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# Toxin Production and Population Dynamics of *Gloeotrichia echinulata* with Considerations of Global Climate Change

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**Toxin production and population dynamics of *Gloeotrichia echinulata***  
**with considerations of global climate change**

Sarah Nicole-Marie Stamann

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Master of Science

Biology Department

August, 2015

## DEDICATION

This thesis is dedicated to the following:

For Michelle, who is intelligent, graceful, and kind.

For Joseph, who is compassionate and steadfast.

For Abigail, who loves fiercely and feels deeply,

For Lucy, who is filled with joy, and

For Jacob, who is still curious.

They have given me all their love, and continuously remind me to approach the world and its problems with wonder and enthusiasm.

*“However, I continue to try and I continue, indefatigably, to reach out. There’s no way I can single-handedly save the world, or perhaps, even make a perceptible difference — but how ashamed I would be to let a day pass without making one more effort.” -Isaac Asimov*

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## ABSTRACT

### TOXIN PRODUCTION AND POPULATION DYNAMICS OF *GLOEOTRICHIA ECHINULATA* WITH CONSIDERATIONS OF GLOBAL CLIMATE CHANGE

By Sarah Nicole-Marie Stamann

Global climate change has been identified as a driver for increasing cyanobacteria blooms world-wide. Blooms of the cyanobacterium, *Gloeotrichia echinulata*, (hereafter, *G. echinulata*) have been observed in Silver Lake (Oceana County, MI), often forming dense surface scums. This organism is known to produce the hepatotoxin, microcystin-LR, and its growth is linked to phosphorus accumulation from sediment and temperature.

A series of experiments at ambient and elevated temperatures (+2° and +6° above ambient) with sediment from four locations in Silver Lake with varying phosphorus concentrations were conducted to examine the effect of these variables on *G. echinulata* growth. These experiments were designed to examine the effects of global climate change and sediment phosphorus concentrations on *G. echinulata* blooms.

Initially, colonies were incubated to induce akinetes. Akinetes then were incubated in growth chambers at three different temperatures (17°C, 19°C, and 23°C) for 30 days in test tubes filled with 30 mL of filtered lake water and 5mL of sediment from one of the four locations. On alternate days, 25 mL of lake water was withdrawn from each test tube, placed into a scintillation vial, and preserved with Lugol's solution until enumeration. Withdrawn water was replaced with 25 mL of filtered lake water. At the end of the incubation period, settling chambers were used to enumerate filaments germinated by each colony. The concentration of microcystins was examined three times throughout the sampling season, using 500 washed and filtered pelagic colonies, and

analyzed by LC-MS. Lastly, time-to-event statistics were performed on temperature and phosphorus treatment data.

The results showed that *G. echinulata* colonies produced low levels of microcystin-LR and microcystin-RR in Silver Lake (0.17 µg/l and 0.09 µg/l, respectively). Results showed that it was possible to induce early akinete development in *G. echinulata* by approximately four days using lowered incubation temperatures and shorter periods of light availability. Results of various temperature and phosphorus treatments showed a significant difference in temperature and phosphorus treatments. A high temperature produced earlier germination, regardless of phosphorus concentrations ( $\chi^2 = 0.2$ ,  $df = 1$ ,  $p = 0.622$ ). However, at low temperatures, phosphorus concentrations became important in germination times ( $\chi^2 = 7$ ,  $df = 1$ ,  $p = 0.01$ ).

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## CHAPTER I

### INTRODUCTION

Cyanobacteria blooms are occurring more frequently in freshwater bodies worldwide as a result of an increasing trend in cultural eutrophication (Chorus et al. 2000; Ahn 2011), the introduction of exotic species (Juhel et al. 2006), and global climate change (Paerl and Paul 2011; El-Shehawy et al. 2012). Recent research, laboratory studies, and field observations show that the combination of anthropogenic nutrient loading, rising temperatures, enhanced vertical stratification of the water column, in which water stratifies based on density, and increased atmospheric CO<sub>2</sub> supplies will favor cyanobacterial dominance in a wide range of aquatic ecosystems (Paerl and Paul 2011). The expansion of cyanobacteria blooms has serious consequences for drinking and recreational water uses, and more research is needed to develop effective management programs to mitigate cultural eutrophication and global climate change.

Cyanobacteria often produce cyanotoxins, which are harmful to both plants and animals (WHO 1998). Because of this, controlling blooms of cyanobacteria is an increasing concern as water becomes scarcer and the demand grows higher. *Gloeotrichia echinulata*, (hereafter, *G. echinulata*) is a bloom forming cyanobacterium that is capable of nitrogen fixation, and originates from the sediments of what was previously thought to be eutrophic lakes (Barbiero 1993; Jacobsen 1994; Karlsson-Elfgren et al. 2004). Recently, Carey et al. (2012) determined that *G. echinulata* can be found in oligotrophic/mesotrophic lakes, which are generally thought to lack the nutrient resources for excessive cyanobacterial growth.

### *Akinetes and Overwintering Life Stage*

As conditions in the water column become unfavorable for continuing growth in late summer, *G. echinulata* forms akinetes, and settles on the sediment (Forsell and Pettersson 1995; Tymowski and Duthie 2000). The settled organism loses the vegetative portion of the colony, and the akinetes of the old colony remain in the thick mucus that surrounds its pelagic colonial form (Forsell and Pettersson 1995). *G. echinulata* akinetes overwinter in their residual colonial mucus. During the spring, germlings will break through the hard cell wall of the akinete, and begin accumulating nutrients from the sediment and decomposing organic matter on the lake bottom (Tymowski and Duthie 2000).

This organism is the only known species of cyanobacteria where growth in the epilimnion is based almost entirely on accumulated phosphorus reserves obtained from sediment (Tymowski and Duthie 2000). Blooms of *G. echinulata* can result in an increase in phosphorus movement in the pelagic zone (Tymowski and Duthie 2000; Carey et al. 2010) and may encourage the growth of other algae and cyanobacteria by increasing the phosphorus in the water column through cell death or “leaking” cells (Carey et al. 2010).

### *Recruitment*

Recruitment is defined as the life cycle of the organism that includes germination of the akinete, nutrient accumulation from the sediment, and migration into the water column once conditions improve for survival of the filaments (Karlsson-Elfgren et al. 2004). Current literature suggests that recruitment is influenced largely by light and temperature (Barbiero 1993; Forsell and Petersson, 1995; Carey et al. 2012). Previous

experiments (Karlsson-Elfgren et al. 2004) determined that temperature and light were the key parameters for the initiation of germination, but that temperature and sediment mixing influenced the magnitude of germination. While Karlsson-Elfgren et al. (2004) found that light was a driving factor of the initiation of germination, Barbiero (1993) determined that at a certain, undetermined point, temperature became a more important recruitment influence than light.

### *Global Climate Change*

As surface temperatures of the Earth rise, harmful algal blooms (HABs) are likely to become an increased threat to water supplies. Based on current and projected conditions, NASA predicts that the surface temperature of the earth will rise between 2°C and 6°C by the end of the century (Riebeek 2010). When compared to NASA's findings that the temperature difference between current times and the last ice age was only 4°C (Riebeek 2010), we see a far more compressed time frame in which the earth will warm. This compressed global rise in temperature could potentially be enough to encourage cyanobacteria such as *G. echinulata* to bloom at increasing rates and frequencies. An experiment on a shallow, hyper-eutrophic lake during the European heat-wave of 2003 showed that a 5°C increase in temperature over one summer was enough to overcome the benefits of artificial intermittent mixing, allowing cyanobacteria blooms to form almost immediately after mixing was stopped (Johnk et al. 2008). This indicates that for lakes with low vertical mixing, an upturn in temperature as predicted by NASA, could result in an increase of HABs frequency and bloom intensity.

### *Nutrient Uptake*

Phosphorus uptake dynamics of *G. echinulata* were examined with laboratory bioassays by Tymowski and Duthie (2000). Results showed that benthic colonies of *G. echinulata* on the sediment had a rapid rate of phosphorus assimilation from sediment porewater, whereas planktonic colonies of the species did not appear to assimilate phosphorus from the water column. Planktonic colonies were found to mainly rely on their sediment-accumulated reserves to sustain summer growth. While Tymowski and Duthie (2000) examined phosphorus uptake, they, like Karlsson-Elfgren et al. (2004), did not study the role of sediment phosphorus concentrations in germination or colony growth. Sediment phosphorus concentration may be an important variable in *G. echinulata* colony growth given its dependence on benthic uptake.

### *Toxin Production*

*G. echinulata* produces the hepatotoxin microcystin-LR (Carey et al. 2007). Hepatotoxins are harmful to the liver of humans and other animals (WHO 1998). With sufficient hepatotoxin intake, the liver begins to shut down and death follows (WHO 1998). To avoid health concerns, the World Health Organization (WHO) has established microcystin exposure guidelines for drinking and recreational water of 1 µg/L. WHO does not establish exposure guidelines for recreational waters. WHO has stated that exposure to toxic microcystin through recreational is a minimal risk, however, exposure to microcystin may occur through ingestion of toxin-laden waters (WHO 1998). Though it has been shown that *G. echinulata* can produce microcystin-LR between 58 and 7,148 ng/g dry weight, which is generally lower than the WHO recommendations, there is concern that levels could rise should blooms be especially large (Carey et al. 2007, 2012).

### *Methods of Analysis*

An enzyme-linked immunosorbent assay (ELISA) was used to identify microcystin-LR as the cyanotoxin produced by the cyanobacterium (Carey et al. 2007). The ELISA test exhibits cross reactivity with other analogues, including microcystin-RR (MC-RR) (Metcalf et al. 2000). Consequently, the use of ELISA may overestimate the MC-LR concentration if other analogues are present. Microcystin analogues exhibit varying toxicities, as the LD50 values for MC-LR and MC-RR are 43 ug/kg and 243 ug/kg, respectively (Gupta et al. 2003). Liquid Chromatography – Mass Spectrometry (LC/MS) analyses would be required to determine the composition of individual microcystin analogues present in a *G. echinulata* bloom.

### *Silver Lake, Oceana Co., MI*

Silver Lake is a 279 hectare water body, located in Oceana County, MI. It is 178.5 m above sea level, and 1.5 m above the mean sea level of Lake Michigan (Fisher et al. 2007). Summer blooms of *G. echinulata* have been reported in Silver Lake and efforts are underway to develop management programs to control nuisance cyanobacteria blooms. The lake drains to Lake Michigan by way of Silver Creek, located on the



Figure 1.1 A map of Michigan, with Oceana County highlighted.



South-Western side of the lake. Silver Creek contains a weir that controls the level of lake (Fisher et al. 2007). The lake receives water from two creeks on the North-Eastern side of the lake. Silver Lake itself is only 7 m deep at its maximum, and is bordered by an active dune on the western edge that is about 2 km wide (Fisher et al. 2007). The active dune separates Silver Lake from Lake Michigan. Silver Lake State Park is located on the eastern edge, as well as a resort, and multiple lakeside cottages.

Four locations around the Silver Lake littoral zone were used to collect sediment, and two locations within the center of the lake were used to collect pelagic zone water (Figure 1.1). Location North was located at (N43° 40.660 W086°30.003) and was near private cottages. Location South was located at (N43° 39.653 W086°30.501) and was near private cottages. Location East was located at (N43° 39.561 W086°30.465) and was near the center of the state park. Location West was located at (N43° 40.308 W086°30.488) and was analogous to the center of the recreational dunes from the lake. These sampling locations were chosen as it was hypothesized that sediment near private cottages, in front of recreational dunes, and near a state park would each contain levels of sediment phosphorus different from one another.

## THESIS OBJECTIVES

The purpose of this research is to examine toxin production and recruitment of *G. echinulata* in Silver Lake, MI. We examined the role of sediment phosphorus concentrations on the recruitment of the organism using methods established by Karlsson-Elfgren et al (2004). Additionally, we will examine the effect that global climate change will have on *G. echinulata* growth by incubation at elevated temperatures.

In Chapter II, we examined toxin production of *G. echinulata* in Silver Lake. LC/MS methods were used to determine the microcystin congeners produced by *G. echinulata* on a temporal basis.

In Chapter III, we examined akinete development from pelagic colonies and the *G. echinulata* life cycle. Our experiment included a novel approach to induce akinete production from growth phase pelagic colonies. The experimental design to induce akinete production and the results of this unique incubation approach are discussed.

In Chapter IV, we examined how sediment pore water phosphorus concentration and temperature could interact singly and together to create increased algal biomass and earlier germination in *G. echinulata* colonies. We also used time-to-event statistics to analyze germination data. This chapter takes into consideration how a global increase in temperature (climate change) could affect blooms of *G. echinulata*.

## CHAPTER II

### MICROCYSTIN PRODUCTION BY *G. ECHINULATA* IN SILVER LAKE

#### ABSTRACT

Toxic secondary metabolite production of microcystin-LR by *G. echinulata* has been previously examined using Enzyme Linked Immunosorbent Assay (ELISA) methods to determine toxin concentration. ELISA methods exhibit cross-reactivity between congeners, producing a concentration that includes multiple microcystin analogues, rather than just the most toxic form, microcystin-LR (MC-LR). In this experiment, pelagic colonies were removed from Silver Lake (Michigan, USA) and examined for microcystin production using Liquid Chromatography-Mass Spectrometry (LC/MS), which can identify and quantify the different analogues of microcystin.

Our results indicate that *G. echinulata* produced MC-LR, as well as MC-RR. The total microcystin (MC-T) concentration measured in early July was 94 ng/g dry weight with MC-RR accounting for 36% of the total. Total MC concentrations declined to 31 ng/g dry weight later in July, however, the % MC-RR increased to 71%. This is the first report of MC-RR and MC-LR production by *G. echinulata* using LC-MS. Since the World Health Organization exposure guidelines are based on MC-LR, the presence of a significant fraction of MC-RR suggests that LC-MS methods may more appropriate to assess the environmental health significance of *G. echinulata* blooms.

## INTRODUCTION

### *Microcystin Toxicity*

Hepatotoxins can include nodularins and cylindrospermopsins (de Figueiredo et al. 2004), but almost exclusively refer to microcystins, which are produced in abundance in freshwater systems, and are especially toxic (WHO 2003). There are an abundance of cyanobacteria that produce microcystins, including *Anabaena*, *Nostoc*, *Cylindrospermopsis*, and *Microcystis spp.* (Rastogi et al. 2014). These cyanobacteria can form blooms, and, when producing toxins, are referred to as harmful algal blooms (HABs).

Microcystin has over 90 congeners (Schmidt et al. 2014) and is a monocyclic peptide (Dawson 1998) that inhibits protein phosphatases PP1 and PP2A (Gupta et al. 2003). Differentiation of microcystin congeners is determined by the amino acid located at the X and Y positions of the peptide (Gupta et al. 2003), and is identified using the one letter abbreviation for the amino acid. The Adda amino acid at position 5 binds to the phosphatases, thereby making microcystin hepatotoxic (de Figueiredo et al. 2004).

Of all the congeners, microcystin-LR (hereafter, MC-LR) is studied the most due to its abundance and high toxicity (Codd et al. 2005). MC-LR is identified by the amino acids leucine (L) and arginine (R) at the 2 and 4 (or, X and Z) positions of the microcystin peptide (Gupta et al. 2003), and is produced by *G. echinulata* (Carey et al. 2007). Microcystin bio-accumulates in aquatic food webs; however, the low log  $K_{ow}$  and rapid depuration rate limit its ability for bio-magnification (Ibelings and Chorus 2007).

MC-LR is widely studied, and has repeatedly been shown to be highly toxic to fish (Ibelings and Chorus 2007; Kopp et al. 2013), plants (Hereman and Bittencourt-Oliveira 2012), and humans (Kozdeba et al. 2014; Poste et al. 2011; Pouria et al. 1998). Microcystin-RR (MC-RR) is differentiated from the other microcystin congeners by the presence of arginine amino acids at both the 2<sup>nd</sup> and 4<sup>th</sup> (or, X and Y) position on the microcystin peptide. MC-RR is toxic (Gupta et al. 2003), is an endocrine disrupter (Xie et al. 2015), and can prolong the growth of other harmful bacteria, such as *E. coli* (Yang et al. 2008). MC-RR is more stable in filtered water than natural water systems (Prakash et al. 2009), and is not removed by modern waste water treatment systems (Vasconcelos and Pereira 2001; Svrcek and Smith 2004).

#### *Health Hazards*

Microcystin is globally distributed, as the toxin has been found in drinking reservoirs in many locations (Rastogi et al. 2014). It is now thought that over 40% of drinking reservoirs and freshwater drinking sources are eutrophic, and favorable to harmful cyanobacterial growth (Bartram et al. 1999). Chronic exposure to low levels of MC-LR is a concern, as reservoirs become filled with microcystin, human populations increase, and fresh drinking water becomes scarce (de Figueiredo et al. 2004). In animals, low level chronic exposure has been shown to cause tumors and decreased reproduction in mice (Gupta, 2003), result in deleterious effects on mice lungs (Soares et al. 2007), and harm the heart muscles of rats (Milutinović et al. 2006). Exposure has also been shown to disrupt thyroid and metabolic function in mice (Zhao et al. 2015). The long term, chronic exposure through fish is thought to be the reason behind China's increasing liver cancer rates (Zhang et al. 2009; Ueno et al. 1996). Multiple human

exposures, though not resulting in death, have been documented globally (Bláha et al. 2009), with health effects including contact dermatitis, fever, liver damage, and respiratory irritation.

Microcystin can be harmful to both human and environmental health. When exposed to microcystin, fish experience decreased survival, behavior modifications, reduced growth, and, in the developing juveniles, curved bodies and tails (Malbrouck and Kestemont 2006). Microcystin exposure in fish that results in a decrease in food for predators can affect multiple trophic levels of an aquatic ecosystem. Microcystin has been found to accumulate in a wide variety of organisms, including bivalves (Song et al. 2007), and fish (Poste et al. 2011; Kopp et al. 2013), both of which are a common food source for many cultures.

Microcystin also can negatively affect aquatic plants that are used as a food source for aquatic organisms. An experiment on watercress showed microcystin exposure reduced growth rates of the plant, at even low doses of  $1 \mu\text{g L}^{-1}$  (Gehring et al. 2003). Previous studies show that microcystin exposure to aquatic plants can result in the uptake of microcystin through root systems (Chen et al. 2012), resulting in decreased or limited growth. Microcystin can accumulate in common edible plants, such as lettuce (Hereman and Bittencourt-Oliveira 2012), when microcystin-laden water is used on agricultural fields.

The World Health Organization (WHO) has set a tolerable daily intake (TDI) limit for microcystin-LR exposure in order to avoid public health effects. WHO established a TDI of  $1 \mu\text{g L}^{-1}$  for exposure through aquatic sources, such as drinking water, and a TDI of 0.04 mg/kg of body weight for exposure through food sources, such

as fish (WHO 2003). Human exposure to microcystin results in skin irritation from recreational contact. Low exposure can result in gastrointestinal distress, and rashes (WHO 2003; Kozdeba et al. 2014).

#### *Light and Temperature Effects on Microcystin Congener Production*

Both light and temperature have been experimentally shown to affect microcystin production. Multiple studies report that microcystin production increases with light intensity (Wiedner et al. 2003; Kaebernick et al. 2000; Rapala and Sivonen 1998) and that red light, as well as white light (WHO 2003), is more important than blue (Kaebernick et al. 2000). Between 2 and 20 meters, red light is most prevalent after passing through water of that depth (Wetzel 2001). Studies also show that temperature can influence microcystin congener production in *Anabaena* through physiological changes in the organism. Temperatures above 25°C tend to produce microcystin-RR (MC-RR), while temperatures below 25°C tend to produce MC-LR (Rapala and Sivonen 1998; Rapala et al. 1997). The World Health Organization reports that pH, nitrogen, phosphorus, and carbon dioxide can also influence the *amount* of toxin produced. Some studies show no positive correlation between surface water nutrients and the total amount of toxins produced (WHO 2003; Wicks and Thiel 1990), while other studies have shown that higher nutrients result in more harmful algal blooms (Davis et al., 2015; Singh et al., 2015).

#### *Removal and Degradation*

Both microcystin-RR and microcystin-LR are able to survive modern waste water treatment plants (Prakash et al. 2009; Svrcek and Smith 2004). However, microcystins can effectively be removed through a variety of specialized methods. It has long been

shown that microcystin toxins will adsorb to activated carbon (Huang et al. 2007; Donati et al. 1994). Bio-degradation in waste water treatment plants has been reported, but efficacy is dependent on the number of microorganisms available to break down toxins (Ho et al. 2010). Microorganisms that have been shown to break down microcystin toxins include *Bordetella sp.* (Yang et al. 2014) and *Stenotrophomonas sp.*, which found to completely consume microcystins LR and RR within one day at varying concentrations of the organism (Chen et al. 2010).

Sand filtration also has been shown to be effective, removing microcystin toxins to below WHO recommendations (Ho et al. 2010; Bourne et al. 2006). UV radiation can have mixed effects on breaking down MC-LR. Previous studies show UV radiation as a toxin removal was ineffective (Gajdek et al. 2004), but later studies show that UV radiation in the presence of a catalyst, such as hydrogen peroxide, effectively remove MC-LR toxin (Qiao et al. 2005). More recent research suggests that combining oxidation with UV radiation not only removes microcystin toxin from drinking water, but other harmful bacteria and organisms (Chang et al. 2015).

#### *Microcystin Production by G. echinulata*

*G. echinulata* was first shown to produce MC-LR by Carey et al. (2007) using Enzyme Linked Immunoassay (ELISA) methods. However, the ELISA test exhibits cross reactivity with other analogues, including MC-RR (Metcalf et al. 2000) and may overestimate the MC-LR concentration if other analogues are present. Most commercial ELISA kits list a 50-60% cross-reactivity with MC-RR. Microcystin analogues exhibit varying toxicities, as the median lethal dose (LD<sub>50</sub>) values for MC-LR and MC-RR are 43 µg/kg and 243 µg/kg, respectively (Gupta et al. 2003). Liquid Chromatography – Mass



Spectrometry (LC-MS) analyses would be required to determine the composition of individual microcystin analogues present in a *G. echinulata* bloom. In this experiment, we examined MC-LR and MC-RR production using LC-MS in *G. echinulata* collected from Silver Lake (MI).

## METHODS

### *Study Site: Silver Lake, MI*

Silver Lake is a 279 hectare water body, located in Oceana County, MI with a maximum depth of 7 m. It is used seasonally for recreational purposes, such as swimming, boating, and jet skiing. The Silver Creek watershed is approximately 22 square miles (Groves et al. 2015). The watershed is primarily forest and wetland, with 1/3<sup>rd</sup> of the watershed consisting of agriculture. Over 2,000 acres of the Silver Lake watershed are classified as wetlands (Groves et al. 2015). The lake has a heavily developed lake basin shoreline. The shoreline consists of a frequently used state park, a hotel, and several private cottages surrounding the lake.

The lake is fed by two creeks, and exits to Lake Michigan via Silver Creek. The lake levels are controlled by a weir on Silver

Creek. Historically, total phosphorus concentrations in

Silver Lake are lower in the spring than in the summer since 1989 (Progressive AE 2011). The summers of 2010 and 2011 showed elevated chlorophyll-a concentrations, which correlated to persistent algal blooms on the lake. The Silver Lake basin is a nutrient rich environment that is trending toward rapid eutrophication (DeMaat et al. 2013).

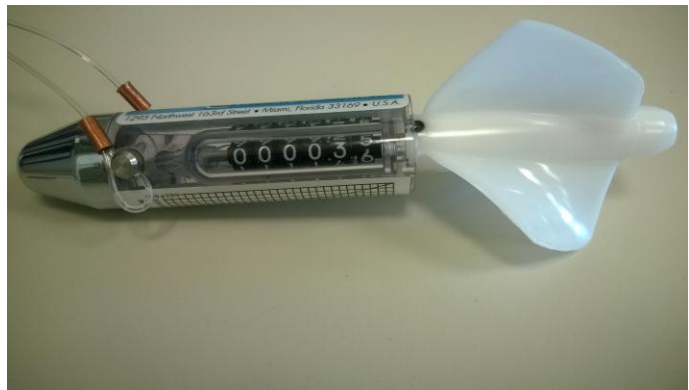


Figure 2.1: General Oceanics mechanical flow meter with counting rotor.

### *Colony Acquisition*

Plankton tows with a 35  $\mu\text{m}$  plankton net (Wildco) were used for colony acquisition. The net was towed alongside a johnboat at idle speed for approximately 5 – 8 meters at a depth of 0 to 30cm within the water column. Actual distance traveled was calculated post-sampling using a mechanical flowmeter (General Oceanics, part 2030R) that was attached to the plankton net (Figure 2.1). The counting rotor was then used post-sampling to determine the distance the tow net traveled through the water and the volume of water sampled (Table 2.1) (General Oceanics mechanical flowmeter operator's manual 2006). Once the plankton net appeared full, it was removed from the water. The plankton was washed from the inside of the net using an aspirator bottle filled with lake water and sprayed on the outside of the mesh so as not to contaminate the catch with new plankton. The plankton net catch was then released into 500 mL Nalgene bottles and placed into a cooler filled with ice for transport back to the lab. Plankton towing occurred five times through the season: June 3<sup>rd</sup>, June 19<sup>th</sup>, July 1<sup>st</sup>, July 14<sup>th</sup>, August 4<sup>th</sup> and August 20<sup>th</sup>.

Table 2.1: Distance traveled and volumes for sampling *G. echinulata* in Silver Lake, including mechanical flowmeter rotor start and end counts.

Date	Rotor Start	Rotor End	Rotor Count Difference	Distance Traveled (m)
6.3.14	150	363	213	5.7
6.19.14	134	350	216	5.8
7.1.14	254	493	239	6.4
7.14.14	764	1044	280	7.5
8.4.14	373	628	255	6.9
8.20.14	2651	2854	203	5.5

### *Plankton Tow Evaluation*

Plankton net catches were evaluated in the laboratory after collection. 20 mL of plankton net catch was placed into the 11x13 inch Pyrex glass dish, illuminated from below using a light box. The plankton net catch was diluted with 250 mL of filtered lake water, and 500 live colonies were pulled from the Pyrex dish by hand and washed. This washing method is explained further in Chapter IV, and was performed to rid the filaments of living zooplankton and phytoplankton. There were other cyanobacteria that produce microcystin-LR that can live within the filaments of *G. echinulata*, such as *M. aeruginosa* (Tymowski and Duthie 2000), and having these cyanobacteria in the same sample as *G. echinulata* can bias the MC-LR results (Carey et al. 2007). Thus, the colonies were washed to remove these extraneous cyanobacteria prior to microcystin analysis.

After washing, colonies were placed on a tared Whatman glass microfiber filter (type GF/A) and allowed to dry at room temperature in a desiccator, and were then weighed again. 500 colonies were found to yield approximately 1.0 g dry weight. Filters were then placed into a freezer for future analysis. From the remainder of the plankton tow, ten colonies were removed and placed in a petri dish to be examined under a microscope for an estimation of colony radius for biovolume calculation. The colonies that were analyzed were collected from the plankton tows on June 19, July 1, and July 14, 2014. There were no other dates that provided enough colonies on which to perform a toxin test, as at least 500 colonies were needed (Table 2.1).

### Bio-volume Estimations

From the three plankton tows that produced sufficient colonies for analysis, 25 colonies for each tow were pulled at random. Colonies were placed under an epifluorescence microscope with a stage micrometer, and the radius of each colony was estimated. The average bio-volume for each plankton tow was estimated (Table 2.4). Bio-volume calculations for spherical algae was used (Hillebrand 1999), using the diameter of the mean average of 25 randomly chosen colonies from each plankton tow. Microcystin production was determined using the following formula as described, explained, and used in previous research (Carey et al. 2012):

$$\left( \frac{\mu\text{g MC-LR}}{\text{L}} \right) = \left( \frac{\text{ng MC-LR}}{\text{g}} \right) \times \left( \frac{\text{g}}{\text{mL}} \right) \times \left( \frac{\text{mL}}{\text{colony}} \right) \times \left( \frac{\text{colony}}{\text{L}} \right) \times \left( \frac{0.001 \mu\text{g}}{1 \text{ ng}} \right)$$

Where:

- $\mu\text{g MC-LR L}^{-1}$  = concentration of MC-LR within the water column that can be attributed to the *G. echinulata* colonies sampled.
- $\text{ng MC-LR g}^{-1}$  = mean MC-LR concentration determined by the instrument (ELISA in the 2012 study, LC-MS in this study).
- $\text{g mL}^{-1}$  = specific gravity of an individual *G. echinulata* colony (assumed to be 1  $\text{g mL}^{-1}$ ; Reynolds 2006).” (As cited in Carey et al. 2012).
- $\text{mL colony}^{-1}$  = mean colony biovolume determined by measuring the radius of 25 randomly chosen colonies.
- $\text{colony L}^{-1}$  = mean water column *G. echinulata* density in each lake.

### *Microcystin Analysis: Extraction*

Filters containing *G. echinulata* colonies were transferred to 10 ml marked vials and placed in an ultralow freezer for a minimum of 12 hours. After freezing, the filters were lyophilized for a minimum of 8 hours. The filters were then extracted by adding 5 ml of 75% methanol and 25% water, and then sonicated for 3 minutes. The extract was filtered using 0.25µm GF syringe filter, and placed in a 2 ml autosampler vial containing 40 ng/ml of Nodularin as an internal standard. Extracts were analyzed for MC-LR, MC-RR, and MC-YR using a Thermo Surveyor MSQ under the following conditions:

<b>MS DETECTOR PARAMETERS</b>						
PROBE TEMP: 500 DEGREES C	MASS	SPAN	TIME	DWELL	POLARITY	CONE (V)
SIM1 - MICROCYSTIN RR	519.99	0.20	5-12 MIN	0.20 MIN	POS	80
SIM2 - INTERNAL STANDARD	825.72	0.20	5-12 MIN	0.20 MIN	POS	80
SIM3 - MICROCYSTIN LR	995.95	0.20	5-12 MIN	0.20 MIN	POS	80
SIM4 - MICROCYSTIN-YR	1045.96	0.20	5-12 MIN	0.20 MIN	POS	80
<b>HPLC Conditions</b>						
	TIME	FLOW	A	B	C	
A) HPLC WATER	0	0.40ML/MIN	90	0	10	
B) ACETONITRILE	1 MIN	0.40ML/MIN	90	0	10	
C) ACETONITRILE (0.10% FORMIC ACID)	6 MIN	0.40ML/MIN	0	90	10	
	12 MIN	0.40ML/MIN	0	90	10	
	12.01 MIN	0.40ML/MIN	90	0	10	
	20 MIN	0.40ML/MIN	90	0	10	

The column used was a Phenomenex Kinetex 100mm x 3.0mm C18 2.6u 100A, and the standard curve was 100/50/25/10/5 ng/ml.

## RESULTS

### *Population Density*

We found that *G. echinulata* populations increased towards the middle of the summer, and peaked on July 1 (Table 2.2). Colonies were not found in the plankton tow on June 3, nor in the plankton tow on August 20.

Table 2.2: Colonies per liter in Silver Lake found per sampling date.

Date	Distance Traveled (m)	Volume Filtered (m <sup>3</sup> )	Volume Filtered (L)	Colonies In Sample	Colonies Per Liter
6.3.14	5.7	0.40	404	0	0.0
6.19.14	5.8	0.41	410	958	2
7.1.14	6.4	0.45	453	15854	35
7.14.14	7.5	0.53	531	733	1
8.4.14	6.9	0.48	484	3	<0.01
8.20.14	5.5	0.39	385	0	<0.01

### *Toxin Analysis*

Microcystin congeners were not detected in the June 19 sample, meaning, there was no MC-LR or MC-RR detectable in these samples. The amount of MC-LR found in the second sample date, July 1, was more than six times higher than the third sample date just two weeks later (Table 2.3). MC-RR concentrations only increased by ~40% during the same period. The highest MC-LR production, measured on July 1, corresponds to sampling date with the highest colonies per liter. Colonies per liter were less on July 14 than on June 19, but MC-LR and RR production were high enough on July 14 to produce measurable results, whereas on June 19, MC congener production was not detectable.

Total microcystin concentrations declined from 94 ng/g dry weight to 31 ng/g dry weight

from July 1 to July 14. The %MC-RR increased from 36% to 71% during the same period. Microcystin-YR was not detected in the Silver Lake samples.

Table 2.3 MC-RR, MC-LR, and total MC expressed as ng/g dry weight for *G. echinulata* on three separate sampling dates in Silver Lake during the 2014 growing season (Total MC = MC-RR + MC-LR).

Sample Date	MC-RR (ng/g dry weight)	MC-LR (ng/g dry weight)	MC-YR (ng/g dry weight)	Total MC (ng/g dry weight)
June 19, 2014	< 0.025	< 0.025	< 0.025	< 0.050
July 1, 2014	34	60	< 0.025	94
July 14, 2014	22	9	< 0.025	31

#### *Bio-volume Averages and Estimated Microcystin Concentration*

Bio-volumes for colonies averaged between 0.03 and 0.35 mm<sup>3</sup>. A higher bio-volume was observed later in the season (Table 2.4). Using a calculation derived from previous experiments (Carey et al. 2012) and described above, total MC-LR, total MC-RR, and total MC concentration attributable to *G. echinulata* was determined. Toxin production was higher on July 14 than June 19, but colonies per liter were lower on July 14 than June 19. Thus, more toxin was produced on July 14 than was produced by a lower colony density sampled June 19. Colony density peaked mid-summer (July 1).



Table 2.4 Average bio-volume and microcystin concentration ( $\mu\text{g MC-LR L}^{-1}$  and  $\mu\text{g MC-RR L}^{-1}$ ) attributable to *G. echinulata*. Plankton tows occurred in Silver Lake between June 19 and July 14, 2014.

Sample Date	Average Bio-Volume ( $\text{mm}^3$ )	Colonies $\text{L}^{-1}$ (Colony Density)	Microcystin-LR Concentration ( $\mu\text{g MC-LR L}^{-1}$ )	Microcystin-RR Concentration ( $\mu\text{g MC-RR L}^{-1}$ )
June 19, 2014	0.0366	2	$2.13 \times 10^{-6}$	$2.13 \times 10^{-6}$
July 1, 2014	0.0788	35	0.1659	0.0930
July 14, 2014	0.3568	1	0.0046	0.0106

## DISCUSSION

### *Population Density*

*G. echinulata* colonies in Silver Lake reached peak densities that were comparable to previously reported results from similar lakes, but much lower than lakes with long-established colony growth. For example, Lake Sunapee (New Hampshire, USA) was found to have a colony density of  $2.96 \text{ L}^{-1}$  (Carey et al. 2007), while Lake Erken, often considered the archetype for *G. echinulata* blooms, can experience colony densities of  $5000 \text{ L}^{-1}$  (Carey et al. 2007). The peak colony density measured in Silver Lake was  $35 \text{ colonies L}^{-1}$ .

The winter preceding my sampling was approximately  $10^\circ\text{C}$  colder than average (NOAA 2014). This may be the reason colony counts were low. Previous research has shown that light and temperature are two of the most important factors in *G. echinulata* germination (Karlsson-Elfgren et al. 2004; Barbiero 1993). An extended winter where ice covered the lake longer than normal would inhibit light filtering to the top layer of sediment where the akinetes overwinter. Temperatures also would be lower, and recruitment into the water column would be delayed.

The bio-volume of the colonies was comparable to that found in previous research. Previous experiments found a colony bio-volume between  $0.01 \text{ mm}^3$  and  $0.34 \text{ mm}^3$  (Carey et al. 2007). My experiment found similar average bio-volumes, being between  $0.03 \text{ mm}^3$  and  $0.37 \text{ mm}^3$ .

### *Microcystin Production*

MC concentration per gram of dry weight was comparable to other experiments. Results were reported by Carey et al. (2007) of 94 ng/g dry weight MC-LR using an Envirologix ELISA kit. Previous research has shown MC concentrations attributed to *G. echinulata* to be  $1.16 \times 10^{-5}$  g MC-LR L<sup>-1</sup> (Carey et al. 2007). Further studies along the Eastern United States coastal states found MC concentrations ranging from 58 to 7,148 ng microcystin-LR g<sup>-1</sup> dry weight (Carey et al. 2012), contributing between  $1.59 \times 10^{-4}$  and  $7.4 \times 10^{-7}$  g MC-LR L<sup>-1</sup>. My study found a concentration of 94 ng/g dry weight MC-T (60 ng/g dry weight MC-LR; 34 ng/g dry weight MC-RR) on July 1. Based on the 54% cross reactivity reported for the Envirologix ELISA kit, our result should correspond to 78 ng/g dry weight by ELISA. MC concentration and average bio-volumes were comparable to other studies, showing that Silver Lake *G. echinulata* do not appear to be morphologically different from other colonies described elsewhere.

At the peak of the bloom on July 1, MC production of MC-LR was still ten times below the WHO recommended limit of 1 µg L<sup>-1</sup> for exposure through aquatic sources. However, chronic exposure to MC-LR has been shown to have negative health effects. Microcystin congener production was variable, and a higher MC-RR fraction was observed on July 14. For all of June and July, temperatures at Silver Lake averaged below 25 °C (NOAA 2015), indicating that congener production should be tilted toward MC-LR (Rapala et al. 1997; Rapala and Sivonen, 1998). However, this was not the case during this experiment. These results show that using LC-MS as a method of microcystin analysis is important, as individual congener concentrations can be variable and unpredictable.

Even though colony density was lower July 14 than June 19, MC concentrations were higher during the lower density sampling. The average bio-volume of colonies was larger on July 14 than on June 19, but colony density was lower. This might suggest that toxin production does not necessarily correspond with colony density, but perhaps corresponds instead with colony size, environmental stress, predation, or competition with other cyanobacteria. However, all the results and toxin data from this experiment should be interpreted cautiously, as colony density sampled was very low. Most northern USA lakes with *G. echinulata* blooms had maximum densities in mid to late August (Carey et al. 2012). Peak density in Silver Lake occurred in early July and rapidly declined as other cyanobacteria became dominant (primarily *Microcystis spp.*).

## CONCLUSION

My experiment showed that MC concentrations produced by *G. echinulata* in Silver Lake during 2014 were not high enough for human health concerns. While this is encourage news, there is the possibility that blooms in future summers may increase due to cultural eutrophication and climate change, resulting in an increase of MC production and greater potential for health hazards. Previous research also indicates that chronic exposure to low levels of both MC-RR (Cazenave et al. 2005) and MC-LR (de Figueiredo et al. 2004) has deleterious health effects, and chronic exposure to MC may become an issue in Silver Lake if blooms persist. Experiments have also shown that MC-RR can increase *E.coli*, which in itself is a hazard to health (Yang et al. 2008).

In addition to chronic exposure concerns, monitoring on Silver Lake should continue, as other studies suggest that toxin production is not related to taxonomic status. Previous research has shown that individual species, while possessing the ability to produce toxins, do not necessarily produce toxins simply as a function of their species (Asencio 2013). This means that while *G. echinulata* has the physiological potential to produce toxins as a species, the organism may produce toxins in one water body, while not producing toxin in another water body. Thus, while *G. echinulata* was found to produce low amount of toxin in this study, future studies in other lakes, whether similar bathymetrically or not, might produce different toxin results.

This experiment is the first to use LC-MS to examine microcystin congener profiles in *G. echinulata*, and the first to report the production of MC-RR using this method of analysis. The presence of a substantial fraction of MC-RR indicates that ELISA methods would overestimate MC-LR concentrations due to cross reactivity.

Implications for management suggest that MC concentrations produced by *G. echinulata* in Silver Lake are not high enough to cause concern, although overall microcystin production should continue to be monitored, as toxic MC-LR could be produced by other cyanobacteria residing in the lake.

## CHAPTER III

### AKINETE DEVELOPMENT FROM PELAGIC COLONIES

#### ABSTRACT

Akinetes are thick-walled reproductive structures, which may serve as a resting stage and ensure survival during adverse growth conditions. *G. echinulata* produces akinetes mid-way through its life cycle in response to unfavorable environmental conditions, usually brought on by seasonal change. This study examined inducing akinete differentiation from vegetative cells in the laboratory earlier than the cyanobacterium's natural cycle by creating adverse environmental conditions within an incubation chamber. We found that by lowering the incubation temperature and decreasing the length of a light source to mimic autumn lake conditions, akinetes could be encouraged to develop from vegetative cells in pelagic colonies after 14 days. Currently, this is the first report of generating akinetes in the lab from pelagic *G. echinulata* colonies that are mid-way through their natural life cycle. This method has implications for future research in that scientists who are unable find overwintering akinetes within the sediment, and have a need to continue experimentation. Whole water colonies are easier to obtain than sediment overwintering akinetes, as they can be found with a simple plankton net tow. Researchers who do not have the time or resources at their disposal to gather sediment and search for akinetes might be able to induce akinetes using this method.

## INTRODUCTION

### *G. echinulata* Life Cycle

In the spring, rising air temperatures warm the water column, and light becomes more available to organisms. *G. echinulata* akinetes contained in senesced filaments and mucilage that were resting on the lake bottom begin to send out germlings from within the akinetes (Karlsson-Elfgren et al. 2004). These germlings form basal heterocysts, which come together with other germlings. Eventually, multiple germlings, which at this point are filaments, will form filament bundles. The filament bundles eventually position themselves so that the heterocysts point inwards, and the bundle then becomes a spherical colony (Karlsson 2003). The colony is held together by a mucilaginous sheath that forms over the heterocysts (Barbiero 1993). As water column temperatures and light increases, the new colony makes its way into the pelagic zone in one of two ways- either passively or actively (Carey et al. 2014). If passive, the colony will enter the pelagic zone as the sediment and water column is stirred by the spring winds. If active, the colony will form gas vacuoles and will rise into the water column itself.

Once the colony is in the water column, it will use its heterocysts to fix elemental nitrogen. All of its phosphorus needs will be met internally, as *G. echinulata* is the only cyanobacteria currently known to obtain its phosphorus requirements from the sediment during the overwintering period (Tymowski and Duthie 2000). Thus, *G. echinulata* often is neither nitrogen nor phosphorus limited.

Eventually, light will become limited and temperatures will drop within the water column during autumn. *G. echinulata*, sensing stressful conditions, will begin to transform a portion of its vegetative cells into akinetes (Forsell and Pettersson 1995;



Barbiero 1993). The gas vacuoles will begin to disappear, and the colony will sink to the bottom of the water column. Eventually, the vegetative cells will either die or be grazed. The dead colony is eventually joined by other dead colonies. The benthos aggregate then consists of akinetes, dead filaments, and mucilage. In the spring, temperatures will again warm the water column, light availability will increase, and the *G. echinulata* life cycle will begin anew.

### *The Akinete*

The akinete is a three walled, hardened cell that forms from the vegetative cells of cyanobacteria in the order Nostocales (Baker 1999). Akinetes are resistant to desiccation, cold, and lack of nutrients (Forsell and Pettersson 1995; Tymowski and Duthie 2000) – forming as environmental conditions become harsh, or as predation increases (Baker 1999). Akinete cells differentiate from the vegetative cells of the organism (Miller and Lang 1967), and new vegetative cells germinate from within the akinete once environmental conditions improve (Kaplan-Levy et al. 2010). Wyman and Fey (1986) discovered that the akinetes of *G. echinulata* begin to differentiate when light quality is poor – and that green light stimulates this differentiation.

The akinete cell can be up to ten times larger than the vegetative cells of the organism (Kaplan-Levy 2010). Akinetes have a thick cell wall that surrounds an inner multi-layered envelope (Nichols and Adams 1982). The akinete itself contains glycogen, lipids, and large cyanophycin deposits, in addition to the thylakoid membranes retained from being a vegetative cell (Kaplan-Levy et al. 2010).

In Chapter IV we examine how sediment phosphorus concentration and temperature may interact to increase germination rates of *G. echinulata*. A previous

experiment that examined the role of temperature used non-germinated akinetes that were collected from overwintering sediment (Karlsson 2004). In my study, a preliminary examination of sediment in Silver Lake, by coring and petite PONAR grabs did not reveal overwintering akinetes. However, sampling of the pelagic zone later in the season found planktonic *G. echinulata* colonies in the lake. In order to continue the remainder of this thesis experiment, a laboratory based approach to induce akinete development was explored.

As previously stated, akinetes develop when environmental conditions become unfavorable. Based on previous research, we hypothesized that by creating conditions in the laboratory similar to what *G. echinulata* might experience within the lake as the summer turned to fall, we could encourage the cyanobacterium to produce overwintering akinetes. These akinetes could be used for recruitment and germination experiments when benthic, overwintering akinetes cannot be found.

## METHODS

### *Live Colony Acquisition*

In order to obtain live colonies, a 35  $\mu\text{m}$  plankton net (Wildco) was towed alongside a johnboat at idle speed between two different locations on opposite sides of the lake, for approximately 5 – 8 meters. Once the net appeared full, it was removed from the water. The plankton within the net was washed from the inside of the net using an aspirator bottle. The aspirator bottle was filled with lake water and sprayed on the outside of the mesh so as not to contaminate the catch with new plankton or new live colonies that were not part of the original catch. The plankton net catch was then released into 500 mL Nalgene bottles and placed into a cooler filled with ice for transport back to the lab.



Figure 3.1: Counting dish above a lightbox, used for sediment evaluation and colony counting.

Plankton towing occurred five

times through the sampling season: June 3, June 19, July 1, July 14, August 4, and August 20, 2014.

Plankton net catches were evaluated at the lab after each plankton tow. 20 mL of plankton net catch was placed into the 11x13 Pyrex glass dish, illuminated from below using a light box. The plankton net catch was diluted with 250 mL of filtered lake water (Whatman GF/A, pore size 1.6  $\mu\text{m}$ ), and live colonies were pulled from the Pyrex dish by hand (Figure 3.1).

### *Filtering Lake Water*

Lake water was collected in a 19 L carboy at the same time a plankton tow occurred (see above for sampling dates). Lake water was filtered through a Whatman glass microfiber filter (type GF/A, pore size 1.6  $\mu\text{m}$ ) to remove zooplankton that might graze on live *G. echinulata* colonies. Filtered lake water was collected below the filter in a 4L glass flask. It was then poured from the flask into 500mL Nalgene bottles. The bottles of filtered lake water were then placed into an incubation room held at 10°C.

### *Washing the G. echinulata*

*G. echinulata* is a filamentous cyanobacterium, which can host zooplankton, such as rotifers, within the filaments (Tymowski and Duthie 2000). Zooplankton living within the filaments that are not removed have been observed to prey on colonies, killing the

colony before the experiment begins.

For this reason, we

“washed” the

colonies after

returning to the lab.

To wash the *G.*

*echinulata*, 10 mL

pipettes of

phytoplankton were removed from the sampling bottles in which they had been stored,

and placed into an 11x13 Pyrex glass dish, which was then placed onto a light box

(Figure 3.1). The 10 mL of phytoplankton sample was then diluted with 250 mL of

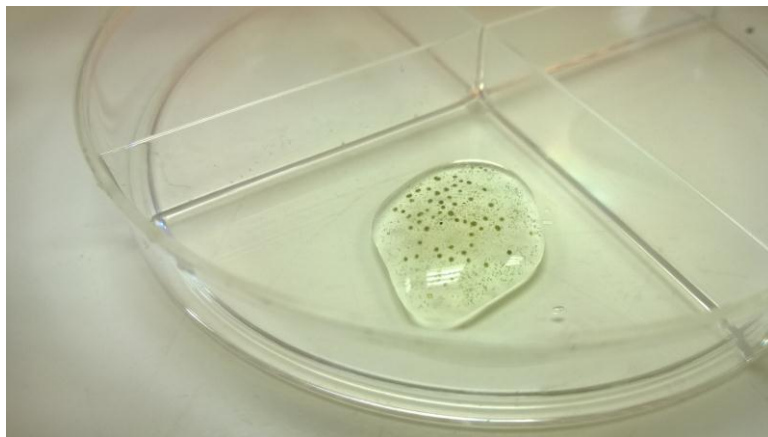


Figure 3.2 *G. echinulata* colonies placed into a 4-quadrant petri dish for washing, to remove predacious zooplankton living within the filaments.

filtered lake water. The *G. echinulata* colonies were removed from the Pyrex dish by hand with the aid of a magnification lens. They were placed into one well of a four-quadrant split petri dish (Figure 3.2). After all the colonies in the 10 mL pipette subsample had been removed from the Pyrex dish and placed in the petri dish, filtered lake water was vigorously added to the quadrant of the petri dish containing the *G. echinulata* colonies using a 1 mL plastic transfer pipette.

The *G. echinulata* were then removed with a micropipette from the quadrant, and placed into the next quadrant of the petri dish, careful to leave any zooplankton or phytoplankton that had been washed from the filaments behind. This washing process was repeated, with rinsing and *G. echinulata* being moved from quadrant to quadrant, until the *G. echinulata* colonies had been rinsed a total of 8 times. One colony was then selected at random and examined under a Nikon stage microscope at 40x to determine if anything was left living in the filaments, as well as to examine the *G. echinulata* for existing akinetes. No akinetes were found to have

developed in any of the randomly selected colonies. If the filaments appeared empty of phytoplankton or zooplankton, the colonies were placed into a separate, non-quadrated



Figure 3.3. Test tube rack with one *G. echinulata* colony and 30mL of filtered lake water per tube.

petri dish for holding. This process was repeated until the 500 mL Nalgene bottle containing the phytoplankton tow was empty.

#### *Akinete Germination*

To set up the akinete development experiment, one hundred 35mL glass test tubes (Pyrex) were placed into two test tube racks. 30mL of filtered lake water was placed in each test tube (Figure 3.3). One washed *G. echinulata* colony was placed in each test tube, for a total of one hundred incubating colonies. The tube racks were placed in an incubation room at 10°C under a 12:12 L:D cycle. The tubes were examined daily to assess if gas vacuoles had disappeared, indicating the colonies were preparing for overwintering. Each day, the colonies were examined from

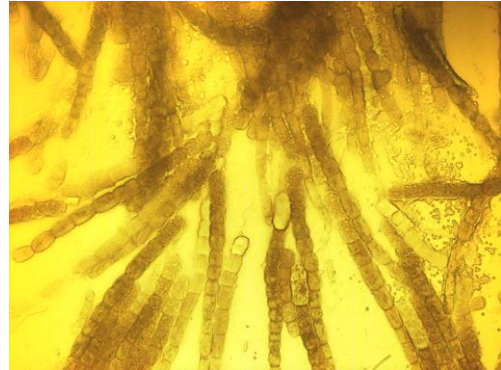


Figure 3.4 *G. echinulata* from Silver Lake at 40x before incubation, taken August 15<sup>th</sup>, 2014. Basal heterocysts are observable, but elongated cells (akinetes) are absent.

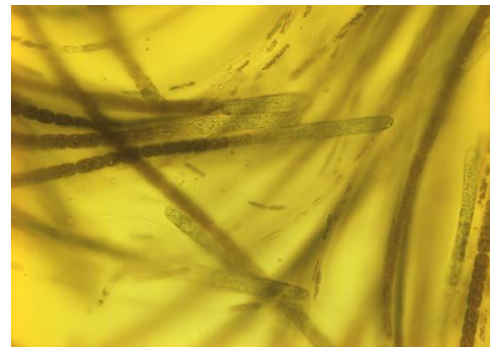


Figure 3.5 Elongated terminal cells (akinetes) observed in *G. echinulata* from Silver Lake after eleven days of incubation.

outside the test tubes. It was concluded that when the colonies went from floating in the water column of the test tube to resting on the bottom of the test tube, the gas vacuoles had disappeared. After 14 days of incubation, two *G. echinulata* were randomly selected, one from each test tube rack, and examined under a Nikon stage microscope under 40x power to examine the colonies for akinete development.

## RESULTS

### *Incubation Results*

The first *G. echinulata* colony to fall to the bottom of the test tube, indicating a lack of gas vacuoles, occurred ten days after incubation had started. Over 1/3 of the *G. echinulata* appeared to have lost a considerable amount of chlorophyll at this point, and appeared as a dull olive green. By eleven days after incubation had started, over 95% of the *G. echinulata* had fallen to the bottom of the test tubes. Previous to beginning the experiment, *G. echinulata* were examined for pre-existing akinetes. As no akinetes were discovered in a random selection of live colonies (Figure 3.4), we were able to compare incubated colonies with pre-incubated colonies. After 14 days, only two colonies were not yet at the bottom of the test tube. The two colonies randomly chosen and examined after 14 days had each developed elongated cells as akinetes (Figure 3.5). It was not known if more akinetes developed over time, as the colonies that were examined appeared to have akinetes on most of their filaments, and more colonies were not sacrificed to observation, as the incubating colonies were to be used for the remainder of the subsequent experiments (see Chapter IV). Incubating colonies that were sacrificed for examination had colony diameters of 550  $\mu\text{m}$  and 600  $\mu\text{m}$ . It can be reasoned that colonies that were used for the experiment would be similar in size, as all the colonies incubated were collected at the same collection time.

## DISCUSSION

By inducing akinetes to develop in pelagic colonies of *G. echinulata*, germination experiments can be performed without the mechanical sorting and separation of senesced colonies from the sediment. Though the life cycle of naturally overwintering akinetes has not been compared to artificially induced akinetes through experimentation, akinetes were successfully used in continuation of this experiment (see Chapter IV). These artificially induced akinetes germinated successfully.

During sampling, colonies per liter were calculated and it was found that these colonies per liter were similar to lakes in other studies. Lake Erken in Sweden has blooms of 50 to 5000 colonies per liter (Carey et al. 2012). A study of 36 east coast oligotrophic and mesotrophic lakes in the United States by Carey et al. in 2013 had between 0.01 colonies per liter and 100 colonies per liter, with some lakes having surface scums as high as 250 colonies per liter. Our results indicated colony density was between 1 and 35 colonies per liter, depending on the season. Thus, Silver Lake did not have as large a colony density as, Lake Erken, but did have results similar to the lakes on the eastern coast of the U.S.

Our study was done in the spring, summer, and fall of 2014. Michigan was below average temperature for 2013 (NOAA 2014). Indeed, the winter of 2013-2014 saw what was colloquially called a “polar vortex” that crossed over the Midwest in January of 2014. This low-pressure system brought unusually bitter cold temperatures and an extended winter to Michigan. Temperatures were, on average, ten degrees colder than “normal” (NOAA 2014). This intense cold the winter preceding sampling may be the reason colony counts per liter were low. Previous research has shown that light and



temperature are two of the most important factors in *G. echinulata* germination (Karlsson-Elfgren et al. 2004; Rengefors et al. 2004; Barbiero 1993). An extended winter where ice and snow covered the lake longer than normal would inhibit light filtering to the top layer of sediment where the akinetes overwinter. Silver lake experiences snow cover for a large portion of the winter. Temperatures would also be lower, and recruitment into the water column could be delayed.

Akinete formation has been discussed in previous literature in both *G. echinulata* and other similar filamentous cyanobacteria, such as *Cylindrospermopsis*. Vegetative cells in *Cylindrospermopsis* were found to undergo drastic morphological changes, which resulted in a multi-cellular, hardened cell that stored nutrients (Miller and Lang 1968). This study on *Cylindrospermopsis* also reported new germlings forming from old akinetes, where the germling “pushes” its way through the wall of the akinete via pores. Only akinetes in this study were observed to germinate, suggesting that germination in akinete-forming cyanobacteria is done only through the akinete. Germination from old akinetes was also observed in this study.

Akinete formation can be “triggered” by a variety of factors, depending on the genus. For example, a lack of potassium in water systems will trigger akinete formation in *Aphanizomenon ovalisporum* (Sukienik et al. 2007). *G. echinulata* akinete formation was previously found to be largely influenced by light (Wyman and Fey 1986) and temperature (Barbiero 1993). Notably, the factors that influence akinete formation in *G. echinulata* are the same as the factors that were found to influence recruitment (see Chapter IV), but at the opposite end of the metric. For example, it was found that a decrease in light and a decrease in temperature induced akinete formation, and that an

increase in light and an increase in temperature facilitated recruitment (Barbiero 1993; Karlsson-Elfgren et al. 2004).

Akinete formation has been discussed previously in literature, but has mostly been focused on germination from overwintering akinetes, rather than induced akinetes from pelagic colonies. There is very little, if any, research done on akinete formation from colonies that are harvested from the water column mid-life cycle. Previous literature has also focused on length of time to germination. Previous research shows that colonies harvested from migration traps and then incubated for germination experiments had germination that varied between 1 and 5 days, and occurred from the old akinetes (Karlsson 2003). However, the experiment published by Karlsson in 2003 did not evaluate colonies for existing akinetes previous to experimentation, so it is unknown if colonies harvested from migration traps had akinetes, or if the incubations performed in the experiment induced akinetes.

There has been previous research on akinete germination from cultured colonies of other cyanobacteria species. Akinetes were induced from cultures of *Anabaena cylindrica* in an iron-limited BG-11 medium. Akinetes developed in this fashion were allowed to form for 35 days, though the experiment does not state when akinete formation first began (Olsson-Francis et al. 2009). An experiment performed on cultured *C. raciborskii* triggered akinete formation using temperature shocks (Moore et al. 2004). This experiment showed germination from akinetes and the emergence of germlings from old akinete colonies after 6 days, but, again, did not discuss the length of time to akinete development from cultured colonies. No experiments with akinete germination from laboratory cultured colonies of *G. echinulata* have been previously reported.

## CONCLUSION

Our experiments have shown that by lowering light duration and lowering temperature, akinetes can be induced in pelagic *G. echinulata* colonies earlier than what would occur in a natural life cycle. This information can be useful in cases where overwintering akinetes cannot be detected in the sediment, but germination experiments must proceed. The method also is more reproducible and requires less time than separating senesced colonies from sediment. There might, however, be physiological differences between forced-formation akinetes and naturally forming akinetes, which might affect the concentration of toxin produced, the congener of toxin produced, and the length of time to germination.

Washing, or rinsing, the *G. echinulata* multiple times with filtered lake water successfully removes zooplankton or phytoplankton living within the *G. echinulata* filaments. Using filtered lake water ensures that the *G. echinulata* will not be subjected to shock from type I DI water, or chemicals from filtered city tap water. This is a necessary step before akinete development experiments, so to avoid predation of the *G. echinulata* by zooplankton, as well as to avoid toxin contributions from other cyanobacteria species.

In a natural system, akinetes will form when conditions become harsh, and knowing when *germination* occurs appears to be more important than knowing when akinetes will develop. Likely, *G. echinulata* colonies may not germinate as successfully in a lake when the germination spring is preceded by an unusually cold winter. This is important to note for natural resources managers, as blooms of *G. echinulata* may not be the primary concern for management after a colder than average winter.

## CHAPTER IV

### PHOSPHORUS AND TEMPERATURE INCUBATIONS

#### ABSTRACT

Phosphorus is accumulated by *Gloeotrichia echinulata* during its benthic phase as an overwintering akinete or germling. The stored phosphorus is used to sustain colony growth throughout the bloom season. However, sediment phosphorus concentration has rarely been examined as a factor when evaluating *G. echinulata* recruitment. Increased temperature has been shown to influence *G. echinulata* recruitment from the sediment, suggesting that global climate change may increase the frequency of blooms. Our experimental objectives were to examine how sediment phosphorus concentrations and temperature could interact singly, and together, to initiate earlier germination in *G. echinulata* colonies. This was accomplished by incubating colonies of *G. echinulata* with laboratory induced akinetes. Colonies were obtained from Silver Lake, in Oceana County, Michigan, USA. Silver Lake is an oligo-mesotrophic (5 to 10 µg/L of total phosphorus) recreational water body that is 7 m at its deepest point, and has predominantly sandy sediment with very little organic matter.

The experiment was conducted for thirty days under three different temperature treatments (17 °C, 19 °C and 23 °C), and at four different sediment total phosphorus concentrations (25 mg/kg, 35 mg/kg, 94 mg/kg, and 225 mg/kg). A secondary objective was to examine how temperature and phosphorus increased germination bio-volume. This was examined by quantifying the number of incubating colonies that germinated

under the previously mentioned temperature and phosphorus treatments. Results, using Kaplan-Meier survival curves and pair-wise comparisons, showed a significant difference in the temperature and phosphorus treatments. A non-statistically significant result ( $\chi^2 = 0.2$ ,  $df = 1$ ,  $p = 0.622$ ) when performing point-wise comparisons between the highest temperatures and various TP-P indicates that high temperatures produced earlier germination, regardless of TP-P. However, at lower temperatures, TP-P became important in germination times ( $\chi^2 = 7$ ,  $df = 1$ ,  $p = 0.01$ ).

Our results suggest that at higher temperatures, sediment phosphorus could become a negligible factor in reducing germination times. However, our results also indicate that even if temperatures do not increase globally, an increase in sediment phosphorus will produce reduced germination times. Reduced germination times will lead to blooms that occur earlier and persist longer. Our data suggest that increased global temperature associated with climate change, as well as an increase in sediment phosphorus from cultural eutrophication, could enhance recruitment of *G. echinulata* and intensify bloom events.

## INTRODUCTION

In algae and cyanobacteria, phosphorous is taken up in a certain atomic ratio, called the Redfield ratio (Redfield, 1934). The Redfield ratio, named after Alfred Redfield in 1934, is an important ratio for all aquatic life. The Redfield ratio was developed to explain the near-same N:P ratio found in marine plankton and seawater. This ratio is 106C: 16N : 1P, meaning that under nutrient sufficient conditions, algal biomass contains 1 phosphorous atom for every 16 nitrogen atoms and 106 atoms of carbon. There are anomalies to this ratio, as marine nitrogen uptake can vary between 16 and 60 (Geider and La Roche 2002); however, this ratio is generally accepted as the composition of phytoplankton. This ratio is the accepted N:P ratio in systems that are stable. However, systems that experience flux would experience a different N:P ratio, depending on the limitation experienced within the system. Absolute concentration levels also play a role in producing differing N:P ratios. A high absolute concentration of DIP and a high absolute concentration of dissolved inorganic nitrogen (DIN) would result in neither N nor P being limited.

Deviation from the Redfield ratio in the water column can result in an ecosystem that is nutrient limited. When the N:P ratio is high ( $> 16$ ), phytoplankton become phosphorous limited, as more nitrogen is in the system than there is phosphorous. When there is a low N:P ratio ( $< 16$ ), phytoplankton become nitrogen limited (Brönmark and Hansson 2005, pp.51). Nitrogen limitation is observed more frequently in marine systems. Conversely, phosphorous limitation is generally observed in freshwater systems (Hecky and Kilham 1988; Schindler 1974). This phenomenon may be due to marine systems having relatively less iron in comparison to freshwater systems. Iron, which

forms precipitates with phosphorous, reduces phosphorus availability to aquatic organisms (Brönmark and Hansson 2005). In freshwater systems, however, there is generally more iron, which binds with phosphorous resulting in conditions unavailable for uptake (Brönmark and Hansson 2005).

### *Forms of Phosphorus and the Phosphorus Cycle*

Phosphorus in its elemental form cannot be used for biological functions. However, phosphorus is rarely found in nature in its elemental form due to its reactive nature. In aquatic systems there are two main forms of phosphorus: particulate and dissolved. Both particulate and dissolved phosphorus exist in organic and inorganic forms (Wetzel 2001). Most phosphate (about 80%) is available as organic phosphate (Brönmark and Hansson 2005).

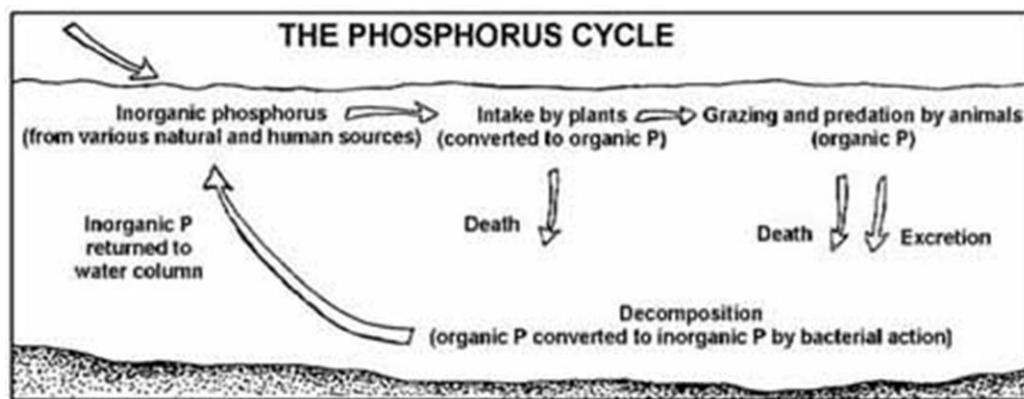


Figure 4.1 A simplified aquatic phosphorus cycle, showing how inorganic phosphorus enters a water system and is taken up by various organisms, and converted to various forms (EPA, 2012).

Particulate phosphorus is defined as the P fraction adsorbed or bound to particles that are retained on a filter. Organic particulate phosphorus can include the phosphorus contained within organisms, including DNA, RNA, ATP, and organism waste, as well as phosphorus that has adsorbed to dead or decaying particulates of organic matter (Wetzel

2001). Conversely, inorganic particulate phosphorus is defined as phosphorus which is bound or adsorbed to minerals, rocks, or clay (Wetzel 2001).

Dissolved phosphorus is defined as the fraction that passes through a filter (usually 0.45  $\mu\text{m}$ ). This is the only usable form of phosphorus for biological processes (Grzybowski and Szydlowski 2013). Dissolved inorganic phosphorus is known by several names, including soluble reactive phosphorus (SRP) and orthophosphate ( $\text{PO}_4^{3-}$ ). SRP, however, could contain colloidal fractions of orthophosphate that pass through the filter. Dissolved inorganic phosphates can also include polyphosphates, which were commonly used as an ingredient in detergents (Grzybowski and Szydlowski 2013). The last soluble phosphorus form, dissolved organic phosphorus, includes organic colloids (Wetzel 2001). In lakes, phosphorus usually is the nutrient limiting growth (Kalf 2002; Brönmark and Hansson 2005; Wetzel 2001). Thus, phosphorus can be considered the limiting factor of production for an entire lake system (Carpenter et al. 1992).

Phosphorus enters a lake either as runoff from terrestrial ecosystems (Bennett et al. 2001; Carpenter 2005), as legacy phosphorus in sediments (Chen et al. 2014; Carpenter 2005), or via atmospheric deposition (Brönmark and Hansson 2005). Phosphorus can also be released by rocks and minerals as soluble phosphorus. This natural weathering occurs slowly, and is the reason why phosphorus is generally limiting in non-polluted systems (Wetzel 2001). Consequently, this released, inorganic phosphorus is taken up by plants and algae and converted to the useable organic phosphorus. Likewise, zooplankton graze on plants and algae, consuming organic phosphorus (Wetzel 2001). When plants and zooplankton die, they are decomposed via bacterial action, and are broken down into inorganic phosphorus, which then either settles



on the sediment or is returned to the water column, where it is again taken up by algae or plants (Figure 4.1).

#### *Phosphorus Release in Sediments*

One consequence of the phosphorus cycle can be increased anoxic conditions on the sediment surface. As plants and animals senesce, the organisms sink to the sediment where bacteria break down the organic matter. As bacteria break down organic matter, oxygen along the surface of the sediment and within the interstitial pores is consumed. The sediment surface becomes anoxic, and redox reactions can then take place. Phosphorus itself does not undergo redox reactions, but its availability in the water column can be influenced by the solubility of iron (Fe), which changes with the redox state of the sediment, the redox state of the sediment surface, and the presence or absence of sulfur (Wetzel 2001).

When water is oxygenated, iron is present as ferric iron ( $\text{Fe}^{3+}$ ), and when joined with phosphorus within the water is present as ferric phosphate. As oxygen levels drop at the benthic layer due to decomposition by microbes, ferric iron is reduced and becomes ferrous iron ( $\text{Fe}^{2+}$ ), which releases the phosphorus into the water column. The ferrous iron then readily binds to sulfides, rather than the phosphorus. The binding of ferrous iron to sulfides reduces the amount of Fe compounds within the water that are available to sequester phosphorus. This phenomenon can result in even larger releases of phosphorus from the sediment. As waters again become oxygenated, either through bio-turbation, wind, or seasonal turnover (in which the cold winter water sinks to the bottom of the lake, pushing the warmer water upwards to the surface), ferrous iron is oxidized

and becomes ferric iron. The ferric iron releases the bound sulfides and it is again available to form compounds with the water column phosphorus.

### *Cultural Eutrophication and Consequences*

The phosphorus cycle is a well-established process of recycling phosphorus within an aquatic ecosystem. However, anthropogenic activity has caused an imbalance of this system, altering the phosphorus cycle and increasing eutrophication of lake systems (Bennett et al. 2001). Eutrophication is defined as the over-enrichment of a system by dissolved nutrients, such as phosphorus or nitrogen, which results in excessive aquatic plant growth and can lead to oxygen depletion. Eutrophication can cause visible surface scums and odors to form in a lake system. Cultural eutrophication is the over-enrichment of lake systems due to anthropogenic causes, including agriculture and urban runoff.

Lake systems were often limited by phosphorus under natural conditions, and therefore depended on new sources of phosphorus from weathering of rocks or atmospheric deposition (Schindler 1977). However, lakes are increasingly becoming less phosphorus limited and more eutrophic, as phosphorus levels rise from agricultural runoff, use of fertilizers, and use of detergents (Bennett et al. 2001). Overall, levels of total phosphorus in global freshwater and terrestrial ecosystems have risen dramatically since the industrial revolution, with at least a 75% increase of phosphorus retention (Bennett et al. 2001).

Eutrophication can lead to a host of issues, including fish kills due to anoxic conditions, increases in harmful cyanobacterial blooms (HABS) (Zurawell et al. 2005), and excessive nuisance plant production (Carpenter 2005). A generally unrecognized

consequence of eutrophication and nutrient over enrichment is persistent eutrophication. Some lakes, such as large, deep lakes, or lakes that have been anthropogenically altered, are slow to recover from eutrophication (Carpenter 2005), and can take hundreds or thousands of years to recover, if they manage to recover at all. This lag in recovery could be due to legacy phosphorus within the sediments (Carpenter 2005).

### *Global Climate Change*

Rising global temperatures have been a topic of concern for several decades. Global climate change is primarily due to trapped atmospheric gases that cause the average temperature of the Earth to rise (Riebeek 2010). These atmospheric gases, also known as “greenhouse gases,” include carbon dioxide, nitrous oxide, ozone, water vapor, and methane. Since the industrial revolution, carbon dioxide in the atmosphere has risen from 280 ppm to 385 ppm, while methane has risen from 715 ppb to 1774 ppb (Boyd and Tompkins 2010). A report released in 2014 by the Carbon Dioxide Information Analysis Center, which is the primary U.S Department of Energy’s climate analysis center, shows a significant increase in these greenhouse gases (Figure 4.2), with methane and nitrous oxide being especially persistent (Blasing 2014). Research by the IPCC in 2007 (IPCC 2007) predicted a global temperature rise between 2°C and 4°C, while newer predictions by NASA predict a rise between 2°C and 6°C over the next decade (Riebeek 2010). This rise is directly responsible for rising sea levels and changing precipitation patterns, leading to localized drought and flooding (National Academy of Sciences 2010).

GAS	Pre-1750 tropospheric concentration <sup>1</sup>	Recent tropospheric concentration <sup>2</sup>	GWP <sup>3</sup> (100-yr time horizon)	Atmospheric lifetime <sup>4</sup> (years)
<b>Concentrations in parts per million (ppm)</b>				
Carbon dioxide (CO <sub>2</sub> )	280 <sup>6</sup>	395.4 <sup>7</sup>	1	~ 100-300 <sup>4</sup>
<b>Concentrations in parts per billion (ppb)</b>				
Methane (CH <sub>4</sub> )	722 <sup>8</sup>	1893 <sup>9</sup> /1762 <sup>9</sup>	28	12 <sup>4</sup>
Nitrous oxide (N <sub>2</sub> O)	270 <sup>10</sup>	326 <sup>9</sup> /324 <sup>9</sup>	265	121 <sup>4</sup>
Tropospheric ozone (O <sub>3</sub> )	237 <sup>1</sup>	337 <sup>2</sup>	n.a. <sup>3</sup>	hours-days

Figure 4.2 A portion of a table from the Carbon Dioxide Information Analysis Center showing a significant post-industrial increase in the concentration of tropospheric greenhouse gases. The troposphere refers to the lower 75-80% of the atmosphere. GWP refers to the “global warming potential” of the various gases relative to an equal amount of released carbon dioxide. Atmospheric lifetime is the persistence of each gas (Blasing 2014).

### *Harmful Cyanobacterial Blooms*

Multiple research articles have predicted that harmful cyanobacterial blooms will increase in frequency and duration with global temperatures (El-Shahawy et al. 2011; IPCC 2007; Paerl and Huisman 2008; Paerl and Huisman 2009; Taranu et al. 2012). As some cyanobacterial blooms produce secondary metabolites known as cyanotoxins, rapid cell growth and reproduction could cause an increase in the production of neurotoxins, skin irritants, or hepatotoxins. . Hepatotoxins and neurotoxins are produced by the cyanobacterial genera that often appear as surface scums, such as *Microcystis*, *Oscillatoria*, *Spirulina*, and *G. echinulata* (Christiansen et al. 2001). *G. echinulata*, for example, produces the hepatotoxin microcystin-LR (Figure 4.3). Toxin producing genera are often found in freshwater sources that are used as drinking water sources (WHO 2003). Therefore, harmful blooms are of especial concern, as drinking water sources could become affected.

Harmful cyanobacterial blooms appear in water that has sufficient nutrients, and are usually over-enriched by nitrogen and phosphorus, making these water bodies eutrophic and hypereutrophic (WHO 2003).

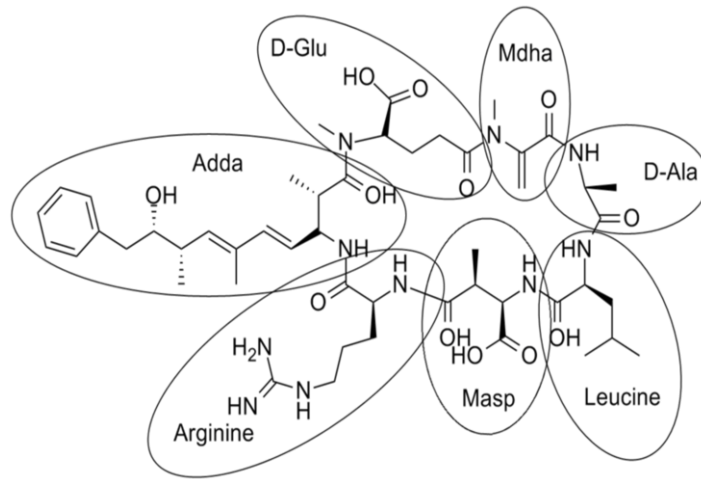


Figure 4.3 Microcystin-LR, which is produced by multiple genera, including *G. echinulata*. Note the amino acids arginine and leucine, which define this as congener —LR, as well as the adda amino acid, which is known to be the reason for the hepatotoxicity in this peptide.

Generally, the temperature of the water that blooms appear in is around 15

and 30°C, with the water column pH level between 6 and 9 (WHO 2003). Blooms usually occur in late summer or early fall (WHO 2003).

#### *The Role of Temperature and Phosphorus in the Recruitment of G. echinulata*

Though most harmful cyanobacterial blooms occur in late summer or early fall, *G. echinulata* begins its migration into the water column as soon as early spring, and can bloom as long as early fall (Barbiero 1993; Karlsson 2003). *G. echinulata* recruitment initiation is based largely on temperature (Barbiero 1993) and light (Karlsson-Elfgren et al. 2004), whereas *G. echinulata* recruitment duration is based largely on sediment mixing and temperature (Karlsson-Elfgren et al. 2004). As climate change is expected to raise global temperatures (Riebeek 2014), it is important to examine how climate change will affect recruitment. Silver Lake is a shallow lake, and experiences sediment mixing

consistently through the cyanobacteria growing season, whether through anthropogenic means, such as boats, swimmers, or jet skis, or natural means, such as wind. The sediment mixing in such a shallow, high-use recreational lake can be considered to be kept constant throughout the season. Thus, we performed recruitment experiments under various temperatures, and maintained sediment mixing by sparging air through a pipette near the sediment surface throughout the duration of the experiment (Karlsson-Elfgren et al. 2004). This method effectively mixed the sediment within the test tube.

*G. echinulata* is the only species of cyanobacteria currently known to uptake all of its phosphorus for seasonal growth from the sediment (Tymowski and Duthie 2000). Sediment-resting *G. echinulata* was found to contain, on average, 45% more phosphorus than needed (Pettersson et al. 1993). A 45% surplus amounted to approximately 81 ng of phosphorus per colony more than the colony needed for benthic growth. This surplus phosphorus was used by the colony to sustain growth in the epilimnion (Pettersson et al. 1993). It is reasonable to conclude that an increase in sediment phosphorus would increase recruitment, as there would be more nutrients available to sustain a larger colonial recruitment. Thus, we performed recruitment experiments under various sediment phosphorus concentrations that occurred in Silver Lake. It was not necessary to examine the effect of water column phosphorus, as it was found in previous research that *G. echinulata* utilizes very little water column phosphorus, regardless of the phosphorus fraction present in the epilimnion (Barbiero and Welch 1992; Istvanovics et al. 1993).

Silver Lake was chosen for this study, as the lake has experienced *G. echinulata* blooms since the summer of 2012. There are concerns about possible toxin exposure due to the heavy recreational use of this lake. This experiment was designed to determine if

increases in sediment phosphorus and temperatures could cause influence bloom events of *G. echinulata* in Silver Lake. Rising phosphorus would be due to anthropogenic inputs, such as that from septic systems, while rising temperatures would be from climate change.

## METHODS

### *Sampling Location: Silver Lake, Oceana Co., MI*

Silver Lake is a 259-hectare water body, located in Oceana County, MI. It is 178.5 m above sea level, and 1.5 m above the mean sea level of Lake Michigan, a Laurentian Great Lake (Fisher et al. 2007). The lake drains to Lake Michigan by way of Silver Creek, located on the southwestern side of the lake. Silver Creek contains a weir that controls the level of lake, and thus nutrient release (Fisher et al. 2007). The lake receives water from three

tributaries: the largest, Hunter Creek, also called Au Sable Creek, as well as two unnamed tributaries, one located near the state park and one located near North Shore Drive. Silver Lake itself is only 7 m deep at its maximum, and is bordered by an active, recreational dune on the

western edge that is about 2 km wide (Fisher et al. 2007). The active dune separates Silver Lake from Lake Michigan. Silver

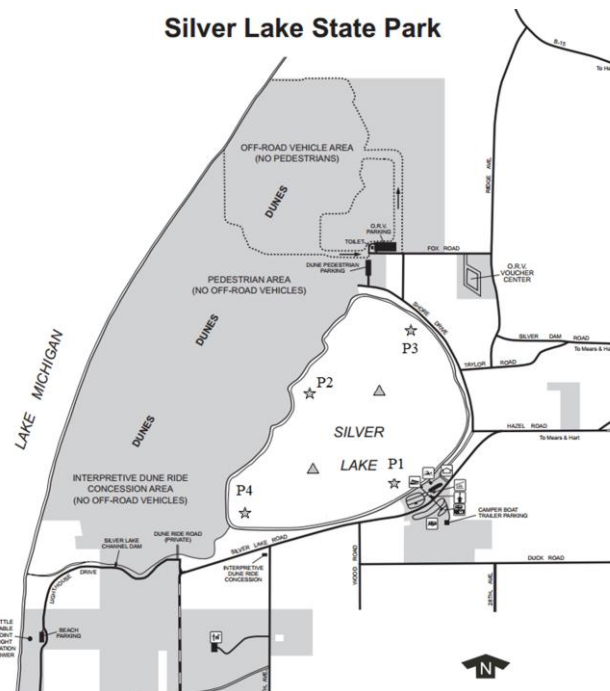


Figure 4.4: Approximate Silver Lake sediment sampling locations (stars) and phytoplankton sampling locations (triangles), Oceana County, MI (adapted from MDEQ 2013). P1 – P4 labeled. Phytoplankton towing occurred five times through the sampling season: June 3, June 19, July 1, July 14, August 4, and August 20, 2014. Sediment sampling occurred August 5, 2014.

Lake State Park is located on the eastern edge of the lake, along with a resort, and multiple lakeside cottages. The lake has predominantly sandy sediment, with very little



deposited organic matter, and has a nutrient and hydraulic residence time of approximately 7 months (Brennan 2015).

Four locations (Figure 4.4) around the Silver Lake littoral zone were used to collect sediment, and two locations within the lake were used to collect pelagic zone water. Sediment was obtained by petite PONAR on 5 August 2014. The sediment PONAR sampling depth was approximately 7.5 cm. Sediment sampling locations were chosen in areas around the lake that were hypothesized to contain varying levels of sediment phosphorus. Sampling locations for the North ( $N43^{\circ} 40.660$ ;  $W086^{\circ}30.003$ ) and South ( $N43^{\circ} 39.653$ ;  $W086^{\circ}30.501$ ) locations were both located near private cottages. The West location ( $N43^{\circ} 40.308$ ;  $W086^{\circ}30.488$ ) was located in front of the recreational dunes, and the East location ( $N43^{\circ} 39.561$ ;  $W086^{\circ}30.465$ ) was located in front of the state park area. Samples were chosen in these areas, as it was expected that phosphorus concentrations in the sediment would differ in areas with high anthropogenic loading, such as the cottages, the state park, and the hotel, all of which are on septic systems, compared to areas with low anthropogenic loading, such as the sand dunes. Each location chosen was also near a USGS sampling well (Brennan et al. 2015).

The sediment samples were placed in individual plastic bags and transported back to the lab on ice. Once in the lab, the sediment from each location was placed into an amber collection jar. Amber collection jars were then placed into freezer storage for one week to kill any living zooplankton within the sediment, and thus inhibit predation of colonies during experimentation.

### *Sediment Evaluation*

Sediment from each location was examined by placing approximately 1 tablespoon of thawed sediment into a 11x13 inch Pyrex glass dish, which was then placed onto a light box. The light box produced light from below the glass dish, illuminating the sediment above. The sediment was then sorted by hand, using a micropipette to move sediment, and with the aid of a magnification lens. If no overwintering akinetes or live zooplankton were found, the sediment was rinsed with 500 mL of filtered lake water, and sorted again by hand. If no akinetes or live zooplankton were found after the third sorting procedure, sediment was placed into a clean amber collection jar.

### *Phosphorus Fraction Testing*

Prior to experimentation, the sediment from all four locations was tested to ensure that total phosphorus concentrations differed. A SEAL analytical Discrete Multi-Chemistry Analyzer AQ-2 was used to analyze the samples for total phosphorus (TP-P), total soluble phosphorus (TSP-P), and soluble reactive phosphorus (SRP-P). Soluble unreactive phosphorus (SUP-P) was determined by subtracting the SRP-P fraction from TSP-P. TP-P was analyzed on whole sediment samples. The remaining phosphorus fractions were analyzed on the porewater fraction. Porewater was obtained from the sediment by centrifugation and filtration through a 0.45  $\mu\text{m}$  syringe filter.

The EPA standard persulfate-digestion ascorbic method was used to determine TP-P and TSP-P (EPA method 119-A Rev.5). 10 mL sample sizes were used and three samples per location were tested. The first test result was used, as the following two tests did not vary from the first at any location. For SRP-P, the acid-molybdate reduction method (EPA method 118-A Rev.4) was used.

Continuing calibration verification (CCV) and continuing calibration blanks (CCB) as QA/QC standards were used, with CCV/CCB standards returning as  $\pm 15\%$  of the target concentration. Matrix spike - matrix spike duplicates (MS-MSD) were also run for QA/QC, and returned within  $\pm 15\%$  of the original spike concentration. Both CCV/CCB and MS-MSD results were within the acceptable lab procedure limits.

#### *Experimental Variables and Incubation Design*

The experiment had two treatment factors (temperature and phosphorus). There were three levels of temperature (ambient temperature (17 °C), +2 (19 °C), and +6 (23 °C)). There were two ambient temperature incubations run, one with each of the increased temperatures, in order to account for possible variation in incubation chambers over two separated months of incubations. The temperature incubations were run as 17 °C and 19 °C from September 2<sup>nd</sup> to October 2<sup>nd</sup>, 2014, and as 17 °C and 23 °C from October 9<sup>th</sup> to November 8<sup>th</sup>, 2014.

There were four levels for sediment phosphorus, one level from each sediment sampling location; P1, P2, P3, and P4, correspond to each of the sampling locations. P1 = east location; P2 = west location; P3 = north location; P4 = south location (Figure 4.4). Colony incubation was set up with one 5x4 test tube matrix in each of two test tube racks. Each matrix contained five replicates of each sediment phosphorus concentration, for a total of twenty colony incubations at 19 °C and 23 °C, and forty colony incubations at 17 °C. Each experimental test tube contained 3 mL of sediment from one of the four locations, one akinete-developed colony, and 25 mL of filtered (Whatman GF/A, pore size 1.6  $\mu\text{m}$ ) lake water.

Germination bio-volume was examined for each test tube in each treatment, and defined as the total number of filaments produced by each incubating colony. The potential for germination of the akinete was called a “germination event.” A germination event was said to occur if filament emerged from a colony.

### *Incubation Procedures*

Incubation at 17 °C was performed in an incubation room. Incubations at 19 °C and 23 °C were run in a Fisher low-temperature incubator (Fisher Scientific, Pittsburgh, PA, U.S.A) set to each respective temperature. Temperature was maintained throughout the experiment within  $\pm 1$  °C. Both the incubation room and the incubators used Sylvania Gro-Lux lights (Osram Sylvania, Danvers, MA, and U.S.A). A Li-Cor LI-193 Quantum Sensor (Li-Cor Biosciences, Lincoln, NB, U.S.A) was used to measure the light output that reached the incubation tubes. Light output (PAR) was set at  $47 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (329.073 foot candles) for both the incubation room and the incubator. Light levels were consistent with shallow-water, pelagic-zone, natural sunlight levels (Wetzel, 2001).

On alternate days of incubation, 20 mL of water was removed from each test tube using a plastic transfer pipette. The water was placed into a plastic 30 mL scintillation vial and was then fixed with two drops of Lugol’s solution. 20 mL of new, filtered lake water was then added to each test tube using a graduated cylinder. Water within each test tube was vigorously mixed every other day with a plastic transfer pipette (Karlsson-Elfgren et al. 2004) to ensure mixing of the sediment, as sediment mixing was found to be one of the most important factors for recruitment (Karlsson-Elfgren et al. 2004). This sediment mixing technique was kept constant for all test tubes through treatments for the entirety of the experiment.

### *Enumeration*

Samples that had been fixed with Lugol's were then placed into Utermöhl settling chambers, and allowed to settle for 24 hours. Samples were then examined under a Nikon Eclipse TE-200 inverted microscope at 40x and 100x. Pictures of samples were taken using a SPOT Insight camera (SPOT Imaging Solutions, Sterling Heights, MI, USA) and Image-Pro Plus software v.6.0 (Media Cybernetics, Rockville, MD, USA). Filaments which had intact vegetative cells were counted as "~~new~~ germination" from the akinete colonies. Filaments which had degraded vegetative cells were considered "~~old~~ filaments" left over from the akinete colony as it began overwintering, and were not counted towards germination. Each sample had new filaments enumerated, recorded, and then disposed of, as it was observed in this experiment that newly germinated filaments soon degraded and were unable to be stored longer than three days.

### *Statistical Analysis*

Statistical software used for analysis was R, v.3.2.0. Kaplan-Meier nonparametric survival curves were estimated for all combinations of temperature and phosphorus levels, and a graph of these estimated survival curves were generated. Survival curves indicate the proportion of *G. echinulata* akinetes that have not yet germinated as a function of time  $t$  since onset of the experiment. Conditions favoring germination will cause the survival curve to decrease faster with time. The null hypothesis of no differences among survival curves was tested using a nonparametric log-rank test of homogeneity (McNair et al. 2012). Potential pairwise differences between treatment groups were also tested using log-rank tests. To assess the quantitative effects of phosphorus and temperature on germination, a semi-parametric Cox proportional hazards

model was used (McNair et al. 2012), with temperature and TP-P, TSP-P, SRP-P, or SUP-P as quantitative explanatory variables (covariates). Preliminary testing was performed prior to the semi-parametric Cox proportional hazards testing, and results and generated graphs (Figures A1 – A4) are reported in the appendix of this thesis. The hazard function is closely related to the slope of the survival function. It is a function of time  $t$  since onset of the experiment, and tells us how likely it is that an akinete which has not germinated by time  $t$  will germinate soon after  $t$ ; the greater the hazard function, the faster the survival function decreases with increasing  $t$ . The Cox proportional hazards model assumes that all akinetes share a common baseline hazard function  $h_0(t)$ , which the covariates increase or decrease. Specifically, the hazard function  $h(t)$  is assumed to have the form

$$h(t) = h_0(t)e^{\beta_1 x_1 + \beta_2 x_2}$$

where  $x_1$  is the phosphorus level,  $x_2$  is the temperature, and  $\beta_1$  and  $\beta_2$  are the corresponding coefficients, analogous to the slope coefficients in a multiple linear regression model. Here,  $h_0(t)$  equals the baseline hazard function, which is estimated by the model, and assumed to be the same for all testing.  $e^{\beta_1 x_1 + \beta_2 x_2}$  represents the regression function that takes into consideration the covariates and the constants. The goal of the analysis is to estimate coefficients  $\beta_1$  and  $\beta_2$  and determine whether they are statistically significantly different from zero. A positive coefficient implies that the corresponding covariate increases the hazard function (which decreases the median time to germination), while a negative coefficient implies the opposite. The assumption of a common hazard function  $h_0(t)$  was assessed using the visual diagnostic plot generated here, and discussed by McNair et al. (2012).

## RESULTS

### *Sediment Testing*

All four sampling locations (Table 4.1) showed different levels of total sediment phosphorus (TP-P). P1 =  $25 \pm 4$  TP-P mg/kg, P2 =  $35 \pm 5$  TP-P mg/kg, P3 =  $94 \pm 14$  TP-P mg/kg, and P4 =  $225 \pm 4$  TP-P mg/kg (Table 4.1). P1 and P2, which were located in front of the state park area and the recreational sand dunes, respectively, had similar TP-P. These locations also had the lowest TP-P, and, thus, the lowest anthropogenic impact. P3, which was located in front of private cottages, and was hypothesized to have a higher anthropogenic impact due to septic system use, showed a total phosphorus concentration that was 2.5 to 3 times higher than P1 and P2. P4, which was also located in front of private cottages and was the closest site to Silver Creek, had a TP-P concentration that was 6.4 to 9 times higher than the P1 and P2, and 2.4 times higher than P3.

Table 4.1: Phosphorus porewater fraction concentrations for all four sampling locations in Silver Lake, as mg/kg. P3 and P4 were both located near private cottages. P1 was located in front of the recreational dunes, and P2 was located in front of the state park area. The EPA standard persulfate-digestion ascorbic method was used to determine total sediment phosphorus (TP-P) and total soluble phosphorus (TSP-P) (EPA method-119-A). The acid molybdate method was used to determine soluble reactive phosphorus (SRP-P). Variation =  $\pm 15\%$ .

Location	P1	P2	P3	P4
SRP-P (mg/L)	$0.0413 \pm 0.0062$	$0.0049 \pm 0.0007$	$0.0346 \pm 0.0052$	$0.0058 \pm 0.0009$
SUP-P (mg/L)	$0.0982 \pm 0.0147$	$0.0427 \pm 0.006$	$0.1374 \pm 0.0206$	$0.9942 \pm 0.1491$
TSP-P (mg/L)	$0.1395 \pm 0.021$	$0.0476 \pm 0.007$	$0.172 \pm 0.0026$	$1 \pm 0.15$
TP-P (mg/kg)	$25 \pm 4$	$35 \pm 5$	$94 \pm 14$	$225 \pm 34$

All four locations also showed different levels of porewater TSP-P and SRP-P (Table 4.1). TSP-P was highest at location P4, near the private cottages and Silver Creek. TSP-P was similar at locations P1 and P3, which were spatially located near one another, and were in front of the State Park and the Northern private cottages, respectively. TSP-P was lowest at P2, which is located in front of the dunes.

Porewater SRP-P was highest at location P1, in front of the state park, but very similar to location P3 which is in front of the Northern Cottages. Again, these two locations are spatially near one another. SRP-P was lowest in front of the Northern private cottages.

### *Germination*

By examining germination, temperature, and phosphorus together, we see that P1 and P2 had similar germination results (Figure 4.5), and similar TP-P (Table 4.1). P3 and P4 had higher germination totals than P1 and P2 (Figure 4.5), and higher TP-P (Table 4.1).

Looking at temperature germination by itself, results show that the 19 °C incubations had more non-germination than the 17 °C and 23 °C incubations (Figure 4.5). The 19 °C and 23 °C incubations individually had higher germination than either 17 °C incubation (Figure 4.5). The first filament produced at the warmest temperature, 23 °C, was on day two of incubation, at the highest TP-P ( $225 \pm 33.75$  TP-P mg/kg). The first filament produced at the lowest temperatures, 17 °C and 19 °C, was at day six of incubation, in the second highest TP-P ( $94 \pm 14.1$  TP-P mg/kg).



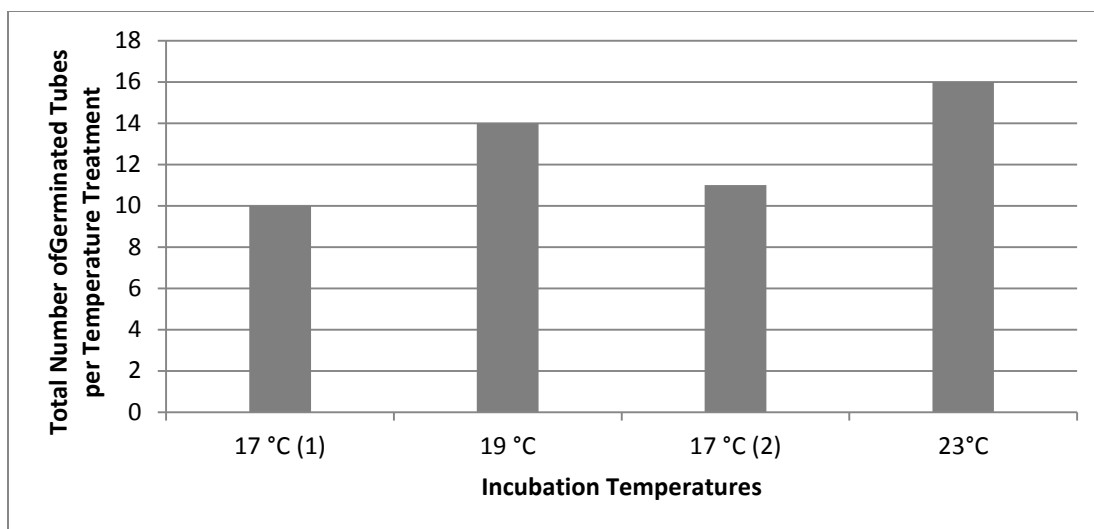


Figure 4.5 Number of *G. echinulata* colonies from Silver Lake that germinated at various incubation temperatures. The highest incubation temperature (23°C) produced the most germination. Each germinated tube represents one germinated colony.

When examining phosphorus germination by itself, results show that P1 had more colonies produce at least one filament than the P2, even though it had a lower total phosphorus concentration. P1 also had nearly the same number of colonies produce at least one filament as P3, even though P1 had less than half the TP-P of the P3 (Figure 4.6).

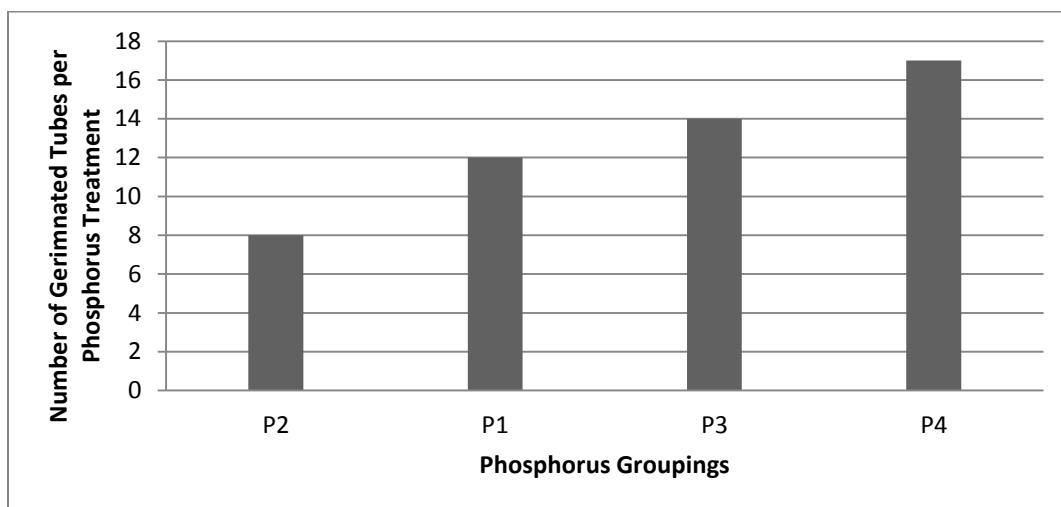


Figure 4.6 Number of *G. echinulata* colonies from Silver Lake that germinated at various phosphorus groupings. P1 =  $25 \pm 4$  TP-P mg/kg, P2 =  $35 \pm 5$  TP-P mg/kg, P3 =  $94 \pm 14$  TP-P mg/kg, and P4 =  $225 \pm 4$  TP-P mg/kg. Each germinated tube represents one germinated colony. Note that P1 had more germinated tubes than P2, but had a lower TP-P.

### *Statistical Testing: Kaplan-Meier Curves and Point-wise Comparisons*

Preliminary tests of the null hypothesis showed very little homogeneity between survival curves among all possible treatment combinations ( $p = 1.51e-05$ ). The p-value indicates that the survival curves for all 12 possible treatment groups were not similar, as  $p \neq 0$ .

Kaplan-Meier survival curves for pair-wise difference testing between all 12 possible variable interactions visually indicate that both temperature and phosphorus affect germination times (Figure 4.7), at different extremes. Consequently, point-wise comparisons were performed for the extreme ends of the treatment groups. At the lowest sediment phosphorus concentration (25 TP-P mg/kg), there is a clear visual difference (Figure 4.6) in germination times between the lowest temperature (Group A) and the highest temperature (Group C). A pair-wise test between Group A and Group C show a statistical difference in germination times ( $\chi^2 = 13$ ,  $df = 1$ ,  $p < 0.001$ ). At the highest sediment phosphorus concentration (225 TP-P mg/kg), there is a visual difference in germination times between the lowest temperature (Group J) and the highest temperature (Group L). A pair-wise test between Group J and Group L confirms this ( $\chi^2 = 6.1$ ,  $df = 1$ ,  $p = 0.0134$ ). These results indicate that temperature has a significant effect on germination times, regardless of sediment concentration. Pairwise comparisons for all potential interactions were analyzed. Only the extremes are shown here, however, the full matrix is located in the appendix of this thesis (Figure A5).

The Kaplan-Meier survival curves also indicate that high temperature had a greater effect than phosphorus concentration. Because the data are not a function of concentration or fraction, but a function of time, there is only one way of characterizing

these groups. There are different ways of characterizing the water (such as by phosphorus fraction), but the water remains the same. Thus, there is only one set of Kaplan-Meier curves to report. Visually, when examining the survival curves at the highest temperature (23 °C) between the lowest sediment phosphorus concentration (Group C) and highest sediment phosphorus concentrations (Group L) we see that the survival curves are very similar. A pair-wise comparison between Group C and Group L shows that there is no statistical difference in germination times at this temperature ( $\chi^2 = 0.2$ ,  $df = 1$ ,  $p = 0.622$ ).

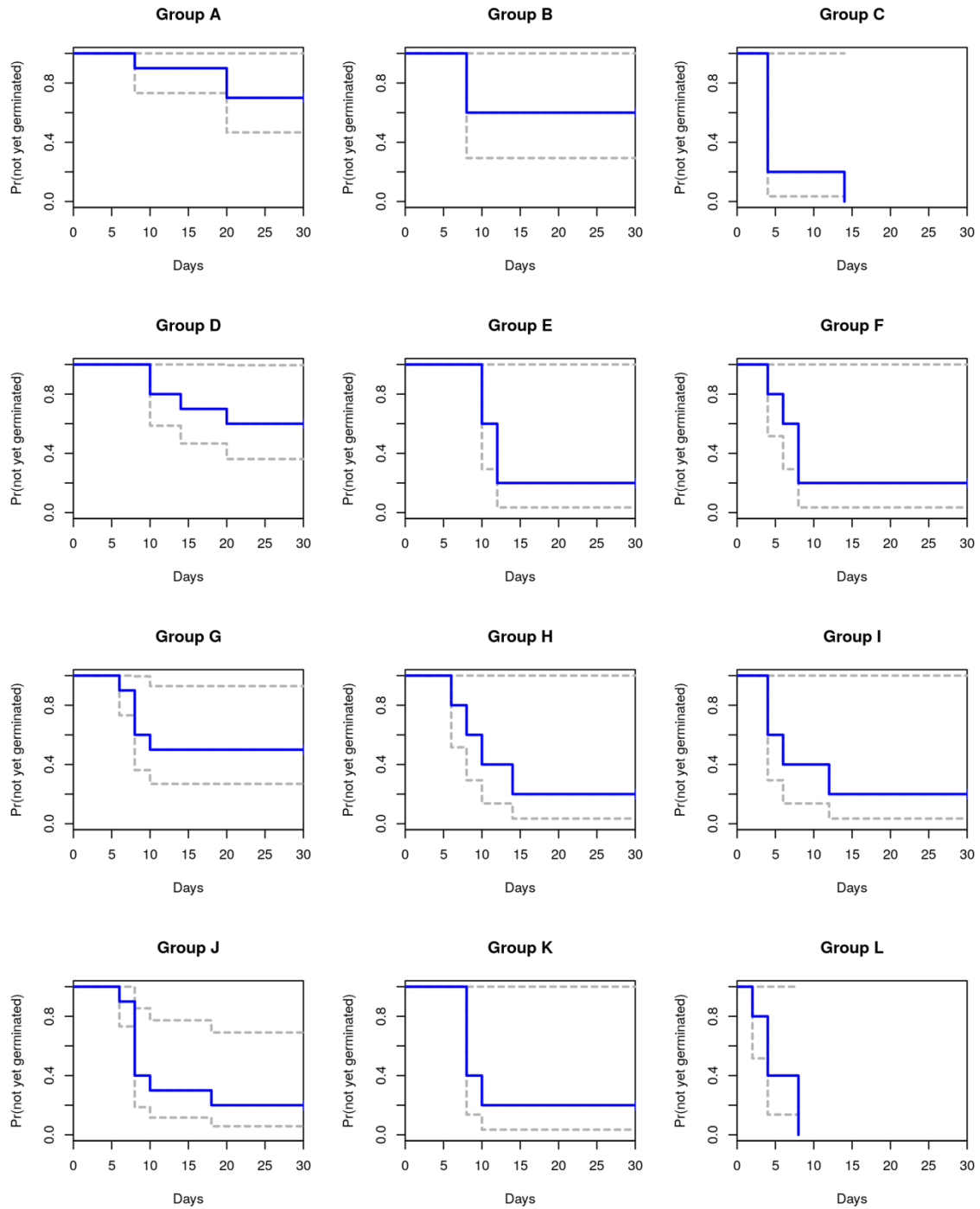


Figure 4.7: Kaplan-Meier survival curves for pair-wise difference testing between all 12 possible variable interactions in *G. echinulata* incubations. The three columns correspond to the three temperature variables (low, medium, high), and the four rows correspond to the four sediment phosphorus concentrations (P1 – P4). Survival curves are indicated by the solid lines, and pointwise 95% confidence intervals are indicated by the dashed lines. Each group represents a different temperature and phosphorus treatment combination. Groupings are discrete, rather than based on predictor variables.

### *Cox Proportionate Hazards Model*

The Cox proportionate hazards model was fit to the TP-P, SRP-P, SUP-P, and TSP-P data (Table 4.2). For clarity in comparison, the survival model used is duplicated below. Because values were very similar, entire values are reported, and no rounding of the reported values occurred.

$$h(t) = h_0(t) \cdot e^{\beta_1 X_1 + \beta_2 X_2}$$

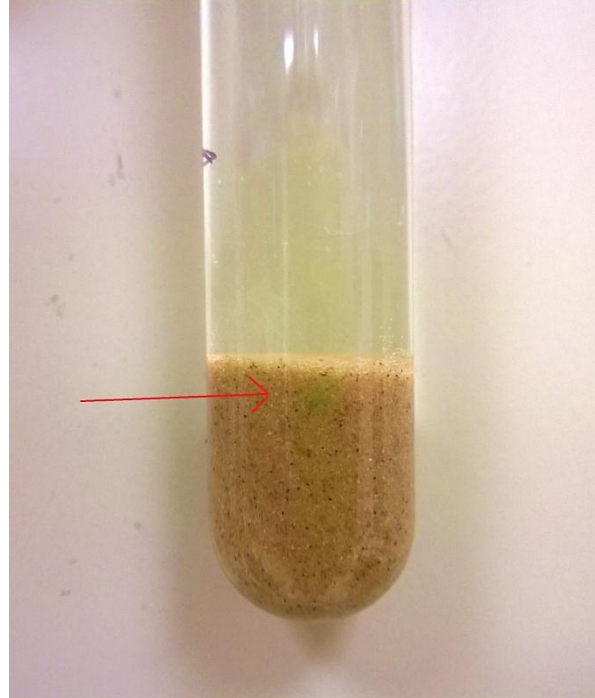
Table 4.2 Results from the Cox proportionate hazards model, fit to TP-P, SRP-P, TSP-P and SUP-P phosphorus fractions from sediment pore water from Silver Lake, MI.

	$\beta_1$	e	S.E. of $\beta_1$	z- score	p- value
X <sub>1</sub> (TP-P)	0.005106	1.005119	0.001712	2.982	0.00286
X <sub>2</sub> (Temp)	0.267969	1.307307	0.055115	4.862	1.16 x 10 <sup>-6</sup>
X <sub>1</sub> (SRP-P)	-10.53	2.677 x 10 <sup>-5</sup>	8.552	-1.231	0.218
X <sub>2</sub> (Temp)	0.2612	1.299	0.05474	4.772	1.82 x 10 <sup>-6</sup>
X <sub>1</sub> (TSP-P)	1.0355	2.8164	0.3441	3.009	0.00262
X <sub>2</sub> (Temp)	0.2748	1.3162	0.0555	4.951	7.38 x 10 <sup>-7</sup>
X <sub>1</sub> (SUP-P)	1.0154	2.7604	0.3368	3.015	0.00257
X <sub>2</sub> (Temp)	0.2747	1.3161	0.0555	4.949	7.45 x 10 <sup>-7</sup>

Results show significance ( $p < 0.05$ ) for all model results, except for SRP-P. SUP-P produced the most significant result of all phosphorus fractions ( $p = 0.00257$ ), and was very similar in significance to TP-P ( $p = 0.00286$ ). Temperature related to all phosphorus fractions was very significant.

## DISCUSSION

Within natural lake systems, researchers often use sediment traps to count germination rates (Carey et al. 2012; Karlsson-Elfgren and Brunberg 2004; Barbiero and Welch 1992). This allows for counting all filaments as they are released within a controlled area. However, even within natural systems, recruitment can vary greatly from year to year. In previous natural recruitment experiments on various cyanobacteria other than *G. echinulata*, recruitment varied between 600 colonies  $\text{m}^{-2} \text{d}^{-1}$  and more than 10 000 colonies  $\text{m}^{-2} \text{d}^{-1}$  over seven years (Rigosi et al. 2014).



Barbiero & Welch (1992) showed in their experiment on Green Lake, Washington, that approximately 40% of the colonies that were in the water column had been recruited from

the sediment (it is assumed that the other 60% were a result of asexual reproduction while in the water column). Thus, my germination and recruitment percentage of 64% was slightly higher than the 40% described by Barbiero and Welch. The difference may be explained by the lack of predation in our experimental set-up. All of my akinete colonies

Fig. 4.8 A *Gloeotrichia echinulata* colony from Silver Lake that has burrowed into the sediment. Burrowing occurred less than 24 hours after the colonies were placed on top of sediment. This may be indicative of gliding motility.

had the potential to germinate and be recruited without predation, which is not representative of a natural water system.

### *Burrowing Behavior*

Colonies that had been incubated to induce akinetes were placed on sediment for this portion of the experiment after they had developed akinetes, and were observed to “burrow” into the top 1 to 2 mm of sediment in the test tubes (Figure 4.8). Though previous research reports akinetes are found in the top 3 – 5 cm of sediment (Pettersson et al. 1993), and previous research suggests akinete aggregates reside on sediment surface (Tymowski and Duthie 2000), there are no reports of intentional burrowing behavior. This burrowing occurred within 24 hours of the experiment initiation and prior to the first removal of lake water. Sediment mixing during this experiment began on day two, 48 hours after the colonies were placed in the incubation tubes.

These findings suggest that sediment burrowing is a natural activity of overwintering akinetes, and was not a result of sediment mixing. Possible explanations for this burrowing behavior could be that *G. echinulata* exhibits “gliding motility.” This phenomenon has been observed previously in cyanobacteria, and is an adaptation that allows cyanobacteria in contact with a solid surface to move (Biddanda et al. 2015, in review; Hoiczky 2000). The mechanics of gliding motility are relatively unknown, and are beyond the scope of this experiment, but mucilage is always present in the translocation (Glagoleva et al. 1980; Hoiczky 2000). As each *G. echinulata* filament is surrounded by a mucilaginous sheath (Carey et al. 2012), it is reasonable to conclude that *G. echinulata* is exhibiting gliding motility at the end of its life cycle, before the filaments die. Other filamentous species that exhibit gliding motility are *Anabaena* sp.

(Hoiczky 2000), *Pseudanabaena* (Tamulonis and Kaandorp 2014), and *Oscillatoriaceae* (Read et al. 2007).

Our observations suggest that germination rates could be influenced by the amount of sediment used. A natural burrowing tendency that precludes germination may not be achievable in small amounts of sediment. The amount of sediment required is important to note, as subsequent experiments may not have successful germination with a limited sediment, and may find even greater success in germination by using larger volumes of water and greater amounts of sediment.

#### *Sediment Storage*

A difference in germination rates between the experiment performed here and previous experiments could also potentially be explained in the way the sediment was stored. Karlsson's 2003 experiment stored sediment at 4 °C in the dark. Sediment stored in this fashion still showed an abundance of active, predatory meiofauna within the sediment. In the experiment performed here, meiofauna activity did not cease until the sediment was frozen. Previous research showed that multiple types of microfauna, including the protozoan *Naeglaria* (Fey et al. 2010), the rotifer *Lindia euchromatica* (Edmondson 1938), and the aquatic crustacean *Daphnia pulex* (Fey et al. 2010), will "graze" *G. echinulata*, which can lead to colony death (Fey et al. 2010). Karlsson-Elfgren does not describe the physical state of her colonies post-experimentation. However, in laboratory settings, there is a possibility that microfauna that remained alive in the sediment could graze on colonies prior to germination, lowering successful germination yields. With only one colony per test tube, it is important to ensure there is



no predation of colonies. In natural systems, however, the microfauna would graze *G. echinulata* within the water column.

Future laboratory experiments may be designed with multiple colonies in larger mesocosms, rather than individual colonies within test tubes, and use sediment with living microfauna to better simulate natural lake conditions. Microfauna predation also is important for lake management, as increasing predatory microfauna naturally occurring within lakes experiencing large *G. echinulata* blooms may mitigate blooms through biological control.

#### *Statistical Evaluation*

The Cox proportional temperature coefficients for TP-P, TSP-P, and SUP-P were positive. This indicates that increasing temperature and/or phosphorus increased the hazard rate, and thus lowered, or decreased, the time to germination rate. The Cox proportional hazard p-values for temperature and TP-P, TSP-P, and SUP-P were all <0.05, indicating that both the null hypotheses of temperature and phosphorus having no effect can be rejected.

The Cox proportional hazard coefficients for phosphorus fraction (TP-P, TSP-P, and SUP-P) were all positive. This indicates that as time increases, the likelihood of germination occurring- if germination has not yet occurred- increases. The TSP-P coefficient (1.0355) was higher than both the TP-P (0.005106) and SRP-P (-10.53) coefficients, and the SUP-P coefficient (1.0154) was just slightly lower than the TSP-P coefficient. This higher TSP-P value indicates that, when using the model, a higher TSP-P concentration will raise the hazard rate; and similarly, the greater the chance germination will occur, thus reducing the time to germination. However, when taking

into consideration the standard error of  $\beta_1$  for both TSP-P and SUP-P, the results become very similar, and both fractions may have similar effects on reducing germination times. Unlike the other fraction coefficients, the SRP-P coefficient (-10.53) was negative. This indicates that the presence of pore water SRP-P could actually lower the hazard rate, and thus increase, or delay, the time to germination.

Our results suggest that TSP-P and SUP-P are the phosphorus fractions that have the greatest likelihood of reducing the time to germination. Previous studies found similar phosphorus fraction results, where the SUP-P fraction remained high while the TSP-P fraction remained low and constant (Barbiero and Welch 1992). In their experiments, a constant TSP-P fraction, in combination with a high SUP-P fraction, may have encouraged a reduction in germination time just due to a persistent presence of TSP-P and SUP-P.

The authors conclusion was that SUP-P utilization occurred as the SUP-P input increased- however, there was no experimental evidence provided to show that the utilization of SUP-P occurred by *G. echinulata*. Utilization of pore water SUP-P may have occurred by any number of organisms within the benthos. Another investigation showed high *G. echinulata* colony counts occurred during high pelagic SRP-P concentrations (Istvanovics 1993), which is the opposite found in 1992 by Barbiero and Welch, and the opposite in this study. Our SRP-P fractions were often an order of magnitude below the TSP-P concentrations and there may be a threshold amount required for recruitment.

Previous studies also report that *G. echinulata* colonies are unable to uptake inorganic P in the epilimnion, and may supplement their P requirements with organic P

while in the epilimnion (Istvanovics 1993). This same study found that colony density in Lake Erken, Sweden, was high during periods of high SUP-P, nearly three times the SUP-P found during low colony times. That study concluded, however, that the high SUP-P concentration was a result of “leaking” organic P from *G. echinulata* while in the epilimnion. It should be noted that the 1992 and 1993 studies examined epilimnion phosphorus fractions, while this study examined sediment pore water phosphorus fractions.

### *Germination Times*

Germination began by day two in the highest temperature treatment (23 °C). Comparatively, previous experiments (Karlsson 2003) performed in 17 °C showed that most colonies that germinated did so within four days of the onset of incubation. Previous research shows that increased temperature greatly decreased the time between germination and recruitment in *G. echinulata* (Karlsson-Elfgren et al. 2004). The experiment performed here showed that temperatures of at least six degrees above ambient had such a large effect in lowering time before germination that sediment phosphorus concentration became almost negligible. This is important for lake managers to note, as emphasis is placed largely, and, arguably, rightly so, on reducing external and internal loading. However, placing efforts towards a reduction in external and internal phosphorus loading may become less important than the physical removal of colonies if global temperatures continue to rise as they are predicted (Riebeek 2014). Though dredging and skimming a lake are financially costly endeavors, management practices that destroy the cells while the colony is in the water column may be a feasible management solution.

The lack of a statistical difference in germination times at the highest temperature ( $\chi^2 = 0.2$ ,  $df = 1$ ,  $p = 0.622$ ) suggests that a high temperature will produce shorter germination times, regardless of sediment phosphorus concentration. A pair-wise comparison at the lowest temperature (17 °C) between the lowest phosphorus concentration (Group A) and the highest phosphorus concentration (Group J) shows statistical significance ( $\chi^2 = 7$ ,  $df = 1$ ,  $p = 0.01$ ). This suggests that at a low, or ambient, lake temperature, sediment phosphorus is important to germination times.

Preliminary statistical testing of the assumptions for porewater SRP-P and temperature provides evidence that the proportionate hazards model may not be a good fit for these particular SRP-P data (see appendix figure, A3). The confidence interval and estimated survival lines do cross, which indicates that the proportional hazards model for SRP-P does not visually pass the assumptions for this test. Here, we still report SRP-P by fitting the Cox proportional hazards model to the data, as the results may still be of significance biologically; however, results of the SRP-P fraction should be interpreted with care, as the assumptions of the model were indicated to have been violated. This is evidenced by SRP-P having both a negative coefficient and a coefficient magnitudes away from the other fractions. Porewater SRP-P was also the only phosphorus fraction for this experiment for which the Cox proportionate hazards model produced an insignificant result ( $p = 0.218$ ). For future experiments, it should be noted that similar SRP-P data in future experiments may pass the assumptions of this test, and produce statistically significant results, with a larger sample size or greater concentrations are used. All other data were successfully fit to the model, were significant, and violated no assumptions.

### *Implications for Lake Management*

Sample locations around the lake produced different sediment TP-P concentrations, and presumably were related to the land features near the sampling location. For example, P1 and P2 were sampling locations in front of a non-habitable sand dune and a seasonally habituated state park, respectively. Sites P1 and P2 had lower sediment TP-P concentrations than sites P3 and P4, which were located in front of both seasonal and year-round cottages. Most Silver Lake residences, as well as the state park, have a septic waste system in place, rather than city sewers. Though more sampling would need to be done to ensure a large enough sample size for accurate results, the results here indicate that septic waste systems might produce lake sediments with higher TP-P than surrounding areas.

However, data from the USGS wells located near our sampling areas have varying information. The East well, located near the state park, had the lowest SRP-P groundwater concentration out of all four groundwater wells. The highest SRP-P and TP-P was located at the South groundwater well. Concentrations of TP-P varied between 0.03 and 0.12 mg L<sup>-1</sup>, while SRP-P concentrations ranged between 0.035 and 0.11 mg L<sup>-1</sup>. TP-P and SRP-P in the West and North wells, located near the dunes and cottages, respectively, never saw concentrations above 0.02 mg L<sup>-1</sup>.

Dune lakes are an important resource for the economic growth of an area, and the local economy might suffer if nuisance blooms take hold. Toxic blooms could also result in an increase in harm to recreational water users, which in turn would adversely affect local economy, as well as the possible disruption of a lake's ecosystem. Climate change

could decrease *G. echinulata* recruitment times, allowing for blooms that arrive earlier in the season and prolong later into the year. The observed gliding motility of *G. echinulata*, and subsequent observed burrowing behavior, indicate that lakes with *G. echinulata* blooms may show lower recruitment if the top 3 – 5 cm of sediment are raked or dredged to remove burrowing akinetes before they have a chance to germinate in the spring. Raking and dredging, however, are not cost effective in most situations. Wind-induced sediment re-suspension can be addressed by using a skimming net to remove colonies as they are lifted up into the water column. This activity may require a permit and, unfortunately, remove beneficial organisms at the same time.

Our results suggest that while water column phosphorus is usually the target for nutrient reduction, sediment total phosphorus also is an important variable to take into consideration when managing a lake system. Larger reserves of sediment phosphorus could mean larger blooms of nuisance algae, like *G. echinulata*, even after management practices for water column phosphorus have been implemented and external loading has been limited and reduced. This experiment and its results are applicable to shallow, temperate-climate lakes that mirror Silver Lake, e.g., lakes with quick nutrient cycling that contain predominantly sandy sediment.

## CONCLUSION

Our experiments were the first to show a positive influence of sediment phosphorus concentration on both the frequency of germination and biovolume of *G. echinulata*. Previous studies have identified temperature and sediment mixing as key variables influencing recruitment. Our recruitment experiments also documented the importance of temperature; at 17 °C and 19°C, both sediment phosphorus concentration and temperature significantly increased recruitment. Sediment phosphorus concentration was not significant at 23°C, indicating temperature was a stronger factor driving recruitment under elevated conditions. These results suggest that global climate change may be a stronger influence than cultural eutrophication in the promotion of *G. echinulata* blooms.

The data of this experiment did not meet parametric assumptions (there was not equal variance). Nor were traditional parametric statistics found to be sufficient. A traditional parametric model could be used, but standard software assumes that there is no delay before germination occurs- which is not true. The length of the delay before germination varies with temperature and environmental conditions, making the delay biologically significant and important. Time-to-event analysis is a semi-parametric model that takes this biologically significant delay into consideration.

Kaplan-Meier survival curves provided excellent visual representations that allowed us to examine patterns or trends in the data. These visual representations, combined with point-wise comparisons, provide both visual and statistical evidence of the effect of experimental treatment. This experiment examines “time to germination”,

or, the proportion of *G. echinulata* that had not yet germinated as a function of time. The Kaplan-Meier survival curves examine, as a function of time, how many colonies had not yet germinated. This function looks at discrete groups, not at predictor variables, whereas the Cox proportionate hazards model looks at predictor variables, and behaves like a regression model. Therefore, it is recommended that both functions be used when examining germination data for future experiments. An in-depth comparison of traditional statistical methods compared to survival analysis can be reviewed in previous studies (McNair et al. 2012).

This study also is the first to document gliding motility in *G. echinulata*. Previous research shows that some mucilaginous, filamentous cyanobacteria exhibit this translocation, but *G. echinulata* has not previously been mentioned. This motility appears to be used to burrow into the top centimeters of sediment, perhaps to access as much sediment phosphorus as possible; however, the true reason for the motility exhibited by *G. echinulata* is yet unknown.



## CHAPTER V

### SUMMARY CONCLUSIONS

In Chapter II, my experiment showed that *G. echinulata* produces both MC-LR and MC-RR, though not in concentrations large enough to be of acute exposure concern. However, there is evidence through previous research that chronic, long-term, low-level exposure to MC-LR can cause serious adverse health problems. Colony densities were on the low end of previously reported results however concentrations expressed as ng/g dry weight were similar to other studies. The presence of a significant fraction of MC-RR (36-71%) shows the importance of using LC-MS instead of ELISA to monitor toxin production during *G. echinulata* blooms. However, if MC-RR is recognized to be extremely low in a lake, MC-RR concentrations may be of more interest academically, rather than recreationally or in a lake management context.

In Chapter III, my experiment showed that it is possible to produce akinetes from *G. echinulata* colonies that are mid-life cycle, which should be beneficial information to academic researchers. We also were able to reproduce evidence that germination does come from the old, overwintering akinete. Future experiments can benefit from this portion of the experiment if researchers are unable to obtain overwintering akinetes.

In Chapter IV, my experiment showed that temperature and phosphorus concentrations do affect germination times and recruited bio-volume. Results indicate that at low temperatures, sediment phosphorus plays an important role in inducing germination. At high temperatures, sediment phosphorus becomes a negligible factor in inducing germination. Results also show that higher sediment phosphorus will produce

an increase in recruited bio-volume, as will a higher temperature. Gliding motility in *G. echinulata* may also have been observed, and showed that *G. echinulata* can burrow within the sediment, perhaps to have access to as much sediment phosphorus as possible.

This study also introduces the use of statistical time-to-event analysis as a tool for modeling the recruitment of cyanobacteria from akinetes to filaments. Though survival analysis has rarely been used in germination experiments, the amount of non-germination, as well as the non-normal distribution of germination data, required a novel statistical approach. Survival analysis, also called “time-to-event” analysis, takes into consideration germination that may have occurred should the experiment have continued, rather than having been terminated. This “missing-data” is called “right-censored data,” and is often overlooked in traditional, non-parametric tests.

Experiment termination occurred in this experiment after 30 days, but germination had the *potential* to occur beyond that under the various treatments in which the akinetes were exposed. This germination potential needs to be taken into consideration when examining our hypothesis, as the germination *may have* occurred if the experiment continued. This class of statistical methods should prove especially useful in larger studies aimed at identifying key variables that can influence cyanobacteria blooms.

Current data suggest that global temperatures are on the rise. This experiment shows that rising temperatures are a stronger variable than cultural eutrophication for the germination of toxic blooms of *G. echinulata*. As a global increase in temperature is most likely inevitable, natural resources managers should consider focusing their efforts

on reducing the physical blooms, such as overwintering akinetes, in addition to reducing nutrient loads.

Persistent cultural eutrophication, an explosion in human population, and the dire and increasing need for clean water all create significant challenges for the future management of freshwater resources. As we have seen, lakes, drinking water sources, and fresh-water reservoirs are becoming increasingly cyanobacterial dominant. Due to cyanotoxin producing blooms, livestock have been killed, birds, fish and freshwater vertebrates have suffered physiologically, and there is evidence that human health has been affected through chronic, long-term exposure. Cultural eutrophication has consistently been shown to be the cause for harmful cyanobacterial blooms, and though not all lakes behave similarly, the end result of cultural eutrophication is a lack of clean, available drinking water. As shown in this experiment and other experiments, along with observations and observed trends, it is vitally important to human health that we manage reservoirs and freshwater sources for microcystin and other cyanotoxins.

## APPENDIX

### *Preliminary Statistical Testing: TP-P*

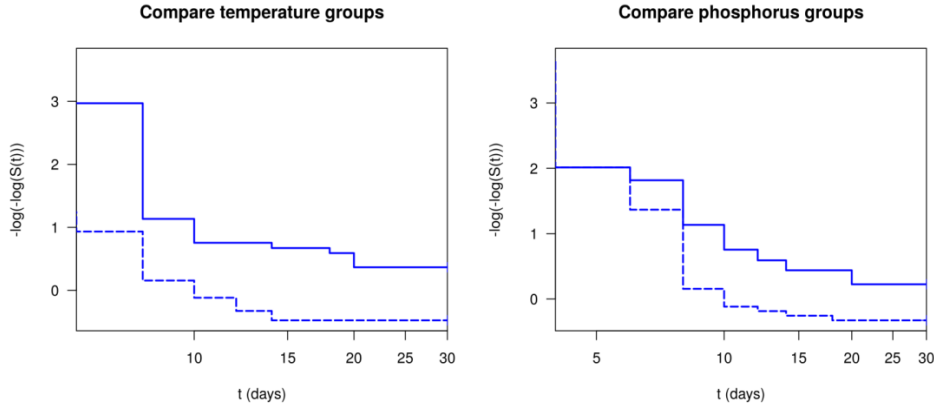


Figure A1: A log-rank test of groups across the three temperature variables and comparisons of groups across the four TP-P variables in germinations of *G. echinulata*. This is a similar method to testing for normality in exchange to a t-test. These comparisons take into consideration the effect phosphorus has on temperature, and the effect temperature has on phosphorus. Survival curves are indicated by the solid lines, and pointwise 95% confidence intervals are indicated by the dashed lines.

### *Preliminary Statistical Testing: TSP*

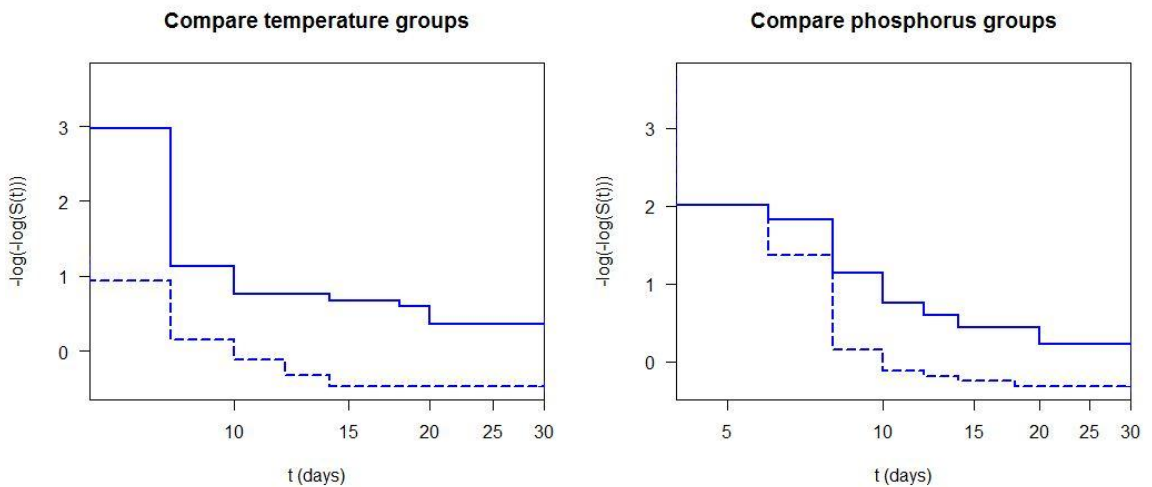


Figure A2: A log-rank test of groups across the three temperature variables and comparisons of groups across the four TSP variables in germinations of *G. echinulata*. Survival curves are indicated by the solid lines, and pointwise 95% confidence intervals are indicated by the dashed lines.

### Preliminary Statistical Testing: SRP-P

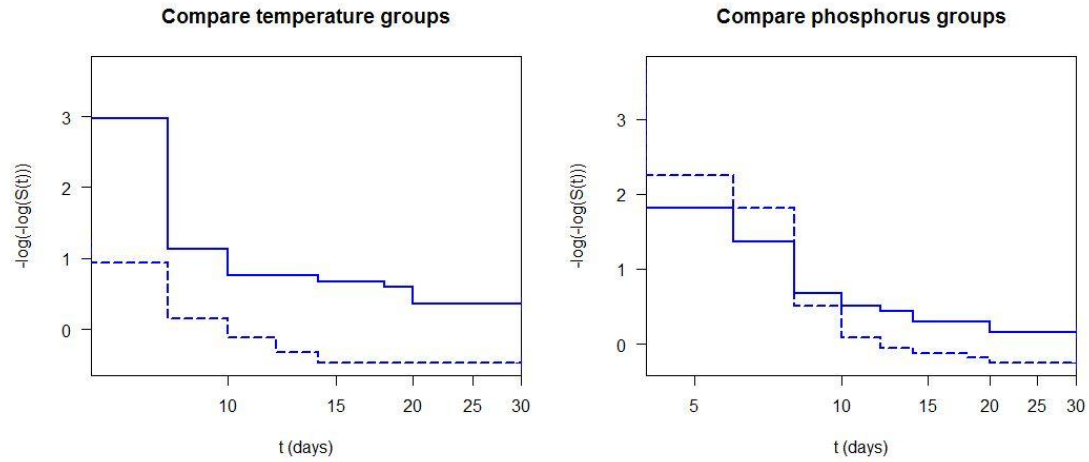


Figure A3: A log-rank test of groups across the three temperature variables and comparisons of groups across the four SRP-P variables in germinations of *G. echinulata*. Survival curves are indicated by the solid lines, and pointwise 95% confidence intervals are indicated by the dashed lines. The survival curve estimates and pointwise confidence interval lines do cross for SRP-P, indicating that this model may not be the best model for these data.

### Preliminary Statistical Testing: SUP-P

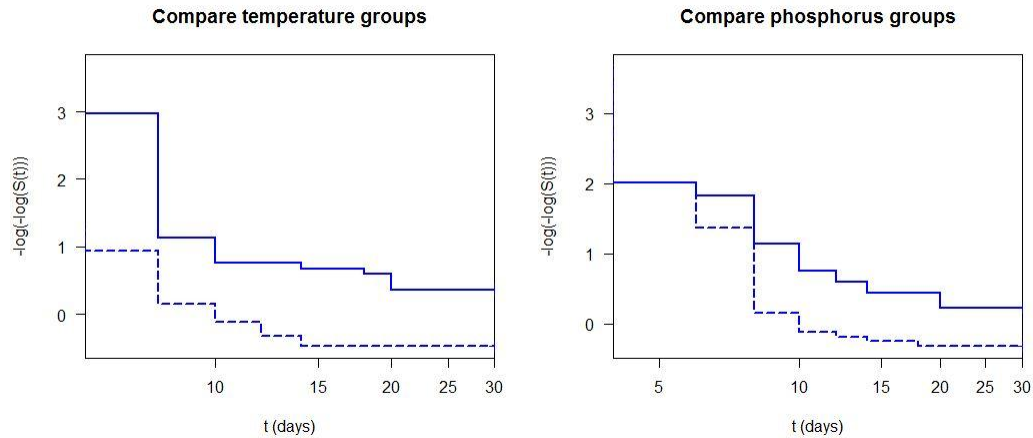


Figure A4: A log-rank test of groups across the three temperature variables and comparisons of groups across the four SUP-P variables in germinations of *G. echinulata*. Survival curves are indicated by the solid lines, and pointwise 95% confidence intervals are indicated by the dashed lines.

Pointwise Comparison Matrix of All Kaplan-Meier Possibilities

	B	C	D	E	F	G	H	I	J	K	L
A	$\chi^2 = 0.3$ p = 0.601	$\chi^2 = 13.9$ p = 0.000196	$\chi^2 = 0.3$ p = 0.601	$\chi^2 = 4.4$ p = 0.0355	$\chi^2 = 6$ p = 0.0142	$\chi^2 = 1.3$ p = 0.259	$\chi^2 = 5.5$ p = 0.0186	$\chi^2 = 6.3$ p = 0.0122	$\chi^2 = 7$ p = 0.00822	$\chi^2 = 5.2$ p = 0.0228	$\chi^2 = 13.8$ p = 0.00209
B		$\chi^2 = 5.4$ p = 0.0198	$\chi^2 = 0.1$ p = 0.78	$\chi^2 = 0.5$ p = 0.502	$\chi^2 = 2.3$ p = 0.126	$\chi^2 = 0.2$ p = 0.684	$\chi^2 = 1.2$ p = 0.266	$\chi^2 = 2.2$ p = 0.141	$\chi^2 = 2$ p = 0.161	$\chi^2 = 1.3$ p = 0.255	$\chi^2 = 5.6$ p = 0.0181
C			$\chi^2 = 10.4$ p = 0.00124	$\chi^2 = 2$ p = 0.157	$\chi^2 = 1.2$ p = 0.264	$\chi^2 = 7$ p = 0.00804	$\chi^2 = 2.1$ p = 0.143	$\chi^2 = 0.6$ p = 0.426	$\chi^2 = 5$ p = 0.0253	$\chi^2 = 2$ p = 0.157	$\chi^2 = 0.2$ p = 0.622
D				$\chi^2 = 2.8$ p = 0.0935	$\chi^2 = 5.6$ p = 0.0175	$\chi^2 = 0.7$ p = 0.415	$\chi^2 = 3.7$ p = 0.05	$\chi^2 = 4.5$ p = 0.034	$\chi^2 = 5.2$ p = 0.0223	$\chi^2 = 4.6$ p = 0.0325	$\chi^2 = 16.6$ p = 4.57e-5
E					$\chi^2 = 1.6$ p = 0.209	$\chi^2 = 0.1$ p = 0.72	$\chi^2 = 0.1$ p = 0.712	$\chi^2 = 0.5$ p = 0.467	$\chi^2 = 0.5$ p = 0.481	$\chi^2 = 1.1$ p = 0.305	$\chi^2 = 9$ p = 0.00264
F						$\chi^2 = 2.2$ p = 0.138	$\chi^2 = 0.3$ p = 0.555	$\chi^2 = 0$ p = 0.888	$\chi^2 = 0.5$ p = 0.471	$\chi^2 = 0.6$ p = 0.443	$\chi^2 = 1.2$ p = 0.273
G							$\chi^2 = 0.8$ p = 0.37	$\chi^2 = 2.2$ p = 0.142	$\chi^2 = 1.4$ p = 0.23	$\chi^2 = 0.8$ p = 0.385	$\chi^2 = 7.7$ p = 0.00552
H								$\chi^2 = 0.3$ p = 0.609	$\chi^2 = 0$ p = 0.989	$\chi^2 = 0$ p = 0.905	$\chi^2 = 4.2$ p = 0.0403
I									$\chi^2 = 0.5$ p = 0.47	$\chi^2 = 0.2$ p = 0.63	$\chi^2 = 1.2$ p = 0.271
J										$\chi^2 = 0$ p = 0.933	$\chi^2 = 6.1$ p = 0.0134
K											$\chi^2 = 4.7$ p = 0.0309

Figure A5: A matrix of the results of all possible point-wise comparisons for the Kaplan-Meier curves. Shown are  $\chi^2$  and p values. Significant results are shaded in peach. Significance indicates a p value less than 0.05.

## BIBLIOGRAPHY

- Ahn, C., Oh, H., Park, Y., 2011. Evaluation of environmental factors on cyanobacterial bloom in eutrophic reservoir using artificial neural networks. *J. Phycol.* 47, 495-504.
- Asencio, A.D., 2013. Determination of microcystins in reservoirs of different basins in a semiarid area. *J. Appl. Phycol.* 25, 1753-1762.
- Baker, P., 1999. Role of akinetes in the development of cyanobacterial populations in the lower Murray River, Australia. *Mar. Freshwater Res.* 50, 265-279.
- Barbiero, R.P., 1993. A contribution to the life-history of the planktonic cyanophyte, *Gloeotrichia echinulata*. *Arch. Hydrobiol.* 127, 87-100.
- Barbiero, R.P., Welch, E.B., 1992. Contribution of benthic bluegreen algal recruitment to lake populations and phosphorus translocation. *Freshwater Biol.* 27, 249-260.
- Bartram, J., Carmichael, W.W., Chorus, I., Jones, G., Skulberg, O.M., 1999. Toxic cyanobacteria: Introduction, in Chorus, I., Bartram, J. (Eds.), *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management*. Spon Press, London, pp. 1-14.
- Bennett, E.M., Carpenter, S.R., Caraco, N.F., 2001. Human impact on erodable phosphorus and eutrophication: a global perspective. *Bioscience* 51, 227-234.
- Biddanda, B.A., McMillan, A.C., Long, S.A., Snider, M.J., Weinke, A.D., 2015 (In review). Seeking sunlight: Rapid phototactic motility of filamentous mat-forming cyanobacteria optimize photosynthesis and enhance carbon burial in Lake Huron's submerged sinkholes. *Frontiers in Aquatic Microbiology*.
- Bláha, L., Babica, P., Maršálek, B., 2009. Toxins produced in cyanobacterial water blooms - toxicity and risks. *Interdiscip. Toxicol.* 2, 36-41.
- Blasing, T.J., 2014. Recent Greenhouse Gas Concentrations. DOI: 10.3334/CDIAC/atg.032  
<[http://cdiac.ornl.gov/pns/current\\_ghg.html](http://cdiac.ornl.gov/pns/current_ghg.html)> Accessed Feb. 15, 2015
- Bourne, D.G., Blakeley, R.L., Riddles, P., Jones, G.J., 2006. Biodegradation of the cyanobacterial toxin microcystin LR in natural water and biologically active slow sand filters. *Water Res.* 40, 1294-1302.
- Boyd, E., Tompkins, E.L., 2010. *Climate Change: A Beginner's Guide*. Oneworld, Oxford.

- Brennan, A., Feb. 25<sup>th</sup>, 2015. Personal Communication. Silver Lake nutrient and hydraulic residence time.
- Brennan, A. Hoard, C.J., Duris, J.W., Ogdahl, M.E., Steinman, A.D. 2015. Water Quality and Hydrology of Silver Lake, Oceana County, MI with Emphasis on Lake Response to Nutrient Loading, 2012-2014.
- Brenneman, C., Hansson, L., 2005. The Biology of Lakes and Ponds. Oxford University Press, Oxford.
- Carey, C.C., Weathers, K.C., Ewing, H.A., Greer, M.L., Cottingham, K.L., 2014. Spatial and temporal variability in recruitment of the cyanobacterium *Gloeotrichia echinulata* in an oligotrophic lake. *Freshwater Science* 33, 577-592.
- Carey, C.C., Ewing, H.A., Cottingham, K.L., Weathers, K.C., Thomas, R.Q., Haney, J.F., 2012. Occurrence and toxicity of the cyanobacterium *Gloeotrichia echinulata* in low-nutrient lakes in the northeastern United States. *Aquat. Ecol.* 46, 395-409.
- Carey, C.C., Haney, J.F., Cottingham, K.L., 2007. First report of microcystin-LR in the cyanobacterium *Gloeotrichia echinulata*. *Environ. Toxicol.* 22, 337-339.
- Carey, C.C., Rengefors, K., 2010. The cyanobacterium *Gloeotrichia echinulata* stimulates the growth of other phytoplankton. *J. Plankton Res.* 32, 1349-1354.
- Carpenter, S.R., Cottingham, K.L., Schindler, D.E., 1992. Biotic feedbacks in lake phosphorus cycles. *Trends Ecol. Evol.* 7, 332-336.
- Carpenter, S.R., 2005. Eutrophication of aquatic ecosystems: biostability and soil phosphorus. *Proc. Natl. Acad. Sci. U. S. A.* 102, 10002-10005.
- Cazenave, J., Wunderlin, D., Bistoni, M., Ame, M., Krause, E., Pflugmacher, S., Wiegand, C., 2005. Uptake, tissue distribution and accumulation of microcystin-RR in *Corydoras paleatus*, *Jenynsia multidentata* and *Odontesthes bonariensis* - A field and laboratory study. *Aquat. Toxicol.* 75, 178-190.
- Chang, J., Chen, Z., Wang, Z., Kang, J., Chen, Q., Yuan, L., Shen, J., 2015. Oxidation of microcystin-LR in water by ozone combined with UV radiation: the removal and degradation pathway. *Chem. Eng. J.* 276, 97-105.
- Chen, J., Hu, L., Zhou, W., Yan, S., Yang, J., Xue, Y., Shi, Z., 2010. Degradation of Microcystin-LR and RR by a *Stenotrophomonas* sp. strain EMS isolated from Lake Taihu, China. *Intern. J. Mol. Sci.* 11, 896-911.
- Chen, J., Han, F.X., Wang, F., Zhang, H., Shi, Z., 2012. Accumulation and phytotoxicity of microcystin-LR in rice (*Oryza sativa*). *Ecotoxicol. Environ. Saf.* 76, 193-199.



- Chen, M., Ye, T., Krumholz, L.R., Jiang, H., 2014. Temperature and cyanobacterial bloom biomass influence phosphorus cycling in eutrophic lake sediments. *PloS one* 9, e93130.
- Chorus, I., Falconer, I., Salas, H., Bartram, J., 2000. Health risks caused by freshwater cyanobacteria in recreational waters. *J. Toxicol. Environ. Health B Crit. Rev.* 3, 323-347.
- Christiansen, G., Dittmann, E., Ordorika, L., Rippka, R., Herdman, M., Borner, T., 2001. Nonribosomal peptide synthetase genes occur in most cyanobacterial genera as evidenced by their distribution in axenic strains of the PCC. *Arch. Microbiol.* 176, 452-458.
- Codd, G.A., Lindsay, J., Young, F.M., Morrison, L.F., Metcalf, J.S., 2005. Harmful cyanobacteria: From mass mortalities to management measures, in Huisman, J., Matthijs, H.C.P., Visser, P.M. (Eds.), *Harmful Cyanobacteria*. Springer, Dordrecht, Netherlands, pp. 1-23.
- Davis, T.W., Bullerjahn, G.S., Tuttle, T., McKay, R.M., Watson, S.B., 2015. Effects of increasing nitrogen and phosphorus concentrations on phytoplankton community growth and toxicity during planktothrix blooms in Sandusky Bay, Lake Erie. *Environ. Sci. Technol.* 49, 7197-7207.
- Dawson, R.M., 1998. The toxicology of microcystins. *Toxicon.* 36, 953-962.
- de Figueiredo, D.R., Azeiteiro, U.M., Esteves, S.M., Gonçalves, F.J.M., Pereira, M.J., 2004. Microcystin-producing blooms—a serious global public health issue. *Ecotoxicol. Environ. Saf.* 59, 151-163.
- DeMaat, M., Cargill, C., Byl, L., Beckman, J., 2013. Silver Lake Nutrient Budget Project Update (Fall 2013). < <http://www.friendsofsilverlake.org/wp-content/uploads/2014/03/December-2013-Silver-Lake-Evaluation.pdf>>
- Donati, C., Drikas, M., Hayes, R., Newcombe, G., 1994. Microcystin-LR adsorption by powdered activated carbon. *Water Res.* 28, 1735-1742.
- Edmondson, W.T., 1938. Three New species of Rotatoria. *Trans. Am. Microsc. Soc.* 57, 153-157.
- El-Shehawy, R., Gorokhova, E., Fernández-Piñas, F., del Campo, F.F., 2012. Global warming and hepatotoxin production by cyanobacteria: What can we learn from experiments? *Water Res.* 46, 1420-1429.
- Fey, S., Mayer, Z., Davis, S., Cottingham, K., 2010. Zooplankton grazing of *Gloeotrichia echinulata* and associated life history consequences. *J. Plankton Res.* 32, 1337-1347.

Fisher, T.G., Loope, W.L., Pierce, W., Jol, H.M., 2007. Big lake records preserved in a little lake's sediment: an example from Silver Lake, Michigan, USA. *J. Paleolimnol.* 37, 365-382.

Forsell, L., Pettersson, K., 1995. On the seasonal migration of the cyanobacterium *Gloeotrichia echinulata* in Lake Erken, Sweden, and its influence on the pelagic population. *Mar. Freshwater Res.* 46, 287-293.

Gajdek, P., Bober, B., Mej, E., Bialczyk, J., 2004. Sensitised decomposition of microcystin-LR using UV radiation. *J. Photochem. Photobiol.* 76, 103-106.

Gehringer, M.M., Kewada, V., Coates, N., Downing, T.G., 2003. The use of *Lepidium sativum* in a plant bioassay system for the detection of microcystin-LR. *Toxicon.* 41, 871-876.

Geider, R., La Roche, J., 2002. Redfield revisited: variability of C : N : P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37, 1-17.

General Oceanics, 2006. General Oceanics Digital Flowmeter Mechanical & Electrical Operators Manual. <[http://epic.awi.de/31495/1/flowmeter\\_general-oceanics.pdf](http://epic.awi.de/31495/1/flowmeter_general-oceanics.pdf)>

Glagoleva, T.N., Glagolev, A.N., Gusev, M.V., Nikitina, K.A., 1980. Protonmotive force supports gliding in cyanobacteria. *FEBS Lett.* 117, 49-53.

Groves, T., Tyning, P., Meyers, T., 2015. Progressive AE. Mapping and understanding your lake's watershed. <[http://michiganlakeinfo.com/uploads/Mapping\\_and\\_Understanding\\_Your\\_Lake\\_s\\_Watershed.pdf](http://michiganlakeinfo.com/uploads/Mapping_and_Understanding_Your_Lake_s_Watershed.pdf)>

Grzybowski, W., Szydlowski, J., 2013. Photodegradation of Detergent Phosphorus in Aquatic Environment. *Water Air Soil Pollut.* 224, 1-5.

Gupta, N., Pant, S.C., Vijayaraghavan, R., Rao, P.V.L., 2003. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. *Toxicol.* 188, 285-296.

Hecky, R.E., Kilham, P., 1988. Nutrient limitation of phytoplankton in freshwater and marine environments: a review of recent evidence on the effects of enrichment. *Limnol. Oceanogr.* 33, 796-822.

Hereman, T.C., Bittencourt-Oliveira, M.d.C., 2012. Bioaccumulation of microcystins in lettuce. *J. Phycol.* 48, 1535-1537.

Hillebrand, H., Dürselen, C., Kirschtel, D., Pollinger, U., Zohary, T., 1999. Biovolume calculation for pelagic and benthic microalgae. *J. Phycol.* 35, 403-424.

Ho, L., Hoefel, D., Palazot, S., Sawade, E., Newcombe, G., Saint, C.P., Brookes, J.D., 2010. Investigations into the biodegradation of microcystin-LR in wastewaters. *J. Hazard. Mater.* 180, 628-633.

Hoiczyk, E., 2000. Gliding motility in cyanobacteria: observations and possible explanations. *Arch. Microbiol.* 174, 11-17.

Huang, W., Cheng, B., Cheng, Y., 2007. Adsorption of microcystin-LR by three types of activated carbon. *J. Hazard. Mater.* 141, 115-122.

Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: A review. *Environ. Pollut.* 150, 177-192.

IPCC, 2007. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change 2007. Cambridge University Press, Geneva, Switzerland.

Istvanovics, V., Pettersson, K., Rodrigo, M.A., Pierson, D., Padis'k, J., Colom, W., 1993. *Gloeotrichia echinulata*, a colonial cyanobacterium with a unique phosphorus uptake and life strategy. *J. Plankton Res.* 15, 531-552.

Jacobsen, B., 1994. Bloom formation of *Gloeotrichia echinulata* and *Aphanizomenon flos-aquae* in a shallow, eutrophic, Danish lake. *Hydrobiol.* 289, 193-197.

Jöhnk, K.D., Visser, P.M., Sharples, J., Huisman, J., Stroom, J.M., Sommeijer, B., 2008. Summer heatwaves promote blooms of harmful cyanobacteria. *Glob. Change Biol.* 14, 495-512.

Juhel, G., Davenport, J., O'Halloran, J., Culloty, S.C., O'Riordan, R.M., James, K.F., Furey, A., Allis, O., 2006. Impacts of microcystins on the feeding behaviour and energy balance of zebra mussels, *Dreissena polymorpha*: A bioenergetics approach. *Aquat. Toxicol.* 79, 391-400.

Kaebernick, M., Neilan, B.A., Börner, T., Dittmann, E., 2000. Light and the transcriptional response of the microcystin biosynthesis gene cluster. *Appl. Environ. Microbiol.* 66, 3387-3392.

Kalff, J., 2002. Limnology: inland water ecosystems, in Anonymous . Prentice Hall, Upper Saddle River, NJ, pp. 94.

Kaplan-Levy, R.N., Hadas, O., Summers, M.L., Rucker, J., Sukenik, A., 2010. Akinetes: dormant cells of Cyanobacteria., in Lubzens, E., Cerd , J., Clark, M. (Eds.), Dormancy and Resistance in Harsh Environments. Springer, Heidelberg, Germany, pp. 5-27.

Karlsson, I., 2003. Benthic growth of *Gloeotrichia echinulata* cyanobacteria. *Hydrobiol.* 506-509, 189-193.

Karlsson-Elfgren, I., Brunberg, A.K., 2004. The importance of shallow sediments in the recruitment of *Anabaena* and *Aphanizomenon* (cyanophyceae). *J. Phycol.* 40, 831-836.

Karlsson-Elfgren, I., Rengefors, K., Gustafsson, S., 2004. Factors regulating recruitment from the sediment to the water column in the bloom-forming cyanobacterium *Gloeotrichia echinulata*. *Freshwat. Biol.* 49, 265-273.

Kopp, R., Palíková, M., Adamovský, O., Ziková, A., Navrátil, S., Kohoutek, J., Mareš, J., Bláha, L., 2013. Concentrations of microcystins in tissues of several fish species from freshwater reservoirs and ponds. *Environ. Monit. Assess.* 185, 9717-9727.

Kozdęba, M., Borowczyk, J., Zimoląg, E., Wasylewski, M., Dziga, D., Madeja, Z., Drukala, J., 2014. Microcystin-LR affects properties of human epidermal skin cells crucial for regenerative processes. *Toxicon.* 80, 38-46.

Malbrouck, C., Kestemont, P., 2006. Effects of microcystins on Fish. *Environ. Toxicol. Chem.* 25, 72-86.

McNair, J., Sunkara, A., Frobish, D., 2013. How to Analyse Seed Germination Data Using Statistical time-to-event analysis: Non-parametric and semi-parametric methods. *Seed Sci. Res.* 22, 77-95.

Metcalf, J.S., Beattie, K.A., Pflugmacher, S., Codd, G.A., 2000. Immuno-crossreactivity and toxicity assessment of conjugation products of the cyanobacterial toxin, microcystin-LR. *FEMS Microbiol. Lett.* 189, 155-158.

Miller, M.M., Lang, N.J., 1968. The fine structure of akinete formation and germination in *Cylindrospermum*. *Arch. Mikrobio.* 60, 303-313.

Milutinović, A., Zorc-Pleskovic, R., Petrovic, D., Zorc, M., Suput, D., 2006. Microcystin-LR induces alterations in heart muscle. *Folia Biol.* 52, 116.

Moore, D., McGregor, G.B., Shaw, G., 2004. Morphological changes during akinete germination in *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria). *J. Phycol.* 40, 1098-1105.

Moore, P., Reddy, K., Fisher, M., 1998. Phosphorus flux between sediment and overlying water in Lake Okeechobee, Florida: Spatial and temporal variations. *J. Environ. Qual.* 27, 1428-1439.

National Academy of Sciences, 2010. Advancing the Science of Climate Change. The National Academies Press, Washington, D.C., USA.

Nichols, J.M., Adams, D.G., 1982. Akinetes, in Carr, N.G., Whitton, B.A. (Eds.), The Biology of Cyanobacteria. Blackwell Scientific Publications, Oxford, pp. 387-412.

NOAA National Climatic Data Center, 2015. Monthly Climatological Summary (2013).

Olsson-Francis, K., de la Torre, R., Towner, M., Cockell, C., 2009. Survival of akinetes (resting-state cells of cyanobacteria) in low earth orbit and simulated extraterrestrial conditions. *Origins Life Evol. B.* 39, 565-579.

Paerl, H.W., Huisman, J., 2008. Blooms like It Hot. *Science* 320, 57-58.

Paerl, H.W., Huisman, J., 2009. Climate change: A catalyst for global expansion of harmful cyanobacterial blooms. *Environmental Microbiology Reports* 1, 27-37.

Paerl, H.W., Paul, V.J., 2012. Climate change: Links to global expansion of harmful cyanobacteria. *Water Res.* 46, 1349-1363.

Pettersson, K., Herlitz, E., Istvánovics, V., 1993. The role of *Gloeotrichia echinulata* in the transfer of phosphorus from sediments to water in Lake Erken. *Hydrobiol.* 253, 123-129.

Poste, A.E., Hecky, R.E., Guildford, S.J., 2011. Evaluating microcystin exposure risk through fish consumption. *Environ. Sci. Technol.* 45, 5806-5811.

Pouria, S., de Andrade, A., Barbosa, J., Cavalcanti, R., Barreto, V., Ward, C., Preiser, W., Poon, G.K., Neild, G., Codd, G., 1998. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* 352, 21-26.

Prakash, S., Lawton, L.A., Edwards, C., 2009. Stability of toxigenic *Microcystis* blooms. *Harmful Algae.* 8, 377-384.

Progressive AE, Silver Lake 2011 Water Quality Monitoring Report, September 2011

Qiao, R., Li, N., Qi, X., Wang, Q., Zhuang, Y., 2005. Degradation of microcystin-RR by UV radiation in the presence of hydrogen peroxide. *Toxicon.* 45, 745-752.

Rapala, J., Sivonen, K., 1998. Assessment of environmental conditions that favor hepatotoxic and neurotoxic *Anabaena* spp. Strains cultured under light limitation at different temperatures. *Microb. Ecol.* 36, 181-192.

Rapala, J., Sivonen, K., Lyra, C., Niemelä, S.I., 1997. Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. *Appl. Environ. Microbiol.* 63, 2206-2212.

Rastogi, R.P., Sinha, R.P., Incharoensakdi, A., 2014. The cyanotoxin-microcystins: current overview. *Rev. Environ. Sci. Biotechnol.* 13, 215-249.

Read, N., Connell, S., Adams, D.G., 2007. Nanoscale Visualization of a Fibrillar Array in the Cell Wall of Filamentous Cyanobacteria and Its Implications for Gliding Motility. *J. Bacteriol.* 189, 7361.

Redfield, A., 1934. On the proportions of organic derivations in sea water and their relation to the composition of plankton, in Daniel, R.J. (Ed.), James Johnstone Memorial Volume. University Press of Liverpool, pp. 177-192.

Rengefors, K., Gustafsson, S., Stahl-Delbanco, A., 2004. Factors regulating the recruitment of cyanobacterial and eukaryotic phytoplankton from littoral and profundal sediments. *Aquat. Microb. Ecol.* 36, 213-226.

Riebeek, H., 2010. How Much More Will Earth Warm?. NASA. Accessed March 15<sup>th</sup>, 2014. <<https://earthobservatory.nasa.gov/Features/GlobalWarming/>>

Rigosi, A., Carey, C.C., Ibelings, B.W., Brookes, J.D., 2014. The interaction between climate warming and eutrophication to promote cyanobacteria is dependent on trophic state and varies among taxa. *Limnol. Oceanogr.* 59, 99-114.

Schindler, D.W., 1977. Evolution of phosphorus limitation in lakes. *Science* 195, 260-262.

Schindler, D.W., 1974. Eutrophication and recovery in experimental lakes: implications for lake management. *Science* 184, 897-899.

Schmidt, J.R., Wilhelm, S.W., Boyer, G.L., 2014. The fate of microcystins in the environment and challenges for monitoring. *Toxins* 6, 3354-3387.

Singh, S., Rai, P.K., Chau, R., Ravi, A.K., Neilan, B.A., Asthana, R.K., 2015. Temporal variations in microcystin-producing cells and microcystin concentrations in two fresh water ponds. *Water Res.* 69, 131-142.

Soares, R.M., Cagido, V.R., Ferraro, R.B., Meyer-Fernandes, J.R., Rocco, P.R.M., Zin, W.A., Azevedo, S.M.F.O., 2007. Effects of microcystin-LR on mouse lungs. *Toxicon.* 50, 330-338.

Song, L., Chen, W., Peng, L., Wan, N., Gan, N., Zhang, X., 2007. Distribution and bioaccumulation of microcystins in water columns: A systematic investigation into the environmental fate and the risks associated with microcystins in Meiliang Bay, Lake Taihu. *Water Res.* 41, 2853-2864.

Sukenik, A., Beardall, J., Hadas, O., 2007. Photosynthetic characterization of developing and mature akinetes of *Aphanizomenon ovalisporum* (cyanoprokaryota). *J. Phycol.* 43, 780-788.

Svrcek, C., Smith, D., 2004. Cyanobacteria toxins and the current state of knowledge on water treatment options: a review. J. Environ. Eng. Sci. 3, 155-185.

Tamulonis, C., Kaandorp, J., 2014. A model of filamentous cyanobacteria leading to reticulate pattern formation. Life (Basel, Switzerland) 4, 433-456.

Taranu, Z.E., Zurawell, R.W., Pick, F., Gregory- Eaves, I., 2012. Predicting cyanobacterial dynamics in the face of global change: the importance of scale and environmental context. Global Change Biol. 18, 3477-3490.

Tymowski, R., Duthie, H., 2000. Life strategy and phosphorus relations of the cyanobacterium *Gloeotrichia echinulata* in an oligotrophic precambrian shield lake. Arch. Hydrobiol. 148, 321-332.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F., Park, H.D., Chen, G.C., Chen, G., Yu, S.Z., 1996. Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis 17, 1317-1321.

USGS, Brennan, A., Water quality and hydrology of Silver Lake, Oceana County, MI with special emphasis on response of the lake to nutrient loading. 2015.

Vasconcelos, V.M., Pereira, E., 2001. Cyanobacteria diversity and toxicity in a wastewater treatment plant (Portugal). Water Res. 35, 1354-1357.

Wetzel, R.G., 2001. Limnology: Lake and River Ecosystems. Academic Press, San Diego.

Wicks, R.J., Thiel, P.G., 1990. Environmental factors affecting the production of peptide toxins in floating scums of the cyanobacterium *Microcystis aeruginosa* in a hypertrophic African reservoir. Environ. Sci. Technol. 24, 1413-1418.

Wiedner, C., Visser, P.M., Fastner, J., Metcalf, J.S., Codd, G.A., Mur, L.R., 2003. Effects of light on the microcystin content of *Microcystis* strain PCC 7806. Appl. Environ. Microbiol. 69, 1475-1481.

World Health Organization, 2003. Cyanobacterial toxins: Microcystin-LR in Drinking-water: Background document for development of WHO Guidelines for Drinking-water Quality.

World Health Organization, 1998. Guidelines for Drinking-Water Quality - Second Edition - Volume 2 - Health Criteria and Other Supporting Information - Addendum. 98.1.

Wyman, M., Fey, P., 1986. Interaction between light quality and nitrogen availability in the differentiation of akinetes in the planktonic cyanobacterium *Gloeotrichia echinulata*. *Brit. Phycol. J.* 21, 147-153.

Xie, L., Yan, W., Li, J., Yu, L., Wang, J., Li, G., Chen, N., Steinman, A.D., 2015. Microcystin-RR exposure results in growth impairment by disrupting thyroid endocrine in zebrafish larvae. *Aquat. Toxicol.* 164, 16-22.

Yang, C.Y., Wang, W.B., Li, D.H., Liu, Y.D., 2008. Growth and antioxidant system of *Escherichia coli* in response to microcystin-RR. *Bull. Environ. Contam. Toxicol.* 81, 427-431.

Yang, F., Zhou, Y., Sun, R., Wei, H., Li, Y., Yin, L., Pu, Y., 2014. Biodegradation of microcystin-LR and-RR by a novel microcystin-degrading bacterium isolated from Lake Taihu. *Biodegradation* 25, 447-457.

Zhang, D., Xie, P., Liu, Y., Qiu, T., 2009. Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake Taihu, China, with potential risks to human health. *Sci. Total Environ.* 407, 2191-2199.

Zhao, Y., Xue, Q., Su, X., Xie, L., Yan, Y., Steinman, A.D., 2015. Microcystin-LR induced thyroid dysfunction and metabolic disorders in mice. *Toxicol.* 328, 135-141.

Zurawell, R., Chen, H., Burke, J., Prepas, E., 2005. Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. *J. Toxicol. Environ. Health B Crit. Rev.* 8, 1-37.