I, Randall S Marshall, hereby submit this original work as part of the requirements for the degree of Master of Science in Biological Sciences.

It is entitled:
Environmental Isolations, Community Nutrient Ratio Effects, and Allelopathy of Microcystis from Grand Lake Saint Mary’s

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Environmental Isolations, Community Nutrient Ratio Effects, and Allelopathy of Microcystis from Grand Lake Saint Mary's

A thesis submitted to the
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of the University of Cincinnati
in partial fulfillment of the
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Master of Science

in the Department of Biology
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by
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Abstract

*Microcystis* is a bloom forming cyanobacteria associated with eutrophic water systems. Debate exists as to how *Microcystis* becomes dominant and forms blooms. Nutrient competition and allelopathic inhibition of other phytoplankton species are possible mechanisms. The genus is resistant to isolation efforts and laboratory culturing. *Microcystis* bloom samples were obtained from Grand Lake St Mary's and several other sources. Samples were agitated, filtered, and grown on solid media and organismal growth monitored by microscopy. *Microcystis* isolates were identified by comparison to established lab strains and by production of microcystin toxins. *Nitzschia* sp. and other phytoplankton organisms were also isolated from plate cultures. Isolates were maintained in semicontinuous batch culture under summer seasonal lighting and temperature conditions for future experimental use. These recently established isolates of *Microcystis* and *Nitzschia* were deprived of nitrogen and phosphorous to deplete internal stores. These were then introduced into modified BG11 media with either high or low nitrogen/phosphorous (N:P) ratios to determine comparative nutrient growth responses. Both batch and semicontinuous long-term microcosm experiments were performed. Populations were assessed regularly using hemocytometry and compound microscopy. Regardless of ratio, *Nitzschia* experienced greater initial population increases followed by *Microcystis* culture dominance, with some resulting in exclusion of the diatom. To test allelopathy, a bioassay was also performed using *Nitzschia* and microcystin at an environmentally relevant concentration and found no significant population impacts.
Dedication

This thesis is dedicated to the faculty of the Biology department of the University of Cincinnati, and my parents, for guidance, patience, support, and understanding. Also my cat, Anne Frank, who was often very bored during the process.

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Table of Contents

Abstract ......................................................................................................................................ii
Dedication and Acknowledgements .........................................................................................iv
Table of Contents ........................................................................................................................v
List of Tables .................................................................................................................................vi
List of Figures .................................................................................................................................vii

Chapter 1 - Review and Overview of Thesis ...........................................................................9
Literature cited .................................................................................................................................22

Chapter 2 - *Microcystis* Isolation and Culturing .................................................................29
Literature cited .................................................................................................................................47

Chapter 3 - *Microcystis* Allelopathy and Bloom Community Response to High and Low N:P Ratios ..............................................................................................................49
Literature cited .................................................................................................................................71

Chapter 4 - Conclusions .............................................................................................................74
Literature cited .................................................................................................................................80

Appendix .......................................................................................................................................81
List of tables

Table 2.1. Microcystin concentration in the water of Grand Lake St Mary's during 2013........30
Table 2.2. ODNR GLSM 2012 sampling data.................................................................32
Table 2.3. Nutrient additions to standard BG11 media..................................................35
Table 2.4 - Cultures and isolates maintained.................................................................39
Table 2.5 - Microcystin ELISA test results.................................................................42
Table 3.1 - BG11Δ media formulation........................................................................55
Table 3.2 - Nitrogen and phosphorous concentrations and molar ratios used in semicontinuous batch experiments.................................................................57
List of figures

Figure 2.1 - Geographical map of Grand Lake St. Mary's ..........................................................31
Figure 2.2 - Growth characteristics of UTEX Microcystis culture #2385 in BG-11+ media........37
Figure 2.3, Grand Lake St. Mary's agarose/BG11+ pour plate...................................................40
Figure 2.4, Grand Lake St. Mary's agarose/BG11+ streak plate..............................................40
Figure 2.5, GLSM Microcystis colony after suspension in BG11+ media.................................41
Figure 2.6. GLSM Microcystis colony 4 weeks after suspension in media.................................41
Figure 2.7, GLSM Microcystis culture 8 weeks after suspension in media.................................42
Figure 2.8. Spectrograph analysis of filtrate from GLSM-UCMA1 isolate.................................42
Figure 3.1 - Model competition for a single resource. .................................................................51
Figure 3.2 - Structure of microcystin-LR..................................................................................53
Figure 3.3 - Growth curves of GLSM-UCMA1 Microcystis (MA) and GLSM-UCNP1 Nitzschia (NP) monoculture isolates in limited phosphorous culture.............................................59
Figure 3.4 - Comparison of mean Microcystis and Nitzschia cell densities in semicontinuous batch competition experiments.................................................................60
Figure 3.5 - Comparison of Microcystis and Nitzschia cell densities by nutrient ratios on day 1 .......................................................................................................................................60
Figure 3.6 - Comparison of Microcystis and Nitzschia cell densities by nutrient ratios on day 18 .......................................................................................................................................61
Figure 3.7 - Comparison of Microcystis and Nitzschia cell densities by nutrient ratios on day 85 .......................................................................................................................................61
Figure 3.8 - Semicontinuous batch experiments between GLSM-UCMA1 and GLSM-UCNP1 at 10:1 and 100:1 N:P ratios..................................................................................................62
Figure 3.9 - Comparison of Microcystis and Nitzschia cell densities on day 1 after inoculation of semicontinuous batch tandem experiments.........................................................63
Figure 3.10 - Comparison of Microcystis and Nitzschia cell densities on day 18 after inoculation of semicontinuous batch tandem experiments.........................................................63
Figure 3.11 - Comparison of Microcystis and Nitzschia cell densities on day 85 after inoculation of semicontinuous batch tandem experiments.........................................................64
Figure 3.12 - Total trial productivity comparison of Microcystis and Nitzschia cell counts throughout the semicontinuous trials.................................................................64
Figure 3.13 - MANOVA testing of factors and interactions affecting cell counts........................65
Figure 3.14 - Microcystin Mc-LR Nitzschia bioassay..........................................................66
Figure 3.15 - Total cells censused during the Mc-LR trial......................................................66
Figure 4.1 - Summer 2012 Grand Lake St. Mary's ODNR water sampling data.........................75
Figure 4.2 - Overview of aquatic food web interactions showing organisms involved in and interacting with the microbial loop (Barber 2007)..........................78
Chapter 1 - Review and Overview of Thesis

Algal blooms

Harmful Algal blooms (HABs), undesirable proliferations of aquatic photosynthetic organisms, are well-documented phenomena attracting attention from regulatory agencies in response to apparent increases in event frequency and severity (Smayda 2008, Anderson 2002, Heisler 2008, Hudnell 2010). These blooms can be marine or freshwater, and of cosmopolitan or remote occurrence. Communities of phytoplankton present can consist of Chlorophytes, Bacillaria (diatoms) Cyanobacteria, Dinoflagellates, Euglenoids and other taxa of photosynthetic eukaryotic organisms (Zingone and Enevoldsen 2000). Phytoplanktonic HABs can produce toxins and anoxia, reduce water clarity and light penetrance to the point of eliminating competitive macrophytes, and have been linked to fish kills, livestock deaths, undesirable water quality, and possible human health effects raging from acute toxicity (Shideh 1998) to tumorigenic effects at chronic subclinical exposures (Huisman 2005).

Bloom formation dynamics are complicated at best, but necessarily involve either increased reproduction or decreased mortality rates that result in dramatic population growth. Reproductive rates are somewhat organism specific and partly determined by effects on nutrient uptake of organism size, and surface area: volume (S:V) ratios (C. S. Reynolds 1989, Lichtman et al. 2010). Smaller organisms with higher S:V ratios are generally expected to have more competitive nutrient uptake and reproductive rates in nutrient poor systems; larger organisms may stay competitive with specific, often attenuate morphologies that increase organismal surface area (Lewis 1976). Major influences on the reproductive rates of these autotrophs include abiotic factors such
astemperature (Huber 2012), inorganic or organic nutrient concentrations (Smayda 2008), and photosynthetically active radiation (Huisman 2005). Cell mortality increases with UV damage (Zamir 2001), grazer, phage, or other biotic activity (Proctor and Fuhrman 1990), and routine environmental insults such as toxins or insufficient resource availability (Paerl and Fulton 2006). Immigration or inoculation from other areas may also contribute to bloom formation. Some bloom organisms have benthic as well as planktonic phases in their life cycles, and many have additional methods of cell motility, allowing for dispersal and migration. In addition to organismal migration, large introductions of organisms can take place via water currents, seasonal turnover, or anthropogenic activity such as ship ballast water dumping (Smayda 2008). Washout and sedimentation are emigration drivers in lotic systems, particularly turbid light-limited ones. Lotic systems tend to experience blooms less frequently (Chalar 2007).

With sufficient resources, populations follow logistic growth until resource limitation causes stabilization at the carrying capacity. Prior to that, a population's biotic interactions tend to influence its size and functional role within a larger community. Phytoplankton communities tend to be diverse, and milliliter samples often have dozens of species present with no dominant organism (Armstrong 1980). This phenomenon seemingly violates predictions of Gause's law of competitive exclusion stating that two species in competition for a limited resource cannot indefinitely coexist. This has been termed the "Paradox of Plankton" by G. Evelyn Hutchinson (Hutchinson 1961, Hardin 1960), and proposed mechanisms generally rely on an assortment of disturbances of the system, presumably with species diversity optimums at intermediate disturbance levels (Connell 1978, Reynolds 1993, Hutchinson 1961, Hambright 2000). A constant
flux of conditions and changes in limiting nutrients ensures neither an environmental equilibrium nor dominant organisms is permanently established in a given system (Scheffer 2003). In contrast, algal blooms represent low diversity and low evenness (Chalar 2007, Kononen 2001, Sedmak and Kosi 1998). In addition to high cell concentrations, blooms are generally low diversity and often referred to by the dominant species present; red tides, *Microcystis* blooms, *Euglena* blooms and so forth (Sedmak and Kosi 1998). Low species diversity HABs may be the result of changes in the normal rate of disturbance, or disturbances of such severity that they trivialize other factors (Rojo and Cobelas 1993).

In phytoplankton communities, a dozen macronutrients and micronutrients are considered essential for the growth of planktonic photosynthetic autotrophs (Anderson 2005). Formulations used for algal culturing tend to be very similar in composition to terrestrial plant hydroponic formulas such as Hoagland’s solution. These nutrients should be essential and for the most part non-substitutable (Tilman 1982). Bloom formation is frequently attributed to exogenous nutrient availability, enrichment, or systemic eutrophication (Smayda 2008, Heisler 2008).

Nutrient enrichment and increases in productivity have been linked to loss of system diversity. This has been long-demonstrated in long-term parkgrass field fertilizer experiments in Rothamsted UK which were started in 1856 and continue to the present day (Virtanen et al. 2000). A number of supporting limnological and phycological studies have also been performed (Velghe, Vermaire and Gregory-Eaves 2012, Dodson, Arnott and Cottingham 2000, Gamfeldt and Helmut 2011, Jeppesen 2000, Worm and Lotze 2006, Van Raalte and Valiela 1975). With short generation times and requiring
comparatively small study areas and resources, phytoplankton species provide promising model organisms for these types of experiments. While established systems may be comparatively resource-poor, with resources equally limited and limiting, artificially or newly enriched systems (with an abundance of some nutrients) will have any unenriched resources brought into comparative scarcity. Species best able to compete for these scarce resources should predominate (Tilman, 1982). In the case of highly productive systems and dense algal blooms, this limiting resource may plausibly be light (Huisman 1991, Mur, Gons and van Liere 1977, Whitelam and Codd 1983), which would present an advantage to buoyant vacuolated, planktonic genera. These organisms should also compete favorably for access to atmospheric gases such as carbon as carbon dioxide, oxygen or nitrogen. More typical limiting resources of freshwater inland systems are fixed nitrogen and phosphorous, particularly phosphorous (Anderson 2002), and enrichment of these two elements has been strongly linked to HAB occurrence. (Heisler 2008, Smayda 2008)

Environmental phosphorous enrichment increases algal productivity (Schindler 1977) and affects community structure, with diversity decreases and increased likelihood of cyanobacteria dominance. These effects were first widely appreciated as a result of numerous whole-lake experiments performed in the late 1960s and 1970s (Schindler 1973, Ahlgren 1971). Phosphorous, considered a macronutrient needed in comparatively large abundance by most organisms, is also one of the three major components of commercially available agricultural fertilizers, with product NPK ratings describing the relative ratios of nitrogen, phosphorous, and potassium. Environmentally available phosphorous in water bodies is usually present as either inorganic
orthophosphate, which may be taken up by all organisms, or organic organophosphorous compounds such as phospholipids, nucleic acids, phosphorylated proteins and other biologically produced molecules (Healy 1982, Wang 2010). Organic phosphorous compounds are scavenged by organisms using a variety of mechanisms, pH-dependent acid and alkaline phosphatases notable among them (Harke and Berry 2012, Wang et al. 2006). The uptake of both organic phosphorous and inorganic orthophosphate is generally considered to be dependent upon external phosphate levels, and may be dramatically increased by phosphorous deprivation (Healy 1982). Deficiency appears to up-regulate production of transport proteins and enzymes involved in phosphorous uptake (Shen and Lirong 2007). At or above 0.1 mM concentrations, analogous to mesotrophic water systems, passive uptake predominates with surface area:volume ratios presumably becoming more important (Carr and Whiton 1982). Phosphate uptake rates have been found to vary with light intensity, Mg$^{2+}$ concentration, and temperature (Carr and Whiton 1982)

Unlike marine systems, nitrogen limitation in freshwater phytoplankton communities is generally believed to be less common than phosphorous limitation (Anderson 2002, Schindler 1977). Dense algal blooms are possible exceptions to this scenario, especially in the vicinity of high-phosphorous inputs from agriculture or wastewater (Kleinman 2011). Changes in available nitrogen have long been believed to play a part in seasonal planktonic species succession (Reynolds 1989). If true, evolving limitation in blooms would provide competitive advantage for nitrogen-efficient or diazotrophic organisms. The chemical species of nitrogen present may also be relevant, with organisms demonstrating greater facility for ammonium, nitrate, nitrite or more
recently anthropogenic urea input, which is believed to be a possible contributor to cyanobacterial dominance (Anderson 2002, Berman and Chava 1999).

**Cyanobacteria**

Cyanobacteria comprise a large and diverse branch of prokaryotic organisms, and were likely the first organisms on Earth to perform oxygenic photosynthesis (Holland 2006). Oxygen production by these organisms is believed to be responsible for Earth’s rising atmospheric oxygen levels prior to and during the Great Oxygenation Event (GOE) approximately 2.4 billion years ago, drastically altering atmospheric, geological, and biological systems (Holland 2006). Despite the age of the phylum they continue to be ubiquitous, contributing a significant estimated proportion of Earth's primary productivity (Liu, Nolla and Campbell 1997). In addition to natural systems, cyanobacteria readily colonize and take advantage of man-made or anthropogenically-altered systems, and the responses of these organisms to anthropogenic environmental changes such as rising carbon dioxide levels, temperatures, and nutrients is a subject of considerable research and interest.

Cyanobacterial dominance in aquatic communities has been associated with comparatively low available N:P in systems (Schindler 1973, Elser 1999, Plinski and Jozwiak 1999, Luttenton and Lowe 2006), particularly below the 15:1 N:P"Redfield ratio" organismal content of phytoplankton experimentally determined by Alfred Redfield (Anderson 2002). This is believed to be true of nitrogen-fixing genera, which use atmospheric N\textsubscript{2} as necessary to address shortages as well as genera incapable of nitrogen fixation (Elser 1999).
Cyanobacteria are generally characterized as poor competitors for phosphorous: with low Vmax/k affinity and uptake values compared to heterotrophic bacteria and eukaryotic algae (Healy 1982, Carr and Whiton 1982). This may be explained and supported by a number of possible mechanisms, including inefficient transport proteins and phosphatases, interference from mucilage sheaths produced by many cyanobacterial genera, spherical or simple morphologies resulting in poor surface area:volume ratios, colonial habits, inefficient usage or storage, and others (Harke and Berry 2012).

Conversely, cyanobacteria as a group can be considered nitrogen-efficient. Some species of cyanobacteria may be diazotrophic and will acquire atmospheric nitrogen under N-limited conditions, using available dissolved nitrogen preferentially due to the high energy requirements of nitrogen fixation. Tracking N:P values of affected water bodies has been proposed and investigated as a potential way to predict algal blooms. Debate exists, however, as to whether nutrient ratios (Rhee 1980) or absolute concentrations (Downing, Watson and McCauley 2001) are more important for water quality and bloom-risk assessments (Miller 2005, Flynn 2010).

Microcystis

*Microcystis* genera, often generalized as *M. aeruginosa*, are well-known bloom-forming cyanobacteria (Carmichael 1997). *Microcystis* is a medium-sized (4.5-5.5 µm) coccoid non-nitrogen-fixing cyanobacteria (Huisman 2005), that forms large grazer and desiccation-resistant colonies up to 8mm across (Jüttner 2010, Yang 2012), surrounded by a collective layer of mucilage (Watanabe 1996). This colonial habit may present
disadvantages as well as advantages. Although *Microcystis* can form exceptionally large blooms, it is also considered one of the worst competitors for nutrients (Holm and Armstrong 1981). This is especially true in oligotrophic environments (Huisman 2005), where colonies sink below the epilimnion quickly in accordance with Stokes' Law unless resources are devoted to maintaining buoyancy (Heisler 2008). Like many cyanobacteria (Walsby 1994), *Microcystis* has rigid proteinaceous gas vesicles that accumulate gas and provide buoyancy in response to reduced irradiance (Walsby 1994). Large colonies of *Microcystis* are able to migrate quickly up and down in the water column in response to lighting conditions (Huisman 2005). The ability of *Microcystis* to rapidly position itself in the water column for optimal lighting conditions and overshadow competitor organisms gives it distinct competitive advantages, particularly in developed blooms and highly eutrophic systems with low water clarity (Xiao 2012). *Microcystis* blooms typically follow a seasonal pattern, being transported to the top of the water column during the spring turnover, occupying the uppermost surface of the water for much of the summer, and then sinking back to the bottom and switching to fermentative metabolism (Moezelaar 1994) in the fall as lower temperatures and reduced metabolism minimize gas production from carbohydrates (Hambright 2000, Huisman 2005, Blomqvist and Brunberg 2002). Overwintering *Microcystis* exposed to light have lower survivorship, possibly due to increased oxygen production, CO$_2$ consumption, and a failure of fermentative metabolism (Blomqvist and Brunberg 2002).

Identification of *Microcystis* species in field samples tends to be based on colony morphology, gas vesicles, cell size and morphology, and coloration. Microcystin toxin production is another defining characteristic, although all strains of toxin producing
species often are not competent toxin producers (Watanabe 1996). Although these characters are heritable, they also are highly plastic and may be affected by environmental conditions. Described Microcystis species include M. aeruginosa, M. viridis, M. wesenbergii, M. ichthyolabe, and M. novacekii, but definitive genetic or metabolic differences have not been established or published (Lepere, Wilmotte and Barbana 2000). The classifications seem questionable (Neilan 1997), and have been referred to as morphospecies by some authors (Komarek and Komarkova 2002). This document will use Microcystis as a general classification.

Microcystis, like many cyanobacteria, is resistant to laboratory isolation and culturing (Shirai 1989). The colonial mucilage sheath traps and holds both Microcystis cells as well as heterotrophic bacteria (Shirai 1989, Nan 2011). These Microcystis associated heterotrophs may perform important symbiotic nutrient acquisition and retention roles (Yuan et al. 2009, Nan 2011, Shen 2011). The growth requirements of Microcystis are relatively fastidious, particularly in axenic cultures, and domestically available cell lines are neither axenic nor recently isolated, with many lab cultures dating back to the 1950s and 1960s (UTEX Algal culture collection).

Microcystins

Most, but not all (Watanabe 1996) strains of Microcystis produce cyclic heptapeptides known as microcystins (Lawton 2001). Microcystins are produced by cyanobacteria via a non-ribosomal translation process involving amino acid reactions on scaffold proteins that complicates investigation of regulation and production (Kaebernick 2001). The scaffold protein genes involved, McyA-McyE, have been investigated for use
as bloom toxicity predictors via methods such as qPCR (Al-Tebrineh 2011, Furukawa 2006). Microcystin’s structure contains a unique and cyanobacterial-specific ADDA amino acid group that fluoresces under 238 \( \mu \)m light which can be used for detection purposes (Westrick and Oehrle 2003).

Toxic effects following microcystin exposure result from permanent inactivation of protein phosphatases 1 & 2 and possibly oxidative stress (MacKintosh 1990). Beyond acute toxicity, the hyper-phosphorylations caused by chronic low-dose exposure to microcystins have been investigated as possible promoters of liver tumors and cancers (Lezcano 2012, Nishiwaki 1991, Westrick and Oehrle 2003).

While allelopathic affects of microcystins are in doubt, their role in antiherbivory seems established. Recent studies have implicated community selection of *Microcystis* cells by *Dreissena polymorpha* mussels, an unwelcome invader to North American and European waterways (Vanderploeg 2002). *D. polymorpha* can reach extremely high population densities in affected areas, in part by consuming phytoplankton. *D. polymorpha* reject *Microcystis* cells (Vanderploeg 2002) in apparent correlation to toxicity in pseudofaeces (Juhel 2006), potentially helping their survivorship selection as part of the phytoplankton assemblage.

**Research overview**

Effects of environmental variables on toxin production have been investigated, including temperature (van der Westhuizen and Eloff 1985), day length and light intensity (Wiedner 2003, Hesse, Dittman and Borner 2001), nitrogen (Orr and Jones 1998), phosphorous (Oh 2000), N:P ratios (Orihel 2012, Vezie 2002), and exposure to grazer
species (Jang, Jung and Takamura 2007). These studies seem to generally positively correlate population growth rate to microcystin production (Long, Jones and Orr 2001). *Microcystis* strains that produce microcystins have reduced mortality under oxidative and high-irradiance photoinhibition conditions, indicating a possible protective effect for an organism that typically inhabits the very surface of the epilimnon (Watanabe 1996). Laboratory studies have been devoted over the years to algal nutrient competition in an effort to understand bloom formation: the most well-known examples of these are likely the *Asterionella* and *Cyclotella* Si:P competition experiments performed by David Tillman and often cited in college ecology texts (Tilman, 1977). Others include competitions for nitrogen as ammonium (Yang 2006), toxin-producing versus nontoxic *Microcystis* competition for carbon relevant to climate change (Van der Waal 2011), light-limited competitions between toxic and nontoxic *Microcystis* (Kardinaal 2007), other cyanobacteria, and chlorophytes (Huisman 1991), phosphorous competition with the Chlorophyte *Chlorella* (Shanqin 2011) and the cyanobacteria *Oscillatoria* (Kromkamp, Van Den Heuvel and Mur 2007), and an appreciable number of others, some of which focused on N:P ratios. Monitoring N:P ratios as well as absolute concentrations in bloom affected water bodies is often a major part of watershed management techniques, under the assumption that *Microcystis* and other cyanobacteria have a competitive advantage over other phytoplankton under high low N:P conditions (Smith 1983).

Lacking amongst these are straightforward, in-vitro phosphorus or nitrogen limited studies between *Microcystis* and other organisms under varying N:P ratios. Although controlled research has not been done, strategies such as silica remediation
(to encourage non-bloom organisms such as diatoms) have been performed at bloom sites such as Grand Lake St. Mary's (ODNR 2010). Silica remediation is a practice which would benefit from lab support prior to widespread field use. Chapter 3 of this thesis reports on batch and semicontinuous nutrient studies between *Microcystis* and *Nitzschia*, another typical *Microcystis* bloom community resident, at high and low N:P ratios, with a goal of investigating the assumption of cyanobacterial dominance expectations at low N:P ratios used in watershed management.

Community experiments with a toxin producing organism should include efforts to assess any allelopathic interactions between the organisms. A moderate number of toxicology studies have been performed with microcystins and have been published as reviews (Babica 2006), however a definite pattern of phylogenetic or ecological sensitivity or relevance has yet to emerge. The seemingly obvious questions are, do microcystins inhibit the proliferation of phytoplankton in close contact with toxin-producing *Microcystis* blooms, and does this play a part in bloom formation or persistence? Chapter 3 of this thesis also compared growth curves of *Nitzschia* in a standard media solution with similar inoculations performed in microcystin-LR (Mc-LR) to investigate allelopathic affects of microcystins on our experimental diatom.

Before any labwork could be conducted, newly isolated strains of both *Microcystis* and its competitor organisms needed to be established, as cultures maintained in vitro for long periods of time appear to have diverged from wild organisms. Loss of gas vesicles and buoyancy, loss of colonial habit, and loss of microcystin production are all typical of long-held lab *Microcystis* strains. Diatoms in-vitro also are known to diverge from environmental counterparts, with
successive generations typically reducing in size and vigor in the absence of sexual reproduction, which usually fails to occur in a lab. Chapter 2 of this thesis reports on the environmental sampling and lab isolation of *Microcystis* and *Nitzschia* strains used in later testing, as well as a number of other phytoplankton organisms identified and cultured during the process.


ODNR. *State Actions for Water Quality Improvement at Grand Lake St. Mary's*. Ohio Department of Natural Resources (ODNR), July, 2010.


Chapter 2 *Microcystis* isolation and culturing

Abstract

*Microcystis* is a bloom forming cyanobacteria associated with eutrophic water systems; however, the genus is resistant to isolation efforts and laboratory culturing. *Microcystis* bloom samples were obtained from Grand Lake St Mary’s and several other sources. These samples were mechanically agitated and then filtered to disrupt *Microcystis* colonies and exclude filamentous organisms. Aliquots of filtrate were cultured on solid media and organismal growth monitored by microscopy. *Microcystisisolates were identified by comparison to established lab strains and by production of microcystin toxins. Nitzschia sp. and other phytoplankton organisms were also isolated from plate cultures. Isolates were maintained in semicontinuous batch culture under summer seasonal lighting and temperature conditions for future experimental use.

Introduction

In addition to being the primary source of potable water for the surrounding communities, Grand Lake St. Mary’s(GLSM) in midstate Ohio hosts a $200 million/year tourism industry threatened by frequent regulatory public health advisory postings(ODNR 2010) resulting from algal blooms, predominantly *Microcystis*, a bloom-forming cyanobacteria known for toxin production. Recurrent visible *Microcystis* blooms and elevated concentrations of cyanotoxins have become an annual event coinciding with peak summer recreation use (Table 2.1). GLSM Microcystin toxin concentrationstest well above the World Health Organization drinking water standard of 1ppb for much of the summer(Gupta 2003)(Table 2.1).
Table 2.1. Microcystin concentration in the water of Grand Lake St Mary’s during 2013.

<table>
<thead>
<tr>
<th>Test date</th>
<th>Sample type</th>
<th>Sample location type</th>
<th>Sampled location</th>
<th>Concentration (ppb)</th>
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Lake Grand St. Mary’s and its tributaries are located in an agriculturally dominated region, and receive exogenous nutrients from fertilizer runoff, as well as from Northmor golf course on the lake’s north shore (Figure 2.1). Like many Ohio lakes, GLSM is a shallow-depth (1.5-2m), man-made, agricultural-use oriented reservoir, suggesting re-entrainment and recycling of nutrients from the bottom (Table 2.2). GLSM total phosphorous levels are often 0.1-0.2mg/L or higher, which along with high nitrogen levels and elevated chlorophyll qualify it as a hypereutrophic system in the Carlson Trophic Index (Carlson 1977) (Table 2.2), putting it at high risk for harmful algal blooms and related undesirable water conditions (Heisler 2008, Smayda 2008). While “Microcystis” blooms and elevated microcystin toxin levels at GLSM have been widely reported and sampled, no efforts at culturing, genotyping or sequencing, or community metagenomic analyses have been reported. The nature of the bloom community is relatively undefined, and responses to water chemistry manipulation are unknown.

Figure 2.1 - Geographical map of Grand Lake St. Mary’s. Shown are approximate locations of ODNR sampling locations shown in Table 2.2. (GLSM ODNR website, personal communication with Rick Wilson, ODNR)
Table 2.2. ODNR GLSM 2012 sampling data. Lake depth, Secchi depth, chlorophyll A and total phosphorus concentrations are shown. Test results consistently exceeded chlor-a, TP, and Secchi disk results consistently exceeded published criteria for Carlson hypereutrophic systems.

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Despite being environmentally ubiquitous and sometimes forming extremely large blooms, cyanobacteria can be difficult to isolate, culture, and maintain in laboratory settings (Shirai 1989, Carmichael and Gorham 1974). Domestically available *Microcystis* cultures are in short supply. Axenic cultures in particular can be difficult or impossible to establish due to the difficulty in separating associated bacteria from the cyanobacterial colonies (Han 2010, Chen 2011). Axenic cultures also tend to grow more
slowly, and are more prone to population crashes than xenic cultures. Bacterial cells which are not actively dividing are believed to be less susceptible to phenol or antibiotic treatment, and dark treatment of phytoplankton samples has been used to minimize bacterial contamination in cyanobacterial isolation efforts (Carmichael and Gorham 1974).

Environmental *Microcystis* samples are primarily identified based on morphology, which can be qualitative and challenging despite the genus’ distinctive combination of visible traits. Production of the toxin microcystin can be used as an additional characteristic in identifying isolates. While microcystin production is not unique to the genus, other cyanobacteria which produce it are morphologically dissimilar to *Microcystis*. Microcystin production was thus used here as an additional necessary isolate characteristic. Enzyme-linked immunosorbent assay (ELISA) testing is the primary commercial testing method used, however the characteristic “adda” amino acid moiety of microcystins strongly absorbs 238nm UV light (Westrick and Oehrle 2003), and may be detectable by spectrometer at sufficient concentrations. UV absorbance is also typically used for microcystin identification by high pressure liquid chromatography (HPLC). Colonial colonies also present additional enumeration challenges (Seung-Hyun 2006), while simultaneously being the most easily identifiable feature of the genus. *Microcystis* cells absent a colony are very difficult or impossible to identify visually.

*Microcystis* a challenge to move from the environment into laboratory culture for experimentation purposes. This chapter reports on the collection of Grand Lake St. Mary's water samples during a *Microcystis* bloom, and on the development of
methodology for successful isolation and culturing of testable organisms.

Methods

Environmental sampling was performed near the North-East boat docks of Grand Lake Saint Mary’s (40.541414,-84.419436). Water samples from visible but diffuse blue-green scums were taken using wide-mouth bottles attached to sampling rods on July 25th and August 25th 2012, at roughly 5:00 pm. Samples were placed on ice, returned to the University of Cincinnati and refrigerated until use the following day. Water samples were visually inspected on a Nikon Eclipse E400 light microscope at 100x magnification. Samples were kept on ice for less than 48 hours before isolation plating efforts were performed.

Nutrient media: Supplemented (table 2.3) BG11 nutrient media, (Anderson 2005) from Sigma Aldrich (Milwaukee, WI) was autoclaved separately from the agar or agarose solidifying agent (200mL of BG11+ media, 50mL of deionized water with 2.0g of gelling agent) and allowed to cool to 60°C before combining. This media was used for routine culture maintenance as well as as a nutrient base for solid media.

Table 2.3. Nutrient additions to standard BG11 media. Supplemented media referred to in the remained of the text as BG11+. Organic additions were according to Watanabe et al. (2000), silica to facilitate diatom growth, and tricine to help stabilize pH values at 7.1-7.3

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<th>Added compound</th>
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<th>Molar mass (g/M)</th>
<th>Concentration in BG11+media (M)</th>
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</tbody>
</table>
Plate culture and isolation: Microcystis containing lakewater samples were homogenized for one minute in 20mL centrifuge tubes, and then filtered through 20µM Whatman filter paper to exclude colonies and filamentous organisms. Autoclaved media was allowed to cool to under 40°C before introduction of homogenized and filtered lake sample (100µL per 250mL of media). The organism-containing mixture was agitated and quickly poured into 10 plastic petri plates (20-25mL per plate). Plates were wrapped in parafilm and placed under 100 µmoles/m²/s, 16:8 hour day:night cycle lighting at 20°C and allowed to incubate until colonies were detectable upon visual inspection. Initial identification of colonies on plates was facilitated using a low-wavelength UV lamp, followed by microscope evaluation for suitable cell size and morphology. Colonies were then transferred by sterile toothpick under a field microscope into 1mL tubes containing BG11+ media, and allowed to incubate another 2 weeks. Ten additional plates were prepared by quadrant streaking on sterile BG11+ media plates and incubated similarly. Homogenized and filtered GLSM water samples were kept in dark conditions for 24 hours. Phenol was added to produce 1.25E-4M and 1.25E-5M concentrations, and the filtrate placed back into dark conditions for 4 hours. 100L of each concentration was then added to 250ML of 40°C and used to produce 10 pour plates each. Ten additional plates were prepared by quadrant streaking on sterile BG11+ media plates and incubated similarly. Accurate enumeration requires that cells be free and not clumped in colonies, therefore preliminary colony separations of isolates were attempted using 5% acetic acid, 5% sodium hydroxide, 20% and 40% ethanol, 15% hydrogen peroxide, and heated to 100°C in Eppendorf tubes for 10 minutes.
Reference cultures: UTEX strain 2385 (UTEX Algal culture collection), a commonly used Microcystis culture, and a sequenced (Kaneko 2008), axenic strain of Microcystis aeruginosa, NIES-843, were purchased from University of Texas and the Japanese National Institute of Environmental Studies respectively for comparison to GLSM organisms. An additional large (2 liter) field sample from an Eastern Lake Erie Microcystis bloom was provided for isolation attempts by Dr. Mike Miller (personal communications). Lab organisms were cultured similarly to field-collected ones. Aseptic technique was used, and all non-sterile equipment and media was autoclaved prior to use.

Microcystin analysis: Putative Microcystis cultures were evaluated for microcystin toxin production by Dr. Armah delaCruz of the Cincinnati Microbiological and Chemical Exposure Assessment Research Division office of the EPA using microplate ELISA assays by EnviroLogix, Inc. (Maine, USA) per published methods (delaCruz et al. 2012). Two mature (30 day old) cell-free filtrates of cultures taken from cultures during nutrient rinsing were evaluated for microcystin-indicative absorbance peaks by spectrophotometry at 238nm.

Results

Reference cultures: Streak plating on DIFCO-2A bacterial agar indicated that reference cultures of UTEX-2385 contained heterotrophic organism contaminants. These Microcystis cultures attained very high cell densities in culture (Figure 2.2), presented no colonial habit or discernible mucilage production, lacked visible gas vesicles, and tested
negative for microcystins (Figure 2.9). UTEX-2385 cultures were also prone to population crashes. In comparison, NIES-843 showed no signs of heterotrophic bacterial contaminants and appeared very similar to the newly collected environmental samples. NIES-843 cultures grew slowly when compared to UTEX-2385, field isolated cultures, and other published strain studies (Watanabe 1996).

![Figure 2.2 - Growth characteristics of UTEX Microcystis culture #2385 in BG11+ media. Cultures were non-colonial and very high populations were attained compared to typical environmental samples and published Microcystis culture experiments.](image)

*Environmental samples*: On visual inspection, Lake St. Mary's water samples contained abundant grazer species such as rotifers and Cladocerans, and a number of algal species typically associated with eutrophic water bodies, but predominantly consisted of Microcystis colonies. Medium sized (1.0-3.0mm) Microcystis colonies were visible to the unassisted eye, and would produce a floating scum up to 0.5cm thick if samples were left undisturbed to settle. Microcystis cell density estimates were not performed because
of the difficulty in identifying the cells in mixed environmental samples after colony separation. *Planktothrix* filaments were the second most common organism present. Lake Erie samples contained predominantly *Microcystis* colonies and *Chlorella*, with minimal active grazers.

**Solid media culture and isolation:** Both pour plates and spread plates took 2-3 weeks to develop algal colonies of bloom organisms visible at 100x magnification. Plates not wrapped in parafilm failed to produce visible colonies before drying to the point of unsuitable growth conditions after roughly 4 weeks. Parafilm wrapped plates produced visible diatom colonies after as few as 2-3 weeks, and other algal colonies after 6-8 weeks. None of the phenol-treated pour plates produced cyanobacterial growth. Diatoms (*Nitzschia*) and Chlorophyte (*Scenedesmus, Chlorella, and Chlamydomonas*) colonies were produced at low concentrations of less than ten per plate.

Cyanobacterial colonies presented as a yellowish color under UV light, in comparison to chlorophytes, which appeared as a deeper orange or reddish color. Putative *Microcystis* colonies were among the later species to appear in the agarose, but not agar, plates, becoming noticeable as bright "traffic light green" colonies (Figure 2.3 and 2.4) with small cells, regular edges, and a visible mucilage sheath (Figure 2.5), as compared to "forest green", large-celled *Chlorophyte* colonies. Organisms were then identified using, visual comparison to strain NIES-843 with the assistance of Dr. Miriam Kannan of Northern Kentucky University. Four unialgal cultures of *Microcystis* were isolated, along with a number of other species (Table 2.4). Unlike other organisms, *Microcystis* colonies always lifted out of the agarose as an intact colony, presumably
due to the mucilage sheath. After transfer to liquid media, isolates adopted typical irregular *Microcystis* colonial structures. All *Microcystis* colonies displayed buoyancy and evidence of gas vesicles on 1000x magnification microscopic examination.

Table 2.4 - Cultures and isolates maintained

<table>
<thead>
<tr>
<th><em>Microcystis</em> species and strain/culture</th>
<th>Source</th>
<th>Notes or identifying characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Microcystis aeruginosa</em>, NIES-843</td>
<td>Japan Prefecture</td>
<td>Sequenced axenic sample</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em>, 2385</td>
<td>UTEX</td>
<td>Not axenic, non-colonial, small cells with no vacuoles</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em>, ERIE-UCB</td>
<td>Isolated from Lake Erie samples provided by Dr. Mike Miller</td>
<td>&quot;Flakey,&quot; chunky, flat colonies</td>
</tr>
<tr>
<td><em>Microcystis sp.</em>, ERIE-UCF</td>
<td>Isolated from Lake Erie samples provided by Dr. Mike Miller</td>
<td>Minimally buoyant, usually very bluish, possibly <em>M. viridis</em></td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em>, GLSM-UCMA1</td>
<td>Isolated from GLSM samples</td>
<td>Visible vacuoles, spherical colonies</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em>, GLSM-UCMA2</td>
<td>Isolated from GLSM samples</td>
<td>Stringy colonies</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em>, GLSM-UCMA3</td>
<td>Isolated from GLSM samples</td>
<td>Comparatively slow growth</td>
</tr>
<tr>
<td><em>Microcystis sp.</em>, GLSM-UCMA4</td>
<td>Isolated from GLSM samples</td>
<td>Minimally buoyant, bluish, possibly <em>M. viridis</em></td>
</tr>
<tr>
<td>Other isolates from bloom samples</td>
<td>Source</td>
<td>Notes or identifying characteristics</td>
</tr>
<tr>
<td><em>Ankistrodesmus</em> sp.</td>
<td>Isolated from Lake Erie samples provided by Dr. Mike Miller</td>
<td>Highly bent shape</td>
</tr>
<tr>
<td><em>Chlorella</em> sp.</td>
<td>Isolated from Lake Erie samples provided by Dr. Mike Miller</td>
<td>Very fast growth</td>
</tr>
<tr>
<td><em>Chlamydomonas</em> sp.</td>
<td>Isolated from GLSM samples</td>
<td>Very motile</td>
</tr>
<tr>
<td><em>Nitzschia</em> sp.</td>
<td>Isolated from GLSM samples</td>
<td>Motile</td>
</tr>
<tr>
<td><em>Oscillatoria</em> sp.</td>
<td>Isolated from GLSM samples</td>
<td>Very motile, bluish coloration, forms mats</td>
</tr>
<tr>
<td><em>Planktothrix</em> sp.</td>
<td>Isolated from GLSM samples</td>
<td>Vacuolated, motile</td>
</tr>
<tr>
<td><em>Pseudo-Nitzschia</em> sp.</td>
<td>Isolated from Lake Erie samples provided by Dr. Mike Miller</td>
<td>Very small, motile</td>
</tr>
<tr>
<td><em>Synechococcus</em> sp.</td>
<td>Isolated from Lake Erie samples provided by Dr. Mike Miller</td>
<td>Very small, bluish coloration</td>
</tr>
<tr>
<td><em>Scenedesmus quadricauda</em></td>
<td>Isolated during experiment from GLSM samples</td>
<td>Average cell size, rounded cells, visible spines</td>
</tr>
</tbody>
</table>

39
Figure 2.3, Grand Lake St. Mary's agarose/BG11+ pour plate prepared with 100μL of homogenized and filtered water sample after 8 weeks of incubation. Cyanobacteria are visible as more blue-green, less yellow, often ovoid colonies.

Figure 2.4, Grand Lake St. Mary's agarose/BG11+ streak plate used for isolation of algal cells after 7 weeks of incubation.
Figure 2.5. GLSM *Microcystis* colony after suspension in BG11+ media presenting a regular margin surrounded by a comparatively thick mucilage layer.

Figure 2.6. GLSM *Microcystis* colony 4 weeks after suspension in media. Voids are evident and colony morphology was more closely identifiable with environmental isolates, which are typically irregularly shaped.
Figure 2.7, GLSM *Microcystis* culture 8 weeks after suspension in media. Discrete, irregular and buoyant colonies are visible after manual agitation.

*Microcystins analysis*: Two cell-free filtrates of GLSM-UCMA1 cultures demonstrated absorbance peaks at 238nm (Figure 2.8). Cultures GLSM-MA1, 2 and 4 returned positive ELISA results for microcystins; GLSM-MAUC3 was not tested. The *Planktothrix* isolate and UTEX 2385 were also tested and returned negative results (Table 2.5).

Figure 2.8. - Representative chromatogram of filtrate from mature (peak phase) GLSM-UCMA1 isolate demonstrating an absorbance peak at approximately 238nm, indicating probable presence of microcystins
Table 2.5 - EPA microcystin ELISA test results. UTEX-2385 returned negative results, as did previously performed Lake Erie isolates and a GLSM *Planktothrix* isolate tested. All GLSM *Microcystis* cultures tested positive for the presence of microcystins

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th>26-Feb-13</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>neat</td>
<td>1/200</td>
<td>neat</td>
<td>1/500</td>
</tr>
<tr>
<td>UTEX 2385</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Erie-UCB</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Erie-UCF</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>GLSM-UCPTX</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>GLSM-UCMA1</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>GLSM-UCMA3</td>
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<td>+</td>
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<tr>
<td>GLSM-UaCMV4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Colony separation:* Acetic acid, ethanol, and sonication did not disrupt colonies sufficiently to allow cell enumeration. Sodium hydroxide and hydrogen peroxide provided complete colony separation, but shriveled cells severely, disrupting their morphology to the point where identification would be difficult in xenic cultures. Boiling for 10 minutes provided complete colony separation with minimal cell morphology disruption. Boiled *Microcystis* cells had a noticeably swollen appearance, to the extent that *Microcystis* cells were not reliably distinguishable from normally much larger spherical eukaryotic cells such as *Scenedesmus* and *Chlorella*.

**Discussion**

While the NIES-843 sequenced isolate served as a good reference and was morphologically in accordance with published species descriptions, UTEX 2385 shared minimal morphological and toxicity characteristics with either GLSM environmental isolates or NIES-843. Similar observations were made of UTEX strain 2386 at the EPA (Dennis Lye, personal observations and communications). Recently published studies of
long-term lab cultures have demonstrated dramatic morphological and metabolic divergence of lab strains over long periods of time (Blount et al., 2008). These two UTEX *Microcystis* samples were first isolated by Wayne Carmichael in 1954 and have had ample opportunity to diverge from environmental organisms. With a doubling time of roughly one day, these isolates have likely had over 20,000 generations to diverge from wild-type organisms. UTEX 2385 has been used in at least one successful toxicology study (Lakshmana, 1998), implying that loss of toxicity may have occurred in the 15 years since then. Isolation and establishing of laboratory strains of *Microcystis*, particularly axenic ones, should continue to be a priority for investigators, despite the challenges involved, and improvements in methodology would greatly facilitate these efforts. Future genetic taxonomy investigations of *Microcystis* in particular will necessitate faster and more reliable isolation of organisms.

Shoreline environmental samples of *Microcystis* blooms were able to be taken from Lake St. Mary’s with a minimum of equipment and time investment. Samples left unrefrigerated for more than 48 hours appeared to quickly degrade, with grazers present visibly eliminating many of the algal cells present, filamentous species overgrowing samples, general chlorosis throughout, and an apparent lowering of the number of *Microcystis* colonies present.

Cyanobacterial colonies probably appeared more yellowish under UV lighting due to the presence of cyanobacteria-specific phycobiliproteins, which have strong emissions in the 580-620nm range (Sun et al., 2003). Unialgal colonies were relatively straightforward to produce on parafilm-wrapped BG11+ plates acceptable apparent separation, but *Microcystis* isolates took a comparatively long time and only grew on
agarose plates, a phenomenon observed by other investigators and sometimes attributed to inhibitors present in typical laboratory agarose products (Shirai 1989). Newly isolated *Microcystis* cultures adopted a colonial habit similar to both environmental observations and sequenced strain NIES-843 (figures 2.6 and 2.7). Generally, cultures tended to have more defined colonies and less diffuse cell scums with apparent agitation, which has been observed by other investigators, and may implicate mechanical forces in colony formation. (Li 2013). Cultures were maintained by monthly inoculation into fresh BG11+ media for over two years with no observed change in traits or growth patterns. Cultures typically reached peak apparent cell density and mass 1-2 weeks after inoculation into fresh media. After 45-60 days cultures were chlorotic and depigmented, but apparently had populations of viable cells, as they could be used to establish new cultures for 4 months or more. Colonies were observed to sink to the bottom after exposure to irradiance, and float to the top of the media during a night cycle. Lowered lighting conditions and temperatures appeared to contribute to culture longevity. Cultures grown in high lighting conditions (200 µmoles/m²/s) produced larger colonies, but less pigmentation, indicating a certain amount of developmental plasticity that could potentially hinder field identification efforts.

Very dense, mature culture filtrates of GLSM-UCMA1 presumably contained high enough concentrations of microcystins that they could be detected even by simple spectrophotometry. All Lake St. Mary’s isolates tested positive for microcystins by ELISA, strongly suggesting the presence of microcystins. ELISA testing does not discriminate among the 80 variants of microcystins known to be produced however, and
these may vary greatly in toxicity. Additional testing to confirm the dominant microcystins produced by these cultures can and should be performed.

No axenic *Microcystis* cultures were successfully established in the time allotted, as is apparently often the case in *Microcystis* laboratory isolation efforts (Shirai 1989, Carmichael and Gorham 1974); cultures believed to be axenic in initial isolations may also have been contaminated during subsequent culture transfers. Subsequent axenic clean-up of environmental isolate cultures to could most simply be performed using repeated quadrant streak-plates of BG11+/agarose media, as opposed to more labor-intensive pour plating.

A number of phytoplankton species were isolated from bloom samples in the process of obtaining *Microcystis* isolates, including species from toxin producing genera that could be the subject of future studies. Six *Microcystis* isolates from different geographic regions were also isolated and maintained along with the two reference cultures, which could be used for comparative analyses. After boiling, swollen *Microcystis* cells were very difficult to differentiate from normally much larger *Scenedesmus* cells. As *Nitzschia* seemed to be a vigorous growing, easily isolated, and readily identifiable organism isolated from bloom samples, it was selected for subsequent nutrient competition experiments with *Microcystis*. 
Citations


ODNR. *State Actions for Water Quality Improvement at Grand Lake St. Marys*. Ohio Department of Natural Resources (ODNR), July, 2010.


Chapter 3- *Microcystis* Allelopathy and Bloom Community Response to High and Low N:P Ratios

Abstract

*Microcystis* blooms are undesirable proliferations of toxin-producing cyanobacteria often associated with highly eutrophic systems. Debate exists as to how *Microcystis* becomes dominant and forms blooms. Nutrient competition and allelopathic inhibition of other phytoplankton species are possible mechanisms. Recently established isolates of *Microcystis* and *Nitzschia* were deprived of nitrogen and phosphorous to deplete internal stores. These were then introduced into modified BG11 media with either high or low nitrogen/phosphorous (N:P) ratios to determine comparative nutrient growth responses. Both batch and semicontinuous long-term microcosm experiments were performed. Populations were assessed regularly using hemocytometry and compound microscopy. Regardless of ratio, *Nitzschia* experienced greater initial population increases followed by *Microcystis* culture dominance, with some resulting in exclusion of the diatom. To test allelopathy, a bioassay was also performed using *Nitzschia* and microcystin at an environmentally relevant concentration and found no significant population impacts.

Introduction

Environmental cyanobacterial dominance in aquatic systems has long been associated with low N:P ratios, resulting from either comparatively low nitrogen input or phosphorous enrichment. (Schindler 1973, Smith 1983, Heisler 2008, Havens, et al. 2003) Theorized reasons for the phenomenon vary, but chief among them is the ability
of cyanobacteria to fix and use atmospheric nitrogen in the absence of sufficient supply, giving them competitive advantages in nitrogen-poor habitats, although this is believed to come at a metabolic cost (Brill 1977). Total phosphorous levels also tend to correlate to total system productivity and reduced light penetration in the system (Carlson 1977). As cyanobacteria tend to require comparatively low light levels for maximal growth rates (Carr and Whiton 1982, Mur, Gons, and van Liere 1977), light limitation in high-productivity systems may provide cyanobacteria with competitive benefits over other photosynthetic organisms (Ahlgren 1971, Mur, Gons, and van Liere 1977, Yang and Xiangcan 2011). Many bloom-forming cyanobacteria are also toxin producers, implying possible allelopathic interactions; the production of these compounds has also been studied under both nitrogen and phosphorous limited cultures (Dai 2008) as well as different N:P ratios (Orihel 2012, Vezie 2002).

Current water management strategies often prioritize phosphorous limitation as one of the more feasible nutrient input reductions (ODNR 2010). Point-source reduction, alum treatments, and dredging are common remediation methods used for phosphorous control (Kalff 2001). In Lake Saint Mary’s and other lakes, recent efforts to reduce *Microcystis* populations and lower microcystin concentrations have also involved addition of silica to encourage growth of diatoms present (ODNR 2010), presumably only effective if silica was in limiting supply to begin with.

As *Microcystis* are non-diazotrophic cyanobacteria, attempting competitive control by limiting phosphorous as a means of raising N:P ratios may be less effective than reducing both nitrogen and phosphorous concomitantly. These experiments sought to evaluate relative nutrient response for typically limiting nutrients, nitrogen and
phosphorous, between two representative genera of phytoplankton present in Lake St. Mary's: the toxin-producing and bloom-forming cyanobacteria *Microcystis*, and *Nitzschia*, a diatom frequently present in *Microcystis* blooms. Experiments used N:P ratios typically associated with either cyanobacterial dominance or *Chlorophyte/Bacillaria* dominance.

Gause's law of competitive exclusion states that two competitors for a limited resource cannot indefinitely exist; if either organism has even a marginal competitive advantage, that organism will displace the less effective competitor over a sufficient period of time as populations increase and the more effective competitor lowers resource concentrations below levels required for the worse competitor to sustain populations (Figure 3.1). As nitrogen and phosphorous are often limiting or near-limiting nutrients in freshwater systems, they are popular subjects for watershed nutrient-control management strategies, and the effects of manipulation worth studying.

![Figure 3.1](image_url)  
*Figure 3.1 - Model competition for a single resource. Left-hand growth curves (A.) show resource requirements for each two species (R*ₐ and R*ₐ) to maintain equilibrium assuming mortality rates of mₐ and mₐ, respectively. Organism A can attain higher reproductive rates (curve B.), but tends to have lower resource-use efficiency than organism B, being competitively displaced as organism B lowers the resource to levels insufficient to sustain species A. (Tilman, Resource Competition and Community Structure 1982)*
Limited-nutrient batch cultures are one of the simpler ways to assess organismal nutrient acquisition and use efficiency. Batch cultures, however, sometimes produce inconclusive results, particularly insufficient growth response to elucidate population dynamics. These drawbacks can be overcome with long-term semicontinuous-supply competition trials to produce more definitive growth responses. This chapter first establishes baseline nutrient use of phosphorous by *Microcystis* and *Nitzschia* grown in phosphorous-limited batch monocultures of Grand Lake Saint Mary’s *Microcystis sp.* and *Nitzschia sp.* isolates. Growth response for both species was then assessed at 5:1 and 50:1 N:P ratios; similar ratios are expected to respectively yield *Microcystis* and *Nitzschia* dominance when employed in watershed management.

*Microcystin* toxins may also play a part in any competition experiments. *Microcystins* are cyclic heptapeptide compounds believed to be unique to certain taxonomic groups of cyanobacteria, typically bloom-forming *Microcystis* species but also *Nostoc, Oscillatoria, Planktothrix* (Dawson 1998, Otsuka 1998), and lichen symbiotes (Kaasalainen 2009). *Microcystins* share structural similarity with nodularins, another group of pentapeptide cyanotoxins, and may share evolutionary pathways (W. Carmichael 1997). Over 60 amino acid substitutions and variants of *microcystins* have been described (Lawton 2001). This conformational variation is believed to be the result of non-ribosomal synthesis involving scaffold proteins (Westrick and Oehrle 2003, Kaebernick 2001). Most conformational variants are of lesser toxicity than the most well-known variant of *Mc-LR* (Figure 3.2), which has leucine and arginine residues at the 2 and 4 positions (Dawson 1998). Toxicity is somewhat attributed to a characteristic "ADDA" residue, and is greatly reduced when this group is removed or
inactivated (Dahlem 1993). This ADDA group has a characteristic absorbance at 238nm, which can be used for assay and testing purposes, particularly in mass spectrometry and high-performance liquid chromatography (HPLC) (Lawton 2001). Enzyme-linked immunosorbent assays (ELISA) are the other predominantly used testing method in both laboratory and field studies, and qPCR tracking of microcystin production genes has recently been investigated for field prediction of toxin levels (Al-Tebrineh 2011, Furukawa 2006).

Figure 3.2 Structure of microcystin Mc-LR showing cyclic structure and characteristic adda residue (Watanabe 1996).

Microcystins' primary mechanism of toxicity is believed to be a result of permanent inactivation of serine/threonine protein phosphatase (PPA) 1 & 2 enzymes in exposed cells, leading to hyperphosphorylation and cell regulation dysfunction (MacKintosh 1990, Nishiwaki 1991). As might be expected of phosphatase inhibitors, subclinical exposure to microcystins has been implicated in liver tumor promotion (Lezcano 2012).
Additional damage by oxidative stress has also been investigated (MacKintosh 1990).

The role of microcystins in ecological communities is not yet definitively known, although studies have been performed on a variety of organisms. While the toxic effects on mammals and other terrestrial vertebrates are of societal relevance, algal interactions with most terrestrial organisms would presumably not be ecologically relevant. As an endotoxin, microcystins are primarily released upon cell lysis (Lawton 2001), bringing into question their proposed roles in allelopathy. Recent studies demonstrate ejection and subsequent selection against Microcystis cells by zebra mussels (Dreissena polymorpha) (Vanderploeg 2002), possibly in response to beta-cyclocitrinal produced by Microcystis, believed to be a poor food quality warning chemical (Jüttner 2010). Negative feeding and growth effects of microcystin intoxication of Cladocera have been well established (Rohrlack 1999, Demott 1991).

The toxic effects of microcystins on macrophytes and other eukaryotic and prokaryotic phytoplankton have been the subject of some study with mixed results (Babica 2006), calling into question proposed allelopathic affects (El-Sheekh, Khairy and El-Shenody 2010). These studies have sometimes been performed with very high concentrations of microcystins that may not be relevant in ecological systems. Phytoplankton typically co-occurring in Microcystis blooms would seem especially likely to be tolerant of microcystins. This thesis reports on response of a Myrocystis bloom community diatom to microcystin exposure, at a concentration similar to environmental sampling results, using batch culture experiments.
Methods

Unialgal cultures of *Microcystis* and *Nitzschia* recently isolated (3-4 months prior) from Grand Lake St. Mary’s Blooms were grown in BG11+Si media (R. Anderson 2005, Chapter 2) with additional silica at a saturation concentration of 0.05g/L (Morey 1964), under 100µmol/m²/s lighting and 25°C to high (10⁶ to 10⁷ cells/mL) density. Cultures were then maintained for 5 days in filtration flasks with media without nitrogen and phosphorous sources (BG11Δ, Table 3.1) to deplete cellular reserves. Light transmission was measured through the length of the serum vials with a Fluke 941 light meter. No light attenuation was detected throughout the 85 day trial; light measurements through inoculated serum vials never differed from control media vials.

Table 3.1 - BG11Δ media formulation. Similar in formulation to standard BG11 media with substitutions for potassium, manganese, EDTA and cobalt, and no inorganic nitrogen or phosphorous. Additional organic nutrients were added to produce sufficient growth response, along with saturation concentration silica and tricine buffer.

<table>
<thead>
<tr>
<th>Component</th>
<th>standard BG11 media molar conc (M)</th>
<th>BG11Δ media molar conc (M)</th>
<th>Component</th>
<th>standard BG11 media molar conc (M)</th>
<th>BG11Δ media molar conc (M)</th>
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<tr>
<td>KCl</td>
<td>substitution</td>
<td>5.12E-04</td>
<td>ZnSO₄·7H₂O</td>
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<td>CuSO₄·5H₂O</td>
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<td>3.16E-04</td>
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<tr>
<td>Citric Acid·</td>
<td>3.12E-05</td>
<td>3.12E-05</td>
<td><strong>CoCl₂·6H₂O</strong></td>
<td>substitution</td>
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<tr>
<td>Ferric Ammonium Citrate</td>
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<td>2.28E-05</td>
<td>SiO₂ (silica gel)</td>
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<tr>
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<td>4.63E-05</td>
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</tbody>
</table>
Batch monoculture trials: Pilot batch trials using environmentally relevant phosphorous concentrations reflective of Carlson Trophic State levels (.01, 0.05, .2mg/L) produced insufficient growth response to enumerate our model organisms accurately. N:P deprived cells were transferred to four replicate serum vials (50mL) containing BG11Δmedia with nitrogen levels of 1.76x10^{-2}M as NaNO₃ (250mg N/L) and available phosphorous limited to 2.13x10^{-4}M (6.0mg P/L) as P₂O₅. Cultures were grown as batch cultures without further nutrient addition (methods per Anderson, 2006). Molar N:P ratios were sufficiently large at 91:1 that these should be considered very low phosphorous trials that may produce phosphorous limitation or stress. Samples were rotated daily on the test shelving and kept under controlled lighting and temperature conditions as previously stated. Regular (daily or every other day) 200 µL samples were taken with wide-mouth pipette tips after 1 minute of agitation (30sec vortexing followed by 30sec manual shaking). Samples were heated to 100°C for 10 minutes in PCR tubes to separate colonies and clumped cells, and to rupture Microcystis gas vacuoles that otherwise cause cells to float and complicate counting. Cell counts and densities were visually evaluated in standard hemocytometers on a Nikon Eclipse E400 microscope. Empty Nitzschia tests absent visible chloroplasts were considered nonviable and ignored. Populations were estimated from cell counts in ten 100nL hemocytometer fields per replicate, evaluated and compared via one-way ANOVA tests in JMP statistical software (SAS Institute, Cary NC).

Semicontinuous tandem trials: N/P depleted cells were transferred to four 50mL replicate serum vials of media representing either low N:P ratio (5:1) or higher ratio (50:1)
nutrient levels (table 3.2). Absolute N and P concentrations were derived from previous studies performed by Rhee and Gotham (Rhee 1980). 10mL aliquots of samples were removed and replaced with fresh media bi-daily for 60 days via pippetteman. After 60 days, media removal and replacement was performed every 5 days for another 30 days. Samples were regularly prepared (daily or bi-daily) and assessed in the same manner as monoculture trials. Populations were determined and analyzed via one-way ANOVA.

Table 3.2 - Inorganic nitrogen and phosphorous concentrations and molar ratios used in semicontinuous batch experiments.

<table>
<thead>
<tr>
<th></th>
<th>N(Molar)</th>
<th>N (PPM)</th>
<th>P (Molar)</th>
<th>P (PPM)</th>
<th>N:P molar ratio</th>
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</thead>
<tbody>
<tr>
<td>Batch trial</td>
<td>1.76E-02</td>
<td>250</td>
<td>2.13E-04</td>
<td>6.0</td>
<td>82.3:1</td>
</tr>
<tr>
<td>Low N:P (5:1)</td>
<td>1.20E-04</td>
<td>1.68</td>
<td>3.91E-05</td>
<td>1.2</td>
<td>3.1:1</td>
</tr>
<tr>
<td>Semicontinuous</td>
<td>1.20E-04</td>
<td>1.68</td>
<td>3.91E-05</td>
<td>1.2</td>
<td>3.1:1</td>
</tr>
<tr>
<td>High N:P (50:1)</td>
<td>3.20E-04</td>
<td>4.48</td>
<td>7.11E-06</td>
<td>0.22</td>
<td>45:1</td>
</tr>
<tr>
<td>Semicontinuous</td>
<td>3.20E-04</td>
<td>4.48</td>
<td>7.11E-06</td>
<td>0.22</td>
<td>45:1</td>
</tr>
</tbody>
</table>

Microcystin toxicity bioassay: Unialgal cultures of *Nitzschia* recently isolated from Grand Lake St. Mary's *Microcystis* blooms were grown in BG11+Si media with SiO₂ at saturation concentration of 0.05g/L (Morey 1964), under 100 μmoles/m²/s lighting at 25°C to high (10⁶ cells/mL) density. Cells were transferred to serum vials containing 25mL of either BG-11+ media, or BG-11+ media with 100μg/mL of microcystin-LR obtained from Thermo Fisher Laboratories (Waltham, MA, USA). Mc-LR was transferred to a nutrient media prep flask using 2.0mL of methanol as a carrier solvent. Methanol was allowed to evaporate before addition of nutrient media. Final media Mc-LR concentrations in assay media were assayed by Greenwater laboratories in Palatka,
Florida using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Eight replicates each of both test and control vials were prepared and positions then rotated in daily on the test shelf.

Additional media was added daily over the experimental period. This was done by drawing off 2.5mL (10% of total volume) samples and replacing with fresh media. Before media replacement, daily 200 microliter samples were taken after 1 minute of agitation (30 sec vortexing followed by 30sec manual shaking). Samples were heated to 100°C for 10 minutes in PCR tubes to separate clumped cells and facilitate counting. Cell counts were visually determined standard hemocytometers on a Nikon Eclipse E400 microscope. Empty *Nitzschia* tests absent visible chloroplasts were assumed to be long-dead cells and ignored. Populations were evaluated and compared via one-way ANOVA and MANOVA tests in JMP statistical software.

**Results**

*Monoculture growth:* Growth curves of *Microcystis* (MA) and *Nitzschia* (NP) are shown in Figure 3.3.*Microcystis* monocultures started at significantly higher (F(1,6)=111.7, p=0.0001*) mean cell densities of 49 cells/µL than *Nitzschia* (1cell/µL) trials. Despite this, *Nitzschia* populations grew to their peak mean cell density (635 cells/µL) by 7 days, significantly higher than the mean cell density of *Microcystis* (129 cells/µL) (F(1,6)=21.1, p=0.0037*). *Nitzschia* cell densities dropped sharply thereafter while *Microcystis* culture densities continued to increase. *Microcystis* cell counts peaked at a significantly higher mean density of 516 cells/µL than *Nitzschia* (F(1,6)=6.7, p=0.0412*).
*Microcystis* populations remained constant with significantly higher mean populations for the remainder of the trial.

Figure 3.3 - Growth curves of GLSM-UCMA1 *Microcystis*(MA) and GLSM-UCNP1 *Nitzschia* (NP) monoculture isolates in limited phosphorous (batch culture). *Nitzschia* experiences a sharp initial population increase, with cell densities eventually decreasing and being exceeded by *Microcystis*.

**Semicontinuous experiment media effects:** Population comparisons of *Microcystis* and *Nitzschia* when grown together at different N:P ratios can be seen in Figure 3.4. Initial mean cell count comparisons were not significantly different between 5:1 and 50:1 N:P media for either *Microcystis* or *Nitzschia* (Figure 3.5). (F(1, 6)=0.2, p=0.6430) and (F(1, 6)=5.5, p=0.0577). Neither organism had significantly different mean densities between treatments by day 18 or for the remainder of the trial (Figures 3.6 & 3.7).
Figure 3.4 - Comparison of mean *Microcystis* and *Nitzschia* cell densities in semicontinuous batch competition experiments.

Figure 3.5 - Comparison of *Microcystis* and *Nitzschia* cell densities by nutrient ratios on day 1 after inoculation of semicontinuous batch competition experiments.
Figure 3.6 - Comparison of Microcystis and Nitzschia cell densities by nutrient ratios on day 12 after inoculation of semicontinuous batch competition experiments.

Figure 3.7 - Comparison of Microcystis and Nitzschia cell densities by nutrient ratios on day 85 after inoculation of semicontinuous batch competition experiments.

Semicontinuous experiment organism growth comparisons: Population growth responses of Microcystis and Nitzschia to nutrient ratios is given in Figure 3.8. Initial mean cell counts were significantly different in 5:1 medium with Microcystis and Nitzschia.
mean cell densities of 23 and 31 cells/µL (F(1,6)=9.3, p=0.0225*) but were not significantly different in 50:1 treatments (F(1,6)=0.3, p=.05882)(Figure 3.9). Cell counts of *Microcystis* initially increased slowly in comparison to *Nitzschia*, which reached peak mean cell counts of 310 cells/µL in 50:1 media and 492 cells/µL in 5:1 media cultures on day 12, exceeding *Microcystis* cell densities despite *Microcystis* starting at numerical advantages. *Microcystis* and *Nitzschia* cell counts were not statistically different in either 5:1 or 50:1 treatments on day 18 (F(1,6)= 0.2, p =0.6487 and F(1,6)=0.4, p=0.5508)(Figure 3.10). Mean *Microcystis* counts were significantly greater than *Nitzschia* on day 22 in 50:1 media cultures (F(1,6)= 54.8, p =0.0003*)and in 50:1 by day 26 (F(1,6)= 10.9,p =0.0163*). *Microcystis* counts remained significantly higher than *Nitzschia* for the remainder of the trial. Mean *Nitzschia* cell density never reached zero, however three-quarters of trial cultures had either no detectable *Nitzschia* cells or a greater than 100 to 1 ratio of *Microcystis* to *Nitzschia* by day 85 (Figure 3.11).

Figure 3.8 - Semicontinuous batch competition experiments between GLSM-UCMA1 and GLSM-UCNP1 at 5:1 and 50:1 N:P ratios. Cultures received 20% daily media replacement and effective mortality
Figure 3.9 - Comparison of *Microcystis* and *Nitzschia* cell densities on day 1 after inoculation of semicontinuous batch competition experiments.

Figure 3.10 - Comparison of *Microcystis* and *Nitzschia* cell densities on day 18 after inoculation of semicontinuous batch competition experiments.
Figure 3.11 - Comparison of Microcystis and Nitzschia cell densities on day 85 after inoculation of semicontinuous batch competition experiments.

Figure 3.12 - Total productivity comparison of Microcystis and Nitzschia for entire 85-day tandem trial.
Overall model of effects: MANOVA effects testing is shown in Figure 3.13. Effects testing indicated that the independent variables with effects on cell concentrations were number of days after inoculations, N:P ratio, and organism. The interactive effect of species, nutrient ratio, and time was not significant, nor were day/N:P, or organism/N:P effects. Significant interactive effects of day*organism were determined.

Microcystin toxicity bioassay: The presence of MC-LR was verified. Media microcystin-LR concentrations were determined to be 46.0 µg/L⁻¹ (appendix). Mean cell densities were not significantly different between the control group and the Mc-LR treatment medium on any day over the course of the bioassay (Figure 3.16), although day 8 neared a significant difference (F(1, 14) = 3.8, p = 0.0720). Total cells counts for the entirety of the trial did not differ significantly between control and Mc-LR media trials (F(1, 222) = 0.6, p = 0.4411) (Figure 3.17).
Figure 3.14 - Semicontinuous 46µg/L microcystin-LR inhibition experiment. Previously isolated GLSM-UCNP1 \textit{Nitzschia} was inoculated in BG-11+Si media with or BG11+Si with 50µg/L Mc-LR added. Cultures had 10% daily medium replacement and mortality.

Figure 3.15 - Total cells censused during the Mc-LR trial demonstrating no significant difference between mean cell densities between control media and medium with microcystins added.
Discussion

In every comparative species trial, *Nitzschia* demonstrated either higher initial reproduction or lower mortality rates and grew rapidly to a maximum population size. This was followed, however, by *Nitzschia* population declines. In contrast, *Microcystis* underwent slow but steady population increases and eventually reached sustained population levels. One of the clearest outcomes was the observation that *Microcystis* populations persisted in all treatments and culture conditions. This pattern was observed in both monocultures and competitive experiments, despite differences in nutrient levels or absolute concentrations. After 85 days, mean *Microcystis* counts greatly outnumbered *Nitzschia*, and subsequent competitive exclusion of the diatom in all cultures seemed likely given sufficient time.

As *Microcystis* had consistently become dominant in nutrient manipulation experiments performed, it was possible that allelopathy may have played a part in the phenomenon. The bioassay of *Nitzschia* indicated no apparent toxicity; *Nitzschia* grew as well in the presence of the toxin as in its absence. It also followed much the same pattern observed in other trials; early, rapid growth followed by decline. This experiment only investigated possible allelopathic effects on competitor organisms, and did not address possible survivorship benefits of microcystins for the organism producing them.

Studies have indicated possible roles for microcystins in cell-survival under high-light and oxidative environments. More effective adaptation of *Microcystis* than co-occurring Chlorophytes to high-light surface layer conditions has been noted in field observations as far back as 1985 (Paerl, 1985). Laboratory studies have demonstrated survival benefits of microcystin production over knockout mutants in high-light
conditions (Phenlan 2011). At roughly the same time other studies investigated intracellular-interactions of microcystins. Transcriptional up-regulation of microcystins in response to high-light conditions has been recently demonstrated (Meissner, 2013), as might be expected of a protectant compound. Detailed molecular investigations have investigated microcystin binding cysteine residues in redox-sensitive proteins, including Rubisco and other Calvin cycle components (Zilliges, 2011).

The mechanism of the Nitzschia strain’s tolerance of Mc-LR is interesting given microcystin’s role as a protein phosphatase inhibitor. Plausible mechanisms of tolerance would be PPA protein conformations that are less prone to inactivation, PPA production upregulation, interior cell conditions that are less conducive to microcystin activity, or most plausibly upregulation of microcystin inactivating enzymes, which has been described in Daphnia(Gustafsson, Renegfors and Hansson 2005) and bacterial species such as Sphingopyxis(Zhang, Pan and Yan 2010).

While treatment concentrations were specifically selected for environmentally relevant phosphorous and nitrogen levels, as well as to give similar total cell densities across treatments, the similarity in population comparisons of both organisms at 5:1 and 50:1 N:P ratios seems notable. In preliminary batch studies, overall initial growth was too low at phosphorous levels under 2.0 ppm, and problematically high in media above 4.0 ppm. The treatment levels for phosphorous were set mid-level for the monoculture study where only one initial supply of nutrients was provided. For semicontinuous, levels were set at levels thought to be inhibiting or luxurious. Nitrogen levels were then dictated by the need to achieve nutrient ratios that approximated 5:1 or 50:1. As might be expected, cell density was higher in high phosphorous media, suggesting that
phosphorous limitation may have been a greater impediment to reproduction or survivorship at the concentrations used, and the primary determinant of populations in either treatment. This is despite decreasing both phosphorous and nitrogen in 5:1 media by 50% from a previous experimental effort that had problematically large cell count disparities between 5:1 and 50:1 media. Alternatively, phosphorous levels may have been insufficiently low to be limiting at either concentration. Field studies of phosphorous enrichment in freshwater systems consistently relate concentrations and availability to chlorophyll-a concentrations and phytoplankton biomass (Carlson 1977).

*Nitzschia*’s initial fast growth and dominance followed by *Microcystis* overtaking the diatom suggests that *Nitzschia* is less adept at competing for limited phosphorous than *Microcystis*, despite the popular perception of cyanobacteria being generally poor competitors for phosphorous (or, conversely, good competitors for limited nitrogen in high-phosphorous conditions.) This is further supported by comparison studies of growth kinetics for assorted species of algae wherein *Nitzschia palea* specifically was predicted to be comparatively poor at phosphorous acquisition or use (Grover 1989). In all trials here, *Nitzschia* was unable to sustain a stable population after reaching a sharp peak population size. Given the range of phosphorous concentrations where this occurred, it is likely that *Nitzschia* has higher phosphorous requirements than *Microcystis*.

As many genera are nitrogen fixers and can thrive in high-phosphorous, nitrogen-limited systems, cyanobacterial dominance in these water bodies would seem to be a result of their effective competition for nitrogen. As a non-nitrogen-fixing genus however, *Microcystis* may have no nutrient-use advantage in nitrogen-limited, high phosphorous
systems. As a vacuolated genus, *Microcystis* could be expected to be at an advantage in low-mixing, eutrophic, highly productive systems where light, carbon dioxide, and oxygen may be limiting - typical of summer conditions in Grand Lake St. Mary’s and other shallow lakes where *Microcystis* blooms are frequently reported. Notably, *Planktothrix*, another vacuolated filamentous genus, was the second most dominant organism in *Microcystis* blooms sampled. *Nitzschia*, like other diatoms, is expected to have good carbon-use efficiency (Raven 1983), and may maintain a competitive advantage and presence in bloom communities as a result of heavy surface carbon dioxide consumption and carbon limitation (Van der Waal 2011).

For water management in systems with frequent *Microcystis* blooms, the implication is that absolute concentrations of nutrient elements, chlorophyll, and light penetrance levels may matter as much or more than slight adjustments of water chemistry ratios. If phosphorous is the nearest to limiting element, as is reported to often be the case in freshwater systems, control of inputs may be the best way to limit bloom formation risks, although as a non-diazotrophic organism, *Microcystis* populations may also be subject to control by nitrogen limitation.

Future field studies investigating these two phytoplankton’s responses to varying N:P concentrations and ratios could further support these findings. These studies could be done using qPCR to establish relative community abundance. *Microcystis*-specific primers have already been widely investigated for use in bloom prediction methods. *Nitzschia*-specific primers using large-subunit rDNA have also been used and could be incorporated (Trabajo, 2009). Broader comparative studies of Cyanobacteria and Bacillaria abundance could be done with similar methods.
Citations


Havens, Karl, R. Thomas James, Therese East, and Val H Smith. "N:P ratios, Light


ODNR. State Actions for Water Quality Improvement at Grand Lake St. Mary’s. Ohio Department of Natural Resources (ODNR), July, 2010.


Rohrlack, Thomas et al. "Role of Microcystins in Poisoning and Food Ingestion Inhibition of Daphnia Galeata Caused by the Cyanobacterium Microcystis Aeruginosa." Applied and Environmental Microbiology 65, no. 2 (Feb 1999): 737-739.


Chapter 4- Conclusions

Grand Lake St. Mary's is a lake with persistently recurrent cyanobacterial blooms and cyanotoxin levels sufficiently elevated to potentially endanger public health and its use as a recreational waterway. Indications are that the lake also experiences highly eutrophied conditions as a result of exogenous nutrient inputs from the surrounding watershed, with hydrogeology that fails to properly sequester these nutrients, instead regularly recycling them into the water column where they can contribute to system productivity.

What is less clear is whether bloom frequency and the particular organism that dominates is determined more by nutrient ratio stoichiometry, absolute concentrations, aspects of the biology of the bloom organisms themselves, or other features of the food web. A review of total nitrogen:total phosphorous ratios derived from GLSM water chemistry testing indicates somewhat low ratios of 20-30:1, but appreciably above the 15:1 or lower "Redfield ratio" one might expect of a cyanobacteria dominated lake (Figure 5.1). Supporting this is the failure of our diatom isolate, Nitzschia, frequently found in Microcystis blooms and ostensibly a competitor for any limiting nutrients, to outcompete Microcystis at either high or low N:P ratios. While merely one strain each of two species was tested, the implication is that Microcystis and other cyanobacteria may be more effective competitors than larger eukaryotic organisms for limited nutrients than is generally appreciated. This could be as simple as smaller cell sizes leading to more competitive surface:volume ratios (sometimes mitigated by complex surface area increasing eukaryotic morphologies (Lewis 1976), or have more complicated mechanisms such as luxury storage, transport proteins, or some aspects of internal
cellular composition. Regardless, the practical assumption is that cyanobacteria are at least "good enough" nutrient competitors to remain ubiquitous in phytoplankton community assemblages, and that conditions occasionally arise where they come to predominate. The relevant question for watershed management then becomes "What conditions encourage cyanobacterial blooms, especially toxic species, and how can they best be avoided?"

Figure 4.1 - Nitrogen (mg/L), phosphorous (µg/L), N:P ratios, chlor-a (µg/L), and microcystin (µg/L) sampling means for Grand Lake St. Mary’s, summer 2012 (ODNR sampling, Personal communications with Rick Wilson)
While nutrient ratio theories remain controversial, the consensus is that algal blooms in general are results of disturbance scenarios, particularly exogenous nutrient input-driven productivity increases. In some instances, even this seemingly safe generalization may not hold true. Aside from the obvious scenarios of algal blooms reported in oligotrophic water bodies, one of the intriguing aspects of *Microcystis* are the general K-strategist aspects of the genus. For a seemingly simple prokaryotic organism, *Microcystis* has surprising plasticity and an extensive toolbox of survival mechanisms. From grazer unpalatability and herbivore avoidance by colony formation and size exclusion, buoyancy control for optimal light, sedimentation avoidance, and atmospheric gas acquisition, benthic fermentation and overwintering, possible allelopathy and potential competitive advantage. Does Grand Lake St. Mary’s experience multiple population dips and increases that result in days where scums are or are not visible, or does the population remain steady with high survivorship, moving around the lake, more visible on some days than others? The latter scenario seems plausible given benthic overwintering and an emphasis on survival mechanisms. If this were the case, it would explain the penchant for lakes with blooms to remain lakes with blooms over months or even years.

From a nutrient control standpoint, the only "weak link" that may stand out about *Microcystis* in comparison to other cyanobacteria is its lack of nitrogen fixing ability. While current watershed management philosophies prioritize phosphorous limitation to lower total system productivity for "algal" control, and under the belief that often-toxic cyanobacteria, in general, prefer low N:P ratios, phosphorous-focused regulation and control may not be as effective as a more balanced approach that also seeks to limit
total nitrogen availability and concentrations. Our experimental data, as well as nutrient level studies from Lake St. Mary's, suggests that this may not be the case, and minor alterations in water chemistry ratios will likely not suffice to disrupt patterns of recurring *Microcystis* blooms.

Little of this would matter were *Microcystis* not a toxic genus with possible impacts on human health; populations of *Microcystis* capable of producing microcystin concentrations of concern may be undetectable without microscopic inspection, and even visible scums tend to be present one day and gone the next. Persistently affected water bodies sometimes are reduced to a monitoring expectation of "detect elevated nutrient levels, measure ensuing microcystin concentration increase," and while this simplification may be of use in regulatory regimes, it overlooks complexity that may address the ecological roles of microcystins and aid in their mitigation or elimination. The implication of our toxicity experiment and others that have failed to find inhibitory or toxic effects of microcystins on algal community organisms is that microcystins may not perform allelopathic functions in the bloom itself or in the surrounding waters, particularly as an endotoxin mostly released upon cell lysis.

What does seem well established are microcystin’s roles in antiherbivory, although even here beta-cyclocitral or others may be the actual labile compounds alerting micro and macroinvertebrates to low food quality/toxic cells and triggering avoidance behaviors prior to consumption. In hypereutrophic systems, avoiding consumption by grazers would be a key aspect to blooms maintaining population size and their hold on system nutrient pools.
A number of studies strongly suggest antiherbivory as an ecological role of microcystins, although controlled in-vitro studies directly comparing grazer consumption rates of toxic and nontoxic cell strains seem to be lacking. While this thesis did not investigate grazing, herbivore rejection and subsequent selection of the toxic *Microcystis* is a plausible mechanism for *Microcystis* community dominance. A number of scenarios have been identified as contributing to failure of grazers to control algal cell populations, including foreign mussel invasion or mortality of native species, overpredation of microinvertebrate grazers by zooplanktivorous fish, either by invasives or when freed of top-down control by piscivores(Kasprzak 2003), environmental effects, viruses, and many other events. In an already eutrophic water body, the results could be dramatic increases in algal biomass and a failure of nutrient pools to move up the food chain, with primary productivity instead remaining in a microbial loop(Barber 2007)(Figure 5.1) and weighting the system's biomass towards unicellular organisms, including bloom forming phytoplankton. In a scenario involving proliferation of unpalatable organisms like *Microcystis*, one could plausibly foresee similar end results.

Figure 4.2 - Overview of aquatic food web interactions showing organisms involved in and interacting with the microbial loop (Barber 2007).
Recent studies hint at a more basic function of microcystins of providing fitness benefits to the producing organism by intracellular interactions, particularly covalent bonding to rubisco and Calvin cycle proteins under high irradiance and oxidative stress conditions, possibly preventing crosslinking of cysteine residues and disadvantageous conformational changes (Zilliges 2011). Microcystins are produced in at least one terrestrial lichen symbiont (in *Peltigera leucophlebia* (Kaasalainen 2009)) and comparatively few closely related cyanobacteria genera, notably *Microcystis* but also *Anabaena*, *Nostoc*, *Oscillatoria*, and *Planktothrix* among them, all of which also produce buoyant vacuoles or structures allowing them to maintain position the very top of the water column under minimally turbulent conditions, but potentially exposing them to damaging amounts of irradiance and photoinhibition, as well as UV radiation. Microcystins may be intracellular protectant compounds first and foremost, grazer deterrence compounds secondarily, and only occasionally or not at all be performing allelopathic functions. *Microcystis' main competitive advantages would then be antiherbivory, light and atmospheric gas monopolization in eutrophic systems with already poor light penetrance, and surprisingly effective nutrient competitive ability compared to other bloom organisms.
Works cited


Appendix

Greenwater laboratories microcystin concentration analysis

Microcystin Data Report
Project: University of Cincinnati

Sample Identification | Sample Collection Date
--- | ---
UC 1 | 9/25/13
UC 2 | 9/25/13

Toxins – microcystin-LR (MC-LR)

Sample Prep –
The sample was filtered through 0.2 μm PVDF and analyzed via LC/MS/MS. A duplicate sample of UC 2 was spiked (lab fortified matrix – LFM) at a concentration of 50 ppb with MC-LR for single point standard addition quantitation.

Analytical Methodology –
Liquid chromatography coupled with mass spectrometry/mass spectrometry (LC/MS/MS) was utilized for microcystin-LR analysis. MC-LR was fragmented and the following transitions were monitored: 995.5→553.3 m/z & 995.5→599.5 m/z. The detection limit for MC-LR is 10 ppb.

Summary of LC/MS/MS MC Results

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<thead>
<tr>
<th>Sample</th>
<th>MC-LR levels (μg/L)</th>
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<tr>
<td>UC 1</td>
<td>ND</td>
</tr>
<tr>
<td>UC 2</td>
<td>46</td>
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<tr>
<td>Detection Limit (μg/L)</td>
<td>10</td>
</tr>
<tr>
<td>ND – Not detected above the detection limit</td>
<td></td>
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</tbody>
</table>

Submitted by: Mark T. Aubel, Ph.D.  
Date: 9/27/13

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