GROWTH RATE OF THE HARMFUL ALGA *PRYMNESIUM PARVUM*: AN ATTEMPT TO IDENTIFY THE SALINITY THRESHOLD FOR ALGAL BLOOMS

by

JAMES WILLIAM CODY BLACK

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ABSTRACT

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James W. Cody Black, M.S.

The University of Texas at Arlington, 2012

Supervising Professor: James P. Grover

Harmful algal blooms due to *Prymnesium parvum* have occurred in Texas for nearly three decades in waters of moderate salinity. More recently, blooms have occurred in locations such as West Virginia, in waters of very low salinity where pollution discharges are suspected to have increased salinity to levels that support growth of *P. parvum*. These observations raise the question, what is the lowest salinity at which this species of algae can cause toxic fish kills? Previous research based on field observations has suggested a salinity threshold of about 1.5 PSU for occurrence of blooms in Texas reservoirs in winter. Other research using laboratory cultures suggest that temperature influences the growth response of *P. parvum* to salinity. Here we used laboratory cultures to attempt to identify the threshold salinity for blooms of this harmful species. We used 12-hour day/night cycles at temperatures of 10° C and 20° C with a salinity range from 0.6 to 5.8 PSU. Population growth of cultures was measured over periods of 1, 2, 3, and 4 weeks. At 10° C, average population growth rates over longer time scales of 21 to 28 days were positive only at 5.8 PSU, but under shorter time scales positive growth was observed at 2.0 PSU. At 20° C positive growth at time scales of 21 to 28 days was produced at 2.0,

2.5 and 5.8 PSU, and at shorter time scales positive growth occurred at salinities as low as 0.6 PSU. A regression analysis indicated that growth may be possible at salinities ranging as low as 1.0 PSU. Previous laboratory studies have measured growth over shorter time scales, and predictive regression models from those studies were evaluated with data obtained here. These results suggest that models based on such short-term measurements might not predict growth very well over longer time intervals. In contrast, the salinity thresholds for positive growth estimated here, of 1.0 to 1.5 at 20° C and 2.2 to ≈ 3.1 PSU at 10° C, are similar to those estimated from field observations.

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CHAPTER 1

INTRODUCTION

Harmful algal blooms that occur in marine estuaries are known for deleterious effects to marine mammals, fish and may cause harm to humans. In inland or aquatic ecosystems harmful algal blooms occur with less notoriety than their marine counterparts (Hudnell 2010). In freshwater systems stream inflows introduce organic material, fluxes of salts from run-off and nutrients into lakes. These chemical additions often occur in pulses of flow and have been known to alter productivity of phytoplankton communities: negatively by removing populations and positively by relieving the stress of nutrient limitation. However, water quality is reduced by the inflow of nutrients and salts, which may make blooms more likely, but these effects are not immediate and may occur over time. Some of these pulsed inflows of salts and nutrients have also been linked to blooms of algae in several river systems in Texas. This includes water bodies, such as Lake Possum Kingdom, Lake Granbury and Lake Whitney which all have experienced harmful algal blooms of *Prymnesium parvum* (Roelke et al. 2011). In this region, the past decade has seen many toxic blooms from this species (Grover et al. 2007, Baker et al. 2009, Roelke et al. 2010, Roelke et al. 2011).

Prymnesium parvum belongs in the phylum Haptophyta and is one of many species of algae causing harmful blooms (HAB's) on a global scale. This species has caused fish kills in the United States (Southard et al. 2010), Norway, (Baker et al. 2007, Johnsen et al. 2010) and in the Baltic Sea (Fistarol et al. 2003, Roelke et al. 2010,). A very recent fish kill in West Virginia may be linked to *P. parvum* (Hopey 2009, Renner 2009, Brooks et al. 2011). It is well documented in Texas and other southwestern states that *P. parvum* is responsible for fish kills in many inland

lakes (Baker et al 2009, Grover et al 2007, Johannson and Graneli 1999). In Texas alone, *P. parvum* has been linked to a number of fish kills ranging in the tens of millions of individuals in 19 lakes (Baker et al 2007). Prymnesins 1 and 2 were the first identified ichthyotoxins released by this species and may be the primary toxins related to fish kills in these brackish inland lakes and marine estuaries. It is believed that the prymnesins are absorbed through the gills of fish and interfere with ion regulation (Brooks et al 2010). More recently, several fatty acid amides produced by *P. parvum* have been identified and found to have ichthyotoxic activity (Bertin et al. 2012a, b).

The bodies of water where inland water HAB's occur typically have low salinity levels compared to seawater, are in temperate regions with warm water, and an abundant supply of nutrients (Fistarol et al. 2003). In Texas reservoirs salinities usually range between less than one and four practical salinity units (PSU), reaching higher levels when there has been low annual rainfall. Toxic blooms from *P. parvum* in Texas reservoirs have a complex relationship with salinity and temperatures, because both can be stressors on this alga when at suboptimal levels (Baker et al. 2007, 2009). A salinity threshold has to be exceeded in order to support population growth, while a critically low temperature must be met to suppress growth of competitors, such as cyanobacteria (Roelke et al. 2010). This being said, low salinity and low temperatures just above the thresholds for growth can stress *P. parvum* and trigger its production of toxins (Baker et al. 2007). In an analysis of field observations attempting to identify salinity thresholds in three Texas lakes, inflows and salinity were studied to see how they effect *P. parvum* bloom formation (Roelke et al. 2011). It was determined that Lake Possum Kingdom has a salinity threshold of ≈1.5 PSU for *P.* parvum growth, whereas Lake Granbury and Lake Whitney both have thresholds of 0.5 PSU. Though blooms do not always occur at salinities above these thresholds, they do not occur when the salinity is below them. Lake Possum Kingdom may have a higher salinity threshold due to its higher position on the Brazos River watershed and higher natural range of salinity variation when compared to the other two lakes (Roelke et al. 2011).

Effects of salinity and temperature on population growth rate of *P. parvum* have been assessed in several laboratory experiments. Generally algal growth decreased as salinities decreased toward those of inland waters (Larsen et al. 1993, Larsen and Bryant 1998, Baker et al. 2007, 2009). The salinity limit for growth in published experiments ranged down to 0.5 PSU. Growth rate also decreased as temperatures decreased toward those of winter conditions during which blooms are observed in Texas (Baker et al. 2009). Although, there have been several laboratory studies that have focused on salinity, temperature and other factors such as light (Larsen and Bryant 1998, Baker et al. 2007, 2009, Lutz-Carrillo et al. 2010), the lower limit threshold of salinity for *P. parvum* population growth has not been precisely identified. This is partly because most studies use a range of high and low salinity, with only a few lower levels of salinity in the experimental design. An additional complication is that different investigators have run experiments for differing lengths of time, and have chosen various time intervals over which to estimate population growth rates. In these ecophysiolgical studies, most of the growth rates estimates are made over relatively short intervals (<10 days). But in natural circumstances, harmful algal blooms might take weeks or even months to develop. Over a short period of time, a low but positive growth rate might be indistinguishable from zero. So not only is there a need to examine growth over a range of low salinity treatments, but there is also a need to examine the potential for population growth over a range of time intervals. The objective to this research was to estimate the salinity threshold for *P.* parvum more precisely by employing several treatments of low salinity and measuring growth over intervals of one to four weeks.

The results of this study will help identify bodies of water susceptible to HAB's caused by *P. parvum*, and set standards for effluent discharges that could raise the salinity of receiving waters. For example, in the Ohio River Valley, disposal of natural gas drilling fluids, and spoils from mountain top removal coal mining may salinize otherwise fresh waters and stimulate the growth of *P. parvum* (Brooks

et al. 2011). Especially after the large fish kill in West Virginia due to *P. parvum*, the question was raised, what is the lowest salinity at which *P. parvum* can bloom?

CHAPTER 2

METHODOLOGY

The cultures used in this experiment were originally obtained from the Culture Collection of Algae at the University of Texas at Austin, strain number UTEX LL 2797. These cultures were maintained in a medium of artificial seawater diluted to 5.8 PSU with ultrapure water (Millipore Milli-Q, 18 MΩ cm^{-1}). The medium was based on artificial seawater prepared in accordance with procedures of Baker et al. (2007). Algal cultures were transferred once a month into new media and kept in a 20° C incubator.

Water for experimental cultures was made by diluting artificial seawater to salinities of 0.6, 0.8, 1.0, 1.5, 2.0, 2.5 and 5.8 PSU. The final concentration of sodium bicarbonate ($NaHCO_3$) added to all treatments was the same as used in the stock cultures (384 μM). That is, the concentration of this salt was not reduced in proportion to that of the other major salts. This was done to provide the same supply of dissolved inorganic carbon dioxide for photosynthesis in all cultures. The same concentrations of nutrients (nitrate, phosphate, vitamins and trace metals) were also added to all salinity treatments as were in the stock cultures (concentrations as for f/2 medium, McLachlan 1973). A volume of 100 mL of media was autoclaved in 250 mL Erlenmeyer flasks. Each salinity level had four replicates at two selected treatment temperatures, 10˚ C and 20˚ C. A total of 56 experimental flasks were prepared and autoclaved. The media was allowed to cool for approximately 24 hours before the flasks were inoculated with *P. parvum* cells from a stock culture.

Inoculation occurred under a laminar flow hood by pipetting 0.5 mL of stock culture into each flask. The flasks were swirled and placed in 10° C and 20° C incubators. Each incubator was set at the appropriate temperature and a 12:12 hour day/night photoperiod.

Flasks were retrieved from the incubators for weekly sampling. Sterile pipettes were used to collect samples under the laminar flow hood. Samples of 5 mL were put into 20 mL scintillation vials and preserved with two to three drops of Lugol's solution. Sampling occurred on days 0, 7, 14, 21 and 28. Each sampling event occurred approximately at 11 am on sampling days.

The cell densities for all samples were determined by microscopic counting. One milliliter of sample was obtained from the sample container and a pipette into a Sedgwick-Rafter cell. Each Sedgwick-Rafter cell had a grid pattern with 1000 grids. Cells settled onto grids projecting one microliter (1 μL) of volume. Randomly selected grids were counted on the microscope at 400X magnification until at least 200 cells were enumerated. The population density in each sample was calculated as

Equation (1)

(Number of cells counted/number of grids counted) X 1000 = number of cells per mL

From population densities as calculated above, the exponential growth rate in each culture (μ) was calculated with the formula $\mu = (\ln N_t - \ln N_0)/t$ where t is the time (days), and N_t and N_0 are the cell densities on days t and 0, respectively. This calculation was done over four time scales including 7, 14, 21 and 28 days of growth.

The goal of this study was to estimate the salinity threshold for positive population growth. There was more than one reasonable way to use the data from this experiment to do so. One approach is based on an analysis of variance (ANOVA). The population growth means of the groups defined by treatment combination of the two experimental factors (salinity and temperature) were calculated, and their standard errors were estimated from the mean-square error of the ANOVA. The standard error was then used to calculate a 95% confidence interval for the mean, and mean growth for each group was deemed to be positive if zero was below the lower limit of this confidence interval. For each temperature,

the lowest salinity level supporting positive growth was taken as the estimated threshold. The advantage of this method was that it is not tied to any particular quantitative model of growth rate in relation to salinity and temperature. The disadvantage was that the estimated salinity threshold could only be equal to one of the salinity levels tested in the experiment.

In addition to providing estimates of mean growth rates and standard errors for each treatment combination, the factorial ANOVA used permits three null hypotheses to be tested concerning the two experimental factors, temperature and salinity. The null hypotheses are (1) that there is no interaction between temperature and salinity, in other words, the average effect of salinity does not depend on temperature and the average effect of temperature does not depend on salinity; (2) that mean growth rate is equal for both temperatures; (3) mean growth rate is equal for all salinities. The corresponding alternative hypotheses are (1) there is an interaction between the two treatments, so that the effect of salinity does depend on temperature and vice versa; (2) there are differences in mean growth rates between both temperatures; and (3) there is at least one difference in mean growth at different salinities.

An alternative method of estimating the salinity threshold was based on fitting a quantitative regression model relating growth rate to salinity and temperature, and identifying salinities at which the fitted growth rate passes from negative to positive. Because only two temperature levels were used in this experiment, temperature (T) was encoded as a binary dummy variable. Previous studies have suggest that growth rate is a unimodal function of salinity (Baker et al. 2007), and that there is a temperature salinity interaction (Baker et al. 2009). Therefore, a second-order regression model for growth rate (μ) was fitted:

Equation 2

$$\mu = \beta_0 + \beta_1 T + \beta_2(S\text{-}Sc) + \beta_3(S\text{-}Sc)^2 + \beta_4 T(S\text{-}Sc) + \text{error}$$

where the β_i terms are regression coefficients. To eliminate the collinearity that can arise in such a model, salinity (S) was centered by subtracting the mean salinity in the experiment (Sc). For each experimental

temperature, the salinity threshold was estimated from Equation 2 as the salinity intercept such that lower salinities produced negative growth and higher salinities produced positive growth.

We accepted estimates by this method only when the intercept in question fell within the range of experimental salinities tested, because estimates beyond this range would have been extrapolated and have high error. For each time interval, the model was fitted to obtain parameter estimates and standard errors, a test of the null hypothesis that each parameter is zero, a test of the null hypothesis that the overall model does not explain variation in growth rate, and R^2, the percentage of variance in growth rate explained by the regression model. Although individual parameters (β_i) were tested for their significance, non-significant parameters were retained because the goal of this analysis was to quantify the relationship between growth rate and temperature and salinity. Further, previous research indicated that a quadratic model of this form (Eq. 2) was appropriate for this type of analyses (Baker et al. 2007, 2009). Note that the regression model included the interaction identified as potentially important in this prior work. Choosing to retain all terms in Equation 2 also assured that growth rates over all time intervals examined were analyzed with the same regression model. The x-intercepts were calculated by inserting the values from each component of the regression model into quadratic equation.

CHAPTER 3

RESULTS

3.1 Growth Rate Analysis

Time series plots indicated that in the 10° C samples 2.0, 2.5 and 5.8 PSU all showed positive growth over the first 7 days. After seven days only 5.8 PSU had a positive growth rate. In the 20° C samples, *P. parvum* did not grow in cultures of less than 1.0 PSU. There was growth over the first three weeks at salinities greater than 1.0 PSU, with the greatest growth rates occurring in salinities greater than 2.0 PSU. Over the last week of the experiment growth rate declined in cultures with salinities of 1.0 to 2.0 PSU (Figure 1). Exponential growth rates calculated over the time scales of 7, 14, 21, and 28 days reflected the differential patterns of growth observed in the different cultures (Table 3.1 and Table 3.2).

Table 3.1: 10-degree centigrade exponential growth rates calculated over different time scales for all replicates.

Temperature (° C)	Salinity (PSU)	Time (Days)	Replicate	Replicate	Replicate	Replicate
			A	B	C	D
10	0.6	7	-0.024	-0.024	-0.005	-0.046
		14	-0.031	-0.081	-0.081	-0.076
		21	-0.181	-0.081	-0.197	-0.211
		28	-0.203	-0.046	-0.199	-0.193
10	0.8	7	-0.106	-0.121	-0.064	-0.122
		14	-0.201	-0.155	-0.145	-0.257
		21	0.208	-0.179	-0.179	-0.224
		28	-0.174	-0.127	-0.184	-0.201
10	1	7	-0.263	-0.183	-0.203	-0.163
		14	-0.045	-0.123	-0.219	-0.194
		21	-0.166	-0.099	-0.146	-0.122
		28	-0.245	-0.022	-0.182	-0.192
10	1.5	7	-0.100	0.033	-0.098	-0.141
		14	-0.161	-0.027	-0.069	-0.031
		21	-0.178	-0.074	-0.102	-0.089
		28	-0.118	-0.091	-0.139	-0.101
10	2	7	0.204	-0.005	0.084	0.0667
		14	0.091	-0.001	0.008	-0.021
		21	0.041	-0.063	-0.003	-0.088
		28	0.020	-0.080	-0.015	-0.124
10	2.5	7	0.003	-0.240	-0.197	-0.202
		14	0.051	-0.119	-0.147	-0.103
		21	0.029	-0.065	-0.039	-0.047
		28	0.018	-0.072	-0.020	-0.013
10	5.8	7	0.268	0.216	-0.004	0.223
		14	0.069	0.039	0.060	0.054
		21	0.098	0.0855	0.116	0.101
		28	0.086	0.088	0.109	0.097

Table 3.2: 20-degree centigrade exponential growth rates calculated over different time scales for all replicates.

Temperature (° C)	Salinity (PSU)	Time (Days)	Replicate	Replicate	Replicate	Replicate
			A	B	C	D
20	0.6	7	0.02	-0.001	0.111	-0.085
		14	0.266	-0.082	0.119	-0.037
		21	-0.408	-0.212	0.036	-0.139
		28	-0.139	-0.221	-0.083	-0.191
20	0.8	7	0.255	0.258	-0.012	0
		14	0.132	0.150	-0.024	0.037
		21	0.058	0.087	-0.042	-0.043
		28	-0.082	-0.035	-0.129	-0.047
20	1	7	-0.040	0.081	0.074	0.125
		14	0.133	0.095	0.129	0.147
		21	0.078	0.063	0.081	0.085
		28	-0.091	-0.092	-0.011	-0.102
20	1.5	7	-0.014	-0.019	0.041	-0.024
		14	-0.002	-0.037	-0.013	-0.047
		21	-0.026	-0.049	-0.047	-0.076
		28	-0.044	-0.053	-0.055	-0.055
20	2	7	0.316	0.364	0.324	0.320
		14	0.215	0.232	0.243	0.230
		21	0.149	0.157	0.151	0.167
		28	0.065	0.079	0.079	0.096
20	2.5	7	0.287	0.213	0.271	0.251
		14	0.254	0.251	0.206	0.154
		21	0.220	0.268	0.204	0.162
		28	0.155	0.168	0.157	0.139
20	5.8	7	0.361	0.384	0.428	0.519
		14	0.283	0.315	0.314	0.314
		21	0.225	-0.207	0.234	0.228
		28	0.170	0.160	0.175	0.166

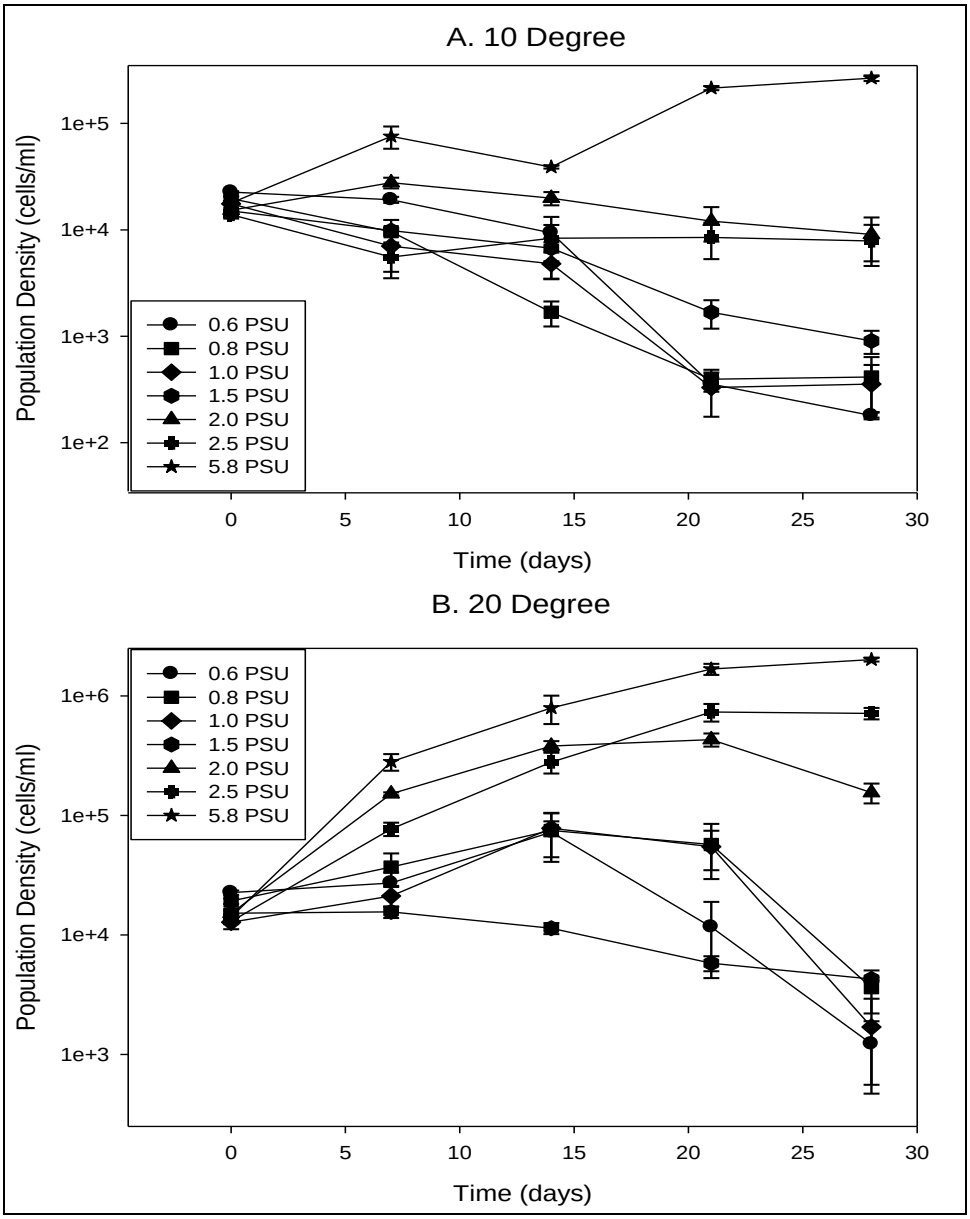

Figure 1: Dynamics of population growth in cultures of *P. parvum* incubated at (A) 10-degrees centigrade and (B) 20-degrees centigrade.

3.2 Analysis of growth over 7 days

For the ANOVA of exponential growth rates calculated over 7 days (Table 3.3), the interaction between salinity and temperature was significant ($F_{6, 42} = 4.76$, $p < 0.01$), as were the main effect of temperature ($F_{1, 42} = 94.7$, $p < 0.01$), and ($F_{6, 42} = 22.3$, $p < 0.01$). Mean 7-day growth rates were higher at 20° C than at 10° C and tended to increase with temperature (Table 3.4). Growth rates were significantly negative for three temperature-salinity combinations, all at 10° C; growth rates were not significantly different from zero for five temperature-salinity combinations; and growth rates were significantly positive for six temperature-salinity combinations.

Table 3.3: Factorial ANOVA for 7-day exponential growth rates.

	Sum of Squares	Degrees of Freedom	Mean of Squares	F	p
Temperature	0.628	1	0.628	94.7	0
Salinity	0.886	6	0.148	22.3	0
Interaction	0.189	6	0.032	4.76	0.000874
Error	0.278	42	0.00663		

Table 3.4: Means and 95% confidence intervals for 7-day exponential growth rates.

Temperature (° C)	Salinity (PSU)	Mean Growth Rate (d^{-1})	Standard Error (d^{-1})	Lower Limit (d^{-1})	Upper Limit (d^{-1})	Sign of Growth Rate
10	0.6	-0.025	0.041	-0.107	0.057	zero
10	0.8	-0.103	0.041	-0.186	-0.021	negative
10	1	-0.180	0.041	-0.262	-0.098	negative
10	1.5	-0.077	0.041	-0.159	0.005	zero
10	2	0.087	0.041	0.005	0.170	positive
10	2.5	-0.159	0.041	-0.241	-0.077	negative
10	5.8	0.176	0.041	0.0938	0.258	positive
20	0.6	0.0114	0.041	-0.071	0.094	zero
20	0.8	0.125	0.041	0.043	0.207	positive
20	1	0.060	0.041	-0.022	0.142	zero
20	1.5	-0.004	0.041	-0.086	0.078	zero
20	2	0.331	0.041	0.249	0.413	positive
20	2.5	0.256	0.041	0.173	0.338	positive
20	5.8	0.423	0.041	0.341	0.505	positive

Mean 7-day growth rates were plotted against salinity to visualize their relationship with temperature and salinity (Fig. 2). A multiple regression fitted to temperature and salinity predicted positive growth rate for the range of salinity tested when temperature was 20° C. In contrast, at 10° C, the regression model predicted positive growth only for salinity above about 2.5 PSU.

For exponential growth rates calculated over 7 days (Table 3.5), the regression indicated that there was significance for the overall model ($F_{1,54} = 25.04$, $p < 0.01$, $R^2 = 0.644$). In this time interval

(Table 3.6), it appeared that only the coefficient for temperature was significant ($t_{51} = 6.74$, $p < 0.01$). All other components of the quadratic equation were not significant all with p-values > 0.05.

Table 3.5: 7-Day overall regression statistics.

	Sums of Squares	Degrees of Freedom	Mean Squares	F	p
Regression	0.627818	1	0.627818	25.03769	0.000006
Residual	1.354046	54	0.025075		
Total	1.981865				
R^2=0.644					

Table 3.6: 7-Day regression coefficients.

Model Term	Coefficient (β)	Standard Error of (β)	t(51)	p
Intercept	**-0.245**	**0.053**	-4.62	**0**
Temperature	**0.021**	**0.003**	6.74	**0**
Center Salinity	**0.031**	**0.034**	0.900	**0.372**
Center Salinity^2	**-0.003**	**0.007**	-0.385	**0.702**
Interaction	**0.003**	**0.002**	1.37	**0.176**

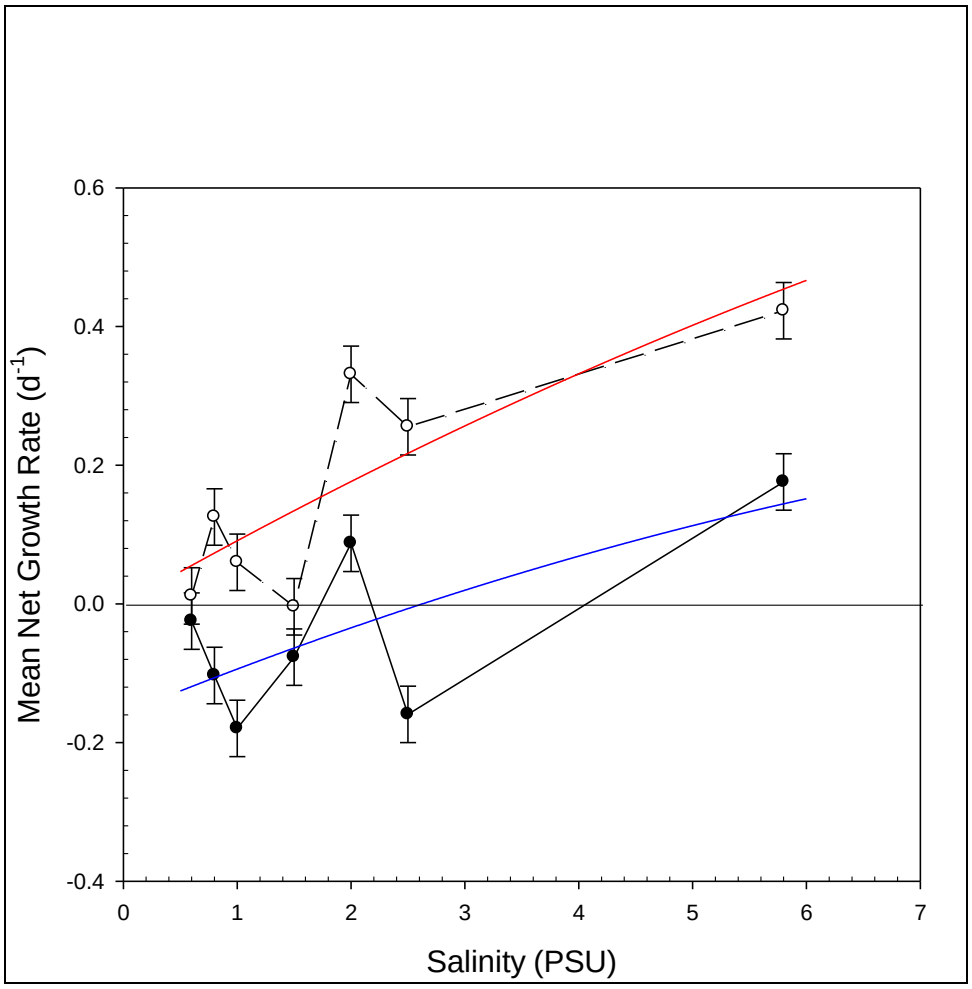

Figure 2: Mean 7-day exponential growth rates, with standard errors: solid circles – 10° C; open circles – 20° C; dotted line – fitted regression model for 10° C; chained line – fitted regression model for 20° C.

3.3 Analysis of growth over 14 days

For exponential growth rates calculated over 14 days (Table 3.7), the interaction between salinity and temperature was significant ($F_{6, 42} = 3.62$, $p < 0.01$), as were the main effects of temperature ($F_{1, 42} = 143.4$, $p < 0.01$), and salinity ($F_{6, 42} = 15.5$, $p < 0.01$). Mean 14-day growth rates were higher at 20° C than

at 10° C and tended to increase with temperature (Table 3.8). Growth rates were significantly negative for five temperature-salinity combinations, all at 10° C; growth rates were not significantly different from zero for three temperature-salinity combinations; and growth rates were significantly positive for six temperature-salinity combinations.

Table 3.7: Factorial ANOVA for 14-day exponential growth rates.

	Sum of Squares	Degrees of Freedom	Mean of Squares	F	p
Temperature	0.588	1	0.588	143.4	0
Salinity	0.382	6	0.0637	15.5	0
Interaction	0.0891	6	0.0148	3.62	0.00551
Error	0.172	42	0.00410		

Table 3.8: Means and 95% confidence intervals for 14-day exponential growth rates.

Temperature (° C)	Salinity (PSU)	Mean (d^{-1})	Standard Error (d^{-1})	Lower Limits (d^{-1})	Upper Limits (d^{-1})	Sign of Growth Rate
10	0.6	-0.067	0.032	-0.132	-0.002	negative
10	0.8	-0.190	0.032	-0.254	-0.125	negative
10	1	-0.107	0.032	-0.171	-0.042	negative
10	1.5	-0.072	0.032	-0.137	-0.007	negative
10	2	0.019	0.032	-0.045	0.084	zero
10	2.5	-0.079	0.032	-0.144	-0.015	negative
10	5.8	0.055	0.032	-0.009	0.120	zero
20	0.6	0.067	0.032	0.002	0.131	positive

Table 3.8-continued

20	0.8	0.074	0.032	0.009	0.139	positive
20	1	0.126	0.032	0.061	0.191	positive
20	1.5	-0.025	0.032	-0.090	0.040	zero
20	2	0.230	0.032	0.166	0.295	positive
20	2.5	0.216	0.032	0.152	0.281	positive
20	5.8	0.307	0.032	0.242	0.371	positive

Mean 14-day growth rates were plotted against salinity to visualize their relationship with temperature and salinity (Fig. 3). A multiple regression fitted to temperature and salinity predicted positive growth rate for the range of salinity tested when temperature was 20° C. In contrast, the regression model predicted positive growth only for salinity above about 3.0 PSU when temperature was 10° C.

For exponential growth rates calculated over 14 days (Table 3.9), the regression analysis indicated that there was significance for the overall model ($F_{1,54} = 49.32$, $p < 0.01$, $R^2 = 0.709$). In this time interval (Table 3.10), it appeared that only the temperature coefficient was significant ($t_{51} = 9.14$ $p < 0.01$). All other components of the quadratic equation were not significant all with p-values > 0.05.

Table 3.9: 14-Day overall regression statistics.

	Sums of Squares	Degrees of Freedom	Mean Squares	F	p
Regression	0.587451	1	0.587451	49.32265	0
Residual	0.64316	54	0.01191		
Total	1.230611				
R^2=0.709					

Table 3.10: 14-Day regression coefficients.

Model Term	Coefficient (β)	Standard Error. of β	t(51)	p
Intercept	**-0.251**	**0.038**	-6.63	**0**
Temperature	**0.021**	**0.002**	9.143	**0**
Center Salinity	**0.033**	**0.024**	1.36	**0.180**
Center Salinity^2	**-0.006**	**0.005**	-1.29	**0.202**
Interaction	**0.002**	**0.001**	1.16	**0.253**

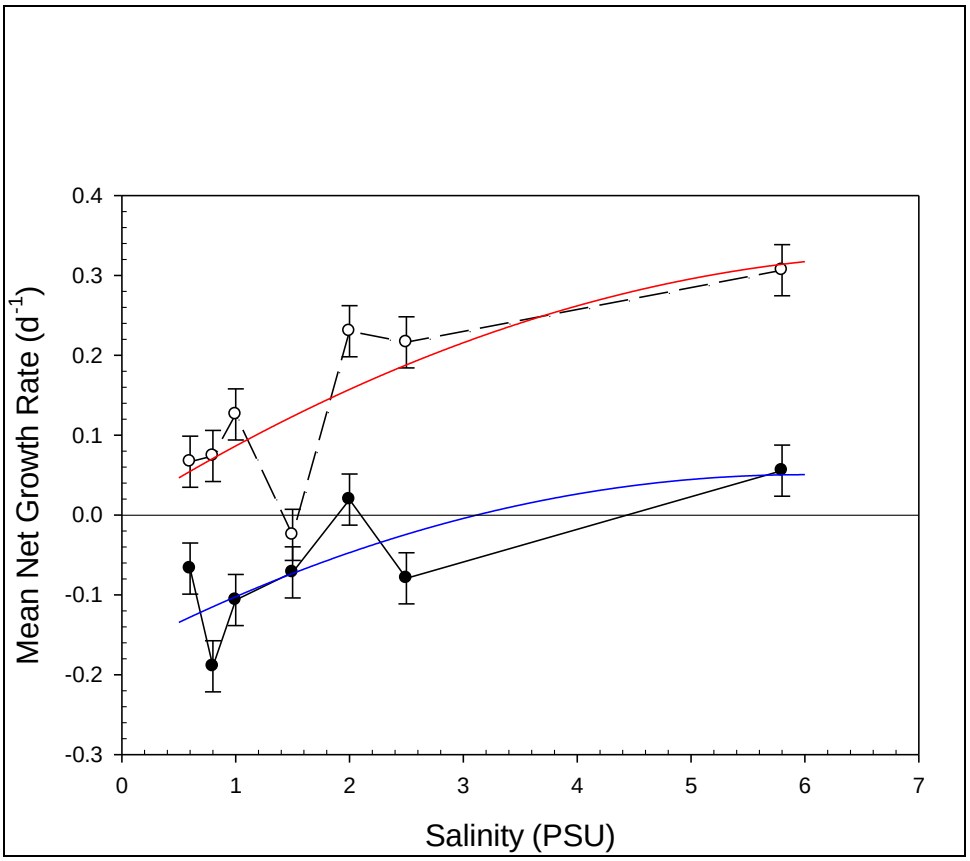

Figure 3: Mean 14-day exponential growth rates, with standard errors: solid circles – 10° C; open circles – 20° C; dotted line – fitted regression model for 10° C; chained line – fitted regression model for 20° C.

3.4 Analysis of growth over 21 days

For exponential growth rates calculated over 21 days (Table 3.11), the interaction between salinity and temperature was significant ($F_{6, 42} = 2.37$, $p < 0.01$), as were the main effects of temperature ($F_{1, 42} = 21.4$, $p < 0.01$), and salinity ($F_{6, 42} = 8.59$, $p < 0.01$). Mean 21-day growth rates were higher at 20° C than at 10° C and tended to increase with temperature (Table 3.12). Growth rates were significantly negative for four temperature-salinity combinations (i.e., 0.6, 1.0, and 1.5 PSU at 10° C and 0.6 PSU at

20° C); growth rates were not significantly different from zero for seven temperature-salinity combinations; and growth rates were significantly positive for three temperature-salinity combinations (i.e., 2.0, 2.5 and 5.8 PSU at 20° C).

Table 3.11: Factorial ANOVA for 21-day exponential growth rates.

	Sum of Squares	Degrees of Freedom	Mean of Squares	F	p
Temp	0.215	1	0.215	21.4	0.380
Salinity	0.519	6	0.0865	8.59	0.000004
Temp*Salinity	0.143	6	0.0239	2.37	0.0462
Error	0.423	42	0.0101		

Table 3.12: Means and 95% confidence intervals for 21-day exponential growth rates.

Temperature (° C)	Salinity (PSU)	Mean (d^{-1})	Standard Error (d^{-1})	Lower Limits (d^{-1})	Upper Limits(d^{-1})	Sign of Growth Rate
10	0.6	-0.167	0.050	-0.269	-0.066	negative
10	0.8	-0.094	0.050	-0.195	0.008	zero
10	1	-0.187	0.050	-0.289	-0.086	negative
10	1.5	-0.111	0.050	-0.212	-0.009	negative
10	2	-0.028	0.050	-0.129	0.073	zero
10	2.5	-0.030	0.050	-0.132	0.071	zero
10	5.8	0.100	0.050	-0.001	0.201	zero
20	0.6	-0.181	0.050	-0.282	-0.080	negative
20	0.8	0.015	0.050	-0.087	0.116	zero
20	1	0.077	0.050	-0.024	0.178	zero

Table 3.12-continued

20	1.5	-0.050	0.050	-0.151	0.052	zero
20	2	0.156	0.050	0.055	0.257	positive
20	2.5	0.214	0.050	0.112	0.315	positive
20	5.8	0.120	0.050	0.019	0.221	positive

Mean 21-day growth rates were plotted against salinity to visualize their relationship with temperature and salinity (Fig. 4). A multiple regression fitted to temperature and salinity predicted positive growth rate for the range of salinity tested when temperature was 20° C. In contrast, the regression model predicted positive growth only for salinity above about 2.5 PSU.

For exponential growth rates calculated over 21 days (Table 3.13), regression analysis indicated that there was significance for the overall model ($F_{1,54}$ = 10.71, $p < 0.01$, R^2 = 0.499). In this time interval, the coefficient for temperature was significant (t_{51} = 4.10, $p < 0.01$). The quadratic coefficient for salinity was significant (t_{51} = -3.16, $p < 0.01$) (Table 3.14). All other components of the quadratic equation were not significant all with p-values > 0.05.

Table 3.13: 21-Day overall regression statistics.

	Sums of Squares	Degrees of Freedom	Mean Squares	F	p
Regress.	0.215101	1	0.215101	10.70537	0.001865
Residual	1.085013	54	0.020093		
Total	1.300115				
R^2=0.499					

Table 3.14: 21-day regression coefficients.

Model Term	Coefficient (β)	Standard Error of (β)	t(51)	p
Intercept	**-0.142**	**0.051**	-2.78	**0.008**
Temperature	**0.012**	**0.003**	4.10	**0**
Center Salinity	**0.112**	**0.033**	3.42	**0. 4**
Center Salinity^2	**-0.020**	**0.006**	-3.16	**0.003**
Interaction	**-0.001**	**0.002**	-0.663	**0.510**

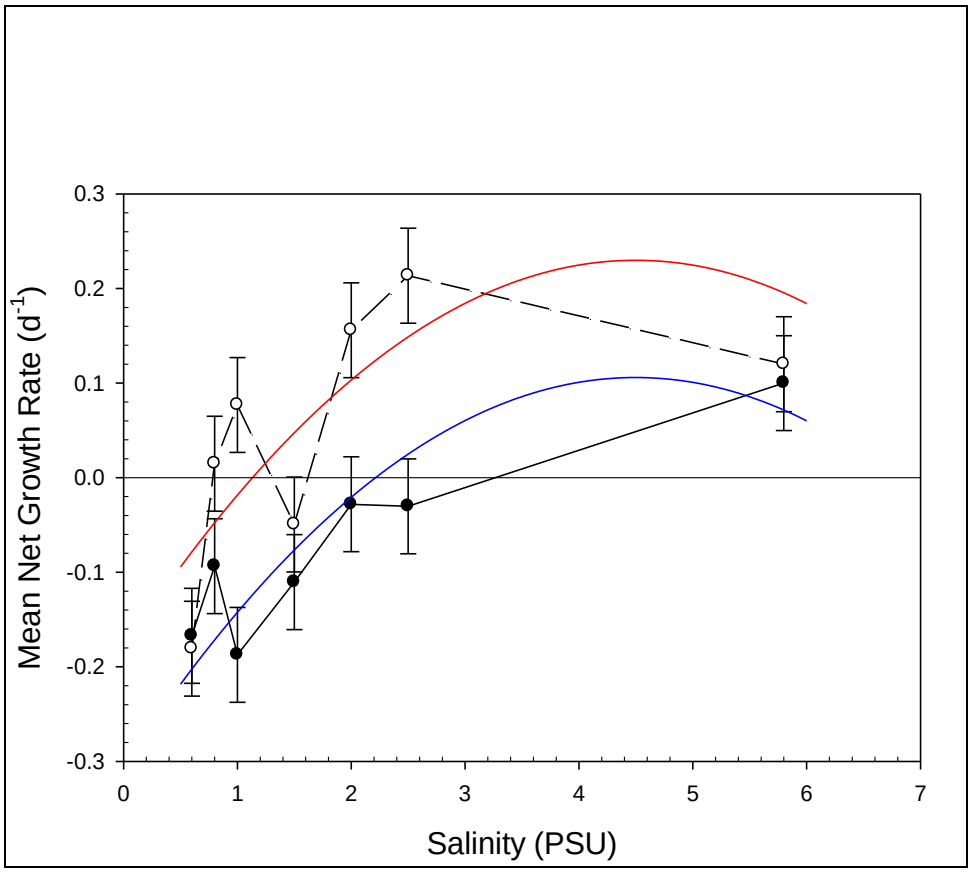

Figure 4: Mean 21-day exponential growth rates, with standard errors: solid circles – 10° C; open circles – 20° C; dotted line – fitted regression model for 10° C; chained line – fitted regression model for 20° C.

3.5 Analysis of growth over 28 days

For exponential growth rates calculated over 28 days (Table 3.15), the interaction between salinity and temperature was significant ($F_{6, 42} = 3.87$, $p < 0.01$), as were the main effects of temperature ($F_{1, 42} = 73.7$, $p < 0.01$), and salinity ($F_{6, 42} = 62.8$, $p < 0.01$). Mean 28-day growth rates were higher at 20° C than at 10° C and tended to increase with temperature (Table 3.16). Growth rates were significantly negative for nine temperature-salinity combinations (i.e., 0.6, 0.8, 1.0, 1.5 and 2.0 PSU at 10° C and 0.6, 0.8, 1.0, and 1.5 PSU at 20° C); growth rates were not significantly different from zero for one temperature-salinity combination (i.e., 2.5 PSU at 20° C); and were significantly positive at 2.0, 2.5, and 5.8 PSU at 20° C (Table 3.16).

Table 3.15: Factorial ANOVA for 28-day exponential growth rates.

	Sum of Squares	Degrees of Freedom	Mean of Squares	F	p
Temp	0.114	1	0.114	73.7	0
Salinity Level	0.585	6	0.0974	62.8	0
Temp*Salinity	0.0361	6	0.00601	3.87	0.00365
Error	0.0652	42	0.00155		

Table 3.16: Means and 95% confidence intervals for 28-day exponential growth rates.

Temperature (°C)	Salinity (PSU)	Mean (d^{-1})	Standard Error (d^{-1})	Lower Limits (d^{-1})	Upper Limits (d^{-1})	Sign of Growth Rate
10	0.6	-0.161	0.0197	-0.200	-0.121	negative
10	0.8	-0.172	0.0197	-0.211	-0.132	negative
10	1	-0.168	0.0197	-0.208	-0.128	negative
10	1.5	-0.112	0.0197	-0.152	-0.073	negative

Table 3.16 continued

10	2	-0.050	0.0197	-0.089	-0.010	negative
10	2.5	-0.022	0.0197	-0.062	0.0180	zero
10	5.8	0.095	0.0197	0.055	0.135	positive
20	0.6	-0.159	0.0197	-0.198	-0.119	negative
20	0.8	-0.073	0.0197	-0.113	-0.033	negative
20	1	-0.074	0.0197	-0.114	-0.034	negative
20	1.5	-0.052	0.0197	-0.092	-0.012	negative
20	2	0.080	0.0197	0.040	0.119	positive
20	2.5	0.155	0.0197	0.115	0.194	positive
20	5.8	0.168	0.0197	0.128	0.208	positive

Mean 28-day growth rates were plotted against salinity to visualize their relationship with temperature and salinity (Fig. 5). A multiple regression fitted to temperature and salinity predicted positive growth rate for the range of salinities tested when temperature was 20° C. In contrast, the regression model predicted positive growth only for salinity above about 2.5 PSU.

For exponential growth rates calculated over 28 days (Table 3.17), regression analysis indicated that there was significance for the overall model ($F_{1,54} = 9.01$, $p < 0.01$, $R^2 = 0.856$). In this time interval (Table 3.18), it appeared that the coefficients were significant ($p < 0.01$), except for the interaction of temperature and salinity ($p > 0.05$).

Table 3.17: 28-day overall regression statistics.

	Sums of Squares	Degrees of Freedom	Mean Squares	F	p
Regress.	0.114408	1	0.114408	9.008584	0.004062
Residual	0.685794	54	0.0127		
Total	0.800202				
R^2=0.856					

Table 3.18: 28-day regression coefficients.

Model Term	Coefficient (β)	Standard Error of (β)	t(51)	p
Intercept	**-0.126**	**0.021**	-5.90	**0**
Temperature	**0.009**	**0.001**	7.12	**0**
Center Salinity	**0.091**	**0.014**	6.56	**0**
Center Salinity^2	**-0.017**	**0.003**	-6.45	**0**
Interaction	**0**	**0**	0.692	**0.492**

Overall the regression model for the growth rates of *P. parvum* shows significance for temperature where all p-values < 0.01 and for some components of the quadratic formula. However, the interaction was expected to be significant, but in each quadratic calculation the p-values were > 0.01.

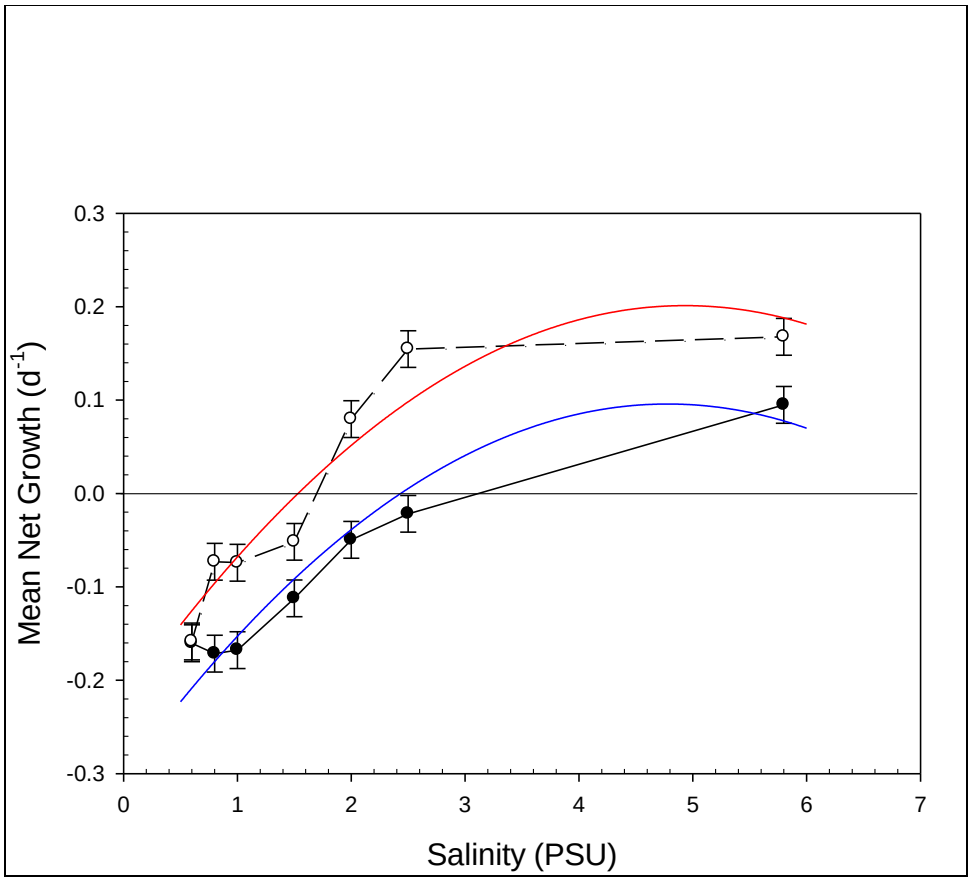

Figure 5: Mean 28-day exponential growth rates, with standard errors: solid circles – 10° C; open circles – 20° C; dotted line – fitted regression model for 10° C; chained line – fitted regression model for 20° C.

3.6 Summary of analyses and salinity thresholds

The ANOVA and regression analyses provided somewhat different perspectives on the results of this experiment, and lead to different estimates of the lower salinity threshold for growth of *P. parvum*. The hypothesis that there was an interaction between temperature and salinity can be evaluated by looking at the p values for the interaction terms in the ANOVA for each time scale examined. For 7, 14, 21, and 28 days the p-values are 0.000874, 0.00551, 0.0462, and 0.00365 respectively. With each p-value

less than the $p \leq 0.01$, these analyses indicated that there was an interaction between the two treatments of temperature and salinity. In contrast, the regression analyses did not result in significant interaction coefficients for any of the time scales examined. The regression analysis was more constrained than the ANOVA, and could only detect interactions that are consistent with the second-order model (Equation 2). The ANOVA could detect a wider variety of patterns of interaction.

The term threshold can be assigned to the lowest salinity at which positive growth was observed at a given temperature. From the ANOVA's thresholds may be assigned as the lowest salinity treatment for which a 95% confidence interval was significantly positive, and from the regressions thresholds may be assigned as the point where a fitted regression line crossed the salinity axis. Different thresholds may also be assigned for the different time intervals over which growth rate was calculated.

Although the two different analyses provided threshold estimates for most treatment combinations (Table 3.19), there were circumstances under with they also failed to provide an estimate. For estimates based on ANOVA and the 95% confidence interval, such failure occurred at 10° C when significantly positive growth was not observed at any salinity for growth over 14 and 31 day time scales. For estimates based on regression analysis, estimation of a threshold failed when the fitted model was positive at all tested salinities, so that a salinity intercept occurred outside of the range of tested salinities. This occurred at 20° C for growth over seven and 14 day time scales. For conditions where threshold estimates were obtained, those estimated from the ANOVA method ranged widely, from 0.6 to 5.8 PSU, while those obtained from regression were more constrained, ranging from about 1 to 3 PSU.

Table 3.19: Summary of thresholds from ANOVA and regression analyses.

Time Scale (Days)	Temperature (°C)	ANOVA Threshold (PSU)	Regression Threshold (PSU)
7-Day	10	2.0	2.61
7-Day	20	0.8	Out of Range
14-Day	10	Out of Range	3.13
14-Day	20	0.6	Out of Range
21-Day	10	Out of Range	2.22
21-Day	20	2.0	1.12
28-Day	10	5.8	2.40
28-Day	20	2.0	1.54

CHAPTER 4

DISCUSSION

The purpose of this research was to attempt to identify the salinity threshold for the harmful alga *Prymnesium parvum*. This species of algae is responsible for large fish kills in Texas and West Virginia. Previous field studies (Roelke et al. 2009) suggested that the salinity threshold in some Texas lakes may be as low as 0.6 PSU up to 1.5 PSU. Here we looked at the growth rates of this alga at two temperatures and at multiple salinities over the course of 28 days, because *P. parvum* is a relatively slow growing alga. Within this time frame, shorter time scales of growth were examined, because prior laboratory studies (e.g. Baker et al. 2007, 2009) used a range of mostly shorter time scales for quantitative analysis of population growth. Salinities in this study ranged from 0.6 PSU to 5.8 PSU, covering the typical ranges of brackish inland waters in Texas.

We employed two different methods of analysis for estimating salinity thresholds for growth from the experimental data. Each method provided a different perspective on the data, and led to different results. Although the ANOVA method was simple in principle, and did not assume any particular quantitative relationship between salinity, temperature and growth rate, it led to highly variable estimated thresholds. The thresholds estimated by this method could only include the tested values of salinity, and in fact covered the range of values tested, from 0.6 to 5.8 PSU. In contrast, at the expense of assuming a second-order quantitative relationship, the regression method provided estimates that ranged about 1 to 3 PSU.

In field studies, it appears that in Texas lakes where blooms from *P. parvum* have occurred, the salinity varied between lakes. In Lake Possum Kingdom the lowest salinity permitting growth was > 1.5 PSU, whereas in Lake Granbury the salinity was > 0.5 PSU (Roelke et al. 2011). The thresholds obtained from regression analysis in this experiment appear to match the field observations better than those obtained by the ANOVA method, especially when longer time scales of 21 to 28 days were considered. At 20° C, the salinity threshold estimated by regression appeared to be between about 1.0 to 1.5 PSU and at 10° C, between about 2.2 and 3.1 PSU. The growth rates observed in this experiment can be compared to previous laboratory experiments using similar methods (Baker et al. 2007, 2009). These studies developed predictive regression models whose predictions can be compared to the observed growth rates in this study. The prediction formulas from Baker et al. (2007) was

Equation (3)

$$\mu = 0.061749 + 0.00694\ (S - 17.8) + 0.860\ \exp[1.87\ (T - 20 / 20)] + 0.000611\ (E - 222) - 0.00080\ (S - 17.8)^2 - 0.218\ \exp[3.74(T - 20 / 20)] - 0.00000573\ (E - 222)^2$$

where μ is the growth rate; exp is the exponential function; S is the salinity in PSU; T is the temperature in degrees C; and E is the irradiance (μmol photons $\times$ m^{-2} s^{-1}). For these experiments, E = 150 μmol photons m^{-2} s^{-1}.

The formula from Baker et al. 2009 was:

Equation (4)

$$\mu = -3.531 + 0.02534\ (S - 1.833) - 0.06311\ (S - 1.833)^2 + 7.468\ \exp[0.7\ (T - 20 / 20)] - 3.414\ \exp[1.4\ (T - 20 / 20)] + 0.1697\ (S - 1.833)\ \exp[0.7\ (T - 20 / 20)]$$

where μ is the growth rate; T is the temperature in degrees C; and S is the salinity in (PSU). Both studies used short periods of 7-10 days to calculate exponential growth rates, with frequent sampling over these times and did not use many low salinity treatments. Mostly the salinities used in the prior work were ≥

2.0 PSU. In contrast, the experiment reported here was sampled weekly over the course of 4 weeks and several tested salinities were well below 2.0 PSU.

When plotted against predictions from previous regression models, the observed growth rates from this experiment should fall along a 1-to-1 line if the predictions are successful. If the observed growth rates do not fall along this line, the two possibilities are either variance in the observations, indicated by points that are spread out from the line, or bias in the predictions, indicated by points falling mostly above or below the line. In the following figures observations from each of the temperatures and time scales of growth are plotted separately.

For observations at 10° C compared to the predicted values of Baker et al. (2007) over the 7-day time scale (Figure 6A) there is high variance of the data points around the 1-to-1 line, but little indication of consistent bias. In contrast, over longer time scales (Figure 6 B-D) the data points show less variance and are closer to the line, but there are indications of bias at the lower range of growth rates. Also at the highest salinity of 5.8 PSU the predicted values and the experimental values are very close. However, there does also appear to be high variance in the 0.8 PSU samples at the 21-day time interval.

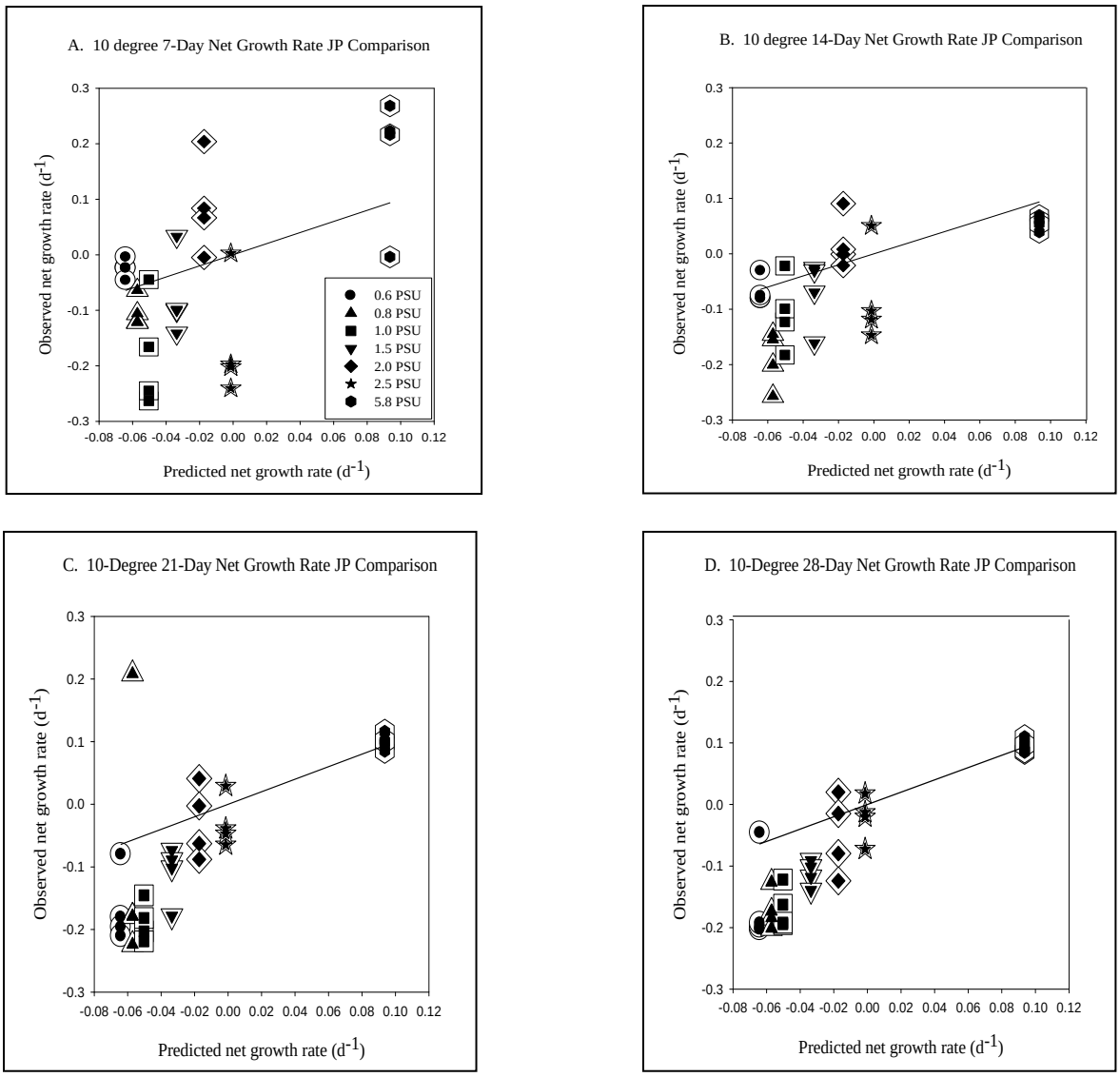

Figure 6: Comparison of observed growth rates over each time interval and for each level of salinity, at 10° C, using prediction from the regression model of Baker et al. (2007).

The predictive model formulated by Baker et al. 2009, was also compared to observed growth rates from this experiment. At 10° C and for all time scales of growth there was a large amount of bias at the lower

range of predicted growth rates, accompanied with high variance and bias at the higher predicted growth rates (Figure 7).

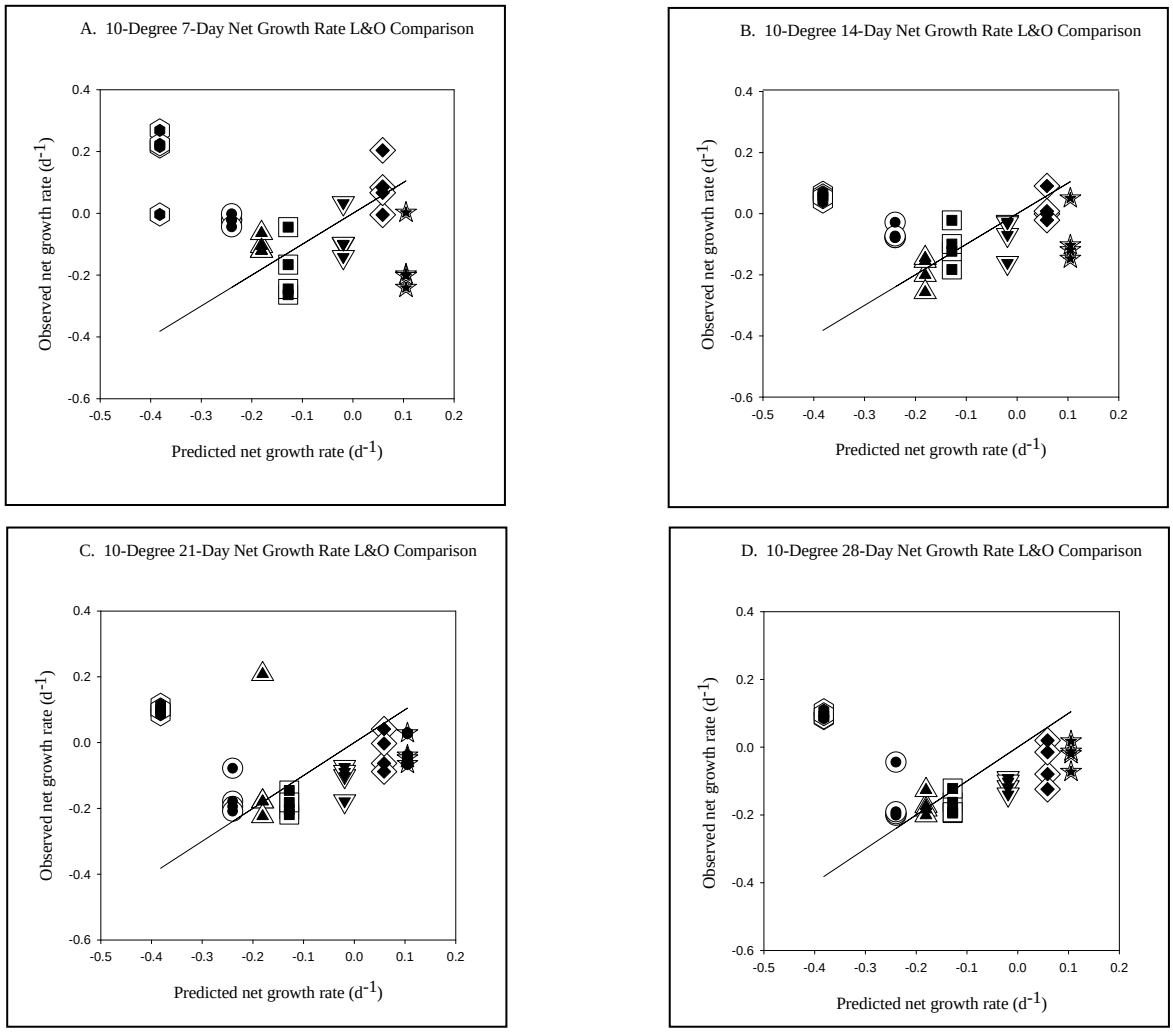

Figure 7: Comparison of observed growth rates over each time interval and for each level of salinity, at 10° C, using prediction from the regression model of Baker et al. (2009).

For observations at 20°C compared to the predictive models of Baker et al. (2007), there was both high variance and high bias at the lower range of predicted growth rates (Figure 8A). Bias was especially

high for the time scales of growth greater than seven days, for which the majority of the experimental data fell below the 1-to-1 line of the predictive models (Figures 8 B-D).

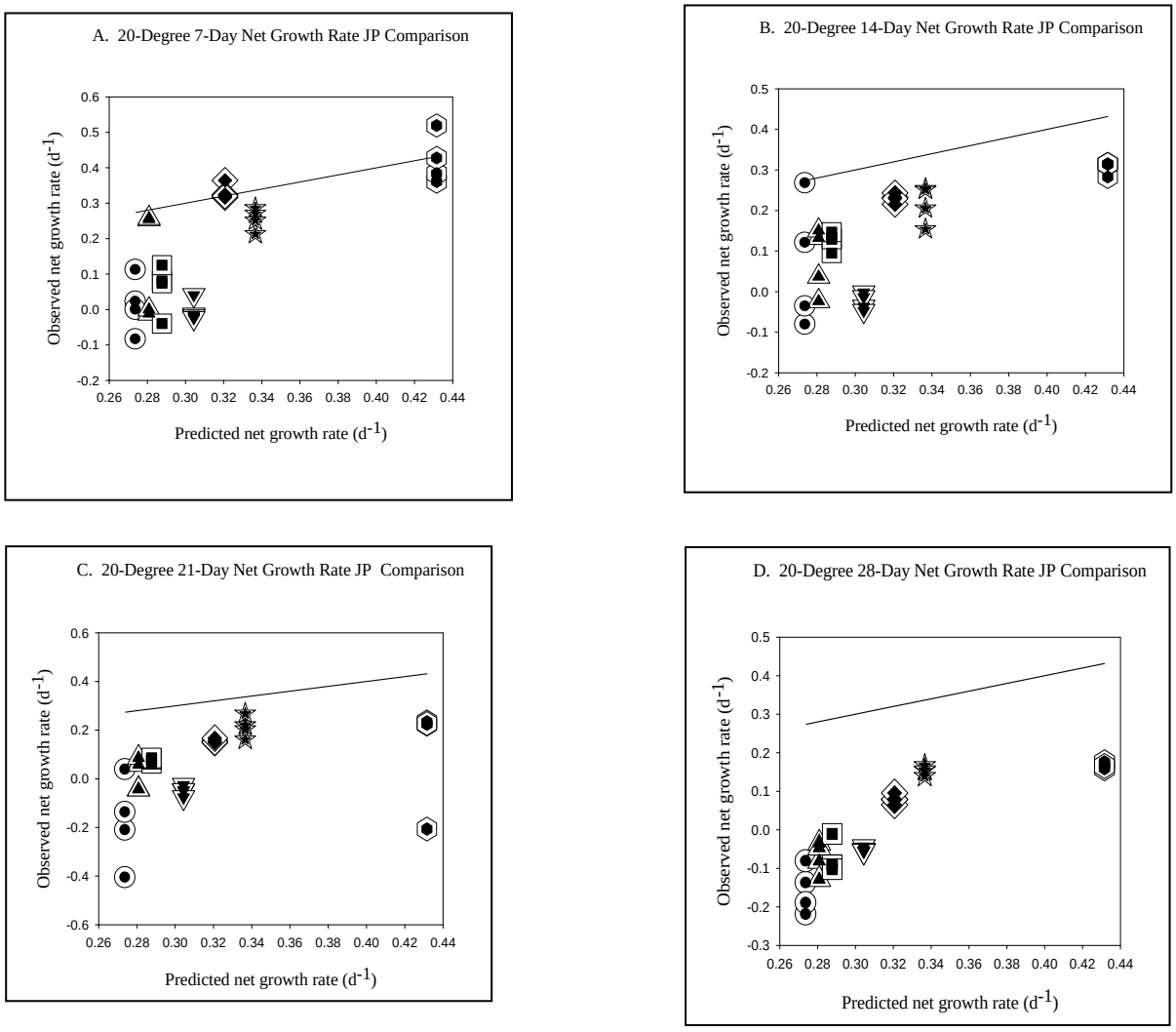

Figure 8: Comparison of observed growth rates over each time interval and for each level of salinity, at 20° C, using prediction from the regression model of Baker et al. (2007).

For observations at 20° C compared to the predictions of the Baker et al. (2009) model, once again there appeared to be bias at most of the salinity levels, however the 5.8 salinity had some variance in the first seven days (Figure 9A). Again the experimental data fell below the predicted values of the model (Figure 9B-D).

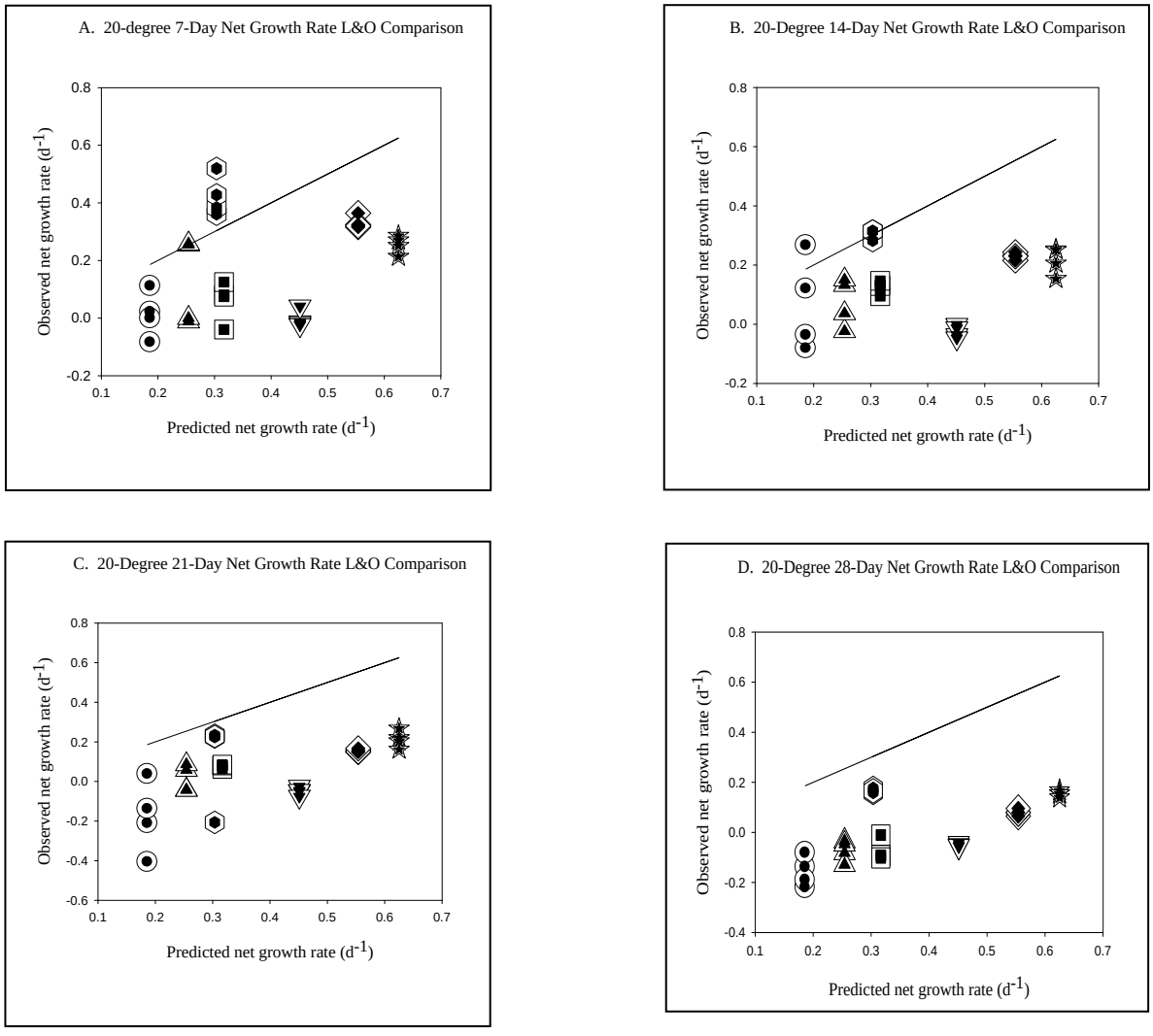

Figure 9: Comparison of observed growth rates over each time interval and for each level of salinity, at 20° C, using prediction from the regression model of Baker et al. (2009).

The regression model formulated by Baker et al. 2009 has become an accepted model for predicting the growth of *P. parvum*. For example, it has been incorporated into simulation model of its dynamics in Texas reservoirs (Grover et al. 2010, 2012). However, it appears that the model may make biased predictions. Although there was high variance of experimental data around the predictions of the earlier model of Baker et al. (2007), there was less bias of its predictions.

There is disagreement among laboratory estimates of the growth rate of *P. parvum*, which is reflected in the error of the predictive models of Baker et al. (2007, 2009). Nevertheless, regression models of growth rate over longer time scales (21-28 days) estimate salinity thresholds for positive growth that are broadly consistent with field observations in Texas, indicative of thresholds of about 1-2 PSU.

With the spread of *P. parvum* across Texas and more recently West Virginia, the study of harmful effects and growth potential of this alga need to be considered further. It would be instructive to conduct field studies in regions such as the vicinity of Dunkard Creek, West Virginia, which differs greatly in climate and background salinity from the Texas region invaded by *P. parvum.* Available information indicates that to prevent further blooms, the salinity of inland water systems should be kept below about 1.0 PSU. However, the broader applicability of such criterion needs to be evaluated.

With the above mentioned caveats in mind, especially those in West Virginia and Pennsylvania where harmful algal blooms from *P. parvum* are now of concern, implications and policy become important. This is where science and watershed management can come together and forge policy change in how freshwater systems that are susceptible to salinization can create a policy on how to manage the salinity levels in vulnerable lakes and streams. These water bodies can be divided into three zones, for example a red, yellow and green zone. The red zones would be areas where salinity levels are already at or above 2.0 PSU, which from previous studies as well as this study determines that *P. parvum* can grow

and form a bloom. Salinity changes in these red zones would be highly susceptible to toxic blooms. These areas are typically found in the semiarid to arid south central and southwestern United States. A yellow zone would be in an area where the salinity levels are those between 1.0 and 2.0 PSU. Such water bodies can be found throughout the regions of the U.S. This zone is susceptible to bloom formation, but no blooms have occurred as of yet. The third region would be the green zone where water bodies are below 1.0 PSU. These areas are typical of water bodies in the relatively humid eastern and northwestern United States. These freshwater bodies are typically below the 1.0 PSU level and no blooms have occurred. However, with natural and human impacts where salinization may occur, management officials can monitor salinity levels and attempt to keep the water bodies below 1.0 PSU. Collaboration between science and management is an attainable goal to help prevent the spread of harmful algal blooms.

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BIOGRAPHICAL INFORMATION

James W. Cody Black is a student and husband who worked a full-time job throughout his undergraduate and graduate careers. Driven by childhood memories of fishing on the San Juan River in northwestern New Mexico, a passion for aquatic ecology was instilled in him at a very young age. In 2008, he earned his Bachelor of Science Degree in Biology with an emphasis on the environment and ecology. As an undergraduate he took a course in Environmental Biological Aspects. The professor for this course researches a species of algae that releases toxins into Texas lakes and causes large fish kills. An interest in harmful algal blooms arose and he became an undergraduate research assistant. In 2009, The State of West Virginia experienced it's very first toxic bloom of *Prymnesium parvum* and a large number of fish were killed. This led Cody to work on his Master's of Science in Environmental and Earth Science with an emphasis on Aquatic Ecology and performed his research in the same professor's lab. During his graduate career, he has had the opportunity to take several field courses at Michigan State University's Kellogg Biological Station. At the end of his academic career, he hopes to work as an aquatic ecologist or fisheries biologist.