two days until day 10 and then weekly from day 14. Samples of about 15 mL were also taken and preserved with several drops of Lugol's solution in 20 mL scintillation vials. These samples were also taken every two days until day 10 and then weekly from day 14. These counts were performed with a Sedgwick Rafter slide and 200-400 cells were typically counted per sample. Weekly throughout the experiment, 10 mL of culture was preserved with 0.5 mL of filtered formalin for bacterial counts. Samples from the end of the experiment were tested for acute lethal toxicity to fish using a 48-hour test with juvenile fathead minnows (*Pimephales promelas*; US EPA 1991).

Table 4.4 Sampling schedule for factorial experiment

Sample	**Day**
Live and preserved counts	Every two days until day 13. Then every day from day 14-28.
Bacteria, Chlorophyll *a*	5,11,28
Particulate phosphate, CHN, acute toxicity to fish	28

4.3.3 Results

The 20 ºC cultures were generally more abundant in cells (Fig. 4.13). The culture with the highest abundance (about 500,000 cells/mL) at the endpoint was at 20 ºC, 4 psu and was aerated with silica added. The 2 psu cultures that were 20 ºC and aerated were low in abundance, regardless of silica addition. This is contrasted with a 2 psu, 20 ºC culture that was neither aerated nor had any silica added; this culture had much greater abundance. The 14 ºC cultures were generally lower in cell abundance.

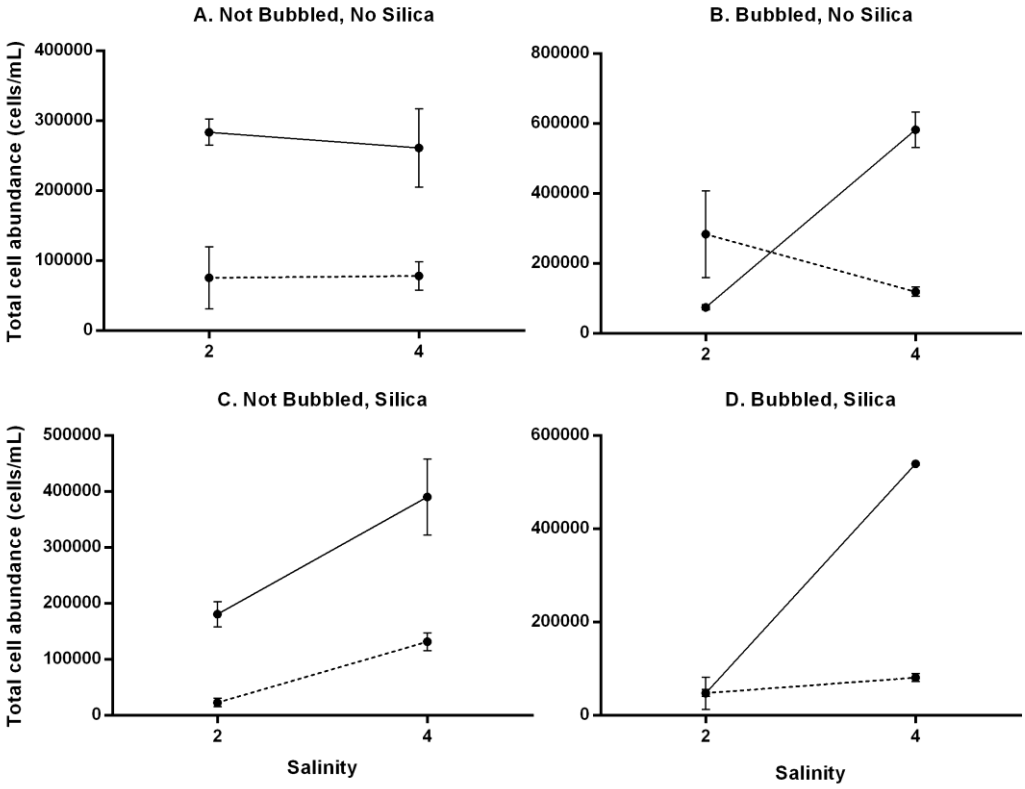

Figure 4.13 Total cell abundance (cells/mL) Solid lines represent 20 ºC and the dashed lines represent 14 ºC. Error bars represent ± standard deviation.

Cultures at the cooler temperature, 14 ºC, were generally more acutely toxic to fish than corresponding cultures at 20 °C (lower LC_{50} on a percent dilution basis), when salinity was also low (2 psu) (Fig. 4.14). The cultures at were generally more toxic than the corresponding

cultures at 2 psu. Aeration tended to reduce acute toxicity to fish, while silica addition appeared to have no consistent effect. Temperature ($F = 23.39$, $p < 0.001$), salinity ($F = 172.19$, $p < 0.001$), and aeration ($F = 43.05$, $p < 0.001$) were all significant factors (Table 4.5). There were also significant interactions between temperature and salinity ($F = 49.02$, $p < 0.001$), salinity and aeration ($F = 38.23$, $p < 0.001$), and salinity and silica ($F = 5.71$, $p = 0.003$). As well as a significant three-way interaction between temperature, salinity, and aeration ($F = 6.01$, $p = 0.026$) (Table 4.5).

When acute toxicity to fish was expressed on the basis of LC_{50} as total cell density, similar patterns emerged (Fig. 4.15), except that salinity did not appear to affect toxicity for low temperature (14 °C), aeration appeared to increase toxicity when silica was added, and temperate appeared to have only a weak effect on the toxicity of aerated cultures ($F = 3.05$, $p = 0.09$). Non-motile cells were scarce in all cultures (< 10%), so a separate analysis of LC_{50} on a motile cell basis was not performed since the pattern of results was very similar.

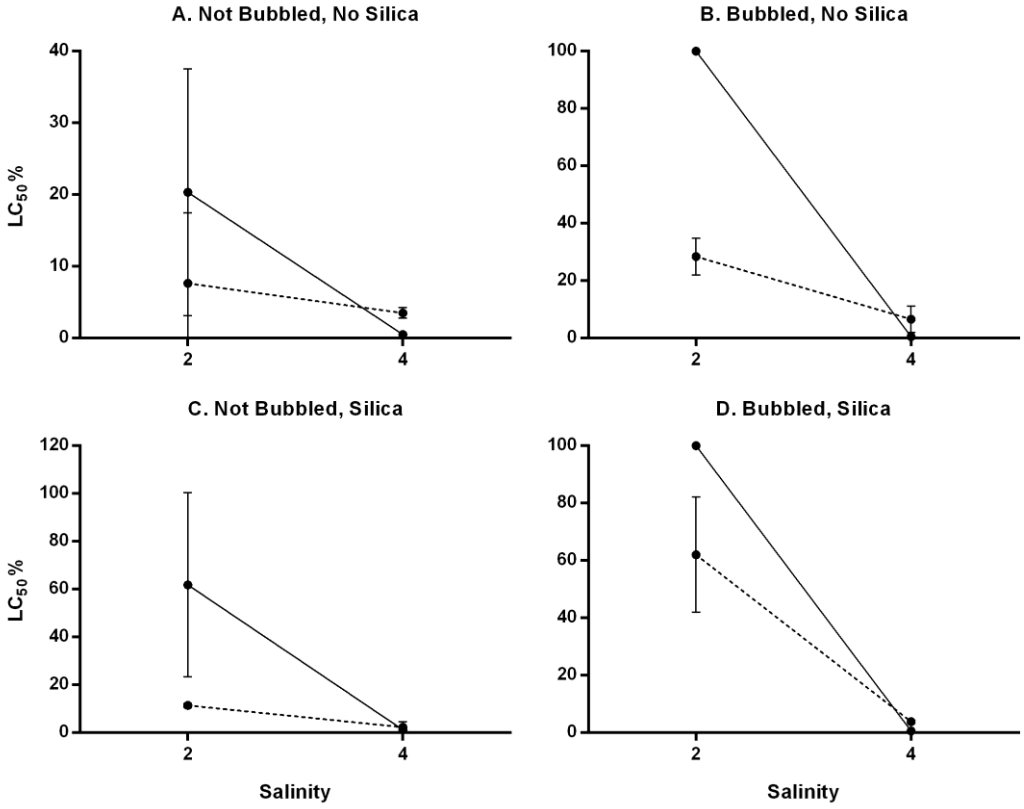

Figure 4.14 LC_{50} on a percentage dilution basis. 14 ^{o}C temperature represented by the dashed line, 20 ^{o}C by the solid line. Data are means ± standard deviation for duplicate cultures.

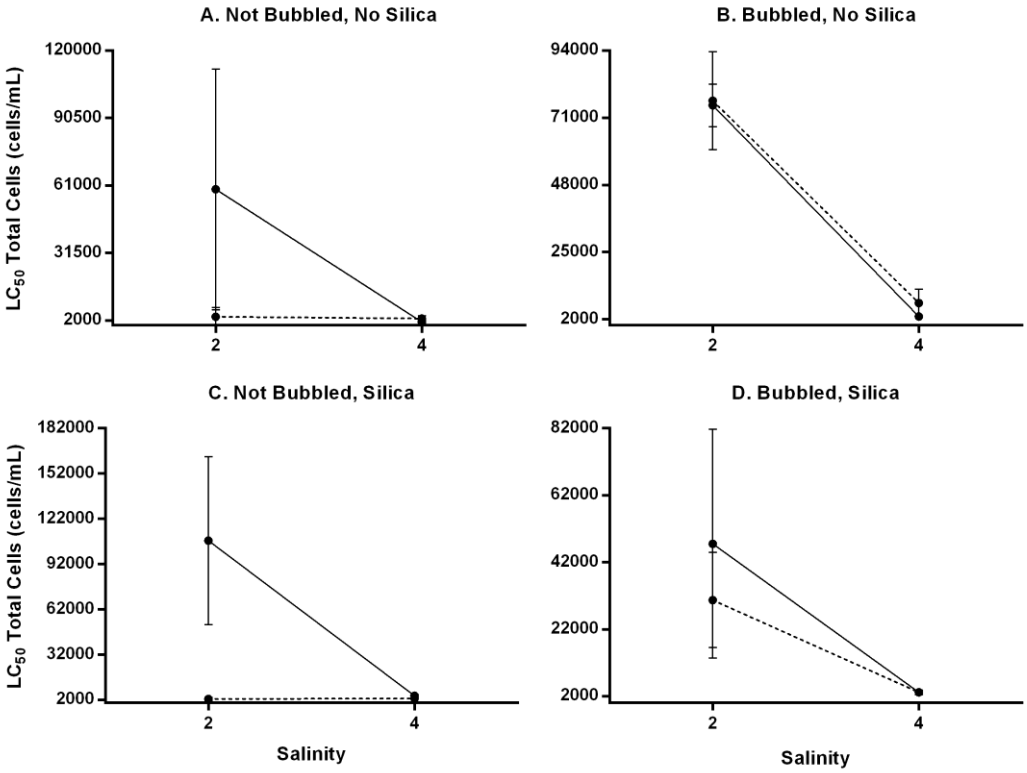

Figure 4.15 LC_{50} on a total cell basis. 14 ^{o}C temperature represented by the dashed line, 20 ^{o}C by the solid line. Data are means ± standard deviation for duplicate cultures.

Table 4.5 ANOVA of LC_{50} on a percentage dilution basis in the factorial experiment. LC_{50} data are arcsine square root transformed.

Source	SS	df	MS	F	P
Temperature (T)	0.507	1	0.507	23.39	**< 0.001**
Salinity (S)	3.733	1	3.733	172.19	**< 0.001**
Bubbling (B)	0.933	1	0.933	43.05	**< 0.001**
Silica (Si)	0.099	1	0.099	4.56	0.048
T × S	1.063	1	1.063	49.02	**< 0.001**
T × B	0.065	1	0.065	2.97	0.104
T × Si	0.003	1	0.003	0.15	0.698
S × B	0.829	1	0.829	38.23	**< 0.001**
S × Si	0.124	1	0.124	5.71	**0.03**
B × Si	0.01	1	0.01	0.45	0.508
T × S × B	0.13	1	0.13	6.01	**0.026**
T × S × Si	0.002	1	0.002	0.07	0.783
T × B × Si	0.07	1	0.07	3.23	0.091
S × B × Si	0.005	1	0.005	0.22	0.644
T × S × B × Si	0.062	1	0.062	2.87	0.109
Error	0.347	16	0.022		

Table 4.6 ANOVA of LC_{50} on a total cell basis in the factorial experiment.

Source	SS	df	MS	F	P
Temp (T)	4.841	1	4.841	11.59	**0.004**
Salinity (S)	35.753	1	35.753	85.58	**< 0.001**
Bubbling (B)	6.325	1	6.325	15.14	**0.001**
Silica (Si)	0.006	1	0.006	0.01	0.908
T × S	7.734	1	7.734	18.51	**0.001**
T × B	6.551	1	6.551	15.68	**0.001**
T × Si	1.265	1	1.265	3.03	0.101
S × B	2.009	1	2.009	4.81	**0.043**
S × Si	0.133	1	0.133	0.32	0.581
B × Si	2.334	1	2.334	5.59	**0.031**
T × S × B	3.813	1	3.813	9.13	**0.008**
T × S × Si	0.203	1	0.203	0.49	0.496
T × B × Si	0.071	1	0.071	0.17	0.685
S × B × Si	0.062	1	0.062	0.15	0.705
T × S × B × Si	0.009	1	0.009	0.02	0.885
Error	6.684	16	0.418		

Table 4.7 ANOVA of Phosphorus cell quotas in the factorial experiment.

Source	SS	df	MS	F	P
Temp (T)	3.486	1	3.486	13.97	**0.002**
Salinity (S)	2.641	1	2.641	10.58	**0.005**
Bubbling (B)	0.235	1	0.235	0.94	0.347
Silica (Si)	3.226	1	3.226	12.92	**0.002**
T × S	4.221	1	4.221	16.91	**0.001**
T × B	1.316	1	1.316	5.27	**0.035**
T × Si	0.318	1	0.318	1.27	0.276
S × B	0.025	1	0.025	0.10	0.757
S × Si	2.477	1	2.477	9.93	**0.006**
B × Si	0.453	1	0.453	1.82	0.196
T × S × B	2.799	1	2.799	11.22	**0.004**
T × S × Si	1.191	1	1.191	4.77	**0.044**
T × B × Si	0.052	1	0.052	0.21	0.653
S × B × Si	0.054	1	0.054	0.22	0.648
T × S × B × Si	0.034	1	0.034	0.13	0.719
Error	3.994	16	0.250		

When not aerated, the 14 ºC cultures had a higher phosphorus quota than the 20 ºC cultures at both salinities ($F = 1.191$, $p = 0.044$). When aerated, the 20 ºC cultures had a higher P quota at 2 psu salinity. This was reversed at 4 psu salinity; the 14 ºC cultures had a higher P quota than the 20 ºC cultures ($F = 11.22$, $p = 0.004$).

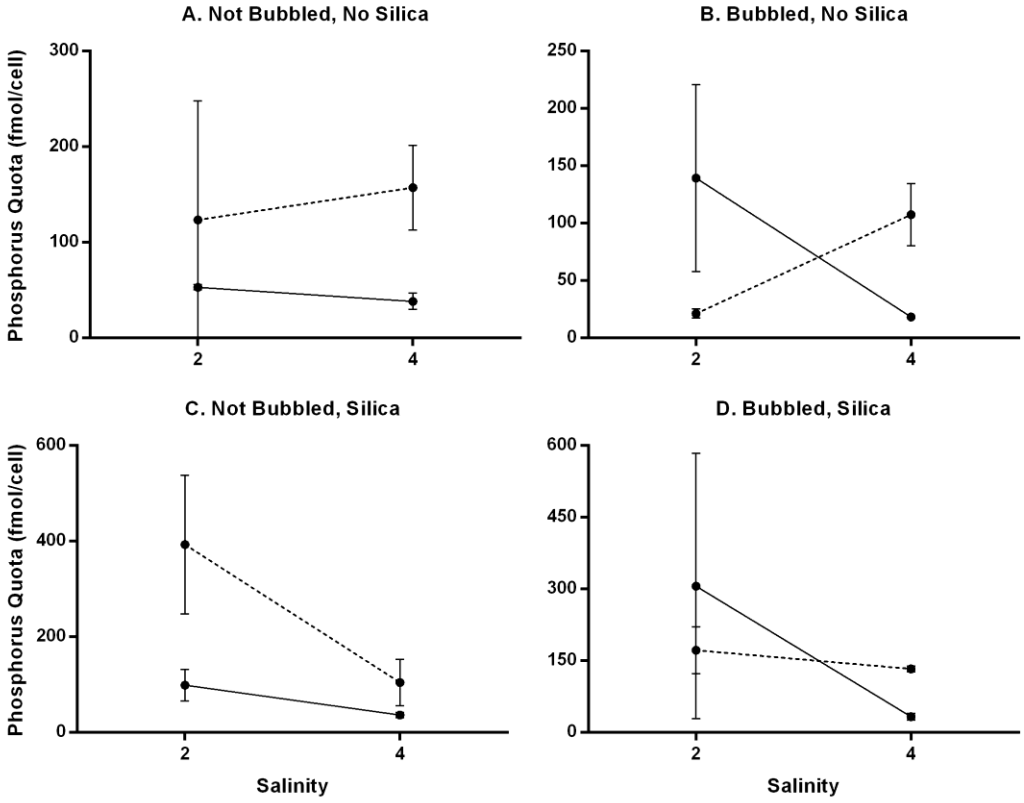

Figure 4.16 Phosphorus quota (fmol/cell). 14 ^{o}C temperature represented by the dashed line, 20 ^{o}C by the solid line. Data are means ± standard deviation for duplicate cultures.

4.3.4 Discussion

The non-motile stage was scarce during this experiment, and motile cells dominated, this experiment does not permit drawing conclusions about motility, but it does permit exploration of other factors affecting toxicity. Cultures grown at 14 ^{o}C generally exhibited greater toxicity. The 4 psu cultures were generally more toxic than those grown at 2 psu salinity. However, bubbling with air and silica additions did provide some exceptions to this. The 4 psu cultures were only moderately toxic without bubbling and without the silica addition. There were significant interactions between: temperature and salinity, temperature and bubbling, salinity and silica additions, and temperature, salinity, and bubbling.

There was no consistent trigger for the non-motile phase identified. There were small numbers of non-motile cells in all cultures. No patterns were apparent and thus there were possibly complex interactions, but observations of non-motile cells were too few to allow statistical analysis.

. These observations support some previous ideas about toxicity of *P. parvum*. In particular, low temperature enhanced toxicity (as in Baker et al. 2007). But low salinity did not (as would also be expected from Baker et al. 2007), probably for a similar reason to Baker et al. (2009). Low salinity might increase cellular stress and thus enhance toxicity as proposed by Baker et al. (2007), but it also decreases abundance and thus perhaps also total toxin production. Aeration did not consistently increase toxicity, as expected from Igarashi et al. (1995, 1998), possibly because the aeration rate used here was comparatively low (Skingel et al. 2010), However, because few non-motile cells were observed, this experiment did not strongly suggest conditions that promote the transition to non-motile stages. The experiments reported in previous sections of this chapter all used nutrient-limited media that prevented a high accumulation of cell density, so comparing nutrient-limited to nutrient-sufficient conditions would be a logical next step.

4.4 Population Density and Nutrient Limitation of *Prymnesium parvum* in Relation to Toxicity and Formation of Non-motile Stages

4.4.1 Introduction

The experiment reported here compared nutrient-sufficient and nutrient-limited cultures. We contrasted nutrient-sufficient with nutrient-limited cultures because nutrient limitation often enhances toxicity in *P. parvum* (Dafni et al. 1972, Johansson and Granéli, 1999). Included is a treatment that added glucose to nutrient-limited cultures to encourage mixotrophy based on a hypothesis that toxicity is related to and enhanced by mixotrophy. In a previous laboratory experiment with glucose supplementation of phosphorus-limited cultures (Harman and Grover

2008); aggregations of cells were seen resembling the “feeding swarms” of mixotrophic *P. parvum* observed by Tillman (2003). Mixotrophy can stimulate toxic activity if the toxin is used for prey immobilization (Brutemark and Granèli 2011).

4.4.2 Methods

The media used for these cultures is a modification of an artificial seawater medium in which the ion composition is similar to that of Texas lakes where *P. parvum* occur (LW medium, Baker et al. 2009). This medium includes higher relative concentrations of calcium and bicarbonate than found in seawater. All cultures were maintained at 20 ^{o}C, a salinity of 3 psu, an irradiance of 150 µE m^{-2} s^{-1}, and a 12:12 photoperiod. All three experimental groups were triplicated.

Prior to inoculation from stock cultures, the flasks of LW media were sterilized through autoclaving. The sodium bicarbonate portion of the media was added aseptically using a 0.2 µm Nalgene nylon syringe filter. This was done to prevent precipitation during autoclaving. The 9 flasks were then separated into the 3 experimental groups and received the appropriate amount of nitrogen and phosphorous (Table 4.6), which was also added aseptically via filter-sterilization. After this process each flask received 2 mL of a *P. parvum* stock culture that was in a late exponential growth phase.

Nitrogen and phosphorous were supplied at f/2 levels for the nutrient-sufficient cultures and 40 uM nitrogen and 1.5 uM phosphorous for the nutrient-limited cultures. The nutrient supplies of these cultures were chosen because they represent those of north Texas lakes where *P. parvum* occurs. Three experimental groups were used: nutrient-sufficient, nutrient-limited, and nutrient-limited with added glucose (Table 4.6). The cultures were 5 liters in volume to accommodate multiple samplings for testing acute toxicity to fish.

Table 4.8 Description of experimental cultures for experiment 3

Experimental Culture	Description
Nutrient-Sufficient	f/2 levels of nitrogen and phosphorous
Nutrient-Limited	40 uM nitrogen and 1.5 uM phosphorous
Nutrient-Limited with Carbon Source	40 uM nitrogen and 1.5 uM phosphorous + glucose (300 µM)

All cultures were sampled for live counts, preserved counts, particulate nutrients, bacteria, chlorophyll, and toxicity determinations. Live counts were performed using Sedgwick rafter slides and a compound light microscope at 400X magnification. These counts were preformed every other day until stationary growth phase was reached at about day 14 and then weekly to day 42. The use of live counting provides a measure of motile and non-motile cells a various stages of cell population growth. Since this method is a rapid and somewhat imprecise measure of cell density, 5 mL samples were also taken and preserved with Lugol's iodine solution to measure total population density more precisely. The time of preserved sampling coincided with the live count sampling schedule. Sedgwick rafter slides were also used for preserved sample counts.

Table 4.9 Sampling schedule for experiment 3

Sample	Day
Live and preserved counts	Every two days until day 10. Then weekly from day 14
Particulate phosphate, CHN, bacteria, chlorophyll, LC50	6, 14, 21,28, 35, 42

4.4.3 Results

Non-motile cells were scarce in the nutrient-limited and nutrient-limited with glucose cultures. On the other hand, the nutrient-sufficient cultures appeared to shift from the motile stage to the non-motile stage around day 28 and then again at the endpoint of the experiment (Fig. 4.17). Motile cells continually increased in the nutrient-limited cultures, but did not reach a steady state. None of the cultures reach an apparent steady state. Motile cells increased in the nutrient-limited cultures with added glucose, but temporarily decreased around week number three and then rose again at the end of the experiment.

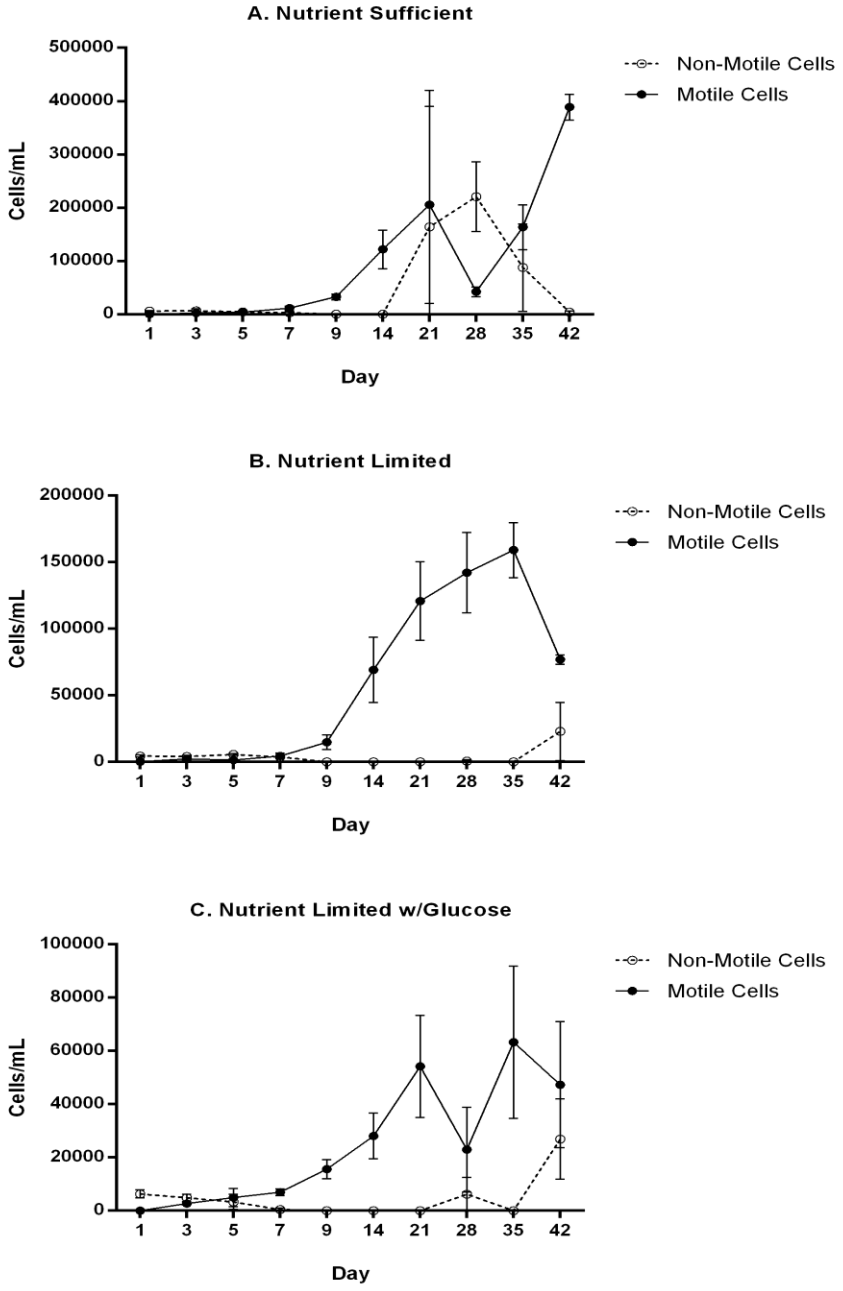

Figure 4.17 Motile and non-motile cells time series. (A) nutrient sufficient, (B) nutrient limited, (C) nutrient limited w/glucose. Error bars are ± standard deviation.

Total densities of *P. parvum* were highest in nutrient-sufficient cultures, followed by nutrient-limited cultures, followed by nutrient-limited cultures supplemented with glucose (Fig. 4.17). When inoculated from stock cultures, populations in all treatments were initially dominated by non-motile cells. By day 9 the majority of cells in all cultures were motile and total population was increasing. Toxicity to fish on day 7 was low for all cultures, but was significantly greater for the nutrient-sufficient cultures than for the other treatments (one-way ANOVA, $F = 8.853$, $p = 0.016$). By day 14, populations of $10^4 – 10^5$ cells/mL had developed in all cultures, and toxicity to fish was high (48-hr LC_{50} < 10% dilution of whole culture samples). Toxicity to fish remained high (low LC_{50}) during the rest of the experiment in nutrient-limited cultures and nutrient-limited cultures supplemented with glucose. From days 21 – 42, toxicity of the nutrient-limited and nutrient-limited carbon-enriched cultures was significantly higher (lower LC_{50}) than toxicity of the nutrient-sufficient cultures (one-way ANOVA, $F = 13.457$, $p = 0.006$). The more toxic treatments were also consistently dominated by motile cells. In contrast to these more toxic treatments, nutrient-sufficient cultures decreased in toxicity (LC_{50} increased) from days 21 – 35, during which time the proportion of non-motile cells was high.

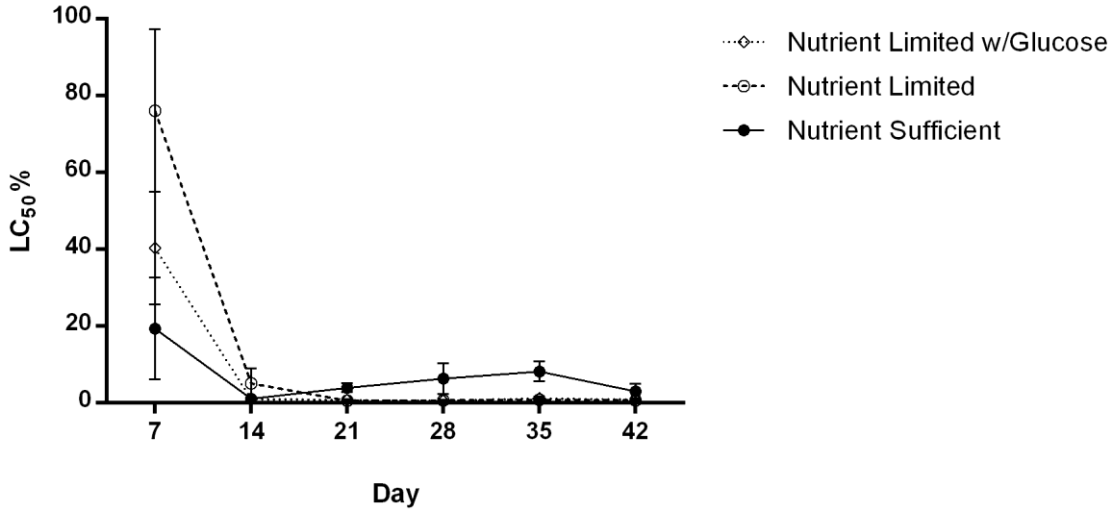

Figure 4.18 LC_{50} on a percentage dilution basis. Black dots are nutrient-sufficient, white dots are nutrient-limited, white diamonds are nutrient-limited w/glucose. Error bars represent ± standard deviation.

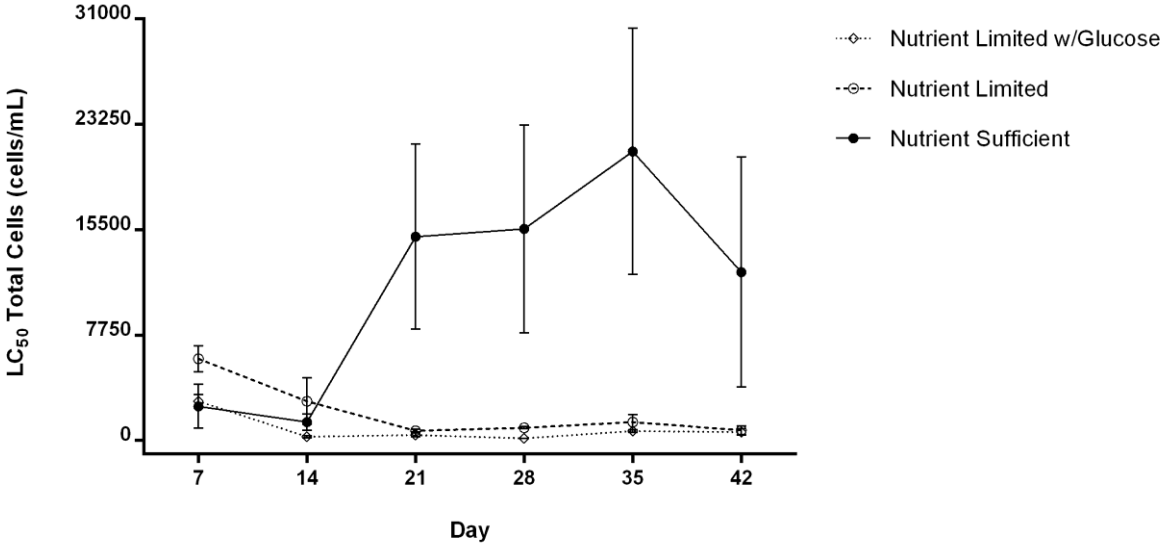

Figure 4.19 LC_{50} on a total cell basis. Black dots are nutrient-sufficient, white dots are nutrient-limited, white diamonds are nutrient-limited w/glucose. Error bars represent ± standard deviation.

Phosphorus quotas were relatively low in all experimental groups. This can be related to the high toxic activity observed. The nutrient-sufficient cultures exhibited the most variation in phosphorus quotas, which can also be related to the variation observed in toxicity (Fig. 4.20). On all days, differences among treatments were statistically significant (Table 4.8).

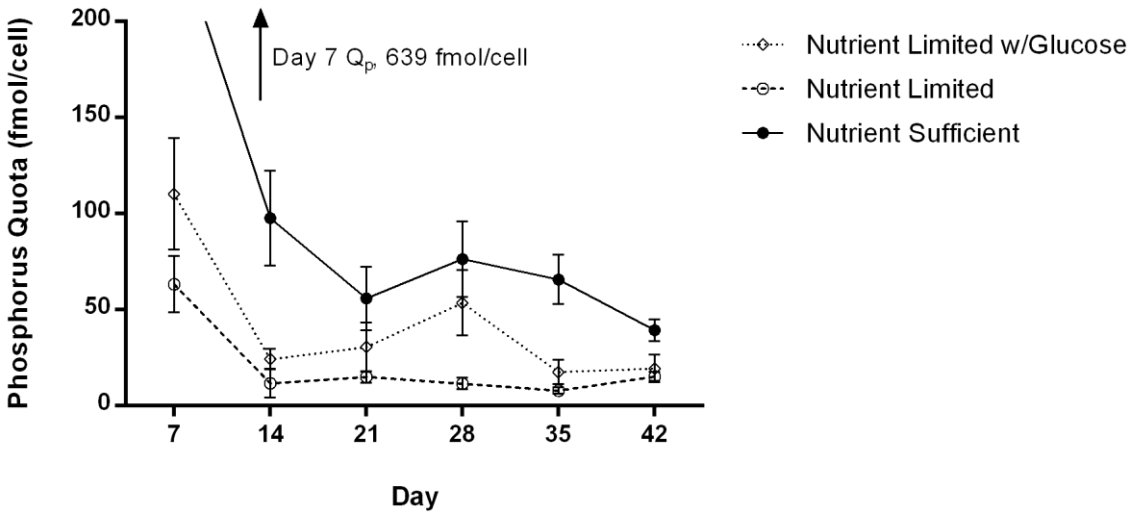

Figure 4.20 Phosphorus cell quota (fmol/cell). Black dots are nutrient sufficient, white dots are nutrient limited, white diamonds are nutrient limited w/glucose. Error bars represent ± standard deviation.

Table 4.10 Statistical analysis, ANOVA for experiment 3

Measure	Day	F-ratio	P-value
Total cells	7	3.83	0.08
	14	9.98	**0.01**
	21	16.66	**0.003**
	28	27.93	**< 0.001**
	35	21.07	**< 0.001**
	42	142.83	**< 0.001**
Non-motile cells	7	5.41	0.05
	14	9.98	**0.01**
	21	1.46	0.30
	28	29.46	**< 0.001**
	35	9.54	**0.01**
	42	278.11	**< 0.0001**
LC_{50}%	7	8.80	**0.01**
	14	3.22	0.11
	21	47.66	**< 0.0001**
	28	24.54	**< 0.001**
	35	38.06	**< 0.0001**
	42	11.40	**0.009**
LC_{50} total cells	7	8.85	**0.01**
	14	4.74	0.06
	21	13.45	**0.006**
	28	11.59	**0.008**
	35	14.93	**0.005**
	42	5.72	**0.04**
Phosphorus cell quota	7	15.56	**0.004**
	14	28.08	**< 0.001**
	21	8.60	**0.01**
	28	14.07	**0.005**
	35	41.28	**< 0.001**
	42	16.95	**< 0.001**

4.4.4 Discussion

Nutrient-sufficient cultures had greater cell densities compared to both the nutrient-limited cultures and the nutrient-limited cultures supplemented with glucose. The LC_{50} remained low in both types of nutrient-limited cultures, but varied over time in the nutrient-sufficient cultures. The proportion of motile cells remained high in both the nutrient-limited cultures, but varied over time in the nutrient-sufficient cultures. Figure 4.17 shows a shift from a population dominated by non-motile cells to one dominated by motile cells. During this same time, the toxicity varied inversely to motile cells decreased as the non-motile cells began to dominate.

Nutrient-sufficient cultures were the least toxic in general, and temporal phasing of toxicity appeared related to motile versus non-motile cells. These observations support the hypothesis that motile cells are more active in producing toxin than non-motile cells, and field observations that blooms are less toxic when there are a lot of non-motile cells (Genitsaris et al. 2009). Higher toxicity of the two nutrient-limited treatments is also expected from previous observations and hypotheses that nutrient stress promotes toxicity.

Higher quotas of nutrient-sufficient cultures were coupled to their lower toxicity, as expected based on Skingel et al. (2010). Low quotas were in low phosphorus cultures. They were higher in low phosphorus cultures with glucose; this would be expected if *P. parvum* were eating bacteria, which are expected to be relatively phosphorus rich.

While both types of nutrient-limited cultures remained very toxic throughout the experiment, the nutrient-sufficient cultures had changes in toxicity. This was correlated with the shift in abundance of motile cells to non-motile cells, with the motile cells more toxic. These results suggest that non-motility or encystment is not a result of stressful conditions, unlike toxicity. Instead, this research can suggest that induction of the non-motile state may be a result of high population density, rather than stress due to non-optimal light, salinity, or temperature. The experiment strongly suggests that high population density promotes the transition to non-motile stages. In light of the other sections of this chapter, other potential triggers of non-motility

have not been supported. Low nutrients do not trigger non-motility, and factors such as temperature, salinity, aeration, silica appear to have weak influence.

CHAPTER 5

CONCLUSIONS

The research presented has provided new information on how toxicity to fish on the part of *P. parvum* is closely related to population shifts from dominance by motile cells to dominance by non-motile cells under certain culture conditions. The experiment described in section 4.4 produced results contrary to the current view of stress related encystment (Green et al. 1982, Johnsen et al. 2010). Instead, the production of large numbers of non-motile cells appears to be related to high population density. Additionally, the manipulation of other conditions such as low temperature or limiting nutrients did not result in high numbers of non-motile cells. These results should also open new lines of study involving the proximate extracellular signals mediating these life history shifts, and may eventually lead to novel bloom control strategies based on the manipulation of life history events.

As presented, the hypothesized bloom cycle attributes termination primarily to encystment after all resources, inorganic and organic, have been depleted (Fig. 1.1). Based on the observations made in this study, it would be reasonable to modify this conceptual model by adding density-dependent non-motility and encystment to other hypothesized mechanisms of bloom termination. However, this does not mean to exclude plausible alternatives for bloom termination, such as hydraulic washout and inflow of fresh water, growth of competitor or grazer populations, or infection by viruses. Some of these alternative explanations of bloom termination have been tested in our field experiments, through treatments manipulating grazers and viruses

(Schwierzke et al. 2010). Bloom termination by grazers, or perhaps viruses, could be especially important. The occurrence of *P. parvum* blooms in winter in Texas inland waters suggests that appropriate natural enemies are not active to attack and prevent or terminate blooms in this region. The invasion of *P. parvum* into Texas inland waters appears to be recent. As many biological invasions proceed, either indigenous species or other invaders eventually proliferate to attack the new invader. It this idea is correct, winter blooms of *P. parvum* could be a transient phenomenon that will eventually occur less frequently, once a competent natural enemy proliferates and actively attacks abundant blooms. The proposed cycle of *P. parvum* blooms, including a possibility for density-dependent non-motility and encystment emphasizes the endogenous population processes orchestrated by life history transitions, but we expect that a complete explanation of this species' ecology will require a complementary theory of the exogenous processes in plankton communities and lake habitats that set the timing for these life history events and the concurrent initiation and termination of blooms.

The proposed cycle of bloom initiation, formation, and collapse is at present only partially supported by available evidence. The cycle begins with a small population of active, motile cells under conditions favorable for growth, which serves to inoculate a bloom. This inoculum could come either from germination of cysts already present at a location, or from the immigration of active cells from elsewhere. Given the presence of an inoculum, a bloom develops from *in situ* growth if circumstances remain favorable for growth for about one or a few weeks. We now have sufficient observations of population growth in both lab and field circumstances to state with reasonable precision the bounds on conditions of temperature, salinity, irradiance, and nutrient concentration that such population growth requires. Observations also suggest that reduced populations of competing algae and grazers must occur to allow this growth phase to take place. However, both field and laboratory observations suggest that bloom termination might follow several pathways whose importance in natural systems remains to be fully assessed.

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