EFFECT OF NUTRIENT LIMITATION, COMPETITORS AND GRAZERS ON THE ABUNDANCE AND TOXICITY OF LABORATORY CULTURES OF *P. PARVUM A,* HARMFUL ALGA

by

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ABSTRACT

EFFECTS OF NUTRIENT LIMITATION, COMPETITORS AND GRAZERS ON THE ABUNDANCE AND TOXICITY OF LABORATORY CULTURES OF *P. PARVUM*, A HARMFUL ALGA

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P. parvum Carter is a haptophyte that occurs world wide in both fresh and brackish waters. Though P. *parvum* blooms occur world wide under a variety of environmental conditions, it occurs during the cooler months of the year in Texas. Various treatment combinations of temperatures, salinity and limiting nutrients were used to test the hypothesis that the interaction of these factors would affect the abundance and toxicity of *P. parvum.* In addition to testing various above mentioned factors and their interactions, *P. parvum* was grown in mixed species cultures with major competitors from a natural lake community (Lake Whitney) to test the hypothesis that competition from other algal species could be partially responsible for the seasonal

dynamics of *P. parvum* blooms in Texas lakes. Furthermore, the mortality rate of *P. parvum* was studied in a preliminary grazing experiment using two cladoceran species.

Findings confirm earlier reports of increase in toxicity during nutrient limiting growth conditions. *P. parvum* batch cultures grown at 3 psu salinity were more toxic than those cultures grown at 1 psu salinity and there was a considerable increase in acute fish toxicity during P limitation rather than in N limitation. A substantial decrease in toxicity was noticed in mixed species cultures, especially those grown at 30 ^{0}C.

It has been found that competition from other algal species can explain at least in part, the seasonal dynamics of *P. parvum* blooms in Texas lakes. *P. parvum* abundance was greater in mixed species cultures at 10 ^{0}C (representing winter conditions) than the corresponding cultures at 30 ^{0}C. At warmer temperatures (30 ^{0}C representing summer conditions) other algal species out-competed *P. parvum.*

Sub-lethal toxicity studies confirm the toxic effects of *P. parvum* on *Daphnia magna*, a common grazer species that cooccurs with *P. parvum* in some habitats. Mortality rate of *P. parvum* under the influence of grazers was studied using a dilution experiment with two cladoceran species *Daphnia magna* and *Ceriodaphnia dubia*. This study demonstrated that cladocerans cause mortality of *P. parvum* suggesting that direct ingestion may be one route by which grazing zooplankton are exposed to toxins.

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CHAPTER 1

INTRODUCTION

Harmful algal blooms (HABs) are caused by microscopic algae when harmful species out-compete other co-existing algal species. Extensive growth of these harmful algal species can become detrimental to aquatic flora and fauna by decreasing light penetration, depleting available resources, by secreting toxins into surrounding water, or by the accumulation of intracellular toxins in herbivores and, eventually, other animals. HABs can occur in a variety of aquatic habitats ranging from marine, estuarine to fresh water under a wide range of salinities. Conditions that favor formation of algal blooms vary widely; hence the prediction of bloom initiation in different aquatic systems is difficult. Alteration of water quality by human-induced cultural eutrophication is considered as one of the major reasons (Hallegraeff 1993) for the initiation of blooms.

A newly investigated HAB species, *Prymnesium parvum* Carter, is a haptophyte that occurs worldwide. Though it is a euryhaline species seen predominantly in brackish waters (Moestrup 1994), tolerance to low salinity levels has allowed it to colonize inland waters also (Shilo 1971, Bjerskov et al. 1990). *P. parvum* was first reported in the 1920s (Liebert and Deerns 1920) where it caused massive fish kills in European coastal waters. Fish kills due to *P.parvum* were subsequently reported from fish-farming ponds in Israel and China (Shilo and Shilo 1953, Guo et al. 1996). Based on anecdotal evidence, fish kills due to *P. parvum* in the state of Texas started as early in 1965, but scientific confirmation came later, in 1984, after fish kills in Pecos River

valley (James and De La Cruz 1989). In later years, blooms dominated by P. parvum and related fish kills were extensively reported from Brazos River, the Colorado River, and the Red River basins. Since 1985, *P. parvum* blooms have caused major fish kills in the aquaculture industry and in natural habitats of rivers and reservoirs. It is estimated that about 7.6 million fish were killed in various rivers in Texas and 5.1 million fish were killed in Dundee fish hatchery during the production season in 2001 alone, causing an economic loss of about 4.5 million dollars (TPWD 2003).

Since the first report of occurrence of *P. parvum* mediated fish kills extensive studies were done investigating various factors (biotic and abiotic) that affect bloom formation and toxicity. As mentioned earlier, *P. parvum* is a euryhaline species and has a wide distribution because of tolerance to lower salinity ranges. Occurrence of *P. parvum* has been documented from a wide range of salinities from 3- 30 psu by different researchers (McLaughlin 1958, Paster 1973, Kaartvedt 1991, Larsen and Bryant 1998). The ability of different *P. parvum* strains to tolerate a wide range of salinities is attributed to its ability to synthesize compatible solutes (Dickson and Kirst 1987).

P. parvum blooms can occur in a wide variety of habitats under varying environmental conditions. In Texas, *P. parvum* blooms are more frequently reported from October through April, which are comparatively cooler than other months of the year. This suggests that temperature is an important ecological factor influencing the growth of *P. parvum.* There are several studies addressing the effect of temperature, light, salinity and nutrients on the effect of exponential growth rate of similar strains of

P. parvum and *P. patelliferum,* considered as same species currently (Larsen and Edvrdsen 1998). Most of these studies were conducted in marine isolates.

Anthropogenic nutrient enrichment of water bodies may explain in part the increasing frequency and intensity of HABs. The state of Texas is continuously experiencing high rates of industrialization, urbanization and agricultural development leading to clearing of forest and grasslands and subsequent application of N-based fertilizers. Thus nutrients can potentially reach the water bodies through surface water runoff, and stimulate the growth of harmful algae such as *P. parvum* (Hallegraeff, 1993).

Prymnesium parvum produces an array of highly potent exotoxins generally known as prymnesins (Shilo and Sarig, 1989, Igarashi et al. 1996). It has a wide range of biological activities including ichthyotoxic, cytotoxic, neurotoxic, haepatotoxic activity that potentially affect a wide range of aquatic organisms. Prymnesins are usually most detrimental to gill-breathing aquatic organisms like fishes and molluscs (bivalves) as they affect the permeability of gill tissues in a two-step process that causes the membrane to become permeable to calcium (Volkanov 1964, Shilo 1971, Ulitzer and Shilo 1964). A structural elucidation of prymnesin done by Igarashi and co-workers (1996) revealed the presence of two glycosidic toxins, prymnesin I and II, having similar biological activities.

Like the growth rate, toxicity is also influenced by a number of environmental factors (salinity, temperature, irradiance, nutrient availability etc.). Previous studies generally show that *P. parvum* is more toxic when it is subjected to physiological stress

such as growth-limitation by environmental conditions (Ulitzur and Shilo 1964; Dafni et al. 1972; Baker et al. 2007). There are contradicting views regarding the effect of temperature on the toxicity of *P. parvum*. Most of the studies have suggested that temperature does not have any significant effect on toxicity within a range of 5- 30 ^{o}C (Shilo and Ashner 1953, Yariv and Hestrin 1961, Larsen and Bryant 1998). In a fish toxicity study conducted by Ulitzer and Shilo (1964), they noticed a positive correlation of temperature and toxicity within a range of 10 ^{0}C to 30 ^{0}C contradicting results from the previous studies.

Several previous studies have documented the effect of light on toxicity (Shilo and Aschner 1953, Parnas, Reich, Bergman 1962) revealing a complete inactivation of toxicity by UV light. The light-sensitive inactivation of toxin could be either due to direct inactivation by light or due to the delayed toxin synthesis because of changes in the rate of photosynthesis and subsequent metabolism.

Larsen and Bryant (1998) concluded that salinity does not have a significant effect on toxicity. Other studies suggest that pH has a significant effect on toxicity. Shilo and Aschner (1953) and McLaughlin (1958) observed high toxicity from neutral to higher pH ranges (7.5- 9) with lower toxicity below pH 7.5. According to Padilla and Martin (1973) different toxic properties respond differently at different pH. Maximum binding of the toxin-receptor occurred in the range of 4.6 to 5.5 where as the maximum hemolytic activity occurred at pH 5.5 and this contradicts results from previous studies. They also noticed a complete arrest of cytotoxicity at a pH of 6.4.

Many previous toxicity studies examined the effect of nutrient limitation. The first reported relationship between low levels of P and high toxicity levels was found by Shilo in 1967. This was later confirmed by Dafni, Ulitzer and Shilo (1972) in their study investigating the mechanism of increase in toxicity during nutrient limitation. They speculated a phosphate limitation in the surrounding environment can cause leakiness of the membrane of the victim, because of malformation of membrane phospholipids. The effect of nutrient limitation on the toxicity was also found in a related species, *Chrysochromulina polylepis* (Edvardsen et al. 1990, Simonsen and Moestrup, 1997). In a field study conducted by Kaartvedt et al. (1991) in the Sandsfjord system in Norway, toxicity was associated with phosphorous limitation. Johannson and Granéli (1999) noticed increased hemolytic activity at both N and P limitation. This observation implies that there is no direct effect of P or N on toxin synthesis, but rather that the physiological stress of nutrient-limited growth is responsible for enhanced toxicity.

Previous experimental results from laboratory cultures of *P. parvum* suggests that blooms could occur in Texas in land waters during summer, because the optimal temperature for growth is relatively high at 27 °C, and growth rates remain high up to 30 ° (Baker et al. 2007). But it has been observed that *P. parvum* blooms occur in Texas inland waters from early winter to spring, suggesting the occurrence of other seasonal factors that limit growth of *P. parvum*, such as competition from other algal species or grazing.

Competition among different algal species has been suggested as one of the important shaping factors of community structure and composition in several algal communities (Dayton 1975, Tilman et al. 1982, Grover 1997). Grazers also have a considerable influence in plankton community dynamics. Several studies have showed a decrease in grazer population can be a contributing factor for the initiation of blooms (Fleming 1939, Martin 1965).

Though several studies have investigated the influence of different environmental factors like temperature, salinity, and irradiation on the growth dynamics and toxicity of *P. parvum,* only a few of them have looked at the effect of interaction of these factors on the growth and toxicity of *P. parvum* (but see Baker et al 2007). In many of the studies reviewed above, toxicity of *P. parvum* was measured with assays based on hemolytic activity or effects on brine shrimp (*Artemia salina*), which do not occur with *P. parvum* in lower salinity ranges characteristic of Texas inland waters. Earlier studies conduced by Shilo's group in Israel, and the recent study by Baker et al. (2007) used bioassays based an acute lethal toxicity to fish. Only one previous study (Roelke et al. 2007) examined toxic effects on cladoceran crustaceans, which are important herbivores in inland waters, finding that field samples dominated by *P. parvum* could reduce the fecundity of *Daphnia magna*.

This study addresses the effects of temperature, salinity, limiting nutrients and their interactions using factorial experimental designs that allow for the presence of interactions among factors. Moreover, the competitive interaction of *P. parvum* is studied by growing it with major competitors collected from a natural lake community

(Lake Whitney) at two different temperatures representing spring and summer conditions. Grazers were also examined, both for their effects on *P. parvum* and for toxic effects of *P. parvum* on grazers. Though there are several studies on the effect of grazers on many algal species, little is known about the effect of the grazers commonly found in inland waters on *P. parvum*. In this study, the effects on *P. parvum* of two cladocera were examined; *Daphnia magna* and *Ceriodaphnia dubia*, and the sub-lethal effects of *P. parvum* on the reproduction of *D. magna* were examined. In this study a model fish, the fathead minnow (*Pimephales promelas*) was also used as a test organism for toxicity assays, which may be of relevance to understanding blooms and fish kills in natural habitats due to *P. parvum.*

1.1. Semicontinuous cultures

In this study, the technique of semicontinuous culture was used to create nutrient-limited cultures of *P. parvum* of sufficient volume and duration to permit examining toxic effects on both fish and cladocerans. Semi-continuous cultures should approximate a chemostat, or truly continuous culture, when maintained with frequent dilutions at a constant volume. True chemostat cultures have a culture vessel of constant volume, into which a sterile nutrient medium is pumped at a constant rate, balanced by an outflow of the whole culture. Semi-continuous cultures are diluted periodically (here every two days) with defined volume of sterile medium, after removing the same volume of whole culture.

Expectations for these experiments were thus based on the conventional theory of chemostat cultures (Monod 1950, Grover 1997). Consider the population density (N)

of a species growing in a chemostat with a growth-limiting resource at a concentration *R*, supplied from an inflow having a concentration *S*. The flow rate as a proportion of culture volume is *D*, the dilution rate. The system of equations describing resource and species dynamics is

$$\frac{dN}{dt} = \mu(R)N - DN \tag{1}$$

$$\frac{dR}{dt} = D(S - R) - \frac{\mu(R)N}{Y} \tag{2}$$

According to eq. (1), population density increases with growth at a per capita rate μ, and decreases with dilution. According to eq. (2), resource concentration increases with supply in the inflow decreases with consumption which is proportional to growth through the yield coefficient (*Y*). This latter quantity is the population density produced by consumption of a unit concentration of the nutrient resource.

The growth rate is modeled as a Monod function of the nutrient concentration (Monod 1950):

$$\mu(R) = \frac{\mu_{\max} R}{K + R}$$

Growth rate increase with nutrient resource concentration as a rectangular hyperbola and saturates at $\mu_{\max}$.

In a chemostat, a steady state is reached in which population density and nutrient concentrations are constant. When operated with a dilution rate less than about ¾ of the maximal growth rate, most of the limiting nutrient is consumed at steady state, and thus its concentration is low. Manipulating the relative concentrations of potential

growth-limiting nutrients permits examining defined physiological states, such as N or P limitation. Other limiting factors, such as temperature or salinity affect the parameters of the growth function (eq. 3), thus altering the steady state population density and physiological status.

In the current study semi-continuous cultures were used to study the effect of various environmental factors on the growth rate of *P. parvum.* Therefore the population is expected to reach a steady state in which the limiting nutrient is depleted to low concentration. The steady state nutrient concentration found in *P. parvum* cultures is theoretically a measure of its competitive ability (Tilman 1982, Grover 1997). If this concentration is low, then few or no competitor species will be able to persist. If it is high, then competitors will thrive and eventually dominate. Given that nutrient limitation is among the stresses previously associated with high toxicity of *P. parvum*, strong toxic effects on fish and crustaceans are expected for cultures maintained in semi-continuous cultures. Semi-continuous cultures were operated for several weeks, both to permit taking periodic samples over the three weeks required for assays of cladoceran reproduction, and to permit characterizing trends of competitive dynamics with other species.

1.2. Objectives

The overall objective of the current study is to further understanding of *P. parvum* bloom dynamics and toxicity using laboratory cultures as an experimental tool. Specifically this research focused on the roles of temperature, salinity, limiting nutrients

and interactions among these factors, interspecific competition among different algal species, and grazing in determining the abundance and toxicity of *P. parvum*.

CHAPTER 2

GROWTH AND TOXICITY OF *PRYMNESIUM PARVUM* IN MONOCULTURES UNDER N AND P LIMITATION AT DIFFERENT LEVELS OF TEMPERATURES AND SALINITY

2.1. Introduction

The primary goal of this experiment was to investigate changes in the abundance, nutrient element composition and toxicity of *Prymnesium parvum* (*P. parvum*) in response to differing N: P supply ratios, temperatures and salinities. All cultures were monocultures of *P. parvum* except for two additional mixed species cultures that were run as a prototype competition experiment.

Similar to terrestrial plants, phytoplankton require both macro and micro nutrients for their growth. Hence biological productivity of an aquatic ecosystem is a direct function of the amount and composition of available nutrients. Algal community structure, biomass and plankton biochemistry are highly dependent on the availability and composition of nutrient. For harmful species of algae toxicity can vary substantially with the supply of nutrients or other factors that limit growth (Plumley, 1997). Blooms dominated by *P. parvum* are reported mainly from highly eutrophic waters (Collins, 1978) characterized by elevated nitrogen (N) and phosphorous (P) levels. *Prymnesium parvum* is a euryhaline algal species occurring in habitats with widely ranging salinity (Shilo, 1971), but historically dense *P. parvum* blooms and associated fish kills are reported mainly from coastal and inland waters at higher salinity ranges (Paranas and

Abott, 1965; Moestrup, 1994). Since the late 1980's, blooms of *P. parvum* and associated fish kills have become common in inland waters of Texas and the south western U. S. with salinities just above fresh water that are probably less saline than habitats where earlier blooms were reported (TPWD website). These observations motivate examining the growth and toxicity of *P. parvum* in relation to nutrients, salinity and temperature.

Previous studies show that *P. parvum* is more toxic when it is subjected to physiological stress by environmental conditions (Ulitzur and Shilo 1964; Dafni et al. 1972; Baker et al. 2007). The first reported relationship between low levels of P and high toxicity levels was found by Shilo in 1967. Later Johansson and Granéli (1999) reported increased hemolytic activity under both N and P limitation. This observation implies that there is no direct effect of P or N on toxin synthesis, but rather that the physiological stress is responsible for enhanced toxicity. A general effect of stress promoting toxicity is consistent with reports that suboptimal temperature or salinity also enhances toxicity (Baker et al. 2007).

Though there are numerous studies addressing the effect of factors such as temperature, salinity, irradiance limiting nutrients etc. on the growth and toxicity of *P. parvum*, most do not address the interacting effects of these factors on the growth and toxicity of *P. parvum*. Moreover, many previous studies have used toxicological assays based on hemolysis of red blood cells, or effects on brine shrimp (*Artemia* spp.), which do not occur with *P. parvum* in habitats throughout the lower salinity range tolerated by this alga. Here this experiment addresses effects of temperature, salinity and limiting

nutrients using a factorial design capable of quantifying interactions. Toxicological assays, conducted by colleagues at Baylor University working under the direction of Dr. Bryan Brooks, used a fish (*Pimephales promelas*) and a crustacean (*Daphnia magna*) considered representative of organisms likely to co-occur with *P. parvum*. A semi-continuous culture design was used in an attempt to approximate steady state growth at a defined rate equal to average dilution rate (Kilham, 1978).

2.2. Materials and methods

2.2.1. Algal strain and culture conditions

The *P. parvum* strain used in this study was obtained from the UTEX culture collection of algae (strain number UTEX LB ZZ181). It was isolated from bloom dominated by *P. parvum* and causing and active fish kill in the Colorado River, Texas. This strain has been used in related studies of *P. parvum* (Baker et al. 2007). Stock cultures were maintained in a medium of Artificial Sea Water (ASW) (Kester et al. 1967) diluted to a working salinity of 5.8 psu with 18 Ω/ cm ultrapure water. The basal ASW medium was enriched with f/2 levels of nutrients, trace metals (ferrous ammonium sulfate was substituted with ferric chloride equimolar in iron) and vitamins (MacLachlan, 1973). These stock cultures were maintained in an incubator on a 12: 12 light: dark cycle with an irradiance of ~150 µmol photons/ m^2/ second. This photoperiod and irradiance were used for all experimental cultures as they are similar to the field conditions in fall and early winter during when *P. parvum* blooms cause fish kills in Texas lakes. Stock cultures were transferred to fresh medium on a monthly basis.

2.2.2. Experimental design

This was a large-volume semi-continuous experiment with three experimental factors each at two levels (2 x 2 x 2 factorial design) (Table 2.1)**.** There were two levels of temperature (10 ^{0}C and 30 ^{0}C) and salinity (1 psu and 3 psu) and two levels of limiting nutrients (high and low N: P supply ratios). There were two replicates for each treatment for a total of 16 cultures. In two additional cultures, *P. parvum* was grown in mixed culture with other species of algae as a prototype competition experiment.

Extensive *P. parvum* blooms and associated fish kills are reported from Texas inland waters and other parts of south western U. S. where the water is harder than ASW medium (i.e. richer in divalent cations). Hence we used a medium designated Lake Water medium (LW) modified from ASW to have salt composition closer to that of the weakly saline inland waters of Texas. A basal LW medium containing only the major ions (Na^+, K^+, Ca^{+2}, Mg^{+2}, Sr^{+2}, B^{+3}, Cl^-, Br^-, F^-, HCO_3^- SO_4^{-2}) was prepared and diluted to the desired working salinity, then enriched with NO_3- N and PO_4- P at f/2 levels or reduced experimental levels, and with f/2 trace metals and vitamins. Table 2.2 reports the element composition of LW medium when prepared at the salinity 5.8 psu used for stock cultures. Major ions are reduced proportionally when lower salinity media are prepared, except that HCO_3^- is maintained at the reported concentration (Table 2.2) to maintain a high alkalinity characteristic of the weakly saline inland waters of Texas. In order to minimize precipitation, separate solutions of most basal salts were prepared from stocks, one containing NaCl, Na_2SO_4, KCl, KBr, NaF and

H_3BO_3 and the other containing $MgSO_4$ and $SrCl_2$. These solutions were mixed and brought to about 80 % of the final volume before adding stock solution of $CaCl_2$ and bringing the complete basal salt solution to its final volume. This basal salts medium with a total salinity of

Table: 2.1. Semi continuous culture experimental design. Temperature, salinity and limiting nutrients represent independent variables. Each treatment combination had duplicates for a total of 16 cultures

Temperature	Salinity	Limiting nutrient
10 ^{0}C	1 psu	N (low N:P)
		P (high N:P)
	3 psu	N (low N:P)
		P (high N:P)
30 ^{0}C	1 psu	N (low N:P)
		P (high N:P)
	3 psu	N (low N:P)
		P (high N:P)

Table: 2.2. Elemental composition of LW base medium

Constituent	Concentration (µM)
Cl	68400
Na	64800
S	10800
Mg	7080
Ca	11000
K	1320
C as HCO_3	2480
Br	110
Sr	12
B	56
F	7
NO_3-N	880
PO_4-P	36
Zn	0.08
Mn	0.9
Mo	0.03
Co	0.05
Cu	0.04
Fe	11.7
Organic C as EDTA	117
Organic C as vitamins	1.82

42.4 psu was then diluted to make culture medium of the desired salinity. LW base medium was always prepared fresh from the working stocks and then diluted to desired levels of salinities (1 psu and 3 psu for the current experiment) by adding ultra-pure water. After making up required basal salts to the required volume of final medium, f/2 or reduced levels of N and P ($NaNO_3$ and Na_2HPO_4), trace metals and vitamins were added before autoclaving. N limited media were prepared with the f/2 level of P (36 μM) and 5 % of the f/2 level of N (44 μM); similarly, P limited media were prepared with the f/2 level of N (880 μM) and 5 % of the f/2 level of P (1.8 μM). Culture media were autoclaved at 120 ^{0}C for 30 minutes. $NaHCO_3$ stock solution was filter sterilized (0.2 μm) and added aseptically after autoclaving to minimize precipitation.

Experimental cultures were grown in 5000 ml glass jars (Kimble® Kimax) containing a 2 liter working volume of either N or P limited medium. Culture vessels were autoclaved at 120 ^{0}C for approximately 30 minutes and the autoclaved culture medium was poured into the vessels under a sterile air laminar flow chamber.

Two different incubators were used to achieve experimental temperatures. The irradiance of experimental cultures was measured using a photon flux meter at different locations in the incubator, and flasks were placed in locations delivering 150 μmol photons/ m^2/ second. In order to produce semi-continuous growth at a defined average rate, experimental medium was diluted every other day with 700 ml of fresh sterile LW medium to achieve an average dilution rate of 0.215 day^{-1}.

2.2.3. Prototype competition cultures

As a prototype for later competition experiments, in two additional culture vessels *P. parvum* was grown along with competing algal species (obtained from the plankton community of Lake Whitney, a lake where *P. parvum* blooms occur). One of these mixed species cultures were grown in the N-limited medium and the other in the P- limited medium. Both competition cultures were grown at the same temperature (30 ^{0}C) and salinity (3 psu). Competitors were obtained from Lake Whitney, Texas in the summer (07/09/2006). Samples of lake water were collected in 1-liter MilliQ-rinsed polycarbonate bottles and brought to the lab in a cooler. The samples were pooled and then sieved through 153 µm mesh to remove large grazers and bubbled with N_2 gas for 2-3 h to asphyxiate small grazers. Two 500 ml aliquots were then prepared and placed in separate incubators for pre-conditioning of algae to the experimental growth conditions. Temperature of the incubator was initially set to 20 ^{0}C and over the next five days temperature of the designated 30 ^{0}C incubator was raised by 2 ^{0}C every day to reach the desired temperatures. Two days into the pre-conditioning period 200 ml of sterile LW medium (N, P co- limited) was added. On the fifth day of pre- conditioning 200 ml of the culture medium was replaced with 200 ml of fresh LW medium. These pre-conditioning cultures provided inocula for the competition cultures, and 10 ml of the inoculum culture was preserved with Lugol's iodine solution (Modification by Throndsen, 1978).

2.2.4. Sampling and counts

Samples were taken from pre-conditioned stock cultures used for inoculation and preserved with Lugol's iodine solution. Samples of experimental cultures were taken on every 4 days. At each sampling, aliquots of 10 ml were preserved with 1 ml of Lugol's iodine solution (Modification of Throndsen, 1978) to enumerate *P. parvum* population density. Aliquots of 10 ml were preserved with 0.5 ml of formalin solution for bacterial counts. For estimation of chlorophyll *a* concentration 10 ml of each experimental culture samples were drawn through Whatman GF/ F filters and frozen in saturated magnesium carbonate. The chlorophyll *a* concentration was estimated fluorometrically after overnight extraction of chlorophyll samples with 90 % acetone (Welschmeyer, 1994). Cell density was determined by direct microscopic counts of at least 200 cells per sample in a Sedgwick Rafter counting chamber. Because we observed both motile and non-motile cells during the experiment, live counts of *P. parvum* were also done for all cultures on the sampling days using Sedgwick Rafter chambers. Both motile and non-motile cells were counted to estimate the proportion of motile cells. Non motile cells had no visible flagella and resembled the cells described as "temporary cysts" of *P. parvum* by Carter (1937).

2.2.5. Chemical analysis

Samples for the analysis of dissolved nutrients (nitrate, nitrite, ammonium, Soluble Reactive Phosphorous, SRP) and particulate nutrients (particulate phosphorous and CHN) were collected on days 12, 28 and 40 of the experiment. Samples for dissolved nutrients were filtered through cellulose nitrate membranes (0.2 µm). Nitrite, ammonium and SRP analysis followed Strickland and Parsons (1972). Spongy cadmium was used to reduce nitrate to nitrite (Jones, 1984), followed by nitrite analysis

2.2.6. Toxicity studies

Lack of standards for prymnesins, the characterized toxins of *P. parvum* (Igarashi, 1996), and the possible occurrence of other toxins hinder the direct chemical analysis of toxins. Therefore bioassays using model organisms were used to quantify toxicity of *P. parvum* cultures. All toxicity tests were performed in climate-controlled chambers at 25 ± 1°C, with a 16:8 h light: dark cycle. Samples for fish toxicity tests were collected on day 12 and day 40 of the experiment and transported to Baylor University for toxicity studies. For this study a model fresh water fish (*P. promelas*) was used to estimate acute toxicity of *P. parvum* cultures grown under varying environmental conditions. Acute toxicity of whole- water samples from cultures was determined using an assay based on the survival of juvenile *P. promelas* over 48 h using EPA method 2000.0 (USEPA, 2002). The fish used were greater than 48 hrs old and were fed newly hatched *Artemia* nauplii approximately 2 hours before initiation of testing. A dilution series of whole culture water was made at 7 levels from 100 % to 6.25 % using a standard hard water formulation for dilutions. Dilution series of sterile

N- and P-limited LW culture media were used as controls. For each dilution level, two replicate chambers with five organisms in each chamber were used to assess toxicity (14 chambers for 7 levels of dilution). Acute LC_{50} values in terms of the percentage dilution of whole water from each culture were estimated using Probit (Finney, 1971) or Trimmed Spearman-Karber (Hamilton et al. 1977) methods as appropriate for data properties.

Samples for crustacean toxicity tests were collected from days 16 to 36 of the experiment and transported to Baylor University for toxicity studies. For this study a model fresh crustacean (*Daphnia magna*) was used to estimate sub-lethal toxicity of *P. parvum* cultures grown under varying environmental conditions. Reproductive output was measured as number of offspring during the test period, and reduced fecundity was interpreted as indicating sub-lethal toxicity. These *Daphnia magna* bioassays followed established US EPA protocols (US EPA 1994). An artificial hard-water medium was prepared according to standard methods (APHA 1998) and used as a control treatment, while experimental treatments consisted of *Daphnia* grown in whole water from *P. parvum* cultures. Five experimental units were used per sample, each consisting of a single individual < 24 h old in its own container. *Daphnia magna* were fed a Cerophyll/green algal suspension daily prepared according Brooks et al. (2004) and Dzialowski et al. (2006).

2.2.7. Data analysis

Cell densities did not follow the normal distribution and were transformed to natural logarithms to reduce heteroscedasticity and skew.

These and other response variables were analyzed with factorial ANOVA, to test main effects of temperature (2 levels), salinity (2 levels), and limiting nutrients (2 levels) and interaction of these independent variables. When significant treatment effects were identified by factorial ANOVA, Tukey's HSD test (Kleinbaum et al. 1988) was used for multiple comparisons. In both ANOVA and Tukey's HSD test statistical significance was concluded when the null hypothesis of no effects could be rejected at a level of $p < 0.05$. All statistical analyses were done with Statistica 7.0 (Statsoft Inc., Tulsa, Oklahoma, USA).

As preliminary analysis of prototype competition cultures, total chlorophyll *a* was compared against the *P. parvum* cell concentration.

2.3. Results

2.3.1. Cell density of *P. parvum in monocultures:*

The majority of cultures did not reach a clear steady state by the end of the experiment (e.g Figure 2.1). Those cultures which reached steady state did so within 20-24 days. In N and P limited cultures the limiting nutrients were not depleted to expected levels (Table 2.9). Total and motile cell densities of *P. parvum* were analyzed in response to experimental factors on days 12 and 40 when samples were taken for toxicity to fish. Total and motile cell densities were also analyzed after averaging over time period from day 16 to 36, when samples were taken for determining sub-lethal toxicity to *Daphnia.* In all cases densities were transformed to natural logarithms to conform to assumptions of factorial ANOVA used.

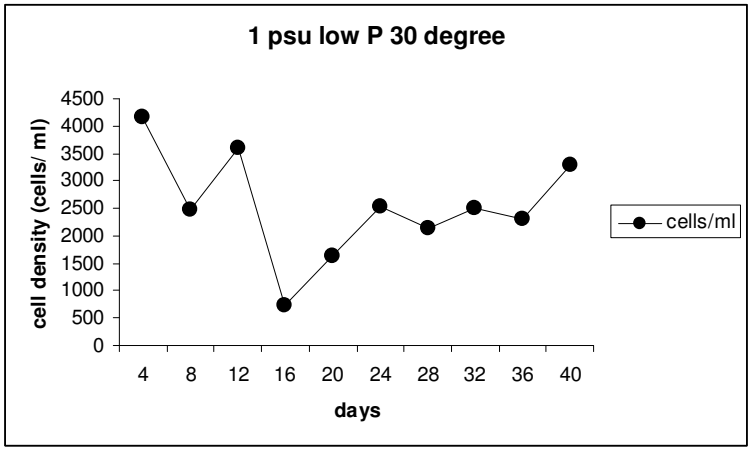

Figure: 2.1. A representative culture which did not reach a steady state

For total cell density of *P. parvum* on day 12, the three-way interaction of all experimental factors was significant (Table 2.3). Most treatment combinations had similar mean densities, and the only significant difference between treatment means was that N limited cultures at 10 ^{0}C with 1 psu salinity had lower density than those with 3 psu salinity (Tukey's HSD, $p < .05$). Though differences were not significant, the N-limited cultures at 10 ^{0}C with 1 psu salinity had lower mean total cell density than other treatment combinations (Figure: 2.3).

Table: 2.3. ANOVA for total cell density of *P. parvum* on Day 12. Boldface indicates significance at $p < 0.05$.

	SS	Degree of freedom	MS	F	P
Temp	**6.13**	**1**	**6.13**	**8.68**	**0.02**
Nutrient	0.0106	1	0.0106	0.015	0.91
Salinity	**4.51**	**1**	**4.51**	**6.39**	**0.04**
Temp*Nutrient	0.110	1	0.110	0.156	0.70
Temp*Salinity	0.814	1	0.814	1.15	0.31
Nutrient*Salinity	0.280	1	0.280	0.397	0.55
Temp*Nutrient*Salinity	**3.99**	**1**	**3.99**	**5.66**	**0.04**
Error	5.65	8	0.706		

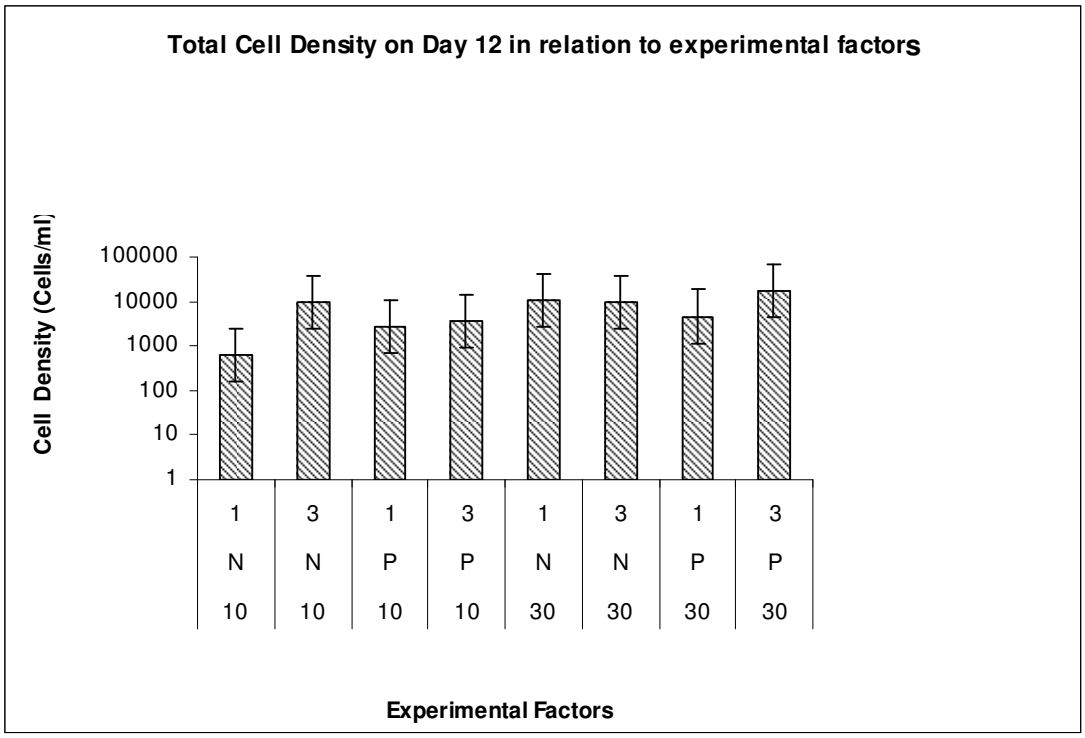

Figure: 2.2. Total cell density on day 12 in relation to experimental factors. X-Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

For motile cell density of *P. parvum* on day 12, the three-way interaction of all experimental factors was again significant (Table 2.4). Generally cultures with 1 psu salinity had lower mean motile cell density than those with 3 psu salinity, and in particular the P-limited cultures at 30 ^{0}C with 1 psu salinity had significantly lower mean motile cell density than all other treatment combinations (Figure 2.4) (Tukey's HSD, $p<0.05$)

Table: 2.4. ANOVA for motile cell density of *P. parvum* on Day 12. Boldface indicates significance at $p< .05$

	SS	Degrees of freedom	MS	F	P
Temp	0.008	1	0.008	0.017	0.90
Nutrient	2.80	1	2.80	5.556	0.05
Salinity	**2.69**	**1**	**2.69**	**5.045**	**0.00**
Temp*Nutrient	**2.96**	**1**	**2.96**	**5.872**	**0.04**
Temp*Salinity	0.919	1	0.919	1.826	0.21
Nutrient*Salinity	1.47	1	1.47	2.934	0.12
Temp*Nutrient*Salinity	**8.79**	**1**	**8.79**	**17.43**	**0.00**
Error	4.03	8	0.504		

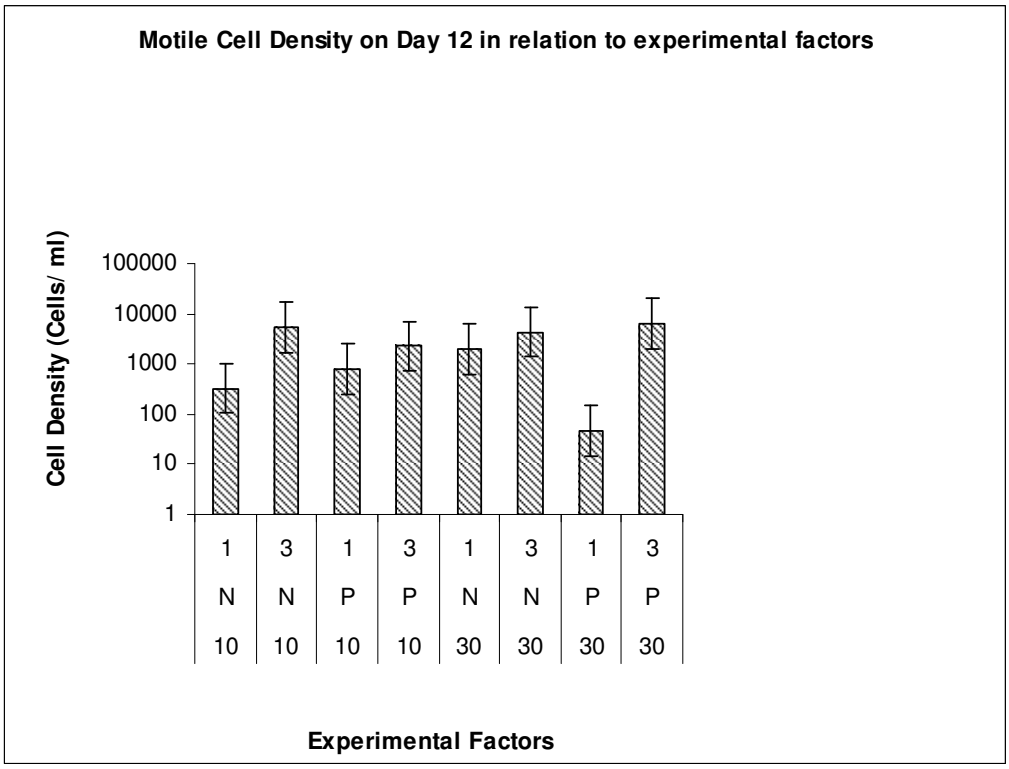

Figure: 2.3. Motile cell density on day 12 in relation to experimental factors. X-Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

For total cell density of *P. parvum* on day 40, the main effects of limiting nutrient and salinity were significant (Table 2.5). Mean total cell density in cultures with 1 psu salinity was lower than in cultures with 3 psu salinity, and mean total cell density in N limited cultures was lower than in P-limited cultures (Figure 2.5).

For motile cell density of *P. parvum* on day 40, the main effect of temperature was significant, as was the interaction between salinity and limiting nutrient (Table 2.6). Mean motile cell density was lower in cultures at 30 ^{0}C than in those at 10 ^{0}C. Mean motile cell density was higher in 3 psu cultures than in 1psu cultures (Figure 2.6)

Table: 2.5. ANOVA for total cell density of *P. parvum* on Day 40. Boldface indicates significance at p< .05

	SS	Degree of Freedom	MS	F	P
Temp	**2.398**	**1**	**2.398**	**18.08**	**0.003**
Nutrient	0.058	1	0.058	0.434	0.529
Salinity	**2.50**	**1**	**2.500**	**18.86**	**0.002**
Temp* Nutrient	0.014	1	0.014	0.102	0.757
Temp* Salinity	0.048	1	0.048	0.364	0.563
Nutrient* Salinity	**0.713**	**1**	**0.713**	**5.38**	**0.049**
Temp*Nutrient* Salinity	0.145	1	0.145	1.09	0.327
Error	1.060	8	0.133		

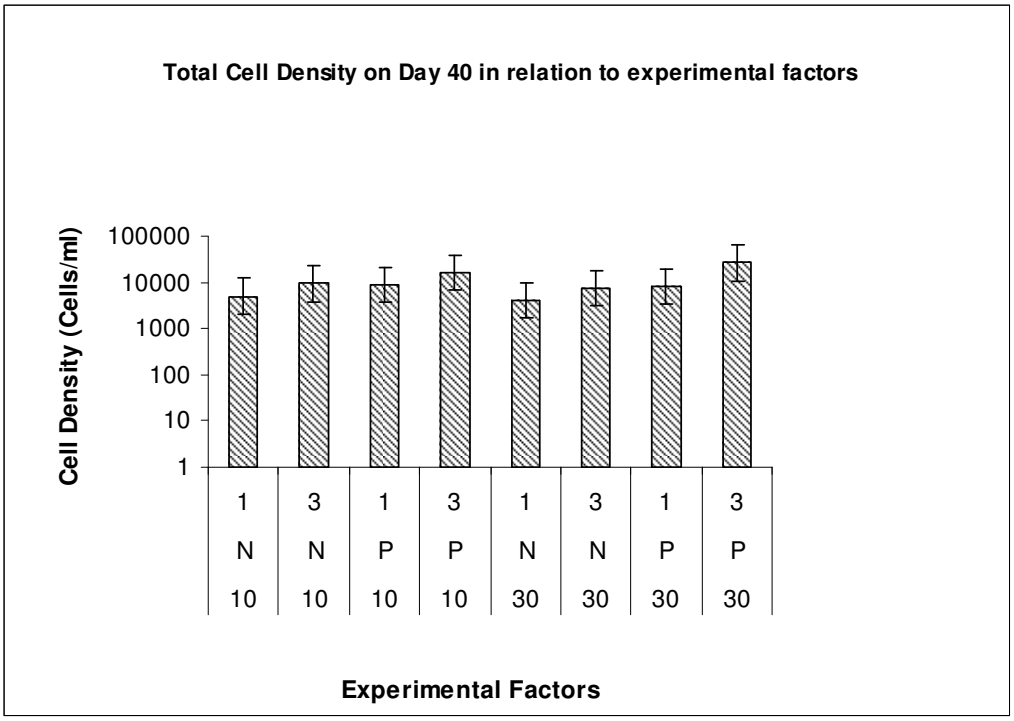

Figure: 2.4. Total cell density on day 40 in relation to experimental factors. X-Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

Table: 2.6. ANOVA for motile cell density of *P. parvum* on day 40.Boldface indicates significance at $p < 0.05$

	SS	Degree Of Freedom	MS	F	P
Temp	0.000	1	0.000	0.001	0.974
Nutrient	**0.438**	**1**	**0.438**	**7.88**	**0.023**
Salinity	**0.429**	**1**	**0.429**	**7.73**	**0.024**
Temp* Nutrient	0.031	1	0.031	0.57	0.473
Temp* salinity	0.014	1	0.014	0.25	0.633
Nutrient* salinity	0.017	1	0.017	0.29	0.600
Temp* Nutrient	0.017	1	0.017	0.31	0.596
Error	0.444	8	0.056		

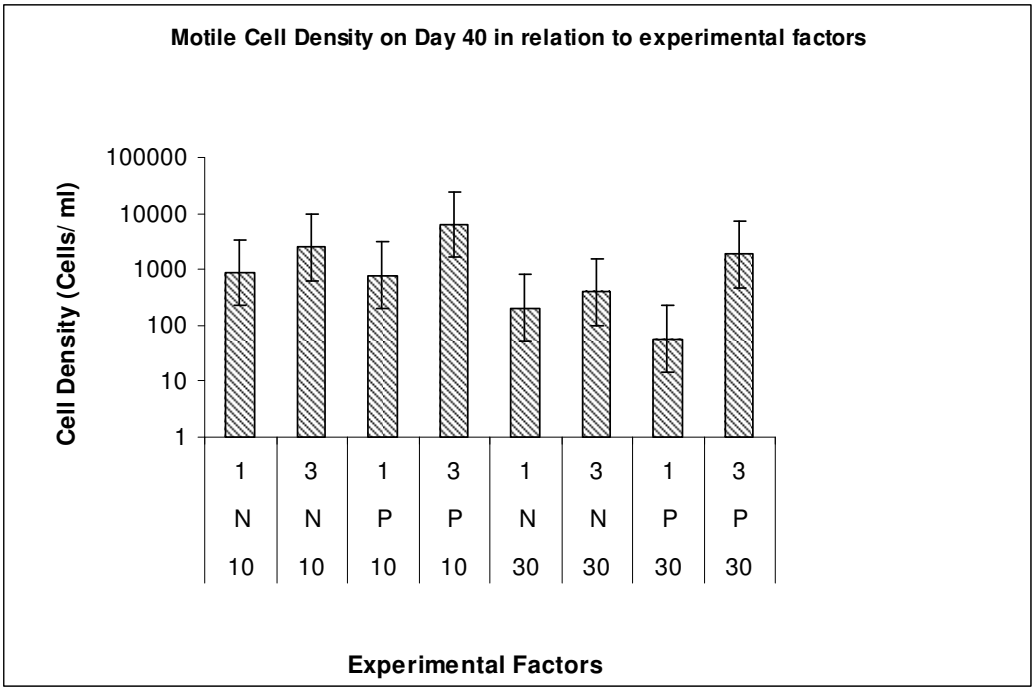

Figure: 2.5. Motile cell density on day 40 in relation to experimental factors. X-Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

Table: 2.7. ANOVA for total cell density of *P. parvum* over days 16- 36. Boldface indicates significance at $p < 0.05$

	SS	Degree of freedom	MS	F	p
Temp	0.009	1	0.009	0.036	0.854
Nutrient	0.219	1	0.219	0.924	0.364
Salinity	2.99	1	2.99	12.64	0.007
Temp* Nutrient	0.016	1	0.016	0.069	0.799
Temp* Salinity	1.34	1	1.34	5.65	0.045
Nutrient* Salinity	0.792	1	0.792	3.34	0.105
Temp*Nutrient*Salinity	0.812	1	0.812	3.43	0.101
Error	1.89	8	0.233		

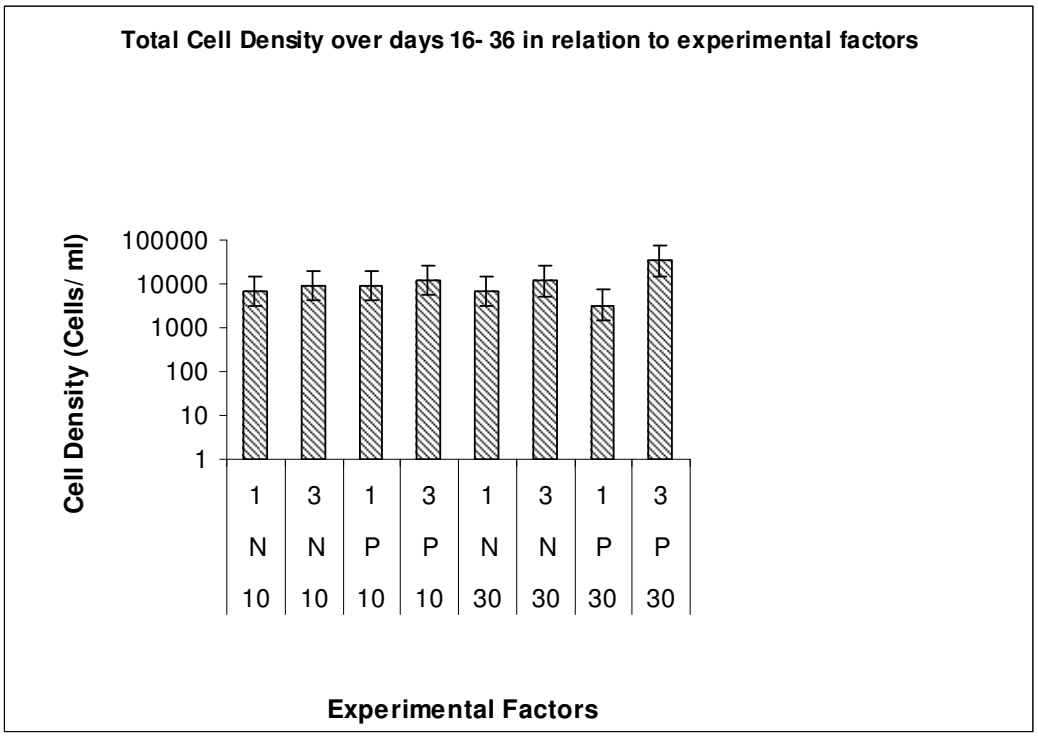

Figure: 2.6. Total cell density over days 16-36 in relation to experimental factors. X- Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

For total cell density averaged over days 16- 36, the interaction of temperature and salinity was significant (Table: 2.7). Mean total cell density in cultures with 1 psu salinity was generally lower than in cultures with 3 psu salinity. However, the only significant difference between treatment combinations was that N- limited cultures at 30 ^{0}C with 3 psu salinity had higher total mean cell density than P-limited cultures at 30 ^{0}C with 1 psu salinity (Figure 2.7)

For motile cell density of *P. parvum* averaged over days 16-36, only the main effect of salinity was significant (Table 2.8). Mean motile cell density was lower in cultures with 1 psu salinity than in those with 3 psu salinity (figure 2.8)

Table: 2.8. ANOVA for motile cell density of *P. parvum* over days 16- 36. Boldface indicates significance at $p < 0.05$

	SS	Degree of Freedom	MS	F	P
Temp	1.11	1	1.11	2.11	0.184
Nutrient	0.024	1	0.024	0.045	0.838
Salinity	**5.06**	**1**	**5.06**	**9.65**	**0.015**
Temp* Nutrient	0.002	1	0.002	0.003	0.957
Temp* Salinity	1.24	1	1.24	2.37	0.162
Nutrient* Salinity	1.44	1	1.44	2.75	0.136
Temp*Nutrient*Salinity	0.365	1	0.365	0.696	0.428
Error	4.19	8	0.523		

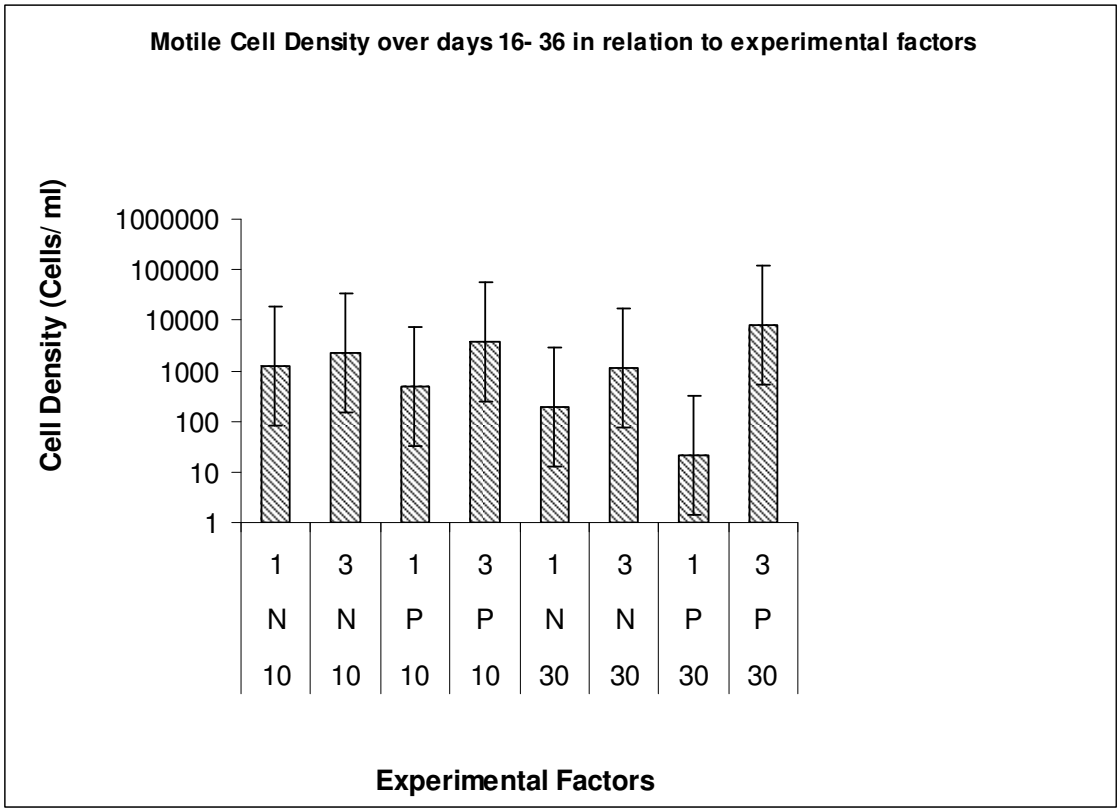

Figure: 2.7. Motile cell density over days 16-36 in relation to experimental factors. X- Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

2.3.2. Nutrient concentration in outgoing medium

In both N- and P-limited cultures, levels of ammonium were below the analytical detection limit on day 12 and there was a consistent increase in ammonium concentration during the rest of the experimental period. In P-limited cultures SRP concentration was similar on days 12 and 28 but was below the analytical detection limit on day 40 (**Table: 2.9**). In N-limited cultures levels of nitrite were low throughout the experiment, but levels of nitrate were high.

Table: 2.9. Concentration of dissolved nutrients in the outgoing medium

Nutrient	DAY 12	DAY 28	DAY 40
NH_4^+ (μM)	N. D.	0.3 ± 0.2	2.7 ± 2.5
SRP (μM)	0.8 ± 0.5	0.7± 0.4	N. D.
NO_2 (μM)	0.3 ± 0.4	0.2 ± 0.3	0.2 ± 0.2
NO_3 (μM)	40.4 ± 15.8	43.3 ± 15.5	45.1 ± 13.9

2.3.3. Toxicity studies

Fish toxicity samples were collected on day 12 and day 40 of the experimental period. Only two cultures showed detectable levels of toxicity and those cultures had LC 50 > 50 %.

For fish toxicity on Day 40, both main effects and two-way interactions between experimental factors were significant (Table 2.10). Mean toxicity of 1 psu cultures was significantly higher than that of 3 psu cultures and mean toxicity of P-limited cultures was higher than the N-limited cultures (Figure 2.9).

Mean reproduction rate of *Daphnia* was analyzed to estimate sub-lethal toxicity of *P. parvum* cultures. For mean reproduction rate of *Daphnia* the main effects of limiting nutrient and salinity were significant (Table 2.11) as was the interaction

between limiting nutrient and salinity (Figure 2.10). Mean reproduction rate of 1 psu cultures were higher than that of 3 psu cultures. In general, samples from P-limited cultures reduced *Daphnia* reproductive output more than samples from N-limited cultures. In hard water controls, *Daphnia* reproduction was 166.8 neonates per female.

Table: 2.10. ANOVA for *P. parvum* fish toxicity on day 40. Boldface indicates significance at $p < 0.05$

	SS	Degree of Freedom	MS	F	P
Temp	**3364.87**	**1**	**3364.87**	**52.35**	**0.00**
Nutrient	**1376.97**	**1**	**1376.97**	**21.42**	**0.00**
Salinity	**10746.9**	**1**	**10746.9**	**167.21**	**0.00**
Temp* Nutrient	**538.12**	**1**	**538.12**	**8.37**	**0.02**
Temp* Salinity	**5278.39**	**1**	**5278.39**	**82.12**	**0.00**
Nutrient* Salinity	**504.56**	**1**	**504.56**	**7.85**	**0.02**
Temp*Nutrient *salinity	73.15	1	73.15	1.14	0.31
Error	514.19	8	64.27		

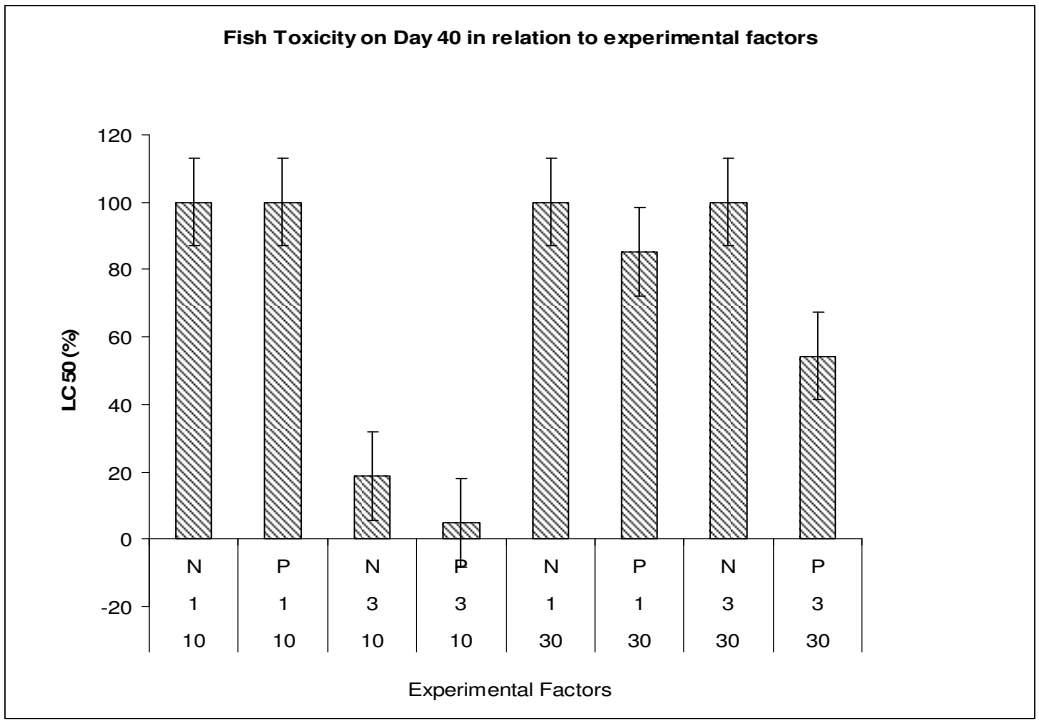

Figure: 2.8. Fish Toxicity on day 40 in relation to experimental factors. X-Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

Table: 2.11. ANOVA for *P. parvum* sub lethal toxicity on day 40. Boldface indicates significance at $p < 0.05$

	SS	Degree of Freedom	MS	F	P
Temp	0.640	1	0.640	0.00	0.98
Nutrient	**6872.4**	**1**	**6872.4**	**7.34**	**0.03**
Salinity	**8317.4**	**1**	**8317.4**	**8.88**	**0.02**
Temp* Nutrient	1036.8	1	1036.8	1.11	0.32
Temp* Salinity	234.09	1	234.09	0.25	0.63
Nutrient* Salinity	6021.8	1	6021.8	6.43	0.03
Temp*Nutrient *salinity	1772.4	1	1772.4	1.89	0.20
Error	7489.8	8	936.22		

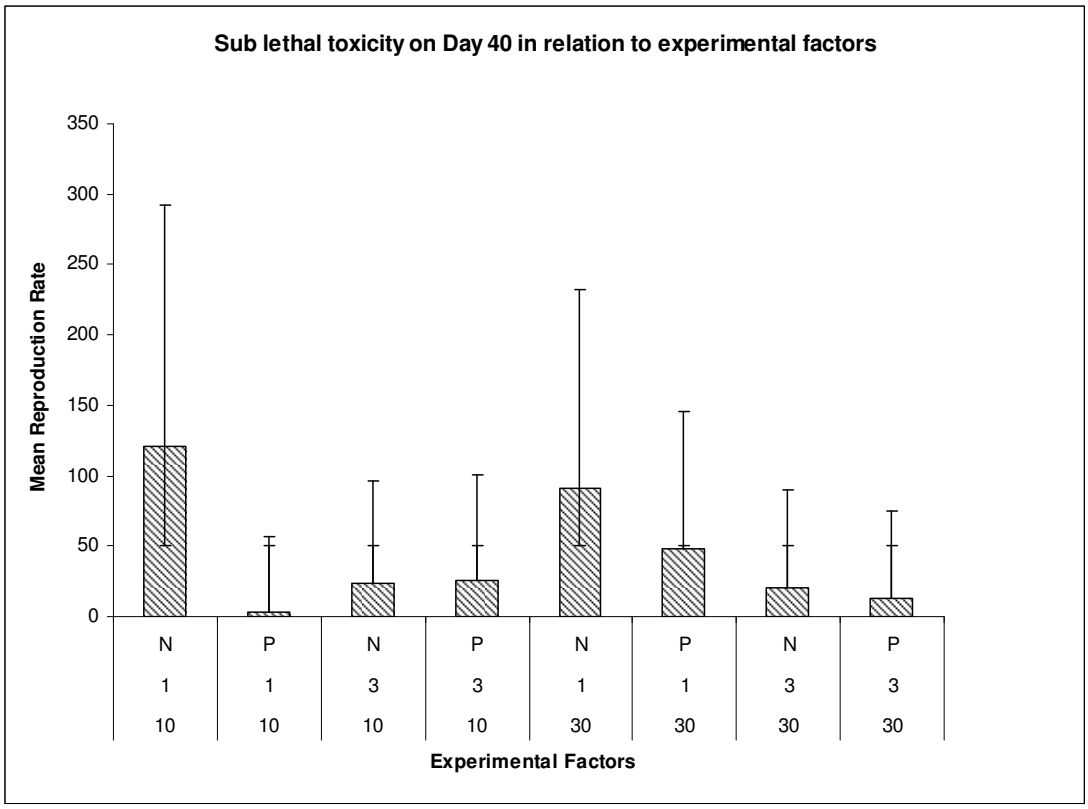

Figure: 2.9.: Sublethal toxicity on day 40 in relation to experimental factors. X- Axis represents different treatment combinations of salinity (1 vs. 3 psu), limiting nutrient (N vs. P), and temperature 10 vs. 30 °C).

2.3.4. Prototype competition cultures

Analysis of end-point N- and P-limited prototype competition cultures shows that *P. parvum* was out-competed by other species. Various filamentous cyanobacteria and *Coelosphaerium* sp. dominated P-limited endpoint cultures where as N-limited cultures were dominated by filamentous cyanobacteria (Figure 2.11). In both N- and P-limited cultures *P. parvum* was out-competed after the first half of the culture period. In both N- and P-limited prototype competition cultures an increase in total biomass composed primarily of competitor species was observed (Figure 2.12)

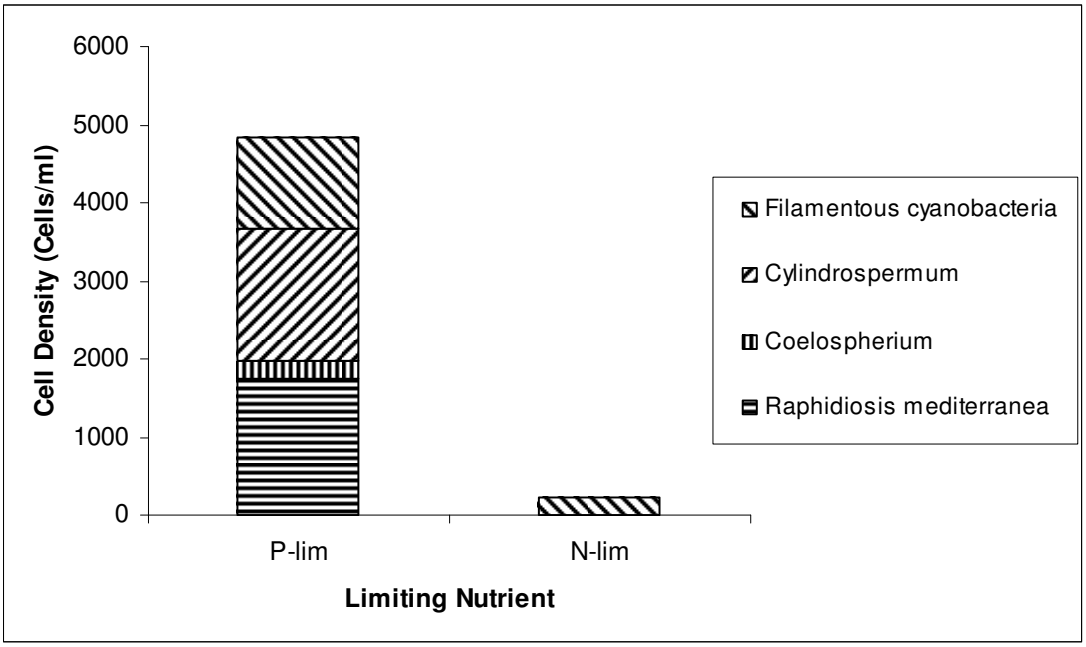

Figure: 2.10. Prototype competition culture results

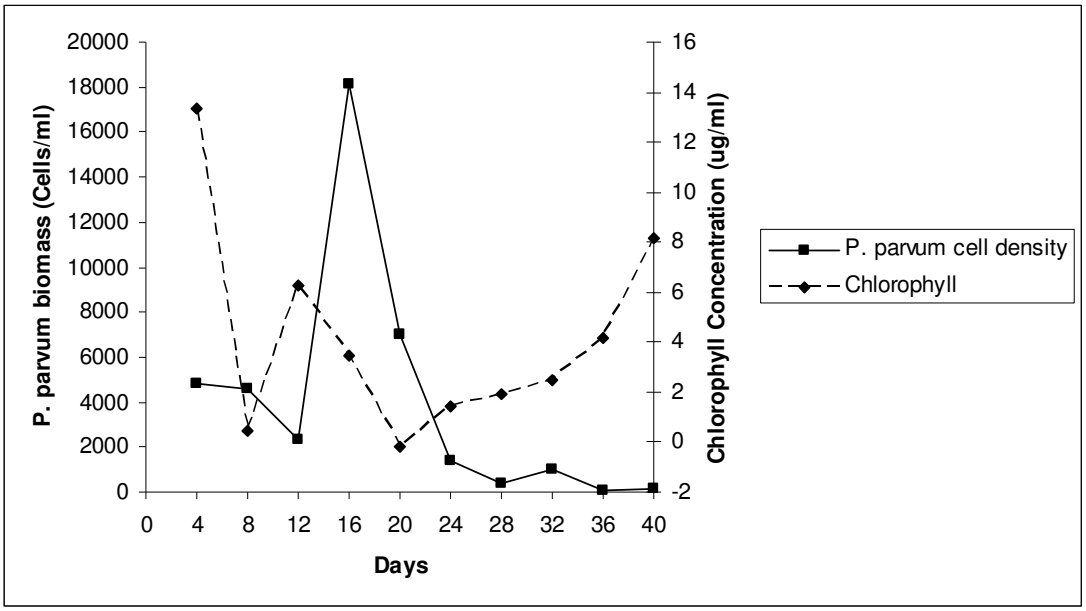

Figure: 2.11. Comparison of *P. parvum* concentration and total algal biomass (Chl *a*). Straight line represents *P. parvum* cell density and the dotted line represents total biomass of N-limited prototype competition culture

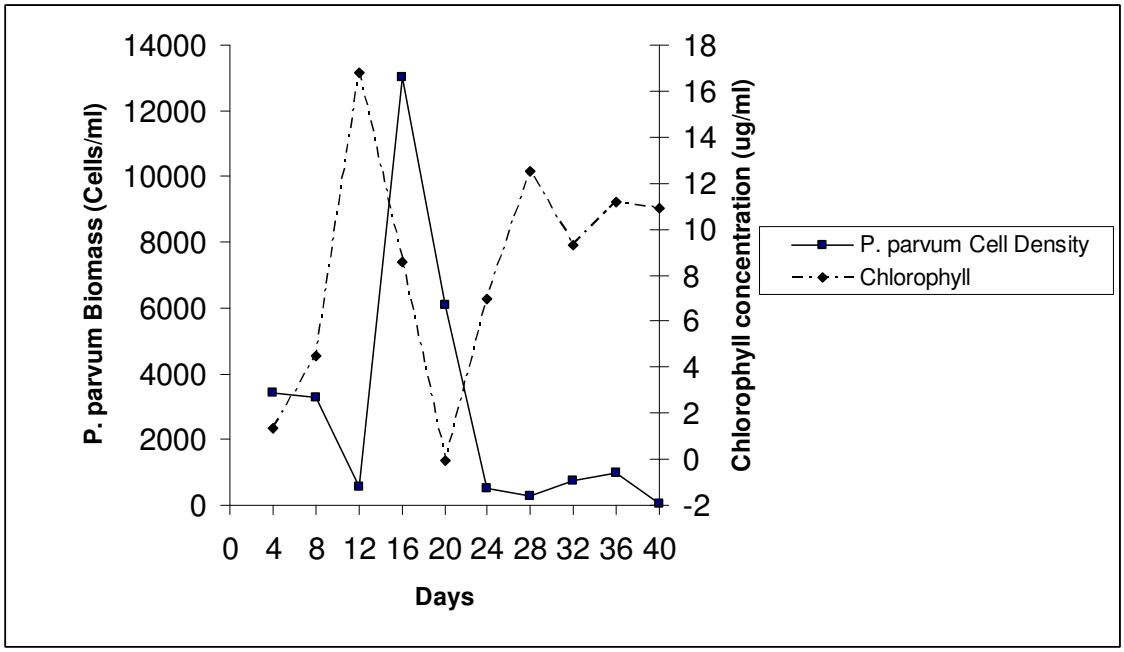

Figure: 2.12. Comparison of *P. parvum* concentration and total algal biomass (Chl *a*). Straight line represents *P. parvum* cell density and the dotted line represents total biomass of P-limited prototype competition culture

2.4. Discussion

Acute toxic activity of *P. parvum* (measured as LC_{50}) on juvenile fathead minnows (*P. promelas*) showed a complex relation with experimental factors. Results from this experiment indicate that acute toxicity to fish was highest under conditions which are unfavorable for the growth of *P. parvum*. Abundance estimates suggest that *P. parvum* grow better under warmer temperatures (30 ^{0}C) within the salinity range examined. Toxicity, however, was higher for cultures grown at 10 ^{0}C, especially under P-limited conditions.

Though there are several studies investigating effect of factors such as temperature, irradiance, salinity and limiting nutrients on measures of toxicity of *P. parvum*, most of them do not address interacting effects of these factors. The enhancement of toxicity under P-limited conditions is in agreement with previous studies showing that low inorganic P supply can enhance toxicity. Johansson and Graneli (1999) reported an increase in hemolytic activity under limitation by either N or P. Earlier works reported enhancement of toxicity under P-limited conditions in both *P. parvum* sensu stricto (Dafni et al., 1972) and in the *patelliferum* form of the species (Larsen et al., 1993). Baker et al. (2007) studied the effect of interaction of temperature, salinity and irradiance on growth and toxicity of *P. parvum* finding that sub-optimal conditions of growth enhanced toxicity. In some of the above mentioned studies toxicity of *P.* parvum was measured with assays based on hemolysis of red blood cells or effects on brine shrimp (*Artemia salina*) which do not occur with *P. parvum* in lower salinity

ranges characteristic of Texas inland waters. This experiment addresses the effects of temperature, salinity, limiting nutrients and their interactions using a factorial design. Moreover in this study a model fish was used as the test organism, which may be of greater relevance to understanding blooms and fish kills in natural habitats due to *P. parvum.*

As part of the current study *Daphnia magna* was tested for sub-lethal toxicity of *P. parvum*. Reproductive output was lower for all samples of *P. parvum* cultures than for control *Daphnia* raised in standard hard water, and was reduced to a greater extent by P-limited *P. parvum* cultures, and those grown at the higher salinity of 3 psu. These observations confirm the sub-lethal toxicity to *Daphnia* recently detected in field samples and the enhancement of such toxicity by nutrient limitation (Roelke et al. 2007). The greater reduction of *Daphnia* fecundity by cultures with 3 psu salinity compared to 1 psu may have been a direct result of high salinity, rather than *P. parvum.* Fecundity of *Daphnia magna* is not affected by salinity up to 2 psu, but is reduced at 4 psu (F. Ureña-Boeck and B. Brooks, personal communication)

In the current study semi-continuous cultures did not work according to theoretical expectations. If dilutions of semi-continuous cultures are frequent, they approximate the true continuous conditions of a chemostat. Therefore the population is expected to reach a steady state in which the limiting nutrient is depleted to low concentration. In these experiments, limiting nutrients were not depleted as much as expected, especially for N, and well defined steady states were not evident in most cultures. These deviations from theoretical expectations could be due to the large

proportion of encysted cells observed. Theoretically, if a population in a chemostat encysts, a steady state is not necessarily reached and the population can oscillate through a cycle of motile cells transforming to cysts, then cysts germinating back to motile cells (Malik and Smith, 2006).

Encystment also reduces the number of cells in a population actively consuming the limiting nutrient, making that population less competitive relative to species that remain completely active. Other experiments in our laboratory suggest that *P. parvum* has low half saturation constant for P-limited growth (Baker, 2007), and would be a good competitor under P-limitation. Yet it was nearly eliminated from N- and P-limited competition cultures and in the monocultures reported here, the majority of the *P. parvum* population was encysted (> 75%). If *P. parvum* encysted to the same extent in competition cultures, its ability to compete with other species was probably much reduced.

Though the experimental results did not conform to what was theoretically expected for semi-continuous cultures, they confirm previous reports that nutrient limitation and other sub-optimal growth conditions increase the toxicity of *P. parvum.* Moreover these results add to a growing literature documenting toxic effects on ecologically relevant representatives of organisms that co-occur with *P. parvum.* Observations of large proportion of encysted cells in these cultures suggest that the life history transitions of *P. parvum* could affect the bloom development and toxicity. Subsequent experiments reported in the following chapters were designed to further

examine semi-continuous dynamics and toxicity, and to continue examining competition with other algae.

CHAPTER 3

EFFECT OF SPRING INOCULUM OF COMPETITORS ON GROWTH AND TOXICITY OF *PRYMNESIUM PARVUM* IN MIXED SPECIES CULTURES AT DIFFERENT TEMPERATURES UNDER N AND P LIMITATION

3.1. Introduction

In this study *P. parvum* populations were both grown in monocultures and exposed to mixed species algal populations derived from a natural lake community (Lake Whitney). The competitor inoculum was sampled during spring, under conditions favoring *P. parvum* bloom formation in this lake. The primary goal of this study was to investigate the competitive interaction of *P. parvum* with other species and its effect on the abundance and toxicity of *P. parvum* at two different temperatures (10 ^{0}C and 30 ^{0}C, representing winter and summer conditions). In order to study the mortality rate of *P. parvum* under the influence of grazers, endpoint monocultures were used to construct a dilution experiment (modified from Landry and Hassett 1982) with two cladoceran species, *Daphnia magna* and *Ceriodaphnia dubia.*

Previous experimental results suggest that summer blooms of *P. parvum* could occur in Texas inland waters due to its relatively high optimal temperature for growth (Baker et al., 2007). But it has been observed that blooms occur from early winter to spring in Texas inland waters (Roelke et al. 2007), suggesting summer blooms might be prevented by factors such as competition from other algae or zooplankton grazing.

The distribution pattern and abundance of algal species is determined to a certain extent by competition along with disturbance and physical stress in the community. Competition would be predicted as the most significant factor which determines the community structure in the absence of environmental stress and disturbances (Grime, 1974, 1977, 1979). For competition to occur in a natural community or in an artificial culture medium, there should be some resources that are limiting and the spatial and temporal distribution of these resources determine the nature and intensity of interaction among algae (Carpenter 1990). Competition among different algal species has been suggested as one of the important shaping factors of community structure and composition in macroalgal communities (Dayton 1975, Hay 1986) and in phytoplankton (Tilman et al 1982, Grover 1997).

Studies show that grazers have considerable influence on phytoplankton growth dynamics in lakes (Sterner 1989). It seems likely that a decrease in abundance of grazers can be a contributing factor for the initiation of blooms. There are contradictory aspects of zooplankton grazing on phytoplankton dynamics: grazing which reduces algal standing crop, and nutrient re-generation which can stimulate algal growth by increasing available nutrients. The response to grazers varies at species level, and the net response of phytoplankton is not known for all species. Classical studies of marine phytoplankton blooms in relation to zooplankton grazing are focused on copepods and diatoms. Fleming (1939) suggested that diatom growth rate can be controlled by grazers. Riley (1947) developed a mathematical model depicting the potential of grazer population to control phytoplankton populations. Martin (1965) in a study conducted at

Narragansett Bay concluded that spring blooms established partially because of the lower abundance of grazers, with grazers becoming dominant and eliminating the bloom by the latter half of spring and summer. Similar dynamics in lakes have been referred to as the "clear water phase" (Sommer 1986).

Though there are several studies on the effect of grazers on many algal species, little is known about the effect of the grazers commonly found in inland waters on *P. parvum*. In this study *P. parvum* was grown in semi-continuous monocultures and mixed species cultures to assess the effects of competing algae on abundance and toxicity. The inoculum of competitors was collected in spring from a Texas reservoir (Lake Whitney). Semi-continuous cultures were grown at two temperatures (10 ^{0}C and 30 ^{0}C) for six weeks to population dynamics and toxicity of *P. parvum*. Monocultures were then used to construct a dilution experiment to estimate the mortality rate of *P. parvum* under the influence of two grazers- *Daphnia magna and Ceriodaphnia dubia.* The temperatures used are typical of winter and summer in Texas lakes, and a salinity of 2 psu was used for all cultures because it is representative of salinities in lakes where *P. parvum* blooms occur and within a range that do not affect the fecundity of *Daphnia magna* (F. Ureña-Boeck and B. Brooks, personal communication). Using the same methods as in chapter 2 this species and the fathead minnow, *P. promelas* were used to assay sub-lethal and lethal toxicity of cultures containing *P. parvum.*

3.2. Materials and Methods

3.2.1. Algal strain and culture conditions

The *P. parvum* strain used in this experiment was obtained from the UTEX collection of algae (Strain number UTEX LB ZZ181). Stock cultures were maintained in a medium of Artificial Sea Water (ASW) (Kester et al., 1967) diluted to a working salinity of 5.8 psu with 18 Ω/ cm ultrapure water. Preparation of stock culture medium was similar to that of the previous experiment (Chapter 2). These stock cultures were maintained in an incubator on a 12: 12 light and dark cycle with an irradiance of 150 µmol photons/ m^2/ second.

3.2.2. Experimental design

This was a large volume semi-continuous experiment with two experimental factors each at two levels (2 x 2) factorial design. There were two levels of temperature (10 ^{0}C and 30 ^{0}C) and competitors (present or absent). There were three replicates of each culture for a total of 12 cultures (Table 3.1).

Table: 3.1. Experimental design. '+' and '-'sign indicates presence and absence of competitors

Temperature	**Salinity**	**Competitors**
10^0 C	2 psu	**+**
		-
30^0 C		**+**
		-

Lake water medium modified to have salt composition closer to that of weakly saline inland waters of Texas (as described in Chapter 2) was used for all experimental cultures. However, in this experiment instead of two levels of salinity, medium of a single salinity (2 psu) was used and the entire experimental medium was prepared to be N-, P- colimited by using 5 % of f/2 levels of both the nutrients (44 μM N, 1.8 μM P).

Experimental cultures were grown in 5000 ml glass jars (Kimble® Kimax) containing a working volume of 2 liters. Culture vessels were autoclaved at 120 ^{0}C for approximately 30 minutes and the autoclaved culture medium was poured into vessels under a sterile air laminar flow chamber.

Two different incubators were used to achieve experimental temperatures. The irradiance of experimental cultures was measured using a photon flux meter, and flasks were placed in locations delivering 150 μmol photons/ m^2/ second. In order to produce a semi-continuous growth at a defined average rate experimental medium was diluted every other day with 700 ml of fresh sterile LW medium to achieve an average dilution rate of 0.215/ day.

3.2.3. Mixed species cultures

In half of the cultures *P. parvum* was grown as mixed species cultures along with major competitors from a natural lake community (Lake Whitney). Half of the cultures were maintained at 10 ^{0}C and the other half at 30 ^{0}C. Competitors were obtained from Lake Whitney sampled in spring (03/02/2007). Samples of lake water were collected in 1-liter MilliQ-rinsed polycarbonate bottles and brought to the lab in a cooler. The samples were pooled and then sieved through 153 μm mesh to remove large

grazers and bubbled with N_2 gas for 2-3 h to asphyxiate small grazers. Two 500 ml aliquots were then prepared and placed in separate incubators for pre-conditioning of algae to the experimental growth conditions. Temperature of the incubators were initially set to 20 ^{0}C and over the next five days, the temperature of the designated 30 ^{0}C incubator was raised by 2 ^{0}C every day to reach the desired temperature; likewise that of the designated 10 ^{0}C incubator was lowered by 2 ^{0}C every day to reach the desired temperature. Two days into the pre-conditioning period 200 ml of sterile LW medium (N, P co- limited) was added. On the fifth day of pre-conditioning 200 ml of the culture medium was replaced with 200 ml of fresh LW medium. These pre-conditioning cultures provided inocula for the competition cultures, and 10 ml of the inoculum culture was preserved with Lugol's iodine solution (Modification by Throndsen, 1978).

3.2.4. Grazing experiment

The primary goal of this experiment was to determine the grazer-induced mortality rate of *P. parvum,* using a modification of the dilution technique pioneered by Landry and Hassett (1982). *Daphnia magna* and *Ceriodaphnia dubia* used as grazers in this experiment were cultured by colleagues at Baylor University and transported to our laboratory on the initial day of the experiment. Grazers were standardized by age, size and condition as best could be managed.

After 42 days of growth, six dilution cultures were prepared from each experimental mono culture for a total of 36 cultures. Out of six dilution cultures from each experimental monoculture two were diluted 1/3 and two 1/18 (culture volume to total volume). All dilution cultures were prepared in 250 ml tissue culture flasks. The

1/3 dilution cultures were inoculated with 12 adult individuals of *Ceriodaphnia dubia* and 6 adult individuals of *Daphnia magna.* The 1/18 dilution cultures were inoculated with 2 adult individuals of *Ceriodaphnia dubia* and 1 of *Daphnia magna*. Compared to the original Landry-Hassett design, this modification thus uses only two dilution levels, corresponding to a hypothetical, undiluted grazer community composed of 144 *Ceriodaphnia dubia* individuals per liter, and 72 *Daphnia magna* individuals per liter. Dilution cultures were incubated for 3 days at 20 °C (a temperature compatible with high grazer survival), and 20 ml was sampled daily for chlorophyll *a* determinations.

This experimental design was a minimal version of the design developed by Landry and Hassett (1982). The logic of this design is to dilute the grazer-phytoplankton assemblage and estimate grazing mortality from differences between trends in phytoplankton abundance at different dilutions. The underlying theory assumes equal phytoplankton growth rate in all dilutions and grazing rates that are strictly encounter-limited.

To increase conformity with these assumptions, dilution of endpoint cultures was done with fresh LW medium containing the full f/2 levels of the potential limiting nutrients (N and P) to ensure saturated growth. These dilutions also reduced *P. parvum* densities to levels where encounter-limited grazing would be more likely. The net per capita rate of change (μ) of *P. parvum* in each experimental bottle was estimated by the regression of the natural log of chlorophyll *a* versus time (days). In the original Landry-Hassett design several dilution levels were used and then grazing mortality was obtained by linear regression of μ in relation to dilution fractions. Following the theory

outlined by Landry and Hassett (1982), grazing mortality rate here was calculated as the difference in μ between the two dilution fractions used, 1/3 and 1/18, divided by the difference between dilution fractions. That is, instead of calculating mortality from a regression slope for many points, a two-point slope calculation was done. For each source monoculture, the average values of μ were calculated for the three 1/3 dilution cultures and the three 1/18 dilution cultures. Thus there was one estimate of grazing rate for each of the six source monocultures, three each grown at 10 °C and 20 °C.

3.2.5. Sampling and counts

Samples were taken from preconditioned stock cultures used for inoculation and preserved with Lugol's iodine solution. Samples of experimental semi-continuous cultures (both mono- and mixed species cultures) were taken on days 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 in order to estimate *P. parvum* cell concentration and relative abundance of various species in mixed species cultures. Algal cell density was enumerated using standard methods as in the previous experiment (Chapter 2). Live counts of *P. parvum* and various other species were also done on the same days for all samples using Sedgwick-Rafter chambers. Both motile and non-motile cells were counted to estimate the proportion of motile cells. Non-motile cells had no visible flagella and resembled the cells described as "temporary cysts" of *P. parvum* by Carter (1937). Grazer counts were done by direct microscopic counts using sedimentation chambers.

3.2.6. Chemical analysis

Samples for the analysis of dissolved nutrients (nitrate, nitrite, ammonium, Soluble Reactive Phosphorous, SRP) were collected on days 12, 28 and 40 of the experiment. Standard methods were followed for the estimation of various dissolved and particulate nutrients (as in Chapter 2). For estimation of chlorophyll *a* samples were drawn through Whatman GF/F filters and frozen in saturated magnesium carbonate. The chlorophyll *a* concentration was measured fluorometrically after overnight extraction with 90 % acetone (Welschmeyer 1994)).

3.2.7. Toxicity studies

Samples for fish toxicity were collected on day 12 and 40 of the experiment and transported to Baylor University for toxicity studies. As in the previous experiment (Chapter 2) a model freshwater fish, *P. promelas* was used to estimate acute toxicity of *P. parvum* and mixed species cultures under varying environmental conditions. Procedures of the fish toxicity studies were similar to that of the previous experiment (Chapter 2). Acute LC_{50} values in terms of the percentage dilution of sample water from each culture were estimated using Probit (Finney, 1971) or Trimmed Spearman-Karber (Hamilton et al. 1977) methods as appropriate for data properties.

Samples for crustacean toxicity tests were collected from days 16 to 36 of the experiment and transported to Baylor University for toxicity studies. For this study a model fresh crustacean (*Daphnia magna*) was used to estimate sub-lethal toxicity of *P. parvum* cultures grown under varying environmental conditions, following procedures described previously (Chapter 2). Reproductive output was measured as number of

offspring during the test period, and reduced fecundity was interpreted as indicating sub-lethal toxicity. These *Daphnia magna* bioassays followed established US EPA protocols (US EPA 1994). An artificial hard-water medium was prepared according to standard methods (APHA 1998) and used as a control treatment, while experimental treatments consisted of *Daphnia* grown in whole water from *P. parvum* cultures. Five experimental units were used per sample, each consisting of a single individual < 24 h old in its own container. *Daphnia magna* were fed a Cerophyll/green algal suspension daily prepared according Brooks et al. (2004) and Dzialowski et al. (2006).

3.2.8. Data analysis

Cell densities did not follow the normal distribution and were transformed to natural logarithm to reduce heteroscedasticity and skew.

These and other response variables were analyzed with factorial ANOVA, to test main effects of temperature (2 levels) and competitors (2 levels) and interaction of these independent variables. When significant treatment effects were identified by factorial ANOVA, Tukey's HSD test (Kleinbaum et al. 1988) was used for multiple comparisons. In both ANOVA and Tukey's HSD test statistical significance was concluded when the null hypothesis of no effects could be rejected at a level of $p < 0.05$. All statistical analyses were done with Statistica 7.0 (Statsoft Inc., Tulsa, Oklahoma, USA).

3.3. Results

3.3.1. Cell density of P. parvum in monocultures

As in the previous experiment (Chapter 2), the majority of semi-continuous cultures did not reach a clear steady state by the end of the experiment (Day 40). Total and motile cell densities of *P. parvum* were analyzed in response to experimental factors on days 12 and 40 when samples were taken for toxicity to fish. Total and motile cell densities were also analyzed after averaging over the time period from day 16 to 36, when samples were taken for determining sub-lethal toxicity to *Daphnia.* In all cases densities were transformed to natural logarithms to conform to assumptions of the factorial ANOVA used.

For total cell density of *P. parvum* on day 12, the two-way interaction of both the experimental factors was significant (Table 3.2). Generally mean total cell density of *P. parvum* was higher in monocultures was than in mixed species cultures at both the temperatures, (Figure 3.1) and in particular 30 ^{0}C mixed species cultures had a significantly lower mean total *P. parvum* cell density than other treatment combinations (Tukey's HSD, $p < 0.05$).

For motile cell density of *P. parvum* on day 12 also, both main effects and the two-way interaction were statistically significant (Table 3.3). Most treatment combinations had similar mean densities, and the only significant difference between treatment means was that mean motile cell density of 30 ^{0}C *P. parvum* monocultures was significantly higher than corresponding 10 ^{0}C cultures (Tukey's HSD, $p < 0.05$) (Figure 3.2).

For total cell density of *P. parvum* on day 40 main effects as well as the interaction of experimental factors were significant (Table 3.4). 10 ^{0}C mono cultures showed a significantly lower mean motile total cell density than 30 ^{0} cultures (Tukey's HSD, $p < 0.05$) (Figure 3.3).

On day 40 abundances of motile cells were negligible in all cultures (counts of zero recorded during live counts), so no further analysis was done.

Table: 3.2. ANOVA for total cell density on Day 12. Bold face indicates significance at p< .05

	SS	Degree of freedom	MS	F	P
Temp.	**2.74**	**1**	**2.74**	**14.70**	**0.00**
Comp.	**9.34**	**1**	**9.34**	**50.47**	**0.00**
Temp* Comp.	**3.05**	**1**	**3.05**	**16.49**	**0.00**
Error	1.48	8	0.185		

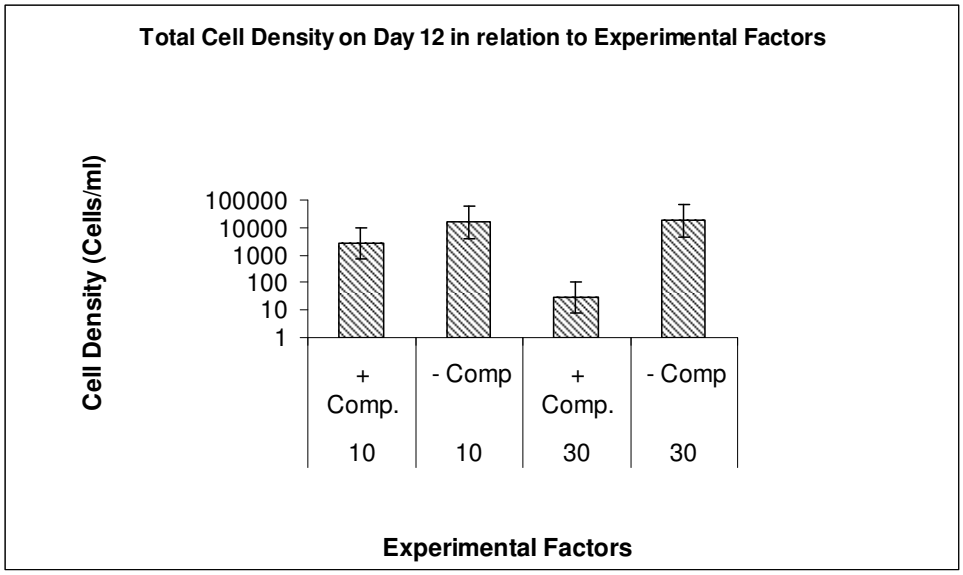

Figure: 3.1. Total Cell Density on day 12 in relation to experimental factors of competitors (+ vs. -) and temperature (10 vs. 30 °C).

Table: 3.3. ANOVA for motile cell density on day 12. Boldface indicates significance at p< .05

	SS	Degree of Freedom	MS	F	P
Temp	**0.909**	**1**	**0.909**	**46.26**	**0.00**
Comp.	**0.909**	**1**	**0.909**	**46.26**	**0.00**
Temp*Comp	**1.51**	**1**	**1.51**	**76.85**	**0.00**
Error	0.157	8	0.019		

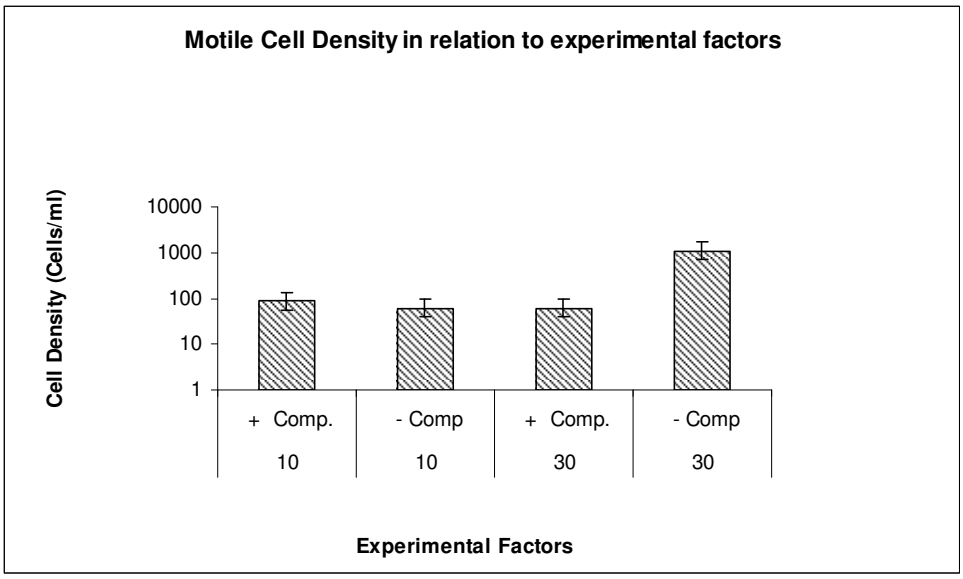

Figure: 3.2. Motile Cell Density on day 12 in relation to experimental factors of competitors (+ vs. -) and temperature (10 vs. 30 °C).

Table: 3.4. ANOVA for total cell density on day 40

	SS	Degree of Freedom	MS	F	P
Temp	**5.43**	**1**	**5.43**	**39.92**	**0.00**
Comp	**2.08**	**1**	**2.08**	**15.30**	**0.00**
Temp*Comp	**8.23**	**1**	**8.23**	**60.53**	**0.00**
Error	1.09	8	0.136		

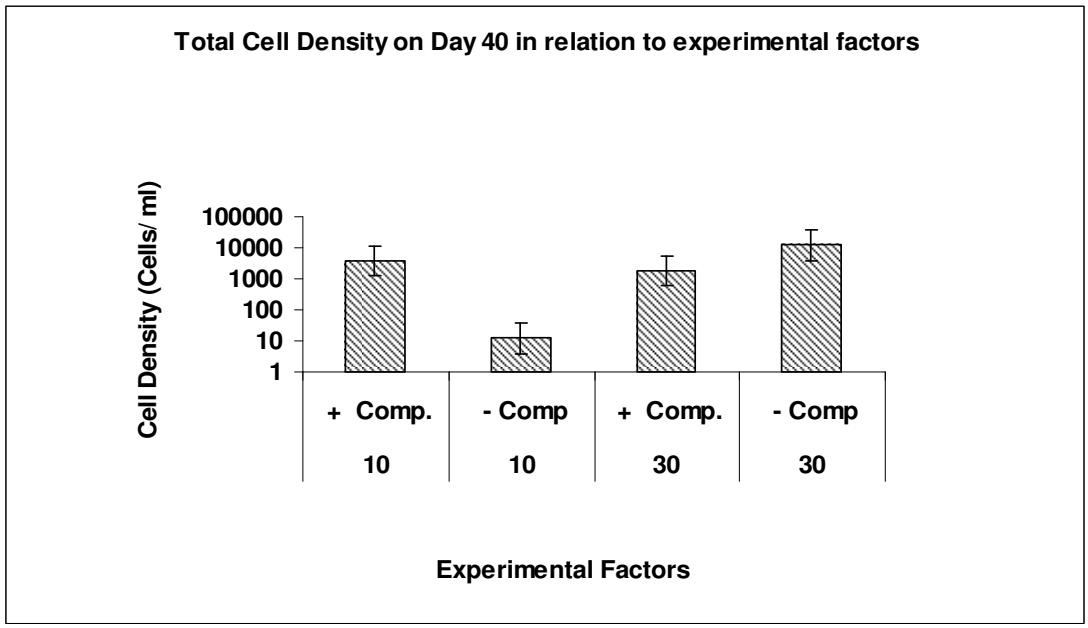

Figure: 3.3. Total density on day 40 in relation to experimental factors of competitors (+ vs. -) and temperature (10 vs. 30 °C).

For total cell density averaged over days 16-36, both the main effects as well as the interactions of experimental factors were statistically significant (Table 3.5). Cultures at 30 ^{0}C showed a higher mean total cell density in the absence of competitors (Figure 3.4) than in their presence.

For motile cell density averaged over days 16-36, only the main effect of temperature was statistically significant (Table 3.6). Cultures at 30 ^{0}C (with and without competitors) showed a higher motile cell density than 10 ^{0}C cultures (Figure 3.5).

Table: 3.5. ANOVA for total cell density over days 16- 36

	SS	Degrees of Freedom	MS	F	P
Temp	**11.90**	**1**	**11.90**	**359.64**	**0.00**
Comp	**2.54**	**1**	**2.54**	**76.70**	**0.00**
Temp* Comp	**6.24**	**1**	**6.24**	**188.40**	**0.00**
Error	0.264	8	0.033		

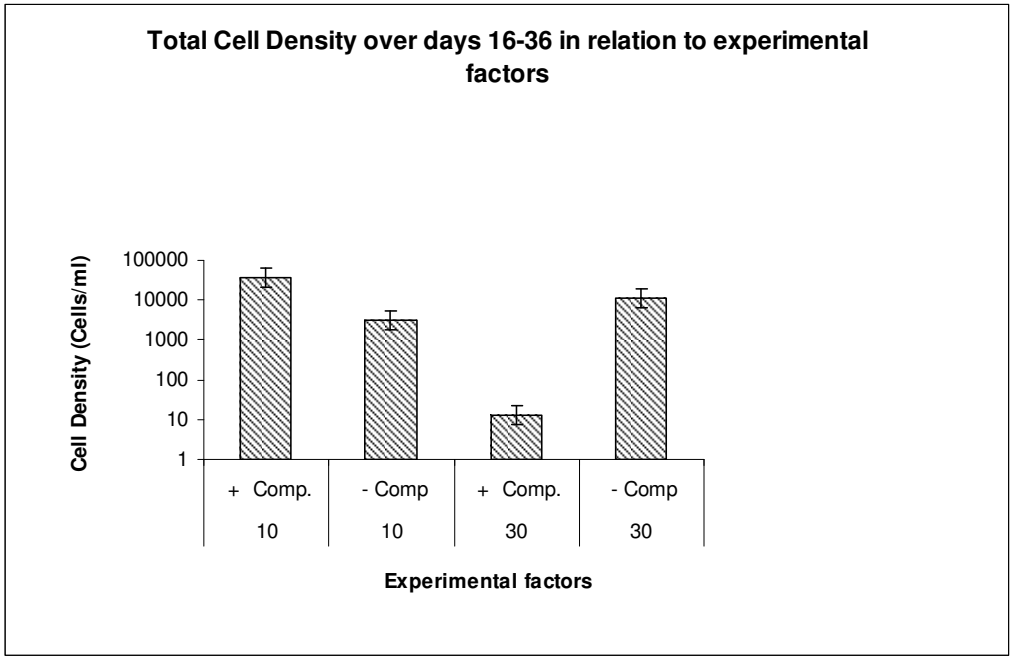

Figure: 3.4. Total density over days 16-36 in relation to experimental factors of competitors (+ vs. -) and temperature (10 vs. 30 °C).

Table: 3.6. ANOVA for motile cell density over day 16- 36

	SS	Degrees of Freedom	MS	F	P
Temp	**23.22**	**1**	**23.23**	**26.39**	**0.00**
Comp	1.76	1	1.76	1.99	0.19
Temp*Comp	1.49	1	1.49	1.69	0.22
Error	7.04	8	0.879		

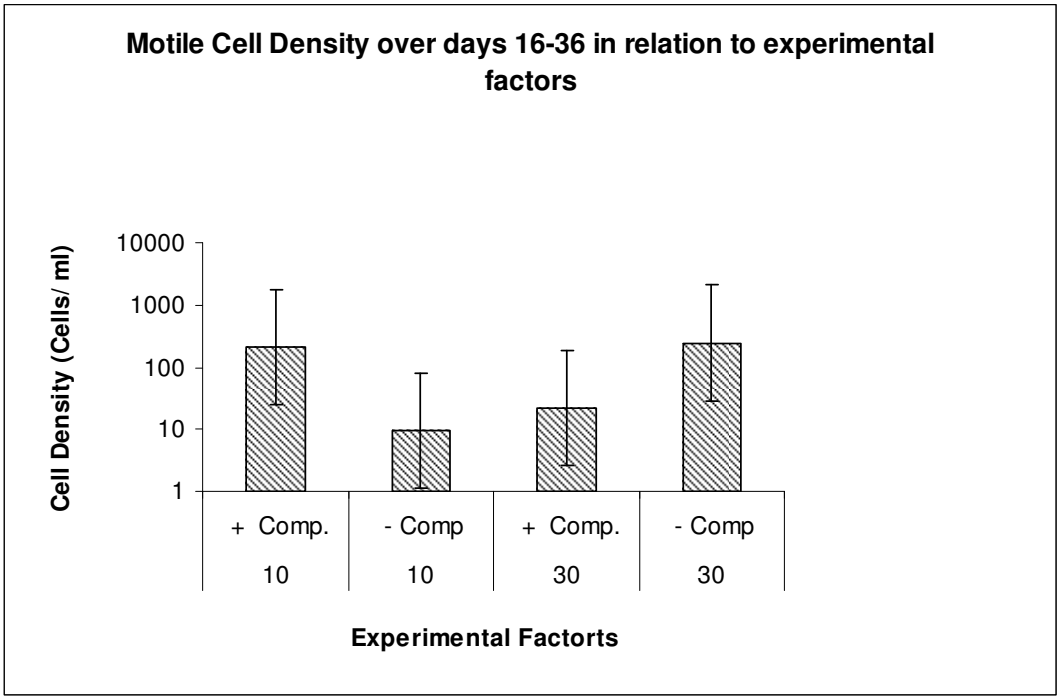

Figure: 3.5. Total density over days 16-36 in relation to experimental factors of competitors (+ vs. -) and temperature (10 vs. 30 °C).

3.3.2. Nutrient concentration of outgoing medium

All culture media were designed to be N-, P-colimited. Concentrations of all dissolved nutrients (NO_3, NO_2 and SRP) showed a decrease from day 12 through day 40 of the experiment. In mixed species cultures (Table 3.7), there was a more extensive depletion of nutrients than in the monocultures (Table 3.8).

Table: 3.7. Inorganic nutrient concentration in the outgoing medium from monocultures

Nutrients	DAY 12	DAY 28	DAY 40
SRP (µM)	0.75 ± 0.43	0.83 ± 0.48	0.53 ± 0.51
NO_3 (µM)	43.91 ± 3.34	38.90 ± 19.18	28.49 ± 15.71
NO_2 (µM)	0.78 ±1.32	0.59 ± 0.60	0.14 ± 0.24

Table: 3.8. Inorganic nutrient concentration in the outgoing medium from mixed species cultures; N.D. – not detectable

Nutrients	DAY 12	DAY 28	DAY 40
SRP (µM)	0.19 ± 0.12	0.09 ± 0.03	0.02 ± 0.01
NO_3 (µM)	20.96 ± 22.79	4.91 ± 5.82	N.D.
NO_2 (µM)	N.D.	0.15 ± 0.18	N.D.

3.3.3. Toxicity studies

Mean reproduction rate of *Daphnia* was analyzed to estimate sub-lethal toxicity of *P. parvum* cultures. In hard water controls, *Daphnia* reproduction was 190 neonates per female. In general, *Daphnia* reproduction equaled or exceeded this value for culture samples, indicating little if any toxicity. For mean reproduction rate of *Daphnia*, only the effect of competitors was significant (Table 3.9). The mean reproduction rate of monocultures was significantly lower than the mixed species cultures at both 10 and 30 °C (Figure 3.6).

Table: 3.9. ANOVA for mean reproduction rate of *Daphnia* over days 16-36

	SS	Degree of Freedom	MS	F	P
Temp	35.4	1	35.4	0.093	0.768003
Comp	**3668.0**	**1**	**3668.0**	**9.662**	**0.014483**
Temp* Comp	1771.5	1	1771.5	4.666	0.062779
Error	3037.2	8	379.7		

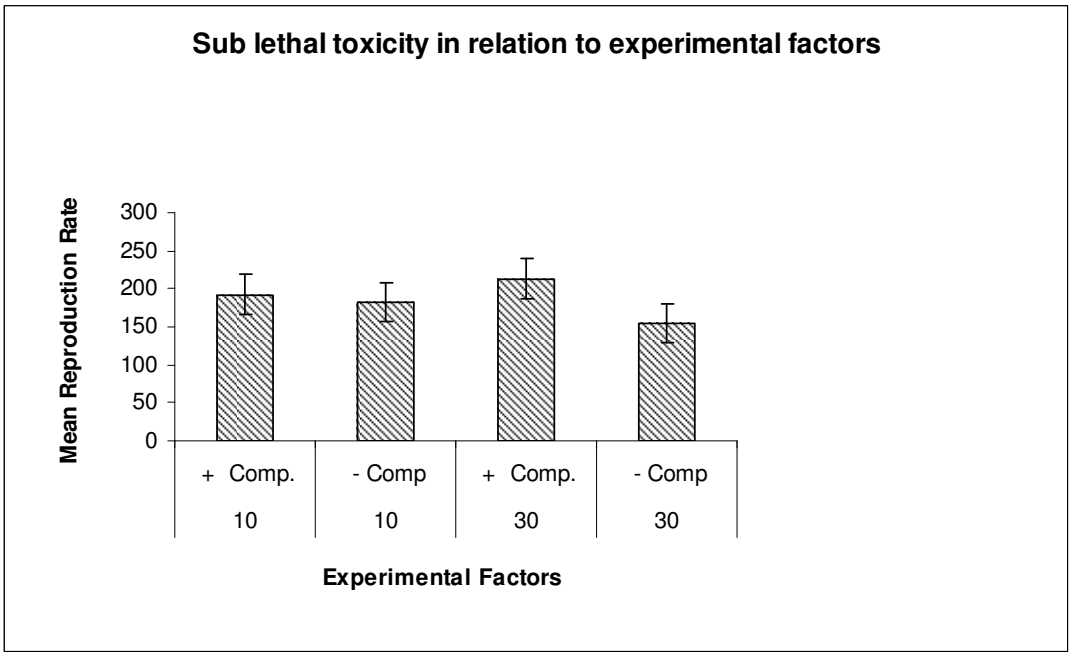

Figure: 3.6. Mean reproduction rate of *Daphnia* in relation to experimental factors

As in the previous experiment acute fish toxicity samples were taken on day 12 and day 40 of the experimental period. For both sampling times, acute toxicity was not detected and survival of test fish was high in all culture samples. No further analysis of these data was done

3.3.4 Mixed species cultures

Half of the cultures were grown as mixed species cultures at two different temperatures, 10 and 30 ^{0}C. Abundances of *P. parvum* and the three other most abundant species were estimated for all the cultures. Though *P. parvum* was not totally outcompeted in 30 ^{0}C cultures its abundance was lowered to a considerable extent, accompanied by the dominance of filamentous cyanobacteria and *Chlorella* species (Figure 3.7). *P. parvum* was dominant and showed higher abundance in 10 ^{0}C mixed species cultures (Figure 3.8). In 30 ^{0}C cultures there was an increase in total biomass relative to the *P. parvum* cell density (Figure 3.9) whereas in 10 ^{0}C cultures *P. parvum* biomass was more or less equal to the total biomass (Figure 3.10).

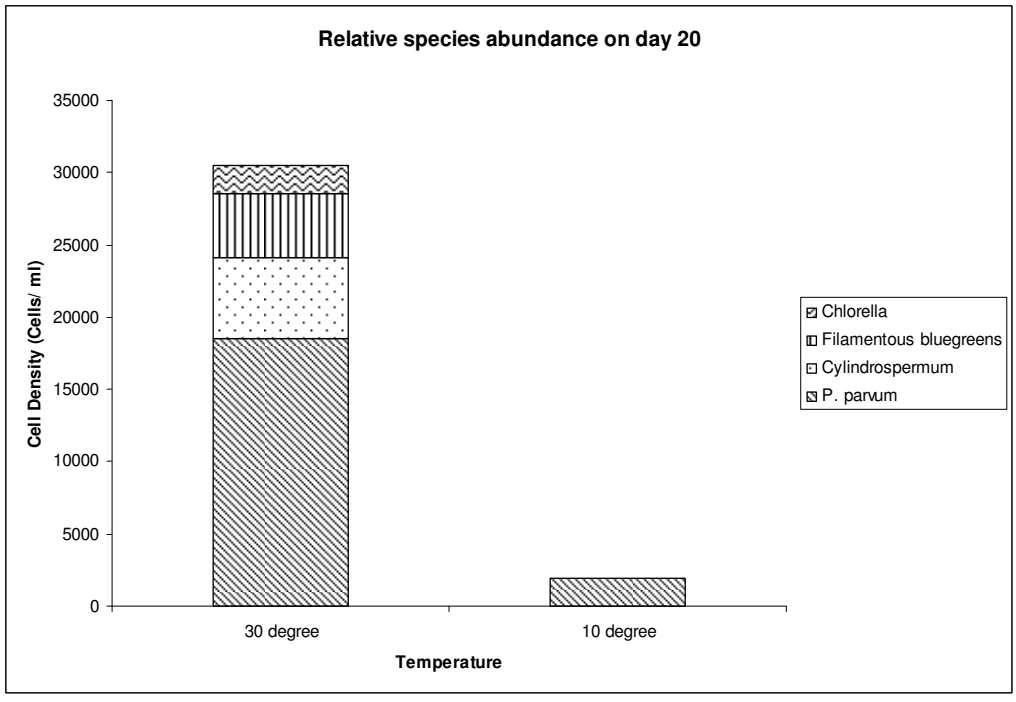

Figure: 3.7. Abundances of different taxa of 10 and 30 ^{0}C cultures on day 20

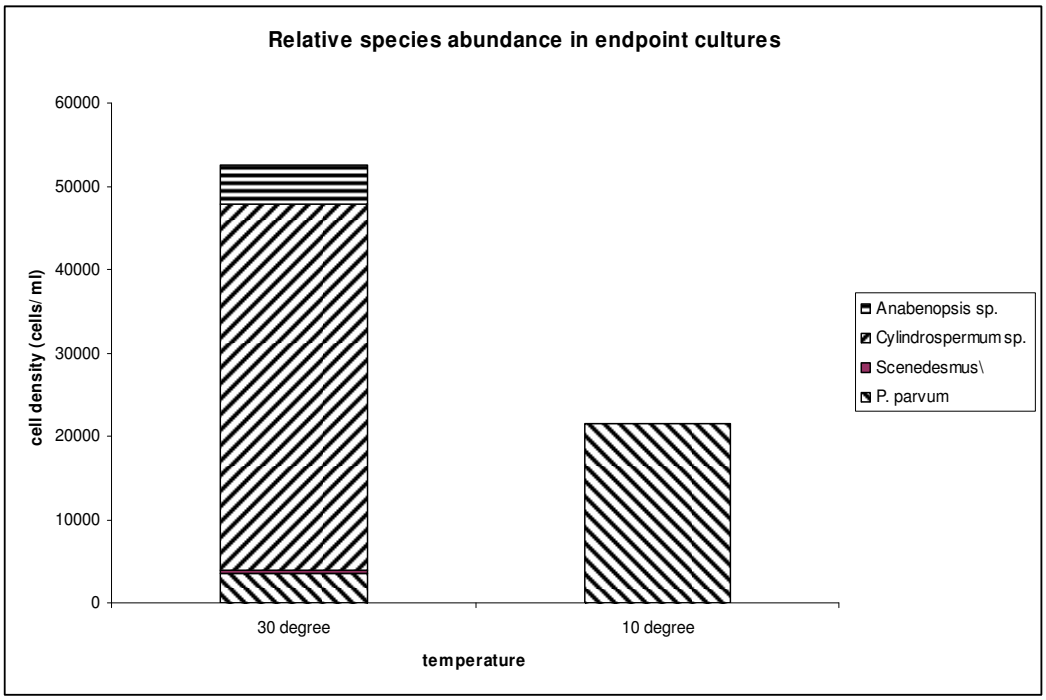

Figure: 3.8. Abundances of different taxa of endpoint culture at 10 and 30 ^{0}C

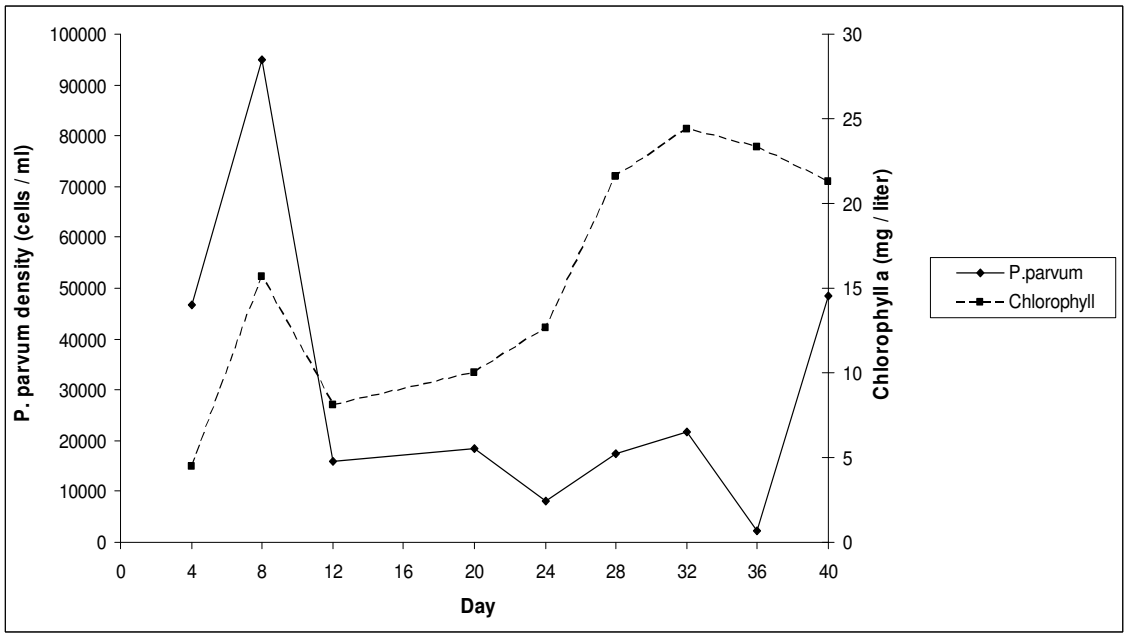

Figure: 3.9. Comparison of total algal biomass with *P. parvum* concentration of a representative 30 ^{0}C mixed species cultures. Straight line represent *P. parvum* concentration while dotted line represent total biomass (Chl *a)* concentration

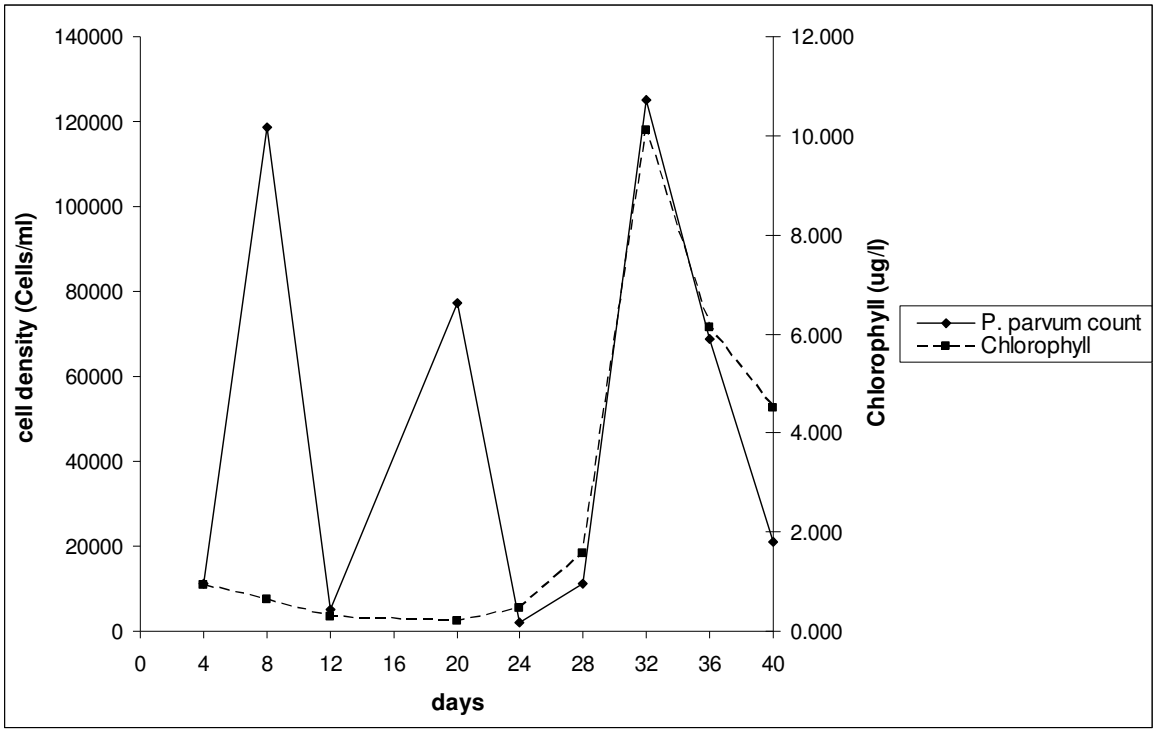

Figure: 3.10. Comparison of total algal biomass with *P. parvum* concentration of a representative 10 ^{0}C mixed species cultures. Straight line represent *P. parvum* concentration while dotted line represent total biomass (Chl *a)* concentration

3.3.5 Grazing experiment

Grazer population abundance varied during the incubation period because of reproduction and death of mature individuals. Grazer populations showed reasonably high survival rates of 77 % and 85 % respectively for *Daphnia magna* and *Ceriodaphnia dubia*

Table 3.10: Grazing experiment results

	Rate of change at dilution fraction (d^{-1})			
10 ^{0}C cultures	**1/3**	**1/18**	**Grazing mortality rate (d^{-1})**	**Average mortality rate (d^{-1}) ± S.D.**
Replicate 1	0.17	0.17	0.01	0.44 ± 0.39
Replicate 2	0.26	0.48	0.78	
Replicate 3	0.22	0.37	0.53	
30 ^{0}C cultures	**1/3**	**1/18**		
Replicate 1	0.06	0.10	0.16	0.66 ± 0.51
Replicate 2	0.11	0.28	0.63	
Replicate 3	0.04	0.36	1.17	

3.4. Discussion

Acute toxic activity of *P. parvum*on juvenile fathead minnows (*P. promelas*) was not detected in this experiment. The lack of toxicity may be related to the low density of motile cells in the cultures. In this experiment, motile cell density was less than about 1000 cells / ml on day 12 and no motile cells were seen during live counts on day 40. In contrast, on day 40 of the experiment reported in chapter 2, acute toxicity to fish was detected and motile cell density ranged up to about 10,000 cells / ml.

Sub-lethal toxic activity of *P. parvum* measured as the effect on reproductive output of *Daphnia magna* was also not detected in this experiment. Reproductive output for *Daphnia magna* exposed to monoculture samples was comparable to that in hard water controls, but higher output was observed for those exposed to mixed culture samples. This indicates that when it is not expressing toxicity, *P. parvum* might be an adequate food source to support reproduction by *Daphnia magna*, but also that a mixed diet containing other algal species provides better nutrition.

The role of temperature as an important ecological factor limiting the distribution of species is well known. Many algal species survive in a wide range of temperature conditions and the optimum temperature for growth varies at species level. *P. parvum* batch cultures grow better in warmer temperatures under laboratory culture conditions (Grover et al. 2007), and a uni-modal relationship with an optimum temperature of 27°C has been found (Baker et al. 2007). However, dominance by competitors in mixed species cultures at 30 °C greatly reduced the abundance of *P. parvum* compared to monocultures at high temperature in this and the previous

experiment. At the lower temperature of 10 °C, *P. parvum* maintained similar, moderately high abundance in both monocultures and mixed species cultures with competitors. Thus abundances in mixed species cultures resembled the seasonal distribution of *P. parvum* in lakes in Texas: low abundances in summer temperatures near 30 °C, and high abundances in winter temperatures near 10 °C (Roelke et al. 2007). Unlike the prototype experimental results (chapter 2), 10 ^{0}C cultures showed higher population abundance throughout the culture period, which is consistent with results from field studies indicating persistence of *P. parvum* populations in mixed species communities at similar, low temperatures (Roelke et al. 2007).

Competitive ability and mortality losses are two main factors which determine the ability of a phytoplankton species to become dominant and form blooms in natural environments. Adaptations of algae to survive grazing pressure would therefore favor the ecological success of the species in that ecosystem. Although it is known that reduced grazer populations can initiate bloom formation, interaction of *P. parvum* and co-occurring zooplankton grazers are poorly studied. In a study conducted by Volkanov (1964) it was demonstrated that copepods show a low sensitivity to *P. parvum.* However, protozoan grazers such as *Euplotes affinis* show high mortality rates with increasing abundance of *P. parvum* (Johansson 2000). Nielsen et al. (1990) reported that *Chrysochromulina polylepis*, a close relative of *P. parvum*, caused mortality of micro-zooplankton and copepods.

This study also demonstrated that cladocerans cause mortality of *P. parvum*, suggesting that direct ingestion may be one route by which grazing zooplankton are exposed to the toxins of this species.

The results from the current study conform to previous studies reporting lower abundance of *P. parvum* at lower temperatures in laboratory monocultures. The different pattern observed in mixed species cultures is consistent with field observations showing a higher abundance of *P. parvum* in during cooler months of the year. These results suggest a hypothesis that competition from other algae prevents formation of *P. parvum* blooms in summer in those Texas lakes sufficiently saline to support the species. Subsequent experiments reported in the following chapter were designed to further examine dynamics in semi-continuous monocultures and mixed species cultures, using a suite of competitors sampled during summer, and to provide additional tests of acute toxicity to fish.

CHAPTER 4

EFFECT OF SUMMER INOCULUM OF COMPETITORS ON GROWTH AND TOXICITY OF *P. PARVUM* IN MIXED SPECIES CULTURES AT DIFFERENT TEMPERATURES UNDER N AND P LIMITATION.

4.1. Introduction

In this study *P. parvum* populations were both grown in monocultures and exposed to mixed species algal populations derived from a natural lake community (Lake Whitney). The competitor inoculum was sampled during summer, under conditions unfavorable to bloom formation by *P. parvum* in this lake. The primary goal of this study was to investigate the competitive interaction of *P. parvum* with other algal species characteristic of those occurring in summer, using low-volume semi-continuous cultures. Both monocultures and mixed species cultures were grown at two different temperatures, 10 °C and 30 °C, representing winter and summer conditions, to test the hypothesis that reducing the temperature to that characteristic of winter would favor *P. parvum* in competition with other algae. Field experiments suggest that high abundance of competing algae reduces the toxicity of *P. parvum* (Roelke et al. 2007). Therefore, acute toxicity of *P. parvum* to a model fish species (*Pimephaes promelas*) was determined to test the hypothesis that competitors would reduce this toxicity.

4.2. Materials and methods

4.2.1. Algal strain and culture conditions

The *P. parvum* strain used in this experiment was obtained from the UTEX collection of algae (Strain number UTEX LB ZZ181). Stock cultures were maintained in a medium of Artificial Sea Water (ASW) (Kester et al., 1967) diluted to a working salinity of 5.8 psu with 18 Ω/ cm ultra pure water. Preparation of stock culture medium was similar to that of the previous experiments (Chapters 2 and 3). These stock cultures were maintained in an incubator on a 12: 12 light and dark cycle with an irradiance of 150 µmol photons/ m^2/ second.

4.2.2. Experimental design

Unlike the previous two experiments, this one was a small volume semi-continuous culture design, in which *P. parvum* was grown in mono cultures and mixed species cultures at two different temperatures. The original plan was for a large volume experiment with sufficient volume to conduct additional studies of interactions with grazers. But because of the unexpected failure of a high capacity ultra-pure water treatment system, we scaled back to small volume semi-continuous cultures (2 X 2 factorial experiment) (Table 4.1). There were two factors two levels of temperatures (10 and 30 ^{0}C) and two levels of competitors (present or absent). Each treatment had triplicates for a total of 12 cultures.

Table: 4.1. Experimental design. '+' and '-'sign indicates presence and absence of competitors

Temperature	Salinity	Competitors
10^0 C	2 psu	+
		-
30^0 C		+
		-

Lake water medium modified to have a salt composition close to that of weakly saline inland waters of Texas (as described in Chapter 2) was used for all experimental cultures. Medium of a single salinity (2 psu) was used and the experimental medium was prepared to be N-, P colimited by using 5 % of f/2 levels of both the nutrients (44 μM for N, 1.8 μM for P). In this experiment, Si was added as $Na_2SiO_3 \cdot 9H_2O$ at a concentration of 134 μM to support growth of diatom competitors.

Experimental cultures were grown in 1000 ml Erlenmeyer flasks containing a working volume of 700 ml of N-, P- colimited medium. Culture vessels were autoclaved at 120 ^{0}C for approximately 30 minutes and the autoclaved culture medium was poured in to vessels under a sterile air laminar flow chamber.

Two different incubators were used to achieve experimental temperatures. The irradiance of experimental cultures was measured using a photon flux meter, and flasks were placed in locations delivering 150 μmol photons/ m^2/ second. In order to produce

semi-continuous growth at a defined average rate experimental medium was diluted every other day with 210 ml of fresh sterile LW medium to achieve an average dilution rate of 0.215 day^{-1}.

4.2.3. Mixed species cultures

In half of the cultures *P. parvum* was grown as mixed species cultures along with major competitors from a natural lake community (Lake Whitney). Half of the mixed species cultures were grown at 10 °C and the other half at 30 °C. Competitors were obtained from Lake Whitney sampled in summer (06/20/2007). Samples of lake water were collected in 1-liter MilliQ-rinsed polycarbonate bottles and brought to the lab in a cooler. The samples were pooled and then sieved through 153 µm mesh to remove large grazers and bubbled with N_2 gas for 2-3 h to asphyxiate small grazers. Two 500 ml aliquots were then prepared and placed in separate incubators for pre-conditioning of algae to the experimental growth conditions. Temperatures of the incubators were initially set to 20 °C and over the next five days temperature of the designated 30 °C incubator was raised by 2 °C every day to reach the desired temperature and that of the designated 10 °C incubator were lowered by 2 °C every day to reach the desired temperature. Two days into the pre-conditioning period 200 ml of sterile LW medium (N, P colimited) was added. On the fifth day of pre-conditioning 200 ml of the culture medium was replaced with 200 ml of fresh LW medium. These pre-conditioning cultures provided inocula for the competition cultures, and 10 ml of the inoculum culture was preserved with Lugol's iodine solution (Modification by Throndsen, 1978).

4.2.4. Sampling and counts

Samples were taken from preconditioned stock cultures used for inoculation and preserved with Lugol's iodine solution. Samples of experimental semi-continuous cultures (both mono- and mixed species cultures) were taken on days 4, 8, 12, 16, 20, 24, 28, 32 and 35 in order to estimate *P. parvum* cell concentration and relative abundance of various species in mixed species cultures. Bacterial and algal cell density was enumerated using standard methods as in the previous experiment (Chapter 2). Live counts of *P. parvum* and various other species were also done on the same days for all samples using Sedgwick-Rafter chambers. Both motile and non-motile cells were counted to estimate the proportion of motile cells. Non-motile cells had no visible flagella and resembled the cells described as "temporary cysts" of *P. parvum* by Carter (1937).

4.2.5. Chemical analysis

Samples for the analysis of dissolved nutrients (nitrate, nitrite, ammonium, Soluble Reactive Phosphorous, SRP) were collected on days 10 and 25 of the experiment and that of particulate nutrients (particulate phosphorous and CHN) were collected on day 16, 28 and 35. Standard methods were followed for the estimation of various dissolved and particulate nutrients. For estimation of chlorophyll samples were drawn through Whatman GF/F filters and frozen in saturated magnesium carbonate. The chlorophyll *a* concentration was measured fluorometrically after overnight extraction with 90 % acetone (Welschmeyer 1994).

4.2.6. Toxicity studies

Samples for fish toxicity were collected on day 35 of the experiment and transported to Baylor University for toxicity studies. As in the previous experiment (Chapter 2) a model freshwater fish, *P. promelas* was used to estimate acute toxicity of *P. parvum* and mixed species cultures under varying environmental conditions. Procedures of the fish toxicity studies were similar to that of the previous experiment (Chapter 2). Acute LC_{50} values in terms of the percentage dilution of sample water from each culture were estimated using Probit (Finney, 1971) or Trimmed Spearman-Karber (Hamilton et al. 1977) methods as appropriate for data properties.

4.2.7. Data analysis

Cell densities did not follow the normal distribution and were transformed to natural logarithm to reduce heteroscedasticity and skew.

These and other response variables were analyzed with factorial ANOVA, to test main effects of temperature (2 levels) and competitors (2 levels) and interaction of these independent variables. When significant treatment effects were identified by factorial ANOVA, Tukey's HSD test (Kleinbaum et al. 1988) was used for multiple comparisons. In both ANOVA and Tukey's HSD test statistical significance was concluded when the null hypothesis of no effects could be rejected at a level of $p < 0.05$. All statistical analyses were done with Statistica 7.0 (Statsoft Inc., Tulsa, Oklahoma, USA).

4.3. Results

4.3.1. Cell density of P. parvum in monocultures

Unlike the previous experiments majority of cultures reached a stationary phase (Figure 4.1) two weeks after dilution had been started. Total and motile cell densities of *P. parvum* were analyzed in response to experimental factors on day 12 for consistency with previous experiments, and on day 35 when samples were taken for toxicity to fish. Total and motile cell densities were also analyzed after averaging over the time period from day 16 to 32 during which period samples were taken for dissolved and particulate nutrient analysis. In all cases densities were transformed to natural logarithms to conform to assumptions of the factorial ANOVA used.

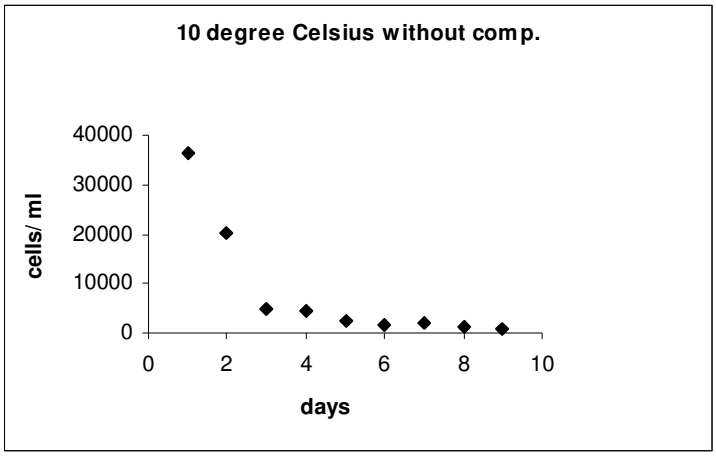

Figure: 4.1. A representative 10 ^{0}C monoculture that has reached stationary phase

For total cell density of *P. parvum* on day 12, none of the experimental factors or their interactions was significant (Table 4.2). 10 °C cultures showed a slightly higher

cell density than the corresponding 30 °C cultures, and *P. parvum* cell concentration was slightly greater in mixed species cultures (Figure 4.2).

Table: 4.2. ANOVA for total cell density on day 12

	SS	Degree of freedom	MS	F	P
Temp	0.079	1	0.079	2.22	0.175
Comp	0.036	1	0.036	0.995	0.348
Temp* Comp	0.037	1	0.037	1.02	0.342
Error	0.288	8	0.036		

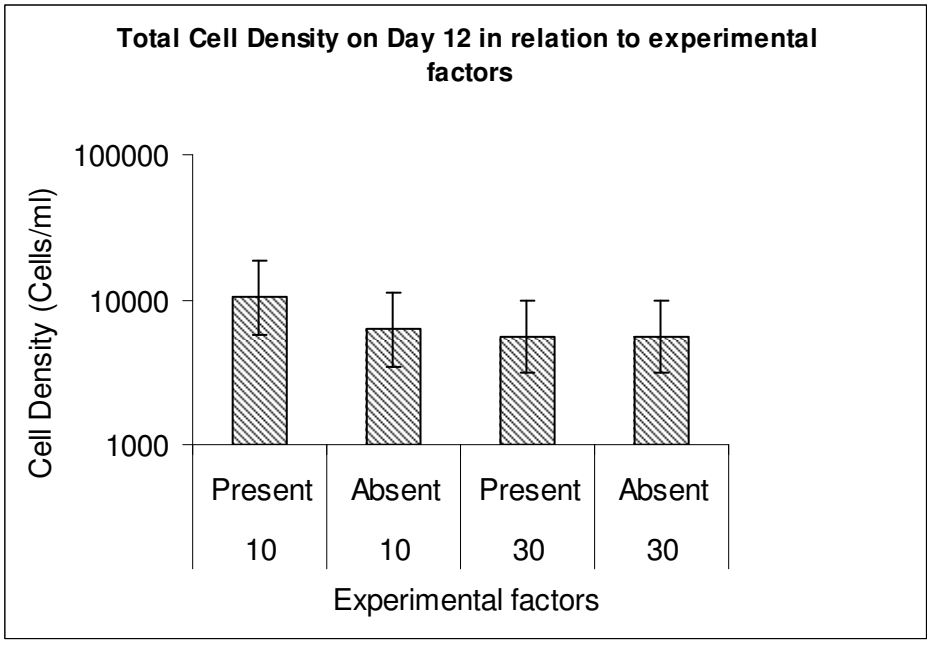

Figure: 4.2. Total density on day 12 in relation to experimental factors of competitors (present vs. absent) and temperature (10 and 30 °C).

For motile cell density of *P. parvum* on day 12 only the main effect of temperature was statistically significant (Table 4.3). Cultures grown at 30 °C showed a significantly lower motile cell density than those growth at 10 °C (Tukey's HSD, $p < .05$) (Figure 4.3).

For total cell density on day 35 both the main effect of temperature and interaction of temperature and competitors were statistically significant (Table 4.4). The mean total density of *P. parvum* was highest in mixed species cultures at 10 °C, lowest in mixed species cultures at 30 °C, and intermediate for monocultures (Figure 4.4).

For live cell density on day 35 there were lot of zero values hence no further analysis was done.

Table: 4.3. ANOVA for motile cell density on day 12. Bold face indicates significance at $p < 0.05$

	SS	Degree of freedom	MS	F	P
Temp	**1.92**	**1**	**1.92**	**16.85**	**0.00**
Comp	0.056	1	0.055	0.48827	0.505
Temp* Comp	0.142	1	0.142	1.24808	0.296
Error	0.909	8	0.113		

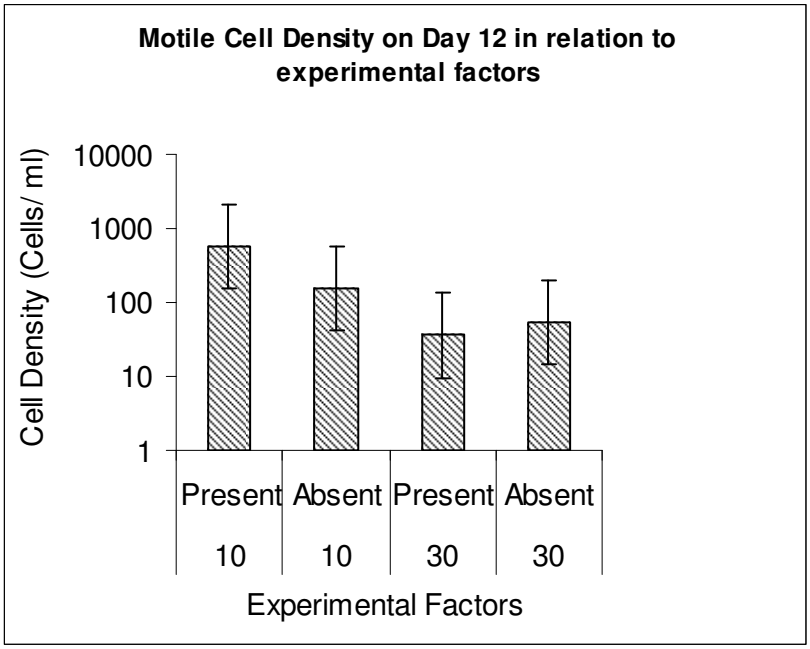

Figure: 4.3. Motile cell density on day 12 in relation to experimental factors

Table: 4.4. ANOVA for total cell density on day 35. Bold face indicates significance at $p < 0.05$

	SS	Degree of freedom	MS	F	P
Temp	**0.382**	**1**	**0.381**	**8.84**	**0.01**
Comp	0.138	1	0.138	3.19	0.11
Temp*Comp	**1.51**	**1**	**1.51**	**34.96**	**0.00**
Error	0.345	8	0.043		

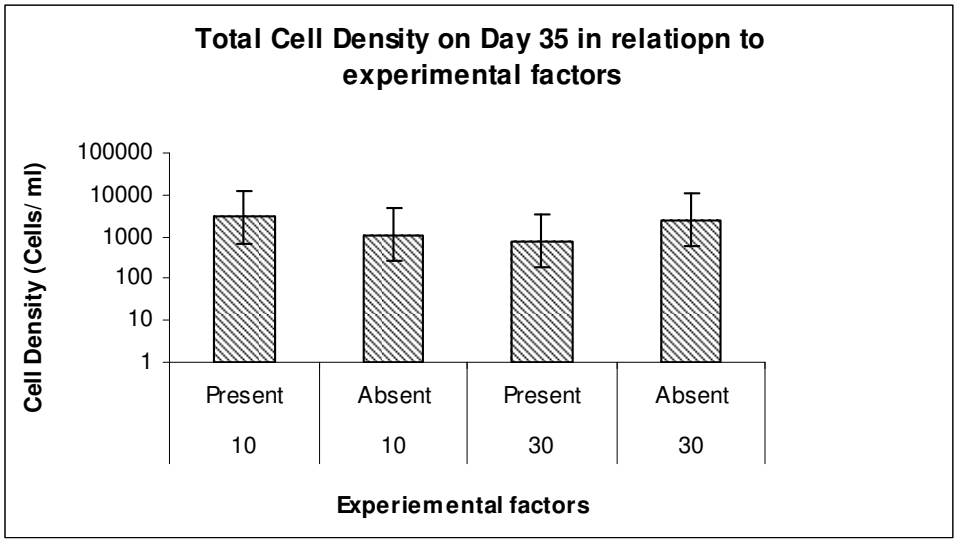

Figure: 4.4. Total cell density on day 35 in relation to experimental factors

For total cell density over days 16- 32 none of the experimental factors or their interactions was statistically significant (Table 4.5).

Table: 4.5. ANOVA for total cell density over days 16- 32. Bold face indicates significance at $p < 0.05$

	SS	Degree of Freedom	MS	F	P
Temp	0.074	1	0.074	0.815	0.393
Comp code	0.002	1	0.002	0.024	0.882
Temp*Comp	0.274	1	0.274	3.00	0.121
Error	0.727	8	0.091		

For motile cell density averaged over days 16-32, the main effect of competitors and two-way interaction between the experimental factors were significant (Table 4.6). Most treatment combinations had similar mean densities (Figure 4.5), and the only significant difference between treatment means was that mixed species cultures at 30 ^{0}C had lower density than those at 10 ^{0}C(Tukey's HSD, $p < .05$).

Table: 4.6. ANOVA for motile cell density over days 16- 32. Bold face indicates significance at $p < 0.05$

	SS	Degree of Freedom	MS	F	P
Temp	0.358	1	0.358	1.38	0.274
Comp	**1.300**	**1**	**1.300**	**5.00**	**0.05**
Temp*comp	**2.207**	**1**	**2.207**	**8.49**	**0.02**
Error	2.08	8	0.260		

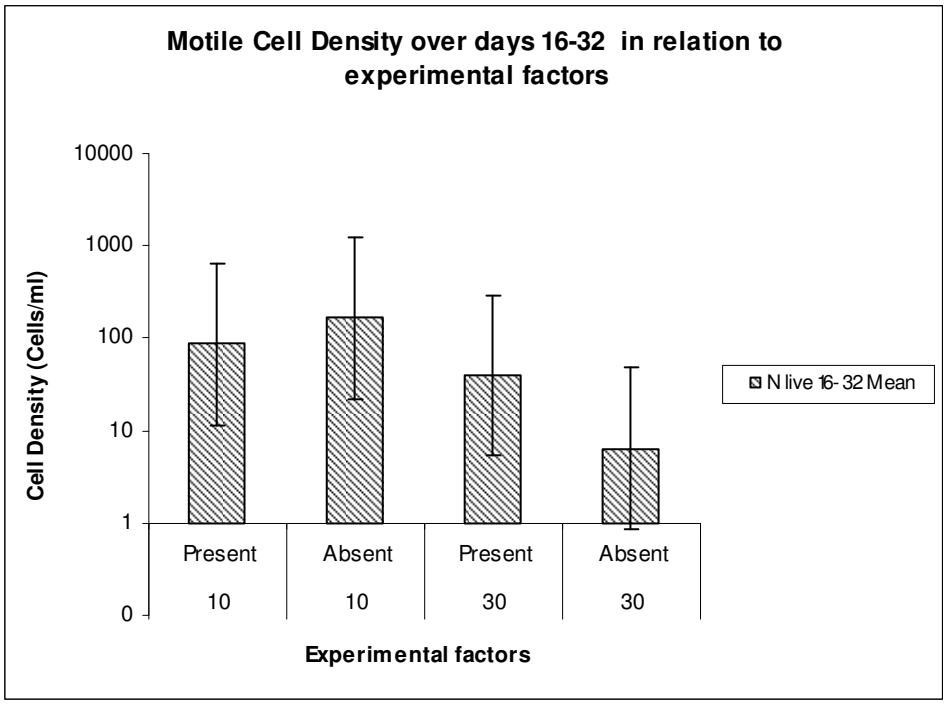

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Figure: 4.5. Motile cell density over days 16- 32 in relation to experimental factors

4.3.2. Toxicity Studies

Fish toxicity samples were collected on the last day of the experimental period. No acute lethal toxicity was detected and survival of test fish was high in all culture samples. No further analysis was done.

4.3.3. *Nutrient concentration of outgoing medium*

In monocultures the concentrations of SRP and nitrite, but not nitrate, showed a decrease from day 16 through day 28 of the experiment (Table 4.7). In mixed species cultures there was a faster and more extensive depletion of nutrients rather than the monocultures (Table 4.8).

Table: 4.7. Inorganic nutrient concentration in outgoing medium from *P. parvum* monocultures

Nutrients	**DAY 16**	**DAY 28**
SRP (µM)	0.07 ± 0.06	0.03 ± 0.07
NO_3 (µM)	20.66 ± 22.99	18.82 ± 20.92
NO_2 (µM)	0.13 ±0.15	0.02 ± 0.13

Table: 4.8. Inorganic nutrient concentration in outgoing medium from mixed species cultures

Nutrients	DAY 16	DAY 28
SRP (µM)	0.14 ± 0.00	0.09 ± 0.01
NO_3 (µM)	41.83 ± 2.69	49.92 ± 1.12
NO_2 (µM)	1.24 ±1.12	0.23 ± 0.20

4.3.4. Mixed species cultures

P. parvum was grown in mixed-species cultures with summer inoculum of competitors from Lake Whitney. In general there was a steady decrease of *P. parvum* abundance at both temperatures. Mixed-species cultures grown at 10 °C showed a higher *P. parvum* concentration than the corresponding 30 °C cultures (Figure 4.6). In cultures at 10 ^{0}C, *P. parvum* dynamics were essentially equivalent to those of total biomass (Chlorophyll *a*) during the last 8 days of the experiment (Figure 4.7), whereas in 30 ^{0}C cultures, *P. parvum* biomass decreased to low levels relative to total biomass (Figure 4.8).

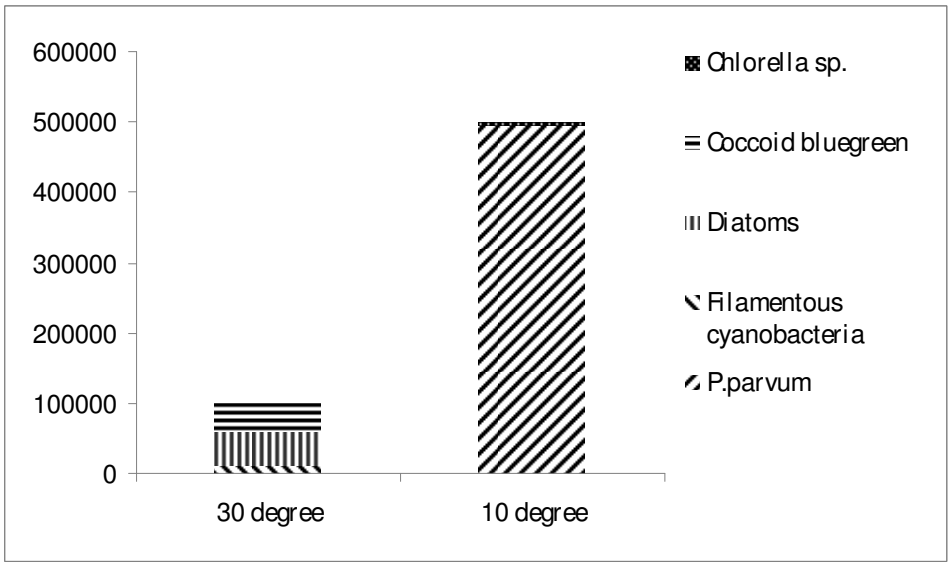

Figure: 4.6. Abundance of different taxa of endpoint cultures at 10 and 30 ^{0}C

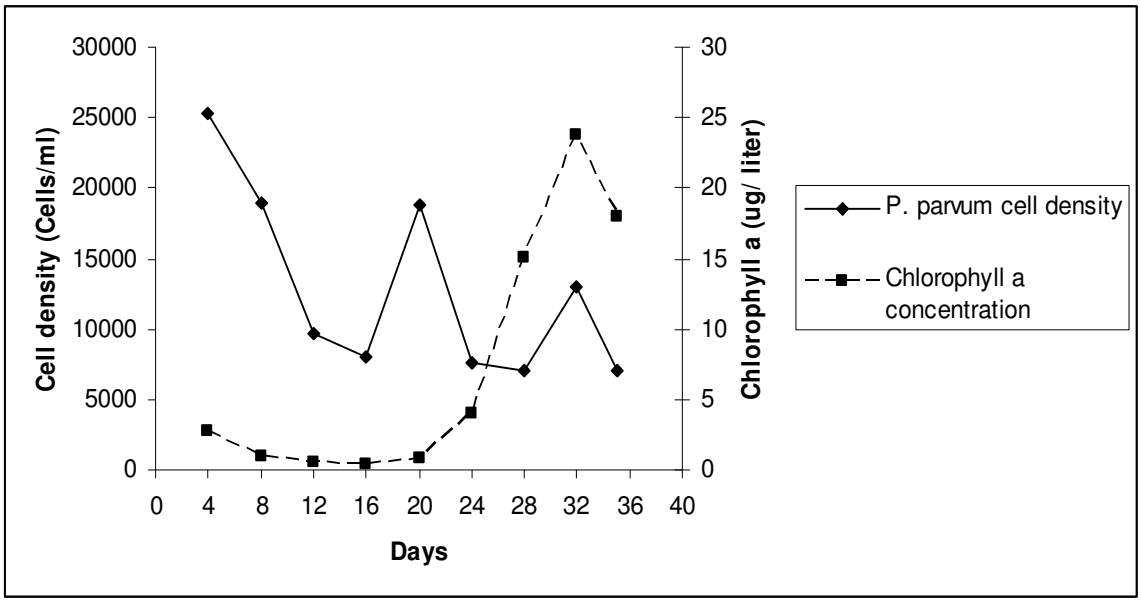

Figure: 4.7. Comparison of total algal biomass with *P. parvum* concentration of a representative 10 ^{0}C mixed-species culture. Straight line represent *P. parvum* concentration while dotted line represent total biomass (Chl *a)* concentration

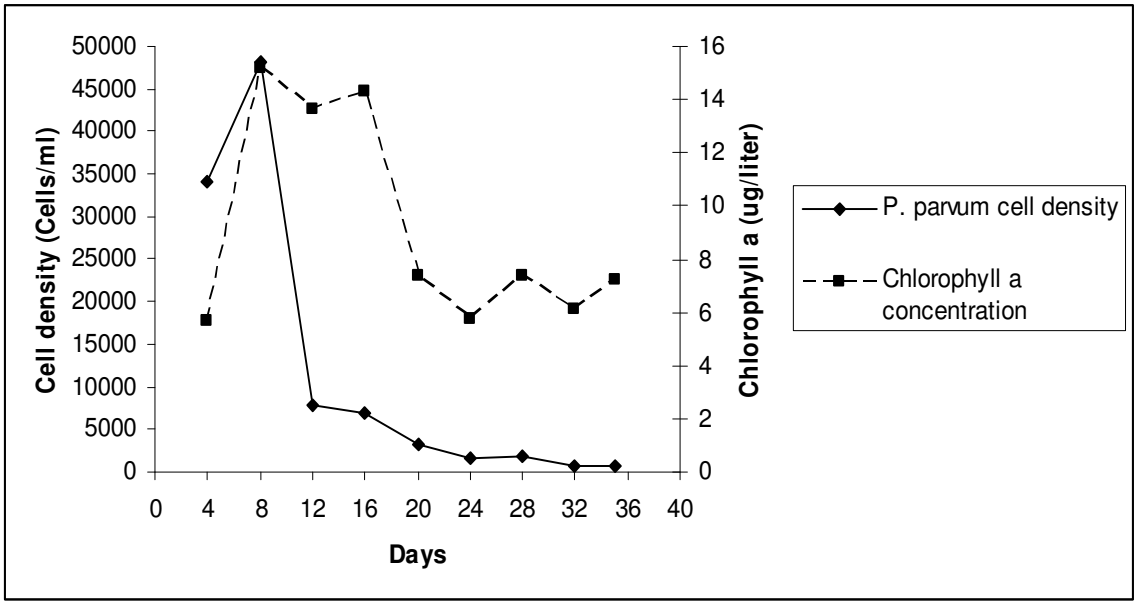

Figure: 4.8. Comparison of total algal biomass with *P. parvum* concentration of a representative 30 ^{0}C mixed-species culture. Straight line represent *P. parvum* concentration while dotted line represent total biomass (Chl *a)* concentration

4.4. Discussion

No acute lethal toxic effects of *P. parvum* on juvenile fathead minnows (*P. promelas*) were detected in this experiment. Again, the lack of toxicity appears related to the low density of motile cells in the cultures. In this experiment, motile cell density was usually less than about 1000 cells / ml over days 16 – 32, and in many cultures no motile cells were seen during live counts on day 35 when samples were taken for tests of toxicity to fish. In contrast, on day 40 of the experiment reported in chapter 2, acute

toxicity to fish was detected and motile cell density ranged up to about 10,000 cells / ml.

Abundance estimates suggest that *P. parvum* monocultures showed a steady decrease in abundance at both temperatures throughout the culture period and reached a stationary state at low abundances with most cells present as cysts. In mixed species culture, those at 10 °C showed a similar abundance to monocultures, but mixed species cultures at 30 ^{0}C cultures had a reduced abundance compared to monocultures.

These results do not completely agree with those from the previous experiment (Chapter 3), since *P. parvum* densities were lower in the small volume cultures reported here. However, they conform well to field observations suggesting increased abundance of *P. parvum* at lower temperature ranges (Roelke 2007), since *P. parvum* retained a higher abundance in the presence of competitors at 10 °C than at 30 °C. It is known that competition can play a significant role in determining the community structure in the absence of environmental disturbances (Grime 1974, 1977, 1979). Several studies have shown that there is a resource mediated demographic variation (species composition and abundance) in natural phytoplankton communities (Threlkeld et al. 1986).

Occurrence of *P. parvum* dominated blooms is quite uncommon in Texas inland waters during summer months suggesting that ecological factor(s) prevents bloom formation. In the previous experiment (Chapter 3), *P. parvum* abundance and toxicity was tested against a suite of competitors collected from a natural lake community sampled in spring, under conditions favorable for the growth of *P. parvum.*

In the current experiment *P. parvum* was grown with a suite of competitors collected from Lake Whitney during summer under conditions in which *P. parvum blooms* are quite uncommon in Texas inland waters. Results show that competitors grow better under warmer temperatures (30 °C), outcompeting *P. parvum*, and thus supporting the hypothesis that competition from other algae might be an important factor preventing blooms of this species in the summer.

CHAPTER 5

CONCLUSION AND SYNTHESIS

In the current study *P. parvum* was grown in semi-continuous cultures to study the effects of temperature, salinity, nutrient limitation, competitive interaction with other coexisting species, and grazers. The semi-continuous cultures did not work according to theoretical expectations. When a semi-continuous culture is maintained with frequent dilutions at constant volume, it should approximate continuous cultures or chemostat. Thus populations of *P. parvum* were expected to reach a stationary steady state where limiting nutrients are depleted to minimal levels. But in these cultures, limiting nutrients, especially N, were not depleted to expected levels, and well defined steady states were not evident in all of the cultures.

Interestingly, in all the experiments, a substantial proportion of *P. parvum* populations showed encystment (resembling the cysts mentioned in the original species description by Carter 1937). The deviations from theoretical expectations that were observed could thus be due to large proportions of encysted cells. Encystment also reduces the number of cells in the population actively consuming the limiting nutrient, potentially making that population less competitive relative to species that remain completely active.

Many algal species grow at wide range of temperatures and the optimum temperature requirement varies at the species level. Previous experimental results show that the growth rate of *P. parvum* in batch cultures is highest under temperatures of 20 –

30 °C (Grover et al. 2007), with a unimodal relationship and an optimum temperature of 27 ^{0}C (Baker et al. 2007). However, in this study, dominance by competitors in mixed species cultures at 30 ^{0}C greatly reduced the abundance of *P. parvum* compared to monocultures at this temperature. In contrast, at lower temperatures *P. parvum* at least maintained or even showed higher population abundance in mixed species cultures with competitors versus monocultures. Thus abundance in mixed species cultures resembled the seasonal distribution of *P. parvum* in lakes of Texas: low abundances in summer temperatures near 30 ^{0}C and high abundance in winter temperature near 10 ^{0}C (Roelke et al. 2007).

Sub-lethal toxic activity of *P. parvum* measured as effect on reproductive output of *Daphnia magna* was found only for monoculture samples, and only in the first experiment. Likewise, lethal toxicity to fish was found only in the first experiment. The apparently reduced toxicity in the second two experiments could again be related to encystment. Presumably, encysted cells do not actively produce toxin, while motile cells potentially do so. Densities of motile ranged up to about 10,000 cells / ml in the first experiment, when toxic effects were detected. In the subsequent two experiments, densities of motile cells were generally an order of magnitude lower, and on many occasions they were undetectable during routine live counts. The greater tendency to encyst in the second two experiments could have been due to use of two nutrients at low concentration. This was intended to produce N- and P-colimited cultures in which a variety of competing species might be successful in mixed species cultures, but it may have induced greater encystment of *P. parvum* than did the use of single limiting

nutrients in the first experiment. Little is known about the factors that induce encystment in *P. parvum*. This process could impact the dynamics of toxic blooms and thus requires further study.

Although it is known that reduced grazer populations can initiate bloom formation, interaction of *P. parvum* and potentially co-occurring zooplankton grazers like *Daphnia magna* and *Ceriodaphnia dubia* is poorly studied. Toxic effects of *P. parvum* blooms on zooplankton grazers are plausible and this study adds to the one and only previous report of sub-lethal reduction in reproduction of a common cladoceran (Roelke et al. 2007). This study also reveals that cladocerans cause mortality of *P. parvum* suggesting that direct ingestion may be one route by which grazing zooplankton are exposed to toxins, and that common cladoceran grazers might limit populations of *P. parvum*. Thus interactions with grazers, like those with competitors, deserve attention as potential causes of the observed seasonal dynamics in Texas inland waters. Moreover this study adds to the growing literature documenting toxic effects on ecologically relevant representatives of organisms that co-occur with *P. parvum*.

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