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Establishing environmental variability in the toxins produced by a single strain of prymnesium parvum

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ESTABLISHING ENVIRONMENTAL VARIABILITY IN THE TOXINS PRODUCED BY A SINGLE STRAIN OF *PRYMNESIUM PARVUM*

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APPROVAL OF THESIS

We, the faculty supervising the work of Amy Parsons-White, affirm that the thesis, *Establishing Environmental Variability In The Toxins Produced By A Single Strain Of Prymnesium Parvum*, meets the high academic standards for original scholarship and creative work established by the Department of Biological Sciences and the College of Science. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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ABSTRACT

Prymnesium parvum (*P. parvum*) is a toxin producing haptophyte that has spread globally over the past 100 years. The algae, initially a marine organism, has moved inland to less saline waters. In addition, there is variability in the efficacy of the toxins produced from bloom to bloom. Variation between strains has been suggested as the reason for bloom differences; however, environmental variability within a single strain must first be established. Here, a single strain of *P. parvum* was cultured at three environmentally relevant salinities, 6psu, 13psu, and 35psu. Three different environmental shocks, decreased temperature, raised pH, and addition of specific ions were applied to cultures of alga at each of the three salinities. Supernatant from each condition was used to test variability in hemolytic, cytolytic, and ichthyiotoxic properties. Culture salinity influenced growth rate of the algae, with increased growth rates at higher, marine salinities. Ichthyotoxicity of toxins produced were also increased with increased culture salinity. Cytolytic and hemolytic properties were found to vary with culture salinity; however, these properties were greatest at a culture salinity of 13psu. Environmental shocks also caused significant variability in efficacy of toxins produced. These tests demonstrate variability in toxic effect within a single strain of *P. parvum* and provide insight into the wide array of toxic effect in blooms worldwide.

CHAPTER 1

INTRODUCTION

Harmful Algal Blooms

Algae occur naturally in both marine and fresh water systems. Under certain conditions, algae have the ability to multiply quickly causing harmful algal blooms (HABs). HABs are a global phenomenon that effect nearly every coastal nation (Hallegraeff, 1993). Over the past three decades, the frequency of HABs have increased dramatically. Though the frequency and severity of HABs has increased, there is an alarming lack of knowledge as to the mechanisms driving these blooms and the variability of strains within a species (Wells, Trainer, Smayda, Karlson,Trick, & Kudela, 2015).

In general, there are three types of HABs. These HABs are classified by their effect to the water and organisms that come in contact with them (Anderson, 1998). They are:

1) Non-toxic blooms that involve single cell algae in a closed water system. They multiply quickly and often discolor the water or leave it anoxic.
2) Toxic blooms that are in both open and closed water systems and are toxic to both humans and wildlife. These blooms consist of algae that produce a potent toxin that can bioaccumulate in fish and other aquatic organisms and enter the food chain. These toxins can cause detrimental health effects or death to those that ingest affected water and/or aquatic organisms.
3) Toxic blooms in open or closed water systems that are toxic only to aquatic organisms and require gills for oxygen exchange.

HABs occur when microscopic algae quickly grow to such numbers that they cause harm due to endogenous toxin production or by simply rendering the water anoxic. Algal blooms have been correlated to changes in temperature, nutrients, or salinity, but biological mechanisms concerning toxicity and exponential growth are poorly understood (Vitousek et al., 1997).

In recent years, there has been an increase in inland freshwater HABs. Though many of these blooms are due to cyanobacteria, or blue-green algae, there are marine algal species that have successfully adapted to inland waters (Paerl et al., 2016). Some species, typically found in brackish waters, are able to survive in inland waters due to an increase in nutrients, salinity, and temperature due to anthropogenic activities and an increase in global temperature leading to evaporation (Vitousek et al., 1997). These invasive species are a growing problem as they have few, if any, natural predators. One species in particular, *Prymnesium parvum,* has not only adapted to inland brackish waters, but has been found in lower salinity rivers, lakes, and ponds (Brooks et al., 2010).

Prymnesium parvum

Prymnesium parvum (*P. parvum*), also known as golden algae, is a toxin producing Haptophyte that has caused massive fish kills around the world. It is considered one of the most harmful algal species in the world for finfish and other gill breathing organisms (Lutz-Carrillo, Southard, & Fries, 2010). In the United States, the alga is known to occur in 16 states. In the state of Texas, P. parvum has had significant ecological and monetary impacts with 17.5 million fish killed with an estimated value of seven million dollars between 1985 and 2003 (Barkoh & Fries, 2005). Recently, a bloom on the border of West Virginia and Pennsylvania killed an estimated 42,997 fish, 15,382 freshwater mussels and 6,447 mudpuppies (Brooks, Grover, & Roelke, 2011). This was the northern most, and most inland toxic bloom to occur in the United

States, which may suggest that the alga is adapting to less brackish waters. This adaptation could also have an effect on how the alga responds to its environment and on the toxins produced (Driscoll, Espinosa, Eldakar, & Hackett, 2013).

Historically, *P. parvum* blooms have occurred in cooler, brackish waters located in temperate and sub-tropic zones. Recently, blooms have shown the euryhaline organism to be moving inland into warmer freshwater reservoirs (Baker et al., 2009) with the range of tolerable conditions still undefined. Despite significant losses worldwide, little is known about the triggers and mechanisms of the toxic compounds produced by the organism. At least seven secondary metabolites with variable toxicity have been identified with the presence of additional toxins being likely (Bertin, Zimba, Beauchesne, Huncik, & Moeller, 2012a). Factors contributing to organism growth and toxicity are characterized by variability, likely due to the fact that different toxins may have been present in literature descriptions of blooms over many years and covering large geographic areas (Manning & La Claire, 2010a). Factors driving the selection of synthesis of one toxin over another are also unknown. While many researchers attribute variability to different genetic strains and to environmental conditions, the actual inherent variability in clonal cultures is not characterized, particularly with respect to the cellular selection of toxin synthesis. The variability inherent in a single organism (clonal culture) needs to be evaluated with respect to the multiple toxins produced before we can attribute variability to genetic diversity. The development of techniques for management and control of the organism are hindered by the lack of understanding of the organism's responses to environmental variables.

Morphology

P. parvum is a single celled alga ranging from 8-12um in length (Lee, 1980). Their shape can be either round or oblong and tend to change according to the abiotic factors of their environment. They have two long flagella ranging in length form 12-15um (Fig. 1), and are used for motility. *P. parvum* is a haptophyte, and therefore has a haptonema that can range from 3-5um in length located between the two flagella (Green, Hibberd, & Pienaar, 1982). Unlike the flagella, the haptonema is slightly more rigid due to its arrangement of microtubules, but is still flexible. The haptonema is used by the alga for sexual reproduction, as well as, phagocytosis (Mclaughlin 1958; Prescott 1968; Tillmann 1998). Because *P. parvum* are mixotrophic, they also have two large chloroplasts located on either side of the midline. They are golden yellow color, giving the alga its nickname golden algae.

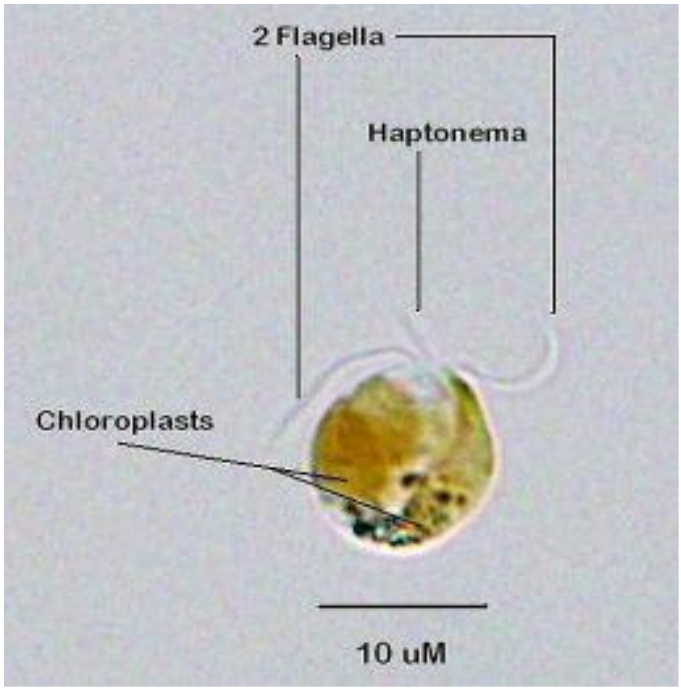


Figure 1. Morphological characteristics of *Prymnesium parvum*
Image used with permission from Texas parks and wildlife.

Toxicity

Until recently, little was known about the alga and the toxin that it produces. Previous studies have characterized two prymnesin toxins (Igarashi, Satake, & Yasumoto, 1996), (Fig.2) while recent studies have shown that a suite of seven fatty acid amides were released during a single toxic bloom (Bertin et al., 2012a). It is known that growth conditions affect the synthesis of secondary metabolites (Manning & La Claire, 2010b), implying that environmental conditions could account for the presence of one *P. parvum* toxin over another when toxicity is induced in clonal culture.

An increase in nitrogen and phosphorous in streams due to agricultural runoff in Texas has a negative impact on the growth of *P. parvum*, but seems to increase the toxicity and feeding

frequency of the organism (Larsen, Bryant, & Båmstedt, 1998.). In contrast, lower nitrogen and phosphorous levels have been shown to decrease *P. parvum* feeding frequency while allowing the alga to grow larger (Carvalho & Granéli, 2010).

The broad osmotic tolerance of *P. parvum* is well documented (Larsen et al., 1998). The algae are found in fresh waters with a salinity of 5ppt as well as more brackish and saline waters with a salinity of 45 ppt (Larsen et al., 1998). This wide range of tolerance may be explained by highly expressed gene transcripts encoding ion transporters (La Claire 2006). The salinity of the water has been shown to have an effect on the type of toxicity exhibited by the alga. For instance, ichthyotoxic properties were found to be inversely related to hemolysis at a salinity of 10psu, while at a salinity of 30psu toxins produced had nearly equivalent ichthyotoxic and hemolytic properties (Shilo & Aschner, 1953).

Temperature and pH have been shown to affect the toxicity of the organism as well. A study by Valenti et al. (2010) showed that lethality of the toxin produced to *Daphnia pulex*, an arthropod, as significantly higher in media with a pH of 8.5 as opposed to media with a pH of 7.5 and 6.5. In the same experiment, when divalent cation concentration was increased, each media became much more toxic with the highest level of toxicity remaining at a pH of 8.5. When cultured under varying conditions, Manning and La Claire (2010b) found that cell swelling and lysis could take less than an hour, or may take several hours, depending on the temperature and pH of the culture. Elevated pH and temperature were also found to be related to an increase in cell lysis resulting in an increase in prymnesin toxin in the culture media. It is important to note that, in this same study, Manning and LaClaire also found that the process of cell swelling could be halted by reversing the culturing conditions. Lysis of *P. parvum* cells only occurs under

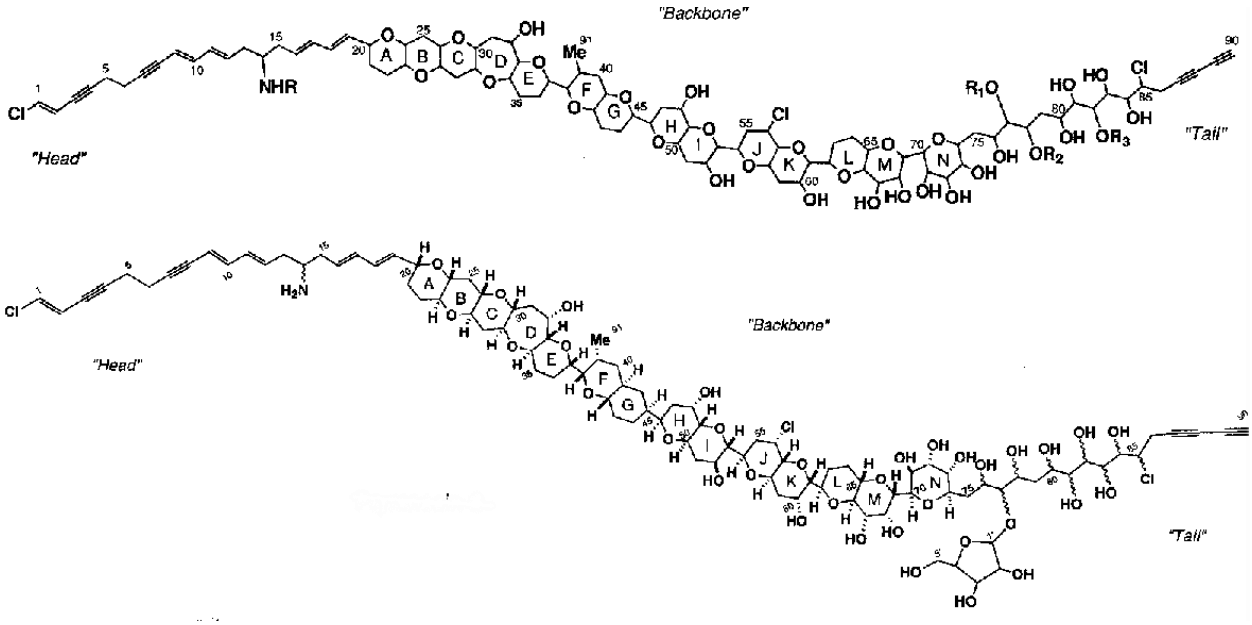
extreme conditions to release both endo and exotoxins which are found to be far more potent. Cells will typically release toxins for protection or food, without harm to themselves. These tests show a correlation between abiotic factors and the potency of the toxin produced by *P. parvum*.

What is not known is whether the same suite of toxins are produced during every toxic event, or if there are factors, biotic or abiotic, that drive the selection or production of one toxin over another. In other words, if multiple toxin templates are available for *P. parvum* to synthesize, are the factors leading to the prevalence of specific toxins genetically selected with variability between strains? Is it that the algae are environmentally selected at the time of growth, or during the toxin release phase? Or, are toxins produced due to a combination of both genetics and environmental circumstances?

Within the last two decades, *P. parvum* has spread into less brackish inland water (Baker et al., 2007). Golden algae blooms have left a trail of ecological devastation, which are threatening the economic and recreational value of freshwater systems throughout the United States (Henrikson et al., 2010). In order to understand the alga and its potential for toxicity, scientists are studying the toxin produced by different strains as well as the toxin produced under different environmental, or abiotic factors (M. Shilo, 1981). It is widely accepted that a variety of biotic and abiotic factors affect *P. parvum* blooms in a variety of ways including growth rate, potency of toxins produced, and survival. Variability in toxicity, indicated by organism response, has been attributed to multiple environmental differences including nutrient availability, salinity, pH, water hardness, divalent ion concentration (including metals), light, and temperature (Manning & La Claire, 2010b). Biotic factors such as growth phase, differences in endo and exotoxin concentrations, and genetic differences in organism strain are also factors which may be

influencing the toxicity of this complex organism (M. F. Freitag, 2011). The recent finding of seven toxic fatty acid amides (Bertin, Zimba, Beauchesne, Huncik, & Moeller, 2012b), in addition to the described polyketides, Prymnesin 1 and 2 (Fig. 2), sheds light on the complexity as each of the described molecules may respond to the biotic and abiotic factors in different ways. In previous published investigations there were discrepancies between numbers and types of toxic compounds isolated. Manning and La Claire (2010a) suggest this may be due to variable extraction techniques and the affinity of compounds for different solvents. Discrepancies may also be due to the variability of production in the numbers and types of compounds produced under a given scenario. Identifying the number of toxins present and the abiotic factors which may influence which toxins are produced under different conditions will be paramount in controlling this destructive species.

Prymnesin-1



Prymnesin-2

Figure 2. Molecular structure of Prymnesin 1 and 2.
Prymnesin 1 and 2 were the first toxic constituents of *P. parvum* toxin to be discovered. Image adapted from Igarashi, Satake, and Yasumoto 1999.

Significance and Aims

Although *P. parvum* has been plaguing lakes, streams, and hatcheries in the United States since 1985 (Baker et al., 2007.), it has been destroying fisheries around the world for much longer. The first known *P. parvum* bloom occurred in 1911 off the coast of Norway (M. Freitag, Beszteri, Vogel, & John, 2011). Since then, scientists have been trying to discover how these micro algae produce toxins that kill gilled organisms in such a variety of ways. The toxin can be hemolytic, cytolytic, ichthyotoxic, or all three (Roelke et al., 2007). Genetic differences between strains of *P. parvum* have been examined with results showing limited genetic diversity (Lutz-Carrillo et al., 2010). Since these results were shown, and with the discovery of fatty acid amides in the toxin (Bertin et al., 2012a), research is turning to abiotic factors as a source of toxin variation.

The salinity at which *P. parvum* is cultured, and abiotic factors the alga is exposed to, will have a significant effect on the molecular make up and efficacy of toxins produced. Laboratory studies conducted thus far have focused on inducing toxicity in cultures grown with a single set of laboratory culture conditions and toxicity induced by some event introduced to the culture. I will work to determine whether toxin released by laboratory cultures has been selected, synthesized, and accumulated in the cells based on the culture conditions (abiotic factors) prior to an event which induces toxicity. The effects of toxins produced by *P. parvum* will be linked with specific abiotic factors, and will demonstrate whether the alga responds to its surroundings with regard to toxin production.

I used a single, cloned strain of *P. parvum*, acquired from the University of Texas, to test the effects of variable salinity and toxin induction methods on the efficacy of toxins produced. The research will answer the following questions:

1) Does the salinity at which the algae are cultured have an effect on the growth rate?

2) Is there a difference in the efficacy of the toxins produced by *P. parvum* cultured at different, environmentally relevant, salinities?

3) Does the toxicity induction method, or type of environmental shock, have an effect of the efficacy of toxins produced?

4) Are the modes of toxicity different due to a combination of environmental conditions such as salinity during culture and toxicity induction methods?

These answers will shed light on the variability present in a single, cloned strain of *P. parvum*. These findings will add to the body of knowledge being used to reduce the frequency of blooms and fish kills.

CHAPTER 2

METHODS

Algal culture

P. parvum (single clone), obtained from The University of Texas at Austin Culture Collection of Algae (strain UTEX LB 2797), was cultured in 375 mL artificial saltwater (ASW) media composed of API aquarium salt and deionized water. The ASW was adjusted to the desired salinity using a conductivity meter and was autoclaved. After autoclaving, Fritz F/2 media, parts A and B, were added in equal parts of 1ml per 500ml ASW. Media and algae were placed in 500 ml beakers and were loosely covered with plastic wrap to prevent evaporation of media while allowing gas exchange (Shilo & Aschner, 1953). Cultures were kept on a slow moving shaker to aerate at an average temperature of 22^{o} C ± 2^{o} C. Stock cultures were maintained in a non-vented, HEPA filtered biosafety hood (Thermoscientific), and were maintained on a 12/12 light/dark cycle with an irradiance of 200 μmol · m−2 · s−1 (cool white light). Media was prepared at three environmentally relevant salinities, 35psu, 13psu, and 6psu to mimic the alga's global distribution. Eighteen beakers of algae were cultured in ASW media at each of these three salinities. Algae were cultured until reaching a density of $2x10^{6}$ cells mL^{-1} as counted using a hemacytometer and protoslo (Carolina Biological, Burlington, North Carolina), a slowing agent to aid in counting of fast moving protists. After this, toxicity was induced by three ecologically relevant methods and efficacy of toxin was assessed using three bioassays as described in Figure 3.

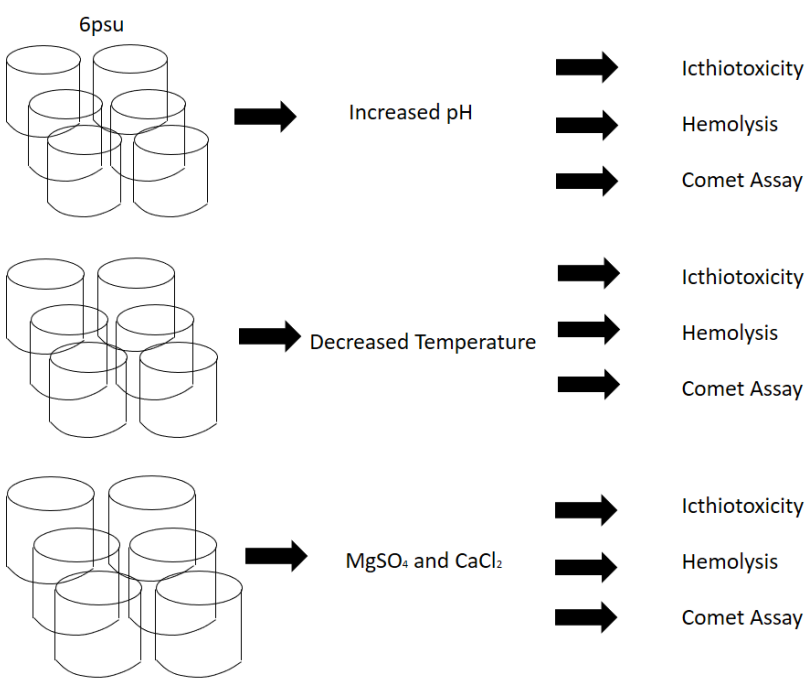


Figure 3. Culture and toxicity methods.

Schematic of algal culture and toxicity methods, showing 18 beakers of independent algal culture at 6psu. Six beakers were shocked to induce toxicity in one of three ecologically relevant ways, and the supernatant of each was used in three bioassays to assess efficacy of the toxin produced. These methods were repeated with algae cultured at 13psu and 35psu.

Growth Curves

Six beakers with 375mL ASW media of each experimental salinity were inoculated with *P. parvum*, strain UTEX LB 2797 at a density of $1x10^2$ cells mL^{-1}. Algae was cultured as previously described and cell counts were conducted daily at 4:00pm for seven days. Cells were counted using light microscopy and a hemacytometer with protoslo (Carolina Biological, Burlington, NC, USA). Cell counts were recorded daily for seven days or until an exponential growth rate was reached for each salinity.

Toxicity Induction

Cell counts were conducted in each algal culture before environmental shock was applied. Cultures were diluted to a count of $2x10^6$ cells/mL and 300mL of each diluted cell culture was transferred to a clean beaker. Algal cultures from each of three salinity groups (n=6) were shocked to induce toxicity by one of three methods. Temperature was reduced by placing cultures on ice until the temperature reached 4°C. Cultures were held at 4°C in the dark for one hour. pH was increased by dropwise addition of NaOH to a pH of 9 as read by a pH meter with gentle stirring. After raising the pH, cultures were placed in the dark at 22° C± 2° C for one hour. Specific ions were added by the addition of 1mL each 1M $MgSO_4$ and $CaCl_2$ solutions. Cultures were gently stirred to mix, then left in the dark for one hour at 22° C ± 2° C.

After one hour, 50mL of each culture was placed in a centrifuge tube and spun at 15°C for 5 minutes at 3000 rpm. Supernatant was removed from the algae pellet and used immediately for toxicity tests.

Hemolysis

Sterile 100% packed sheep erythrocytes were obtained from Innovative Research, Inc. (Southfield, MI, USA). Homogenized buffer medium (HBM) was prepared by diluting sterile RPMI 1640 culture medium (Sigma-Aldrich, USA) by 10% with autoclaved distilled water and 0.005mg/ml heparin sodium salt (Sigma-Aldrich, USA). Cell counts were conducted using a hemocytometer to insure an erythrocyte count of 1×10^7 cells/mL when placed in HBM. Serial dilutions of each algal supernatant, ranging from 25% to 1% toxic algal media, was added to 330µl sheep erythrocyte suspension in HBM in 1.5mL microcentrifuge tubes (Schug et al., 2010). Positive controls were conducted by addition of 20μl Triton X-100 (Sigma-Aldrich, USA) to 330µl sheep erythrocyte suspension in HBM. Negative controls contained 330µl sheep erythrocyte suspension in HBM with 20μl ASW growth media. Centrifuge tubes were placed in the dark at 22^o C ± 2^o C for 1 hour. Tubes were then centrifuged at 2,000 rpm for 5 minutes. 200µl supernatant was transferred to 500µl well of a 96-well plate. Absorbance at 450 nm was read by a plate reader (ELx808 Absorbance Reader; BioTek Instruments, Inc., Winooski, Vermont, USA) and background was determined by the mean of negative control values. The background value was subtracted from each data point before being normalized by the mean positive control value to determine percent lysis.

Rainbow trout gill cell culture

Complete growth medium was made by adding 10% fetal bovine serum (ATCC 30-2020, Manassas, Virginia, USA) to Leibovitz's L-15 medium (ATCC 30-2008, Manassas, Virginia, USA). Rainbow trout gill cells (RTGC) (RT-W1, ATCC CRL-2523, Manassas, Virginia, USA) were cultured in 75 mL polyethylene tissue culture flasks (Falcon, Corning, New York, USA) in

4mL complete Leibovitz's L-15 medium at 37 º C until 80% confluent. Media was aspirated and replaced two times per week. Once confluent, media was aspirated, cells were lifted by addition of 3mL 0.25% Trypsin-EDTA solution for 10 minutes. 8mL complete Leibovitz's L-15 media was added to cell culture to inhibit trypsin. Cells were then split into two culture flasks and returned to the incubator. This process was continued until there were a total of 30 flasks of confluent RTGC.

Treating RTGC with *P. parvum* culture supernatant

Three flasks of RTGC were used as control cells and were treated with 25% ASW medium. All other flasks were treated with three concentrations of each *P. parvum* supernatant collected as described above. Toxic supernatant concentrations used were 25%, 6%, and 1% in 4mL complete Leibovitz's L-15 medium. Treated cells were incubated at 37 º C for 1 hour before being lifted with trypsin, tested for membrane integrity, and used for comet assay.

Comet Assay

A comet assay kit (Trevigen Inc., Gaithersberg, Maryland) containing prepared lysis solution (Cat # 4250-050-01) and comet LMAgrose was used for comet assays. Treated and control RTGC that had been lifted with trypsin as explained in the culture methods were harvested by centrifugation and resuspended in 4º C Ca^{2+} and Mg^{2+} free 1X PBS. Cell counts were performed for each treatment to ensure a cell density of $1x10^5$ cells/mL. LMAgrose was melted in a beaker of boiling water and cooled for 20 min in a 37 º C water bath with the cap loosened. Cells from each treatment group were combined in separate aliquots with molten LMAgrose at a ratio of

1:10. 30µl of mixture was immediately pipetted onto each well of a prepared two well comet assay slide (n=6).

After agrose was allowed to gel, slides were immersed in 4º C prepared lysis solution overnight. Alkaline unwinding solution (pH>13) was prepared using 0.4g NaOH pellets (Fisher Scientific, USA), 250µL 200mM EDTA (Sigma Aldrich, USA), and 49.75L DI water and was chilled to 4º C for 20 minutes before use. Lysis buffer was drained from slides and was replaced with chilled unwinding buffer for 1 hour in the dark. Unwinding solution was drained, and slides were placed horizontally in an electrophoresis tank. Alkaline electrophoresis buffer was prepared using 8g NaOH pellets (Fisher Scientific, USA), 500mM EDTA, pH8 (Sigma Aldrich, USA), and 1 L DI water and was poured gently over the slides. Power supply was set to 21 volts and voltage was applied for 30 minutes at 4º C.

Slides were then immersed twice in DI water 5 minutes each and then dehydrated with 70% EtOH for 5 minutes. Slides were then dried at 45º C for 15 minutes to insure all cells were on a single plane for visualization. Samples were stained with 50µL SYBR® Green (Fisher Scientific, USA) for 5 minutes at 4º C. Excess stain was removed and slides were allowed to dry completely at room temperature. Slides were visualized on an epifluorescence microscope (Zeiss USA, Monument, Colorado) and images were analyzed with Comet Assay IV software (Perspective Instruments, UK). Tail %DNA, Olive Moment, Tail Moment, and Tail length were recorded and used for statistical analysis.

Ichthyotoxicity

Larval fathead minnows (*Pimephales promelas*), <48 hours were obtained from in-house cultures and Aquatic BioSystems, Inc. in Fort Collins, Colorado. All tests were performed immediately upon their arrival to ensure appropriate larval stage. Synthetic fresh water was prepared and tests were conducted in accordance to the USEPA acute toxicity bioassay methods (USEPA, 2002). Six-well plates were prepared with 3mL synthetic fresh water in each well with a control well and five wells with serial dilutions of *P. parvum* supernatant ranging from 25% to 1%. Plates were made for each of the toxic supernatant collected as previously described (n=6). After serial dilutions were made and plates were prepared, four larval fathead minnows were placed in each well and monitored for death at 24 and 48 hours. All surviving larval fish were chemically euthanized following the conclusion of the test.

Statistical Analysis

Median lethal concentration (LC_{50}) and inhibitory concentration (IC25) for larval fish, blood cell and gill cell toxicities were calculated using linear interpolation with the WET Analysis Spreadsheet v1.6.1 as provided by the Environmental Protection Agency's WET test methods under the NPDES program. LC_{50} and IC_{25} means were calculated with 95% confidence intervals and means were given with upper and lower limits. Results were considered significant at $p<0.05$ when limits did not overlap. Normality of growth data was assessed using the Shapiro-Wilks test of normality. Growth rates of each culture salinity were analyzed by two-way ANOVA. ANOVA was performed in, and all graphs were constructed using Prism 7 (Graphpad, La Jolla, Ca). Results were considered significant at $p<0.05$.

CHAPTER 3

RESULTS

Culture salinity affects growth rate

There was a difference in growth rates over a seven-day period in the clonal algal culture between 6psu cultures and 35psu cultures (Fig. 4). Algae in all three salinities grew at the same rate until day two, when the algae cultured at salinities of 13 and 35psu began to outgrow algae in the 6psu culture. By day five, there was a significant difference in growth between the three cultures. Algae cultured at 35psu had significantly more cells than the 13psu culture and the 6psu culture ($p<0.0001$).

Efficacy of toxins varies with culture salinity

When the efficacy of the toxin produced was tested via ichthiotoxicity bioassay, the same trend in toxicity was observed across all three toxicity induction methods and varied with salinity of the culture (Fig.5). The supernatant of algae cultured at 6psu was less toxic than those of algae cultured at 13 and 35psu across all induction methods ($p<0.05$). When toxicity was induced with the addition of $MgSO_4$ and $CaCl_2$ and with the reduction of temperature, the calculated LC50 was >25. When toxicity was induced by raising the pH, the supernatant was more toxic with an LC50 of 20.86 ($p<0.05$).

Algae cultured at 13psu produced significantly more toxic supernatant than that of algae cultured at 6psu across all induction methods ($p<0.05$). When toxicity was induced with the addition of $MgSO_4$ and $CaCl_2$ and with an increase in pH, the LC50 was >25. However, when toxicity was induced by a decrease in temperature, the toxin produced was significantly more toxic with an LC50 of 8.54 ($p<0.05$). When cultured at 35psu, the supernatant produced was

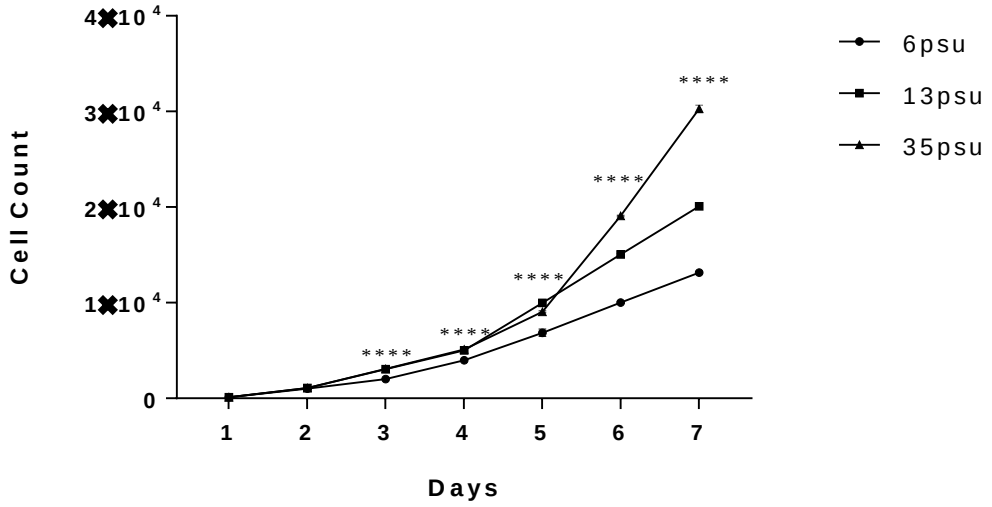


Figure 4. Growth rates of *P. parvum*
Growth rates of *P. parvum* cultured at three different salinities over seven days. Cell counts remained consistent through day two before beginning to deviate. At day three, cultures at 13 and 35psu had significantly more cells than the 6psu culture. At day five, there was a significant difference in growth rates between the three salinities. Algae cultured at 35psu grew the fastest, followed by 13psu and 6psu. Two-way ANOVA, $p<0.0001$ with Tukey's *post-hoc* multiple comparisons test, ****$p<0.0001$.

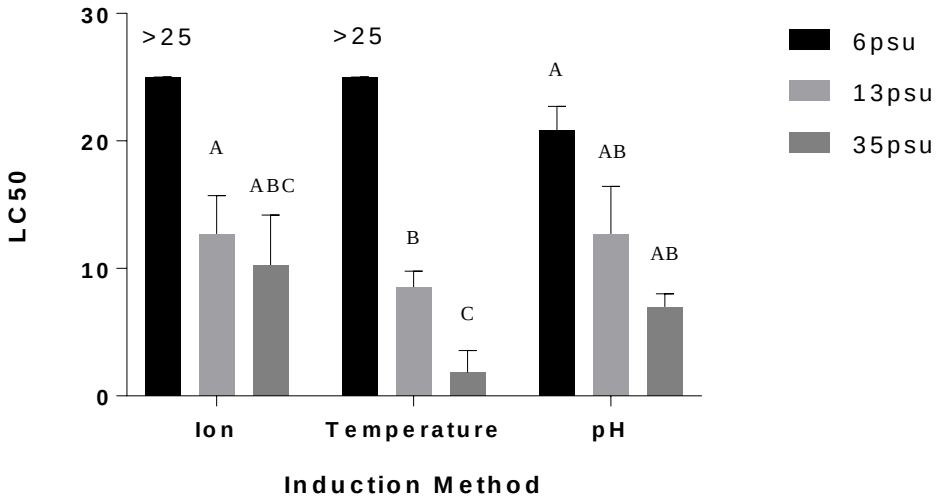


Figure 5. LC50 of ichthyotoxic properties of *P. parvum* supernatant
Toxins produced by *P. parvum* were significantly less toxic across all induction methods when cultured at 6psu. Toxins were found to increase in potency with increasing culture salinity. This trend was consistent across all induction methods. Bars with identical letters are not significantly different from each other.

more toxic than both 6psu and 13psu cultures across all three induction methods. When toxicity was induced with the addition of $MgSO_4$ and $CaCl_2$, the calculated LC50 was 10.27. When toxicity was induced by raising the pH, the LC50 decreased to 6.99. Inducing toxicity by a sudden decrease in temperature was the most toxic with an LC50 of 1.83 ($p<0.05$) when compared to the other induction methods.

Cellular toxicity varies with induction method

To test whether induction method had an effect on toxin produced, an IC25 was calculated for hemolysis and comet assay data and compared to the LC50 for overall ichthyotoxicity for each of the three induction methods. When toxicity was induced by addition of $MgSO_4$ and $CaCl_2$, the algae cultured at 6psu were the least toxic across the three bioassays (Fig.6). Both ichthyotoxicity and comet assay had an LC50 and IC25 of >25, respectively. However, the toxin produced did have greater hemolytic properties than the 35psu culture, with an IC25 of 12.12 ($p<0.05$).

When toxicity was induced with $MgSO_4$ and $CaCl_2$, the 13psu culture had hemolytic properties that were significantly greater than those cultured in either 6 or 35psu, with an IC25 of 0.56 ($p<0.05$). Both double stranded DNA breaks and ichthyotoxicity were significantly greater in organisms cultured at 13psu than those cultured at 6psu, with an IC25 of 0.5 ($p<0.05$) and LC50 of 12.71($p<0.05$), respectively.

Algae cultured at 35psu did not have hemolytic properties, LC50>25. However, the culture did produce the most ichthyotoxic effect and caused greatest DNA fragmentation of the three salinities when induced by addition of specific ions, with an LC50 10.27 and IC25 0.45. These effects were statistically significant when compared to the 6psu culture ($p<0.05$).

When toxicity was induced by sudden drop in temperature (Fig.7), the algae cultured at 6psu produced toxins that were neither hemolytic or ichthyotoxic LC50>25. The culture caused more DNA fragmentation than the other cultures when induced with temperature change, with an IC25 0.6. The 13psu culture was the most hemolytic when induced by temperature change, with an IC25 of 0.55 (p<0.05). The culture had a comet assay IC25 of 1.02 and an ichthyotoxicity LC50 of 8.54, significantly lower than the 6psu culture when induced by the same method.

The 35psu culture, when induced by drop in temperature, did not produce hemolytic toxin, IC25>25. The toxin did, however, cause DNA fragmentation, with an IC25 of 0.82. The 35psu culture was also the most ichthyotoxic of the three salinities induced by temperature reduction with an LC50 of 1.83.

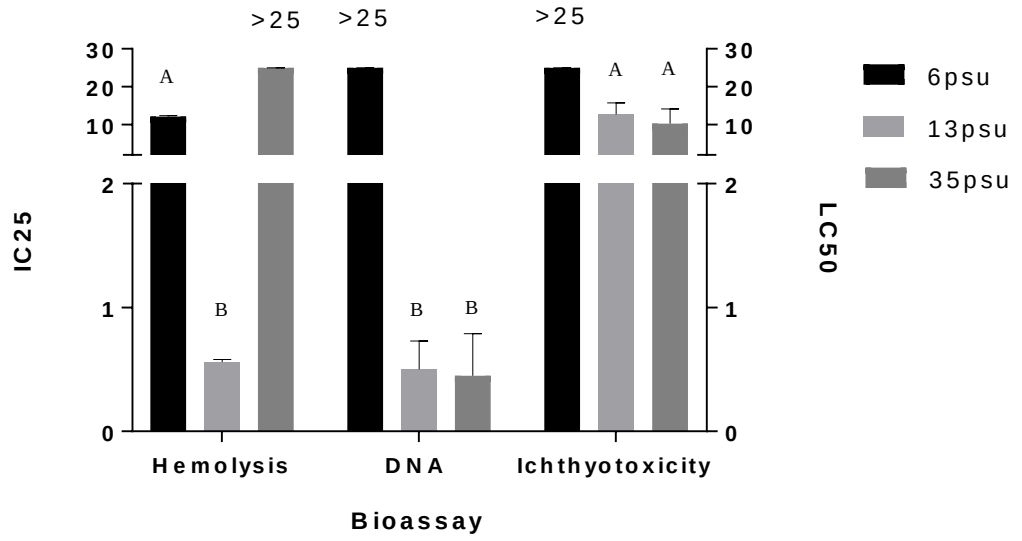


Figure 6. Toxicity of cultures when induced by addition of specific ions
Comparison of IC25 and LC50 from toxin produced by algae cultured at three salinity levels and with toxicity induced by addition of 1M $MgSO_4$ and $CaCl_2$ solutions. Algae cultured at 6psu produced toxin that caused a significant amount of hemolysis; however, the toxin did not cause DNA fragmentation or ichthyotoxicity. When cultured at 13psu *P. parvum* produced toxin that caused a significant amount of hemolysis and DNA fragmentation. Algae cultured at 35psu were not hemolytic, but did cause DNA fragmentation and fish death. Bars with identical letters are not significantly different from each other.

Toxicity was induced by raising the pH to 9 in algal cultures by dropwise addition of NaOH (Fig. 8). In the 6psu culture, the toxin produced was not hemolytic IC25>25. The culture did, however, cause significant DNA damage in rainbow trout gill cells with an IC25 of 0.6 (p<0.05). The toxin produced was also toxic to fish with an LC50 of 20.85.

The 13psu culture reacted differently to the raise in pH than the 6psu culture. The toxin produced by raising the 13psu culture to 9 was hemolytic with an IC25 of 0.89. It also fragmented DNA with an IC25 of 0.59. The ichthyotoxicity assay showed that the toxin produced had an LC50 of 12.71.

When cultured at 35psu, toxins produced by raising pH to 9 did not have hemolytic properties, LC50>25. The toxins produced did fragment DNA, with an IC25 of 3.93. The toxin produced was also more lethal to fish than the other culture salinities induced by raising pH, with an LC50 of 6.99 (p<0.05).

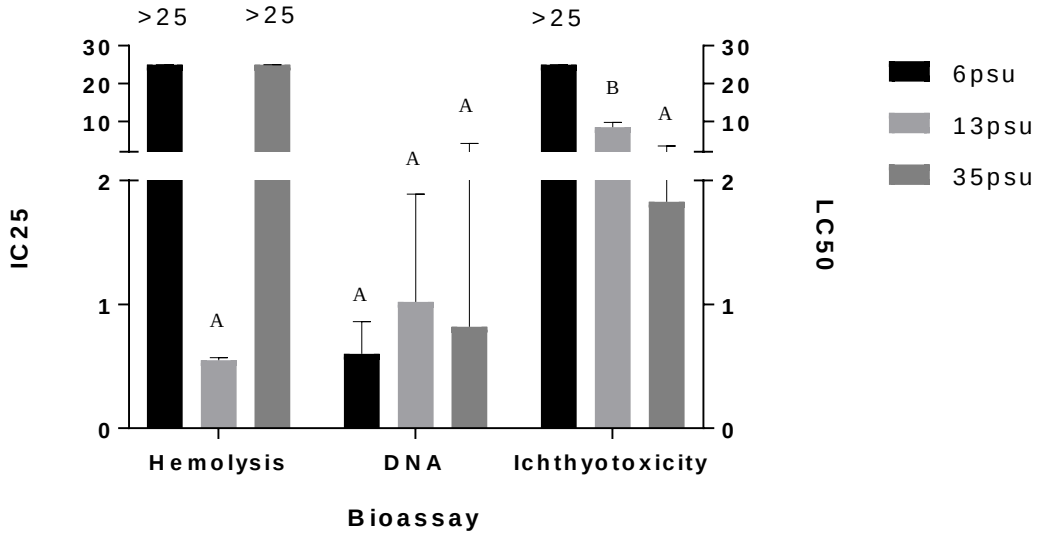


Figure 7. Toxicity of cultures when induced by decreased temperature
Comparison of IC25 and LC50 from toxin produced by algae cultured at three salinity levels and with toxicity induced by sudden drop in temperature. Only algae cultured at 13psu displayed hemolytic properties, while all three culture salinities caused fragmentation of DNA. Algae induced by temperature change and cultured at 6psu were not ichthyotoxic, while the 13psu culture was significantly more toxic than the 6psu culture. Algae cultured at 35psu were significantly more ichthyotoxic than that cultured at 13psu, with an LC50 of 1.83. Bars with identical letters are not significantly different from each other.

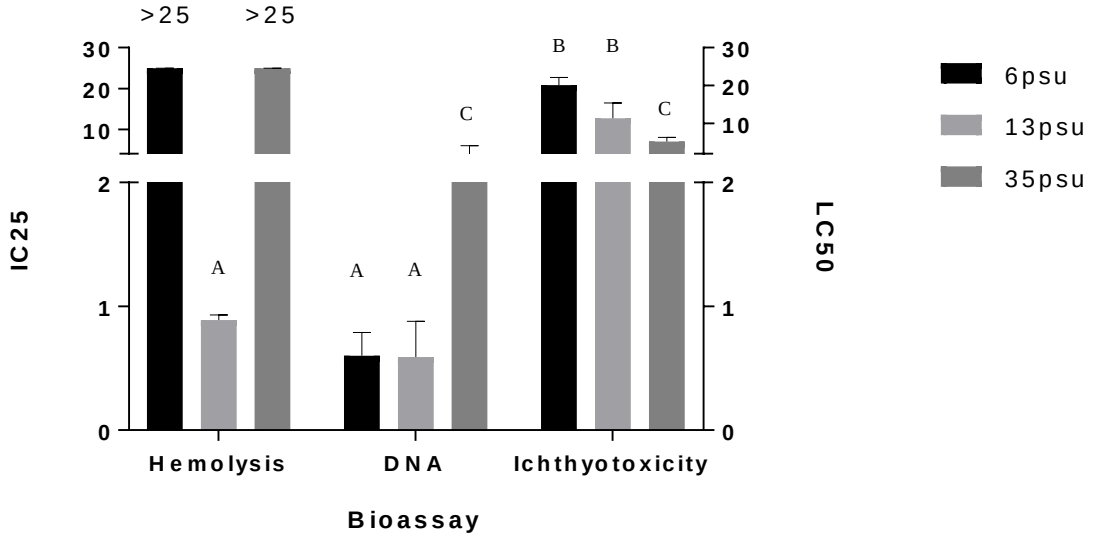


Figure 8. Toxicity of cultures when induced by increased pH
Comparison of IC25 and LC50 from toxin produced by algae cultured at three salinity levels and with toxicity induced by raising the pH to 9. Algae cultured at 6psu did not display hemolytic properties and were the least toxic to fish. It did, however, fragment significantly more DNA than the 35psu culture. Toxins produced by algae cultured at 35psu were not hemolytic. They did, however, cause fragmentation of DNA with an IC25 of 3.93. They were also the most ichthyotoxic with an LC50 of 6.99. Bars with identical letters are not significantly different from each other.

Toxicity varies with culture salinity

Toxin produced at 6psu was generally less toxic across all bioassays than the other culture salinities (Fig. 9). When toxicity was induced by a sudden drop in temperature and an increase in pH, the toxin had no hemolytic effect, IC25>25. When induced by the addition of specific ions, the toxin displayed hemolytic effects with an IC25 of 12.12.

However, the opposite effect was found when looking at DNA fragmentation. The toxin produced by addition of specific ions did not induce apoptosis in rainbow trout gill cells IC25>25. On the other hand, the toxins produced by a sudden drop in temperature and raised pH, both, fragmented DNA with an IC25 of 0.6.

Fish death did not occur with specific ion or decreased temperature induction methods, LC50>25. When the pH was raised, the algal toxins had ichthyotoxic properties, with an LC50 of 20.86.

Algae cultured at 13psu produced toxin that was hemolytic across all induction methods (Fig. 10). When toxicity was induced by addition of specific ions and reduction of temperature, the calculated IC25 was 0.56 and 0.55, respectively. When induced by raising the pH, the toxins produced were only slightly less toxic with an IC25 of 0.89.

The toxins produced also induced apoptosis in gill cells and fragmented DNA. When toxicity was induced with the addition of specific ions, the IC25 was 0.56. Similarly, the culture induced by raising the pH produced toxins that fragmented DNA with an IC25 of 0.55. The culture induced by a sudden drop in temperature was only slightly less toxic with an IC25 of 1.02.

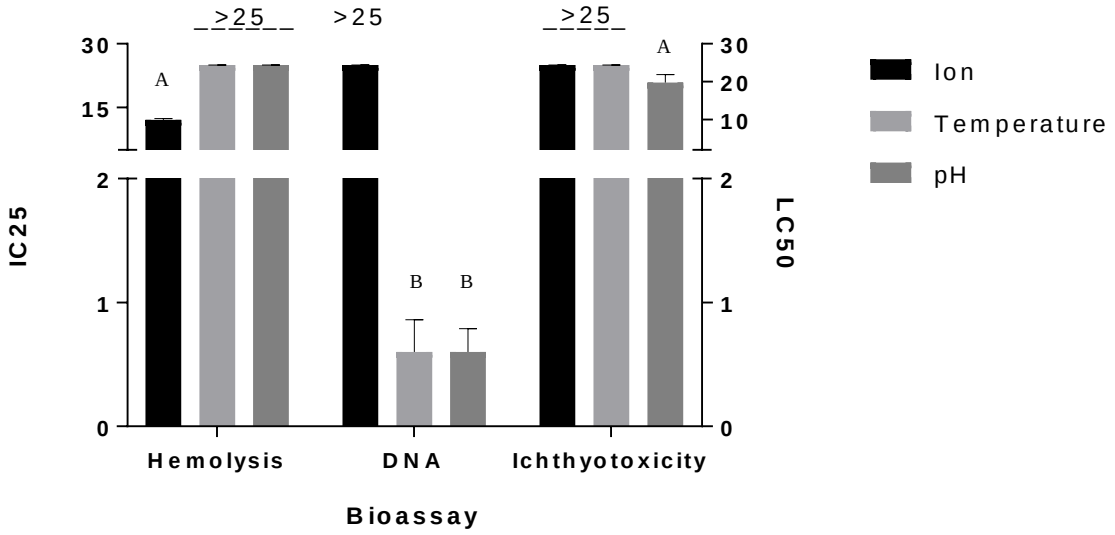


Figure 9. Bioassays of algae cultured at 6psu
Algae cultured at 6psu were only hemolytic when induced by addition of specific ions. However, this induction method neither caused apoptosis in gill cells or caused fish death. Cultures induced by temperature change and raised pH were not hemolytic, but caused apoptosis in gill cells at low concentrations. Only cultures induced by raising pH had ichthyotoxic properties. Bars with identical letters are not significantly different from each other.

Though the toxins produced in the 13psu culture were ichthyotoxic, the toxicity between induction methods was not significant. Both specific ion addition and raised pH induction produced toxins with an ichthyotoxic LC50 of 12.71. Inducing toxicity by temperature drop was slightly more toxic with an LC50 of 8.54.

Algae cultured at 35psu were not hemolytic across all toxicity induction methods, IC25>25 (fig. 10). The toxins did, however, fragment DNA when induced by addition of specific ions IC25 0.45 When temperature was dropped rapidly, the toxins produced were possibly less toxic with an IC25 of 0.82. When toxicity was induced by raising pH, the toxic effect was significantly less with an IC25 of 3.93 ($p<0.05$).

The ichthyotoxicity assay showed that when cultured at 35psu, the algae produced toxins that were ichthyotoxic with all induction methods (fig. 11). When induced by addition of specific ion, the toxins produced an LC50 of 10.27. The culture induced by a reduction of temperature was the most ichthyotoxic with an LC50 of 1.83, and raising the pH produced toxin with an LC50 of 6.99. Though the calculated LC50 means were vastly different across induction methods, there was no significance due to variability in toxic effect.

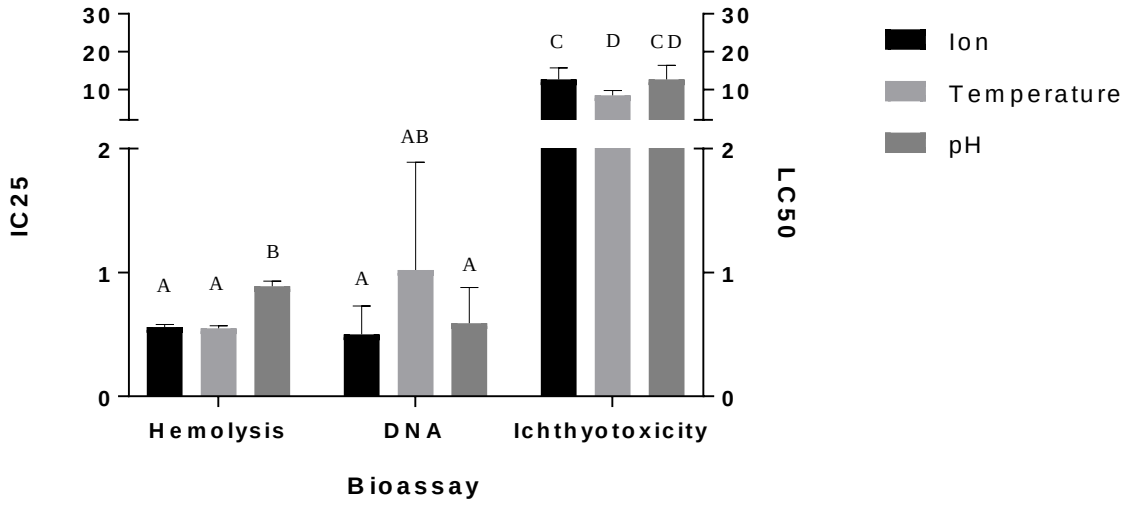


Figure 10. Bioassays of algae cultured at 13psu
Algae cultured at 13psu produced toxins that were highly toxic to erythrocytes, rainbow trout gill cells, and larval fish at low concentrations. The effect of the toxin was similar with each bioassay regardless of toxicity induction method. Bars with identical letters are not significantly different from each other.

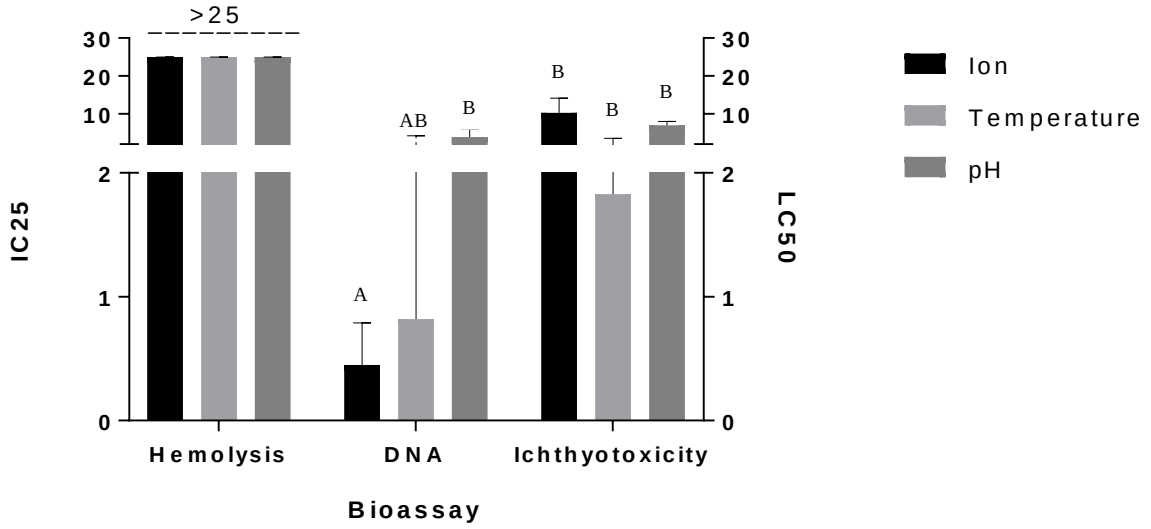


Figure 11. Bioassays of algae cultured at 35psu
Algae cultured at 35psu produced toxins that were not hemolytic across all toxicity induction methods. They did, however, cause apoptosis in rainbow trout gill cells and cause fish death at low concentrations across all induction methods. Bars with identical letters are not significantly different from each other.

CHAPTER 4

DISCUSSION

Growth rate of *P. parvum* increases with culture salinity

Optimal growth rate occurs in highest salinity. When examining the growth rate of *P. parvum*, I looked at growth as a function of salinity under identical light, temperature, and nutrient load. I found that increased growth rate was directly correlated with increased salinity of the culture. These results were similar to previous studies that looked at growth as functions of salinity combined with other variables such as light and nutrients (Baker et al., 2007; Hambright et al., 2014). However, these studies found an inverse relationship between the increased growth rate at high salinity and efficacy of the toxin produced in high salinity culture. Though the lowest culture salinity of 6psu did exhibit slight hemolytic and apoptotic properties under certain toxicity induction methods, it was the least toxic culture to fish under all induction methods. The algae cultured at 13psu with a median growth rate were shown to elicit the most effect across all three toxicity induction methods. These results may indicate that though culture salinity affects growth rate, the efficacy of toxins produced by the algae may not be determined by optimal growth conditions. Instead toxicity could be higher in suboptimal conditions due to stress or as a survival mechanism when cell numbers are low (Remmel & Hambright, 2012; Tillmann, 2003).

Ichthyotoxicity increased with culture salinity

When measuring ichthyotoxicity, we found the same trend across all induction methods. Algae cultured at 6psu were least toxic, followed by 13psu, with 35psu culture being the most toxic regardless of induction method. These findings are opposite of what was expected and what has been found in previous studies (Van Landeghem, Farooqi, Southard, & Patino, 2015;

Andersen, Lorenzen, Snogdal Boutrup, Hansen, & N Lorenzen, 2016). Increased salinity has been shown to increase the toxic effect of some environmental toxins (Dyer, Coats, Bradbury, Atchison, & Clark, 1989). The Ca^{+2} content of an aquatic system may also lead to increased toxicity of sulfates and other environmental toxins that may not typically be toxic to fish (Soucek & Kennedy, 2005). Increased Ca^{+2} content in the culture media may have led to increased toxicity and a biphasic dose responses of high salinity algal cultures that were induced by the addition of $MgSO_4$ and $CaCl_2$ (Fig.12). Though the exact toxins produced were not isolated or characterized, this type of toxic response could also indicate the presence of two different toxins, one that causes death at low concentrations and one that causes death at higher concentrations. The increased toxicity in high salinity cultures could also be due to cumulative stress due to toxins and the effect of the salinity on the fish. Each fat head minnow was exposed to a maximum of 25% culture media with a salinity of 35psu. Indeed, no death was observed in control minnows exposed to 25% culture media. However, there is a possibility of the fish becoming more susceptible to the toxins produced due to being compromised by high salinity. To determine whether death from algae cultured at 35psu resulted from toxins produced or from a combination of high salinity and toxins, a brackish fish species such as the inland silverside should be used and the test repeated.

Toxicity induction method alters efficacy of toxins produced

The method by which toxicity was induced did not play a key role in driving efficacy of toxins produced, though it did influence toxicity under certain conditions. Hemolytic and ichthyotoxic properties displayed the same trend across all induction methods and culture salinities. However, DNA in gill cells was fragmented at greater rate across all salinity cultures

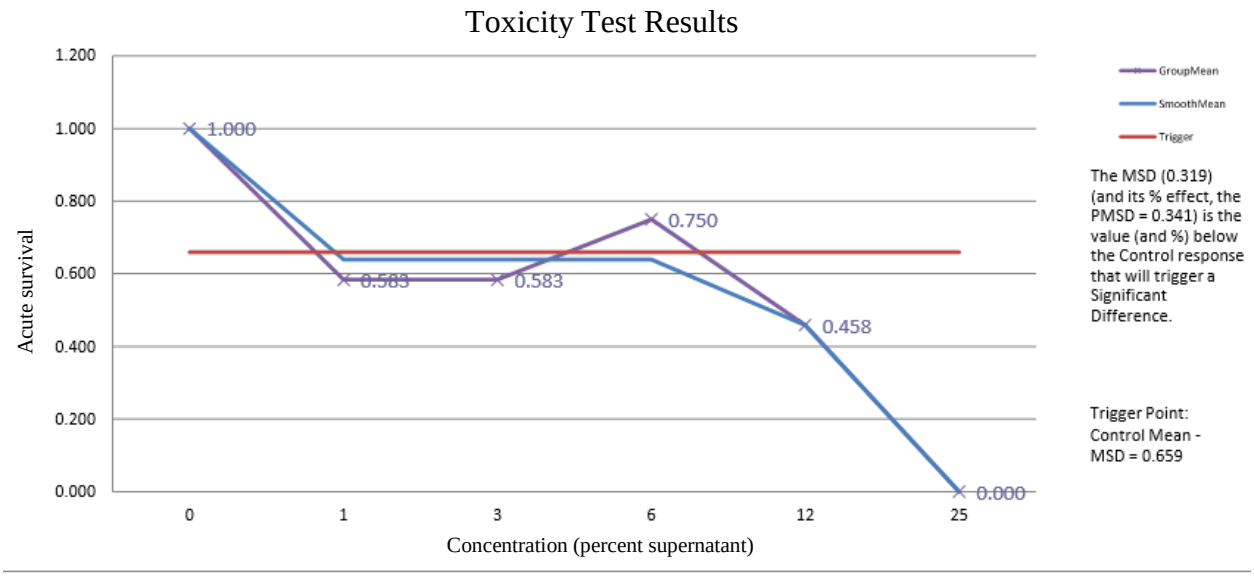


Figure 12. Biphasic toxicity response
An example of EC50 output for ichthyotoxic response when algae were cultured at a salinity of 35psu and toxicity was induced by addition of $MgSO_4$ and $CaCl_2$. The red line represents the control mean, or trigger point, indicating a significant difference. The blue line indicates the smooth mean, which is the smooth observed proportion of mortality per concentration. The purple line represents the group mean of the six replicates at each concentration.

when toxicity was induced by a sudden reduction of temperature and increased pH. Environmental stress that reduces growth rate, such as low temperature, has been shown to enhance the toxicity of *P. parvum* toxin in both field and lab studies (Grover, Roelke, & Brooks, 2012; Hambright et al., 2014). Increased pH is known to alter the toxicity of lipid compounds produced by *P. parvum* causing them to express more cytolytic and hemolytic properties (Bertin, Voronca, Chapman, & Moeller, 2014). Additionally, a Hungarian strain of *P. parvum* was recently found to be highly toxic at high salinities when ponds had pH of 9 along with a low temperature (Vasas et al., 2012). In that study, a suite of 20 gelatinolytic proteases were found in the toxin isolated from several ponds where fish kills had occurred. It would stand to reason that these proteases would cause damage to the membrane of the gill cells and supports the findings that the toxins released when induced by temperature reduction and increased pH caused apoptotic DNA fragmentation in gill cells.

Culture salinity drives toxic efficacy

The salinity at which the algae were cultured predicted the outcome of the bioassays across all induction methods with few exceptions. As previously discussed, hemolysis and ichthyotoxicity results varied only with salinity. Meanwhile, toxins producing apoptotic DNA fragmentation seem to be driven by a combination of salinity and environmental shock with variability between all culture salinities and induction methods.

While the 13psu culture salinity was the only culture to produce toxins with significant hemolytic properties, 6psu cultures were only hemolytic when induced by addition of specific ions, and 35psu cultures had no hemolytic properties. The findings that toxicity from algae cultured at 35psu is less associated with hemolysis are supported by studies that also used the Texas strain (Schug et al., 2010; Skingel et al., 2010). These tests demonstrated that algae

cultured at high salinities were less toxic overall, with no hemolytic properties when compared to algae cultured at lower salinities. Though the finding that algae cultured at 6psu produced less toxic effect than higher culture salinities is contrary to other studies, this may be due to the exact molecular makeup of the toxins produced in this particular strain (Freitag et al., 2011).

The salinity at which *P. parvum* is cultured, and abiotic factors the alga is exposed to as environmental shock had a significant effect on the efficacy of toxins released. These findings may suggest that the alga produces, or selects its toxin according to the conditions in the environment around it at the time of a toxic event. However, the toxins produced could be made more potent due to interactions with specific ions that are available in the water column (Holdway, Watson, & Moss, 1978). There are many discrepancies in current literature regarding hemolytic and ichthyotoxic properties of *P. parvum.* Fatty acids, gelatinolytic proteases, and polyketide prymnesins have all been isolated from various *P. parvum* cultures and blooms. Even so, the combination of constituents of the toxin seems to vary with a vast array of environmental and genetic influences. Variation in toxicity between strains has been shown to be a major source of discrepancy in the literature; however, variation within a single strain is proving to make the identification of toxic mechanisms even more difficult (J.W. La Claire, Manning, & Talarski, 2015). Further studies are needed regarding genetic variations that may occur in different environmental conditions that may be the cause of the apparent evolution of the species from a brackish, saltwater alga to a more inland, freshwater alga (Driscoll et al., 2013). Identifying the role of environmental factors in toxin efficacy within a single strain is a first step in understanding recurrent toxic blooms and fish kills.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY®
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Office of Research Integrity

May 1, 2013

Amy Beth White
825 Hudson St.
Saint Albans, WV 25177

Dear Ms. White:

This letter is in response to the submitted thesis abstract titled "Establishing Variability in the Toxins Produced by *Prymnesium Parvum* Exposed to Differing Culturing and Toxicity Inducing Conditions." After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) Chair has also deemed this not to be animal research requiring their approval. The information in this study is not considered human subject or animal research as set forth in the definitions contained in the federal regulations. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director