THE EFFECTS OF NUTRIENT RATIOS AND FORMS ON THE GROWTH OF
MICROCYSTIS AERUGINOSA AND ANABAENA FLOS-AQUAE

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Abstract

Cyanobacteria are ancient prokaryotic organisms capable of performing oxygenic photosynthesis. An increase in the temporal and spatial distribution of cyanobacteria blooms worldwide has drawn considerable research attention in recent decades because of the health risks cyanobacteria pose to humans and wildlife through the production of cyanotoxins, interference with recreation, and ecosystem changes.

A variety of hypotheses have sought to explain the increasing frequency and severity of cyanobacteria blooms around the world, with the relationship between cyanobacteria abundance and eutrophication receiving considerable attention. While the impacts of phosphorus concentration on cyanobacteria success are relatively well-studied, less is known about how nutrient stoichiometry and nitrogen uptake kinetics of different species contribute to cyanobacteria dominance. The underlying mechanism for the impacts of nitrogen to phosphorus (N:P) ratio and nitrogen form on cyanobacteria involves internal cycling of nitrogen within lakes and aspects of cyanobacteria cell physiology. The primary objective of this study was to assess the impacts of N:P ratios and nitrogen form on the growth of Microcystis aeruginosa and Anabaena flos-aquae in both axenic cultures and natural phytoplankton assemblages from Missisquoi Bay, Lake Champlain. A second objective was to determine whether treatment condition affected the production of the cyanotoxin microcystin. A final objective was to document the presence of benthic ammonium in Missisquoi Bay and the vertical migration of cyanobacteria throughout the water column in the bay, to provide evidence in support of the underlying mechanisms that might provide advantages to cyanobacteria in the bay.

In laboratory culture experiments with M. aeruginosa and A. flos-aquae alone and in a mixed community, N:P ratios were varied between 5, 15, 30 and 45:1, and nitrogen was supplied as both nitrate and ammonium at each ratio. Triplicate samples were preserved after one, three and six days for cell enumeration using the standard Útermohl method. Differences in density between initial and later times were used as an estimate of growth. Microcystin concentration was measured with the ELISA method. Weekly field sampling was conducted in the summer of 2006 in Missisquoi Bay to measure benthic nitrogen concentrations. Nocturnal sampling at varied depths in the bay was used to explore the vertical migration of cyanobacteria throughout the water column.

There were weak associations between ammonium-nitrogen and M. aeruginosa growth and nitrate-nitrogen and A. flos-aquae growth, while the effects of N:P ratio on growth was highly variable across time and treatment condition. Ammonium-nitrogen was documented in the benthic water of Missisquoi Bay throughout the growing season, and M. aeruginosa dominated the vertical migration of cyanobacteria throughout the water column. The lack of clear trends visible within the data from laboratory experiments can be in part attributed to high variability of cell density within treatment conditions and the limitations of the methodology used for cell enumeration. Taken together these data suggest that the distribution of nitrogen within an aquatic system and the ability of M. aeruginosa to vertically migrate may contribute to the M. aeruginosa dominance of the summer phytoplankton community.
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Chapter 1: Literature Review – Cyanobacteria in Temperate, Shallow, Eutrophic Lakes

INTRODUCTION

Toxic cyanobacteria blooms have been documented for centuries (i.e., Francis, 1878; Fogg et al., 1973; Codd & Beattie, 1991), but the spatial and temporal distribution of such events has increased recently (i.e., Cronberg, 1999; Whitton & Potts, 2000; Watzin et al., 2003; 2006; Johnston & Jacoby, 2003). The dramatic discoloration of a water body and other unpleasant aesthetic characteristics caused by dense cyanobacterial growth, and the production of potent toxins that can be fatal to humans (Codd, 1995; Carmichael et al., 2001; Codd et al., 2004) and wildlife (Porter & Orcutt, 1980; Agusti, 1991a; Langmuir, 1997; DeMott, 1999; Downing et al., 2001; Scheffer, 2004) have made people keenly aware of the presence of blooms.

Increased abundances of cyanobacteria are commonly associated with eutrophic conditions (Vitousek et al., 1997; Smith et al., 1999; Bianchi et al., 2000). Eutrophication is the naturally occurring process of lake succession, stimulated by increasing nutrient concentrations with time in a water body. However, human activities and land use patterns have increased the rate at which nutrient accumulation in a water body occurs, causing lakes, ponds, reservoirs, wetlands and slow-flowing rivers worldwide to experience cultural eutrophication. Elevated concentrations of suspended sediments are also found in conjunction with elevated nutrient concentrations because many nutrients, such as phosphorus, commonly enter water sorbed onto soil particles (Novonty & Chesters, 1981). Upon reaching the water, suspended soil particles can cause a rise in water temperature (Ringler & Hall, 1975) because suspended sediments absorb solar
energy that would otherwise have been reflected by water molecules. Consequently increased water temperatures are associated with both eutrophication and cyanobacteria blooms (Fujimoto et al., 1997; Kotak et al., 2000; Elliott et al., 2006).

From a biological perspective, eutrophication translates into increased growth of aquatic vegetation. Phosphorus and nitrogen are commonly limiting nutrients in aquatic systems, so loading with these elements stimulates primary production, especially increasing phytoplankton growth (Smith et al., 1999). Given the direct correlation between primary productivity of a water body and the \textit{in situ} concentrations of nitrogen and phosphorus, considerable work has been done to increase understanding about how these nutrients contribute to the regulation of cyanobacteria in freshwater systems.

The combination of severe human and wildlife health threats from cyanotoxins, and the unfavorable aesthetics caused by cyanobacteria surface scums, has provided the stimulus for the research on cyanobacteria ecology. Gains in understanding made by research will enable water quality experts and environmental policy makers to better manage watersheds to minimize cyanobacterial growth. The following chapter explores the ecological significance of cyanobacteria through time, hypotheses explaining the success of cyanobacteria in freshwater systems, the production of cyanotoxins, nitrogen speciation as it is regulated by redox chemistry in lakes, and the role that algae culture experiments play in studying cyanobacteria.
Cyanobacteria

Cyanobacteria represent a unique group of ancient organisms having had tremendous environmental significance throughout time for a number of reasons. These organisms perform oxygenic photosynthesis, fix atmospheric nitrogen and produce toxins (Whitton & Potts, 2000; Berman-Frank et al., 2003; Canfield et al., 2005; Codd et al., 2005; Visser et al., 2005; Huisman & Hulot, 2005). Cyanobacteria are one of the approximately 20 major clades of species comprising the phylogenetic domain Bacteria (Madigan et al., 2003), and are the only prokaryote capable of oxygenic photosynthesis. Cyanobacteria are found in a wide array of habitats, ranging from terrestrial to marine to freshwater environments (Garcia-Pichel et al., 2003). This literature review will focus on aquatic cyanobacteria, with special emphasis on the freshwater genera *Microcystis* and *Anabaena*, though much of the information pertains to cyanobacteria in general.

**Historic Significance of Cyanobacteria**

It is generally agreed that cyanobacteria are responsible for the dramatic increase in oxygen concentrations on Earth, as they are the oldest, and only known, prokaryotic oxygenic phototrophs (Des Marais et al., 1992; Kasting, 2001). Cyanobacteria play an invaluable role in freshwater ecosystems because of their abilities to produce oxygen via oxygenic photosynthesis and convert atmospheric nitrogen to the biologically available form, ammonium (NH$_4^+$) (i.e., Whitton & Potts, 2000; Berman-Frank et al., 2003; Canfield et al., 2005; Codd et al., 2005; Visser et al., 2005; Huisman & Hulot, 2005). Additionally their status as primary producers strengthens their connection to the aquatic
environment, as they contribute to the foundation of the food web, especially stimulating bottom up food web shifts in shallow, eutrophic lakes (Muylaert et al., 2002).

**Oxygenic Photosynthesis**

Cyanobacteria, like all higher order photosynthetic plants, have dual photosystems that enable them to perform oxygenic photosynthesis (Canfield et al., 2005). It is thought that photosystem I and II (PS I and II) likely existed independently in separate organisms, but combined via lateral gene transfer into a single organism, giving rise to cyanobacteria (Canfield et al., 2005 and references therein). The coupled photosystem of cyanobacteria and resulting oxygenic photosynthesis was unique to the Proterozoic Era, giving rise to the oxygenation of Earth’s oceans and atmosphere (Sergeev et al., 2002). The marked increase in oxygen concentrations that ensued gave way to the development of higher eukaryotic life forms (Kasting 2001; Canfield et al., 2005).

Photosynthesis is the photolithoautotrophic process through which inorganic carbon, \( \text{CO}_2 \), is converted to organic carbon and glucose, producing oxygen, \( \text{O}_2 \) (Madigan et al., 2003). Oxygenic photosynthesis performed by cyanobacteria utilizes the reductive pentose phosphate cycle, or the Calvin-Benson-Bassham cycle (Wedel & Sol, 1998; Canfield et al., 2005). This process employs solar energy to make organic forms of carbon and gaseous oxygen from carbon dioxide and water (Madigan et al., 2003). Solar energy is obtained through a series of light-sensitive pigments, such as chlorophyll \( a \), and the cyanobacteria-specific accessory blue pigment, phycocyanin (Agusti & Philips, 1992; Madigan et al., 2003; Canfield et al., 2005).
Nitrogen Fixation

Nitrogen fixation refers to the conversion of nitrogen gas (N₂) to ammonium (NH₄⁺). The process can be performed by naturally occurring biological and chemical processes, as well as by anthropogenic mechanisms (Postgate, 1978). Biological nitrogen fixation by cyanobacteria requires colonial growth because of the specialization of cells that is required, demanding cooperative efforts by groups of cells (Chan et al., 2004). The specialization of cells in nitrogen-fixing cyanobacteria is a result of the fact that nitrogenase, the primary enzyme involved with nitrogen fixation, is irreversibly inhibited by the presence of oxygen (i.e., Fay, 1992; Canfield et al., 2005). Nitrogen fixation is performed in cells called heterocysts, which can account for approximately one in ten cells within a colony (Thiel & Pratte, 2001), though this percentage changes dramatically with in situ nitrogen conditions and the need for nitrogen fixation (i.e., Findlay et al., 1994; Ferber et al., 2004). Heterocysts are cells that are converted from standard cells; they are characterized by multiple thick cell membranes that dramatically retard the passive diffusion of oxygen into the cell, while nitrogen is admitted readily (Canfield et al., 2005). Not all species of cyanobacteria are capable of performing the energetically costly process of biological nitrogen fixation (Andersen & Shanmugam, 1977). For instance, the genus Anabaena uses heterocysts to fix nitrogen, while Microcystis does not have heterocysts (Oliver & Ganf, 2000; Ferber et al., 2004; Henson et al., 2004), although both of these genera of cyanobacteria can grow either unicellularly or in large colonies.

According to Canfield et al. (2005), the process of nitrogen fixation proceeds as follows: nitrogenase reduces the triple bonded N₂ molecule into two molecules of
ammonium, NH$_4^+$, a process initiated by the donation of an electron from low redox potential compounds. In order to do this, the two proteins comprising the nitrogenase enzyme unite into an active enzyme complex where the actual reduction to ammonium takes place. Electrons are transferred individually, though, so the entire process of a donated electron stimulating the unity of the two nitrogenase proteins must happen eight times in order to reduce a single nitrogen molecule to two ammonium molecules. Consequently, nitrogen fixation is energetically costly, making it undesirable when appreciable pools of biologically available nitrogen, such as nitrate, nitrite, ammonium, or organic nitrogen, are present. The reduction of one molecule of nitrogen to two molecules of ammonium takes approximately 1.25 seconds, which is an incredibly long time compared to the duration of time required for other enzymes to perform their duties (Canfield et al., 2005). The tedious nature of this reduction reaction largely can be attributed to the strong triple bond between the two nitrogen atoms of a nitrogen molecule.

**Current Role of Cyanobacteria in Freshwater Systems**

Presently cyanobacteria continue to contribute oxygen and ammonium to aquatic environments via oxygenic photosynthesis and nitrogen fixation (Oliver & Ganf, 2000; Stal & Walsby, 2000). The contribution of oxygen and ammonium to most freshwater systems by cyanobacteria is less significant than it once was, though, as other organisms and processes are capable of producing both essential elements. Cyanobacteria play an especially important part in freshwater systems where phytoplankton dominate primary
production and cyanobacteria blooms occur (Scheffer, 2004). Cyanobacteria can help to establish the higher trophic levels of the food web because of their relatively low nutritional value to higher order organisms (i.e., Porter & Orcutt, 1980; Nizan et al., 1986; DeMott, 1999). Dense cyanobacterial growth can also dramatically impact light conditions (Agusti, 1991a), and nutrient availability, form and stoichiometry (i.e., Langmuir, 1997; Elser, 1999; Elser et al., 2000; Dowing et al., 2001; Paterson et al., 2002; Scheffer, 2004). Blooms require high nutrient concentrations to support abundant phytoplankton growth, which can lead to nutrient depletion and changes in in situ stoichiometry (Langmuir, 1997; Elser, 1999; Elser et al., 2000; Dowing et al., 2001; Paterson et al., 2002; Scheffer, 2004). Redox chemistry, and thus nutrient speciation and availability, can also change as a result of increased decomposition of dead organic matter when cells senesce and settle to the bottom, depleting benthic oxygen (Langmuir, 1997).

Although some of these impacts are beneficial to aquatic environments, many are considered to be problematic from ecosystem and human perspectives. Changes in redox chemistry impact nutrient cycling and conditions at the sediment-water interface (Langmuir, 1997). In some cases these changes further promote cyanobacterial growth by releasing sediment-bound nutrients (phosphate) and converting nutrients to bioavailable forms (reduction of nitrate to ammonium) (Langmuir, 1997; Canfield et al., 2005). Altered light conditions can change the foundation of an aquatic food web from rooted macrophytes to phytoplankton, clearly having impacts on higher trophic levels (Elser, 1999; Gophen et al., 1999; Scheffer, 2004). Nutrient availability, form and stoichiometry
are also strongly correlated with food web structure (Elser et al., 2000). Some species of cyanobacteria are capable of producing a suite of toxins known as cyanotoxins, which are harmful to humans and wildlife alike (i.e., Porter & Orcutt, 1980; Agusti, 1991a; Codd, 1995; Langmuir, 1997; DeMott, 1999; Kaerbenick & Neilan, 2000; Carmichael et al., 2001; Downing et al., 2001; Codd et al., 2004; Scheffer, 2004). In order to understand the impacts that cyanobacteria can have on their environment, it is important consider why blooms occur.

**THEORIES BEHIND CYANOBACTERIAL DOMINANCE**

Numerous studies have been conducted in an attempt to address the question of why cyanobacteria dominate in lakes that are experiencing noxious cyanobacteria blooms. As a result of these studies, an assortment of theories have been formulated that seek to explain cyanobacterial dominance in temperate, freshwater lakes. Some of the more popular theories are described by Blomqvist et al. (1994) and Reynolds (1998) in papers that review findings and suggestions from other studies. Theories reviewed include the effects of water temperature, light, N:P ratios, buoyancy, and zooplankton grazing on the success of cyanobacteria populations. Nitrogen and phosphorus concentrations, as well as the relative abundance of nitrogen species, are also suspected to play a role in the ability of cyanobacterial species to become overly abundant (i.e., Trimbee & Prepas, 1987; Herrero et al., 2001; Von Ruckert & Giani, 2004). These alleged predictors of cyanobacterial dominance can be divided into three distinct categories: physical, chemical and biological factors.
Physical Factors

Studies have shown that cyanobacteria, especially *Microcystis* and *Anabaena* species, are tolerant of warmer water temperatures than most other phytoplankton species and may even require them for optimal growth (Fujimoto et al., 1997; Kotak et al., 2000; Elliott et al., 2006). Fujimoto et al. (1997) demonstrated that *M. aeruginosa* tended to out-compete the filamentous blue-green algae, *Phormidium tenue*, under laboratory conditions exceeding 25°C, especially at low N:P ratios when nitrogen was the limiting nutrient. Elliot et al. (2006) used the PROTECH model for phytoplankton communities to make predictions about the effect of water temperature, and nutrient load on temperate lake phytoplankton communities. The model considers physical and chemical characteristics, as well as growth rates and unique aspects of cyanobacteria cell physiology (i.e., ability to fix atmospheric nitrogen), for up to eight phytoplankton species at a time (Reynolds et al., 2004). Model runs predict the phytoplankton community over a set period of time based on information about how different species will respond to a host of environmental conditions. Results from the PROTECH model suggest a decrease in density of all temperate phytoplankton species except *Anabaena* at water temperatures above 15°C, lending support to the theory that cyanobacterial species are better adapted to thrive in warm water (Elliott et al., 2006).

Cyanobacteria have also been shown to require less light than many other phytoplankton species, allowing them to be more successful in low-light environments (Jensen et al., 1994; Huisman et al., 1999; Presing et al., 1999). In a study conducted by Floder et al. (2002), species composition was followed in phytoplankton communities
from Lake Biwa, Japan, which were held under continuous culture conditions and subjected to varying light conditions. Cyanophytes were found to dominate under rapidly varying light conditions and were successful under low-light conditions as well (Oliver & Ganf, 2000; Floder et al., 2002). The variable size and quantity of phycobilisomes (light harvesting antennae), possession of phycobillins (accessory light harvesting pigments specific to cyanobacteria) (Agusti & Philips, 1992; Madigan et al., 2003; Canfield et al., 2005), and the ability to self-shade by way of colonial growth forms, thereby minimizing photooxidative stress (Duarte et al., 1990; Agusti, 1991b), are thought to contribute to the ability of cyanobacteria to dominate in low-light environments (Oliver & Ganf, 2000).

Phycobillins, a group of three accessory pigments unique to cyanophytes, help to promote light absorption by cyanobacteria by increasing the range of the electromagnetic spectrum over which absorption can occur (Agusti & Philips, 1992; Madigan et al., 2003; Canfield et al., 2005). The ability to adjust the number and size of phycobilisomes within cells enables cyanobacteria to minimize photooxidative stress, yet still meet their light requirements in low-light environments by maximizing phycobilisomes (Oliver & Ganf, 2000). Cyanobacteria benefit from the colonial growth form because they reduce light quantity for other phytoplankton, while manipulating their own phycobilisomes to meet their light requirements under low-light conditions (Huisman et al., 1999; Oliver & Ganf, 2000). Through this mechanism, cyanobacteria gain a competitive advantage over other phytoplankton by way of self-induced shading from colonial growth.
Chemical Factors

Nitrogen:Phosphorus Ratios

The hypothesis that N:P ratios factor into cyanobacterial dominance has arguably received the most attention of any single hypothesis in the literature. It is generally accepted that low N:P ratios are favorable for cyanobacteria (i.e., Smith, 1983; Michard et al., 1996; Fujimoto et al., 1997). This is commonly explained by the efficient uptake of nitrogen by cyanobacteria (Horne & Commins, 1987; Blomqvist et al., 1994) due to their high surface area to volume ratio and small size. Discrepancies exist in the literature as to what N:P ratio serves as the threshold for cyanobacterial dominance, however, a ratio of 30:1 by weight is most commonly cited (Smith, 1983; Bulgakov & Levich, 1999; Downing, 2001). A classic study by Smith (1983) examined data from 17 lakes worldwide comparing the proportion of cyanobacteria within the epilimnetic phytoplankton throughout the growing season. Based on this analysis, Smith (1983) arrived at the conclusion that an N:P ratio of 29:1 is the upper limit for cyanobacterial dominance. Other studies have yielded similar threshold N:P ratios (Bulgakov & Levich, 1999; Downing, 2001).

Below the N:P ratio threshold of approximately 30:1, different cyanobacteria species are thought to dominate under different conditions. At very low N:P ratios, heterocystous cyanobacteria species (i.e., Anabaena spp.) are thought to be more likely to dominate because of their ability to fix atmospheric nitrogen (Findlay et al., 1994; Levine & Schindler 1999). Following this line of reasoning, a ratio closer to 30:1 would be advantageous for non-heterocystous cyanobacteria (i.e., Microcystis spp.) because, they
cannot fix atmospheric nitrogen, but are still more efficient at nitrogen uptake than other phytoplankton (Horne & Commins, 1987; Blomqvist et al., 1994). However, this story is further complicated by the form and relative concentration of nitrogen present in the water body, a topic to be discussed later in the section titled *Nitrogen Speciation*. Other studies have yielded contradictory results, suggesting that there is either no correlation between N:P ratio and cyanobacterial abundance or that high N:P ratios are actually predictive of blue-green algae dominance (Harris, 1986; Canfield et al., 1989). The prevalence of conflicting information regarding the effects of N:P ratios on cyanobacterial dominance in the literature warrants further investigation and provides a piece of the foundation for my study.

**Nitrogen and Phosphorus Concentrations**

The supposition that nitrogen and phosphorus concentrations, independent of one another, regulate cyanobacteria dominance is closely linked to the idea that N:P ratios play a role in their dominance of phytoplankton communities. A number of studies have been conducted to tease apart the relative importance of N:P ratios versus nutrient concentration to cyanobacterial growth (i.e., Downing et al., 2001; Kim et al., 2007). These questions are directly related to one another because N:P ratios change with fluctuating N and P concentrations. For instance, low N:P ratios can be a result of nitrogen scarcity in an ecosystem or phosphorus abundance, therefore it is important to understand whether the stoichiometric relationship between the nutrients or their actual concentrations is the underlying mechanism promoting cyanobacterial growth. Results of
studies such as that by Berman (2001) suggest that nutrient concentrations, and, in this case, nitrogen limitation, may be a driving force behind cyanobacterial dominance as opposed to the N:P ratio itself, although the ratio of N and P also correlate as nitrogen concentrations decrease.

Many studies have documented a positive association between phosphorus concentration and cyanobacteria biomass (i.e., Kotak et al., 2000; Scheffer, 2004, Dignum et al., 2005 and sources therein). Furthermore, phosphorus is commonly a limiting nutrient in freshwater systems (Hecky & Kilham, 1988; see Dignum et al., 2005), and excessive addition to an aquatic environment is often indicative of more complex, system-wide environmental problems, like anthropogenic eutrophication. As a result, management efforts in watersheds like Lake Champlain have targeted phosphorus reduction as a means of mitigating water quality problems, such as cyanobacterial blooms (Vermont Agency of Natural Resources & New York State Department of Environmental Conservation, 2002). Because nitrogen is less frequently a limiting nutrient in freshwater systems (Scheffer, 2004; see Dignum et al., 2005), control of nitrogen additions to water bodies has received less attention from water quality managers. Further exploration of the relationship between nitrogen loading and cyanobacterial blooms will undoubtedly provide environmental policy makers with greater insights into the most effective way to manage watersheds with the goal of reducing cyanobacterial blooms. For this reason, nitrogen is the focus of my study.

The underlying mechanism behind changes in N:P ratios is likely to favor different phytoplankton communities because some species may thrive from phosphorus
loading, while others are more efficient at nitrogen uptake, enabling them to succeed during times of nitrogen limitation. Downing et al. (2001) examined the correlation between cyanobacterial abundance (as a percentage of total phytoplankton biomass) and variables such as TN:TP, TN and TP. TN was found to be the best predictor of cyanobacterial abundance, with TP following closely. Both TP and TN better explained the occurrence of cyanobacterial dominance within the phytoplankton community than the ratio of TN:TP. However, Downing et al. (2001) did not consider what nutrient concentration regimes correspond to dominance by specific cyanobacterial genera.

A study by Blomqvist et al. (1994) provides more detailed information about the species composition associated with varying nitrogen concentrations and form (see Nitrogen Speciation). Environments characterized by low nitrogen concentrations, or nitrogen limitation, will support a predominantly heterocystous cyanobacteria phytoplankton community. Although the experiments did not directly compare Microcystis and Anabaena species, it can be inferred that low nitrogen concentrations would favor heterocystous Anabaena spp. over non-heterocystous Microcystis spp.

Nitrogen Speciation

Nitrogen can be found in a variety of forms in freshwater systems; however, most studies pay particular attention to nitrate, nitrite and ammonium. Emphasis is placed on these particular nitrogen species both because of the frequency with which they are found in appreciable amounts in aquatic environments, and the effects they have on phytoplankton communities. Of the three nitrogen forms considered here, ammonium is
the only one that can immediately be incorporated into cellular biomass by photosynthetic organisms. Cyanobacteria must reduce nitrate and nitrite to ammonium through a series of reactions catalyzed by the nitrate and nitrite reductase enzymes, respectively (Von Ruckert & Giani, 2004; Herrero et al., 2001). Given the metabolic inefficiency of reducing nitrate and nitrite to usable ammonium, direct absorption of ammonium is energetically favorable for cyanobacteria species (Raven et al., 1992).

Relatively high uptake rates and low half saturation constants for ammonium compared to nitrate among cyanobacteria species enable them to utilize ammonium-nitrogen when it is present in a system (Lipschultz, 1995; Gu et al., 1997). But nutrient uptake rates and half saturation constants differ among cyanobacteria, making some species more adept at utilizing different forms of nitrogen than others. Heterocystous cyanobacteria species such as *Anabaena spp.* tend to have higher uptake rates for nitrate, while non-heterocystous species like *Microcystis spp.* have high uptake rates and low half saturation constants for ammonium compared to nitrate (Halterman & Toetz, 1984; Kappers, 1984). Nitrogen uptake rates and half saturation constants are highly dependent upon environmental or culture conditions, so values for each vary dramatically within the literature (Suttle & Harrison, 1988; Dortch, 1990; Vincent, 1992; Kudela et al., 1997).

In a study by Blomqvist et al. (1994), the effects of nitrogen speciation on phytoplankton communities was analyzed. Greater concentrations of ammonium were found to correspond to higher densities of cyanobacteria, while nitrate-nitrogen dominated conditions favored other phytoplankton species, such as green algae. Earlier research (Moss, 1973; Kappers, 1984), and subsequent studies (Hyenstrand, 1999) have
also found similar correlations between nitrogen form and phytoplankton community, an observation this is explained by the high ammonium uptake rates by cyanobacteria (Zevenboom, 1980).

**Biological Factors**

The abilities of some cyanobacterial species to regulate their height in the water column, fix nitrogen, grow colonially, and produce toxins are also referred to as potential explanations for their success (Andersen & Shanmugam, 1977; Visser, 1995; Oliver & Ganf, 2000; Thiel & Pratt, 2001; Chan et al., 2004). Diurnal fluctuations of cyanobacteria height in the water column usually follow the pattern of sinking in the late afternoon and evening and rising during the early morning (Wallace et al., 2000). Vertical migration has been explained by daily photosynthesis-respiration patterns as well as turgor pressure-mediated gas vesicle collapse (Walsby, 1969; van Rijn & Shilo, 1985). However, most literature suggests that photosynthesis plays a primary role in diurnal vertical migration of cyanobacteria (Oliver, 1994; Walsby, 1994; Visser, 1995; Oliver & Ganf, 2000).

During the day, when photosynthetic rates are high, cells accumulate dense molecules such as carbohydrates, which cause the cells to sink. Once cells have sunk out of the photic zone, respiration utilizes carbohydrates and other dense metabolites, reducing the cell’s density, allowing these cells to rise.

Vertical migration is thought to assist cyanobacteria with competition among phytoplankton for light and the utilization of significant pools of ammonium (Oliver & Walsby, 1984; Huisman et al., 2005). Higher concentrations of biologically available
forms of nitrogen are typically found at greater depth within the water column. Ammonification and the metabolic process of dissimilatory nitrate reduction release ammonium into pore water in the sediments (Canfield et al., 2005). Ammonium accumulates because fewer primary producers are below the photic zone to take it up, and dissolved oxygen concentrations are typically lower, reducing the rates of denitrification (Canfield et al., 2005). High ammonium-nitrogen uptake rates for cyanobacteria further enable them to utilize benthic ammonium pools. Abundant nitrogen promotes cyanobacterial growth because it enables timely construction and repair of nitrogenous compounds, such as amino acids and proteins that are primary building blocks of cells (Madigan et al., 2003). Nitrogen is also important for the synthesis of the nitrogen-based cyanotoxin, microcystin (see next section). Thus vertical migration allows cyanobacteria to both maximize light available to them at the surface during the day and utilize nutrients (i.e., ammonium) that are only present in appreciable pools at the sediment-water interface, giving them a competitive advantage over other phytoplankton species.

While vertical migration is an example of how the cell physiology of cyanobacteria fosters their competitive dominance over other members of the phytoplankton, cyanobacteria-zooplankton relationships are important as well. Physical characteristics of cyanobacteria, such as their ability to form large colonies with mucilaginous membranes, and their relatively low nutritional value, have been cited as reasons for reduced grazing by zooplankton (Porter & Orcutt, 1980; see Sterner & Schultz, 1998). Highly unsaturated fatty acids (HUFA’s) are very important in zooplankton diets (DeMott & Muller-Nevarra, 1997), but cyanobacteria are particularly
deficient in HUFA’s (Ahlgren et al., 1990). Cyanobacteria may also discourage predation by zooplankton through the production of cyanotoxins that may be harmful to predatory organisms (Nizan et al., 1986; DeMott, 1999; Gobler et al., 2007). Grazers have developed a series of adaptations enabling them to avoid toxins, including the avoidance of depths where toxin-producing species are abundant (Forsyth et al., 1990, Haney et al. 1994), and actively selecting non-toxic cyanobacteria (DeMott & Moxter, 1991). Jang et al. (2003) also found that cyanobacteria respond to the presence of zooplankton by increasing toxin production. It was even reported by Jang et al. (2003) that Microcystis strains thought previously to be non-toxic produced microcystin when exposed to zooplankton. These zooplankton-cyanophyte interactions enable cyanobacterial species to be more successful than their phytoplankton competitors due to decreased grazing pressure from zooplankton.

**Cyanotoxin Production**

The potential for many species of cyanobacteria to produce toxins that are potentially fatal to humans and wildlife has drawn considerable attention from researchers (i.e., Carmichael, 2001; Ouellette & Wilhelm, 2003). Cyanotoxin is an umbrella term used to describe a suite of toxic secondary metabolites produced by some cyanobacterial species, the most common of which are hepatotoxins and neurotoxins.
Figure 1. Molecular structure of the cyanotoxins, microcystin and anatoxin, respectively.

A.

Microcystins are a group of frequently found hepatotoxins, or liver toxins, with approximately 65 chemical variants (Carmichael, 2003; Song et al., 1998). The


microcystin molecule is a large cyclic hepatopeptide (Carmichael, 2003; Song et al., 1998). Microcystins are named after the genus of cyanobacteria *Microcystis*, which most commonly produces these hepatotoxins. However, other genera, such as *Anabaena*, *Nodularia*, *Oscillatoria* and *Planktothrix* can also produce microcystins (Carmichael, 2003; Codd et al., 2005). Hepatotoxins affect consumers at a variety of levels, ranging from gastrointestinal distress to causing blood accumulation in an organism’s liver. The latter action induces circulatory system shock and liver failure, which is often fatal (NSW Department of Natural Resources, 2000; Kotak et al., 2000; Kaebernick & Neilan, 2001). Cylindrospermopsins are another group of hepatotoxins that can be produced by *Aphanizomenon spp*. Cylindrospermopsins function slightly differently than microcystins, but the effect on the liver is the same.

The neurotoxins anatoxin and saxitoxin are two other major classes of cyanotoxins. Of the cyanobacteria genera responsible for the production of these toxins, *Anabaena* and *Aphanizomenon* are among the most common culprits, although other genera are known to produce both anatoxins and saxitoxins (Carmichael, 2003; Codd et al., 2005). Neurotoxins operate by inhibiting specific functions of the nervous system through mechanisms such as molecular mimicry and enzyme inhibition (see Kaebernick & Neilan, 2001). Both microcystin and anatoxin molecules are nitrogenous compounds, suggesting that their production requires large amounts of nitrogen to be available (Song et al., 1998; Codd et al., 2005; Borner & Dittmann, 2005). Nitrogen availability is especially closely linked to microcystin production because microcystins are much larger nitrogenous molecules than anatoxins and saxitoxins, and because microcystin production
may serve as a nitrogen storage mechanism for cyanobacteria during times of nitrogen abundance (Downing et al., 2005) (see Potential Functional Roles of Microcystin).

One of the most intriguing and challenging aspects of cyanotoxin production is understanding what mechanisms trigger activation of toxin-producing genes; not all organisms capable of producing cyanotoxins do so at all times (Codd et al., 2005 and sources therein). An organism, colony or population may belong to a species that can produce toxins, but it may not actually be biosynthesizing these molecules at any given point in time. Some organisms, colonies or populations seem to be able to regulate toxin production, but environmental conditions may also trigger or suppress toxin production. Understanding the mechanisms responsible for toxin production is the focus of many research efforts.

A number of environmental and genetic studies have been conducted to elucidate the underlying means of toxin production within cyanobacteria (i.e., Jang et al., 2003; Borner & Dittmann, 2005 and sources therein). Environmental studies have focused on the allelopathic effects and food web dynamics of cyanotoxins (Gilbert, 1994; Jang et al., 2003; Gross, 2003; Sarnelle & Wilson, 2005), the influence of nitrogen and phosphorus concentrations on cyanotoxins, and the potential for toxins to serve as a cellular nitrogen storage mechanism during times of nitrogen abundance (Gilbert, 1994; Song et al., 1998; Downing et al., 2005).

Genetic studies have emphasized identification of the genes responsible for toxin production and locating these genes across the phylogenetic tree (Borner & Dittmann, 2005 and sources therein). Polymerase chain reaction (PCR) is being used to detect
marker genes within the DNA of cyanobacteria which can encode for enzymes important to toxin production. For instance, peptide and polyketide synthases that are responsible for the production of microcystin, among other cyanotoxins, are being identified by PCR in organisms in hopes of predicting their toxicity (Codd et al., 2005). Presently all genes known to be involved with toxin production belong to the peptide and polyketide groups of metabolites (Codd et al., 2005). Given that genetic work surrounding the location of toxin producing genes (the mcy gene cluster) has revealed a sporadic distribution of the mcy gene cluster within the phylogenetic tree, two primary hypotheses for the evolution of these genes amongst species have emerged (Borner & Dittmann, 2005 and sources therein). The first hypothesis is that the distribution of toxin-producing genes among species is the product of lateral gene transfer between genera, species and geneotypes within a given species. The second hypothesis suggests that all genes evolved from a common ancestor and subsequent loss of these genes from some organisms and genera has resulted in the gene patchiness observed today (Borner & Dittmann, 2005).

While the functional roles of cyanotoxins in the environment provide a suite of compelling reasons for why toxic strains of cyanobacteria might have been selected throughout history, leading to the evolution of entire populations containing toxin-producing genes like the mcy gene cluster, phylogenetic studies do not support this hypothesis (Rantala et al., 2004; Jungblut & Neilan, 2005; Borner & Dittmann, 2005 and sources therein). Decreased grazing pressure from zooplankton (Porter & Orcutt, 1980; see Sterner & Schultz, 1998; Kaebernick & Neilan, 2001), the potential for intracellular nitrogen storage (Downing et al. 2005) and regulation of cellular iron-II concentrations
through iron chelation (Utkilen & Gjolme, 1995) are very beneficial to cyanobacteria, likely contributing greatly to their success relative to their phytoplankton competitors. However, Rantala et al. (2004) report that the broad, sporadic distribution of microcystin synthetase genes amongst cyanobacteria provides evidence for the theory that the mcy gene cluster is ancestral and has been repeatedly lost from non-toxic genera and genotypes. Data from Jungblut & Neilan (2005) agree with findings of Rantala et al. (2004) because the phylogeny of mcy gene cluster group with genera known to produce microcystin on a standard phylogenetic tree.

**Potential Functional Roles of Microcystins**

Many hypotheses exist to explain why some cyanobacteria produce toxins. Microcystin will be used to illustrate the array of proposed functions for cyanotoxins because it is part of my research.

Among the most common reasons cited for microcystin production are that its nitrogenous structure serves as a nitrogen storage mechanism for cells, which can be used during times of nitrogen shortage (Dowing et al., 2005), that the toxic effects of microcystins serve as an anti-grazing strategy by cyanobacteria (Nizan et al., 1986; DeMott, 1999; Kaebernick & Neilan, 2001; Gobler et al., 2007), and that microcystin can act as an iron chelator, facilitating iron uptake by cyanobacteria (Utkilen & Gjolme, 1995). Understanding the production of microcystins is complex, because of the wide
range of genera and even genotypes of a particular species capable of producing toxins as discussed previously (see Borner & Dittmann, 2005).

The nitrogenous structure of microcystin can be seen in Figure 1, where its most common chemical variant is depicted (Kaebernick & Neilan, 2001). The hypothesis that microcystin-producing cyanobacteria produce toxin in order to store nitrogen within their cell is supported by strong positive correlations between cellular protein content, nitrogen availability in culture media and microcystin levels (i.e., Kaebernick & Neilan, 2001; Vezie et al., 2002; Downing et al., 2005a; 2005b). Based on results of Kaebernick & Neilan (2001) and Vezie et al. (2002), one could postulate that the correlation between toxin-producing strains of cyanobacteria and nitrogen concentration occurs because microcystin is a nitrogenous compound requiring a sufficiently abundant nitrogen pool for production. The nitrogen stored in microcystin could serve as a nitrogen source in subsequent times of nitrogen limitation. This particular hypothesis for why microcystins are produced is of special interest to my study because of the general emphasis on nitrogen as a primary agent in the success of cyanobacteria.

The significance of toxin production as an anti-grazing strategy has been explored as well (Jang et al., 2003; Kaebernick & Neilan, 2001 and sources therein). As previously mentioned, Jang et al. (2003) conducted experiments in which *M. aeruginosa* were directly and indirectly exposed to zooplankton species. Toxin production was measured in each exposure scenario. Findings from the study included increased microcystin levels in all strains of *M. aeruginosa* (even those previously reported to be non-toxic) when placed in direct contact with zooplankton. Zooplankton have also been reported to feed
preferentially on non-cyanobacterial phytoplankton, and declines in zooplankton populations have been reported when other phytoplankton species have been depleted as a food source (Kaebernick & Neilan, 2001 and sources therein). While toxicity of cyanobacteria may explain the selective predation on non-cyanobacterial phytoplankton by zooplankton, it is important to note that colonial growth and low nutritional value of cyanobacteria may also factor into the observed avoidance of cyanobacteria by zooplankton grazers.

The role of microcystin as an iron chelator was studied by Utkilen & Gjolme (1995) because microcystins have been found to bind with iron and other cations in previous studies (Utkilen & Gjolme, 1995 and sources therein). Utkilen & Gjolme (1995) found that toxin-producing strains of *M. aeruginosa* have much more efficient iron-uptake systems than non-toxic strains. The hypothesis that microcystin minimizes free intracellular iron(II) concentrations during iron(II) limitation by functioning as an iron chelator can explain why iron(II)-limited conditions were found to induce toxin production. This hypothesis is based on the presumed regulation of toxin production by iron availability.

**Environmental Factors Contributing to Cyanotoxin Production**

A variety of studies (i.e., Utkilen & Gjolme, 1992; 1995; Kaebernick et al., 2000; Wiedner et al., 2002; Kameyama et al., 2004) have been conducted to examine the effect of different environmental parameters and cell physiology on toxin production. Nitrogen (Vezie et al., 2002; Ahn et al., 2002; Downing et al., 2005; Gobler et al., 2007),
phosphorus (Kotak et al., 2000), iron (Utkilen & Gjolme, 1995), light (Utkilen & Gjolme, 1992; Song et al., 1998; Kaebernick et al., 2000; Wiedner et al., 2002), temperature (Kaebernick & Neilan 2001 and sources therein), and cell physiology (Kameyama et al., 2004) are among the most studied parameters.

In general, studies considering the effects of nutrients on toxin production find a positive correlation between the nutrient of interest and cellular toxin content. Research focusing on nitrogen, cyanobacterial growth, and subsequent toxin production, report that increased nitrogen corresponds to increased toxin production (Vezie et al., 2002; Ahn et al., 2002; Downing et al., 2005; Gobler et al., 2007). Similarly, Kotak et al. (2000) found a correlation between increased phosphorus concentrations and increased microcystin production, as observed by a positive relationship between total phosphorus and microcystin-LR cellular content, and negative relationship between total nitrogen: total phosphorus and microcystin-LR cellular content. As mentioned previously, Utkilen & Gjolme (1995) found that toxin-producing strains of *M. aeruginosa* had higher iron requirements than non-toxic strains, which was mediated by increased efficiency of iron-uptake systems in toxin-producing cells.

Studies comparing light and temperature to cyanobacterial growth and toxin production also tend to find an increase in toxin production when growing conditions are favorable. Utkilen & Gjolme (1992) compared microcystin production with light quality, selected regions of the electromagnetic spectrum, and quantity. They reported that toxin production increased with light quantity up to 40 microeinstein/m²/s and decreased with higher light intensity. Light quality was found to have only minimal effects on toxin
production. Wiedner et al. (2002) also report a positive relationship between toxin production by *Microcystis* and light quantity up to the point of photosynthetically active radiation (PAR) saturation, at which point a negative relation was observed. Kaebernick et al. (2000) compared the relationship between the transcription rate of the microcystin (mcy) gene cluster and light. Transcriptional rates were shown to increase with increasing light intensities, especially in the red light zone of PAR, while a decrease was observed with high intensity blue and green light. Temperatures within the range of 18-25 °C were found to enhance microcystin production in microcystin-producing genera, while temperatures below 18 °C and above 25 °C were found to reduce toxin production (Kaebernick & Neilan, 2001 and sources therein).

The results of these studies provide evidence for the theory that microcystin production is greatest when growth is optimal for cyanobacteria. High nutrient concentrations eliminate the potential for nutrient limitation of cell growth. Cool or hot temperatures are also unfavorable for cyanobacteria growth, resulting in highest growth and toxin production at moderate temperatures. Furthermore, high light conditions appear to be unfavorable for cyanobacteria that tend to have lower light requirements than many of their phytoplankton competitors (i.e., Jensen et al., 1994; Huisman et al., 1999; Presing et al., 1999; Oliver & Ganf, 2000). The theory that optimal growing conditions and growth during the exponential phase of population expansion is ultimately responsible for toxin production is recognized in studies such as Kameyama et al. (2004) and the review of literature on toxin production by Kaebernick & Neilan (2001).
**Nitrogen Speciation as a Function of Redox Chemistry at the Sediment-Water Interface**

The benthic zone of a water body is the portion of the water column immediately overlying the sediment-water interface. Even in shallow lakes, the benthic zone typically has low light intensities, and is heavily influenced by both the overlying water column and processes occurring within the sediments (Scheffer, 2004). Chemical dynamics of the benthos are governed by the balance between wind-driven mixing of gases within the water column and the consumption of oxygen by primary production and interaction with reduced chemical species (Langmuir, 1997). These competing processes determine where the boundary between oxic and anoxic conditions lies, thereby controlling the location of the redox front (Langmuir, 1997). The redox front describes the region of the sediments where there is a transition between oxidized and reduced ions (Langmuir, 1997). For example, when anoxic redox conditions occur in benthic waters, the redox front can move up to the sediment-water interface (Langmuir, 1997).

The chemical form of nutrients in benthic water is governed by the presence of oxygen, and thus the location of the redox front, as well position of the system on the redox ladder. Nitrogen is no exception, as nitrogen ions change from their reduced forms such as ammonia and ammonium, to oxidized forms, including nitrate and nitrite, in the presence of oxygen. The conversion between oxidized and reduced forms of nitrogen can occur by chemical and biological mechanisms. Ammonium is most commonly generated through the processes of ammonification and dissimilatory nitrate reduction (Canfield et al., 2005). Ammonification is the term used to describe degradation of nitrogenous biomolecules by heterotrophic microorganisms and the subsequent release of ammonium
Microbial dissimilatory nitrate reduction is an energy-gaining metabolic process in which nitrate is reduced with various electron donors to ammonium in the absence of oxygen (Canfield et al., 2005).

While it is inherently difficult to quantify the distribution of the various forms of nitrogen throughout the water column, the literature does suggest that ammonium originating in the sediments and diffusing to benthic waters is important for the aquatic nitrogen budget (i.e., Verdouw & Dekkers, 1982; Scheffer, 2004). Ammonium exists throughout the water column as a result of waste excretion by heterotrophs and lysis of cells; although it is scavenged quickly, it may contribute significantly to the nitrogen demand of primary producers (Verdouw & Dekkers, 1982). However, upward diffusion of ammonium generated in the sediments is also thought to contribute significantly to the water column nitrogen budget in times of hypolimnetic anoxia (Verdouw & Dekkers, 1982). For this reason, cyanobacteria have a dramatic competitive advantage over their non-vertically migrating phytoplankton counterparts because their diurnal trips out of the photic zone to benthic waters provide them with access to otherwise inaccessible pools of ammonium.

**CULTURE EXPERIMENTS**

Entire ecosystems are inherently difficult to study, and performing *in situ* experiments on isolated portions of them is challenging as well. Confounding factors may mask trends and complicate even simple studies. To obtain information about a specific organism or the effects of a particular environmental parameter on an outcome measure
like growth or toxin production, laboratory studies are often required. Performing experiments on cyanobacteria cultures makes it possible to measure the effects of nutrient form, concentration and ratio on the growth of a specific species of cyanobacteria. Culture experiments are not perfect replications of environmental conditions though, and they can have complications as well. Studies performed under simplified environmental conditions can yield data with questionable environmental relevance. Therefore, it is important for studies to involve some mechanism for assessing the ecological relevance, such as paired laboratory and field studies or comparison with similar field studies (i.e., Caron et al., 1991; Ahern et al. 2007).

Different types of culture experiments are used to measure different parameters of cell growth (see Kadouri & Spier, 1997 for a review of such methods). Continuous cultures receive constant supplies of nutrients and aeration through the addition of new media and continual mixing. These culture conditions are ideal for studies designed to measure prolonged growth of a culture at a steady growth phase (Cleveland Biotech, 2006). By contrast batch cultures can be used to measure changes in growth phase with time. Batch culture experiments consist of vessels containing a set amount of culture that is monitored over time (Cleveland Biotech, 2006). Cell densities increase as nutrient concentrations are depleted.

Despite the flaws associated with culture experiments, they do offer a means to observe individual species and measure very specific environmental parameters that otherwise would be far too complex to assess in situ. The ability to simplify field conditions often outweighs the potential drawbacks of culture experiments, explaining
why many researchers choose to use this experimental design (i.e., Caron et al., 1991; Huisman et al., 1999; Downing et al., 2005a).

**Cyanobacteria in Missisquoi Bay**

Missisquoi Bay is the northern-most segment of Lake Champlain, and is bordered by Vermont, New York and Quebec, Canada (Figure 2).

Figure 2. Missisquoi Bay is the northern-most portion of Lake Champlain and is surrounded by Vermont, New York & Quebec, Canada.
The geography of the region is such that Missisquoi Bay drains to the south and is a distinct embayment that is highly isolated from the rest of Lake Champlain, which drains to the north. With limited water exchange with the main lake, many of the physical and ecological characteristics that define Missisquoi Bay differ dramatically from other parts of the lake. The bay is shallow, with a maximum depth of ≤5 meters. It is also surrounded by a heavily agricultural watershed, which contributes sediment and nutrient loading to the bay by way of its tributaries (Newcomb & Watzin, unpublished data).

Long term monitoring data from the Vermont Department of Environmental Conservation (VTDEC) show increasing summer water temperatures from 1992 through 2006 (mean summer temperature from 1992-2006 = 18.3°C). High, and perhaps increasing, nutrient concentrations, especially phosphorus, have also been measured in the bay since the early 1990’s (mean TP from 1992-2006 = 0.047mgP/L) (Lake Champlain Basin Program, 2006). Consequently low N:P ratios (30:1 or lower by weight) have been documented in Missisquoi Bay during the growing season (Watzin et al., 2004; 2005; 2006). There have also been shifts in the species composition and food web of Missisquoi Bay (Watzin et al., 2003; 2006; Couture & Watzin, in review). Historically the phytoplankton community in Missisquoi Bay was diverse (Meyers & Grundling, 1979; Brown et al., 1992; 1993), but now it is dominated by cyanobacteria species (Watzin et al., 2003; 2006). A population of the invasive white perch (*Morone americana*) has also become established in the bay within the last decade (Couture & Watzin, in review). Due to the dietary preferences of white perch for large zooplankton,
top-down shifts in the Missisquoi Bay foodweb may be further favoring cyanobacteria success as large grazing zooplankton populations are reduced.

Since the focus of my study is cyanobacteria, it is important to consider how trends observed in Missisquoi Bay relate to the literature describing factors controlling cyanobacterial dominance. Cyanobacterial blooms have been associated with warm water temperatures (Fujimoto et al., 1997; Kotak et al., 2000; Elliott et al., 2006), low light environments and high turbidity (i.e., Jensen et al., 1994; Huisman et al., 1999; Presing et al., 1999; see Oliver & Ganf, 2000), elevated phosphorus concentrations (Kotak et al., 2000; Dignum et al., 2005 and sources therein), and low N:P ratios (i.e., Smith, 1983; Michard et al., 1996; Fujimoto et al., 1997). The ability of cyanobacteria to vertically migrate beneath the photic zone to depths where benthic pools of ammonium are present (Ganf & Oliver, 1982; Oliver & Walsby, 1984; Oliver & Ganf, 2000) may also contribute to their success in Missisquoi Bay because the bay is so shallow. However, vertical migration of cyanobacteria and the presence of benthic ammonium have not actually been documented in Missisquoi Bay.

The goal of my study was to document relationships between *M. aeruginosa* and *A. flos-aquae* growth and N:P ratios and nitrogen form, using nitrogen concentrations relevant to Missisquoi Bay, Lake Champlain. The underlying hypothesis for this study is that the cyanobacteria community in Missisquoi Bay is, at least in part, governed by N:P ratio and nitrogen form and that vertical migration of *M. aeruginosa* to depths with appreciable ammonium concentrations gives it access to increased concentrations of this form of nitrogen. A series of research questions fall logically from my goal and
underlying hypothesis: (1) What N:P ratios support higher growth of *M. aeruginosa* rather than *A. flos-aquae*? (2) Does ammonium-nitrogen or nitrate-nitrogen give *M. aeruginosa* or *A. flos-aquae* a competitive advantage? (3) Are the particular N:P ratios and nitrogen forms associated with higher *M. aeruginosa* cell densities in axenic culture also associated with higher cell densities of *M. aeruginosa* in the natural Missisquoi Bay phytoplankton assemblage? (4) Is ammonium present in measurable concentrations in the benthic zone (within 20cm of the sediment-water interface)? (5) Can diurnal vertical migration of cyanobacteria be observed in Missisquoi Bay?

Results from this study will contribute to a growing body of knowledge of the environmental factors influencing cyanobacteria species composition and their dominance in freshwater systems. A better understanding of how N:P ratios and nitrogen form impact cyanobacteria species, as well as further documentation of processes occurring in Missisquoi Bay, will help water quality managers to control cyanobacteria blooms in the future.
Chapter 2: Article – The Effects of Nutrient Ratios and Forms on the Growth of *Microcystis aeruginosa* and *Anabaena flos-aquae*

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ABSTRACT

An increase in the temporal and spatial distribution of cyanobacteria blooms worldwide has drawn considerable research attention in recent decades because of the health risks cyanobacteria pose to humans and wildlife through the production of cyanotoxins, interference with recreation, and ecosystem changes. The role of nitrogen concentration and form, as well as N:P ratios, have been the topic of many studies, but results are varied. Redox chemistry in lake sediments (and thus ammonium production) and the ability of cyanobacteria to migrate vertically throughout the water column are thought to factor into their success in freshwater systems. The objective of this study was to test the effect of N:P ratio and nitrogen form on the growth of two common cyanobacteria species, *Microcystis aeruginosa* and *Anabaena flos-aquae*, in laboratory experiments using axenic cultures and a natural phytoplankton assemblage collected from Missisquoi Bay, Lake Champlain. N:P ratios ranged from 5 to 45:1 by weight and nitrogen form included both nitrate and ammonium at each ratio. Microscopic cell counts were used to determine growth of the cultures over the course of a six-day experiment, and microcystin concentrations were measured at the end of the experiment. Limited sampling of benthic water for nitrogen speciation and to determine the nocturnal location of cyanobacteria in the water column of Missisquoi Bay showed that benthic pools of ammonium exist in Missisquoi Bay and that vertically migrating species of cyanobacteria are reaching depths where ammonium is present. High variability in the cell count data from the laboratory experiments presented difficulties in drawing strong conclusions, but results suggested that *M. aeruginosa* growth was highest with ammonium-nitrogen, while
*A. flos-aquae* growth was greater with nitrate-nitrogen. Both trends were most apparent at N:P ratios of 5:1 and 45:1. Microcystin concentrations corresponded most closely with cell density across all treatments. *M. aeruginosa* dominated the vertical migration in Missisquoi Bay during our sampling event, and ammonium was documented near the sediment-water interface throughout the course of the summer. Taken together these data suggest that the distribution of nitrogen within an aquatic system and the ability of *M. aeruginosa* to vertically migrate efficiently may contribute to the *M. aeruginosa* dominance of the summer phytoplankton community in Missisquoi Bay.
INTRODUCTION

Cyanobacteria are ancient prokaryotic organisms whose presence in temperate fresh water bodies has been documented for centuries (i.e., Francis, 1878; Fogg et al., 1973; Codd & Beattie, 1991). However, the spatial and temporal distribution of dense cyanobacteria blooms has increased recently (Cronberg, 1999; Watzin et al., 2003; 2006; Johnston & Jacoby, 2003). Cyanobacteria blooms are concerning because of their ability to produce potent toxins that are harmful to humans, pets and wildlife (Codd, 1995; Carmichael et al., 2001; Codd et al., 2004), and the impacts blooms can have on aquatic ecosystems (Porter & Orcutt, 1980; Agusti, 1991a; Langmuir, 1997; DeMott, 1999; Downing et al., 2001; Scheffer, 2004).

A variety of hypotheses exist to explain why cyanobacteria blooms are becoming increasingly prevalent (Vitousek et al., 1997; Bianchi et al., 2000; Scheffer, 2004; Reynolds, 2006 and sources therein). The most common hypotheses focus on nutrient conditions (Smith, 1983; Vitousek et al., 1997; Hyenstrand, 1999; Bianchi et al., 2000; Berman, 2001; Downing et al., 2001; Von Ruckert & Giani, 2004) and nutrient cycling (Scheffer, 2004; McCarthy et al., 2007a) within a water body, as well as aspects of cyanobacteria cell physiology, such as their ability to migrate vertically within the water column, fix atmospheric nitrogen and produce cyanotoxins (Andersen & Shanmugam, 1977; Visser, 1995; Thiel & Pratt, 2001; Chan et al., 2004). Cyanobacterial blooms are often associated with eutrophic conditions (Vitousek et al., 1997; Smith et al., 1999; Bianchi et al., 2000), so many studies have documented the relationship between nitrogen and phosphorus concentrations, speciation and stoichiometry, and cyanobacteria success.
(Smith, 1983; Raven et al. 1992; Blomqvist et al., 1994; Fujimoto et al., 1997; Hyenstrand, 1999; Levine & Schindler, 1999; Kotak et al., 2000; Downing et al., 2001).

Although inconsistencies exist among results from the various studies, there is general agreement that elevated phosphorus concentrations (Kotak et al., 2000; Scheffer, 2004; Borner & Dittmann, 2005), N:P ratios below approximately 30:1 by weight (Smith, 1983; Vitousek et al., 1997; Bianchi et al., 2000; Downing et al., 2001), and availability of ammonium-nitrogen and low nitrogen concentrations (Blomqvist et al., 1994; McCarthy et al., 2007b) favor cyanobacteria over other phytoplankton. High surface area to volume ratio (Horne & Commins, 1987; Blomqvist et al., 1994; Reynolds, 2006) and low ammonium-nitrogen half saturation constants (Berman et al., 1984; Horne & Commins, 1987; Blomqvist et al., 1994; Sterner & Grover, 1998) of cyanobacteria are thought to contribute to their ability to thrive under low N:P ratio conditions, when nitrogen may be limiting for competing organisms. Nitrogen speciation and availability can also dramatically influence cyanobacteria species composition (Moss, 1973; Halterman & Toetz, 1984; Kappers, 1984; Blomqvist et al., 1994; Hyenstrand, 1999; Downing et al., 2001; Berman, 2001; McCarthy et al., 2007b).

Two ubiquitous cyanobacteria species, *Microcystis aeruginosa* and *Anabaena flos-aquae*, have specific characteristics that may give them advantages when nitrogen is limiting. Both of these species migrate vertically within the water column, and *Anabaena flos-aquae* can fix atmospheric nitrogen. Because *Microcystis spp.* generally forms larger colonies (Ganf, 1974; Watzin et al., unpublished data), it may be better at vertical migration than *Anabaena spp.* (Reynolds, 1984; Kromkamp & Walsby, 1990; Oliver &
Ganf, 2000). *M. aeruginosa*’s capability to vertically migrate quickly has also been observed in other studies, though direct comparisons between *M. aeruginosa* and *A. flos-aquae* have not been made (Ganf, 1974; Walsby & McAllister, 1987; Ibelings et al., 1991).

Once *M. aeruginosa* has sunk beneath the photic zone, to the benthic zone cells are exposed to nutrient pools that are unavailable in the upper regions of the water column (van Rijn & Shilo, 1985; Hyenstrand et al., 1998; Pearre, 2003). Ammonium is especially important because it is a form of nitrogen that can be immediately incorporated into cells, and is quickly and efficiently scavenged in the upper reaches of the water column (Herrero et al., 2001; Von Ruckert & Giani, 2004). However, near the sediment-water interface, ammonium production by ammonification, dissimilatory nitrate reduction, and denitrification-nitrogen fixation can exceed its uptake by microorganisms and oxidation to nitrate (Graetz et al., 1973; Scheffer, 2004; Canfield et al., 2005). The energetic efficiency of ammonium uptake compared to other aqueous nitrogen forms provides the foundation for the argument that vertical migration, combined with exposure to benthic ammonium pools, is highly advantageous for cyanobacteria species such as *M. aeruginosa* that are more adept at vertical migration than their competitors (Berman et al., 1984; Horne & Commins, 1987; Blomqvist et al., 1994; Reynolds, 2006).

The ability of heterocystous cyanobacteria species like *A. flos-aquae* to fix atmospheric nitrogen further contributes to the argument that nitrogen form factors into cyanobacteria species composition because nitrogen fixing species have a guaranteed nitrogen source, making them less reliant on uptake of ammonium (Andersen &
Shanmugam, 1977; Fay 1992; Blomqvist et al., 1994; Chan et al., 2004, Canfield et al., 2005). Although uptake rates and half saturation constants are highly dependent upon environmental or culture conditions, and therefore vary dramatically (Suttle & Harrison, 1988; Dortch, 1990; Vincent, 1992; Kudela et al., 1997), heterocystous cyanobacteria species such as *Anabaena spp.* tend to have higher uptake rates for nitrate, while non-heterocystous species like *Microcystis spp.* tend to have high uptake rates and low half saturation constants for ammonium compared to nitrate (Halterman & Toetz, 1984; Kappers, 1984).

These hypothesized, yet relatively little studied, theories of the relationship between nitrogen and cyanobacteria growth are particularly interesting when the ability of cyanobacteria to produce potent toxins is considered. *M. aeruginosa* and *A. flos-aquae* are capable of producing the hepatotoxins and neurotoxins, microcystin and anatoxin, respectively (Carmichael, 2003). Both secondary metabolites are nitrogenous compounds, though by virtue of size, microcystin is more nitrogen-intensive (Kaebernick & Neilan, 2001). Because of its nitrogenous structure and the fact that microcystin production has been found to increase under nitrogen abundance, it has been proposed that microcystin serves as a nitrogen storage mechanism for cyanobacteria (Downing et al., 2005).

In shallow, eutrophic Missisquoi Bay, Lake Champlain (Figure 2) dense cyanobacterial blooms of both *M. aeruginosa* and *A. flos-aquae* have become an annual occurrence since 2002 (Watzin et al., 2003; 2006). Anoxic conditions are also thought to occur in the sediments and low oxygen concentrations in the bottom waters during the
growing season in Missisquoi Bay, although few data exist to unambiguously document this. These characteristics of Missisquoi Bay make it ideal for examining the relationship between nutrient speciation and stoichiometry, and cyanobacterial growth and cyanotoxin production.

Our primary objectives were to test the effects of N:P ratios (ranging between 5 and 45:1 by weight) and nitrogen form (ammonium and nitrate) on the growth of *M. aeruginosa* and *A. flos-aquae* under laboratory conditions using nitrogen concentrations relevant to Missisquoi Bay. A mixed species experiment was specifically designed to test the competitive interactions of the species under different nutrient regimes. A second objective was to determine whether treatment condition affected the production of the cyanotoxin microcystin. A final objective of the study was to document both the presence of appreciable ammonium concentrations in the benthic zone of Missisquoi Bay and that *M. aeruginosa* does indeed engage in diurnal vertical migrations throughout the water column in the bay. Results from this study will contribute to a growing body of knowledge of the environmental factors influencing cyanobacteria species composition and their dominance in freshwater systems. A better understanding of how N:P ratios and nitrogen form impact cyanobacteria species, as well as further documentation of processes occurring in Missisquoi Bay, will hopefully help water quality managers to control cyanobacteria blooms in the future.
METHODS

Culture Experiments

Maintenance

Axenic cultures of *M. aeruginosa* (LE-3) and *A. flos-aquae* (UTCC 2391) were acquired from Dr. Greg Boyer’s laboratory at the State University of New York for Environmental Science and Forestry in May 2006, and were maintained in a laboratory at the University of Vermont using Sigma Aldrich Co. BG-11 growth media. Cultures were grown in 650mL Erlenmeyer flasks covered with aluminum foil on a lab bench, relying on natural light. Temperatures in the room where cultures were housed ranged between 19 and 25°C. All glassware and other supplies exposed to cultures or their media were acid washed and autoclaved. All other equipment (i.e., disposable pipette tips) directly exposed to cultures or autoclaved media were aseptic. Before conducting experiments the cultures were grown for several months until there were at least 1.5 liters of healthy, vibrant green stock of each species.

Experiments

Modified BG-11 media (Table 1) was made using four variations of nitrogen to phosphorus ratios with environmental relevance to Missisquoi Bay, Lake Champlain. Phosphorus concentrations were held constant between the four media, while nitrogen form and concentration were adjusted to ratios of 5, 15, 30 and 45:1 by nitrogen and phosphorus weight for both nitrate and ammonium, yielding eight different experimental
media, or treatments. The phosphorus concentration, 1.93 μg/L, was determined by averaging soluble reactive phosphorus (SRP) concentrations found in Missisquoi Bay between 2002 and 2005 (Watzin et al. unpublished data), and calculating the associated weight of phosphorus from the phosphate ion. Each medium was made in 1L batches in Erlenmeyer flasks and adjusted to pH 7.5 using 0.2 M sodium hydroxide. Media were then autoclaved and treated aseptically. Prior to beginning experiments with newly created medium, each media was tested for both nitrate and ammonium concentrations with a DR/890 Datalogging HACH Colorimeter using manufacturer Method No. 8192, a cadmium reduction method for low range nitrate, Method No. 10020, a chromotopic acid method for high range nitrate, and Method No. 10023, a salicylate method for low range ammonia. These tests were conducted to verify that desired nitrogen concentrations and form were present in the experimental media.

Experiments were conducted in acid washed, autoclaved 125mL Erlenmeyer flasks with inverted 50mL beakers as lids following the design in Figure 3. In each experimental run triplicate flasks were established for each combination of time, nitrogen form and N:P ratio. Three additional flasks were established and sampled immediately to provide a measure of initial cell density.

Replicate flasks were filled with 50mL of each of the eight different media. Each flask was then inoculated with 10mL of concentrated stock culture of the desired cyanobacteria community. The volume of 10mL for inoculation was selected based upon rapid cell counts at 100x using a Sedgewick-Rafter cell. The goal was to establish cell densities that were comparable among experimental runs, and sufficiently dense that
accurate cell counts could be performed using the Ütermohl settling chamber method described in further detail below. The Ütermohl method underestimates cell densities if the spatial distribution of cells on the slide is too sparse (APHA, 1995; Lund et al., 1958).

The 72 experimental flasks were then placed on a counter near a window under natural light in a haphazard arrangement. Flasks established for initial cell density determination and triplicates for each time interval were sampled by removing 35mL and preserving in 50mL plastic centrifuge tubes using 1% Lugol’s potassium iodine solution. The remaining approximately 25mL of sample not preserved in Lugol’s for cell enumeration was used to test microcystin concentrations in experiments containing M. aeruginosa. Samples for microcystin analysis were vacuum filtered, preserved with 50% methanol and stored at -80°C until analysis.

The entire experimental procedure described above was repeated four times with different cyanobacteria communities. The first three experimental runs employed axenic cultures of M. aeruginosa and A. flos-aquae in the following combinations: (1) M. aeruginosa independently; (2) A. flos-aquae independently; and (3) a mixture of M. aeruginosa and A. flos-aquae. During the single-species experiments the entire inoculation volume was drawn from a single stock of the desired culture. The mixed community received inoculation of equal volume, 5mL, from each species’ stock culture. The goal of this design was to be able to compare trends in growth observed for a single species to its growth observed in a mixed community with the potential for competitive interactions.
The fourth experiment used a phytoplankton community collected from Missisquoi Bay during a *M. aeruginosa* bloom in August, 2006. For this experiment, phytoplankton were collected mid-morning on a calm, clear day, while cyanobacteria were concentrated at the surface of the water column. A five-gallon bucket was used to collect the initial sample, which was allowed to sit so the mixed populations of phytoplankton could rise to the surface of the bucket. One-liter glass reagent jars were then used to collect samples of the concentrated algae at the surface of the bucket. Phytoplankton were transported back to the lab, where they were transferred into a three-liter, clean Erlenmeyer flask. This flask was used to inoculate the experimental flasks the same day as phytoplankton were collected. Using this method, we limited the impacts of zooplankton grazing on phytoplankton and any stress that organisms experienced in the process of collection and transport to the lab.

Microcystin concentrations were measured from one flask per treatment at day six for the Missisquoi Bay, *M. aeruginosa*, and *M. aeruginosa + A. flos-aquae* experiments. For this analysis, a measured volume of experimental culture was filtered onto Whatman 934-AH glass fiber filters, and placed into a 15mL conical glass centrifuge tube with Teflon-lined cap. Four milliliters of 50% methanol were added to each centrifuge tube, after which samples were shaken and stored at –80°C until analysis. Microcystin concentration was determined using an Envirologix™ QuantiPlate™ competitive Enzyme-Linked ImmunoSorbent Assay (ELISA). Samples were thawed, shaken and re-frozen two more times before beginning analysis to ensure rupture of cell walls and release of the toxin. Extracts were diluted with deionized water until methanol represented less than 5%
of the total volume (Metcalf et al., 2000) and were run in duplicate following manufacturer’s instructions on a KC Jr. plate reader (Biotek Instruments), utilizing standards provided in the kit. QA analyses of results using this method have shown good accuracy and precision (Watzin et al., 2003; 2004).

**Laboratory Analysis**

Phytoplankton samples preserved in 1% Lugol’s iodine solution were stored in the dark until being analyzed using Ütermohl settling chambers and slides (Lund et al., 1958). Ütermohl slides were counted using an Olympus IX 70 inverted microscope with phase contrast at a magnification of 400x. Slides were scanned prior to commencement of counting to minimize inconsistencies in cell counts as a function of an uneven distribution of settled cells on the slide. If obvious irregularities in the pattern of settled cells on the slide were evident, we made appropriate adjustments to the pattern by which the required 100-5x5 Whipple grids were counted.

Ten percent of samples analyzed were recounted to estimate precision throughout the cell enumeration process. Samples to be recounted were selected using random numbers, making sure that samples from a diversity of treatment conditions and preservation times were included. Due to the high variability associated with counting individual cells of species that grow in large, dense colonies, samples and their corresponding recount (cells/milliliter) that were more than 40% different were counted a third time. Most sample counts differed by 5 to 30%, which is common for cyanobacteria.
species, with colonial species having variability of an even greater magnitude (Burch et al., 2003).

**Statistical Analysis**

Changes in cell densities of the cultures during the experiments were used as the dependent variable in all statistical analyses. Mean cell densities found for the three initial flasks preserved immediately after inoculation were used as the starting density; growth in each experimental replicate was determined by subtracting the initial density from the density measured at later times. All statistical analyses utilized this calculated growth variable.

Statistical analysis was performed using SAS (SAS Systems 2003). Normality and homogeneity of variance were assessed by time for each experimental run using the Wilk-Shapiro and Brown-Forsythe tests, respectively. Common transformations, such as log, sine, inverse, square root, and power two and three, were applied to variables that did not meet the normality assumption for ANOVA and transformations were retested using the Wilk-Shapiro test. Parametric analyses of variance (ANOVA) were used to test the hypotheses that cell densities varied as a function of growing time, nitrogen form and N:P ratios of the experimental media.

Initially three-way ANOVA’s were performed for each experimental run to assess the combined effects of time, nitrogen form and N:P ratio on cyanobacteria growth. These were followed by two-way ANOVA and one-way ANOVA to isolate the effects of nitrogen form and N:P ratio on cyanobacteria growth at individual times because interaction terms were frequently significant. Bonferroni adjusted multiple comparisons
tests were used in conjunction with the one-ANOVA using data sorted by time and nitrogen form to find differences among ratio treatments. This test controls for otherwise high Type I error rates incurred by performing multiple one-way comparisons.

Microcystin concentrations were analyzed both visually using scatter plots and with the regression procedure in SAS to explore relationships between cell density and microcystin concentration. Cell densities included in the analyses represented both *M. aeruginosa* and *A. flos-aquae* cells within a sample because both are capable of producing microcystins. Although the University of Texas Culture Collection, where our *A. flos-aquae* culture originated, has no record of *A. flos-aquae* (UTCC 2391) producing microcystins, genetic tests for the gene cluster responsible for microcystin production (Wilhelm et al., unpublished data) and ELISA analyses on the axenic culture (Watzin et al., unpublished data) confirm this strain to be capable of producing microcystin.

**Field Methods**

**Sample Collection**

To monitor nutrient conditions in Missisquoi Bay, samples were collected during the summer of 2006 on an approximately weekly basis from a site near Highgate Springs, VT (Figure 2). This sampling site is located at 44° 59.21’ N and 73° 07.12’ W, where the depth is approximately 3 meters. A total of 58 samples were collected from the sediment water-interface and at the surface of the water column for nitrogen speciation analysis. Surface water samples were collected by submersion of a 50mL plastic centrifuge tube into the water from the side of a boat. Bottom samples were collected using two methods: a Glew gravity corer and an improvised syringe apparatus. Clean 50mL plastic centrifuge
tubes were used to hold samples from both methods of collection. To obtain water samples from the Glew gravity corer, a core was taken and water immediately above the sediments in the core was siphoned into a collection vial. The syringe apparatus consisted of a syringe clamped to the bottom of a five-meter threaded metal rod. A rope connected to the syringe plunger was used to suction a water sample from near the sediment-water interface. The sediment water interface was located by carefully lowering the threaded metal rod until we could feel it come in contact with the sediments. Samples were transported on ice back to the laboratory. Nitrate and ammonium analyses were performed within 24 hours of collection using the HACH colorimetric methods described above.

Temperature and pH were measured periodically for use in subsequent ammonium calculations. Average temperature at the sediment-water interface in Missisquoi Bay was 19°C, while pH fluctuated around 7.0. These values were used to adjust the ammonia concentrations measured by HACH method no. 10023 by calculating a temperature-dependent equilibrium constant.

Nocturnal water samples were also collected on September 11, 2006 at depths of 15, 30 and 45cm above the sediment-water interface as well as at the water surface in order to document diurnal vertical migration of cyanobacteria. Samples from near the sediment-water interface were collected by the modified syringe method described above using a syringe with a cut-off tip to allow large colonies of cyanobacteria to be sampled. Samples were collected in 50mL plastic centrifuge tubes and preserved immediately with
1% Lugol’s iodine solution for microscopy using the Ütermohl settling methods outlined previously.

RESULTS

Culture Experiments

Media Nitrogen

Media used in each of the experimental runs contained close to the desired concentrations of nitrate- and ammonium-nitrogen (Table 2). All proportional differences between nitrogen concentrations over our range of N:P ratios were correct, and concentrations did not vary by more than 0.03 mg/L nitrogen. Some deviation from the exact desired amount of nitrate- or ammonium-nitrogen can be explained by the fact that low nitrogen concentrations were very close to the detection limits of the equipment (HACH estimated limit of detection: 0.08mg/L ammonium and 0.01mg/L nitrate). Small concentrations of nitrate in some ammonium-nitrogen media can be attributed to the unavoidable oxidation of some ammonium to nitrate, since we did not attempt to perform this experiment under anoxic conditions.

Growth

Homogeneity of variance was always met using the Brown-Forsythe test, although variability among the triplicate samples resulted in a lack of normality; only 43 percent of data sets were normally distributed. The common transformations did little to help data meet the normality assumption of parametric statistical tests. However,
ANOVA is moderately robust to non-normality (Ott & Longnecker, 2001). Failure to meet the normality assumption is unlikely to yield false conclusions of significant differences, but rather will yield more conservative ANOVA results (Ott & Longnecker, 2001). Therefore, we relied on parametric tests for our analyses.

Cell growth was visible during all experimental runs as flasks generally became greener with time. Three-way ANOVA’s supported this observation, with mean cell growth varying significantly with time in each experimental run (Table 3). Statistical evidence of cell growth over time validated the experimental procedures used in this study, but also made assessment of the impacts of nitrogen form and N:P ratio difficult because many interaction terms containing time were significant, and growth was not uniform across treatments.

*M. aeruginosa* grew consistently over time, both by itself and in the experimental run where it was combined with *A. flos-aquae*. *M. aeruginosa* contained within the Missisquoi Bay community experiment reached maximum cell densities after three days in all treatments, with growth declining on the sixth day. *A. flos-aquae* grew slowly but relatively consistently when by itself and in the mixed axenic culture experiment with *M. aeruginosa*. *A. flos-aquae* cell densities decreased continually with time in the Missisquoi Bay experiment, although its cell densities were considerably lower than those of *M. aeruginosa* and *Aphanizomenon* in this experiment. *Aphanizomenon* followed a similar pattern to *M. aeruginosa* in the Missisquoi Bay community experiment in that maximum cell densities were reached on the third day, after which growth decreased markedly.
The impacts of nitrogen form and N:P ratio on mean cell growth were apparent in some runs and at some times in the two-way ANOVAs by time (Table 4). Significant differences were observed between mean cell densities as a function of nitrogen form, N:P ratio and their interaction terms, although not in each experimental run or consistently across time. The experimental run consisting of *M. aeruginosa* independently had no significant two-way ANOVA results at any time. The experimental run containing both *M. aeruginosa* and *A. flos-aquae* had the most statistically significant results. In this experiment, the mean cell growth of *M. aeruginosa* was significantly different as a function of nitrogen form, N:P ratio or both at each time.

In two sets of one-way ANOVAs performed on those experimental runs with significances in the two-way ANOVAs, only sporadic statistically significant results were found. In the *M. aeruginosa* only experiment, cell densities were similar between nitrate and ammonium, and generally greatest at low N:P ratios (5 and 15:1), but differences were small, especially at one and three days (Figure 4). At day six, greatest growth was seen at ratios of 15:1, while 30 and 45:1 had the lowest growth.

When *A. flos-aquae* was grown independently, cell densities generally declined with time. Inconsistent patterns were seen among N form and ratio. After one day, *A. flos-aquae* growth was greatest at an N:P ratio of 15:1, and lowest at a ratio of 30:1 (Figure 5). However, after three and six days, growth was highest at a ratio of 5:1, and lower with ratios of 15:1 and greater, especially at day six.

The experiment containing a mixture of *M. aeruginosa* and *A. flos-aquae* showed a significant effect of both nitrogen form and ratio, but also significant interactions (Table
4). In one-way ANOVAs (Figures 6 and 7) both cyanobacteria species showed a pattern of response that was more consistent. *M. aeruginosa* growth was generally higher in ammonium-nitrogen treatments after one and six days (Figure 6 a-c), although declines in cell densities were observed after one and three days. Ammonium-nitrogen treatments exhibited the least compromised growth after one day, but after the third day, ammonium-nitrogen was only associated with less compromised growth at a ratio of 5:1 and nitrate-nitrogen yielded higher growth values at higher N:P ratios. Differences between nitrogen forms are small after both one and three days. After six days, *M. aeruginosa* growth was again highest with ammonium-nitrogen at low N:P ratios, whereas differences between nitrate and ammonium-nitrogen were not significant at N:P ratios of 30 and 45:1.

*A. flos-aquae* showed some indication of higher growth with nitrate-nitrogen (Figure 7a-c). This pattern was most pronounced after three days when nitrate-nitrogen was associated with higher growth at all N:P ratios (Figure 7c). After one and six days, this pattern only appears at N:P ratios of 5 and 45:1, and is only statistically significant at an N:P ratio of 30:1. *A. flos-aquae* also grew better at higher N:P ratios (Figure 7c) at most times.

In the Missisquoi Bay community *M. aeruginosa* growth followed a pattern similar to the independent *M. aeruginosa* experiment, with few significant trends. At day three, when cell densities had increased, *M. aeruginosa* growth was similar in nitrate- and ammonium-nitrogen treatments and across all N:P ratios (Figure 8). *A. flos-aquae* cell densities decreased under all treatment conditions and no real differences were visible
between nitrate- and ammonium-nitrogen treatments, although growth was less compromised in nitrate-nitrogen treatments after the first day (Figure 9a). There was also little variation among ratios visible; however, an N:P ratio of 5:1 did correspond most consistently with the lowest growth (Figure 9b). *Aphanizomenon* cell densities also decreased at all times for both nitrate- and ammonium-nitrogen treatments (Figure 10). At day three and day six, *Aphanizomenon* generally had higher cell densities in higher N:P ratio treatments.

**Microcystin Production**

No significant results were found when using regression analyses to assess the effect of nitrogen form and N:P ratio on microcystin production, but this is not surprising, since there were only four data points for each experimental run. Visual assessment of the data suggests a relationship between cell density of potentially toxic cyanobacteria cells and microcystin production (Figure 11), but no pattern is evident between toxin production and treatment condition. Therefore, it is difficult to evaluate the hypothesis that microcystin serves as a cellular nitrogen storage mechanism by cyanobacteria. *M. aeruginosa* did, however, produce greater amounts of microcystin when it was grown independently from other cyanobacteria species.

**Field Data**

**Field Nitrogen**

Ammonium was found in 42 of the 58 samples taken in Missisquoi Bay throughout the summer of 2006 and in all of those taken at the sediment-water interface;
concentrations ranged up to 1.24mg/L NH$_4^+$ (Figure 12a). Benthic ammonium concentrations were consistently higher than their surface counterparts. Nitrate was also found throughout the water column in concentrations up to 0.7mg/L NO$_3$ (Figure 12b). Again, surface concentrations were generally lower than corresponding benthic samples. For both nitrate and ammonium a clear decline in nitrogen concentrations can be seen throughout the course of the growing season except for two samples taken in late September 2006.

**Vertical Migration**

*M. aeruginosa* was the most abundant species present near the sediment-water interface in nocturnal samples (Table 6). Cell densities were two to three orders of magnitude greater than cell densities of either *Anabaena flos-aquae* or *Aphanizomenon*. *M. aeruginosa* cell densities were comparable across the four depths sampled, while *Anabaena flos-aquae* was only found at a depth of 30cm above the sediment-water interface and *Aphanizomenon* was only found at the surface and at 15cm above the sediment-water interface.

**DISCUSSION**

**Patterns of response in the lab experiments**

Although high variability in the data made differences in cell densities between the treatments difficult to quantify with statistics, there was a general trend in the response of *M. aeruginosa* and *A. flos-aquae* growth to N:P ratios and nitrogen form.
Especially in the mixed axenic culture experiment, *M. aeruginosa* demonstrated higher growth in ammonium-nitrogen treatments, and *A. flos-aquae* growth was highest with nitrate-nitrogen.

The original intent of the pairing of individual and mixed axenic culture communities in the experimental design was to enable comparisons between trends in growth observed for a single species versus the same species in a community with potential for competitive interactions to influence growth patterns. Using this experimental design, we hoped to determine whether *M. aeruginosa* was more successful than *A. flos-aquae* when growing under its preferred conditions of nitrogen form and N:P ratio. Because trends observed in these data were only weakly apparent and patterns were inconsistent between experiments, we could not evaluate competitive interactions between *M. aeruginosa* and *A. flos-aquae* in a substantive manner. In the mixed axenic culture experiment there is some suggestion that competitive interactions do control how nitrogen-form and N:P ratio influence the growth of cyanobacteria species. *M. aeruginosa* growth is generally greatest with ammonium-nitrogen, a trend that is most consistent at an N:P ratio of 5:1. *A. flos-aquae* growth is highest with nitrate-nitrogen, especially after three and six days, and when nitrogen concentrations were low (N:P ratios of 5-30:1, Figure 7b). However, definitive conclusions about the nature of competitive interactions between *M. aeruginosa* and *A. flos-aquae* cannot be made with these data.

The trend of *M. aeruginosa* growth being greatest when ammonium-nitrogen is present has been reported in a number of studies (Blomqvist et al., 1994; Jacoby et al.,...
2000; Von Ruckert & Giani, 2004), and can be attributed to the exceptionally low half saturation constant and high maximum uptake rate of ammonium by *M. aeruginosa* (Kappers, 1984; Von Ruckert & Giani, 2004). Blomqvist et al. (1994) also reported that ammonium-nitrogen would favor non-heterocystous cyanobacteria species like *M. aeruginosa* over heterocystous species like *A. flos-aquae*. *M. aeruginosa* growth data from the mixed axenic culture experiment was also consistent with Blomqvist et al. (1994). The low half saturation constant and high maximum uptake rate of ammonium by *M. aeruginosa* enables it to efficiently scavenge ammonium at low concentrations, and utilize large amounts of ammonium when it is abundant. In our data, the trend of *M. aeruginosa* growth being highest with ammonium-nitrogen is most consistent at an N:P ratio of 5:1 (Figure 6 b & c).

Instances where *A. flos-aquae* growth is greater in nitrate-nitrogen treatments, such as the third and sixth days in the independent experiment and the third day of the mixed axenic culture experiments, can be explained by the efficiency of nitrate uptake by *A. flos-aquae*. Halterman & Toetz (1984) found *A. flos-aquae* to have the lowest half saturation constant for nitrate uptake of all 18 phytoplankton species considered in the study. Nitrogen uptake rates and half saturation constants are highly dependent upon environmental or culture conditions, so values for each vary dramatically within the literature (Suttle & Harrison, 1988; Dortch, 1990; Vincent, 1992; Kudela et al., 1997). There is also a general lack of studies making direct comparisons of *M. aeruginosa* and *A. flos-aquae*, so it is difficult to comment on the relative uptake of nitrate and ammonium by the two species. However, our data does show some evidence of the
nitrogen uptake differences between *M. aeruginosa* and *A. flos-aquae* (Figures 6c and 7b and c). Greatest differences in growth of each cyanobacteria species is observed at low N:P ratios, where there is a significant difference between the uptake kinetics for different nitrogen forms. However, as N:P ratios increase, the half saturation constants and maximum uptake rates for the different nitrogen forms by the cyanobacteria species becomes more equal and differences in growth between treatment conditions decreases.

Nitrogen uptake kinetics by cyanobacteria can be influenced by a variety of environmental factors, including nitrogen concentration and form (Gilbert & Ray, 1990; Oliver & Ganf, 2000; Ferber et al., 2004; Flores & Herrero, 2005), temperature (Whalen & Alexander, 1984; Gu & Alexander, 1993; Reay et al., 1999), light (Whalen & Alexander, 1984; Gu & Alexander, 1993), and the presence of micronutrients (Utkilen & Gjolme 1995, Oliver & Ganf, 2000). Some cyanobacteria, like *Microcystis*, can quickly adjust their rate of nitrogen uptake based on its availability (Watanabe & Miyazaki 1996; Oliver & Ganf, 2000). It is generally agreed that ammonium is the most easily assimilated form of nitrogen, followed by nitrate and nitrite, followed by atmospheric nitrogen (Oliver & Ganf, 2000; Ferber et al., 2004; Flores & Herrero, 2005) and that the presence of ammonium inhibits nitrate and nitrite uptake, and the presence of either ammonium or nitrate/nitrite inhibits nitrogen fixation (Gilbert & Ray, 1990; Oliver & Ganf, 2000, Ferber et al., 2004). However, some studies have questioned these inhibitions of nitrogen fixation, finding that the severity is less than is often reported (Dortch, 1990). Temperature has especially pronounced effects on nitrate uptake, causing decreased uptake rates at lower temperatures (Whalen & Alexander, 1984; Gu & Alexander, 1990;
Reay et al., 1999). Light impacts nitrogen uptake at least in part because the process requires energy that must ultimately be produced by photosynthesis (Whalen & Alexander, 1984; Gu & Alexander, 1990).

Not all of the data presented here demonstrate trends that we would expect to see given reports from previous studies, especially with respect to the effects of N:P ratio and nitrogen form on the growth of *M. aeruginosa* and *A. flos-aquae*. In some instances, nitrate-nitrogen appears to have supported higher growth by *M. aeruginosa*, while other times *A. flos-aquae* had higher growth with ammonium-nitrogen. While these results could indicate different responses of *M. aeruginosa* and *A. flos-aquae* to treatment conditions than have previously been reported, the inconsistent scattering of such results suggests that variability in responses is great. Further studies, (see recommendations below), would be necessary to sort out this variation.

Although our results showed very little overall trend with respect to N:P ratio, based on the relevant literature we would have expected to see good cyanobacteria growth at lower N:P ratios (Smith, 1983; Bulgakov & Levich, 1999; Downing, 2001). Smith (1983) suggests that cyanobacteria are most successful at N:P ratios below 30:1 by weight, especially when in competition with other organisms. Therefore we expected to see a low N:P ratio advantage most clearly Missisquoi Bay community where there was competition with other phytoplankton. The fact that we did not observe this trend is most likely a function of the high variability within our data.

In the mixed axenic culture communities, we expected the predictions put forth by Blomqvist et al. (1994) to be apparent: that at low nitrogen concentrations, and thus low
N:P ratios, nitrogen fixing cyanobacteria (i.e., *A. flos-aquae*) should dominate because their ability to fix atmospheric nitrogen eliminates the threat of nitrogen limitation. Combining this argument with the threshold N:P ratio of 30:1 (Smith, 1983), we would have expected *M. aeruginosa* to have its highest growth in the mid-ranged N:P ratios (15 and 30:1).

While the nitrogen concentrations in each of the four treatment media are considerably lower than standard cyanobacteria growth medium. We based out N:P ratios are based on Missisquoi Bay phosphorus concentrations, but this made seeing patterns related to nitrogen concentration more challenging. Nutrient limitation is unlikely the cause of varied growth patterns of cyanobacteria communities in the four experimental runs, as cell densities were highest in the individual *M. aeruginosa* experiment, where the culture appears to have only reached its exponential growth phase.

**Methodological Challenges**

While the trends in our data described above are supported by relevant literature, the high variability amongst cell densities in our experimental treatments made data analysis challenging. Variability within the data can be attributed in part to (1) colonial growth of cyanobacteria (Burch et al., 2003; Bernard et al., 2004) creating irregularities in cell densities in an Ütermohl settling chamber; and (2) the standard methods used to enumerate cells on an Ütermohl slide (Lund et al., 1958); (3) insufficient replication within treatments; and (4) the relatively fine-scale differences among the nitrogen concentrations used in the N:P ratios.
The growth of cyanobacteria in large colonies, particularly in the field, present challenges for accurate cell enumeration. Dense colonies are almost impossible to accurately count because many planes of cells exist, and not all of these planes can be brought into clear focus using an inverted microscope. Consequently, estimations are made either by predetermined conventions for a given mass of cells at a certain magnification, or by trying to count all the cells one can see, but realistically not knowing whether some cells were missed or counted multiple times (Burch et al., 2003). Colony size in this study was generally smaller than would be anticipated in field samples (Watzin et al., unpublished data), but colonies were still large enough to present challenges during the enumeration process. Axenic *M. aeruginosa* grew primarily unicellurally, but colonies up to approximately 300 cells were encountered. Axenic *A. flos-aquae* grew as a mixture of single cells and filamentous colonies with up to 200 cells. Samples collected from Missisquoi Bay contained *M. aeruginosa* colonies with upwards of 1000 cells, while *A. flos-aquae* colonies were relatively small, given their low density within the phytoplankton community at the time of sampling. *Aphanizomenon* grew almost entirely in filamentous colonies of approximately 40 cells, but filaments aggregate, making enumeration more challenging.

Methods used to count cells in an Ütermohl slide are also the source of variability (Lund et al., 1958). By convention, only 100 grids from the Ütermohl slide are actually counted in order to determine cell density, which is problematic for two reasons. First, cells are known to settle unevenly (Lund et al., 1958). Cells typically settle most densely towards the perimeter of the slide, leaving less dense regions in the center of the slide.
While suggested patterns for selecting fields and grids of the slide to count attempt to minimize biases associated with uneven settling, (Lund et al., 1958), it is impossible to eliminate this source of variability completely. This source of error is compounded by the reality that the settling pattern on a slide can be easily disrupted by subtle movements of the slide or cylinder, so the suggested patterns of how to count grids will not always capture the differences in cell densities across the slide. Second, when cells grow in large colonies, the counting or missing of a large colony can dramatically change a cell count.

These problems are compounded by the fact that final cell densities are estimated using a conversion between cells actually counted and the number of cells per milliliter (APHA, 1995; Lund et al., 1958). This conversion yields cell densities many times the number of cells actually counted. Therefore, any variability within the actual cell counts is multiplied many times in the conversion to cells per milliliter.

Insufficient replication within treatment conditions also contributed to variability within the data. Three replicates provided only minimal power to determine the source of variation in ANOVA. More replicates would enable a better estimate of the true mean cell density in a treatment when sample estimates are scattered.

Lastly, the relatively small differences in nitrogen concentrations between the different N:P ratios, when combined with high variability among cell counts, made differences in growth between treatments challenging to discern. Variation among cell counts from a given sample ranged from 0.97 to 524%, with a mean variation of 39%, which is not uncommon for cyanobacteria enumeration (Burch et al., 2003). Therefore,
difference in cell densities between treatments must be larger than our average variation in order to be detected by ANOVA.

N:P ratios, and therefore nitrogen concentrations, were selected based on actual Missisquoi Bay concentrations of nutrients to provide further environmental relevance to the study, but greater differences between nitrogen concentrations would likely have yielded differences in cell growth dramatic enough to quantify statistically, despite the variability within the data. If the differences between nitrogen concentrations had been greater, we may have been able to detect a statistically significant pattern, and not just a subtle trend, between N:P ratio and nitrogen form and cyanobacteria species composition.

A few recommendations can be made for future research of this nature. Increasing replication within a more limited number of treatments would likely yield the greatest improvement to this study. Decreasing the number of N:P ratios or the number of times at which samples were preserved for cell enumeration would have allowed a higher number of replicates sampled from each treatment. Separating large colonies into either smaller fragments or single cells would also dramatically improve accuracy of cell counts. Previous studies have reported disaggregating colonies, especially *Microcystis* colonies, by suspension in deionized water, ultrasonication, alkaline hydrolysis, heating/vortexing and grinding (see Box, 1981 and Bernard et al., 2004 for reviews of these methods), with heating and grinding methods producing the greatest results (i.e., most reduction of colony size and least potential for cell loss and destruction). Greater differences in nitrogen concentrations between treatments would further improve the chances of a similar study producing statistically significant results.
Overall our results provide some suggestive evidence that nitrogen form and N:P ratios may indeed factor into the success of cyanobacteria in temperate freshwater lakes and the species composition that ensues. Documentation of benthic pools of ammonium in Missisquoi Bay and the diurnal vertical migration of *M. aeruginosa* provides support for the hypothesized mechanism by which *M. aeruginosa* acquires ammonium during the growing season, when photic zone ammonium is depleted (Graetz et al., 1973; Kromkamp & Walsby, 1990; Scheffer, 2004; Canfield et al., 2005). While we have only preliminary data, we can infer that benthic ammonium and vertical migration by *M. aeruginosa* to the benthic zone are influencing their success in Missisquoi Bay, as has been reported in other water bodies (Graetz et al., 1973; Verdouw & Dekkers, 1982; van Rijn & Shilo, 1985; Kromkamp & Walsby, 1990; Hyenstrand et al., 1998; Herrero et al., 2001; Pearre, 2003; Von Ruckert & Giani, 2004; Scheffer, 2004; Canfield et al., 2005; Wang et al., 2007).

Given the trends seen in this study and in the literature, water quality improvement efforts should target nitrogen along with other nutrient pollutants to be most successful. Phosphorus often receives a lot of attention in watershed management plans for water quality improvement because of its well-documented, positive correlation with phytoplankton growth (Kotak et al., 2000; Bianchi et al., 2000). While elevated phosphorus concentrations may indeed be stimulating phytoplankton growth, other factors, like nitrogen concentration and form, may be leading to more favorable growing conditions for cyanobacteria as well (Blomqvist et al., 1994; Herrero et al., 2001; Von Ruckert & Giani, 2004).
WORK CITED


Codd, G. and Beattie, K. 1991. Cyanobacteria (blue-green algae) and their toxins: awareness and action in the United Kingdom. Public Health Laboratory Service Microbiology Digest. 8: 82-86.


Tables and Figures

Table 1. Modified BG-11 media used in culture experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Media Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Nitrate</td>
<td>NaNO3</td>
<td>30.000</td>
</tr>
<tr>
<td>Potassium Phosphate . 3H2O</td>
<td>K2HPO4.3H2O</td>
<td>1.050</td>
</tr>
<tr>
<td>Magnesium Sulfate . 7H2O</td>
<td>MgSO4.7H2O</td>
<td>1.500</td>
</tr>
<tr>
<td>Calcium Chloride . 2H2O</td>
<td>CaCl2.2H2O</td>
<td>0.720</td>
</tr>
<tr>
<td>EDTA (disodium salt)</td>
<td>C10H14O8N2Na2.2H2O</td>
<td>0.001</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>NaHCO3</td>
<td>0.400</td>
</tr>
<tr>
<td>Ferric Chloride (anhydrous)</td>
<td>FeCl3</td>
<td>0.011</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>H3BO3</td>
<td>0.057</td>
</tr>
<tr>
<td>Manganese Chloride . 4H2O</td>
<td>MnCl2.4H2O</td>
<td>0.036</td>
</tr>
<tr>
<td>Zinc Chloride</td>
<td>ZnCl2</td>
<td>0.002</td>
</tr>
<tr>
<td>Molybdcic Oxide</td>
<td>MoO3</td>
<td>0.009</td>
</tr>
<tr>
<td>Cupric Sulfate</td>
<td>CuSO4</td>
<td>0.001</td>
</tr>
<tr>
<td>Cobalt Chloride</td>
<td>CoCl2</td>
<td>4.4x10^-4</td>
</tr>
</tbody>
</table>

Table 2. Observed and expected nitrogen concentrations within experimental media.

<table>
<thead>
<tr>
<th>Community</th>
<th>Media</th>
<th>Nitrate (mg/L)</th>
<th>Ammonium (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Form</td>
<td>Ratio</td>
<td>Observed</td>
</tr>
<tr>
<td>Missisquoi Bay &amp; M. aeruginosa</td>
<td>5</td>
<td>0.06</td>
<td>0.0292</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.11</td>
<td>0.0795</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.18</td>
<td>0.2050</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.25</td>
<td>0.2378</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.01</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.03</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.01</td>
<td>0.0000</td>
</tr>
<tr>
<td>A. flos-aquae &amp; M. aeruginosa + A. flos-aquae</td>
<td>5</td>
<td>0.03</td>
<td>0.0292</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.09</td>
<td>0.0795</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.19</td>
<td>0.2050</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.23</td>
<td>0.2378</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.02</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
Table 3 a & b. Three-way ANOVA results from axenic culture and Missisquoi Bay experiments, respectively. All F-statistics and p-values reported represent type III sums of squares. Values in bold are statistically significant at a significance level of 0.1. M represents results from the experiment containing *M. aeruginosa* independently; M (+A) and A (+M) are the species-specific results from the mixed axenic culture experiment for *M. aeruginosa* and *A. flos-aquae*, respectively; and A represents the culture experiment containing *A. flos-aquae* by itself. M, A, and Aph represent results from the Missisquoi Bay experiment for *M. aeruginosa*, *A. flos-aquae* and *Aphanizomenon spp.*, respectively.

### 3a. 3-Way ANOVA on Axenic Culture Experiments

<table>
<thead>
<tr>
<th>Tests</th>
<th>M</th>
<th>M (+A)</th>
<th>A (+M)</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Time</td>
<td>70.1</td>
<td>&lt;.0001</td>
<td>235.55</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Nform</td>
<td>2.19</td>
<td>0.1457</td>
<td>6.54</td>
<td>0.0138</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.66</td>
<td>0.1895</td>
<td>6.13</td>
<td>0.0013</td>
</tr>
<tr>
<td>Time*Nform</td>
<td>0.75</td>
<td>0.4782</td>
<td>3.79</td>
<td>0.0297</td>
</tr>
<tr>
<td>Time*Ratio</td>
<td>1.54</td>
<td>0.1884</td>
<td>2.6</td>
<td>0.0295</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>2.05</td>
<td>0.1203</td>
<td>5.51</td>
<td>0.0025</td>
</tr>
<tr>
<td>Time<em>Nform</em>Ratio</td>
<td>1.39</td>
<td>0.2379</td>
<td>2.66</td>
<td>0.0264</td>
</tr>
</tbody>
</table>

### 3b. 3-Way ANOVA on natural Missisquoi Bay Community

<table>
<thead>
<tr>
<th>Tests</th>
<th>M</th>
<th>A</th>
<th>Aph</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
</tr>
<tr>
<td>Time</td>
<td>25</td>
<td>&lt;.0001</td>
<td>31.24</td>
</tr>
<tr>
<td>Nform</td>
<td>1.12</td>
<td>0.2947</td>
<td>3.3</td>
</tr>
<tr>
<td>Ratio</td>
<td>3.66</td>
<td>0.0188</td>
<td>2.78</td>
</tr>
<tr>
<td>Time*Nform</td>
<td>0.95</td>
<td>0.3955</td>
<td>1.35</td>
</tr>
<tr>
<td>Time*Ratio</td>
<td>1.59</td>
<td>0.1712</td>
<td>0.32</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>2.44</td>
<td>0.0759</td>
<td>0.72</td>
</tr>
<tr>
<td>Time<em>Nform</em>Ratio</td>
<td>0.88</td>
<td>0.5162</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Table 4 a & b. Two-way ANOVA results from axenic culture and Missisquoi Bay experiments, respectively. All F-statistics and p-values reported represent type III sums of squares. Values in bold are statistically significant at a significance level of 0.1.

4a.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Time</th>
<th>M</th>
<th>M (+A)</th>
<th>A (+M)</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Nform</td>
<td></td>
<td>1.1</td>
<td>0.3132</td>
<td>7.82</td>
<td>0.0129</td>
</tr>
<tr>
<td>Ratio</td>
<td>t1</td>
<td>0.4</td>
<td>0.7546</td>
<td>1.4</td>
<td>0.2798</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>t1</td>
<td>1.12</td>
<td>0.3759</td>
<td>0.54</td>
<td>0.6633</td>
</tr>
<tr>
<td>Nform</td>
<td></td>
<td>0.17</td>
<td>0.6588</td>
<td>0.03</td>
<td>0.873</td>
</tr>
<tr>
<td>Ratio</td>
<td>t3</td>
<td>1.3</td>
<td>0.31</td>
<td>12.13</td>
<td>0.0002</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>t3</td>
<td>1.9</td>
<td>0.1705</td>
<td>10.35</td>
<td>0.0005</td>
</tr>
<tr>
<td>Nform</td>
<td></td>
<td>1.52</td>
<td>0.2352</td>
<td>4.46</td>
<td>0.0518</td>
</tr>
<tr>
<td>Ratio</td>
<td>t6</td>
<td>1.94</td>
<td>0.1632</td>
<td>3.54</td>
<td>0.0406</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>t6</td>
<td>1.84</td>
<td>0.1805</td>
<td>3.43</td>
<td>0.0444</td>
</tr>
</tbody>
</table>

4b.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Time</th>
<th>M</th>
<th>A</th>
<th>Aph</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
</tr>
<tr>
<td>Nform</td>
<td></td>
<td>1.52</td>
<td>0.2352</td>
<td>5.38</td>
</tr>
<tr>
<td>Ratio</td>
<td>t1</td>
<td>3.38</td>
<td>0.0445</td>
<td>1.65</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>t1</td>
<td>2.47</td>
<td>0.0992</td>
<td>0.7</td>
</tr>
<tr>
<td>Nform</td>
<td></td>
<td>1.04</td>
<td>0.3233</td>
<td>0.21</td>
</tr>
<tr>
<td>Ratio</td>
<td>t3</td>
<td>0.17</td>
<td>0.9149</td>
<td>1.05</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>t3</td>
<td>5.14</td>
<td>0.0092</td>
<td>0.86</td>
</tr>
<tr>
<td>Nform</td>
<td></td>
<td>0.59</td>
<td>0.454</td>
<td>0.11</td>
</tr>
<tr>
<td>Ratio</td>
<td>t6</td>
<td>1.3</td>
<td>0.3049</td>
<td>0.7</td>
</tr>
<tr>
<td>Nform*Ratio</td>
<td>t6</td>
<td>0.18</td>
<td>0.9059</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Figure 3. Experimental design for laboratory culture experiments with eight treatment media. The “0 Day” replicates were harvested immediately after inoculation to estimate initial cell densities.

<table>
<thead>
<tr>
<th>Time</th>
<th>NH₄ 5:1</th>
<th>NH₄ 15:1</th>
<th>NH₄ 30:1</th>
<th>NH₄ 45:1</th>
<th>NO₃ 5:1</th>
<th>NO₃ 15:1</th>
<th>NO₃ 30:1</th>
<th>NO₃ 45:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
</tr>
<tr>
<td>3 Days</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
</tr>
<tr>
<td>6 Days</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
<td>BG-11</td>
</tr>
</tbody>
</table>

Figure 4. Average *M. aeruginosa* growth across time by N:P ratios in the individual *M. aeruginosa* experiment.
Figure 5. Average *A. flos-aquae* growth across time by N:P ratio in the individual *A. flos-aquae* experiment.

Figure 6 a, b & c. Average *M. aeruginosa* growth after one, three & six days, respectively, in the mixed axenic culture experiment. Starred values represent significant differences between cell growth in nitrate and ammonium treatments.

6a.
Figure 7a, b & c. Average *A. flos-aquae* growth after one, three & six days, respectively, in the mixed axenic culture experiment.

7a.

![Average *A. flos-aquae* Growth After One Day in the Mixed Axenic Culture Community](image)

7b.

![Average *A. flos-aquae* Growth After Three Days in the Mixed Axenic Culture Community](image)
Figure 8. Average *M. aeruginosa* growth in the Missisquoi Bay community over time by nitrogen form.
Figure 9 a & b. Average *A. flos-aquae* growth in the Missisquoi Bay community over time by nitrogen form and N:P ratio, respectively.

9a.

![Graph showing average growth by time and nitrogen form](image)

9b.

![Graph showing average growth by time and N:P ratio](image)
Figure 10. Average *Aphanizomenon* growth in the Missisquoi Bay community over time by nitrogen form.

![Average Aphanizomenon Growth by Time & Nitrogen Form in a Missisquoi Bay Community](image)

Figure 11. Microcystin concentration as a function of cell density. Microcystin concentration is strongly correlated with cell density of microcystin-producing cells. “MB” indicates the Missisquoi Bay experiment, while “M” and “MA” represent the axenic culture experiments of *M. aeruginosa* independently and mixed with *A. flos-aquae*, respectively.

![Variation Between [Microcystin] and Cell Density of Microcystin-producing Cells](image)
Figure 12 a & b. Ammonium-nitrogen and nitrate-nitrogen concentrations, respectively, in Missisquoi Bay in 2006. Benthic samples are reported separately for the two methods used in collection of the samples: a modified syringe method and a Glew gravity corer. Samples collected at the surface of the water column are also reported.

12a.

![Missisquoi Bay Ammonium-Nitrogen 2006](chart1)

12b.

![Missisquoi Bay Nitrate-Nitrogen 2006](chart2)
Figure 13. Average cell densities of *M. aeruginosa* (*M*), *Anabaena flos-aquae* (*A*), and *Aphanizomenon* (*Aph*) from duplicate samples collected at varied distances above the sediment-water interface in Missisquoi Bay on September 11, 2006 at 10:15pm.
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