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Effects of nutrients, temperature, and zooplankton grazing on toxic and non-toxic strains of the harmful cyanobacterium *Microcystis* spp.

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Timothy Walter Davis

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree,
hereby recommend acceptance of this dissertation

Christopher J. Gobler, Dissertation Advisor
Associate Professor, School of Marine and Atmospheric Sciences

Nicholas S. Fisher, Chairperson of Defense
Professor, School of Marine and Atmospheric Sciences

Darcy J. Lonsdale
Professor, School of Marine and Atmospheric Sciences

Jackie L. Collier
Assistant Professor, School of Marine and Atmospheric Sciences

Gregory L. Boyer
Professor, Department of Chemistry
State University of New York
College of Environmental Science and Forestry

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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In temperate latitudes, toxic cyanobacteria blooms are commonly formed by *Microcystis*, a well-known producer of the hepatotoxin, microcystin. One complexity in the study of *Microcystis* blooms has been the co-existence of toxic and non-toxic strains that are morphologically and microscopically indistinguishable. However, recent molecular advances have facilitated the discrimination between these strains as toxic *Microcystis* cells possess a suite of microcystin synthetase genes (*mcyA* - *mcyJ*), while non-toxic strains do not. I strove to better understand the ecology of these strains by examining the effects of nutrients, temperature, and zooplankton grazing on toxic and non-toxic *Microcystis* via quantification of the microcystin synthetase gene (*mcyD;* an indicator of toxic *Microcystis*) and the small subunit ribosomal RNA gene, 16S (an indicator of total *Microcystis*). I found that the molecular quantification of toxic (*mcyD*-possessing) *Microcystis* was a better predictor of *in situ* microcystin levels than total cyanobacteria, total *Microcystis*, chlorophyll *a*, or other factors, being significantly correlated with the toxin in every ecosystem studied ($n = 5$). Regarding the effects of nitrogen (N) and phosphorus (P) on *Microcystis* blooms, I found that first, *Microcystis* blooms displayed flexibility with regard to N assimilation being able to utilize both inorganic and organic forms of N. Second, toxic and non-toxic strains were promoted by

different forms of nitrogen with nitrate and ammonium stimulating the growth of toxic strains and potentially supporting more toxic blooms. Third, P concentrations played a key role in determining whether toxic or non-toxic strains of *Microcystis* dominated and thus may also influence the toxicity of blooms. The earth's temperatures have risen measurably during the past century and are expected to continute to rise through this century. Experimentally enhanced temperatures yielded consistently higher growth rates of toxic *Microcystis*, but did so for non-toxic *Microcystis* less frequently. This finding suggested that elevated temperatures may yield more toxic *Microcystis* cells and/or cells with more *mcyD* copies per cell, with either scenario potentially yielding more toxic blooms. Concurrent increases in temperature and P concentrations often yielded growth rates for toxic *Microcystis* cells which exceeded either individual treatment suggesting that future P-loading and climatic warming may additively promote toxic *Microcystis* blooms. Furthermore, my research showed that natural populations of microzooplankton and mesozooplankton were able to graze on both toxic and non-toxic strains of *Microcystis* with equal success suggesting that the ability to produce microcystin does not provide a defense from grazing. Finally, I observed that natural mesozooplankton communities were able to graze on *Microcystis* more consistently than cultured mesozooplankton suggesting that zooplankton reared in bloom environments have developed a tolerance to these toxic cyanobacteria. In summary, this dissertation has provided significant new insight into the ecology of toxic and non-toxic strains of the harmful cyanobacterium *Microcystis*.

To my wife, Lindsay, my mother and sister

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CHAPTER ONE:

Dissertation introduction

Introduction

Harmful algal blooms (HABs) are a global phenomenon (Hallegraeff, 1993). Studies have shown that the frequency and intensity of these events have increased over the past few decades (Hallegraeff, 1993; Chorus & Bartrm, 1999; Anderson et al., 2002) as has the global economic loss and the cost associated with HABs has also increased (Van Dolah et al., 2001, Anderson et al., 2002, Hoagland et al., 2002). Understanding the mechanisms responsible for the expansion of these HABs has been a multi-decadal undertaking. To date, some of the potential factors driving this global expansion include increased nutrient inputs, the transport of cells or cysts via natural events or anthropogenic activities such as ballast water transport or shellfish seeding, as well as overfishing and increased aquaculture both of which have the potential to alter food webs and allow HAB species to dominate algal communities (GEOHAB, 2001; HARRNESS, 2005; Heisler et al., 2008). In addition, an increase in surface water temperatures due to a changing global climate could play a role in the further proliferation of these HABs (Peperzak, 2003; Paerl & Huisman, 2008; Paul, 2008). An improved ability to detect and monitor HABs as well as increased scientific and public awareness of these events has also lead to more such events being recorded (GEOHAB, 2001; HARRNESS, 2005). Importantly, a consensus has emerged that the proliferation and ecology of these HABs is very complex and typically not caused by a single environmental driver but a number of them occurring simultaneously (Heisler et al., 2008).

Cyanobacteria are a large and diverse group of prokaryotic photosynthetic organisms, which can be found in marine, freshwater, and terrestrial environments. Scientists have recognized the occurrence of harmful cyanobacterial blooms for more

than a century (Francis, 1878). Cyanobacteria blooms in freshwater systems are broadly associated with eutrophic and poorly flushed waters (high residence time; Paerl, 1988, 2001; Philipp et al., 1991; Carmichael, 1994; Rapala, 1997; Oliver & Ganf, 2000). While it is clear that the occurrence of toxic cyanobacteria blooms around the world have increased during recent decades (Chorus & Bartram, 1999; Hudnell & Dortch, 2008), the underlying causes of such blooms and their increased frequency are poorly understood.

Blooms formed by toxic cyanobacteria can include the genera *Anabaena*, *Aphanizomenon*, *Nodularia*, *Oscillatoria*, and *Microcystis* (Carmichael, 1994; Chorus & Bartram, 1999; Fleming et al., 2001). Cyanotoxins associated with these genera fall into two broad categories. Microcystins and cylindrospermopsins are cyclic peptides (hepatotoxins) which inhibit eukaryotic protein serine/threonine phosphatases (Honkanen et al., 1990; Chorus & Bartram, 1999). Anatoxin-a, anatoxin-a(s), and saxitoxins are three types of neurotoxins which block neuronal signal transmission and can lead to the paralysis of the heart and lungs resulting in rapid death in terrestrial vertebrates (Duy et al., 2000; Briand et al., 2003).

Of all the known cyanotoxins, microcystins are the most widespread and common. To date, over 80 microcystin varients have been identified (Hudnell & Dortch, 2008). They are produced in the majority of blooms formed by *Microcystis* spp., but also can be produced by *Anabaena* spp., *Oscillatoria*/*Planktothrix* spp., and *Nodularia* spp. (Chorus & Bartram, 1999). Microcystin toxicity can accumulate within the liver of vertebrates causing chronic liver damage (Fitzgeorge et al., 1994; Falconer et al., 1988). They bind irreversibly to protein phosphatases 1 and 2A which play a key role in maintaining homeostasis in plant and mammal cells (Cohen, 1989; Chorus & Bartram,

1999). The inhibition of these compounds can lead to increased phosphoralation of tumor suppressor proteins which can result in increased signaling and promote cell proliferation, transformation, and tumor promotion (Fujikia & Suganuma, 1993; Chorus & Bartram, 1999). Although the mechanism of toxicity towards in vertebrates has been discovered (see above), the biological function of microcystin has yet to be elucidated. Multiple hypotheses have been put forth regartding the function of microcystin in cyanobacteria including cell signaling (Schatz et al., 2007), iron transport (Lukač & Aegerter, 1993), deterrence of zooplankton grazing (Jungmnn & Benndorf, 1994), and allelopathic deterrence of competing phytoplankton (Vardi et al., 2002).

Human interaction with cyanotoxins is primarily from drinking water; however recreational activities such as swimming or boating can also lead to exposure (Turner et al., 1990; Carmichael, 1994; Falconer, 1999). Although direct consumption of lake water in the United States is rare, a number of cases of cyanotoxin contamination of raw and treated drinking water have been reported within a number of municipalities (Boyer et al., 2004). Moreover, in developing nations, due to a lack of water treatment plants, raw lake water may be consumed. Symptoms of short term recreational exposure to microcystin can include vomiting, diarrhea, central abdominal pain, sore throats, and blistering of the lips (Turner et al., 1990). On the other hand, long term exposure to cyanotoxins can be associated with severe health effects. Worldwide, reports of human deaths, liver and colorectal cancers, neurological disorders, such as Alzhiemer's disease, and other illnesses have been associated with water contaminated with cyanotoxins (Falconer et al., 1988; Carmichael & Falconer, 1993; Bell & Codd, 1994; Carmichael, 1994; Chorus & Bartram, 1999; Zegura et al., 2003; Cox et al., 2005). For example, in 1994 and 1995 in

rural China, where residents depend on surface water contaminated with cyanotoxins for their drinking water, Primary Liver Cancer (PLC) was one of the most common cancers reported in those regions (Yu, 1995). It is likely that the ingestion of microcystin along with aflatoxin exposure from corn and the hepititus B virus acted to initiate and promote PLC (Yu, 1995).

More commonly reported, especially in countries that do not rely on surface water for their primary source of drinking water (e.g. the United States), are animal deaths relating to microcystin poisoning. The first report of animal poisonings due to cyanotoxins was in Australia in 1878 when multiple farm animals died after ingesting water from a lake contaminated with a bloom of *Nodularia spumigena* (Francis, 1878) Over the past 130 years, cyanotoxin poisonings have been reported worldwide in animals of various sizes ranging from ducks to rhinoceros (Carmichael, 1992). However, in the United States most reports of cyanotoxin poisonings involve water fowl, cattle, and domestic pets which are exposed to cyanotoxins by ingestion of contaminated water. For example, there have been more than five reported cases of canine death due to the ingestion of cyanobacterial scum on the shores of Lake Champlain this decade (Rosen et al., 2001; Rolland et al., 2005). Even, as recently as 2009, two canine deaths from liver and kidney failure attributed to ingestion of *Microcystis* bloom water occurred on the shores of the Transquaking River located on Maryland's eastern shore (C. Orano-Dawson, Maryland Department of Natural Resources, pers. comm.).

Cyanotoxins such as microcystin and anatoxin are generally more harmful to mammals and birds then they are to aquatic animals (Oberholster et al., 2006). However, fish deaths during multiple cyanobacterial blooms have been reported (Davidson, 1959;

Koon, 1960; Ochumba, 1990; Sevrin-Reyssac & Pletikosic, 1990). A study conducted in the United Kingdom suggested that damage to gills, digestive tract, and liver were responsible for fish kills during cyanobacteria blooms (Rodger et al., 1994; Oberholster et al., 2006). Moreover, recent findings suggest cyanotoxins accumulate throughout the food web and within in fish (Ibelings et al., 2005).

In addition to high concentrations of potentially lethal toxins, blooms of cyanobacteria can have numerous other negative impacts on aquatic environments. Dense blooms can cause a dramatic decrease in light penetration which can shade out benthic aquatic plants (Paerl et al., 2001). The demise of dense cyanobacteria blooms can rapidly increase bacterial respiration. I observed this phenomenon in Lake Agawam, NY in 2006 as well as in Mill Pond, NY in 2008 when dense cyanobacteria blooms ended abruptly, dissolved oxygen levels decreased to hypoxic and anoxic levels and thousands of fish died while others were "gulping" for air at the surface. Toxic cyanobacteria blooms can also alter the structure of the food web. Research has demonstrated that cyanobacteria are often not readily grazed upon by zooplankton (Paerl et al., 2001; Gobler et al., 2007). Whether it is because of the toxin synthesis, colony size or shape, or poor nutritional value, cyanobacteria do not seem to be an optimal pray item for many zooplankton (Paerl et al., 2001; Wilson et al., 2006). This shift in the structure of the base of the food web can disrupt the flow of energy and carbon to the higher trophic levels (Paerl et al., 2001). Lastly, another negative, yet probably less detrimental, impact these blooms can have on an environment are the foul odors that can be associated with them (Chorus & Bartram, 1999; Carmichael, 2001; Paerl et al., 2001). Since many cyanobacteria have the ability to control their buoyancy and congregate in surface waters,

during high wind events they can be carried to the leeward (or downwind) shore of lakes where an algal scum line can occur (Chorus & Bartram, 1999). These cells eventually die and begin to get broken down creating noxious and sulfidic odors (Chorus & Bartram, 1999; Carmichael, 2001; Paerl et al., 2001).

Toxic and non-toxic strains of cyanobacteria

One complexity in the study of cyanobacteria blooms in the field has been the existence and often co-existence of toxic and non-toxic strains of the same species which are morphologically and taxonomically indistinguishable. Nearly every major species of toxic cyanobacteria has both toxic and non-toxic strains (Chorus & Bartram, 1999). Historically, scientists have used light microscopy to determine the density of cyanobacteria. While this technique is adequate for determining overall population density, it is insufficient for determining the densities of the toxic and non-toxic subpopulations that comprise the overall population. Advances in molecular biology such as polymerase chain reaction (PCR), and the ability to quantify specific DNA sequences using quantitative polymerase chain reaction (qPCR) have provided the means to study these potentially harmful blooms in greater detail (Scholin, 1998; Coyne et al., 2001; Popels, 2003). At present, the dynamics of toxic and non-toxic strains of cyanobacteria blooms are poorly understood (Rapala & Sivonen, 1998). The variation in toxicity of blooms is probably influenced by environmental conditions, the presence of toxic or non-toxic cells, the physiological condition of the cells and the overall plankton community composition (Vézie et al., 2002). Blooms of microcystin producing cyanobacteria are usually comprised of toxic and non-toxic strains (Ohtake et al., 1989; Vézie et al., 1998; Welker et al., 2003; Welker et al., 2007; Kardinaal et al., 2007).

Recent molecular advances have identified the genes responsible for the cellular synthesis of the hepatotoxin microcystin (the *mcy* genes (A-J); Moore et al., 1991; Arment & Carmichael, 1996; Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004) have allowed for the detection of toxic and non-toxic cyanobacteria species as only toxic strains of microcystin producing genera contain the mcy gene cluster (Rinta-Kanto et al., 2005). For my dissertation, I quantifed the the *mcyD* gene as a marker of the toxic (microcystin-synthesizing) *Microcystis* subpopulation. The *mcyD* gene is involved with the formation of the ADDA (3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-diene acid) moiety (Fig 1), the functional portion of the microcystin molecule, which causes the inhibition of the protein phosphatases 1 and 2A. Since previous laboratory studies have revealed important ecological differences among these strains beyond toxin content (Sivonen, 1990; Rapala et al., 1997; Vezie et al., 2002), the recently developed ability to quantify toxic and non-toxic strains of *Microcystis* opens the door for ecological field studies of these populations during blooms events.

Nutrients

Of all of the potential environmental drivers behind HABs, the one that has received the most attention among the global scientific community has been the increase in nutrient pollution of various marine, estuarine, and freshwater ecosystems. Research indicates that cultural eutrophication associated with the increased global human population has stimulated the occurrences of HABs (Anderson et al., 1989; Hallegraeff, 1993; Burkholder, 1998; Glibert et al., 2005; Glibert & Burkholder, 2006). Sewage treatment plants, fertilizers, increased fossil fuel burning (a source of atmospheric nitrogen), storm drain runoff, and septic tanks are all sources of external nutrients to

aquatic ecosystems (Puckett, 1994; Mueller & Helsel, 1996; Carpenter et al., 1998). As bodies of freshwater become enriched in nutrients, especially phosphorus, there is often a shift in the phytoplankton community towards dominance by cyanobacteria (Smith, 1986; Trimbee & Prepas, 1987; Watson et al., 1997; Paerl & Huisman, 2008). Examples of these changes are the dense blooms often found in newly eutrophied lakes, reservoirs, and rivers previously void of these events (Fogg, 1969; Renoylds & Walsby, 1975; Renolds, 1987; Paerl, 1988b; Paerl, 1997). Empirical models predict that summer phytoplankton communities will be potentially dominated by cyanobacteria at total phosphorus (TP) concentrations of ~100-1000 μg L^{-1} (Trimbee & Prepas, 1987; Jensen et al., 1994; Watson et al., 1997; Downing et al., 2001). Higher phosphorus levels have been shown to yield higher microcystin levels per cell in *Anabaena* (Rapala et al., 1997). Also, some studies have found that an increase in phosphorus concentrations can lead to an increase in microcystin to dry weight ratio (Utkilen & Gjølme,1995). The only field work conducted relating the toxicity of cyanobacteria blooms to nutrients have been correlative field studies which found microcystin to be both positively and negatively correlated with various P pools (Wicks & Theil, 1990; Kotak et al., 1995; Lahti et al., 1997). No study has directly examined the role of P in the growth and toxicity of wild cyanobacteria populations.

There is evidence to suggest that nitrogen (N) may be equally or more important in the occurrence of toxic cyanobacteria blooms, particularly in non-diazotrophic cyanobacteria such as *Microcystis* spp. The sum of laboratory evidence indicates that increasing N loads will increase the growth and toxicity of non-diazotrophic cyanobacteria such as *Microcystis* spp. and *Oscillatoria agardhii* (Watanabe & Oishi,

1985; Codd & Poon, 1988; Orr & Jones, 1998). Laboratory studies have shown that an increase in nitrogen can also lead to an increase in cellular microcystin to dry weight ratio in *Microcystis* spp. (Utkilen & Gjølme, 1995). By contrast, N-fixing, toxic cyanobacteria such as *Anabaena* may actually experience depressed toxicity under increasing N loads (Rapala et al., 1997). Importantly, non-toxic strains of *Microcystis* as well as *Anabaena* require lower nutrient concentrations to achieve maximal growth rates compared to toxic strains (Sivonen, 1990; Rapala et al., 1997; Vezie et al., 2002). Moreover, previous laboratory research shows toxic strains of *Microcystis* are able to outgrow non-toxic strains at high nitrogen levels (Vézie et al., 2002). These results suggest toxic strains of cyanobacteria could dominate bloom events in eutrophic environments and that continued N-loading in coastal ecosystems may foster more toxic cyanobacteria blooms in the future.

DON is the largest pool of nitrogen in many aquatic ecosystems (Bronk, 2002) even in oligotrophic systems where nitrogen is limiting the primary production which led to the paradigm that this pool of nutrients is refractory and is not a source of nutrition to phytoplankton. Furthermore, it was thought that the bacterial populations thrived on DON and did so to the exclusion of phytoplankton (Bronk et al., 2007). Recent studies have shown that DON can be classified into three categories. The truly refractory compounds, such as humics, that can remain in the ecosystems anywhere from several months to centuries (as reviewed in Bronk, 2002). The semi-labile compounds (Carlson & Ducklow, 1995) such as proteins, dissolved combined amino acids, and amino polysaccharides that have turnover times ranging from hours to days. The final category is comprised of the labile compounds which include dissolved free amino acids (DFAA),

nucleic acids, as well as urea. These compounds can have very fast turnover times that range from minutes (DFAA; Fuhrman, 1987) to days (nucleic acids and urea; Jørgensen et al., 1993; Bronk et al., 1998).

Since recent research has pointed to the importance of DON as a source of nitrogen for primary producers, a greater understanding of how these compounds can affect phytoplankton species composition is important. Seitzinger & Sanders (1997) estimated that up to 90% of nitrogen entering coastal zones from rivers was in the form of DON. Therefore, phytoplankton that have developed the ability to directly assimilate DON may have an advantage over other species of phytoplankton especially when concentrations of dissolved inorganic nitrogen are low. Since neither the ratio nor quantity of inorganic nitrogen alone can sufficiently explain the extended duration of dense HAB blooms (Vargo et al., 2004; Heisler et al., 2008), research of these events must consider the impacts of all species of N on these events.

Previous research has shown that many genera of HABs, including dinoflagellates and pelagophytes (Burkholder & Glasgow, 1997; Granéli et al., 1997, 1999; Berg et al., 1997, 2002; Lewitus et al., 1999; Stoecker, 1999; Kudela & Cochlan, 2000; Berman, 2001; Glibert et al., 2001, 2006a,b, 2007; Lomas et al., 2001, 2004; Mulholland et al., 2002, 2004; Gobler et al., 2005; Glibert & Legrand, 2006; Lewitus, 2006; Herndon & Cochlan, 2007; Kudela et al., 2008; Cochlan et al., 2008), as well as cyanobacteria (Paerl, 1988; Paerl & Millie, 1996; Pinckney et al., 1997; Berman & Chava, 1999), can utilize various forms of dissolved and particulate organic nitrogen. Among the harmful cyanobacteria two field studies conducted by Takamura et al. (1987) and Presing et al. (2008) used ^{15}N-labeled nitrogenous compounds and found that *Microcystis* was able to

take up nitrate, ammonium, and urea as N sources. Berman & Chava (1999) found that non-axenic cultured *Microcystis aeruginosa* consistently grew better using urea as a nitrogen source than either nitrate or ammonium but did not grow well on other forms of organic N (i.e. amino acids). On the other hand, Dai et al. (2009) found that a clone of *Microcystis aeruginosa* was able to take up and utilize DFAA, such as alanine, leucine, and arginine to support growth and toxin production. However, they also found that other DFAA such as glutamic acid, aspartic acid, and lysine, were unsuitable for *M. aeruginosa* to support growth or microcystin production even though the cells were able to take up these compounds rapidly. Since both inorganic and organic forms of N can be used by cyanobacterial HABs, it is important to understand how increase in various forms of N (nitrate, ammonium, L-glutamine, and urea) will influence the dynamics of toxic and non-toxic strains of *Microcystis* during natural bloom events.

Several studies have indicated that a seasonal succession of *Microcystis* populations from toxic to non-toxic cells occurs during the duration of the bloom event (Fastner et al., 2001; Welker et al., 2007). Often field populations are dominated by non-toxic strains of *Microcystis* during the height of the bloom event (Welker et al., 2003; Welker et al., 2007; Kardinaal et al., 2007). Since nutrient levels will be drawn down at the peak of a bloom, non-toxic strains might be better competitors for nutrients at low concentrations due to intracellular differences between the two strains which allow the non-toxic strains to more efficiently scavenge for nutrients (Vézie et al., 2002). The process of producing the microcystin compound is very complex and requires the use of a multi-enzyme complex and many atoms of nitrogen (Nishizawa et al., 1999; Nishizawa et al., 2000; Tillett et al., 2000). This would suggest that the nutrient quota for toxic strains

of *Microcystis* is significantly higher then that of their non-toxic counterparts (Vézie et al., 2002), thus favoring the non-toxic strains at times of low nutrient concentrations. During recent decades, anthropogenic nutrient loading and occurrences of *Microcystis* blooms have increased worldwide. However, comprehensive field studies examining the direct impact of nitrogen on the growth of toxic and non-toxic cyanobacteria during bloom events have not been conducted.

Temperature

The earth's temperature has increased by 0.6 + 0.2^{o}C during the 20th century, with most of the increase having occurred during the last 40 years (IPCC, 2001). In the current century, global air temperatures are expected to increase between 1.5^{o}C and 5^{o}C and could also lead to an increase in ocean surface water temperature of 1^{o}C to 7^{o}C (Houghton et al., 2001). Changes in temperature could alter phytoplankton growth rates (Eppley, 1972; Goldman & Carpenter, 1974). A study conducted by Canale & Vogel (1974) showed that with increasing temperatures the highest growth rates for general groups of phytoplankton shifts from diatoms to green algae to cyanobacteria. Fu et al. (2007) found that the effects of increasing temperatures had opposite effects on two different species of marine picocyanobacteria (*Synechococcus* and *Prochlorococcus*). Their results showed an increase in the growth rate for *Synechococcus* with an increase of 4^{o}C above ambient (20^{o}C) temperature while *Prochlorococcus* showed no significant increase in its growth rate (Fu et al., 2007). Another study conducted correlated *Microcystis* rates of photosynthesis to temperature (overall range of 2.6 - 31.2^{o}C; Takamura et al., 1985). Harmful cyanobacteria such as *Microcystis* have been found to have an optimal temperature for growth and photosynthesis rates at temperatures around

25°C (Robarts & Zohary, 1987). Moreover, the cellular toxin content of multiple genera of cyanobacteria increases with increasing temperature to a maximum which occurs at ~ 25°C (Van der Westhuizen & Eloff, 1985; Codd & Poon, 1988; Sivonen, 1990; Rapala et al., 1997). Other studies have found that cyanobacteria dominate phytoplankton assemblages in temperate freshwater environments during the warmest periods of the year, particularly in eutrophic systems (Paerl, 1988, 2001; Paerl & Huisman 2008; Paul, 2008). Although prior studies have examined the effects of increased temperatures on cyanobacterial growth rates and photosynthesis rates for general groups of phytoplankton and some specific species, there have been no field studies conducted to evaluate how the toxic and non-toxic strains of *Microcystis* are affected during blooms.

Zooplankton Grazing

Mesozooplankton

Zooplankton are a critical link in transferring energy and carbon from phytoplankton to higher trophic levels. The interaction between zooplankton and cyanobacteria is a crucial aspect to understanding and effectively managing these harmful cyanobacterial blooms as they may act as a top down control on algal blooms or may allow blooms by failing to graze (Paerl, 1988; DeMott & Moxter, 1991; Demott et al., 2001; Gobler et al., 2002; Sarnelle, 2007; Hudnell & Dortch, 2008). It has been reported that many species of cyanobacteria (Porter & Orcutt, 1980; Holm & Shapiro, 1984; Sellner et al., 1994; Camacho & Thacker, 2006), including *Microcystis* (Lampert, 1981, 1982; Fulton & Paerl, 1987 a,b, 1988 a,b; Gobler et al., 2007), are not readily grazed by mesozooplankton. However, the mechanism by which grazing is disrupted is unknown. To date, hypotheses which have been put forward have included colony morphology,

toxin-producing abilities, or the absence of essential nutritional compounds in cyanobacteria that zooplankton need to survive (Porter & Orcutt, 1980; Lampert, 1987; DeMott, 1989; Wilson et al., 2006). However, drawing a comprehensive conclusion as to why zooplankton do not readily graze on cyanobacteria has proven difficult. There have been experiments conducted where zooplankton were given a diet that either did or did not contain cyanobacteria (Arnold, 1971; Porter & Orcutt, 1980), but these studies were largely inconclusive since they did not account for the size and shape of the colony or the ability to produce toxins (Wilson et al., 2006). Other studies have focused on one species with different morphologies to assess these impacts on zooplankton grazing, but contradictory results have been obtained. In one study, it was found that the zooplankton grazed at a greater rate on the larger colonies (Chan et al., 2004) where another study found the opposite result (Hartmann, 1985). Some studies have focused on the effects of dissolved toxins on grazing ability. Jungmann & Benndorf (1994) found that *Daphina* were only affected by dissolved microcystin at concentrations several orders of magnitude higher than environmentally realistic concentrations. However, zooplankton usually encounter toxins via grazing cells containing toxin rather than in the dissolved state (Chorus & Bartram, 1999). Finally, other studies have examined differences in grazing on a microcystin-producing wild-type and non-toxic mutants of the same species (Rohrlack et al., 1999; Kaebernick et al., 2001; Lűrling, 2003), but these studies have been inconclusive in determining whether microcystin can act as a grazing deterrent as similar growth rates were found for populations of zooplankton fed either the toxic strain or the mutant non-toxic strain of *Microcystis* (Lűrling, 2003). Furthermore previous research has found that populations of the cladoceran *Daphnia galeata* exposed to dense

cyanobacteria blooms in Lake Constance were more resistant to *Microcystis* than populations reared under non-bloom conditions suggesting a genetic shift occurs in wild zooplankton populations towards strains able to actively graze toxic cyanobacteria such as *Microcystis* (Hairston et al., 1999, 2001). To date, the ability of mesozooplankton grazers to consume toxic or non-toxic *Microcystis* cells during natural bloom events are unknown.

Microzooplankton

Although toxic cyanobacteria blooms substantially reduce grazing rates in some mesozooplankton, these events do not seem to have the same negative impact on microzooplankton (Nishibe, 2002; Kim et al., 2006; Gobler et al., 2007). Microzooplankton are known to be the primary source of mortality for phytoplankton in aquatic ecosystems (Calbet & Landry, 2004), and microzooplankton densities have been shown to be well-correlated with densities of toxic cyanobacteria in the Potomac River of Chesapeake Bay (Lacouture et al., 1993). Since it has been suggested that microzooplankton may graze on toxic cyanobacteria (Dryden & Wright, 1987; Paerl et al., 2001), microzooplankton may act as an important trophic link between mesozooplankton and toxic cyanobacteria during bloom events. To date, community microzooplankton grazing rates on toxic and non-toxic *Microcystis* have yet to be quantified. Previous research has evaluated species specific grazing rates of microzooplankton on *Microcystis* spp. A study conducted by Nishibe et al. (2002), found that the flagellate, *Collodictyon triciliatum*, was able to actively graze upon *Microcystis* cells as both single cells and small colonies were found in its food vacuole. However, for this particular flagellate, the grazing rate was low (Nishibe et al., 2002). Kim et al.

(2006) found that when the flagellate *Diphylleia viridis* was fed both toxic and non-toxic strains of *Microcystis* spp. as well as other species of phytoplankton such as *Chlorella*, *D. viridis* yielded the highest growth rate as well as the highest ingestion rate when fed the most toxic strain of *Microcystis*. These specific examples demonstrate the importance of determining grazing rates at a community level as individual species of microzooplankton will have varying grazing rates depending on their ability to tolerate *Microcystis* cells. There have been no prior field studies conducted to calculate a community microzooplankton grazing rate on specific, toxic and non-toxic strains of harmful cyanobacteria.

Preliminary results comparing microzooplankton grazing (via dilution experiments; Landry et al., 1995) and mesozooplankton grazing (via *Daphnia sp*. enrichment experiments) in Lake Agawam suggest that during intense toxic cyanobacteria blooms, mesozooplankton do not actively feed, while microzooplankton continue to graze at a substantial rate (Gobler et al., 2007). This preliminary finding correlates well to other studies that have evaluated the ability of microzooplankton, primarily flagellates, to graze on cyanobacteria blooms of *Microcystis* spp. (Cole & Wynne, 1974; Sugiura et al., 1992; Klaveness, 1995; Zhang et al., 1996; Nishibe et al., 2002; Kim et al., 2006). However, my proposed methods should, for the first time, yield specific micozooplankton grazing rates on toxic and non-toxic *Microcystis* spp.

Objectives

An understanding of the conditions that favor the growth and proliferation of the toxic strains of *Microcystis* is crucial to the development of mitigation-focused management strategies for systems experiencing chronic *Microcystis* blooms. To date,

the dearth of investigations on this subject precludes the formation of conclusions regarding causes of toxic and non-toxic blooms. Factors which differentially regulate the dynamics of toxic and non-toxic strains of bloom-forming *Microcystis* are not well known. This dissertation represents a comprehensive ecological, molecular, and chemical investigation of the proliferation of toxic and non-toxic strains of *Microcystis* in multiple aquatic ecosystems. The objectives of my dissertation were as follows:

1. Establish the spatial and temporal dynamics of toxic and non-toxic *Microcystis* densities, within multiple ecosystems across the eastern United States.

2. Evaluate the impact of nitrogen and phosphorus containing nutrients on the growth of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms.

3. Evaluate the impact of temperature on growth of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms.

4. Quantify mesozooplankton and microzooplankton grazing rates on toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms.

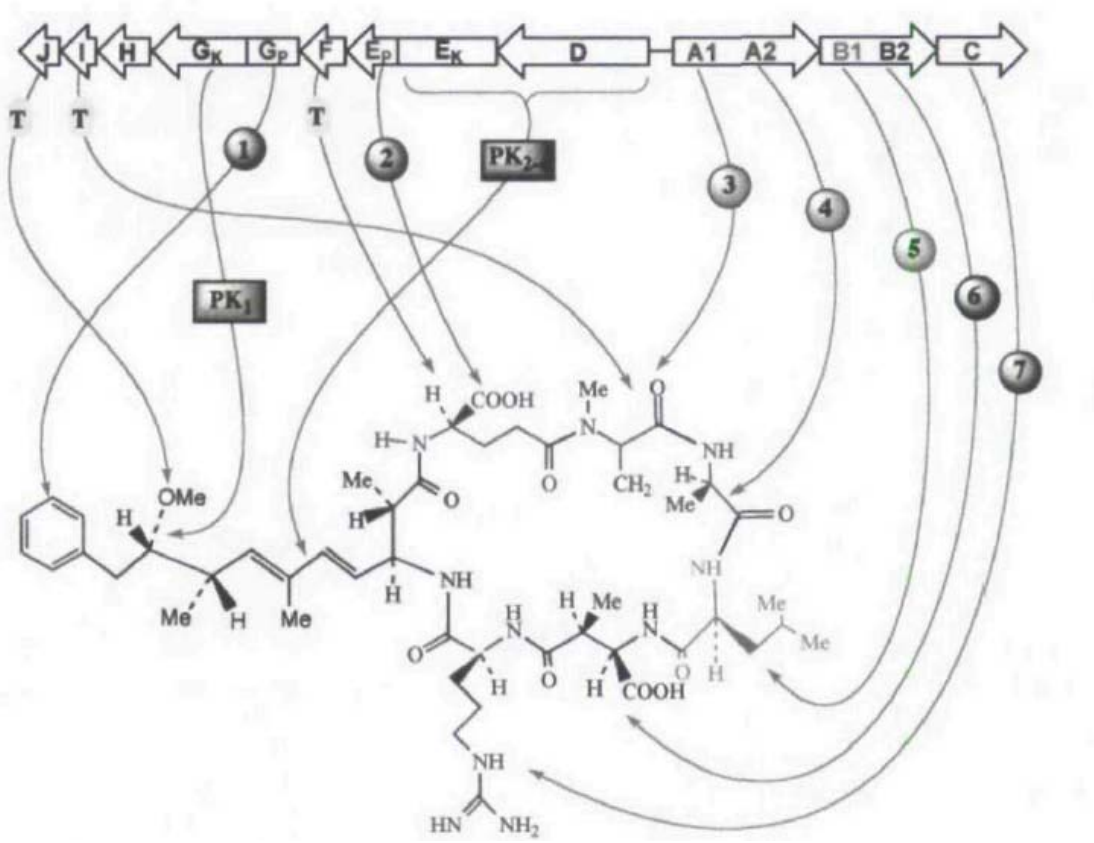

Figure 1: Proposed biosynthetic model for microcystin-LR, showing the organization of the gene clusters *mcyA-J* and microcystin (Kaebernick & Neilan, 2001). Numbered circles indicate the order of amino acids incorpordtcd into the growing peptide chain synthesized by NRPS genes (*mcyA. B, C, E_p, G_p*). Numbered rectangles show the order of polyketide synthesis in the formation of ADDA (*mcyG_k, E_k, D*). "T" indicates additional open reading frames (ORFs) of putative microcystin tailoring function. The relative sizes of the *mcy* ORFs have been approximated, with the entire gene cluster comprising some 55 kb. Figure from Kaebernick & Neilan, 2001.

CHAPTER TWO

Effects of nitrogenous compounds and phosphorus on the growth of toxic and non-toxic strains of *Microcystis* during cyanobacterial blooms

Abstract

Since the mid-twentieth century, both nutrient delivery rates and the frequency of harmful algal blooms (HABs) in coastal aquatic ecosystems have intensified. Recent studies have shown that nitrogen (N) or phosphorus (P) can limit primary production in some freshwater systems and the common freshwater HAB, *Microcystis,* is able to utilize both inorganic and organic forms of N. Here I present a field study during which I quantified microcystin synthetase genes (*mcyD*) and ribosomal RNA genes (16S) to assess how various N and P sources affected the growth of toxic and non-toxic strains of *Microcystis* during blooms while concurrently using ^{15}N isotopes to quantify N assimilation rates by *Microcystis*-dominated plankton communities. During the study, dense *Microcystis* blooms (> 10^7 cell equivalents L^{-1}) were observed within two contrasting ecosystems, a tidal tributary and a eutrophic lake. Overall, the toxic strains of *Microcystis* were stimulated by N or P enrichment more frequently than the non-toxic strains. However, when toxic strains of *Microcystis* were stimulated by N, it was due to inorganic N more frequently than organic N (89 and 33% of experiments, respectively). Conversely, when non-toxic strains were stimulated by N, it was more frequently by organic N compounds (all experiments) than inorganic N compounds (33% of experiments). Inorganic P stimulated toxic strains of *Microcystis* more frequently than non-toxic strains (40 and 17% of experiments, respectively) suggesting that toxic strains require more P than their non-toxic counterparts. Therefore, toxic and non-toxic strains of *Microcystis* were promoted by different forms of nutrients with inorganic N and P preferentially stimulating the growth of toxic strains and thus potentially supporting more toxic blooms. *Microcystis* displayed flexibility in N assimilation, as plankton

communities > 20 μm dominated by this genus (98 ± 4% of cells) obtained the majority of their N from either nitrate, ammonium, or urea on different dates with compound-specific uptake rates being correlated with ambient nutrient concentrations. This flexibility with regard N assimilation during blooms could partly account for the dominance of *Microcystis* in a variety of aquatic habitats.

Introduction

Harmful algal blooms (HABs) are a significant threat to fisheries, public health, and economies around the world. HABs are a recurrent problem in many areas of the US and evidence suggests that the frequency and distribution of HABs is increasing (Heisler et al., 2008). The average annual economic losses in the U.S. from HABs is approaching $100 million (Hoagland & Scatasta, 2006).

The mobilization of nitrogen (N) and phosphorus (P) into coastal zones has been one of the most recognizable impacts of man on the planet (Howarth & Paerl, 2008) and there are strong links between nutrients and HABs (Heisler et al., 2008; Anderson et al., 2008), particularly within freshwater ecosystems (Paerl, 1988, 2001). Traditionally, rates of primary production in freshwater ecosystems have been thought to be limited by phosphorus (Schindler, 1977; Smith, 1983; Hecky & Kilham, 1988) and increases in P-loading (mainly due to anthropogenic influences) has often been associated with eutrophic conditions within these systems (Likens, 1972; Paerl, 1988). This paradigm is partly based on the assumption that diazotrophic cyanobacteria such as *Anabaena* and *Aphanizomenon* tend to dominate P-enriched systems that are depleted in nitrogenous nutrients (Pearl, 1982, 1988).

Nitrogen may also play an important role in the occurrence of freshwater cyanobacteria blooms, particularly in non-diazotrophic cyanobacteria such as *Microcystis* spp. The sum of laboratory evidence indicates that increasing N loads will increase the growth and toxicity of non-diazotrophic cyanobacteria such as *Microcystis* spp. and *Oscillatoria agardhii* (Codd & Poon, 1988; Watanabe & Oishi, 1985; Orr & Jones, 1998). Laboratory studies have shown that an increase in N can also lead to an increase

in cellular microcystin to dry weight ratio in *Microcystis* spp. (Utkilen & Gjølme, 1995). By contrast, N-fixing, toxic cyanobacteria such as *Anabaena* may actually experience depressed toxicity under increasing N loads (Rapala et al., 1997). Furthermore, some freshwater systems which have either large external P supplies or are shallow and have strong benthic mobilization of P from sediments can host levels of dissolved N which limit primary production (Vollenweider & Kerekes, 1982; Paerl, 2009). Finally, recent ecosystems studies have demonstrated that N loading can promote *Microcystis* blooms (Gobler et al., 2007; Moisander et al., 2009).

Many cyanobacteria (both diazotrophic and non-diazotrophic) are able to utilize both organic and inorganic forms of N (Paerl, 1988). Cyanobacteria can form blooms in N-limited ecosystems when concentrations of DON and ammonium are elevated (Pinckney et al., 1997, 1998). Furthermore, a recent study conducted by Dai et al. (2009) found that a toxic clone *Microcystis aeruginosa* was able to take up and utilize some amino acids, such as alanine, leucine, and arginine, to support growth and toxin production. Studies examining ^{15}N assimilation by cyanobacteria blooms have observed uptake rates were highest for ammonium, followed by urea, then nitrate suggesting that reduced forms of N may promote cyanobacteria blooms (Takamura et al., 1987; Mitamura et al., 1995; Présing et al. 2008). Overall, these previous studies suggest both organic and inorganic N can be important in promoting cyanobacteria blooms.

Wild populations of *Microcysits* are comprised of toxic and non-toxic strains which are distinguishable only via quantification of the microcystin synthetase gene (*mcyA - J*; Kurmayer & Kutzenberger, 2003; Rinta-Kanto & Wilhelm, 2006; Yoshida et al., 2006; Davis et al., 2009; Ha et al., 2009). Previous laboratory studies have found that

non-toxic strains of *Microcystis* and *Anabaena* require lower nutrient concentrations to achieve maximal growth rates compared to toxic strains (Rapala et al., 1997; Vezie et al., 2002). Further laboratory research suggests toxic strains of *Microcystis* are able to outgrow non-toxic strains at high N levels (Vezie et al., 2002). While the role of nutrients in the growth of total *Microcystis* populations in culture and in the field has been studied (Watanabe & Oishi, 1985; Codd & Poon, 1988; Blomqvist et al., 1994; Fujimoto et al., 1997; Orr & Jones, 1998; Baldia et al., 2007; Moisander et al., 2009), the manner in which nutrients may promote toxic and non-toxic strains of *Microcysits* within an ecosystem setting is unknown.

The goal of this study was to investigate the role of various forms of organic and inorganic nitrogenous compounds as well as orthophosphate in the growth of toxic and non-toxic strains of *Microcystis* during natural bloom events. The dynamics of *Microcystis* blooms and nutrients in a lake and a tidal tributary were monitored. Concurrently, nutrient amendment experiments were conducted to understand how organic and inorganic N and orthophophate affected the dominance of toxic and non-toxic strains of *Microcystis.* Finally, uptake rates of organic and inorganic N compounds were determined using ^{15}N-labeled compounds.

Methods

Sampling site & water quality monitoring

During this study two contrasting ecosystems were studied. Transquaking River (Latitude: 38º 30’ N; Longitude: 75º 58’ W), a tributary to the Chesapeake Bay, spans 37 km along Maryland’s (USA) eastern shore. It has a watershed area of 70,992 acres, of

which roughly one-third is used for agricultural purposes (MD DNR, 2000), and had previously been host to toxic cyanobacteria blooms (Tango & Butler, 2008). Lake Agawam (Latitude: 40.88ºN; Longitude: 72.39ºW; 1 km^2) is a small (0.5 km), shallow (4 meters maximum depth), eutrophic system that often experiences dense and toxic cyanobacterial blooms (Gobler et al., 2007). During 2008, Lake Agawam (LA) and Transquaking River (TR) were sampled bi-weekly to monthly before, during, and after cyanobacteria blooms (May – November). At each site, surface temperature, dissolved oxygen, and pH were measured using a YSI 556 sonde. Twenty liters of surface water was collected in acid cleaned carboys and taken to the laboratories where triplicate extracted chlorophyll *a* and *in vivo* phycocyanin (as a proxy for total cyanobacteria) were measured with Turner Designs fluorometers using standard techniques (Parsons et al., 1984; Watras & Baker, 1988; Lee et al., 1994). For microcystin analysis, whole water was filtered onto triplicate 47 mm glass fiber filters (GF/F) and stored at -80°C until analysis. Duplicate whole water samples were preserved with Lugol's iodine solution (5% final concentration) to quantify the phytoplankton assemblage. For molecular analysis of cyanobacteria, water was filtered onto triplicate 2 μm polycarbonate filters and immediately placed in CTAB lysis buffer. The samples were heated at 50°C for ten minutes than flash frozen in liquid nitrogen and stored at -80°C until analysis. Triplicate dissolved nutrient samples were collected by filtering lake water through combusted GF/F glass fiber filters and stored at -20°C until analysis. All nutrient analyses were conducted using wet chemistry methods. Nitrate was analyzed by reducing the nitrate to nitrite using spongy cadmium as per Jones (1984). Ammonium, phosphate, and silicate were analyzed using techniques modified from Parsons et al. (1984). Urea was analyzed

using techniques from Price & Harrison (1987). Dissolved free amino acids were measured in duplicate by high performance liquid chromatography (HPLC) (Lindroth & Mopper, 1979; Cowie & Hedges, 1992). Total dissolved nitrogen and phosphorus (TDN & TDP, respectively) were analyzed using persulfate digestion (Valderrama, 1981). Dissolved organic N and P (DON & DOP) were determined by subtracting the DIN (dissolved inorganic N = nitrate, nitrite, and ammonium) from TDN and by subtracting DIP (orthophosphate) from TDP, respectively. The degree to which individual biological and environmental variables were correlated was evaluated by a Pearson's Correlation Matrix.

Impacts of inorganic and organic nitrogen on toxic and non-toxic *Microcystis*

Experiments were conducted to assess the impact of increased organic and inorganic N or orthophosphate concentrations on toxic and non-toxic *Microcystis* populations in TR and LA. Sets of triplicate, 1L bottles (n = 21) were filled with surface water from each experimental site and were either left unamended to serve as a control, or amended with various forms of nitrogen (20 μM NO_3^-, 20 μM NH_4^+, 10 μM (= 20 μM N) Urea, 10 μM (= 20 μM N) L-glutamine), phosphorus (1.25 μM orthophosphate), or a combined treatment of NO_3^- and orthophosphate. For the experiments conducted in LA, the bottles were placed in an *in situ* incubator in Old Fort Pond at the Stony Brook-Southampton marine station located ~1 km west of Lake Agawam, assuring experiments were conducted using ambient light and temperatures. For the experiments conducted in the TR, experimental bottles were placed in an incubator (REVCO©) with light and temperature levels matching conditions in TR. Light intensity and temperature during experiments were measured every minute with *in situ* loggers (Onset Computer

Corporation, Massachusetts, USA) indicated incubation temperatures remained within the same range as those found in each ecosystem. After 48 h, samples were filtered as described above to preserve samples for determination of chlorophyll *a* and densities of toxic and non-toxic *Microcystis* with molecular methods. For the three communities measured (total *Microcystis*, toxic *Microcystis*, and non-toxic *Microcystis*) differences in abundances among nutrient treatments were compared by means of one-way ANOVAs or a non-parametric Kruskal-Wallis test and differences among individual treatments were subsequently assessed with post-hoc Tukey multiple comparison tests.

Uptake rates of nitrogenous nutrients during bloom events

In parallel with the *in situ* nutrient amendment experiments in Lake Agawam, ^{15}N tracer experiments were conducted. Nitrogen uptake was measured using tracer additions (35 ± 4% ambient concentration) of highly enriched (98%) ^{15}N (Mulholland et al., 2002). Six replicate, 50 ml polycarbonate bottles per treatment were filled with whole lake water and inoculated with ^{15}N-labeled nitrate, nitrite, ammonium, glutamic acid or urea and incubated for 30 minutes under ambient conditions in order to avoid any isotope dilution or recycling associated with longer incubations. While bacteria are known to rapidly degrade reduced N compounds, their uptake and degradation rates of urea, ammonium and amino acids (0.06, 2.4, and 2.5 nM-N h^{-1} respectively; Cho et al., 1996; Coffin, 1989; Hoch & Kirchman, 1995) are small relative to the ambient pools and incubation times. Since *Microcystis* colonies were the dominant phytoplankton in the > 20 μm size fraction on every day sampled (~88% of cells were *Microcystis* during this study; Table 2), ^{15}N-amended experimental water was filtered on pre-combusted (2 h @ 450ºC) 25mm GF/F glass fiber filters with and without pre-filtration with a 20μm mesh. Removing

phytoplankton > 20 μm facilitated the measurement of N uptake rates by the total and < 20 μm phytoplankton communities. The difference between the total phytoplankton community uptake rates and the < 20 μm uptake rates was ascribed to the > 20 μm size fraction which was dominated by *Microcystis*. PON samples were analyzed by David Harris at the UC-Davis Stable isotope Laboratory (Davis, CA, USA). A one-way analysis of variance with post-hoc Tukey multiple comparison tests was performed to assess differences among uptake rates for each compound and differences in uptake rates among each plankton group during each experiment.

Sample Analysis

Microscopic analysis

Densities of *Microcystis* and other co-occurring cyanobacteria were quantified using gridded Sedgewick-Rafter and Utermohl counting chambers. Utermohl chambers were used to quantify populations with low cell densities. For dense bloom populations, a gridded Sedgewick-Rafter chamber allowed for accurate assessment of cell densities without the layering of cells which can occur when high biomass samples are concentrated within an Utermohl chamber. For all samples, at least 200 cells were enumerated. To quantify *Microcystis*, *Anabaena*, as well as *Aphanizomenon*, the number of colonies per chamber, as well as the number of cells in 20 colonies, was determined. For eukaryotic plankton, groups such as diatoms, dinoflagellates, and chlorophytes cells were enumerated. This approach provided good reproducibility (< 15% relative standard deviation) on preserved samples, as well as comparability between live and preserved samples.

Microcystin Analysis

Filters for microcystin analyses were extracted in 49% methanol containing 1% acetic acid using ultrasound (four, 20 second bursts with a 20 second pause between bursts). Previous work has shown that this extraction protocol gives > 90% recovery of microcystin-LR (Boyer et al., 2004). Following extraction, the methanolic extract was stored at –80ºC until analysis. Before analysis, the microcystin extract was diluted to 5% methanol and buffered to a pH of 7 using a 5% Tris-EDTA buffer solution. Microcystin concentrations were measured using a microcystin enzyme-linked immunosorbent assay (Abraxis LLC; Warminster, PA, USA) following the methodologies of Fischer et al. (2001). This assay is congener-independent as it is sensitive to the ADDA moiety which is found in almost all microcystins. These analyses yielded a detection limit of 0.10 µg L^{-1}, a relative standard deviation of 10 ± 1% for environmental samples, and 99.5 ± 8.2% recovery from environmental samples spiked with 5 µg L^{-1} microcystin-LR.

Molecular Analyses

Total cellular nucleic acids were extracted from field and experimental samples using methods described in Coyne et al. (2001). Filtered environmental or experimental samples were submersed in CTAB buffer (Dempster et al., 1999), and supplemented with 20 µg L^{-1} pGEM-3z(f+) plasmid (Promega; Table 1) which served as an internal control for extraction efficiency and PCR inhibition (Coyne et al., 2005). The filters were then flash frozen and stored at -80°C until extraction. Nucleic acids were extracted after an initial heating step at 65ºC, followed by a double chloroform extraction, and an isopropanol precipitation. Extracted nucleic acids were resuspended in 20µl of LoTE (a

low salt Tris-EDTA buffer). The quantity and quality of nucleic acids was assessed with a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Two *Microcystis*-specific genetic targets were used during this study, the *Microcystis* 16S rRNA gene (*Microcystis* 16S rDNA) and *mcyD* gene. The *Microcystis* 16S rRNA gene is specific to the *Microcystis* genus and permitted quantification of the abundance of the total *Microcystis* population. The *mcyD* gene is found within the microcystin synethtase gene operon which is responsible for the production of microcystin and is only found in toxic strains of *Microcystis* (Tillett et al., 2000), which allowed us to quantify the toxic population of *Microcystis* (Davis et al., 2009). QPCR was carried out using an ABI 7300 Real Time PCR instrument using TaqMan ® labeled probes (Applied Biosystems) and *Microcystis*-specific *mcyD* and 16S rDNA primers (Table 1). Each 10µL reaction included 5uL of 2x TaqMan® Master Mix (Applied Biosystems), 10µM each primer (Integrated DNA Technologies), 10µM TaqMan® probe (Table 1) and 1µl of a 1:25 dilution of the unknown DNA or standard. For amplification of the pGEM and 16S targets, the cycling conditions were 95ºC for 10 minutes, followed by 55 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. For the *mcyD* gene, the cycling conditions were 95ºC for 10 minutes, followed by 55 cycles of 95ºC for 15 seconds, followed by 50ºC for 1 minute, then 60ºC for 1 minute. To prepare standard samples, cultured toxic *Microcystis aeruginosa*, clone LE-3 (Rinta-Kanto et al., 2005), was enumerated by standard microscopy and collected on polycarbonate filters which were prepared and extracted as outlined above. A standard curve of dilutions of the extracted LE-3 genomic DNA was run with each analytical run to serve as a reference for numbers of toxic *Microcystis* spp. cells. Since some *Microcystis* spp. cells may carry

multiple copies of the 16S rDNA gene and *mcyD* gene, data was expressed as "cell equivalents" rather than cell number (Davis et al., 2009). The numbers of toxic and total *Microcystis* cells were determined using the ΔΔCT method (Livak & Schmittgen, 2001; Coyne et al., 2005). The difference between the number of *mcyD* cell equivalents (toxic cells) and 16S rDNA cell equivalents (total cells) indicated the number of non-toxic cell equivalents (Davis et al., 2009).

Results

Transquaking River cyanobacteria blooms

In 2008, TR hosted dense cyanobacteria blooms co-dominated by *Microcystis* and *Aphanizomenon* (Table 2). Peak algal and cyanobacteria densities occurred on 10 June while peak *Microcystis* densities, as measured by the 16S rRNA gene ($3.1 \pm 0.63 \times 10^8$ cell equivalents L^{-1}) occurred on 24 June (Fig 1). Within the total *Microcystis* population, toxic strains dominated from late May through early July with peak densities of $2.8 \pm 0.23 \times 10^8$ cell equivalents L^{-1} coinciding with peak total *Microcystis* densities (Fig 1). After mid-July, dominance shifted towards non-toxic strains of *Microcystis* which achieved peak densities of $3.9 \pm 1.9 \times 10^7$ cell equivalents L^{-1} in late August (Fig 1). Microcystin concentrations ranged from 0.36 to 12.1 μg L^{-1} and peaked in unison with toxic *Microcystis* densities on 24 June (Fig 1). Microcystin concentrations were significantly correlated with toxic *Microcystis* densities ($p < 0.01$) but were not with non-toxic *Microcystis* densities, chlorophyll *a*, phycocyanin, or the total cyanobacterial densities ($p > 0.05$). Concentrations of inorganic nitrogen were highest during May and June (> 1 μM) but dropped to < 1 μM during the summer and early fall (Table 3) and DIN:DIP ratios were low (3.2 ± 1.8). Silicate levels were high in TR in 2008 (25 ± 8.3

μM) while urea and DFAA concentrations were quite low (0.1 ± 0.03 μM). Dissolved organic nitrogen (DON) was the largest aqueous N pool with concentrations ranging from 14.1 ± 6.59 to 44.8 ± 1.85 μM (Table 3), while DOP concentrations range from 0.4 – 1.4 μM (Table 3). Non-toxic strains of *Microcystis* were significantly correlated with DON concentrations from June through October ($p < 0.01$). Temperatures in TR rose from 13.4ºC in May to 29.2ºC in July and dropped to 21.4ºC by October (Fig 1).

Lake Agawam cyanobacteria blooms

Lake Agawam (LA) hosted cyanobacterial blooms which differed from the TR in community composition and intensity (18.5 to 123 μg L^{-1} chl *a*; Table 2; Fig 2). The LA blooms were dominated by *Microcystis* on every date sampled (Table 2). Total *Microcystis* densities, as enumerated by the 16S rRNA gene, ranged from 1.59 ± 0.09 to 17.3 ± 0.10 x10^6 cell equivalents L^{-1}, with peak densities occurring on 15 October (Fig 2). Unlike the TR, the total *Microcystis* community was dominated by non-toxic strains throughout the 2008 field season (84 ± 4% of total *Microcystis* cells; Fig 2). Non-toxic strains of *Microcystis* ranged from 1.38 ± 0.05 to 15.3 ± 0.06 x 10^6 cell equiv. L^{-1} (Fig 2), while toxic *Microcystis* strains ranged from 0.12 ± 0.02 to 2.81 ± 0.13 x10^6 cell equiv. L^{-1}, with peak densities occurring on 15 October (Fig 2). As was the case in TR, microcystin concentrations (3.10 - 17.8 μg L^{-1}) were significantly correlated with toxic *Microcystis* ($p < 0.01$), but not with total or non-toxic *Microcystis* cell densities ($p > 0.05$). DIN concentrations in LA were high in the early summer (4 – 7 μM), lower in July and August (< 2 μM), and elevated again in the fall (4 – 7 μM; Table 3). In contrast, silicate levels were always high (20 – 70 μM) in LA (Table 3), while DIP, urea, and DFAA were always low (< 0.4, 0.1, and 0.1 μM respectively; Table 3). DON and DOP

were the largest dissolved N and P pools in LA, with mean concentrations of 10 ± 2 μM and 1.2 ± 0.2 μM (Table 3). Temperatures in LA rose from 23.3ºC in June to 27.5ºC in August and dropped to 10.3ºC by the end of October (Fig 2).

Growth responses of toxic and non-toxic strains of *Microcystis* during nutrient amendment experiments

Transquaking River

The total phytoplankton community in the TR was stimulated by one form of N in every experiment conducted and was most consistently stimulated by nitrate ($p < 0.05$; Table 4). Similarly, the total *Microcystis* population in the TR was stimulated by both inorganic and organic forms of N throughout the field season ($p < 0.05$; Table 4; Fig 3). Interestingly, the total *Microcystis* community was more frequently stimulated by urea and glutamic acid (66% and 83% of experiments, respectively) than nitrate or ammonium (Table 4; Fig 3). Furthermore, nitrate and ammonium only stimulated the total *Microcystis* population in June and early July, increasing cell equivalents, on average, 2-times above the control ($p < 0.05$; Table 4; Fig. 3). On the other hand, urea and/or glutamic acid stimulated total *Microcystis* in all experiments conducted, increasing cell equivalents between 2 and 3-times above the control. ($p < 0.05$; Table 4, Fig 3). Increased phosphate concentrations also yielded significantly increased *Microcystis* cell equivalents in experiments conducted in June and early July ($p < 0.05$; Table 4; Fig 3).

Toxic strains of *Microcystis* were stimulated more often by N enrichment (83% of experiments) than their non-toxic counterparts ($p < 0.05$; Table 4; Fig 3). Similarly to the total *Microcystis* community, the toxic strains were stimulated by both inorganic and organic forms of N during the experiments conducted in June and early July, increasing

cell equivalents 1 to 4-times above the control ($p < 0.05$; Table 4; Fig 3). P additions also yielded increased toxic *Microcystis* cell equivalents in half of the all experiments conducted in the TR increasing toxic *Microcystis* between 2 and 5-times above the control ($p < 0.05$; Table 4; Fig 3). Finally, on two dates (29 July & 9 September), N and P stimulated *Microcystis* populations ($p < 0.05$; Table 4; Fig 3). Non-toxic strains of *Microcystis* were never significantly affected by the addition of any individual inorganic N compound compared to control treatments ($p > 0.05$; Table 4; Fig 3). In contrast, these strains were stimulated by organic N compounds during the final three experiments increasing non-toxic cell equivalents between 2 and 6-times above the control, yielding the greatest increase of any treatment for this population ($p < 0.05$; Table 4; Fig 3).

Lake Agawam

None of the phytoplankton populations monitored were limited by any form of N in LA until early July (Table 4; Fig 4). From early July to late September, the total phytoplankton community was stimulated by both inorganic and organic forms of N ($p < 0.05$; Table 4). The densities of the total *Microcystis* community were not affected by nutrients until late July when both inorganic and organic N additions yielded densities significant highly than the control treatment ($p < 0.05$; Table 4; Fig 4). Similarly to the TR, the total *Microcystis* population was stimulated by organic N (urea) more frequently than either inorganic form of N, increasing cell equivalents 1.5- to 5-times above the control. ($p < 0.05$; Table 4; Fig 4). The addition of P only enhanced total *Microcystis* population to abundances significantly greater than the control treatment on one date (23 July; $p < 0.05$; Table 4; Fig 4).

Since the non-toxic strains of *Microcystis* comprised most (84%) of the total *Microcystis* community, their growth responses to nutrients were similar to the total *Microcystis* trends. Non-toxic strains were enhanced by urea more frequently than any other form of N, increasing cell equivalents 2 to 5-times above the control in three experiments ($p < 0.05$; Table 4; Fig 4). Again, the addition of P during the 23 July experiment yielded the greatest increase (9-fold) of any treatment for this population ($p < 0.05$; Table 4; Fig 4).

Densities of toxic strains of *Microcystis* were significantly enhanced over the controls ($p < 0.05$) by nutrients more frequently than the non-toxic population (early July to the end of September; Table 4; Fig 4). Unlike their non-toxic counterparts, toxic *Microcystis* in LA was only enhanced by inorganic forms of N (nitrate and ammonium), yielding densities that were 1.5 to 2-times above the control ($p < 0.05$; Table 4; Fig 4). This population was also enhanced by P additions in two experiments, increasing the cell equivalents 2 to 4-times above the control ($p < 0.05$; Table 4; Fig 4). Furthermore, on two dates (1 July & 18 August), the combined N and P yielded toxic *Microcystis* densities 2 to 4-times above the control ($p < 0.05$; Table 4; Fig 4).

N assimilation rates of plankton communities during cyanobacterial blooms dominated by *Microcystis*

During N-uptake experiments in Lake Agawam, *Microcystis* cell densities ranged from 9.9 ± 0.76 to $306 \pm 2.69 \times 10^6$ cells L^{-1}, representing > 99% of the > 20 μm community, on all dates sampled but one when it was 88% (Table 2). The > 20μm community comprised between 63 and 79% of the particulate organic nitrogen (PON) in

LA in 2008, with an average of 71 ± 3%. Total N uptake for all N species ranged from 0.89 to 5.2 μmol N L^{-1} hr^{-1}, and averaged 2.1 ± 0.69 μmol N L^{-1} hr^{-1} (Fig 5a). The total uptake rates were highest on 5 June, when concentrations of DIN peaked (Fig 5a). The > 20μm plankton community uptake rates followed similar trends as the total community uptake rates with an average uptake rate of 1.30 ± 0.33 μmol N L^{-1} hr^{-1}, and ranging from 0.75 to 2.8 μmol N L^{-1} hr^{-1}.

The N compound assimilated at the highest rate differed among dates. On average, inorganic nutrients (nitrate and ammonium) comprised 69 ± 9 % of the total N uptake, where as the organic nutrients (urea and glutamic acid) represented, on average, 31 ± 9% (Fig. 5d). In the experiments conducted from June through August (n = 4), nitrate and ammonium dominated the total N uptake (81 ± 6%; Mean: 2.34 ± 0.49 μmol N L^{-1} hr^{-1}; Fig 5d) and were taken up at rates that were significantly higher than the organic nitrogen uptake rates (0.38 ± 0.06 μmol N L^{-1} hr^{-1}; t-test; $p < 0.05$). During the experiments conducted in September and October, urea represented the majority of the N uptake (55 ± 1%), followed by ammonium (38 ± 8%; Fig 5d) with both uptake rates being significantly higher than those for either nitrate or glutamic acid ($p < 0.05$).

Trends in N compound assimilation by the > 20μm *Microcystis*-dominated phytoplankton community were somewhat similar to the total plankton community, as this size fraction comprised 68 ± 6% of the total N uptake (Fig 5c). Ammonium, nitrate, urea, and glutamic acid represented, on average, 42 ± 12, 25 ± 10, 28 ± 10, and 4 ± 2% of the total N uptake rate in this larger size fraction (Fig. 5c,f). The larger phytoplankton community obtained a substantially greater fraction of its total N from organic compounds (32%) than the smaller phytoplankton did (24%) (Fig 5d,e). This was most

striking in July, September, and October when organic compounds represented 41%, 61%, and 58% of the total N uptake by *Microcystis*-dominated phytoplankton, where as organic N accounted for 36 ± 4% of the total N uptake in the nano- and picophytoplankton (< 20 μm; Fig 5e). Nitrite uptake was below the limit of detection on every date sampled (< 0.005 μmol N L^{-1} hr^{-1}).

Discussion

Eutrophication is considered a primary cause of many HABs (Heisler et al., 2008; Anderson et al., 2008), especially within freshwater ecosystems (Paerl, 1988, 2001, 2008). Because freshwater ecosystems are traditionally viewed as P-limited, management plans for such systems are commonly aimed toward reducing P loads (Howarth & Paerl, 2008). However, this study demonstrated that N enrichment can promote freshwater blooms of *Microcystis* and inorganic forms may favor toxic strains over those which cannot produce microcystin. Combined with the flexibility in N uptake displayed by *Microcystis*, this study provides new insight into the role of nutrients in the occurrence of toxic and non-toxic *Microcystis* blooms.

Microcystis community composition differed between the two ecosystems studied. Toxic strains of *Microcystis* dominated the TR community during the early summer months (May – June) before non-toxic strains became dominant, whereas non-toxic strains dominated the LA *Microcystis* community (84 ± 4%) on every date sampled. The dominance of toxic strains in TR rather than LA could be related to the availability of phosphorus in each system. Vézie et al. (2002) reported that the growth rates of toxic *Microcystis* exceeded non-toxic strains under high orthophosphate concentrations, and concentrations of orthophosphate were nearly an order of magnitude higher in TR (1.3

μM) compared to LA (0.19 μM; $p < 0.05$; T-test). Changes in the dominance of toxic strains through the year further evidenced the importance of P in TR, as orthophosphate concentrations were significantly higher during May through mid-July (2.2 ± 0.71 μM) when toxic strains dominated the *Microcystis* population (62 ± 20%) compared to late July through October (0.45 ± 0.06 μM; $p < 0.01$; T-test) when non-toxic strains were most abundant (85 ± 5%; Table 3, Fig 1). Hesse et al (2001) reported that microcystin-producing *Microcystis* strain PCC 7806 had a higher content of light-harvesting pigments than the non-toxic mutant strains. The RNA and DNA required for the synthesis of both microcystin and light-harvesting pigments by toxic strains of *Microcystis* may represent a significant P requirement which would account for their association with higher P levels in this and other studies (Vézie et al. 2002).

For both study sites, the total *Microcystis* community was significantly enhanced by at least one form of N in 75% of experiments whereas it was enhanced by increases in P concentrations in 33% of experiments, indicating that *Microcystis* in both systems were more nitrogen limited (Table 5). This finding is contrary to the long held view that freshwater ecosystems are exclusively P-limited (Schindler, 1977; Smith 1983; Hecky & Kilham, 1988; Paerl, 1982, 1988), but consistent with more recent studies of *Microcystis* blooms (Gobler et al., 2007, Moisander et al., 2009). Interestingly, the total *Microcystis* community was stimulated by organic N compounds (urea: 58% of experiments, glutamic acid 50%) more frequently than inorganic N compounds (nitrate 33%, ammonium, 17%; $P < 0.05$; Table 5). Similarly, Berman & Chava (1999) found that cultured *Microcystis* grew noticeably faster on urea than either nitrate or ammonium. However, a recent study conducted by Moisander et al. (2009) found that *Microcystis* blooms were promoted by

nitrate, ammonium, and urea. The finding that *Microcystis* was more frequently stimulated by urea than any other N compound is similar to the harmful marine dinoflagellates, *Prorocentrum minimum* and *Lingulodinium polyedrum* as well as the harmful pelagophyte *Aureococcus anophagefferens*, which also displayed a high affinity for urea over other N forms (Fan et al., 2003; Kudela & Cochlan, 2000; Gobler & Sãnudo-Wilhelmy, 2001a; Baker et al., 2009). These results support the idea that harmful algal blooms (HABs) often exploit a variety of N species, including organic forms, and may do so with faster rates which could provide a competitive advantage over other phytoplankton species especially at times of low DIN concentrations (Paerl, 1997; Seitzinger & Sanders, 1997; Berman & Chava, 1999, Heisler et al., 2008).

The toxic strains of *Microcystis* were stimulated by N enrichment more frequently than the non-toxic strains during this study. This difference was strongest in TR where N enrichment enhanced the abundance of toxic *Microcystis* in all but one experiment, but did so for the non-toxic strains in only half the experiments (Table 4; Fig 4). The stronger response of toxic *Microcystis* to N is consistent with laboratory studies which have reported that toxic strains of *Microcystis* and *Anabaena* require higher nitrogen concentrations to achieve maximal growth rates compared to non-toxic strains, partly due to the high N demand of microcystin synthesis (Rapala et al., 1997; Vezie et al., 2002). This difference between strains is also consistent with the N requirements for microcystin synthesis, as microcystin is a N-rich compound (10 N atoms per molecule) and studies have found that microcystin can represent up to 2% of cellular dry weight (Nagata et al., 1997). Based on the densities of *Microcystis* and the levels of PON > 20μm on dates when *Microcystis* represented 99% of cells > 20 μm, I estimate a cellular N quota of 3.5

$\pm$ 1.3 x 10^{-13} mol N $cell^{-1}$, a value which is within the range of an estimate of its N content based on cell biovolume and a Refield C:N ratio (1.9 x 10^{-13} mol N $cell^{-1}$; Stoecker et al., 1994). Based on this cellular N content of *Microcystis*, the N associated with microcystin was 1 – 2% of the total N content of toxic *Microcystis* cells in Lake Agawam. Furthermore, Hesse & Kohl (2001) found that the microcystin content of *Microcystis* could vary 3-fold with changing environmental conditions, suggesting this N quota could be even higher. Beyond this N in the toxin, toxic *Microcystis* strains will have additional N requirements associated with the enzymes involved in the synthesis of microcystin (Tillett et al., 2000) as well as with additional light-harvesting pigments they may possess (Hesse et al., 2001). Although the precise mechanism is unclear, it does seem as though toxic *Microcystis* cells have a higher N requirement than non-toxic cells.

. The type of N added during field experiments influenced whether toxic or non-toxic strains of *Microcystis* dominated total populations. For example, when non-toxic strains were stimulated by N during experiments (n = 6), it was more frequently by organic N compounds (100%) than inorganic N compounds (33%; Table 5). Interestingly, non-toxic *Microcystis* densities were significantly correlated with DON concentrations in TR, further supporting the hypothesis that these strains dominate when concentrations of labile DON are elevated. Conversely, when toxic strains of *Microcystis* were stimulated by individual N species (n = 7), it was more frequently due to inorganic N than organic N (89 and 33%, respectively; Table 5). This is consistent with Vézie et al. (2002) who found that increases in nitrate concentrations yielded faster growth rates for toxic *Microcystis* cultures compared to non-toxic cultures.

During half of the experiments conducted across both sites, N *or* P increased the abundance of one or more of the *Microcystis* populations relative to control treatments and there were two occasions at each site when increases in N *and* P concentrations did so, suggesting these populations were often co-limited by N and P (Table 4; Fig 4, 5). These results evidence two separate types of co-limitation of *Microcystis* populations: A traditional co-limitation where both nutrients are at levels which restrict growth (response only from adding N and P) and a biochemical nutrient limitation where one nutrient aids in the assimilation or processing of the other (N *or* P; Arrigo, 2005; Saito et al., 2008). Importantly, these results demonstrate that the dual management of N and P (Howarth & Paerl 2008; Paerl, 2009) will be required to control the future occurrence of *Microcystis* blooms in these, and likely other, systems. Finally, P more frequently promoted higher densities of toxic *Microcystis* (~40% of experiments) than non-toxic populations (17% of experiments; Table 4, 5). This observation is consistent with our previous work (Davis et al., 2009) and suggests microcystin synthesis creates a larger cellular P quota for toxic *Microcystis* populations.

The differing response of toxic and non-toxic strains of *Microcystis* during to N and P enrichment and our observations of these populations and nutrients in TR provides evidence for a hypothesis to explain the seasonal dynamics of these strains in temperate ecosystems. In TR, we observed a seasonal transition from higher inorganic nutrients, lower organic (DON, DOP, urea, DFAA) nutrients (Table 3) and dominance by toxic *Microcystis* strains during early summer (May – July; Fig 1) followed by a depletion of inorganic nutrients, an elevation in organic nitrogen, and dominance by non-toxic *Microcystis* in late summer (Table 3; Fig 1). This pattern of nutrients is common for

aquatic ecosystems, as warming summer temperatures bring decreases in freshwater-based nutrient delivery rates (Gobler & Sañudo–Wilhelmy, 2001b) and increases in phytoplankton nutrient assimilation rates (Goldman & Carpenter, 1974). As such, while toxic *Microcystis* thrives on the abundant sources of inorganic nutrients during early summer, the depletion of these nutrients by mid-summer likely contributes toward to the demise of this population, a pattern observed in TR in 2007 and 2008. The remineralization of dead cells and higher pelagic and benthic remineralization rates within warmer summer waters (Boynton et al., 1995) both likely contribute toward a water column which is enriched dissolved organic nitrogen during late summer. This study demonstrated that non-toxic *Microcystis* experiences increased growth rates after enrichment with organic compounds and that non-toxic *Microcystis* densities were significantly correlated with DON concentrations in TR, two findings which may account for their dominance during late summer. This population shift between toxic and non-toxic strains concurrent with decreases in DIN has been found in other systems such as Lake Ronkonkoma, NY, in 2005 (Davis et al., 2009). Furthermore, Briand et al. (2009) found a similar seasonal shift in genotypes from toxic strains to non-toxic strains in a French reservoir. This trend was not seen in LA possibly due to the low P (mean = 0.19 μM DIP) concentrations which may have inhibited toxic strains from becoming dominant within this system due to their high P requirement (Vézie et al., 2002); toxic strains were always < 20% of the total *Microcystis* community in this system.

Measurement of N uptake rates for phytoplankton > 20μm during blooms in Lake Agawam dominated by *Microcystis* (98 ± 4% of the > 20μm plankton community) allowed the N uptake characteristics for this genus and any associated microbes to be

described. The > 20µm size-fraction displayed flexibility in N assimilation, obtaining the majority of its N from either nitrate, ammonium, *or* urea on different dates (Fig 5c, f). This group also obtained significantly more of its total N from organic compounds than did smaller plankton (< 20µm; Fig 5). This observation is consistent with the facts that most of the *Microcystis* cells in LA were non-toxic (85%) and that non-toxic populations were frequently stimulated by organic N compounds during incubation experiments. Uptake rates of ammonium and urea by the > 20µm plankton community were significantly correlated with the ambient concentrations of these nutrients ($p < 0.05$) indicating that N utilization by *Microcystis* was dependent on nutrient availability. This finding is consistent with Takamura et al. (1987) who reported that nitrate uptake during a *Microcystis* bloom paralleled ambient concentrations. In general, our results are consistent with the study of other HABs which have been shown to be capable of utilizing both inorganic and organic N as well as having a preference for organic forms over inorganic (Lomas et al., 1996; Berg et al., 1997; Herndon & Cochlan, 2007; Mulholland et al., 2002, 2009). Our results are also consistent with genomic evidence that *Microcystis* is capable of transporting and metabolizing variety of N sources (Kaneko et al., 2007; Frangeul et al., 2008). The ability to utilize different N compounds under differing environmental conditions likely facilitates *Microcystis* dominance in a variety of ecosystems across the globe (Chorus & Bertram 1999; Hudnell & Dortch 2008). Indeed, Lake Agawam is a stagnant, eutrophic lake with relatively high inorganic N concentrations, whereas Transquaking River is a tidal, estuarine tributary with < 1µM DIN during much of the year. Despite these stark differences, *Microcystis* formed dense blooms in both systems, likely due in part to its flexibility in N assimilation.

In conclusion, *Microcystis* populations in LA and TR were frequently stimulated by N and, to a lesser extent, P. Toxic strains of *Microcystis* were more frequently promoted by N and P than non-toxic strains, but non-toxic strains were more frequently stimulated by organic N. Therefore, dominance of toxic *Microcystis* and ultimately the toxicity of *Microcystis* blooms may be influenced by both the concentration and species of nutrients, with increases in inorganic N and/or P loading likely promoting blooms dominated by toxic strains and potentially yielding higher microcystin concentrations (Davis et al., 2009). Finally, *Microcystis* blooms were able to utilize a variety of N species and shifted nutrient preference with the availability of each N pool. Such flexibility likely contributes toward its dominance in a diversity of freshwater ecosystems.

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DNA Target	Primer	Sequence(5'-3')	Reference
pGEM plasmid DNA	M13F	CCCAGTCACGACGTTGTAAAAACG	Coyne *et al.* 2005
	pGEM R	TGTGTGGAATTGTGAGCGGA	Coyne *et al.* 2005
	pGEM probe	(Taq) FAM[a]-CACTATAGAATACTCAAGCTTGCATGCCTGCA-BHQ-1[b]	Coyne *et al.* 2005
***Microcystis* 16S rDNA**	184F	GCCGCRAGGTGAAAMCTAA	Neilan *et al.* 1997
	431R	AATCCAAARACCTTCCTCCC	Neilan *et al.* 1997
	Probe	(Taq) FAM[a]-AAGAGCTTGCGTCTGATTAGCTAGT-BHQ-1[b]	Rinta-Kanto *et al.* 2005
***Microcystis* mcyD**	F2	GGTTCGCCTGGTCAAAGTAA	Kaebernick *et al.* 2000
	R2	CCTCGCTAAAGAAGGGTTGA	Kaebernick *et al.* 2000
	Probe	(Taq) FAM[a]-ATGCTCTAATGCAGCAACGGCAAA-BHQ-1[b]	Rinta-Kanto *et al.* 2005

Table 1: A list of primers (Integrated DNA Technologies, Iowa, USA) and probes (Applied Biosystems, Foster City, CA, USA) used in the qPCR analysis; a= 6-Carboxyfluorescein b= Black Hole Quencher-1 (quenching range 480-580 nm)
F= forward primer R= reverse primer.

	Microcystis	% *Microcystis*	*Anabaena*	*Aphanizomenon*	Diatoms	Chlorophytes	Dinoflagellates
Transquaking River 2008							
13-May	10,000 (800)	89	0 (0)	0 (0)	1,100 (25)	125 (30)	0 (0)
10-Jun	72,000 (5,000)	17	350,000 (1,100)	0 (0)	130 (9)	0 (0)	210 (80)
24-Jun	190,000 (3,900)	75	7,200 (1,200)	51,00 (2,800)	2,600 (220)	1,100 (100)	3,000 (180)
9-Jul	120,000 (4,000)	45	1,300 (1,300)	120,000 (7,100)	16,000 (1,100)	3,900 (580)	130 (130)
29-Jul	220,000 (3,700)	91	150 (150)	20,000 (1,400)	1,400 (10)	990 (50)	210 (70)
26-Aug	310,000 (2,700)	92	2,400 (300)	21,000 (600)	1,500 (60)	15 (15)	100 (10)
9-Sep	47,000 (340)	44	8,500 (120)	44,000 (1,100)	2,800 (10)	4,800 (210)	160 (10)
30-Sep	23,000 (1,000)	44	0 (0)	24,000 (180)	1,600 (70)	3,000 (50)	95 (5)
Lake Agawam 2008							
3-Jun	95,000 (9,500)	99	0 (0)	0 (0)	200 (20)	540 (50)	0 (0)
5-Jun	140,000 (14,000)	99	0 (0)	0 (0)	170 (20)	250 (30)	0 (0)
1-Jul	81,000 (8,100)	99	530 (50)	133 (1)	20 (2)	67 (7)	0 (0)
23-Jul	130,000 (13,000)	88	7,600 (760)	8,200 (800)	180 (20)	160 (0)	0 (0)
18-Aug	180,000 (18,000)	99	0 (0)	300 (30)	0 (0)	40 (4)	0 (0)
23-Sep	500,000 (50,000)	99	930 (90)	800 (80)	890 (90)	20 (2)	0 (0)
15-Oct	410,000 (41,000)	99	0 (0)	400 (40)	170 (20)	27 (3)	0 (0)
30-Oct	490,000 (49,000)	99	1,300 (130)	2,200 (220)	290 (30)	40 (40)	0 (0)

Table 2: Mean autotrophic plankton densities (cells ml^{-1}) (SE in parentheses) as quantified via light microscopy for the Transquaking River and Lake Agawam 2008.

	Inorganic					
	Nitrate	Ammonium	Phosphate	Silicate	DIN	DIP
Lake Agawam						
5-Jun	5.48 (0.13)	2.45 (0.28)	0.06 (0.00)	22.6 (0.01)	7.94 (0.34)	0.06 (0.00)
1-Jul	1.18 (0.08)	3.44 (0.77)	0.38 (0.05)	20.7 (1.23)	4.62 (0.70)	0.38 (0.05)
23-Jul	1.25 (0.03)	1.23 (0.15)	0.12 (0.01)	45.9 (10.7)	2.06 (0.57)	0.12 (0.01)
18-Aug	0.43 (0.25)	0.48 (0.01)	0.30 (0.08)	59.4 (2.79)	0.78 (0.33)	0.30 (0.08)
23-Sep	4.14 (0.12)	0.47 (0.04)	0.21 (0.10)	68.5 (1.30)	4.61 (0.15)	0.21 (0.10)
15-Oct	6.85 (0.14)	0.68 (0.04)	0.18 (0.02)	68.1 (5.80)	7.53 (0.14)	0.18 (0.02)
Transquaking River						
13-May	10.25 (0)	6.36 (0.13)	3.87 (0.38)	16.0 (3.28)	16.6 (0.23)	3.87 (0.38)
10-Jun	1.99 (0.68)	3.56 (0.02)	2.92 (0.39)	1.23 (0.04)	3.77 (0.35)	2.92 (0.39)
24-Jun	0.08 (0.02)	0.84 (0.13)	1.09 (0.06)	48.5 (7.21)	0.92 (0.12)	1.09 (0.06)
9-Jul	0.17 (0.10)	0.74 (0.07)	0.97 (0.08)	71.6 (3.02)	0.91 (0.06)	0.97 (0.08)
29-Jul	0.07 (0.02)	0.57 (0.03)	0.63 (0.05)	11.9 (0.27)	0.65 (0.04)	0.63 (0.05)
26-Aug	0.30 (0.16)	0.62 (0.02)	0.37 (0.04)	10.0 (1.07)	0.91 (0.17)	0.37 (0.04)
9-Sep	0.59 (0.03)	0.57 (0.03)	0.41 (0.05)	27.6 (4.99)	0.82 (0.23)	0.41 (0.05)
30-Sep	0.26 (0.08)	0.57 (0.08)	0.39 (0.01)	15.8 (0.03)	0.69 (0.20)	0.39 (0.01)

	Organic				Ratios	
	Urea	DFAA	DON	DOP	DIN:DIP	DON:DOP
Lake Agawam						
5-Jun	0.06 (0.06)	0.13 (0.003)	17.0 (8.27)	1.09 (0.37)	262	16
1-Jul	0.05 (0.02)	0.18 (0.01)	13.4 (2.81)	0.68 (0.13)	12	20
23-Jul	0.04 (0.00)	0.10 (0.003)	14.0 (2.66)	1.15 (0.10)	17	12
18-Aug	0.06 (0.04)	0.05 (0.002)	7.94 (0.78)	0.60 (0.06)	3	13
23-Sep	0.35 (0.02)	0.09 (0.002)	12.6 (1.59)	0.72 (0.23)	22	17
15-Oct	0.26 (0.14)	0.11 (0.003)	-	0.97 (0.29)	42	5
Transquaking River						
13-May	0.38 (0.02)	0.1 (0.002)	14.1 (6.59)	0.61 (0.06)	4	23
10-Jun	0.42 (0.01)	0.28 (0.005)	19.4 (1.78)	1.06 (0.05)	1	18
24-Jun	0.45 (0.01)	0.1 (0.002)	24.1 (1.44)	1.05 (0.16)	1	23
9-Jul	0.31 (0.01)	0.06 (0.002)	28.5 (2.82)	0.86 (0.27)	1	33
29-Jul	0.38 (0.09)	0.07 (0.002)	21.9 (0.99)	0.31 (0.06)	1	71
26-Aug	0.13 (0.02)	0.09 (0.006)	44.8 (1.85)	0.56 (0.10)	2	79
9-Sep	0.27 (0.05)	0.05 (0.002)	24.6 (1.71)	0.47 (0.09)	2	52
30-Sep	0.14 (0.08)	0.07 (0.002)	23.9 (6.69)	0.55 (0.25)	2	43

Table 3: Mean dissolved inorganic and organic nutrient concentrations (μM with SE in parentheses) for the Transquaking River and Lake Agawam 2008. DIN = dissolved inorganic nitrogen, DIP = dissolved inorganic phosphorus, DON = dissolved organic nitrogen, DOP = dissolved organic phosphorus, DFAA = dissolved free amino acids A dash indicates the sample was not available for that date.

	Total Phytoplankton	Total *Microcystis*	Non-toxic *Microcystis*	Toxic *Microcystis*
Transquaking River				
10-Jun-08	NO_3	NO_3, U, P	--	NO_3, U, P
24-Jun-08	N+P	GA	--	GA
9-Jul-08	NO_3, N+P	NO_3, NH_4, U, GA, P	--	NO_3, NH_4, U, GA, P
29-Jul-08	NO_3, NH_4, U, GA	GA, P, N+P	U, P, N+P	NO_3, NH_4, P, N+P
26-Aug-08	NO_3, NH_4, U, GA, N+P	U, GA	U, GA	--
9-Sep-08	NO_3, GA, N+P	U, GA, N+P	U, GA	N+P
Lake Agawam				
5-Jun-08	--	--	--	--
1-Jul-08	NH_4, P, N+P	--	--	NH_4, P, N+P
23-Jul-08	N+P	U, P	U, P	NO_3
18-Aug-08	NO_3, U, GA, N+P	NO_3, U, N+P	NO_3, U, N+P	--
23-Sep-08	N+P	--	--	NH_4
15-Oct-08	--	NO_3, NH_4, U, GA	NO_3, NH_4, U, GA	NO_3, NH_4 P

Table 4: Treatments which significantly stimulated the total cyanobacterial community, total *Microcystis* community, non-toxic *Microcystis*, and toxic *Microcystis* relative to control treatments ($p<0.05$) during nutrient amendment experiments in the Transquaking River (top) and Lake Agawam (bottom) 2008. NO_3 = nitrate, NH_4 = ammonium, GA = glutamic acid, U = Urea, P = orthophosphate, N+P = nitrate and orthophosphate. A double dash indiciates no treatment significantly increased the population over the control.

	Total Phytoplankton	Total *Microcystis*	Non-toxic *Microcystis*	Toxic *Microcystis*
Any N compoud	83% (10/12)	75% (9/12)	50% (6/12)	75% (9/12)
Nitrate	75% (9/12)	33% (4/12)	17% (2/12)	50% (6/12)
Ammonium	25% (3/12)	17% (2/12)	8% (1/12)	42% (5/12)
Urea	25% (3/12)	58% (7/12)	50% (6/12)	17% (2/12)
Glutamic Acid	33% (4/12)	50% (6/12)	25% (3/12)	17% (2/12)
Orthophosphate	8% (1/12)	33% (4/12)	17% (2/12)	42% (5/12)

Table 5: The percentage of experiments in which N compounds significantly increased the density of the total phytoplankton community, total *Microcystis* community, non-toxic *Microcystis*, and toxic *Microcystis* relative to control treatments ($p<0.05$) during nutrient amendment experiments. Percentages and number of significant treatments out of total number of experiments (in parentheses) shown.

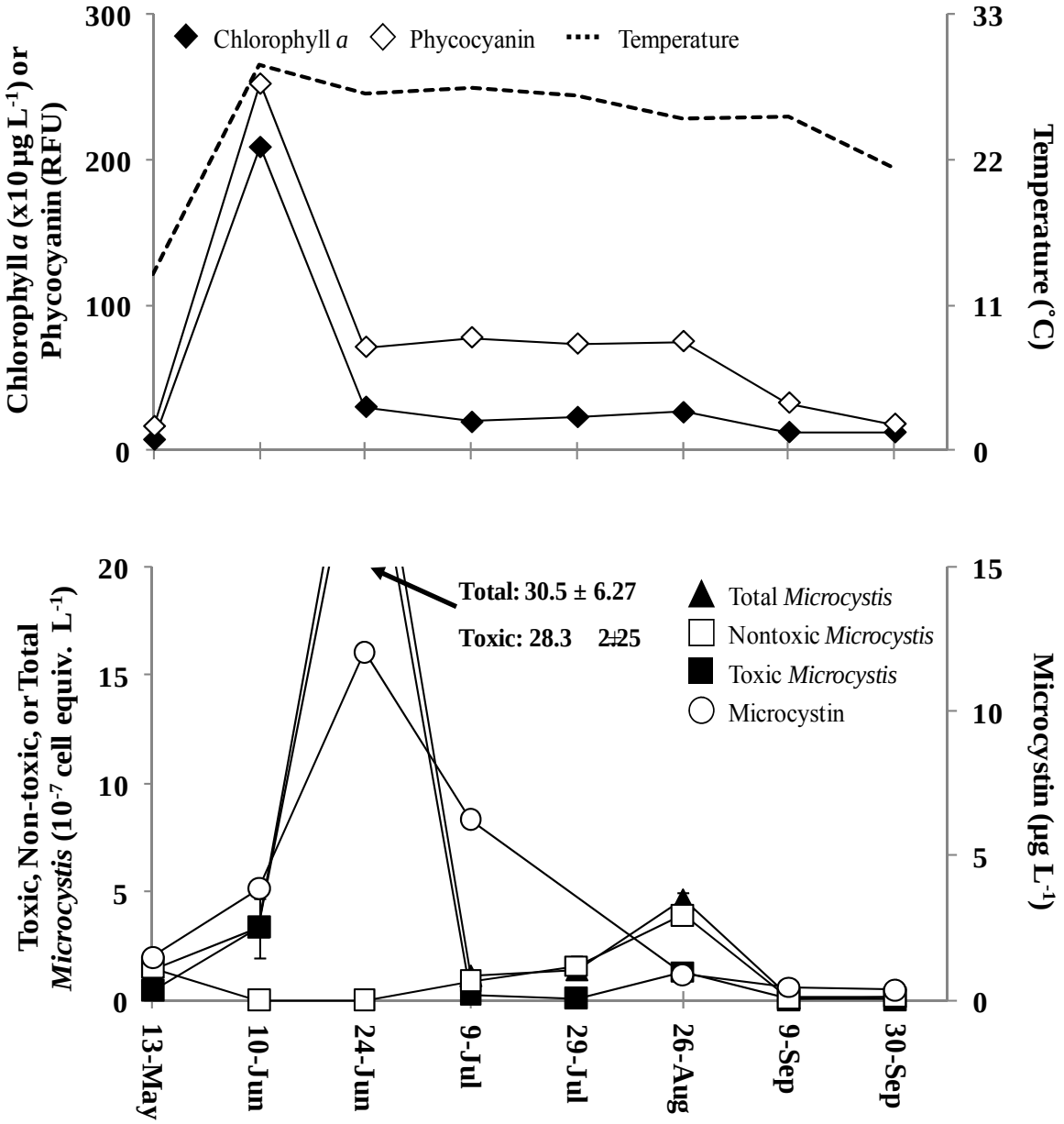

Figure 1: Time series of parameters measured in the Transquaking River, 2008. Top graph: Levels of total chl *a*, phycocyanin and temperature. Bottom graph: Densities of total, toxic and non-toxic *Microcystis* as well as concentrations of microcystin. Error bars represent ± 1 SE of replicated samples.

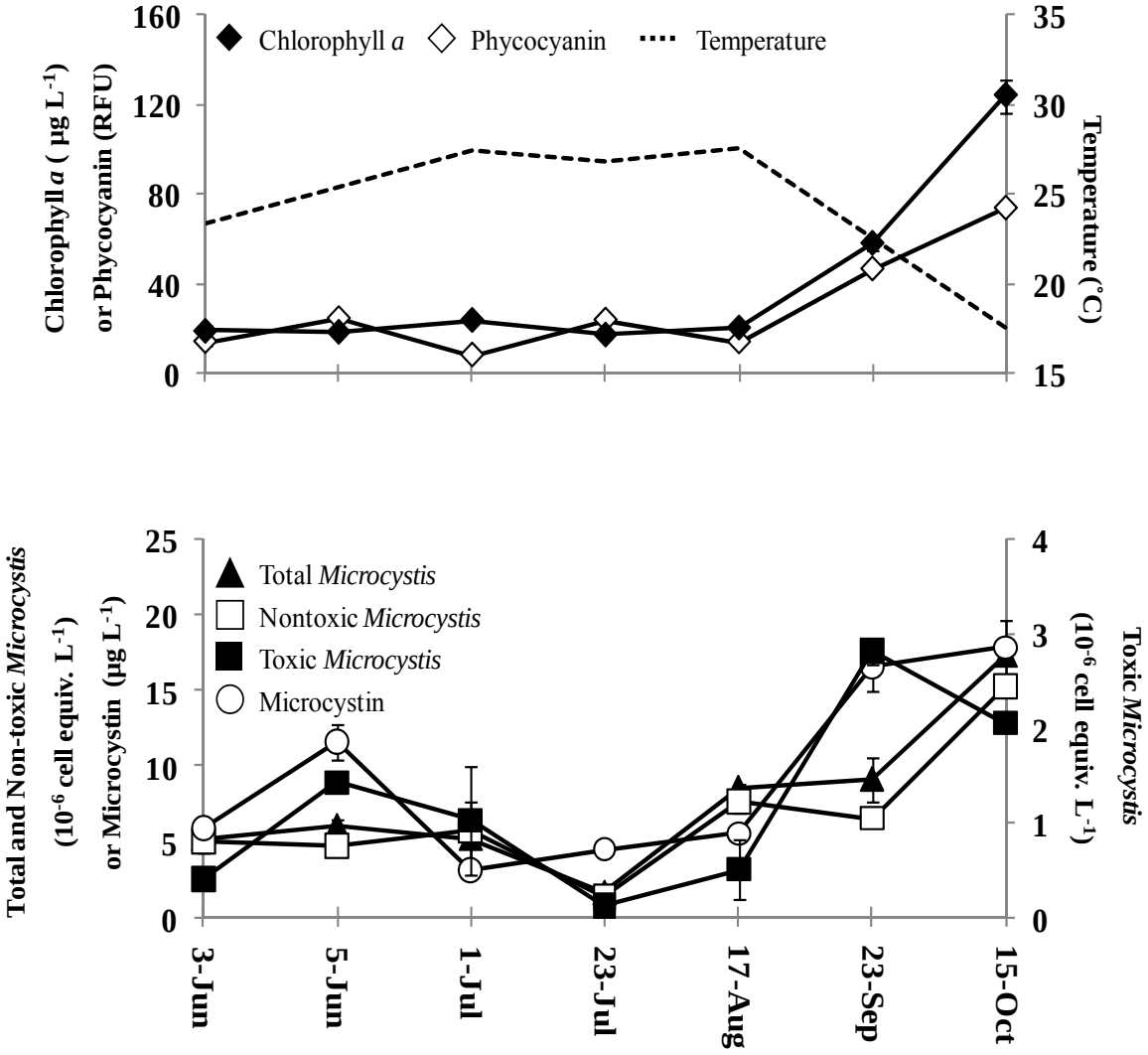

Figure 2: Time series of parameters measured in Lake Agawam, 2008. Further details as in Fig. 1.

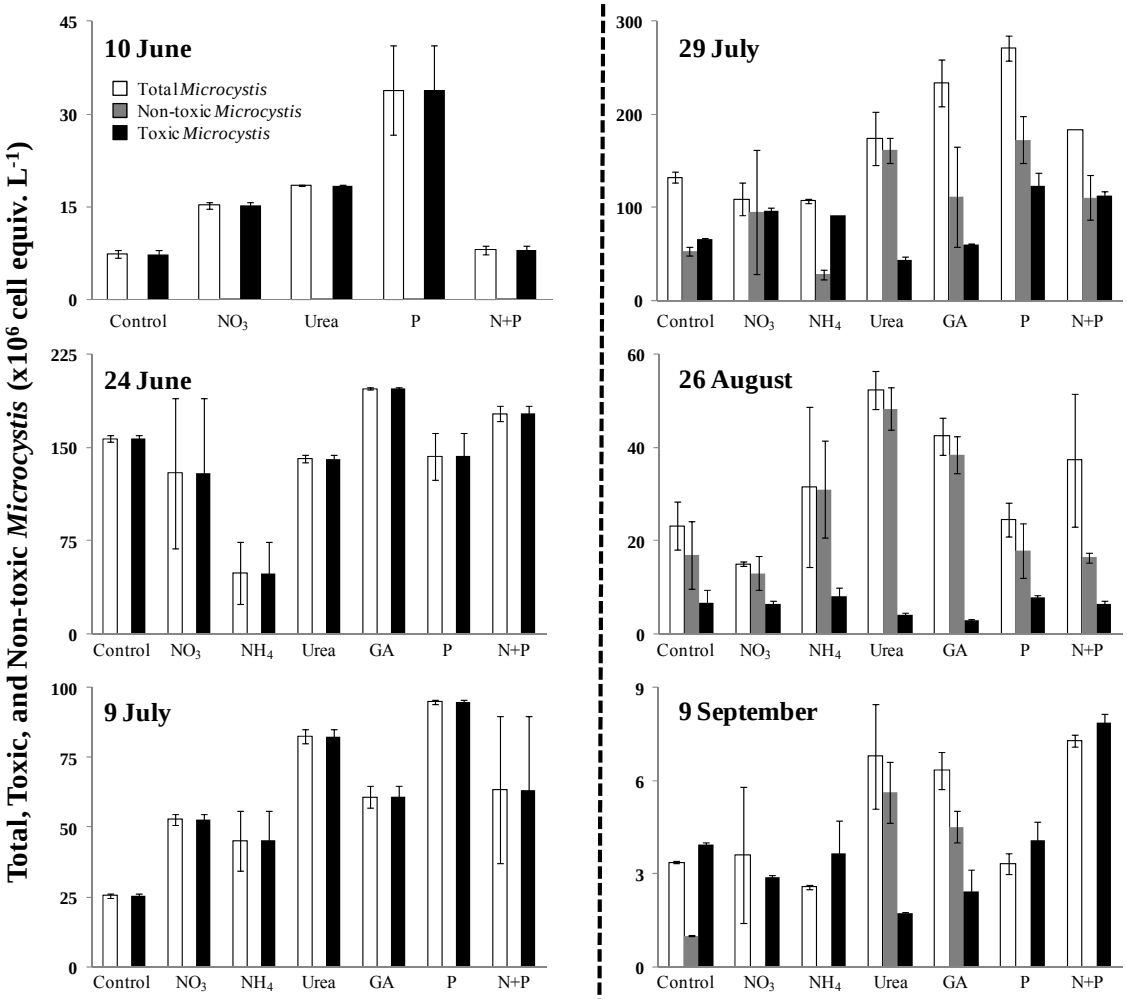

Figure 3: Total, non-toxic, and toxic *Microcystis* densities during nutrient amendment experiments conducted in the Transquaking River dung the summer of 2008. Error bars represent ± 1SE of triplicate experimental bottles. NO_3 = nitrate, NH_4 = ammonium, GA = glutamic acid, U = urea, P = orthophosphate, N+P = nitrate and orthophosphate. During the 10 June, 24 June, 9 July, and 9 September experiments the non-toxic *Microcystis* population was below the dectection limit. During the 10 June experiment, there was no NH_4 or GA treatment.

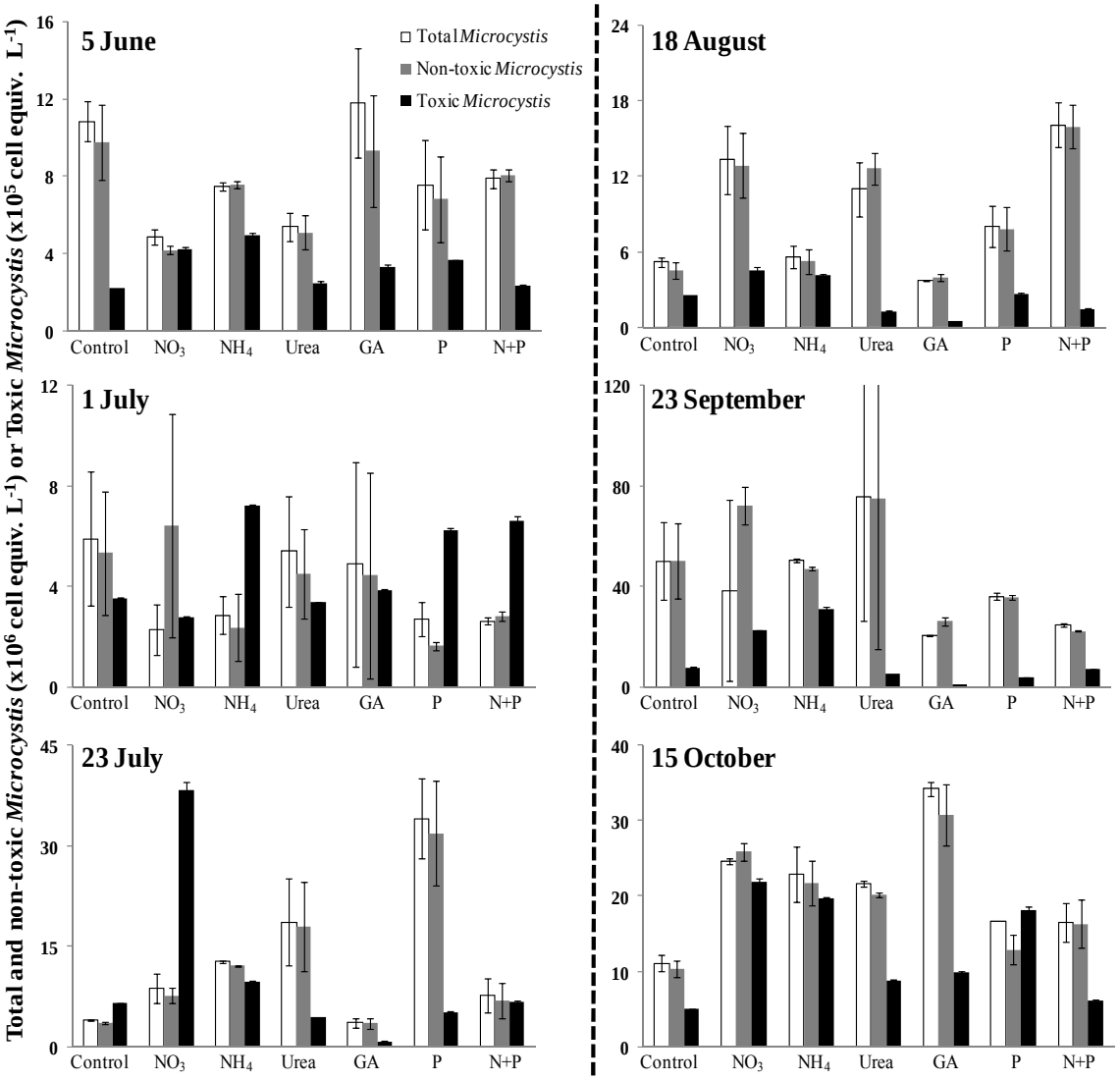

Figure 4: Total, non-toxic, and toxic *Microcystis* densities during nutrient amendment experiments conducted in Lake Agawam during the summer of 2008. Error bars represent ± 1SE of triplicate experimental bottles. NO_3 = nitrate, NH_4 = ammonium, GA = glutamic acid, U = urea, P = orthophosphate, N+P = nitrate and orthophosphate.

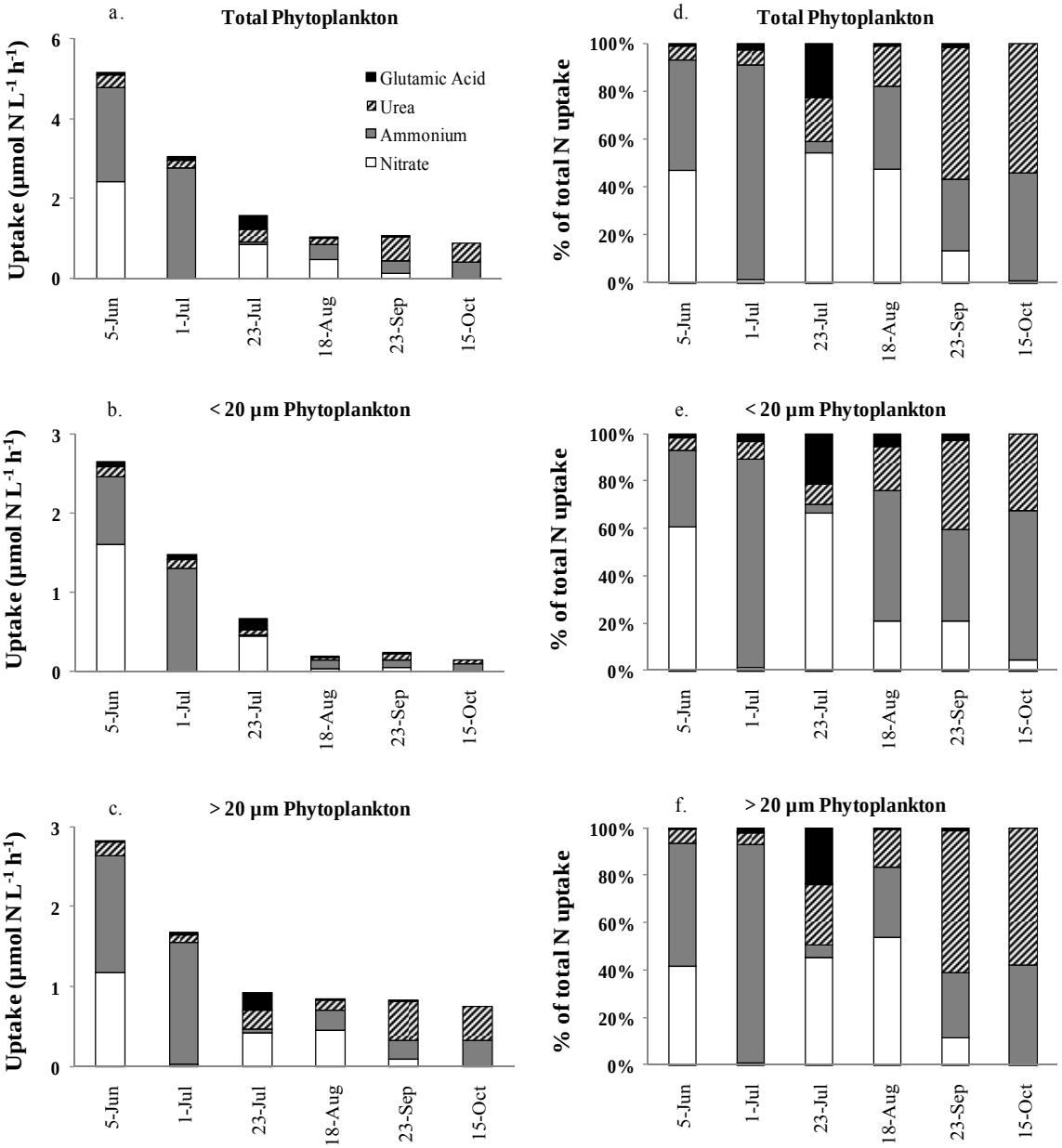

Figure 5a-f: Uptake (µmol N l^{-1} h^{-1}) and % of total uptake of ^{15}N-labeled nitrogen compounds by three plankton size fractions (total, < 20 µm, and > 20 µm) during *Microcystis* blooms. Water was obtained from Lake Agawam during the summer of 2008. Mean relative standard error of uptake rates measurements for all experiments was 14%.

CHAPTER THREE:

Effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms

(Published in Harmful Algae, June 2009)

Abstract

In temperate latitudes, toxic cyanobacteria blooms often occur in eutrophied ecosystems during warm months. Many common bloom-forming cyanobacteria have toxic and non-toxic strains which co-occur and are visually indistinguishable but can be quantified molecularly. Toxic *Microcystis* cells possess a suite of microcystin synthesis genes (*mcyA - J*), while non-toxic strains do not. For this study, we assessed the temporal dynamics of toxic and non-toxic strains of *Microcystis* by quantifying the microcystin synthetase gene (*mcyD*) and the small subunit ribosomal RNA gene, 16S (an indicator of total *Microcystis*), from samples collected from four lakes across the Northeast US over a two-year period. Nutrient concentrations and water quality were measured and experiments were conducted which examined the effects of elevated levels of temperatures (+ 4°C), nitrogen, and phosphorus on the growth rates of toxic and non-toxic strains of *Microcystis*. During the study, toxic *Microcystis* cells comprised between 12 and 100% of the total *Microcystis* population in Lake Ronkonkoma, NY, and between 0.01 and 6% in three other systems. In all lakes, molecular quantification of toxic (*mcyD*-possessing) *Microcystis* was a better predictor of *in situ* microcystin levels than total cyanobacteria, total *Microcystis,* chlorophyll *a*, or other factors, being significantly correlated with the toxin in every lake studied. Experimentally enhanced temperatures yielded significantly increased growth rates of toxic *Microcystis* in 83% of experiments conducted, but did so for non-toxic *Microcystis* in only 33% of experiments, suggesting that elevated temperatures yield more toxic *Microcystis* cells and/or cells with more *mcyD* copies per cell, with either scenario potentially yielding more toxic blooms. Furthermore, concurrent increases in temperature and P concentrations yielded the

highest growth rates of toxic *Microcystis* cells in most experiments suggesting that future eutrophication and climatic warming may additively promote the growth of toxic, rather than non-toxic, populations of *Microcystis*, potentially leading to blooms with higher microcystin content.

Introduction

Blooms of toxic cyanobacteria are a global public health and environmental concern. Toxic blooms are most commonly formed by *Microcystis*, a well-known producer of the hepatotoxin, microcystin (Carmichael, 1992; Carmichael, 1994; Fleming et al., 2002; Chorus & Bartram, 1999; Pearl, 2008). Long term exposure to microcystin has been associated with severe human health effects, including liver and colorectal cancers (Falconer et al., 1988; Carmichael & Falconer, 1993; Bell & Codd, 1994; Carmichael, 1994; Ito et al., 1997; Chorus & Bartram, 1999; Zegura et al., 2003). One complexity in the field studies of harmful cyanobacteria blooms has been the existence and often co-existence of toxic and non-toxic strains of the same species within a genus that are morphologically and taxonomically indistinguishable (Otsuka et al., 1999; Fastner et al., 2001; Kurmayer et al., 2002). While it is clear that the occurrence of toxic cyanobacteria blooms around the world has increased during recent decades (Chorus & Bartram, 1999; Hudnell & Dortch, 2008), the underlying causes of such blooms and the dynamics of toxic and non-toxic strains within them are poorly understood.

Cyanobacteria blooms are typically associated with eutrophic and poorly flushed waters (Paerl, 1988, 2001; Philipp et al., 1991, Carmichael, 1994; Rapala et al., 1997; Oliver & Ganf 2000). Due to increases in human population density, agriculture, and industrial activities nutrient loading to many freshwater ecosystems has increased (Carpenter et al., 1998). As surface waters become enriched in nutrients, particularly phosphorus (P), there is often a shift in the phytoplankton community towards dominance by cyanobacteria (Smith, 1986; Trimbee & Prepas, 1987; Watson et al., 1997; Paerl & Huisman 2008). Higher phosphorus levels have been shown to yield higher microcystin

content per cell in some cyanobacteria (i.e. *Microcystis*; Utkilen & Gjølme, 1995; Rapala et al., 1997). However, nitrogen (N) may be equally important in the occurrence of toxic, non-diazotrophic cyanobacteria blooms, such as *Microcystis* spp. Laboratory studies have indicated that increasing N loads increases the growth and toxicity of this species (Codd & Poon, 1988; Watanabe & Oishi, 1985; Orr & Jones, 1998). Moreover, previous laboratory research suggests toxic strains of *Microcystis* are able to outgrow non-toxic strains at high nitrogen levels (Vezie et al., 2002). However, no field study has examined how nutrients directly affect the growth of toxic and non-toxic strains of cyanobacteria within wild populations.

The burning of fossil fuels and subsequent rise in atmospheric carbon dioxide has caused the earth's surface temperature to increase by approximately 1^{o}C during the 20th century, with most of the increase having occurred during the last 40 years (IPCC, 2001). In the current century, global temperatures are expected to increase an additional 1.5 to 5^{o}C (Houghton et al., 2001). Frequently, cyanobacteria dominate phytoplankton assemblages in temperate freshwater environments during the warmest periods of the year, particularly in eutrophic systems (Paerl, 1988, 2001; Paerl & Huisman 2008; Paul, 2008). Harmful cyanobacteria such as *Microcystis* have been found to have an optimal temperature for growth and photosynthesis at, or above, 25^{o}C (Konopka & Brock, 1978; Takamura et al., 1985; Robarts & Zohary, 1987, Renyolds, 2006; Jöhnk et al., 2008; Paerl & Huisman, 2008). Furthermore, the cellular toxin content of multiple genera of cyanobacteria increases with increasing temperature to a maximum above 25^{o}C (Van der Westhuizen & Eloff, 1985; Codd & Poon, 1988; Sivonen, 1990; Rapala et al., 1997). However, the growth response of toxic and non-toxic strains of cyanobacteria to

increasing water temperature in an ecosystem setting has yet to be explored.

The purpose of this study was to elucidate the conditions that favor the growth and proliferation of the toxic and non-toxic strains of *Microcystis*. A two-year field study was established in four diverse lake systems across the Northeast US to determine the dynamics of toxic and non-toxic strains of *Microcystis* using molecular quantification of total *Microcystis* cells and compared to *Microcystis* cells possessing the microcystin synthetase gene. Levels of microcystin, nutrients, and other environmental parameters were assessed concurrently. Experiments examined the impacts of elevated nutrient concentrations (N and P) and increased temperature on the growth rates of toxic and non-toxic *Microcystis* populations.

Methods

Study sites

During this study, four sites within the Northeast US were studied. Lake Champlain lies between New York and Vermont, and is connected to the Richelieu River to the north and the Hudson River to the south. It is the largest lake in the Northeast United States other than the Great Lakes. Lake Champlain serves as a drinking water supply to millions of individuals, has been subjected to eutrophication since the 1970's, and is dominated by cyanobacteria in some regions during warm months (Myer & Gruendling, 1979; Shambaugh et al., 1999; Boyer et al., 2004). Our sampling site was Missisquoi Bay (Lat: 44.62 N; Long: 73.37 W), a basin in the northeast extent of Lake Champlain that experiences annual toxic cyanobacteria blooms (Boyer et al., 2004).

Our other sampling sites were located on Long Island, NY, USA, which has recently seen its population expand beyond seven million people. Lake Agawam (Lat:

40.88 N; Long: 72.39 W) is a small (0.5 km^2), shallow (4 m maximum depth) system which experiences annual toxic cyanobacteria blooms dominated by *Microcystis* (Gobler et al., 2007). Mill Pond, Watermill, NY (Lat: 40.91 N; Long: 72.36 W) is a deeper (8 m maximum depth), hypereutrophic system (mean chlorophyll *a* = 200 µg L^{-1}) which also experiences dense cyanobacteria blooms dominated by *Microcystis* cells during summer months. Finally, Lake Ronkonkoma (Lat: 40.83 N; Lon: 73.12 W) is the largest body of freshwater on Long Island (area = 1.5 km^2, maximum depth = 27 m), and experiences summer cyanobacteria blooms comprised of *Microcystis*. In all systems sampled on Long Island, lake transects indicated that blooms were spatially similar with regard to chlorophyll *a*, phycocyanin, and toxin concentrations (data not shown). As such, our sampling sites were representative of each system.

Water quality sampling

Field sampling was conducted bi-weekly before, during, and after cyanobacteria blooms (May – November). In 2005, Lake Ronkonkoma and Lake Agawam were sampled whereas in 2006, Lake Champlain, Lake Agawam, and Mill Pond were studied. At each site, general water quality was evaluated using a handheld YSI 556 sonde to determine surface and bottom temperatures and dissolved oxygen. Twenty liters of surface water was collected and taken to the lab where triplicate chlorophyll *a* and *in vivo* phycocyanin (as a proxy for total cyanobacteria) were measured with Turner Designs fluorometers using standard techniques (Parsons et al., 1984; Watras & Baker, 1988; Lee et al., 1994). For microcystin analysis, whole water was filtered onto triplicate 47 mm GFF glass fiber filters and placed in 5 ml cryovials which were stored at -80°C until analysis. Water samples were filtered through 0.2 µm capsule filters to obtain samples for

dissolved nutrient analysis. Nitrate was analyzed by reducing the nitrate to nitrite using spongy cadmium as per Jones (1984). Nitrite, ammonium, phosphate, and silicate were analyzed using techniques modified from Parsons et al. (1984). Duplicate whole water samples were preserved with Lugol's iodine solution (5% final concentration) to quantify phytoplankton assemblages. For molecular analysis of cyanobacteria, bloom water was filtered onto triplicate 0.22 μm polycarbonate filters, immediately placed in CTAB lysis buffer, and stored at –80°C.

Impacts of nutrients and temperature on toxic and non-toxic cyanobacteria

On selected dates (Table 3), experiments were conducted to assess the impact of increased nutrient concentrations and temperature on toxic and non-toxic *Microcystis* populations. For each of the six experiments, two sets of 12 triplicate, 1L bottles (n = 24) were filled with surface water from each experimental site and were either left unamended to serve as a control, or amended with nitrogen (20 μM NO_3^-), phosphorus (1.25 μM orthophosphate), or both N and P in order to determine which nutrient may favor proliferation of toxic and non-toxic *Microcystis*. One set of bottles (n = 12) was placed in an outdoor incubator receiving ambient light and circulating water maintained at the temperatures found in each lake using commercially available heaters and chillers (Aquatic Eco-systems, Inc., Florida, USA) for 48 - 72 hours. The other set of bottles (n = 12) was incubated at natural light levels and circulating water maintained at elevated temperatures (+4°C above ambient water temperature) achieved with heaters, approximating levels projected for the coming century (+4°C; Houghton et al., 2001). Exact water temperatures administered during experiments were monitored every minute with *in situ* loggers (Onset Computer Corporation, Massachusetts, USA). At the end of

the incubation period, aliquots from experimental bottles were filtered for levels of chlorophyll *a*, and analyzed for *in vivo* phycocyanin. Samples were also filtered at the end of the incubation period as described above to preserve samples for determination of densities of toxic and non-toxic cyanobacteria using molecular methods. Net growth rates of each population were determined as follows: $\mu = \ln [N_t / N_o] / t$ where μ is the rate of population growth (d^{-1}), N_o and N_t are initial and final cell densities, and t is the duration of incubation in days.

Sample Analysis

Microscopic analysis

Densities of *Microcystis* and other co-occurring cyanobacteria were quantified using gridded Sedgewick-Rafter and Utermohl counting chambers. Utermohl chambers were used to quantify populations with cell densities that were low. For dense bloom populations, a gridded Sedgewick-Rafter chamber allowed for accurate assessment of cell densities without the layering of cells which can occur when high biomass samples are concentrated within an Utermohl chamber. For all samples, at least 200 cells were enumerated. To quantify *Microcystis* as well as *Anabaena*, the number of colonies per chamber, as well as the number of cells in 20 colonies, was determined. Such a counting approach provided good reproducibility (< 15% relative standard deviation) on live and preserved samples, as well as precise comparability between live and preserved samples. For this study, only *Aphanizomenon* colonies per chamber were counted.

Microcystin Analysis

Filters for microcystin analyses were extracted in 50% methanol containing 1% acetic acid using ultrasound (four 20 second bursts with a 20 second pause between

bursts). Previous work has shown that this extraction protocol gives > 90% recovery of microcystin-LR (Boyer et al., 2004). Following extraction, the methanolic extract was stored at –80ºC until analysis. Microcystin concentration was measured using the protein phosphatase inhibition assay (PPIA; Carmichael & An, 1999). This analytical protocol does not allow for specific congeners of microcystin to be distinguished but rather provides an indication of the potential biological impact of the microcystins, specifically their ability to inhibit protein phosphatases (Carmichael & An, 1999). A certified microcystin-LR standard (Alexis Biochemicals, San Diego, USA) was used to create the standards for this analysis. This assay yielded a 99.5 ± 8.2% recovery of samples spiked with known amounts of microcystin, a methodological relative standard deviation of 9.4%, and a detection limit of 0.05 $\mu g\ L^{-1}$.

Molecular Analyses

Total cellular nucleic acids were extracted from field and experimental samples using methods described in Coyne et al. (2001). Filtered environmental or experimental samples were submersed in CTAB buffer (Dempster et al., 1999), supplemented with 20 $\mu g\ L^{-1}$ pGEM-3z(f+) plasmid (Promega; Table 1) which served as an internal control for extraction efficiency and PCR inhibition (Coyne et al., 2005). The filters were then flash frozen and stored at –80°C until extraction. Nucleic acids were extracted after an initial heating step at 65˚C, followed by a double chloroform extraction, and an isopropanol precipitation. Extracted nucleic acids were resuspended in 20µl of LoTE buffer. The quantity and quality of nucleic acids was assessed with a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Two *Microcystis*-specific genetic targets were used during this study, the 16S rRNA gene (16S rDNA) and *mcyD* gene. The *Microcystis* 16S rRNA gene is specific to the *Microcystis* genus which allowed us to quantify the abundance of the total *Microcystis* population. The *mcyD* gene is found within the microcystin synethtase gene operon which is responsible for the production of microcystin and is only found in toxic strains of *Microcystis* (Tillett et al., 2000) which allowed us to quantify the toxic population of *Microcystis* (Rinta-Kanto et al., 2005). QPCR was carried out using an ABI 7300 Real Time PCR instrument using TaqMan® labeled probes (Applied Biosystems) and *Microcystis*-specific *mcyD* and 16S rDNA primers (Table 1). Each 10µL reaction included 5µL of 2x TaqMan® Master Mix (Applied Biosystems), 10µM each primer (Integrated DNA Technologies), 10µM TaqMan® probe (Table 1) and 1µl of a 1:25 dilution of the unknown DNA or standard. For amplification of the pGEM and 16S targets, the cycling conditions were 95ºC for 10 minutes, followed by 55 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. In order to amplify the *mcyD* gene, the cycling conditions were 95ºC for 10 minutes, followed by 55 cycles of 95ºC for 15 seconds, followed by 50ºC for 1 minute, then 60ºC for 1 minute. To prepare standard samples, cultured *Microcystis aeruginosa*, clone LE-3 (Rinta-Kanto et al., 2005), was enumerated by standard microscopy and collected on polycarbonate filters which were prepared and extracted as outlined above. A standard curve of dilutions of the extracted LE-3 genomic DNA was run with each analytical run to serve as a reference for numbers of toxic *Microcystis* spp. cells. Since some *Microcystis* spp. cells may carry multiple copies of the 16S rDNA gene and *mcyD* gene, data was expressed as "cell equivalents" rather than cell number (Rinta-Kanto et al., 2005). The numbers of toxic and total

Microcystis cells were determined using the ΔΔCT method (Livak & Schmittgen, 2001; Coyne et al., 2005). The difference between the number of *mcyD* cell equivalents (toxic cells) and 16S rDNA cell equivalents (total cells) indicated the number of non-toxic cell equivalents (Rinta-Kanto et al., 2005). Toxic *Microcystis* spp. cell abundances were determined for all locations and on all dates. Total *Microcystis* cells (16S rDNA) were quantified for all systems except Lake Agawam 2006 and Mill Pond 2006. For these two data sets, total *Microcystis* cells equivelents were only quantified on the days of experiments

Statistical Analysis

All time series data sets from each location (chlorophyll *a*, phycocyanin, toxic *Microcystis* cell densities, non-toxic *Microcystis* densities, total *Microcystis* densities, total cyanobacteria cells, microcystin concentration, dissolved inorganic phosphorus (DIP), dissolved inorganic nitrogen (nitrate + ammonium = DIN), and temperature were statistically analyzed using a Pearson's correlation matrix to establish the degree to which individual variables were correlated. Student t-tests were used to determine if the means of different populations (toxic and non-toxic) were significantly different from each other. The effects of nitrogen, phosphorus, and temperature on the net growth rate of toxic and non-toxic *Microcystis* populations were analyzed using three-way ANOVAs with nitrogen, phosphorus, and temperature considered treatment effects ($\alpha = 0.05$). Post-hoc comparisons of significant impacts were elucidated with Tukey's multiple comparison tests. For all results the standard variance presented was ± one standard deviation (SD).

Results

Dynamics of toxic *Microcystis* blooms

Lake Agawam, Long Island, NY

During 2005, Lake Agawam hosted mixed cyanobacteria blooms numerically dominated by *Microcystis* from June through September (Table 2). Blooms contained mean chlorophyll *a* levels of 97 ± 28 μg L^{-1} (mean ± SD) and coincided with peaks of *in vivo* phycocyanin (represented the total cyanobacterial community; Fig 1). Densities of non-toxic *Microcystis* ranged from 3.9 x 10^6 to 1.0 x 10^8 cells L^{-1} during blooms with peak densities achieved on 20 October (Fig 1). Concurrently, toxic *Microcystis* cell densities (possessing the *Microcystis*-specific *mcyD* gene) ranged from 5.0 x 10^2 to 5.0 x 10^5 cell equivalents L^{-1} (Fig 1), peaking on 21 June and representing between 0.01% and 1.56% of total *Microcystis* cells. Microcystin was detectable in the water column from May through October ranging from 0.86 to 11.8 μg L^{-1}, with the highest levels occurring at the peak in toxic *Microcystis* cell densities (Fig 1). Concentrations of microcystin were significantly correlated with toxic *Microcystis* cell equivalents ($p < 0.02$). DIN levels during the summer months (July - August) ranged from 2.67 to 6.52 μM, while DIP concentrations never exceeded 0.38 ± 0.08 μM (mean = 0.25 ± 0.06 μM; Fig 1). During September when algal biomass declined, DIN and DIP levels increased (Fig 1). Temperatures in Lake Agawam during 2005 rose from 13ºC in May to 29ºC in August, and then declined to 15ºC in late October.

During 2006, the temporal dynamics of cyanobacterial blooms in Lake Agawam differed from 2005. Lake Agawam hosted cyanobacteria blooms dominated by *Anabaena* during June and early July with maximal concentrations reaching 2.79 x 10^8

cells L^{-1} (Table 2). This bloom was succeeded by a bloom of *Microcystis* during mid-July which remained dominant through October (Table 2). *Aphanizomenon* was present throughout the sampling period but was never dominant (Table 2). Cyanobacteria blooms contained mean chlorophyll *a* levels of 209 ± 17.0 μg L^{-1} and coincided with peaks of *in vivo* phycocyanin (Fig 2). Densities of toxic *Microcystis* ranged from 5.3 x 10^3 to 3.5 x 10^6 cell equivalents L^{-1}, peaking on 10 October (Fig 2). Particulate microcystin was detectable throughout the study period ranging from 0.69 to 81.1 μg L^{-1}, with the highest levels occurring concurrently with the peak in toxic *Microcystis* cells (Fig 2). Similar to 2005, microcystin concentrations were significantly correlated with toxic *Microcystis* densities ($p < 0.001$). DIN levels during the bloom months (June-October) ranged from 3.03 to 48.81 μM and were inversely related to phycocyanin concentrations, being higher before and after bloom events (Fig 2). During June and early July DIP concentrations were 1.54 ± 0.44 μM but rose later in the sampling season (Fig 2). Surface temperatures in 2006 ranged from 22ºC in June to 29ºC in July, declining to 13ºC by October.

Lake Ronkonkoma, Long Island, NY

Lake Ronkonkoma was the only system examined which hosted cyanobacteria blooms that were first dominated by *Microcystis* and were succeeded by *Anabaena* (Table 2). *Aphanizomenon* was either absent, or at low densities (i.e. < 50,000 colonies L^{-1}; Table 2). Blooms (June-August) contained mean chlorophyll *a* levels of 20.2 ± 8.0 μg L^{-1} and coincided with peaks of *in vivo* phycocyanin (Fig 3). May through June *Microcystis*-dominated blooms (Table 2) were comprised exclusively of toxic cells (1.98 x 10^8 cell equivalents L^{-1} maximum on 27 May; Fig 3), until late June bloom composition

shifted toward dominance by non-toxic cells (peak densities 1.20 x 10^6 cell equivalents L^{-1} on 25 July) before declining during the late summer (Figure 3). Through the sampling period, toxic *Microcystis* cells comprised between 12% and 100% of the total *Microcystis* population and microcystin levels ranged from 1.28 to 78.8 μg L^{-1} (Fig 3). In a manner similar to Lake Agawam, there was a significant correlation between microcystin concentrations and densities of toxic *Microcystis* cells ($p < 0.001$). Nutrient concentrations during the bloom months (June- September) ranged from 4.17 to 11.13μM DIN and 0.19 to 0.71 μM DIP (Fig 3). Finally, surface temperatures ranged from 16ºC in May to 30ºC in August and decreased to 25ºC by September.

Mill Pond, Long Island, NY

Cyanobacterial bloom dynamics within Mill Pond during the summer of 2006 were similar to that of Lake Agawam during the same year. Mill Pond hosted cyanobacteria blooms dominated by *Anabaena* and *Aphanizomenon* from June through early July (Table 2). This bloom subsided and a bloom of *Microcystis* occurred and remained dominant throughout the summer and early fall (Table 2). Blooms (June - October) contained mean chlorophyll *a* levels of 96.9 ± 57.3 μg L^{-1} and coincided with peaks of *in vivo* phycocyanin (Fig 4). Densities of toxic *Microcystis* ranged from 3.3 x 10^3 to 4.1 x 10^6 cell equivalents L^{-1}, achieving the highest concentration on 12 October coinciding with the highest levels of microcystin (Fig 4). Microcystin levels ranged from 5.50 to 154 μg L^{-1}, and were significantly correlated with toxic *Microcystis* cells during the sampling period ($p < 0.001$). DIN concentrations varied widely (6.82 to 81.0 μM), with the highest concentrations present during late spring and fall (Fig 4). DIP concentrations in Mill Pond were also elevated with a mean concentration of 4.93 ± 0.06

μM (Fig 4). Surface temperatures in this system ranged from 22ºC in June to 28ºC in July declining to 12ºC by October.

Lake Champlain

Lake Champlain's Missisquoi Bay was devoid of cyanobacteria until July and was never numerically dominated by *Microcystis* in 2006 (Table 2). A cyanobacteria bloom which occurred during late summer contained mean chlorophyll *a* levels of 52.0 ± 27.5 μg L^{-1}, and coincided with peaks of *in vivo* phycocyanin (mid-July through mid-October; Fig 5), and were dominated by *Anabaena* at a density of 4.91 ± 0.21 x10^7 cells L^{-1} on 1 August (Table 2). *Microcystis* was present in the water column from July through October (Table 2) with densities of non-toxic *Microcystis* ranging from 1.4 x 10^4 to 2.1 x 10^7 cells L^{-1} during blooms (peak densities on 1 August; Table 2). Toxic *Microcystis* cells were detectable from August through September (0.24 - 9.79 x 10^5 cell equivalents L^{-1}; Fig 5) representing approximately 6% of total *Microcystis* cells. Microcystin was present in the water column from May through October at levels that were lower than other systems (0.10 to 1.95 μg L^{-1}, Fig 5) and that peaked on 1 August during the maximum densities of toxic *Microcystis* cells (Fig 5). As was found in other systems, microcystin concentrations in Lake Champlain were significantly correlated with toxic *Microcystis* cell equivalents L^{-1} ($p<0.04$). Nutrient levels were elevated before the onset of *Microcystis* blooms (May – early July DIN = 11.95 ± 4.97 μM; DIP = 0.32 ± 0.08 μM), but were lower during the peak of the bloom (mid July – September DIN = 8.12 ± 3.91 μM; DIP = 0.26 ± 0.19 μM; Fig 5). Nutrient levels increased again in October. The bloom peak also corresponded with the highest temperatures of the sampling campaign (26ºC), with initial and final sampling temperatures close to 10ºC.

Effects of increased temperature and nutrients on toxic and non-toxic strains of *Microcystis*

Lake Agawam

During the July 2005 experiment in Lake Agawam, ambient water temperature was 26.9 ± 1.7ºC while the elevated temperature treatment was 30.6 ± 1.4ºC. Elevated temperature was a significant treatment effect which enhanced both toxic and non-toxic *Microcystis* growth rates by 2 – 3 fold ($p<0.01$; Table 3; Fig 6). Nutrients did not significantly alter growth rates and there were no significant interactions between treatments for either sub-population of *Microcystis* (Table 3; Fig 6).

During the October 2006 experiment in Lake Agawam, temperature was a significant treatment effect which doubled toxic *Microcystis* growth rates compared to the control treatment ($p < 0.05$; Table 3; Fig 6). Furthermore, although the interaction between temperature and phosphorus was not significant, this dual treatment yielded a growth rate that was 125% greater than the control and was the highest growth rate of either population in any treatment (1.87 ± 0.13 d^{-1}; Fig 6). Interestingly, increased temperatures decreased the growth rate of the non-toxic population relative to the control ($p < 0.05$; Table 3; Fig 6). Similarly, the interaction of temperature and phosphorus yielded a growth rate for non-toxic *Microcystis* that was significantly lower than the control ($p < 0.05$; Table 3; Fig 6). Ambient and elevated temperatures were 10.6 ± 0.98 and 14.9 ± 0.81ºC, respectively, for this experiment.

Lake Ronkonkoma

During the Lake Ronkonkoma experiment, ambient water temperature was 20.9 ± 0.97ºC. Experimentally enhanced temperatures (26.7 ± 0.7ºC) significantly increased

growth rates of toxic *Microcystis* strains by 89% ($p < 0.05$; Table 3; Fig 6). N enrichment lead to a significant decrease in the growth rate of the non-toxic subpopulation of *Microcystis* ($p < 0.05$; Table 3; Figure 6), but did not alter the growth rate of the toxic strains (Table 3; Fig 6). In addition, P enrichment yielded a significant, ~2-fold increase in the growth rates of both toxic and non-toxic strains of *Microcystis* ($p < 0.001$; Table 3; Fig 6). There was a significant interaction between N and P on toxic *Microcystis* growth rates ($p < 0.01$; Table 3; Fig 6), likely due to the slightly lower growth response in the N and P treatments compared to P only. There was a significant interaction between temperature and P on the non-toxic strains of *Microcystis* ($p < 0.01$; Table 3; Fig 6). There was also a significant interaction between N, P, and temperature for the non-toxic population of *Microcystis* ($p < 0.02$) perhaps due to the growth rate being lower than that of the temperature / P enrichment (Fig 6).

Mill Pond

During the June experiment in Mill Pond, ambient water temperature was 23.6 ± 2.7˚C and elevated temperature was 26.4 ± 1.9˚C. Temperature was a significant treatment effect which increased the growth rate of toxic *Microcysits* by 22% relative to the control ($p < 0.05$; Table 3; Fig 6). Higher phosphorus concentrations also increased the growth rate of toxic *Microcystis* (by 33% relative to the control), but not significantly ($p > 0.05$; Fig 6). However, temperature and phosphorus acted in unison to yield a growth rate for toxic *Microcystis* that was 63% higher then the control and was the highest growth rate of either population in any treatment (1.55 ± 0.36 d^{-1}, Fig 6). The growth rate of the non-toxic *Microcystis* population was not significantly altered by any

treatment or interaction (Fig 6). Moreover, growth rates of this population were lower than the growth rates for toxic *Microcystis* in all treatments (Fig 6).

During the July experiment, water temperatures were similar to the June experiment (23.8 ± 3.1 and 28.9 ± 1.6ºC for ambient and elevated, respectively). Although none of the individual treatments yielded a significantly increased growth rate for either population, the interaction between temperature and phosphorus was significant for toxic *Microcystis* cells as this dual treatment yielded toxic population growth rates that were 20% higher than the control ($p < 0.01$; Table 3; Fig 6). Furthermore, although not statistically significant, concurrent enrichment of temperature, N and P yielded a growth rate for toxic *Microcystis* that was 40% greater then the control and was the highest growth rate for either population among all the treatments (1.54 ± 0.11 d^{-1}; Fig 6). On the other hand, the interaction between N and P was significant for the non-toxic population yielding growth rates which were enhanced 3-fold over unamended controls ($p < 0.01$; Table 3; Fig 6).

Lake Champlain

During the Lake Champlain experiment, ambient water temperatures were 24.9 ± 2.0ºC. Experimentally enhanced temperatures (29.1 ± 1.3ºC) significantly increased the growth rates of both toxic and non-toxic *Microcystis* populations by 80% and 101% respectively ($p < 0.001$; Table 3; Fig 6). Increased nitrogen concentrations stimulated growth rates of the toxic *Microcystis* population, increasing them 40% over the unamended controls ($p < 0.01$; Table 3; Fig 6). Furthermore, phosphorus enrichment significantly increased the growth rates of both toxic and non-toxic populations by 60% and 68%, respectively ($p < 0.001$; Table 3; Fig 6). Finally, nutrients (N or P) interacted

with temperature, to enhance toxic *Microcystis* growth rates ($p < 0.01$; Table 3; Fig 6) with the enhanced P and temperature treatment yielding the highest growth rates of any population among all treatments (1.17 ± 0.03 d^{-1}).

Discussion

Harmful cyanobacterial blooms have increased globally in frequency and intensity in recent decades. Eutrophication and warmer temperatures are often cited as key factors which promote these events (Paerl, 1988; Chorus & Bartram, 1999; Hudnell & Dortch 2008; Paerl & Huisman, 2008). Previous studies have investigated the effects of singular environmental factors on the growth and/or abundance and/or photosynthesis of total *Microcystis* populations such as light (Codd & Poon, 1988; Wiedner et al., 2003; Kim et al., 2005), nutrient enrichment (Watanabe & Oishi, 1985; Codd & Poon, 1988; Fujimoto et al., 1997; Orr & Jones, 1998; Lee et al., 2000; Oh et al., 2000; Paerl et al., 2001; Vézie et al., 2002; Downing et al., 2005; Gobler et al., 2007), salinity (Tonk et al., 2007) and temperature (Konopka & Brock, 1978; Takamura et al., 1985; Robarts & Zohary, 1987). Other studies have found that *Microcystis* can out-compete other species of phytoplankton at high temperatures (≥ 30ºC; Fujimoto et al., 1997). However, to our knowledge, this is the first field study to investigate the effects of increased temperature and nutrient concentrations on the growth rates of toxic and non-toxic subpopulations of *Microcystis*. Our ability to examine this phenomenon along with the *in situ* dynamics of these two populations within four distinct ecosystems during two years has generated a data set which provides new insight regarding the ecology of toxic *Microcystis* blooms.

Microcystin was detected in all four ecosystems on every date analyzed (Figs 1 – 5). Toxic *Microcystis* cells comprised between 0.01% and 100% of the total *Microcystis*

population among the four systems, a range similar to those found in prior studies (0.01% and 100%; Kurmayer & Kutzenberger, 2003; Rinta-Kanto & Wilhelm, 2006; Yoshida et al., 2006; Rinta-Kanto et al., 2009). However, there were notable differences, even among the systems studied here. In Lake Ronkonkoma, toxic *Microcystis* comprised between 12 and 100% of total cells, whereas the range was between 0.01 and 6% in other systems. The seasonal dynamics of toxic and non-toxic *Microcystis* observed in Lake Ronkonkoma, the only stratified lake studied, were consistent with the findings of Kardinaal et al. (2007) who also found toxic strains of *Microcystis* were succeeded by non-toxic strains in a deep stratified lake. However, in the well-mixed systems we studied (Lake Agawam, northeastern Lake Champlain, Mill Pond), toxic strains comprised a small portion of total cells where as Kardinaal et al. (2007) found the toxic strains dominated *Microcystis* populations in the two unstratified lakes. The seasonal dynamics of cyanobacterial blooms in general, and toxic and non-toxic strains of *Microcystis* in particular, likely vary based on system-specific physical and/or environmental conditions.

The World Health Organization (WHO) currently recommends monitoring chlorophyll *a* concentrations and total cyanobacterial cell counts to protect against human exposure to high levels of microcystin (Chorus & Bartram 1999). However, of the five field data sets generated by this study (Fig 1 – 5), only two displayed mildly significant correlations between chlorophyll *a* concentrations and concentrations of microcystin ($p < 0.05$) while three showed mildly significant correlations between total cyanobacterial cell counts and microcystin concentrations ($p < 0.05$). This is not surprising as all phytoplankton contain chlorophyll *a* and nearly every major species of cyanobacteria has

both toxic and non-toxic strains (Chorus & Bartram, 1999). However, in all five of the time series data sets generated by this study, densities of toxic *Microcystis* cells were significantly correlated with microcystin levels ($p < 0.05$ for all; Fig 1 – 5), often at a very high level of significance (Lake Ronkonkoma, Lake Agawam, Mill Pond; $p < 0.001$). Obviously, the predominance of other microcystin producing genera will influence the relationship between toxic *Microcystis* cells and microcystin (Rantala et al., 2006) and the presence of the *mcyD* gene does not necessarily translate into the synthesis of microcystin *in situ* (Gobler et al., 2007). Despite this, our data demonstrates that *mcyD*-containing *Microcystis* cells were a better predictor of pelagic microcystin concentrations than the total cell counts of cyanobacteria or chlorophyll *a* and thus may be a better predictor of microcystin in aquatic ecosystems than parameters currently recommended to be monitored by the WHO.

As seasonal temperatures increase from 10 to 30ºC in freshwater ecosystems, the phytoplankton group with the highest growth rate generally shifts from diatoms to green algae to cyanobacteria (Canale & Vogel, 1974). Furthermore, it has generally been noted that cyanobacteria dominate phytoplankton assemblages in temperate freshwater environments during the warmest periods of the year, particularly in eutrophic systems (Canale & Vogel, 1974; Reynolds & Walsby, 1975; Konopka & Brock, 1978; Tilman & Kiesling, 1984; Paerl, 1988, Paerl et al., 2001; Paerl & Huisman, 2008). Consistent with this idea, cyanobacteria in general, and *Microcystis* in particular, dominated all of our study sites as temperatures reached their annual peak (Figs 1 – 5). While toxic *Microcystis* cells densities peaked during peak temperatures in half of our study sites (Lake Agawam, Lake Champlain; Fig 1, 5) they were slightly off-set in two other sites

(Mill Pond, Lake Ronkonkoma; Fig 3, 4) likely reflecting the additive role other factors such as nutrients can play in bloom dynamics. More importantly, during five of the six (83%) experiments conducted, a ~4ºC increase in experimental temperatures yielded significantly higher (22 – 115%) growth rates for the toxic (*mcyD*-containing) *Microcystis* cells (Fig 6). In contrast, the growth rates of non-toxic *Microcystis* were significantly increased by higher temperature in only a third of experiments conducted (Fig 6). Furthermore, in five of six experiments, the growth rate of toxic *Microcystis* cells exceeded those of non-toxic cells within the enhanced temperature only treatment (Fig 6). All experimentally elevated temperatures were between 15 and 30ºC, the optimal range for *Microcystis* (Chorus & Bartram, 1999), and in all but one experiment (Lake Agawam, 2006; temperature = 15ºC), the elevated temperatures fell between 25 and 30ºC, a range which yields maximal cellular toxin content in *Microcystis* (Van der Westhuizen & Eloff, 1985; Codd & Poon, 1988). Kim et al. (2005) found that toxic *Microcystis* strains cultured at 25ºC had more *mcyB* transcripts than cultures reared at 20ºC or 30ºC. Our observed increase in the abundance of 'toxic' *Microcystis* during higher temperatures could represent cells with more gene copies per cell, more toxic cells, or both. However, any of these scenarios could lead to higher levels of microcystin in aquatic ecosystems (Tillett et al., 2000; Rinta-Kanto & Wilhelm, 2006). Hence, as surface water temperatures continue to rise (Houghton et al., 2001), toxic *Microcystis* may out-grow non-toxic *Microcystis* or may synthesize more microcystin, yielding blooms that are comprised of a larger proportion of toxic cells and/or have higher microcystin concentrations. Moreover, as predicted by Paerl & Huisman (2008), future

warming of temperate aquatic systems could lead to toxic *Microcystis* dominating for longer time periods than they presently do so long as there is an adiquite nutrient supply.

During two of six experiments conducted (Lake Ronkonkoma, Lake Champlain), phosphorus enrichment yielded significantly higher growth rates for both toxic and non-toxic populations of *Microcystis*, although growth rates were not significantly different between these populations relative to control treatments (Table 3; Fig 6). These findings are contrary to those of Vézie et al. (2002) who found that at higher P concentrations the growth rates of toxic *Microcystis* exceeded non-toxic strains. However, our results were reasonable in light of the ambient nutrient levels as DIP was low (0.2 and 0.6 μM, respectively) and DIN concentrations were greater than 11 μM during these experiments (Fig 3, 5). DIP levels were substantially higher within the two shallower and more eutrophic systems we studied (Mill Pond, Lake Agawam; Fig 1, 2, 4) and P never affected *Microcystis* growth in these systems. Nitrogen enrichment significantly increased the growth rate of toxic *Microcystis* in Lake Champlain (Table 3; Fig 6), a result consistent with Vézie et al. (2002) who found that increasing N concentrations in N-limited cultures significantly increased the growth rates of both toxic and non-toxic subpopulations of *Microcystis*. This result is also consistent with Gobler et al. (2007) who found that nitrogen can promote the growth and microcystin production of *Microcystis* during bloom events.

During many experiments we conducted, nutrients (N or P) and temperature interacted to promote the growth of toxic *Microcystis*. In Lake Champlain, Mill Pond (July 2006), and Lake Agawam (2006), the enhancement of temperature and P yielded growth rates for toxic *Microcystis* that not only increased 170%, 125%, and 20%,

respectively, relative to the controls but also yielded the highest growth rate of either population in any treatment (Fig 6). Similarly, during the Mill Pond experiment conducted during July 2006, the enrichment of N, P, and temperature yielded a growth rate of toxic *Microcystis* that was 40% greater than the control and was the highest growth rate of either population in all treatments (Fig 6). These results are somewhat consistent with the findings of Jiang et al. (2008), who documented that higher temperature and P yielded greater abundance of total *Microcystis* cells. More importantly, these results suggest that future nutrient loading coupled with climatic warming may promote toxic, rather than non-toxic, populations of *Microcystis* and thus may lead to more toxic blooms.

This study has demonstrated that temperature and nutrients can interact to strongly influence the abundance and relative dominance of toxic and non-toxic strains of *Microcystis* within bloom events across the Northeast US. However, our previous research has demonstrated that even toxic populations of *Microcystis* show seasonal changes in expression of the microcystin synthetase gene (Gobler et al., 2007). As such, an important open question is the degree to which environmental factors such as nutrient loading and temperature change influence microcystin synthetase gene expression and cellular microcystin synthesis. Moreover, whether other microcystin synthesizing strains of other toxic cyanobacteria genera are promoted by warming and eutrophication remains unknown.

In conclusion, I found that the portion of wild *Microcystis* populations that were comprised of toxic cells varied seasonally and by location from 0.01 to 100%. Molecularly quantifying toxic (*mcyD*-containing) *Microcystis* was a better predictor of *in*

situ microcystin levels than proxies currently recommended by the WHO to protect against human exposure to microcystin (total cyanobacteria cell densities or chlorophyll *a*; Chorus & Bartram, 1999). Warmer temperatures frequently (83% of experiments) shifted *Microcystis* toward populations comprised of a larger percentage of toxic *Microcystis* and/or cells with more *mcyD* copies per cell, scenarios which could yield more toxic blooms in an ecosystem setting. Finally, this study demonstrated that higher temperatures coupled with elevated P concentrations frequently yielded growth rates of toxic *Microcystis* cells which exceeded all other treatments and populations. Therefore, continued climatic warming and increased eutrophication could lead to a shift towards more toxic *Microcystis* blooms.

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DNA Target	Primer	Sequence(5'-3')	Reference
pGEM plasmid DNA	M13F	CCCAGTCACGACGTTGTAAAAACG	Coyne *et al.* 2005
	pGEM R	TGTGTGGAATTGTGAGCGGA	Coyne *et al.* 2005
	pGEM probe	(Taq) FAM[a]-CACTATAGAATACTCAAGCTTGCATGCCTGCA-BHQ-1[b]	Coyne *et al.* 2005
***Microcystis* 16S rDNA**	184F	GCCGCRAGGTGAAAMCTAA	Neilan *et al.* 1997
	431R	AATCCAAARACCTTCCTCCC	Neilan *et al.* 1997
	Probe	(Taq) FAM[a]-AAGAGCTTGCGTCTGATTAGCTAGT-BHQ-1[b]	Rinta-Kanto *et al.* 2005
***Microcystis* mcyD**	F2	GGTTCGCCTGGTCAAAGTAA	Kaebernick *et al.* 2000
	R2	CCTCGCTAAAGAAGGGTTGA	Kaebernick *et al.* 2000
	Probe	(Taq) FAM[a]-ATGCTCTAATGCAGCAACGGCAAA-BHQ-1[b]	Rinta-Kanto *et al.* 2005

Table 1: A list of primers (Integrated DNA Technologies, Iowa, USA) and probes (Applied Biosystems, Foster City, CA, USA) used in the qPCR analysis; a= 6-Carboxyfluorescein b= Black Hole Quencher-1 (quenching range 480-580 nm)
F= forward primer R= reverse primer.

	*Microcystis**	*Anabaena**	*Aphanizomenon***
Lake Agawam 2005			
26-May-05	68,666,000 (3,433,000)	1,100,000 (141,000)	0 (0)
7-Jun-05	-	14,667,000 (1,604,000)	0 (0)
21-Jun-05	28,300,000 (1,415,000)	700,000 (141,000)	0 (0)
5-Jul-05	53,300,000 (2,665,000)	8,733,000 (2,810,000)	30 (0)
18-Jul-05	58,700,000 (2,935,000)	17,400,000 (3,504,000)	0 (0)
1-Aug-05	60,600,000 (3,030,000)	0 (0)	0 (0)
12-Aug-05	201,300,000 (10,065,000)	3,467,000 (611,000)	140,000 (14,000)
23-Aug-05	52,000,000 (2,600,000)	3,067,000 (1,007,000)	260,000 (30,000)
20-Sep-05	2,000,000 (100,000)	1,700,000 (141,000)	0 (0)
13-Oct-05	-	-	-
20-Oct-05	-	-	-
8-Nov-05	-	-	-
Lake Agawam 2006			
2-Jun-06	76,945,000 (3,847,000)	279,000,000 (13,950,000)	520,000 (26,000)
16-Jun-06	1,200,000 (60,000)	45,500,000 (2,275,000)	30,000 (2,000)
22-Jun-06	55,491,000 (2,775,000)	201,500,000 (10,075,000)	0 (0)
27-Jun-06	3,755,000 (188,000)	265,000,000 (13,250,000)	370,000 (19,000)
6-Jul-06	14,850,000 (743,000)	60,000,000 (3,000,000)	30,000 (2,000)
11-Jul-06	122,960,000 (6,148,000)	28,500,000 (1,425,000)	90,000 (5,000)
19-Jul-06	693,000,000 (34,650,000)	50,000,000 (2,500,000)	390,000 (20,000)
25-Jul-06	429,418,000 (21,471,000)	8,500,000 (425,000)	340,000 (17,000)
10-Aug-06	1,483,227,000 (74,161,000)	15,000,000 (750,000)	570,000 (29,000)
12-Sep-06	76,691,000 (3,835,000)	35,000,000 (1,750,000)	140,000 (7,000)
28-Sep-06	625,527,000 (31,276,000)	12,500,000 (625,000)	150,000 (8,000)
10-Oct-06	436,582,000 (21,829,000)	15,000,000 (750,000)	0 (0)
24-Oct-06	332,000,000 (16,600,000)	16,500,000 (825,000)	40,000 (2,000)
Lake Ronkonkoma 2005			
27-May-05	1,000,000 (0)	0 (0)	0 (0)
8-Jun-05	113,000,000 (6,062,000)	0 (0)	0 (0)
23-Jun-05	-	-	-
5-Jul-05	67,667,000 (6,506,000)	8,000,000 (1,000,000)	0 (0)
25-Jul-05	7,000,000 (2,000,000)	9,500,000 (1,000,000)	25,000 (7,000)
5-Aug-05	2,000,000 (0)	2,500,000 (0)	50,000 (10,000)
18-Aug-05	4,000,000 (0)	5,167,000 (764,000)	20,000 (10,000)
15-Sep-05	-	-	-
Lake Champlain 2006			
22-May-06	0 (0)	0 (0)	0 (0)
19-Jun-06	293,000 (15,000)	0 (0)	0 (0)
3-Jul-06	0 (0)	0 (0)	0 (0)
17-Jul-06	953,000 (48,000)	1,067,000 (53,000)	0 (0)
1-Aug-06	11,480,000 (574,000)	41,867,000 (2,093,000)	213,000 (11,000)
19-Sep-06	1,027,000 (51,000)	2,400,000 (120,000)	5,893,000 (295,000)
3-Oct-06	200,000 (10,000)	0 (0)	80,000 (4,000)
17-Oct-06	60,000 (3,000)	0 (0)	0 (0)
Mill Pond 2006			
2-Jun-06	9,082,000 (454,000)	27,000,000 (1,350,000)	1,050,000 (53,000)
16-Jun-06	18,600,000 (930,000)	78,500,000 (3,925,000)	1,970,000 (99,000)
22-Jun-06	22,636,000 (1,132,000)	46,000,000 (2,300,000)	620,000 (31,000)
27-Jun-06	5,155,000 (258,000)	0 (0)	840,000 (42,000)
6-Jul-06	61,600,000 (3,080,000)	3,000,000 (150,000)	13,970,000 (699,000)
11-Jul-06	244,873,000 (12,244,000)	0 (0)	490,000 (25,000)
19-Jul-06	268,736,000 (13,437,000)	0 (0)	2,370,000 (119,000)
25-Jul-06	181,909,000 (9,095,000)	1500,000 (75,000)	4,410,000 (221,000)
10-Aug-06	264,955,000 (13,248,000)	5,000,000 (250,000)	60,000 (3,000)
12-Sep-06	158,782,000 (7,939,000)	69,000,000 (3,450,000)	1,630,000 (82,000)
28-Sep-06	75,164,000 (3,758,000)	2,000,000 (100,000)	2,020,000 (101,000)
10-Oct-06	529,200,000 (26,460,000)	0 (0)	390,000 (20,000)
24-Oct-06	17,338,000 (867,000)	0 (0)	690,000 (35,000)

Table 2: Mean cyanobacterial densities (cells* or colonies** L^{-1}) (SD in parentheses) for all systems sampled. Counts were made using lightmicroscopy. Dashed lines indicate samples were not available.

	Treatment Effects		Interactions	
	Toxic	Non-toxic	Toxic	Non-toxic
Lake Agawam				
18-Jul-05	T**	T**	-	-
24-Oct-06	T*	-T***	-	-T&N**, -T&P**
Lake Ronkonkoma				
5-Jul-05	T*,P***	P***	N&P**	T&P**, T&N&P*
Lake Champlain				
1-Aug-06	T***, N**, P***	T***, P***	T&N**, T&P***, N&P***	-
Mill Pond				
27-Jun-06	T*	-	-	-
11-Jul-06	-	-	T&P**	N&P**

Table 3: Experimentally significant treatment effects and interactions on the growth rates of toxic and non-toxic *Microcystis* as determined by a 3-way ANOVA. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. A negative sign before a nutrient indicates that the treatment yielded a significant decrease in growth rates, whereas all others yielded significantly higher growth rates.

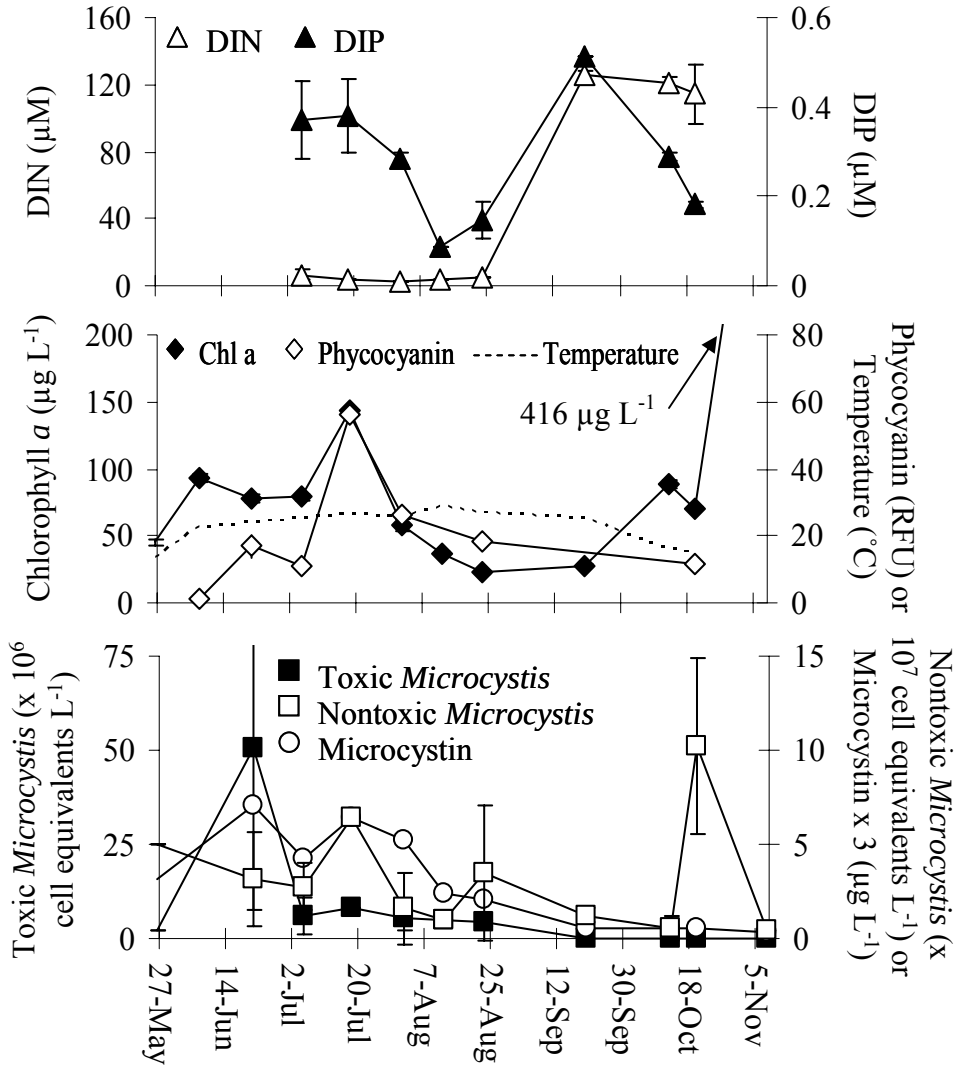

Figure 1: Time series of parameters measured in the Lake Agawam, 2005. (A) Concentrations of dissolved inorganic nitrogen (DIN △) and orthophosphate (DIP ▲). (B) Levels of total chl *a* ♦, phycocyanin ◊, and temperature (---). (C) Densities of toxic ■ and non-toxic *Microcystis* □ as well as concentrations of microcystin ○. Error bars represent ± 1 SD of replicated samples

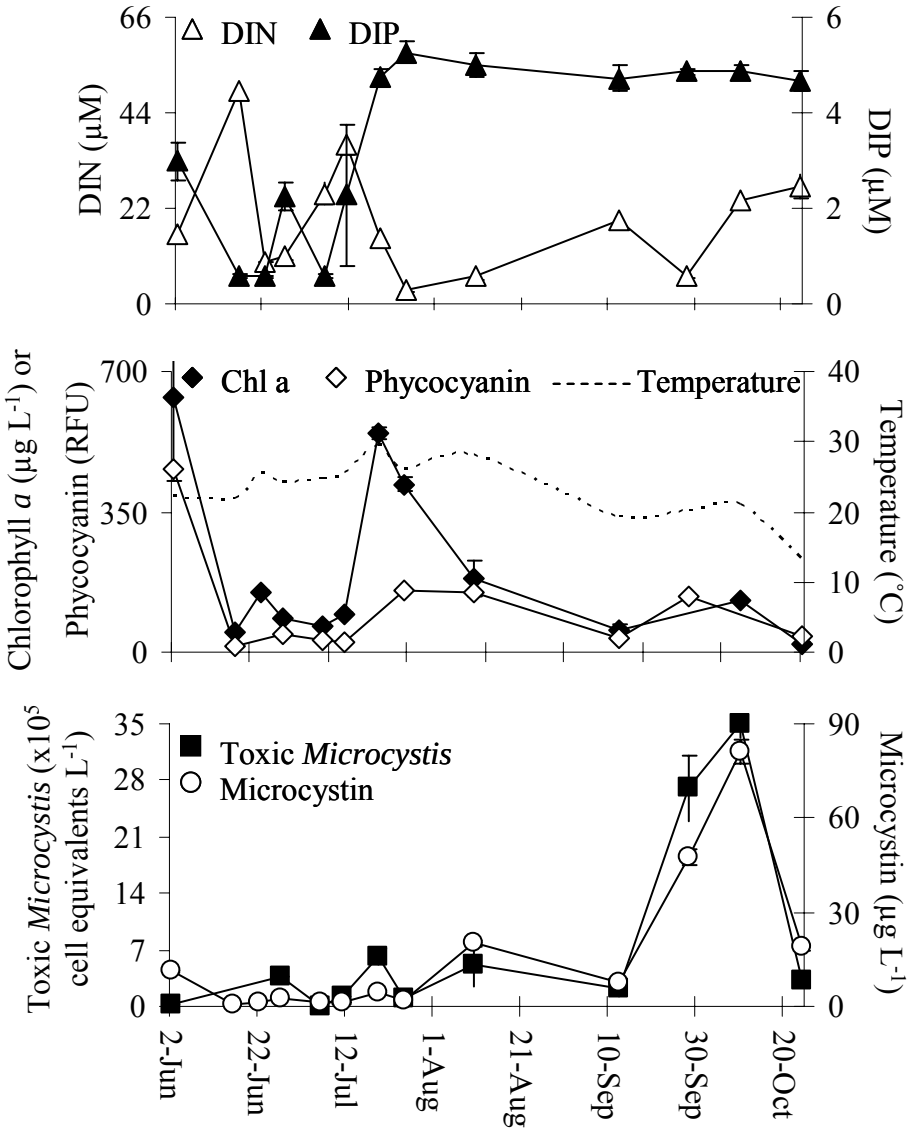

Figure 2: Time series of parameters measured in Lake Agawam, 2006. Further details as in Fig. 1

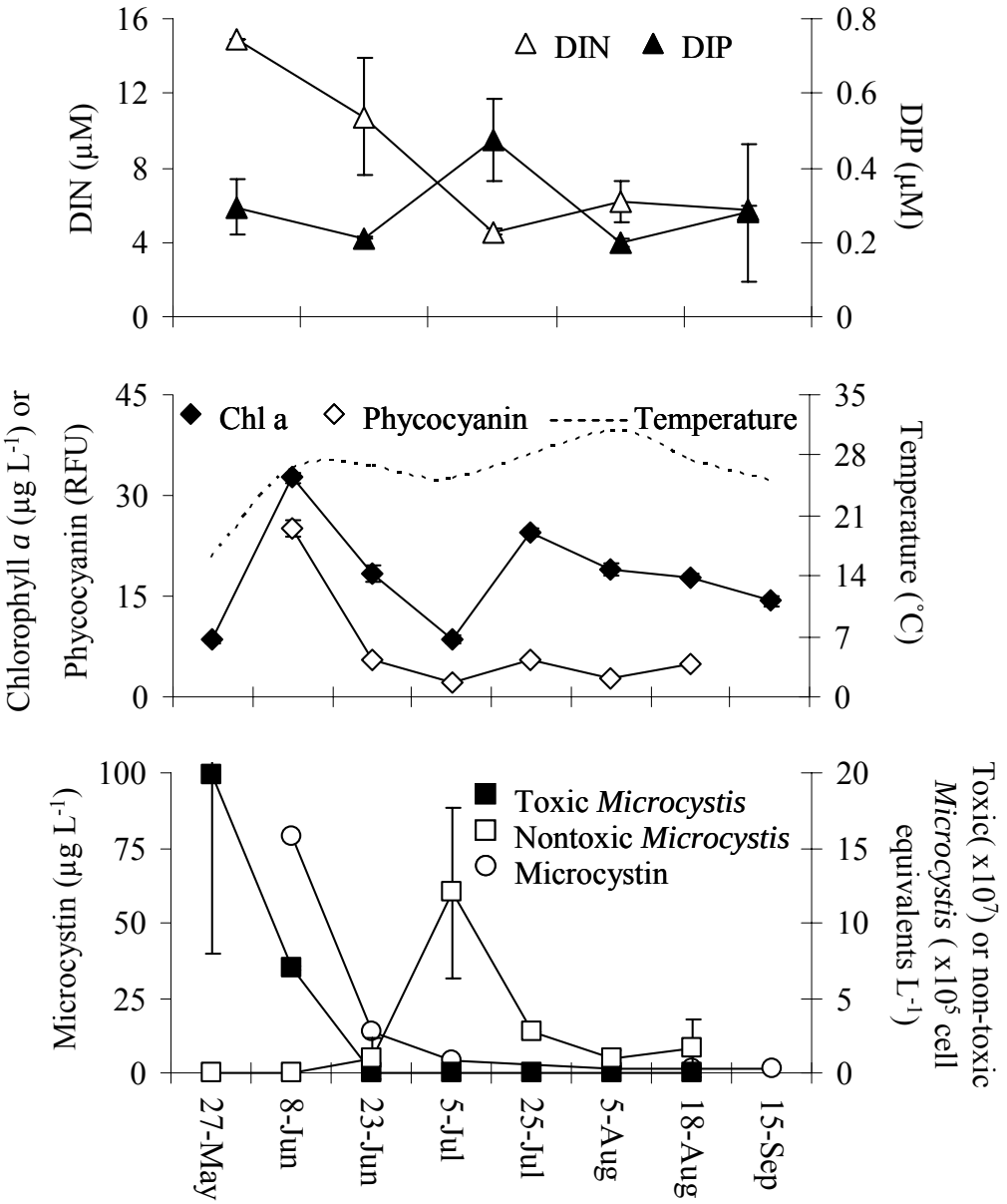

Figure 3: Time series of parameters measured in Lake Ronkonkoma, 2005. Further details as in Fig. 1

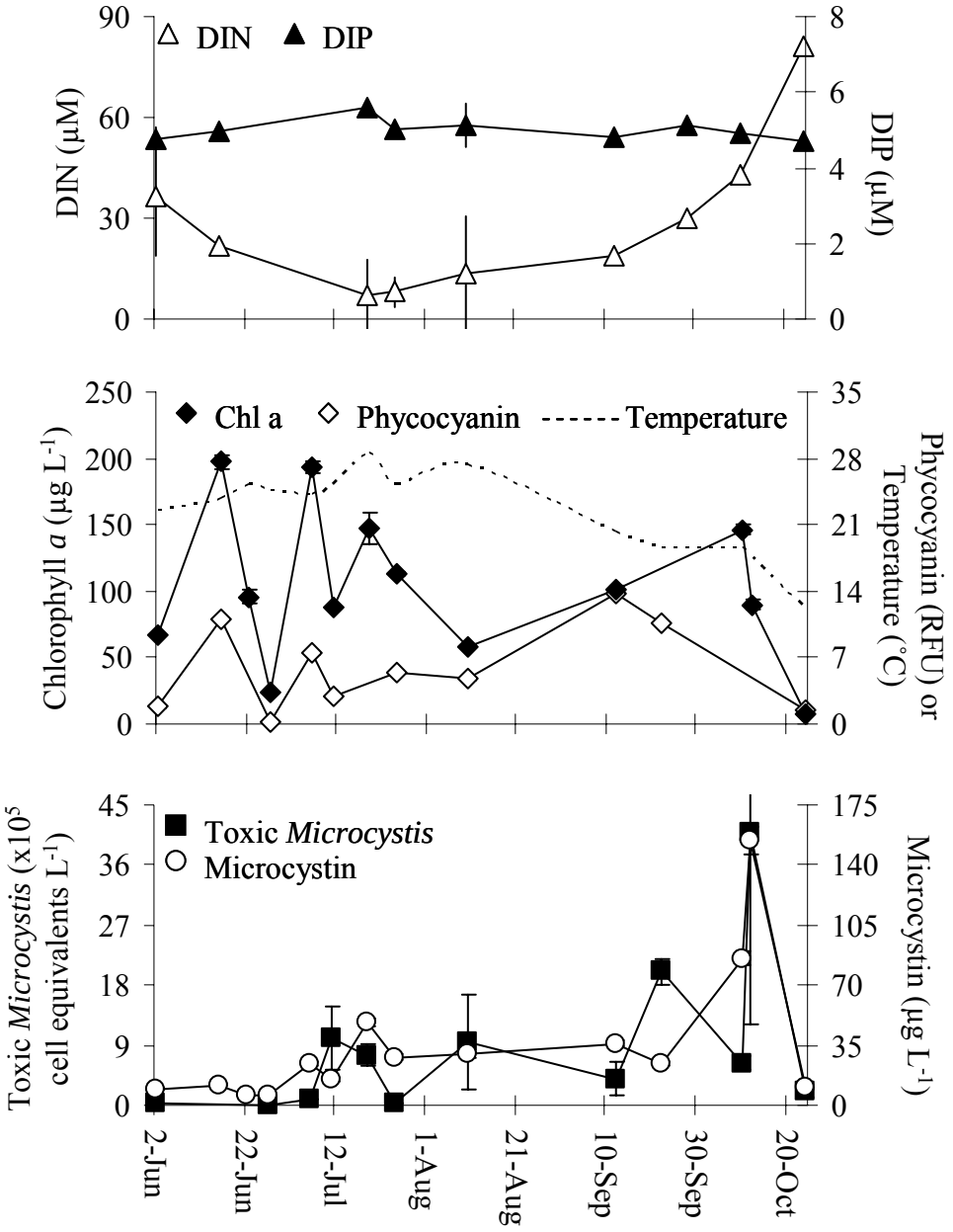

Figure 4: Time series of parameters measured in Mill Pond, 2006. Further details as in Fig. 1

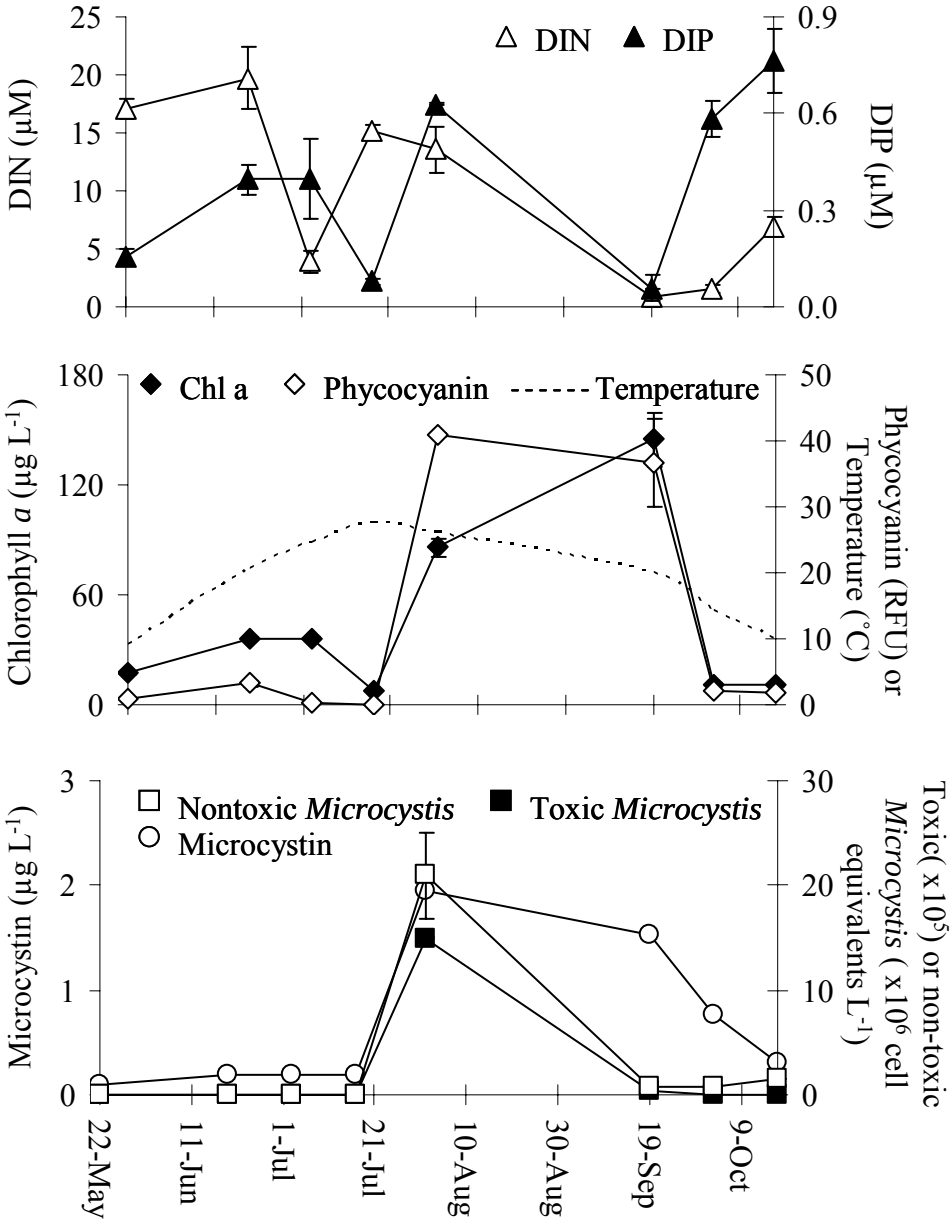

Figure 5: Time series of parameters measured in Lake Champlain, 2006. Further details as in Fig. 1

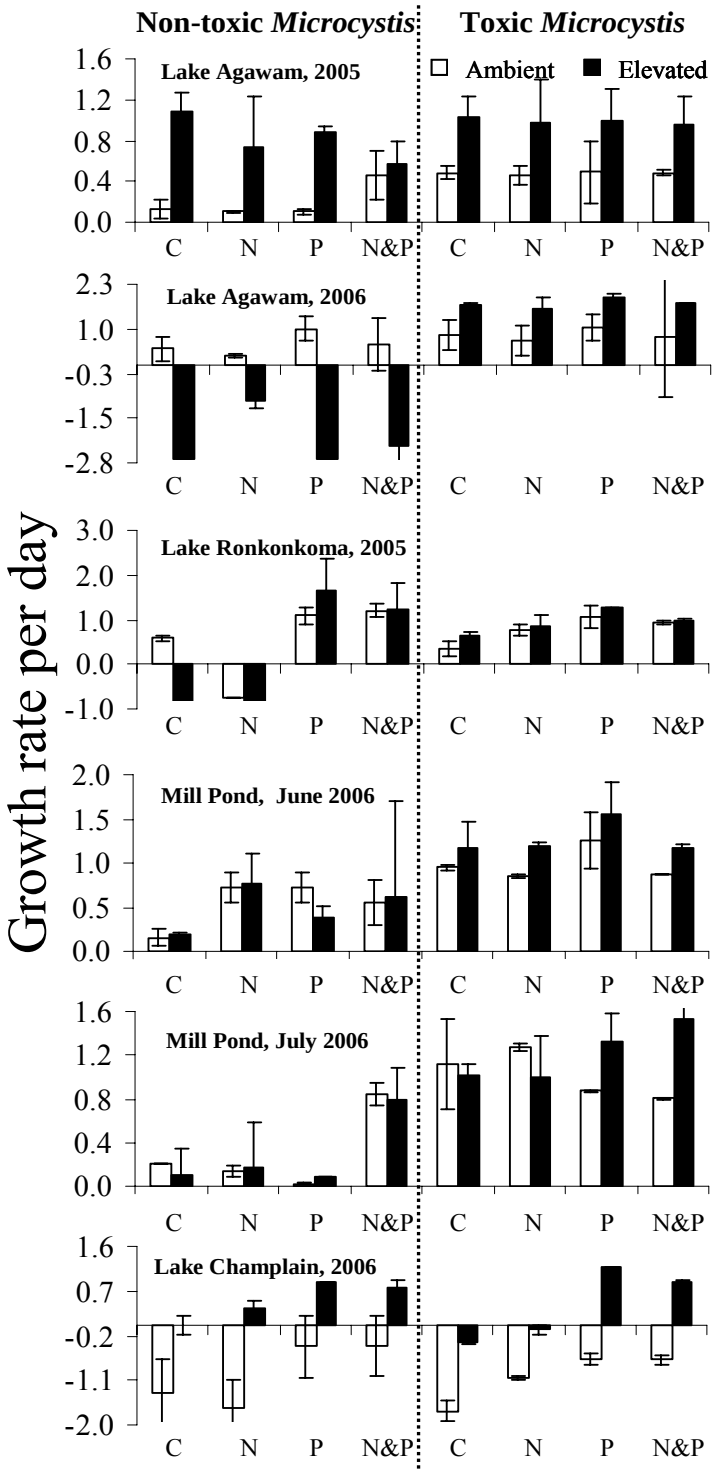

Figure 6: Net growth rates of toxic *Microcystis* (right half of figure) and non-toxic *Microcystis* (left half of figure) during nutrient amendment experiments (t =72 hours) conducted in various systems during the 2005 and 2006 field seasons at ambient (white bars) and elevated (black bars) temperatures. C: control; N: nitrate; P: ortho-phosphate. Error bars represent ± 1 SD of triplicate experimental bottles

CHAPTER FOUR:

Grazing by mesozooplankton and microzooplankton on toxic and non-toxic strains of the harmful cyanobacterium *Microcystis* during bloom events

Abstract

The ability of the harmful cyanobacteria *Microcystis* to synthesize microcystin, coupled with observed reduced zooplankton grazing rates and survival on this species has led to the hypothesis that this compound may inhibit zooplankton grazing. Testing this hypothesis in an ecosystem setting has proved challenging as *Microcystis* blooms are comprised of microscopically indistinguishable toxic and non-toxic strains which can only be resolved molecularly. The objective of this study was to assess the ability of microzooplankton as well as mesozooplankton, including laboratory-reared cladocerans (*Daphnia pulex*), amphipods (*Hyalella azteca*), and natural communities, to graze on toxic and non-toxic strains of *Microcystis* during bloom events. A two-year field campaign was established in the Transquaking River, a tributary discharging into Chesapeake Bay, in which the dynamics of toxic and non-toxic strains of *Microcystis* were determined via quantification of the microcystin synthetase gene (*mcyD*) and ribosomal RNA gene, 16S. *Microcystis* blooms comprised of > 10^7 cell equivalents L^{-1} of toxic and non-toxic strains were present during experiments conducted in both years of study. The microzooplankton community was able to graze on toxic and non-toxic *Microcystis* in 50% and 83% of experiments, respectively, at rate of 1.5 ± 0.52 d^{-1} and 0.98 ± 0.27 d^{-1}, respectively. In experiments using cultured mesozooplankton, *D. pulex* was able to graze both strains of *Microcystis* in 33% of experiments with mean clearance rates of 0.16 ± 0.04 ml $individual^{-1}$ h^{-1} and 0.11 ± 0.05 ml $individual^{-1}$ h^{-1} for toxic and non-toxic strains. Furthermore, *H. azteca* was able to graze on both toxic and non-toxic strains of *Microcystis* in 33% and 16% experiments, respectively, at mean clearance rates of 1.20 ± 0.44 ml $individual^{-1}$ h^{-1} and 0.17 ± 0.26 ml $individual^{-1}$ h^{-1}, respectively. In

contrast to the cultured mesozooplankton, the wild mesozooplankton community was able to graze on toxic and non-toxic *Microcystis* in most experiments conducted (75%). Mean grazing rates on toxic and non-toxic strains were 0.19 ± 0.06 d^{-1} and 0.09 ± 0.02 d^{-1}, respectively. In conclusion, while toxic and non-toxic strains of *Microcystis* are not always grazed, both microzooplankton and mesozooplankton are capable of grazing these strains with similar frequencies and at similar rates. As such, the ability of *Microcystis* to synthesize microcystin does not seem offer populations significant defense against zooplankton grazing.

Introduction

Zooplankton are a critical link in transferring energy and carbon from phytoplankton to higher trophic levels. They may act as a top down control on algal blooms or may allow blooms by failing to graze (Paerl, 1988; DeMott & Moxter, 1991; Demott et al., 2001; Gobler et al., 2002; Sarnelle, 2007; Hudnell & Dortch, 2008). It has been reported that many species of cyanobacteria (Porter & Orcutt, 1980; Holm & Shapiro, 1984; Sellner et al., 1994; Camacho & Thacker, 2006), including *Microcystis* (Lampert, 1981, 1982; Fulton & Paerl, 1987 a,b, 1988 a,b, Gobler et al., 2007), are not readily grazed by mesozooplankton. Previous studies have shown that the grazing ability of large cladocerans, such as *Daphnia*, are more inhibited by the presence of cyanobacteria than small-bodied cladocerans (Gliwicz, 1977), rotifers (Orcott & Pace, 1984; Fulton & Paerl, 1987b), and copepods (Richman & Dodson, 1983). However, the mechanism by which zooplankton grazing is disrupted by cyanobacteria remains unresolved. To date, hypotheses put forth to account for the absence of, or reduction in, grazing by mesozooplankton on cyanobacteria have included colony size and/or morphology (i.e. large filamentous or colonial forms are not well-grazed), or the absence of nutritional compounds (fatty acids or lipids) in cyanobacteria that zooplankton need to survive (as reviewed by Wilson et al., 2006). A third hypothesis is that compounds synthesized by cyanobacteria serve as grazing deterrents. For example, the ability of *Microcystis* to synthesize microcystin, coupled with observed low zooplankton grazing and survival rates on this species has led to the hypothesis that this compound inhibits zooplankton grazing and/or may cause zooplankton mortality (Arnold, 1971; Porter &

Orcott, 1980; Lampert, 1981, 1982; Nizan et al., 1986; Fulton & Paerl, 1987b; Lampert, 1987; de Bernardi & Giussani, 1990; DeMott et al., 1991; Rohrlack et al., 1999).

Although toxic cyanobacteria blooms substantially reduce mesozooplankton grazing rates, these events do not seem to have the same negative impact on microzooplankton (Nishibe et al., 2002; Kim et al., 2006; Gobler et al., 2007). A study conducted by Nishibe et al. (2002), found that the flagellate, *Collodictyon triciliatum*, was able to actively graze upon individual *Microcystis* cells as well as small *Microcystis* colonies. On the other hand, Kim et al. (2006) found that when the flagellate *Diphylleia viridis* was fed both toxic and non-toxic strains of *Microcystis* spp. as well as other species of phytoplankton, such as *Chlorella*, *D. viridis* fed the most toxic strain of *Microcystis* yielded the highest growth and ingestion rates. Gobler et al. (2007) found rates of microzooplankton herbivory did not vary significantly over the course of an extended toxic *Microcystis* bloom, despite large fluctuations in *Microcystis* densities, microcystin levels, and microcystin synthetase gene expression. Therefore, microzooplankton may graze on toxic cyanobacteria (Dryden & Wright, 1987; Paerl et al., 2001) and may act as an important trophic link to upper trophic levels during toxic cyanobacteria blooms.

One complexity in investigating the interaction of zooplankton and toxic cyanobacteria blooms in an ecosystem setting is the co-existence of toxic and non-toxic strains of the same species within a genus, such as *Microcystis*, which are visually indistinguishable (Otsuka et al., 1999; Fastner et al., 2001; Kurmayer et al., 2002). Recent advances in molecular techniques have facilitated the differentiation and quantification of toxic and non-toxic strains of *Microcystis* by the presence or absence of

the microcystin synthetase gene (Tillett et al., 2000; Rinta-Kanto & Wilhelm, 2005; Davis et al., 2009; Ha et al., 2009). I will refer to *Microcystis* cells containing the microcystin synthetase gene (*mcyD*) as 'toxic'. There have been previous laboratory studies investigating zooplankton grazing on toxic and non-toxic strains of cyanobacteria cultures (Kim et al., 2006), but to our knowledge, no study has quantified micro- and mesozooplankton grazing rates on toxic and non-toxic strains of *Microcystis* during bloom events.

The objective of this study was to quantify grazing rates of microzooplankton and mesozooplankton on toxic and non-toxic strains of *Microcystis* during bloom events. A two-year field campaign was established in the Transquaking River, a tributary discharging into Chesapeake Bay, in which the dynamics of toxic and non-toxic strains of *Microcystis* were determined by molecular quantification of total *Microcystis* cells (using the 16S rRNA gene) and toxic *Microcystis* cells possessing the microcystin synthetase gene (using the *mcyD* gene). Concurrently, grazing rates of microzooplankton and mesozooplankton (laboratory-reared cladocerans and amphipods, as well as natural communities) on toxic and non-toxic strains of *Microcystis* were quantified, along with electivity indices for each strain of *Microcystis* by each group of zooplankton. The grazing rates and electivity indices were compared to multiple environmental measurements including cyanobacteria bloom densities, microcystin levels, and phytoplankton and zooplankton community composition.

Methods

Sampling site and water quality monitoring

The Transquaking River (Latitude: 38˚ 30' N; Longitude: 75˚ 58' W) is a tributary to the Chesapeake Bay which spans 37 km along Maryland's (USA) eastern shore and hosts annual toxic *Microcystis* blooms with elevated levels of microcystin (Tango & Butler, 2008). It has a watershed area of 287 km^2, roughly one third of which is used for agricultural purposes (MD DNR, 2000). During 2007 and 2008, Transquaking River (TR), was sampled bi-weekly before, during, and after cyanobacteria blooms (May – November). At each site, general water quality was evaluated using a YSI 556 sonde to determine surface temperatures, dissolved oxygen, and pH. Twenty liters of surface water was collected and taken to the lab where triplicate extracted chlorophyll *a* and *in vivo* phycocyanin (as a proxy for total cyanobacteria) measurements were made with Turner Designs fluorometers using standard techniques (Parsons et al., 1984; Watras & Baker, 1988; Lee et al., 1994). For microcystin analysis, whole water was filtered onto triplicate 47 mm GFF glass fiber filters and placed in cryovials which were stored at -80°C until analysis. Duplicate whole water samples were preserved with Lugol's iodine solution (5% final concentration) to quantify phytoplankton as well as microzooplankton assemblages. To identify and quantify the mesozooplankton community, 4L of whole water was concentrated over a 200 µm sieve, rinsed into a small bottle, and preserved with formalin (1% final concentration). For molecular quantification of *Microcystis* strains, water was filtered onto triplicate, 2 µm polycarbonate filters, immediately placed in CTAB lysis buffer, and stored at –80°C.

Mesozooplankton experiments

The cultured cladoceran *Daphnia pulex* (Aquatic Research Organisms, New Hampshire, USA) and the cultured amphipod *Hyalella azteca* were maintained in separate 40-L aquaria filled with 0.2µm filtered mineral water and fed a diet of *Selenestrun capricornutum* (~$1x10^5$ cells ml^{-1}) and Yeast-Cereal-Trout food (YCT) at a temperature of 23ºC, under 25 µEin m^{-2} s^{-1}. Zooplankton were bubbled with air and fed every other day and aquaria water was exchanged weekly. For experiments, *D. pulex* and *H. azteca*, were individually added to experimental bottles using a modified transfer pipette at abundances which have previously been found during cyanobacteria blooms (100 L^{-1} and 12 L^{-1}, respectively) in Chesapeake Bay tributaries (Sellner et al., 1993) and elsewhere (George & Edwards, 1974; Threlkeld, 1979; Camacho & Thacker, 2006). Natural populations of mesozooplankton (>200 µm) were concentrated to 8-times ambient concentrations over a submerged 200 µm sieve preventing desiccation and death of the zooplankton (Deonarine et al., 2006). An aliquot of this concentrate was preserved in formalin for quantification, while the remaining volume was immediately used for experiments.

To commence mesozooplankton experiments, a set of ten 330 ml acid-cleaned polycarbonate bottles were filled with whole river water to a total volume of 250 ml. One bottle was immediately processed to quantify all phytoplankton and zooplankton groups described below, and the nine remaining bottles were established as unammended controls (n = 3), a *D. pulex* treatment (n = 3), and a *H. azteca* treatment (n = 3). Four additional bottles were filled to a total volume of 250 ml with 8-times the natural mesozooplankton concentration (concentrated as described above). One 8x zooplankton

enrichment bottle was immediately sacraficed for the quantification for all phytoplankton and zooplankton groups at the commencement of experiments. To minimize the effects of nutrients from zooplankton excretion during experiments, saturating nutrients (20 μM nitrate, 1.25 μM orthophosphate) were added to all experimental bottles. Bottles were incubated for ~ 48 h in an environmental control chamber with light and temperature levels which matched *in situ* conditions. The duration of these experiments (~48 h) should have been long enough to observe any negative effects of *Microcystis* on mesozooplankton which have been shown to occur after 12 h of exposure (Rohrlack et al. 2005). Final samples from each incubation bottle were obtained to quantify zooplankton grazing rates on the total phytoplankton community (chlorophyll *a*), total cyanobacteria (phycocyanin), and densities of toxic and non-toxic *Microcystis*. Growth rates for the total phytoplankton community (chlorophyll *a*), the total cyanobacterial comunity (phycocyanin), and toxic and non-toxic *Microcystis* were calculated using the equation: $\mu = \ln [N_t / N_o] / t$ where μ is the rate of population growth (d^{-1}), N_o and N_t are initial and final cell densities or pigment levels, and t is the duration of incubation. Clearance rates of each cultured zooplankton species on each algal prey were calculated according to Frost (1972) as follows: $g = (\mu_c - \mu_g)$ where g is the grazing rate on toxic or non-toxic *Microcystis*, μ_c is the rate of population growth in nutrient control treatments (treatments with N and P addition but no zooplankton) and μ_g is the rate of population growth in zooplankton addition (cladocerans or amphipods) treatments. Using the calculated g value, clearance rate (CR) was determined using the equation: $CR = (g * (V/(t*n)))$ where V is the bottle volume, t is the time of incubation (in hours), and n is the number of zooplankton added. However, since the natural mesozooplankton community was

comprised of multiple genera with differing clearance rates, a community grazing rate was obtained using the Frost (1972) equation, ($g = (\mu_c - \mu_g)/8$), to account for the 8 times increase in natural zooplankton abundance. Negative grazing rates (increases in algal populations upon the addition of the grazers) were interpreted as an absence of grazing by the added zooplankton (Richman et al., 1977; Vanderploeg & Scavia, 1979b). Clearance rates or grazing rates by each zooplankton grazer group on each algal prey group (total cyanobacteria = phycocyanin-specific growth rates, total, toxic, and non-toxic *Microcystis* cells) were compared by means of one-way ANOVAs and multiple comparison tests (Tukey test) with $p < 0.05$ used as the significance level.

Microzooplankton grazing experiments

To estimate microzooplankton grazing, the dilution technique described in Landry et al. (1995) was utilized. Filtered sample water (0.2 μm) was used to create a dilution series consisting of four dilutions (100%, 75%, 50%, 25% whole water WW; n = 3 for each) in acid-washed, 250 mL acid-cleaned polycarbonate bottles, all with complete nutrient enrichment (20 μM nitrate, 1.25 μM orthophosphate; Landry et al., 1995). Experimental bottles were incubated in an incubator with light and temperatures levels matching *in situ* conditions. After 24 – 48 h, levels of phycocyanin and densities of total, toxic, and non-toxic *Microcystis* in each experimental bottle were quantified. Net growth rates of each population at each dilution were calculated from changes in cell densities and pigment using the formula: $\mu = [\ln(B_t / B_o)] / t / \%\ WW$ where μ is the net growth rate, B_t is the amount of biomass (cell density or pigment) present at the end of the experiments, B_o represents the amount of biomass at the beginning of experiments, t is the duration of the experiment in days and % WW is the percent of whole water used.

Grazing mortality rates (m) of populations were determined using the slope of a linear regression of the dilution of sample water (x-axis) versus apparent net growth rates (y-axis), while nutrient enriched growth rates (μ_n) were determined from the y-intercept of these plots (Landry et al., 1995). The net nutrient enriched growth rates (net μ) for each population was calculated by the equation (Net $\mu = \mu_n - m$).

Electivity Indices

For all microzooplankton and mesozoplankton experiments, electivity indices were calculated to determine the magnitude of prey (toxic or non-toxic *Microcystis*) preference by each grazer population (Ivlev, 1961). I used a modified selection coefficient developed by Vanderploeg & Scavia (1979a), $W_i = F_i / \Sigma F_i$, where W_i is the selection coefficient and F_i is the grazing rate on the prey item (toxic or non-toxic *Microcystis*) and ΣF_i is the sum of the grazing rates on both strains of *Microcystis* by the specific zooplankter. W_i was used in the electivity index (E_i*) equation developed by Vanderploeg & Scavia (1979b), $E_i^* = [W_i-(1/n)]/[W_i+(1/n)]$ where n is the total number of prey types. This method is preferable when the prey densities are unequal (Lechowicz, 1982). The range of this index is from -1 to 1. Positive indices indicate a selection for a particular prey type and negative indices indicate avoidance of the prey item. The closer the number is to either -1 or 1 stronger the degree to which the prey item was either selected for or avoided. For this study, if only one of the two populations was grazed during an experiment the grazed upon strain was scored an index of 1 and the avoided strain was scored -1.

Daphnia pulex* grazing on cultured, toxic *Microcystis

Since this study was, to my knowledge, the first to utilize molecular techniques to investigate specific grazing rates on toxic and non-toxic strains of *Microcystis* during natural bloom events, a laboratory experiment was established to quantify *Daphnia pulex* grazing on *Microcystis* using both microscopic and gene quantification techniques. The toxic *M. aeruginosa* clone LE-3 (isolated from Lake Erie by Dr. Wayne Carmichael and provided by Dr. Steven Wilhelm) was grown in BG-11 media at 21ºC under 100 µEin m^{-2} s^{-1} with a light:dark cycle of 12:12h. During this experiment, the culture consisted of either individual cells or small colonies ($\leq$ 4 cells) which were easily counted under a light microscope. The *D. pulex* were grown as described above. To commence the experiment, a set of seven, sterile, 50 ml experimental flasks were filled with *M. aeruginosa* LE-3 culture at a density of 5.7 x 10^5 cells ml^{-1} which was similar to the mean density of *Microcystis* found in TQR during the summer of 2008. The green alga *Selenestrun capricornutum* was added as an alternative food source at a biomass level equal to that of the *M. aeruginosa* culture (as measured by in vivo fluorescence on a Turner Designs, model-10 fluorometer). One flask was immediately processed for the preservation of samples for microscopic cell enumeration in Lugol's iodine solution (5% final concentration) and samples were collected for the quantification of the 16S and *mcyD* rRNA genes (as described below). Of the remaining six bottles, three were left unammended to serve as controls and three had densities of *D. pulex* added to them (4 individuals 50 ml^{-1}) that were identical to the densities added in the field experiments. The bottles were incubated for 24 hours under the conditions described above at the end

of which samples were obtained from each bottle for microscopic and molecular quantification of cells as described above.

Microscopic analysis

Densities of *Microcystis* and other co-occurring cyanobacteria were quantified using gridded Sedgewick-Rafter chambers enumerating at least 200 cells per sample. To quantify *Microcystis* as well as *Anabaena*, the number of colonies per chamber was determined. The number of cells per colony were determined according to Watzin et al. (2006). The size of *Microcystis* colonies within each sample was determined from digital images and SPOT^{TM} Advanced software (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Only colonies of *Aphanizomenon* were quantified in this study. Microzooplankton (> 20 μm) were identified to the genus level and quantified by concentrating Lugol's preserved samples in settling chambers and enumerating them on an inverted microscope (Hasle, 1978) For this manuscript, we categorized the dinoflagellates as part of the microzooplankton community based on the fact that many genera of dinoflagellates are facilitative phagotrophs (Spero & Morée, 1981; Gaines & Elbrächter, 1987). Mesozooplankton in formalin-preserved samples were enumerated and assigned to major taxonomic categories using a dissecting microscope for the 2008 sampling year only (Harris et al., 2000).

Microcystin Analysis

Filters for microcystin analyses were extracted in 50% methanol containing 1% acetic acid using ultrasound (four 20 second bursts with a 20 second pause between bursts), and an extraction protocol which yields > 90% recovery of microcystin-LR (Boyer et al., 2004). Following extraction, the methanolic extract was stored at –80ºC

until analysis. Before analysis, the microcystin extract was diluted to 5% methanol and buffered to pH 7 with 5% Tris-EDTA. Microcystin concentrations were measured using a microcystin (ADDA) enzyme-linked immunosorbent assay (Abraxis LLC; Warminster, PA, USA) following the methodologies of Fischer et al. (2001). This assay is congener-independent as it is sensitive to the ADDA moiety which is found in a majority of microcystins. These analyses yielded a detection limit of 0.10 μg L^{-1}, a relative standard deviation of 15%, and 99.5 ± 8.2% recovery of environmental samples spiked with 5 μg L^{-1} microcystin.

Molecular Analyses

Total cellular nucleic acids were extracted from field and experimental samples using methods described in Coyne et al. (2001). Filtered environmental or experimental samples were submersed in CTAB buffer (Dempster et al., 1999), supplemented with 20 μg L^{-1} pGEM-3z(f+) plasmid (Promega; Table 1) which served as an internal control for extraction efficiency and PCR inhibition (Coyne et al., 2005). The filters were then flash frozen and stored at –80°C until extraction. Nucleic acids were extracted after an initial heating step at 65°C, followed by a double chloroform extraction, and an isopropanol precipitation. Extracted nucleic acids were resuspended in 20μl of LoTE buffer. The quantity and quality of nucleic acids was assessed with a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Two *Microcystis*-specific genetic targets were used during this study, the 16S rRNA gene (16S rDNA) and *mcyD* gene. The *Microcystis* 16S rRNA gene is specific to the *Microcystis* genus which allowed us to quantify the abundance of the total *Microcystis* population. The *mcyD* gene is found within the microcystin synethtase gene

operon which is responsible for the production of microcystin and is only found in toxic strains of *Microcystis* (Tillett et al., 2000) which allowed us to quantify the toxic population of *Microcystis* (Rinta-Kanto et al., 2005). QPCR was carried out using an ABI 7300 Real Time PCR instrument using TaqMan® labeled probes (Applied Biosystems) and *Microcystis*-specific *mcyD* and 16S rDNA primers (Table 1). Each 10uL reaction included 5uL of 2x TaqMan® Master Mix (Applied Biosystems), 10uM each primer (Integrated DNA Technologies), 10uM TaqMan® probe (Table 1) and 1µl of a 1:25 dilution of the unknown DNA or standard. For amplification of the pGEM and 16S targets, the cycling conditions were 95°C for 10 minutes, followed by 55 cycles of 95°C for 15 seconds and 60°C for 1 minute. In order to amplify the *mcyD* gene, the cycling conditions were 95°C for 10 minutes, followed by 55 cycles of 95°C for 15 seconds, followed by 50°C for 1 minute, then 60°C for 1 minute. To prepare standard samples, cultured *Microcystis aeruginosa*, clone LE-3 (Rinta-Kanto et al., 2005), was enumerated by standard microscopy and collected on polycarbonate filters which were prepared and extracted as outlined above. A standard curve of dilutions of the extracted LE-3 genomic DNA was run with each analytical run to serve as a reference for numbers of toxic *Microcystis* spp. cells. Since some *Microcystis* spp. cells may carry multiple copies of the 16S rDNA gene and *mcyD* gene, data was expressed as "cell equivalents" rather than cell number (Rinta-Kanto et al., 2005). The numbers of toxic and total *Microcystis* cells were determined using the ΔΔCT method (Livak & Schmittgen, 2001; Coyne et al., 2005). The difference between the number of *mcyD* cell equivalents (toxic cells) and 16S rDNA cell equivalents (total cells) was assumed to represent the number of non-toxic cell equivalents (Rinta-Kanto et al., 2005).

Statistical Analysis

For the four algal communities studied, total cyanobacteria (phycocyanin), total *Microcystis*, toxic *Microcystis*, and non-toxic *Microcystis*, net growth rates among treatments (control, *D. pulex* addition, *H. azteca* addition, and 8x natural zooplankton enrichment) were compared by means of ANOVAs or a non-parametric Kruskal-Wallis test. The degree to which individual biological and environmental variables were correlated was evaluated by a Pearson's Rank Order Correlation Matrix. In all cases, a significance level of 0.05 was applied to justify statistically significant differences or correlations.

Results

Field observations for Transquaking River 2007 & 2008

During 2007, Transquaking River (TQR) hosted high biomass (64.5 to 386 μg chl *a* L^{-1}; Fig 1) cyanobacteria blooms numerically dominated by *Microcystis* and *Aphanizomenon* from late July through September (Table 2). Total *Microcystis* densities ranged from 1.1 ± 0.6 $x10^6$ to 3.1 ± 0.85 $x10^7$ cell equivalents L^{-1} with dominance shifting between toxic and non-toxic strains throughout the sampling period (Fig 1). During October toxic and non-toxic strains reached peak densities of 4.0 ± 1.5 $x10^6$ cell equivalents $L^{-1}$and 2.7 ± 0.59 $x10^7$ cell equivalents L^{-1}, respectively (Fig 1). Microcystin concentrations ranged from 0.75 to 6.8 μg L^{-1} during blooms (Fig 1) and levels were significantly correlated with toxic *Microcystis* densities ($p < 0.05$) but were not correlated with non-toxic *Microcystis*, total *Microcystis*, or total cyanobacterial densities. *Microcystis* colonies varied in size from 20 ± 3 to 61 ± 15 cells $colony^{-1}$ with an average

size of 36 ± 6 cells colony^{-1}, while water temperature ranged from 8.6 (November) to 27.8ºC (July; Fig. 1). The microzooplankton community was dominated by dinoflagellates on six of the eight dates sampled (Table 3). Densities of dinoflagellates ranged from 0 to 460 ± 0 individuals ml^{-1}, reaching peak densities on 2 October (Table 3). Rotifers, aloricate and loricate ciliates, and copepods were also found during the summer of 2007 but at lower densities than the dinoflagellate community (0 – 34 ± 3 individuals ml^{-1}, Table 3). Interestingly, adult copepod densities were positively correlated with total cyanobacterial densities during the 2007 field season ($p < 0.05$).

In 2008, TQR again hosted cyanobacteria blooms of mixed genera. Among toxic genera, similar to 2007, *Microcystis* and *Aphanizomenon* numerically dominated the community on every date except 10 June when *Anabaena* was more abundant and 13 May when *Aphanizomenon* was not present (Table 2). Peak densities of *Microcystis* (3.1 ± 0.63 x10^8 cell equivalents L^{-1}) occurred on 24 June and were an order of magnitude higher than peak densities in 2007 (Fig 2). Within the total *Microcystis* population, toxic strains dominated from late May through early July with peak densities of 2.8 ± 0.23 x10^8 cell equivalents L^{-1} coinciding with peak total *Microcystis* densities (Fig 2). After mid-July, dominance shifted towards non-toxic strains of *Microcystis* which remained dominant throughout the remainder of the field season reaching peak densities of 3.9 ± 1.9 x10^7 cell equivalents L^{-1} in late August (Fig 2). Microcystin concentrations ranged from 0.36 to 12.1 µg L^{-1} with peak concentrations occurring on the same date (24 June) as peak toxic *Microcystis* densities (Fig 2). Again, similar to 2007, microcystin concentrations were significantly correlated with toxic *Microcystis* densities ($p < 0.01$) but were not correlated with non-toxic *Microcystis* densities, chlorophyll *a*, phycocyanin,

or the total cyanobacterial densities ($p > 0.05$). *Microcystis* colonies ranged in size from 14 ± 2 to 180 ± 51 cells colony^{-1} with the average colony size during the summer of 2008 being slightly larger than 2007 at 47 ± 19 cells colony^{-1}. Temperature during the 2008 field season ranged from 13.4 in October to 29.2ºC in July (Fig 2).

The mesozooplankton community in TQC during 2008 was dominated by small-bodied cladocerans on almost every date sampled whereas large-bodied cladocerans such as *Daphnia*, were either absent or at very low concentrations throughout the sampling period (Table 4). Total cladoceran densities ranged from 2 to 60 ± 3 individuals L^{-1} (Table 4). Genera such as *Bosmina* and *Moina* tended to dominate the cladoceran subpopulation with concentrations ranging from absent to 42 ± 12 and 36 ± 3 individuals L^{-1}, respectively (Table 4). Copepods were less abundant than cladocerans and were comprised of a single genus *Acanthocyclops*. The large ciliate *Climacostomum virens* (> 200 μm) was present on every date except 30 September with peak densities of 98 ± 12 individuals L^{-1} occurring on 10 June (Table 4). Rotifers were absent from the mesozooplankton community until late August when *Brachionus calyciflorus* was seen at densities that ranged from 1 to 7 ± 1 individuals L^{-1} (Table 4). During 2008, the total microzooplankton community densities ranged from 274 ± 21 to 5,882 ± 410 individuals ml^{-1}, with peak densities occurring on 24 June (Table 4). Similarly to 2007, the microzooplankton community was dominated by dinoflagellates on most (88%) dates sampled. Densities of dinoflagellates ranged from 0 to 2,983 ± 298 individuals ml^{-1} with peak densities occurring on 10 June (Table 4). Aloricate and loricate ciliates, rotifers, and copepods were present at lower densities (0 – 74 ± 7 individuals ml^{-1}, Table 4). Unlike the correlations found in 2007, densities dinoflagellates were positively correlated

with the total *Microcystis* community (as determined by the 16S r RNA gene), the toxic *Microcystis* community (as determined by the *mcyD* gene) as well as the microcystin concentrations ($p < 0.01$ for all). Furthermore, the total microzooplankton community was positively correlated with total *Microcystis* densities, toxic *Microcystis* densities, as well as microcystin concentrations ($p < 0.01$).

Microzooplankton Experiments

Dilution experiments conducted in 2007 showed that the microzooplankton community was able to successfully graze on the total cyanobacterial community (based on phycocyanin) in all experiments conducted (n = 6; Table 5). Grazing rates ranged from 0.13 ± 0.02 to 0.45 ± 0.07 d^{-1}. Similarly, in 83% (5 of 6) of experiments conducted, microzooplankton grazed on the total *Microcystis* community with grazing rates ranging from 0.46 ± 0.21 to 1.72 ± 0.52 d^{-1} (Table 5). Furthermore, the microzooplankton community grazed on both toxic and non-toxic strains of *Microcystis*. Non-toxic *Microcystis* was successfully grazed in 83% of experiments whereas toxic *Microcystis* was grazed in only 50% of experiments (Table 5). The mean grazing rates on the toxic and non-toxic strains were 0.98 ± 0.27 d^{-1} and 1.5 ± 0.52 d^{-1}, respectively.

Net growth rates for the total cynobacterial population were always positive except on one date (23 July) and ranged from -0.02 ± 0.04 to 0.67 ± 0.03 d^{-1} (Table 5). Total *Microcystis* net growth rates were positive during the experiments conducted in late July and August (0.79 ± 0.14 to 1.74 ± 0.17 d^{-1}) but were negative durng the experiments conducted in October and November (-0.07 ± 0.33 to -0.47 ± 0.37). Similarly, non-toxic *Microcystis* net growth rates were positive during the July and August experiments (0.81 ± 0.28 to 1.08 ± 0.17d^{-1}; Table 5) and negative in all but one (7 Nov) of the late fall

experiments (0.05 ± 0.53 to -0.87 ± 0.61 d^{-1}; Table 5). In all of the experiments where grazing occurred, toxic strains of *Microcystis* yielded positive net growth rates ranging between 0.04 ± 0.20 and 0.53 ± 0.24 d^{-1} (Table 5).

Overall, electivity indices (E.I.) showed that in all experiments where at least one population was grazed the microzooplankton community preferentially grazed on the non-toxic population over the toxic population 60% of the time (3 of 5; E.I. = 0.11 to 1.0; Table 6). However, electivity indices also showed that in experiments where both toxic and non-toxic populations were grazed (n = 3), there was no clear preference for either. The toxic and non-toxic populations each had a positive index in one of the three experiments (23 July & 17 October, respectively; Table 6), whereas, in the third experiment (7 November) both electivity indices were zero indicating that the microzooplankton showed no preference for one population over the other (Table 6). Neither the ability of microzooplankton to successfully graze *Microcystis* nor the rate at which they grazed *Microcystis* varied as a function of colony size ($p > 0.05$).

Mesozooplankton Experiments

Cultured *D. pulex* were able to graze on the total phytoplankton community and / or the total cyanobacterial community in all experiments (n = 6) with clearance rates ranging from 0.003 ± 0.04 ml individual^{-1} h^{-1} to 0.06 ± 0.01 ml individual^{-1} h^{-1} (Fig 3). In contrast, toxic and non-toxic strains of *Microcystis* were successfully grazed by *D. pulex* in 33% (2 of 6) of experiments conducted with mean clearance rates of 0.16 ± 0.04 ml individual^{-1} h^{-1} and 0.11 ± 0.05 ml individual^{-1} h^{-1}, respectively (Fig 3). Electivity indices indicated that when grazing did occur on at least one of the populations (toxic or non-toxic), *D. pulex* preferentially selected for the non-toxic strains of *Microcystis* 75% of the

time (3 of 4 expts.; Table 7). Similar to *D. pulex*, cultured *H. azteca* was able to successfully graze on the total phytoplankton and/or total cyanobacterial community in all experiments conducted (n = 6) with clearance rates ranging from 0.06 ± 0.003 ml individual^{-1} h^{-1} to 0.52 ± 0.04 ml individual^{-1} h^{-1}. *H. azteca* also consumed toxic and non-toxic strains of *Microcystis* but at a lower frequency, 33% (2 of 6) and 16% (1 of 6) experiments, respectively (Fig 3). Mean clearance rates on toxic and non-toxic strains were 1.20 ± 0.44 ml individual^{-1} h^{-1} and 0.17 ± 0.26 ml individual^{-1} h^{-1}, respectively. In contrast to *D. pulex*, *H. azteca* exhibited a selective preference towards the toxic strains of *Microcystis* in two experiments and selective preference towards the non-toxic *Microcystis* in another (Table 7).

Experimental results obtained with the *in situ* mesozooplankton community differed from the cultured zooplankton. The ambient community of mesozooplankton grazed both the toxic and non-toxic strains of *Microcystis* in most experiments (75%; 6 of 8; Fig 4) with mean grazing rates of 0.19 ± 0.06 d^{-1} and 0.09 ± 0.02 d^{-1}, respectively. In 86% (6 of 7) of the experiments where at least one of the populations (toxic or non-toxic) was successfully grazed, electivity indices were positive for toxic strains of *Microcystis* (0.04 – 1.0) and negative for non-microcystin strains (-0.05 to -1.0) indicating that in most experiments the *in situ* population of mesozooplankton had a selective preference for toxic stains of *Microcystis* (Table 7). Finally, in a manner similar to the cultured mesozooplankton, no relationship was found between *Microcystis* colony size and the ability of zooplankton to graze this species or the measured grazing rates ($p > 0.05$).

D. pulex grazing of toxic *Microcystis* quantified with genetic and microscopic techniques

Daphnia pulex was able to graze on the toxic strain of *Microcystis aeruginosa* LE-3, with mean grazing rates of 0.56 ± 0.12 d^{-1}, 0.58 ± 0.25 d^{-1}, and 0.36 ± 0.13 d^{-1} as quantified microscopically and with qPCR (16S rRNA gene and *mcyD* gene respectively;Fig 5). There was no difference between the grazing rates of *D. pulex* fed *M. aeruginosa* cells as quantified microscopically, by 16S rRNA gene copies, or by *mcyD* gene copies ($p > 0.05$; one-way ANOVA) indicating that the molecular quantification approach I have utilized accurately quantified the changes in *Microcystis* densities in the presence of zooplankton such as *D. pulex*.

Discussion

There are multiple hypotheses regarding why zooplankton are poor grazers of cyanobacteria (Wilson et al., 2006) including the roles of colony size (Wilson et al., 2006), morphology (Fulton & Paerl, 1987a; Chan et al., 2004) and toxins (Nizan, 1986; Rohrlark, 1999; Lürling, 2003) as anti-grazing defenses. However, prior studies have produced contradictory results regarding the ability of the microcystin compound to serve as a grazing defense against zooplankton (Wilson et al., 2006). Rohrlark et al. (1999) reported that microcystin was toxic to *Daphnia* whereas other studies have come to opposing conclusions (Jungmann, 1992, 1995). Furthermore, a laboratory study conducted by Lürling (2003) found that the growth of *Daphnia* was similar between animals fed microcystin-producing *Microcystis* cells or genetically modified mutants which did not produce microcystin. However, to my knowledge, this is the first study to

investigate the ability of micro- and mesozooplankton (natural and cultured) to successfully graze on toxic and non-toxic strains of *Microcystis* during natural bloom events

During the summer of 2008, the mesozooplankton community was comprised of mainly small-bodied cladocerans such as *Bosmina* and *Monia*, copepods (*Acanthocyclops*), and ciliates (*Climacostomum*) but was relatively void of large cladocerans such as *Daphnia* spp. (Table 3) a finding that is consistent with prior studies of zooplankton and toxic cyanobacteria (Gilwicz, 1977; Edmondson & Litt 1982; Richman & Dodson, 1983; Orcutt & Pace, 1984; Infante & Riehl, 1984). Similar to a study conducted by Hansson et al. (2007) in Swedish lakes, we found the total copepod concentrations were significantly correlated with microcystin concentrations ($p < 0.001$). However, conversely to their findings I did not find any negative correlations between microcystin concentration and *Daphnia* densities. Furthermore, for both field seasons, on most dates, the microzooplankton community was primarily comprised of dinoflagellates (Tables 3 & 4). This finding is consistent with a previous study that found dinoflagellates were the dominant group of eukaryotes during a *Microcystis* bloom in a Turkish estuary (Taş et al., 2006).

Although previous studies have shown that individual protozoan grazers were able to graze on *Microcystis* populations (Nishibe et al., 2002, 2004; Ou et al., 2005), this study is the first to report that natural communities of microzooplankton are capable of grazing on both toxic (*mcyD*-containing) and non-toxic strains of *Microcystis*. Electivity indices showed that, in the three experiments where both toxic and non-toxic *Microcystis* strains were grazed, the micozooplankton community displayed no clear preference for

either population, suggesting the ability to produce microcystin did not seem to provide toxic (microcystin-producing) strains of *Microcystis* a defense from grazing by microzooplankton. Consistent with this hypothesis, the ability of microzooplankton to graze on the toxic strains and the rates at which they grazed varied independent of microcystin concentrations and toxic *Microcystis* densities as the highest grazing rate on toxic strains was found on the same date as the highest microcystin concentration (Fig 1, Table 5). Moreover, in 2008, abundances of total microzooplankton were significantly correlated with toxic *Microcystis* densities, as well as microcystin concentrations ($p < 0.01$). These findings were similar to Kim et al. (2006) who found that the flagellate *Diphylleia rotans* yielded the highest growth rate and ingestion rate on the most toxic of five strains of *Microcystis*. All strains of *Microcystis* were ungrazed by microzooplankton during some experiments when the total cyanobacteria population was actively grazed (Table 5). Since microzooplankton grazing rates on all strains of *Microcystis* varied independently of colony sizes, I hypothesize that a low nutritional value or other bioactive compounds besides microcystin (Falconer, 2007) might account for the absence of grazing of *Microcystis* by microzooplankton during these experiments.

I observed large differences in the ability of laboratory-reared mesozooplankton and the natural community of mesozooplankton to graze on toxic and non-toxic strains of *Microcystis*. Both laboratory-reared mesozooplankton, *D. pulex* and *H. azteca*, were poor grazers of *Microcystis*, successfully grazing on various strains in only 16 – 33% of experiments. The differences between the cultured and natural zooplankton could be due natural selection during blooms favoring the zooplankton which are able to effectively graze on *Microcystis*. The mesozooplankton community found in the TR was dominated

by zooplankton which have previously been shown to actively graze on *Microcystis* including small-bodied cladocerans (*Bosmina*, *Mona*; Gliwicz, 1977), rotifers (Orcott & Pace, 1984; Fulton & Paerl, 1987b), and copepods (Richman & Dodson, 1983). In contrast, *Microcystis* is not well grazed by large-bodied cladocerans such as *Daphnia* (Fulton & Pearl, 1988a; Rohrlark et al., 2005; Gobler et al., 2007). Electivity indices indicated that when grazing did occur, *H. azteca* displayed a slight preference for toxic strains over the non-toxic strains of *Microcystis*, while *D. pulex* showed the opposite preference, suggesting that the amphipod, *H. azteca*, was more tolerate of toxic strains *Microcystis* than the cladoceran, *D. pulex* (Table 7). The poor grazing rates of *Daphnia* on toxic cyanobacteria could partly explain why large-bodied cladocerans (i.e. *Daphnia*) were absent in TQR from the beginning of July through the end of September (Table 3; Hansson et al., 2007).

Multiple factors can influence the impacts of toxic *Microcystis* on zooplankton including pre-exposure to cyanobacteria (Hairston et al., 1999), genetic differences (Sarnelle & Wilson, 2005), maternal effects (Gustafsson et al., 2005), as well as how the zooplankton were exposed to the *Microcystis* (i.e. pure culture versus mixed cultures (Rohrlack et al., 2005)). Although cultured *H. azteca* and *D. pulex* rarely grazed *Microcystis*, the natural community of mesozooplankton in TQR was able to graze on total, toxic, and non-toxic strains of *Microcystis* in most of the experiments conducted (75 - 88%; Fig 4). These results indicate that the natural community of mesozooplankton, which was dominated by small-bodied cladocerans, such as *Bosmina* and *Moina*, as well as the cyclopoid copepod *Acanthocyclops* (Table 3) were better grazers of *Microcystis* than cultured mesozooplankton. This conclusion is consistent with

previous studies that found these zooplankton are capable of actively grazing during *Microcystis* blooms (Fulton & Pearl, 1988a; Hansson et al., 2007). Hairston et al. (1999, 2001) found that populations of *Daphnia galeata* exposed to dense cyanobacteria blooms in Lake Constance were more resistant to *Microcystis* than populations reared under non-bloom conditions suggesting a genetic shift occurs in wild zooplankton populations towards strains able to actively graze toxic cyanobacteria such as *Microcystis*. Furthermore, natural populations exposed to cyanobacteria may actively produce glutathione *S*-transferase which detoxifies microcystin (Pflugmacher et al., 1998).

Since the natural mesozooplankton community in TQR was able to graze on both strains of *Microcystis* with equal success and rates, the tolerance for *Microcystis* displayed by the mesozooplankton was for the genus as a whole and was independent of the ability of strains to produce microcystin. Furthermore, in most experiments (86%) where at least one strain of *Microcystis* was grazed, the *in situ* community preferentially grazed on the toxic strains of *Microcystis*. This finding is similar to Guo & Xie (2006) who found that a few small-bodied cladocerans developed a tolerance to toxic strains of *Microcystis* and Kim et al. (2006) who reported some zooplankton graze toxic *Microcystis* cultures at a higher rate than non-toxic clones. This further indicates that the ability to produce microcystin does not protect *Microcystis* cells from predation by wild mesozooplankton.

Microcystis colony size did not seem to influence the ability of or the rate at which microzooplankton or mesozooplankton were grazed on the *Microcystis* populations. Colony size did not co-vary with grazing rates nor did it differ between experiments when there was or was not grazing on any strain of *Microcystis.* Although

this finding is contrary to some previous studies that have shown that zooplankton are less able to graze on larger cyanobacteria colonies (Gliwicz & Siedlar, 1980; Thompson et al., 1982; Hartmann, 1985) the colonies in TQR were smaller than the sizes previously documented as being inhibitory to zooplankton grazers (36 ± 6 cells colony^{-1} in 2007; 47 ± 19 cells colony^{-1} in 2008; O'Brian and DeNoyelles, 1974; Nishibe et al., 2002). The flagellate *Collodictyon triciliatum* is able to graze on *Microcystis* colonies up to 51 cells per colony (Nishibe et al., 2002) and *Microcystis* colonies ranging up to 50 cells did not alter the filtration rate of the mesozooplankton *Ceriodaphnia reticulata* (O'Brian & DeNoyelles, 1974). While I cannot discount the importance of large colony size (> 50 cells per colony) in discouraging zooplankton grazing, it did not seem to affect this process, perhaps due to the small colonies within our study site.

While zooplankton grazing rates on toxic and non-toxic strains of *Microcystis* did not differ significantly, there were multiple experiments when there was active grazing by micro- and/or mesozooplankton on other phytoplankton and/or cyanobacteria, but not on any *Microcystis* population. There are a suite of biologically active compounds that bloom-forming cyanobacteria produce which have a wide range of negative effects on animals including enzyme inhibition and cellular disruptors (Carmichael, 1992; Codd, 1995). To date, multiple protease inhibitors have been discovered including micropeptins, cyanopeptolins, oscillapeptin, and aeruginosins (Namikoshi & Rinehart, 1996). Prior studies have shown that roughly half of all cyanobacteria blooms comprised of microcystin-producing genera are toxic to mammals but almost 100% of these blooms contain protease inhibitors (Carmichael, 1992; Reshef & Carmeli, 2001). A study conducted by Agrawal et al. (2005) found that an extract from a cultured *M. aeruginosa*

strain contained protease inhibitors that targeted the two main digestive proteases of *Daphnia magna*, trypsin and chymotrypsin. Von Elert et al. (2005) described a protease inhibitor produced by *Microcystis*, cyanopeptolin 954, that was able to inhibit chymotrypsin. Furthermore, Rohrlark et al. (2003) has shown that at least one other compound synthesized by *Microcystis*, known as microviridin, can have negative impacts on zooplankton (i.e. *Daphnia*) and could act as an anti-grazing compound. Hence, although the ability to synthesize microcystin does not seem to discourage zooplankton grazing on *Microcystis*, these and other similar compounds may. Overall, this study has shown that the natural communities of mesozooplankton and microzooplankton can actively graze on the total *Microcystis* population within the natural assemblage of phytoplankton even when other prey items are available. Also, although not always grazed, both toxic and non-toxic strains of *Microcystis* can be grazed by cultured as well as natural communities of mesozooplankton and microzooplankton. This study also found that the natural community of mesozooplankton are better grazers of both strains of *Microcystis* than their cultured counterparts which could account for the inconsistent results found by previous laboratory studies as many of the previous studies have been conducted with cultured grazers which the previous studies indicate do not compete well during times of cyanobacterial dominance (i.e. *Daphina*; Fulton & Pearl, 1988a; Hansson et al., 2007). The field results demonstrate that the ability to produce microcystin does not offer *Microcystis* cells refuge from grazing by either mesozooplankton or microzooplankton.

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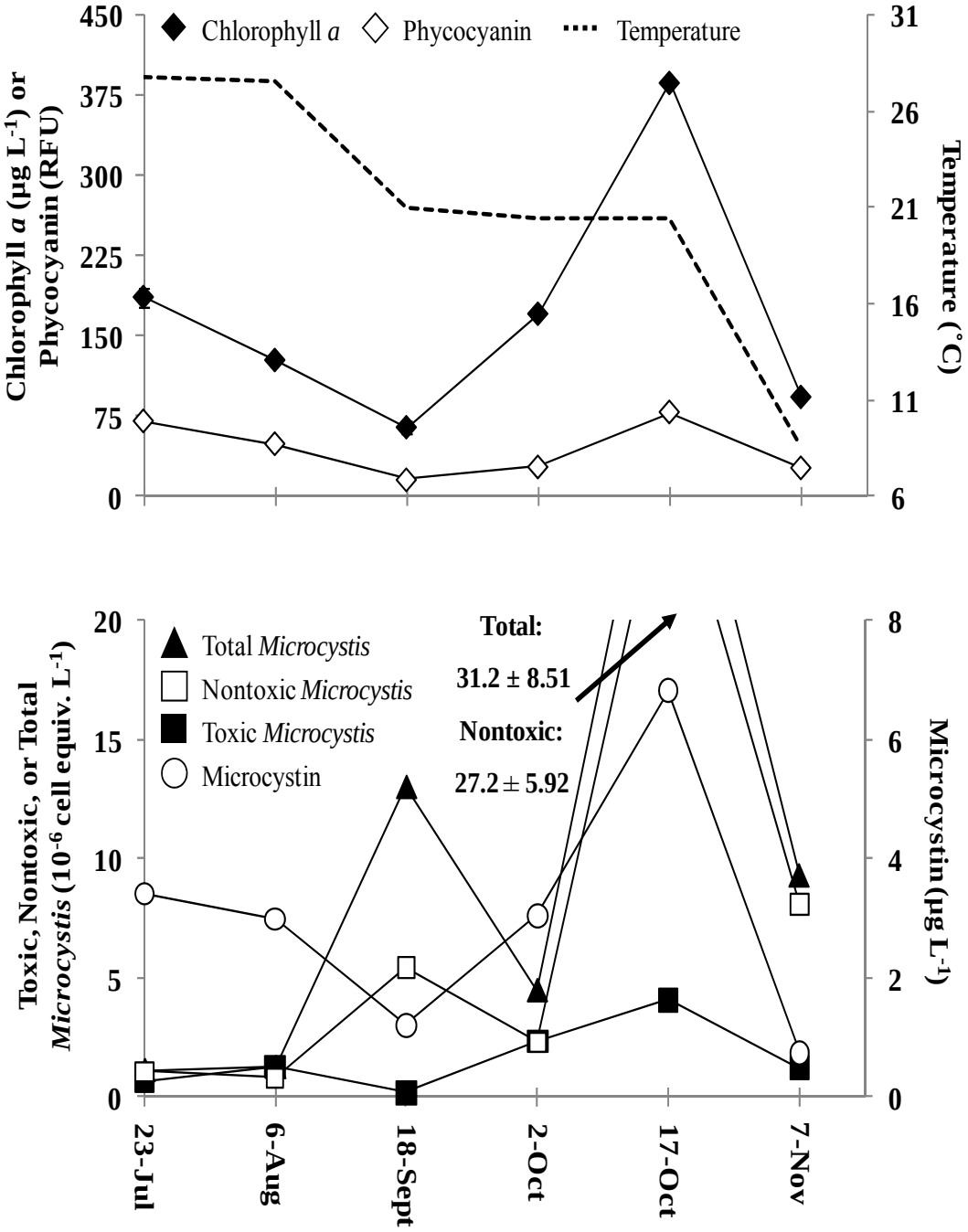

Figure 1: Time series of parameters measured in the Transquaking River, 2007. Top graph: Levels of total chl *a*, phycocyanin and temperature. Bottom graph: Densities of total, toxic and non-toxic *Microcystis* as well as concentrations of microcystin. Error bars represent ± 1 SE of replicated samples.

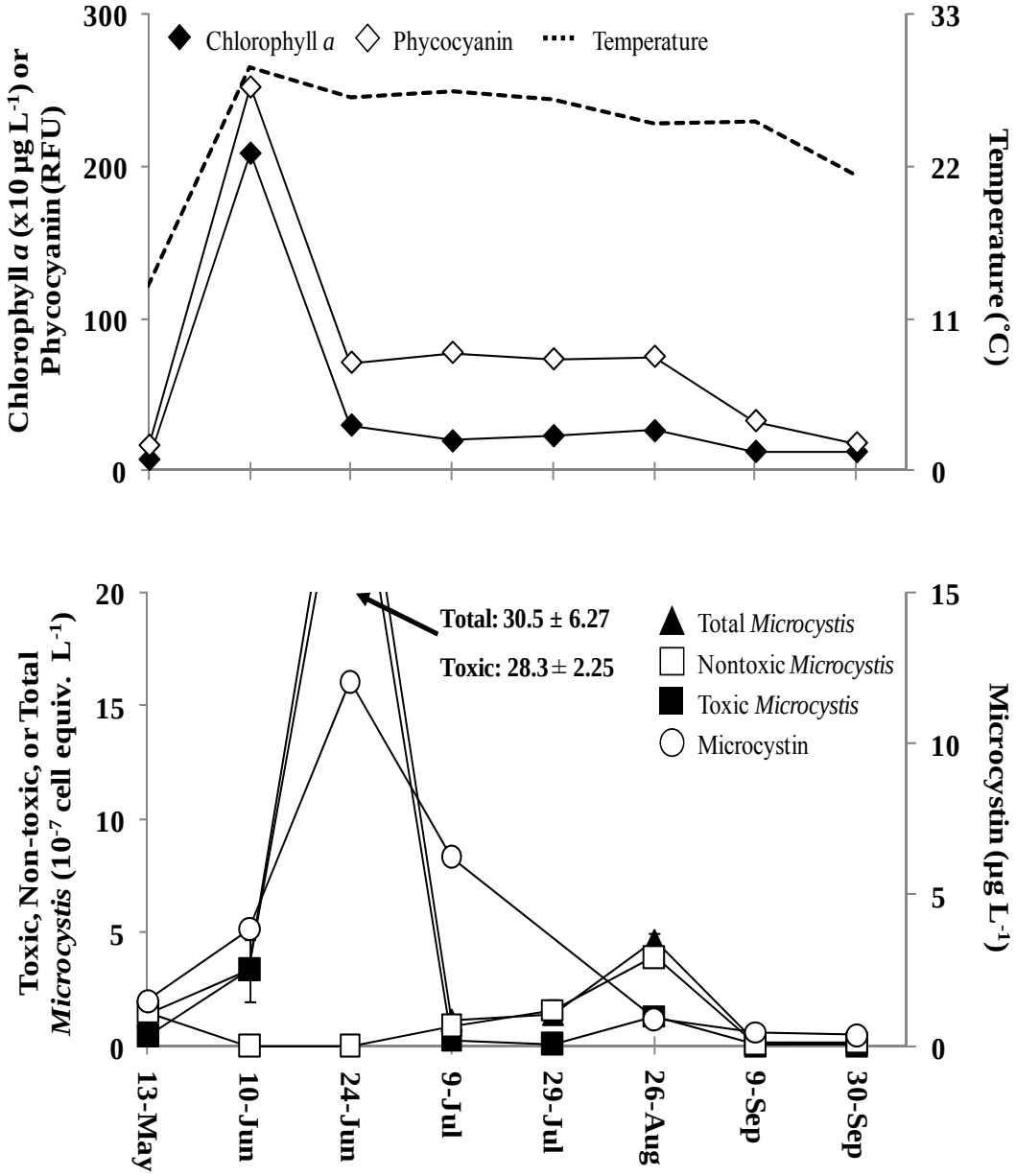

Figure 2: Time series of parameters measured in Transquaking River, 2008. Further details as in Fig. 1.

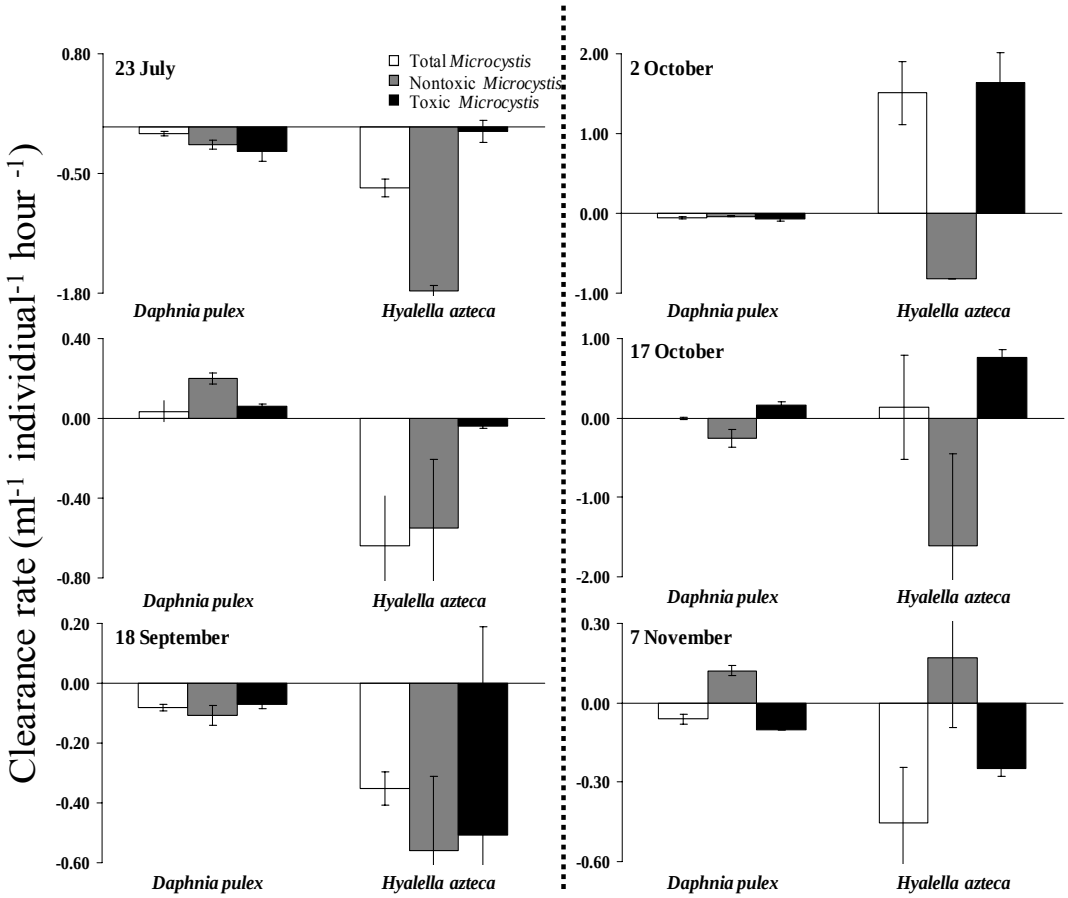

Figure 3: Clearance rates of total, toxic, and non-toxic *Microcystis* by *D. pulex* and *H. azteca* during mesozooplankton addition experiments conducted in the Transquaking River, 2007. Error bars represent ± 1 SE of triplicate experimental bottles.

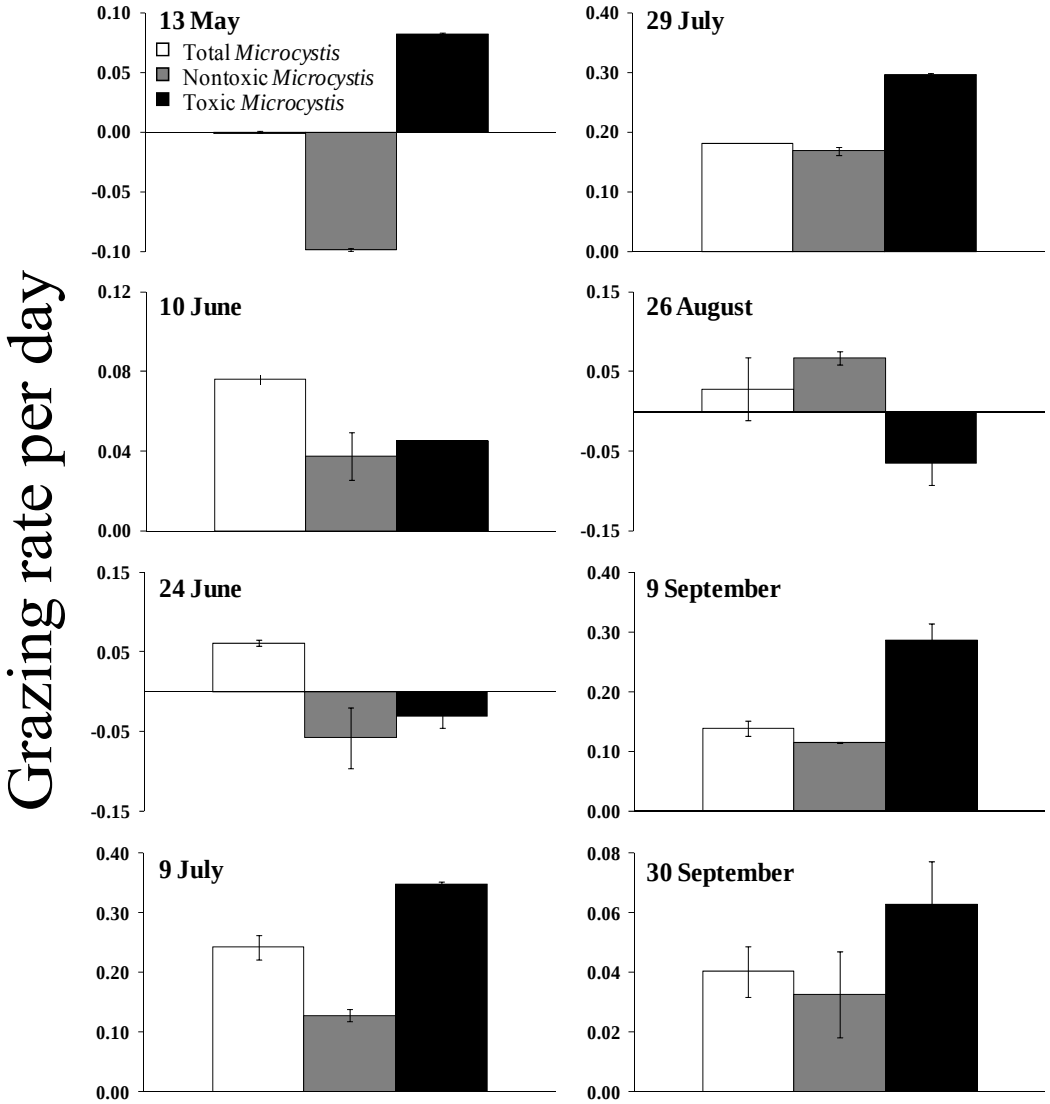

Figure 4: Grazing rates on the total, toxic, and non-toxic *Microcystis* populations by the *in situ* mesozooplankton community during 8x mesozooplankton addition experiments conducted in the Transquaking River, 2008. Error bars represent ± 1 SE of triplicate experimental bottles.

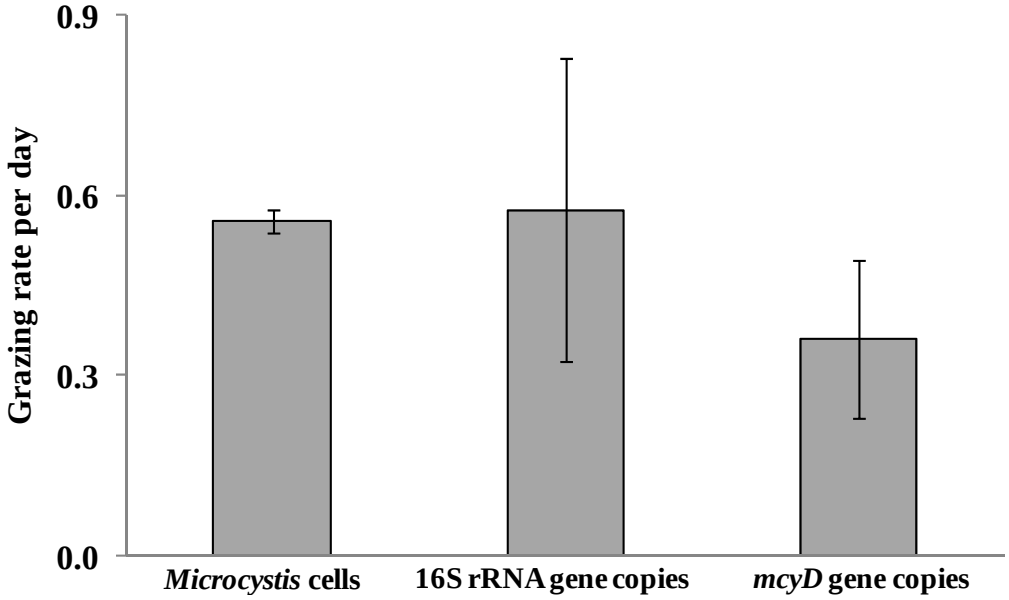

Figure 5: Grazing rates by *D. pulex* on *Microcystis* clone LE-3 densities, as quantified by microscopic cell counts, 16S rRNA gene copies, and *mcyD* gene copies during a 48 hour grazing experiment. Error bars represent ± 1 SD of triplicate experimental bottles.

DNA Target	Primer	Sequence(5'-3')	Reference
pGEM plasmid DNA	M13F	CCCAGTCACGACGTTGTAAAAACG	Coyne *et al.* 2005
	pGEM R	TGTGTGGAATTGTGAGCGGA	Coyne *et al.* 2005
	pGEM probe	(Taq) FAM[a]-CACTATAGAATACTCAAGCTTGCATGCCTGCA-BHQ-1[b]	Coyne *et al.* 2005
***Microcystis* 16S rDNA**	184F	GCCGCRAGGTGAAAMCTAA	Neilan *et al.* 1997
	431R	AATCCAAARACCTTCCTCCC	Neilan *et al.* 1997
	Probe	(Taq) FAM[a]-AAGAGCTTGCGTCTGATTAGCTAGT-BHQ-1[b]	Rinta-Kanto *et al.* 2005
***Microcystis* mcyD**	F2	GGTTCGCCTGGTCAAAGTAA	Kaebernick *et al.* 2000
	R2	CCTCGCTAAAGAAGGGTTGA	Kaebernick *et al.* 2000
	Probe	(Taq) FAM[a]-ATGCTCTAATGCAGCAACGGCAAA-BHQ-1[b]	Rinta-Kanto *et al.* 2005

Table 1: A list of primers (Integrated DNA Technologies, Iowa, USA) and probes (Applied Biosystems, Foster City, CA, USA) used in the qPCR analysis; a= 6-Carboxyfluorescein b= Black Hole Quencher-1 (quenching range 480-580 nm)
F= forward primer R= reverse primer.

	*Microcystis**	*Anabaena**	*Aphanizomenon***
Transquaking River 2007			
23-Jul	570,000,000 (1,200,000)	5,200,000 (100,000)	910,000 (15,000)
6-Aug	130,000,000 (1,300,000)	4,800,000 (250,000)	1,100,000 (95,000)
18-Sep	170,000,000 (17,000,000)	2,700,000 (270,000)	4,500,000 (450,000)
2-Oct	57,000,000 (400,000)	6,000,000 (600,000)	860,000 (70,000)
17-Oct	61,00,000 (700,000)	59,000,000 (200,000)	0 (0)
7-Nov	17,000,000 (1,200,000)	24,000,000 (200,000)	210,000 (5,000)
Transquaking River 2008			
13-May	10,000,000 (760,000)	0 (0)	0 (0)
10-Jun	72,000,000 (4,800,000)	350,000,000 (1,000,000)	0 (0)
24-Jun	190,000,000 (3,900,000)	7,200,000 (1,200,000)	5,100,000 (280,000)
9-Jul	120,000,000 (4,000,000)	1,300,000 (1,250,000)	12,000,000 (710,000)
29-Jul	220,000,000 (3,700,000)	150,000 (150,000)	2,000,000 (140,000)
26-Aug	310,000,000 (2,700,000)	2,400,000 (250,000)	2,100,000 (60,000)
9-Sep	48,000,000 (340,000)	8,500,000 (120,000)	4,400,000 (110,000)
30-Sep	23,000,000 (990,000)	0 (0)	2,400,000 (18,000)

Table 2: Mean cyanobacterial densities (cells* or colonies** L^{-1}) (SE in parentheses) for Transquaking River, 2007 and 2008. Counts were made using light microscopy.

	23-Jul-07	6-Aug-07	18-Sep-07	2-Oct-07	17-Oct-07	7-Nov-07
Rotiphera	34 (3)	27 (3)	9 (1)	29 (3)	30 (3)	1 (1)
Aloricate ciliates	5 (1)	34 (3)	26 (3)	14 (1)	5 (1)	25 (3)
Loricate ciliates	3 (1)	13 (1)	1 (1)	4 (1)	8 (1)	2 (1)
Total ciliates	8 (3)	47 (4)	27 (3)	18 (3)	14 (3)	27 (3)
Adult copepods	16 (2)	7 (1)	5 (1)	4 (1)	11 (1)	0 (0)
Copepod nauplii	13 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dinoflagellates	0 (0)	445 (95)	305 (15)	460 (0)	0 (0)	40 (10)
Total microzooplankton	71 (4)	526 (95)	346 (15)	511 (3)	55 (3)	68 (11)

Table 3: Mean microzooplankton densities in individuals ml^{-1} (SE in parentheses) for the Transquaking River 2007.

	13-May-08	10-Jun-08	24-Jun-08	9-Jul-08	29-Jul-08	26-Aug-08	9-Sep-08	30-Sep-08
Cladorcera								
Bosmina longirostris	42 (12)	2 (2)	13 (2)	16 (3)	5 (2)	11 (0)	1 (1)	1 (0)
Diphanosoma brachyurum	1 (1)	1 (0)	7 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Daphnia parvula	6 (2)	1 (1)	2 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Eubosmina coregoni	2 (1)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Daphnia magna	3 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Daphnia lumholtzi	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Leptodora kindtii	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ceriodaphnia spp.	0 (0)	18 (3)	4 (1)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Moina spp.	0 (0)	10 (3)	33 (3)	20 (2)	17 (1)	36 (3)	6 (1)	1 (0)
Eurycercus spp.	0 (0)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total cladocerans	56 (13)	36 (5)	60 (4)	37 (4)	22 (3)	47 (3)	7 (1)	2 (0)
Copopoda								
Acanthocyclops robustus	3 (2)	8 (0)	37 (4)	19 (2)	19 (5)	11 (0)	5 (1)	1 (0)
Acanthocyclops vernalis	2 (1)	2 (1)	18 (1)	6 (3)	4 (0)	4 (2)	1 (1)	0
Total copepods	5 (2)	10 (1)	55 (4)	25 (4)	23 (5)	15 (2)	6 (1)	1 (0)
Ciliates								
Climacostomum virens	5 (3)	98 (12)	1 (0)	3 (1)	9 (0)	2 (1)	1 (1)	0 (0)
Rotifera								
Brachionus calyciflorus	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	7 (1)	2 (0)	1 (0)
Asplanchna priodonta	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1)
Arthropoda								
Mochlonyx spp.	0 (0)	4 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total Mesozooplankton	65 (13)	147 (13)	116 (6)	66 (5)	54 (6)	70 (3)	16 (1)	4 (0)
Rotiphera	1 (1)	0 (0)	7 (1)	50 (5)	22 (2)	32 (3)	9 (1)	9(1)
Aloricate ciliates	26 (3)	31 (3)	31 (3)	31 (3)	31 (3)	19 (2)	30 (3)	53 (5)
Loricate ciliates	74 (7)	4 (1)	7 (1)	7 (1)	5 (1)	7 (1)	4 (1)	4 (1)
Total ciliates	100 (8)	35 (3)	38 (3)	38 (6)	36 (4)	26 (4)	33 (3)	57 (5)
Dinoflagellates	0 (0)	205 (21)	2983 (298)	125 (13)	210 (21)	100 (10)	155 (16)	95 (10)
Total Microzooplankton	101 (8)	240 (21)	3028 (298)	213 (14)	268 (11)	158 (11)	198 (11)	161 (11)

Table 4: Mean microzooplankton (individuals ml^{-1}) and mesozooplankton densities (individuals L^{-1}) (SE in parentheses) for the Transquaking River 2008.

	Total cyanobacteria			Total *Microcystis*			Nontoxic *Microcystis*			Toxic *Microcystis*		
Date	Grazing rate	Growth rate (μ)	Net μ	Grazing rate	Growth rate (μ)	Net μ	Grazing rate	Growth rate (μ)	Net μ	Grazing rate	Growth rate (μ)	Net μ
23-Jul-07	0.45 (0.07)	0.43 (0.04)	-0.02 (0.04)	0.46 (0.21)	0.79 (0.14)	0.33 (0.13)	0.5 (0.27)	1.6 (0.18)	1.1 (0.17)	0.76 (0.31)	0.80 (0.22)	0.04 (0.20)
6-Aug-07	0.2 (0.05)	0.39 (0.04)	0.19 (0.03)	0.72 (0.26)	1.7 (0.17)	1.0 (0.16)	0.9 (0.44)	1.7 (0.29)	0.81 (0.28)	0.54 (0.25)	1.1 (0.20)	0.51 (0.16)
18-Sep-07	0.17 (0.01)	0.56 (0.01)	0.39 (0.01)	ND	ND	-	ND	ND	-	ND	ND	-
2-Oct-07	0.23 (0.03)	0.24 (0.02)	0.01 (0.02)	2.5 (0.56)	2.1 (0.38)	-0.47 (0.37)	3.1 (0.85)	2.2 (0.60)	-0.87 (0.61)	ND	ND	-
17-Oct-07	0.13 (0.02)	0.24 (0.02)	0.11 (0.01)	1.7 (0.52)	1.3 (0.35)	-0.39 (0.34)	2.5 (0.46)	1.7 (0.31)	-0.79 (0.30)	1.5 (0.38)	2.1 (0.26)	0.53 (0.24)
7-Nov-07	0.2 (0.04)	0.67 (0.03)	0.47 (0.02)	1.0 (0.50)	0.9 (0.35)	-0.07 (0.33)	0.88 (0.34)	0.93 (0.23)	0.05 (0.53)	0.65 (0.25)	0.82 (0.17)	0.17 (0.15)

Table 5: Mean microzooplankton grazing rates, intrinsic algal growth rates (μ), and net algal growth rates (Net μ), SE in parentheses, on the total cyanobacterial community (phycocyanin), total *Microcystis* community non-toxic *Microcystis*, and toxic *Microcystis* during microzooplankton grazing experiments in the Transquaking River, 2007. ND = grazing was not detectable via the dilution method.

Treatment	Date	*Microcystis*	Electivity Index	+/-
Natural microzooplankton community	23-Jul-07	Toxic	0.09	+
		Non-toxic	-0.12	-
	6-Aug-07	Toxic	-0.20	-
		Non-toxic	0.05	+
	18-Sep-07	Toxic	-1.00	-
		Non-toxic	-1.00	-
	2-Oct-07	Toxic	-1.00	-
		Non-toxic	1.00	+
	17-Oct-07	Toxic	-0.13	-
		Non-toxic	0.11	+
	7-Nov-07	Toxic	0.00	0
		Non-toxic	0.00	0

Table 6: Electivities indicies for the *in situ* microzooplankton community on toxic *Microcystis* and non-toxic *Microcystis* during dilution experiments in the Transquaking River 2007. (+) = selection for the pray item. (-) = avoidance of the prey item. (0) = no preference for or against the pray item.

Treatment	Date	*Microcystis*	Electivity Index	+/-
Daphnia pulex	23-Jul-07	Toxic	-1.00	-
		Non-toxic	-1.00	-
	6-Aug-07	Toxic	-0.37	-
		Non-toxic	0.21	+
	18-Sep-07	Toxic	-1.00	-
		Non-toxic	-1.00	-
	2-Oct-07	Toxic	-1.00	-
		Non-toxic	1.00	+
	17-Oct-07	Toxic	1.00	+
		Non-toxic	-1.00	-
	7-Nov-07	Toxic	-1.00	-
		Non-toxic	1.00	+
Hyalella azteca	23-Jul-07	Toxic	-1.00	-
		Non-toxic	-1.00	-
	6-Aug-07	Toxic	-1.00	-
		Non-toxic	-1.00	-
	18-Sep-07	Toxic	-1.00	-
		Non-toxic	-1.00	-
	2-Oct-07	Toxic	1.00	+
		Non-toxic	-1.00	-
	17-Oct-07	Toxic	1.00	+
		Non-toxic	-1.00	-
	7-Nov-07	Toxic	-1.00	-
		Non-toxic	1.00	+
Natural mesozooplankton community	13-May-08	Toxic	1.00	+
		Non-toxic	-1.00	-
	10-Jun-08	Toxic	0.04	+
		Non-toxic	-0.05	-
	24-Jun-08	Toxic	-1.00	-
		Non-toxic	-1.00	-
	9-Jul-08	Toxic	0.19	+
		Non-toxic	-0.30	-
	29-Jul-08	Toxic	0.12	+
		Non-toxic	-0.16	-
	26-Aug-08	Toxic	-1.00	-
		Non-toxic	1.00	+
	9-Sep-08	Toxic	0.18	+
		Non-toxic	-0.27	-
	30-Sep-08	Toxic	0.14	+
		Non-toxic	-0.19	-

Table 7: Electivities indicies for *D. pulex*, *H. azteca*, and the *in situ* mesozooplankton community on toxic *Microcystis* and non-toxic *Microcystis* during mesozooplankton addition experiments in the Transquaking River 2007 & 2008. (+) = selection for the pray item. (-) = avoidance of the prey item.

CHAPTER FIVE:

Dissertation summary

Dissertation Summary

Microcystis is a unique harmful algal bloom genus (HAB) to study as it is one of the only phytoplankton genera for which both the compound responsible for its toxicity (microcystin) and the gene operon that responsible for the biosynthesis of that compound (microcystin synthetase genes *A* - *J*; Tillett et al., 2000) are known. This is extremely important as toxic and non-toxic strains of *Microcystis* often co-occur during bloom events and they are visually indistinguishable. Thus, previous field studies of *Microcystis* were only able to draw conclusions regarding how various environmental factors affected the total *Microcystis* population. For my dissertation, I was able to utilize molecular tools to assess how environmental factors, such as temperature, nutrients and grazing by meso- and microzooplankton affected the abundance of toxic and non-toxic strains of *Microcystis* within ecosystem settings.

From a management perspective, being able to accurately predict microcystin concentrations in a body of water is essential for protecting humans from adverse health effects due to cyanotoxin exposure. To date, the World Health Organization (WHO) has recommended using either chlorophyll *a* concentrations or total cyanobacterial cell densities to manage lakes against human exposure to cyanotoxins (Chorus & Bartram, 1999). This study found that toxic (*mcyD*-containing) *Microcystis* cells were a universally better predictor of microcystin concentrations than either of the current parameters recommended by the WHO. During my dissertation, I studied five distinct freshwater systems from Canada to Maryland and generated eight different time series data sets (Table 1). Within every system sampled and during each year of study (2005 – 2008) abundances of toxic (*mcyD*-containing) *Microcystis* cells were significantly

correlated with microcystin concentrations (Table 1), including systems within which *Microcystis* was not the dominant genera (i.e. Lake Champlain, Transquaking River; Chapters Two – Four; Table 1). Furthermore, the correlations of microcystin with *mcyD*-containing *Microcystis* cells were generally stronger than with any other parameter measured (Table 1). In contrast, parameters suggested by World Health Organization (WHO) to manage lakes against human exposure to cyanotoxins (chlorophyll *a* concentrations and total cyanobacterial cell densities) were only correlated with concentrations of microcystin in half of the time series gathered (Table 1). Abundances of non-toxic *Microcystis* were correlated with concentrations of microcystin in only one time series (Table 1). Since I found that molecularly quantified abundances of toxic (*mcyD*-containing) *Microcystis* cells are the best predictor of microcystin concentrations, monitoring this population may be the best approach for protecting against exposure to this toxin. However, I found that the correlation between microcystin concentrations and toxic *Microcystis* cell densities broke down if I combined all of the data sets from every system. I hypothesize that this lack of correlation is due to different populations of toxic *Microcystis* having different cellular microcystin quotas (Lukač & Aegerter, 1993; Orr & Jones, 1998). Furthermore, I found that for each system sampled multiple years (Lake Agawam & Transquaking River) the correlation between microcystin and toxic *Microcystis* cell densities was maintained (Table 1) indicating that if toxic (*mcyD*-containing) *Microcystis* cells were to be used as a predictor of microcystin concentrations, the regulatory protocols should be system specific. Due to the speed, relatively low cost, and accuracy of these molecular techniques, this qPCR approaches can quickly and accurately predict toxin concentrations, therefore enabling more effective

management against human exposure to these compounds. Because of these advantages, other countries, such as Australia, are now beginning to shift monitoring practices towards molecular approaches (B. Neilan, pers. comm.).

My dissertation focused on the molecular quantification of toxic and non-toxic *Microcystis* via qPCR and my results affirm the accuracy of this method for quantifying toxic *Microcystis* populations (Table 1). However, there were instances when total *Microcystis* densities measured by the quantification of *Microcystis* 16S gene copies differed from densities measured with traditional light microscopy (Chapters Two – Four). On occasion, 16S gene copies were lower than microscopic *Microcystis* cell counts, potentially due to incomplete recovery of DNA from *Microcystis* cells. While I used an internal control when conducting qPCR (pGEM) this plasmid was in solution and therefore allowed me to assess extraction efficiency of *Microcystis* DNA in solution but not the removal of DNA from cells via lysis. Alternatively, 16S gene copies numbers were sometimes greater than *Microcystis* densities determined by light microscopy. This could happen if *Microcystis* populations were single cells (4 - 6 μm) or very small colonies (2 – 4 cells). These populations were probably not quantified via light microscopy since the *Microcystis* populations quantified were almost always larger colonies (> 5 cells). My use of a 2 μm filter to collect *Microcystis* for molecular quantification ensured I quantified all *Microcystis* cells, regardless of colony prescence or size. Finally, the number of 16S gene copies per *Microcystis* may differ widely among strains and this could lead to over- or underestimations of cell densities. Bacteria can contain up to 12 copies of the 16S gene per cell (Fogel et al., 1999) while cultured *Microcystis aeruginosa* NIES 102 strain contained 250 16S gene copies (Ha et al., 2009).

Furthermore, Rinta-Kanto et al. (2005) found that DNA copy numbers could be up to two orders of magnitude greater than cell densities estimated by light microscopy.

My dissertation established that nutrients can play a central role in promoting the densities of toxic *Microcystis* within multiple freshwater ecosystems (Chapter Two; Fig 1). Toxic and non-toxic strains of *Microcystis* were both stimulated by organic and inorganic N and P compounds (Chapter Two). However, non-toxic strains were more often stimulated by organic N whereas toxic strains were primarily stimulated by inorganic N and P (Chapter Two; Fig 1). This difference in affinity for nutrients between the two strains could allow toxic and non-toxic stains to co-occur on an annual basis. Inorganic forms of nutrients are often elevated during early summer months, therefore toxic strains may have adapted the ability to rapidly assimilate these compounds and dominate when inorganic nutrient levels are elevated, which is often in early summer. However, as inorganic N and P are depleted, non-toxic strains become more abundant (Chapter Two). The entire *Microcystis* population showed great flexibility regarding N assimilation (Chapter Two). This may be a mechanism which allows *Microcystis*, as a genus, to dominate under diverse environmental conditions.

As the human population continues to grow and have an ever increasing impact on the surrounding environment, it is important to understand how these changes could impact both toxic and non-toxic strains of *Microcystis*. The burning of fossil fuels and subsequent rise in atmospheric carbon dioxide has caused the earth's surface temperature to increase by approximately $1^{o}C$ during the 20^{th} century, with most of the increase having occurred during the last 40 years (IPCC, 2001). In the current century, global temperatures are expected to increase an additional 1.5 to $5^{o}C$ (IPCC, 2001). During this

study, experimental increases in water temperatures of 4^{o}C above ambient frequently yielded higher growth rates for toxic *Microcystis* strains, but did so less frequently for their non-toxic counterparts (Chapter Three; Fig 1). Furthermore, the additive effect between enhanced temperature and phosphate (P) often yielded the highest growth rate for toxic strains of *Microcystis*, greater than that of any population monitored or treatment administered (Chapter Three; Fig 1), suggesting that the simultaneous increasing in water temperature along with increased P-loading could create conditions where toxic strains are able to out-compete non-toxic strains of *Microcystis* which could potentially lead to blooms of greater toxicity in the future.

During my dissertation research, I documented *Microcystis* population shifts from toxic to non-toxic strains during summer as DIN levels transitioned from high to low levels, independent of *in situ* temperatures (Lake Ronkonkoma, Transquaking River 2008; Chapters Two & Three). I also found that in systems with low DIP concentrations toxic strains never dominated the total *Microcystis* community (Lake Agawam 2008; Chapter 2). Moreover, the highest densities of toxic *Microcystis* did not coincide with absolute, annual maximal temperatures when dissolved inorganic nutrient concentrations were very low (Chapters Two – Four). However, enhanced temperatures *combined with* elevated nutrient concentrations did often yield the highest densities of toxic *Microcystis* (Chapter 3). Therefore, I conclude that increases in temperature will only further enhance toxic *Microcystis* blooms when there are elevated ambient nutrient concentrations. While global temperatures are prediceted to increase 1 - 5˚C this century (IPCC, 2001), management strategies which reduce nutrient levels may avoid increases in toxic *Microcystis* blooms due to increased water temperatures.

The ability of toxic strains of *Microcystis* to dominate systems enriched in inorganic nutrients during early summer months may be a mechanism which permits *Microcystis* dominace throughout summer and early fall. Microcystin-producing strains of *Microcystis* can have allelopathic effects on other phytoplankton (Pflugmacher, 2002; Vardi et al., 2002, Schatz et al., 2007) while non-toxic strains do not (Vardi et al., 2002). Also, previous studies have found that microcystin content per cell is often highest at the onset of blooms and decreases towards the peak of the bloom (Weissing & Huisman, 1994; Kardinaal et al., 2005; Welker et al., 2007). Therefore, allelopathic effects of toxic *Microcystis* on competitors together with grazing deterrence during early summer could promote *Microcystis* blooms through positive feedback whereby higher toxic *Microcystis* cell densities yield fewer competitors and predators which, in turn, facilitate higher cell densities. While declining inorganic nutrient levels may cause a shift from toxic to non-toxic *Microcystis* (Chapter 2), the displacement of competing phytoplankton, higher levels of organic nutrients (Chapter 2), and continued grazing deterrence by non-toxic *Microcystis* (Chapter 4) may allow the genus to bloom throughout the summer and early fall.

This study was the first to investigate the ability of both cultured and natural mesozooplankton as well as the natural community of microzooplankton to graze on toxic and non-toxic strains of *Microcystis*. Both natural populations of meso- and microzooplankton were able to graze on both toxic and non-toxic strains of *Microcystis* at similar rates (Chapter Four; Fig 2). However, there were differences in the grazing ability of the cultured and natural mesozooplankton, as the cultured mesozooplankton were poorer grazers of both strains of *Microcystis* (Chapter Four; Fig 2). These findings

suggest there is a tolerance developed by natural populations of zooplankton to cyanobacteria (Hairston et al., 1999, 2001). Furthermore, this study provides the first evidence that, while not always grazed, both toxic and non-toxic strains can be grazed by meso- and microzooplankton at equal rates and frequencies indicating that the production of microcystin does not provide a defense against grazing. I therefore conclude that grazing by zooplankton does not play a significant role in affecting the dynamics of toxic and non-toxic *Microcystis* strains (Fig 2). Previous studies which have established that *Microcystis* produces a suite of protease inhibitors including micropeptins, cyanopeptolins, oscillapeptin, and aeruginosins (Namikoshi & Rinehart, 1996) some of which can inhibit the two main digestive proteases of mesozooplankton, trypsin and chymotrypsin (Agrawal et al., 2005; Von Elert et al., 2005). As such, future research could be directed towatd the role of these compounds in discouraging zooplankton grazing.

Although microcystin does not seem to discourage zooplankton grazing, recent research suggested that microcystin may have other functions such as being an interspecific signaling compound or as an allelopathic compound (Dittmann et al., 2001; Pflugmacher, 2002, respectively). Specifically focusing on the interaction between microcystin and phytoplankton, a study conducted by Schatz et al. (2007) found that when toxic *Microcystis* cells came in contact with extracellular microcystin it enhanced the expression of the *mcyB* gene in those cells. Furthermore, studies have found that when *Microcystis* competitors such as the dinoflagellate, *Peridinium* were exposed to either toxic strains of *Microcystis* or microcystin extracts, it initiated oxidative stress as well as apoptosis in those cells (Vardi et al., 2002, Schatz et al., 2007) This did not occur

when *Peridinium* was exposed to non-toxic *Microcystis* cells (Vardi et al., 2002). This suggests microcystin may have multiple functions for *Microcystis*. First, microcystin may be as a signaling compound between *Microcystis* cells where its presence in the water column, perhaps due to the lysis of other *Microcystis* cells, causes toxic strains to increase microcystin production. Next, this release can have allelopathic impacts on competing phytoplankton and contribute towards its dominance. Further research is required to definiatively confirm the universal nature of microcystin allelopathy (Babica et al. 2006).

Overall, this study has provided a new understanding of the ecology of toxic and non-toxic strains of *Microcystis*. For the first time, I have been able to study how various environmental factors promote toxic strains of *Microcystis* during natural bloom events providing novel insight into how the toxicity of blooms can be affected by various environmental and ecological processes. Future work exploring the expression of the microcystin synthetase (*mcy*) gene cluster could also prove extremely valuable, as in this study only genomic (DNA) gene copies were quantified. While this approach provided an understanding of factors influencing the dynamics of toxic *Microcystis* cells, ultimately it is the expression of the *mcy* genes which will lead to toxin production. As such, future studies should examine how expression of this gene is affected by various environmental and ecological processes. Also, this study provided a broad overview of how toxic and non-toxic strains are affected by different environmental parameters. In the future, it may be important to investigate the competition within the two sub-populations as increases in toxic cell densities could be due to the stimulation of a single strain or a shift in dominance from a strain that contained one *mcy* gene copy toward

strains with multiple copies. Hence, future studies of *Microcystis* investigating how different strains compete with each other during various environmental conditions will help clarify some of the broad findings of this study. Finally, elucidating the genes responsible for the production of other *Microcystis* metabolites such as protease inhibitors as well as the regulation of those genes may provide a great insight into why *Microcystis* is not readily grazed upon by zooplankton as recent research indicates the production of these compounds could offer a defense against grazing.

Table 1: Correlations between microcystin concentrations and four algal populations quantified in all systems sampled during my dissertation research. Significant correlations ($p<0.05$) are bolded in the table.

	Toxic *Microcystis*	Non-toxic *Microcystis*	Chlorophyll *a*	Total cyanobacterial cells
Lake Agawam 2005	**< 0.01**	0.49	0.86	0.58
Lake Agawam 2006	**< 0.001**	--	0.78	0.35
Lake Agawam 2008	**< 0.01**	0.11	**< 0.05**	**< 0.01**
Lake Agawam (All years)	**< 0.001**	0.71	0.99	0.84
Mill Pond 2006	**< 0.01**	--	0.63	**< 0.001**
Lake Ronkonkoma 2005	**< 0.001**	0.50	**< 0.05**	**< 0.05**
Lake Champlain 2006	**< 0.05**	**< 0.05**	**< 0.05**	**< 0.05**
Transquaking River 2007	**< 0.05**	0.45	**< 0.001**	0.83
Transquaking River 2008	**< 0.001**	0.47	0.84	0.59
Transquaking River (All years)	**< 0.01**	0.77	0.56	0.59

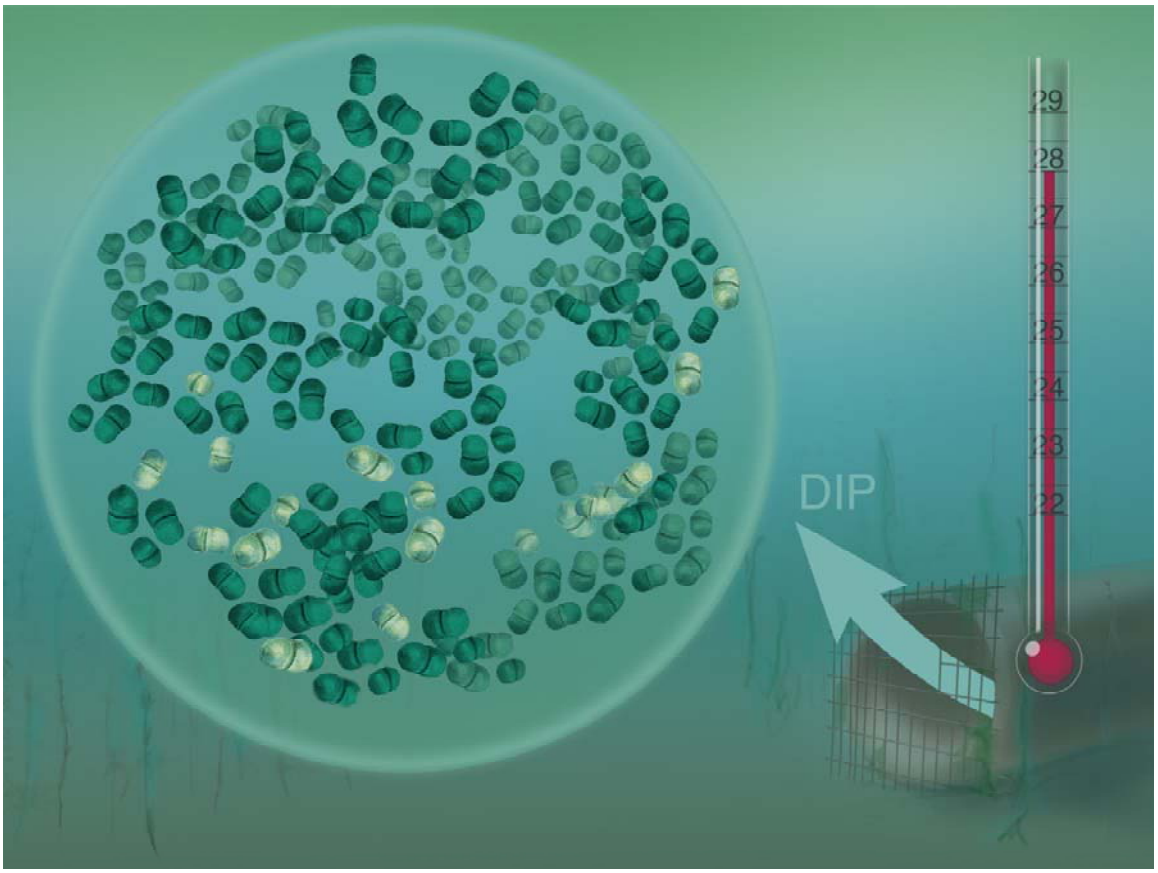

Figure 1: An overview of the abilities of inorganic nutrients and higher temperatures to specifically promote toxic strains of *Microcystis*. Dark colored cells are toxic *Microcystis* and light colored cells are non-toxic *Microcystis*. Dissolved inorganic phosphorus (DIP) was found to promote toxic strains in chapters 2 and 3, while dissolved inorganic nitrogen was also found to promote blooms in chapter 3. Also, in chapter 3, higher DIP and temperatures yielded growth rates for toxic strains of *Microcystis* which were higher than all other treatments and all other populations. Figure was designed by Lindsay K. Moore (lindsay@kozastudios.com)

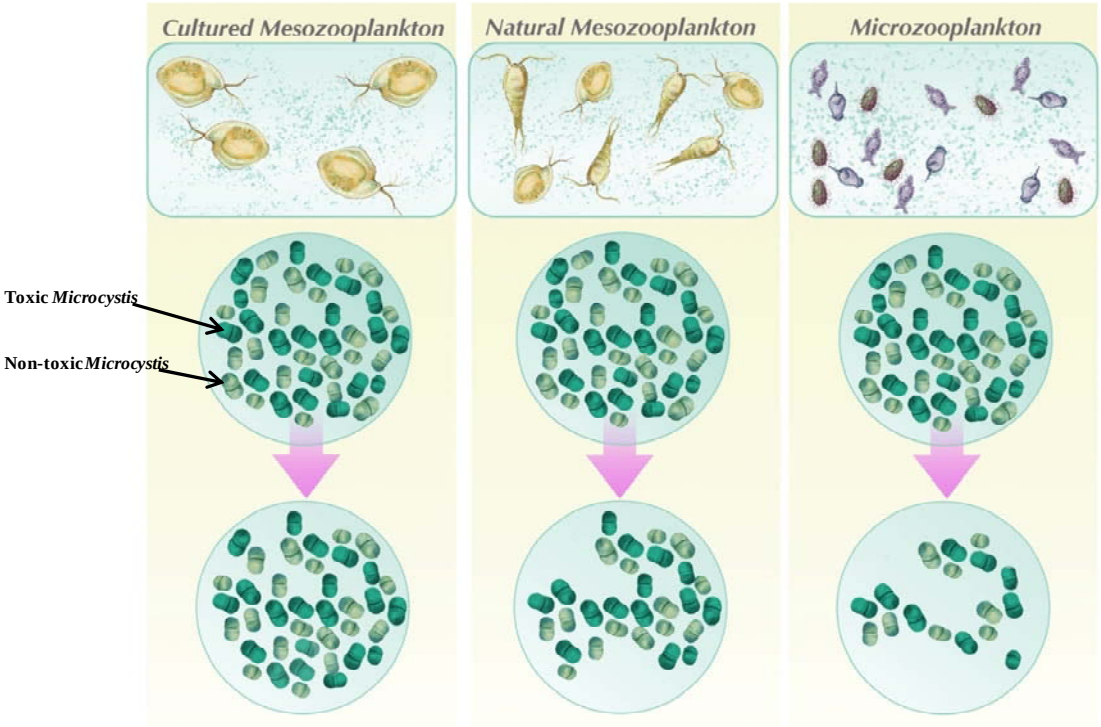

Figure 2: An overview of the abilities of cultured and natural populations of meso- and microzooplankton to graze on both toxic and nontoxic strains of *Microcystis*. Dark colored cells are toxic *Microcystis* and light colored cells are non-toxic *Microcystis*. The figure was designed by Lindsay K. Moore (lindsay@kozastudios.com)

References:

Agrawal, M. K., Zitt, A, Bagchi, D., Weckesser, J., Bagchi, S. N., von Elert, E., 2005. Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. Environ Tox. 20: 314-322.

Anderson, D. M., 1989. Toxic algal bloom and red tides: a global perspective. In: Okaichi, T., Anderson, D. M., Nemoto, T. (Eds.), Red Tides: Biology, Environmental Science and Technology. Elsevier, pp. 11–16.

Anderson, D. M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition and consequences. Estuaries 4B: 704–726.

Anderson, D. M., Burkholder, J. M., Cochlan, W. P., Glibert, P. M. Gobler, , C. J., Heil, C. A., Kudela, R., Parsons, M. L., Renseli, J. E. J., Townsend, D. W., Trainerk, V. L., Vargo, G. A., 2008. Harmful algal blooms and eutrophication: Examples of linkages from selected coastal regions of the United States. Harmful Algae 8: 39-53.

Arment, A. R., Carmichael, W. W., 1996. Evidence that microcystin is a thiotemplate product. J. Phycol. 32: 591–597.

Arnold, D. E., 1971. Ingestion, assimilation, survival, and reproduction by *Daphnia pulex* fed seven species of bluegreen algae. Limnol. Oceanog. 16: 906–920.

Babica, P., Bláha, L., Maršálek, B., 2006. Exploring the natural role of microcystins-A review of effects on photoautotrophic organisms. J. Phycol. 42: 9-20.

Baldia, S. F., Evangelista, A. D., Aralar, E. V., Santiago, A. E., 2007. Nitrogen an phosphorus utilization in the cyanobacterium Microcystis aeruginosa isolated from Laguna de Bay, Philippines. J. Appl. Phycol. 19: 607-613.

Bell ,S.G., Codd, G.A., 1994. Cyanobacteria toxins and human health. Rev. Med. Microbiol. 5: 256-264.

Berg, G. M., Glibert, P.M., Lomas, M.W., Burford, M., 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. Mar. Biol. 129: 377–387.

Berg, G. M., Repeta, D.J., LaRoche, J., 2002. Dissolved organic nitrogen hydrolysis rates in axenic cultures of *Aureococcus anophagefferens* (Pelagophyceae): comparison with heterotrophic bacteria. Appl. Environ. Microbiol. 68: 401–404.

Berman, T., Chava, S., 1999. Algal growth on organic compounds as nitrogen sources. J. Plankton Res. 21: 1423-1437.

Berman, T., 2001. The role of DON and the effect of N:P ratios on occurrence of cyanobacterial blooms: implications from the outgrowth of *Aphanizomenon* in Lake Kinneret. Limnol. Oceanogr. 46: 443–447.

Bertram, P. E., 1993. Total phosphorus and dissolved oxygen trends in the central basin of Lake Erie, 1970–1991. J. Great Lakes Res. 19: 224–236.

Blomqvist, P., Pettersson, A., Hyenstrand, P., 1994. Ammonium-nitrogen: a key regulatory factor causing dominance of non-nitrogen-fixing cyanobacteria in aquatic systems. Arch. Hydrobiol. 132: 141–164.

Boyer, G. L., Matzin, M.C., Shambaugh, A. D., Satchwell, M. F., Rosen, B. H., and Mihuc, T. 2004. The occurrence of cyanobacterial toxins in Lake Champlain. In: Partnerships and Research in the New Millenium. Ed: T. Manley. Kluwer Academic/Plenum Publishers. p. 241-257.

Boynton, W. R., Garber, J. H., Summers, R., Kemp, W. M., 1995. Inputs, transformations, and transport of nitrogen and phosphorus in Chesapeake Bay and selected tributaries. Estuaries 18: 285-314.

Briand, J. F., Jacquet, S., Bernard, C., Humbert, J. F., 2003. Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. Vet. Res . 34: 361–377.

Bronk, D. A., Glibert, P. M., Malone, T. C., Banahan, S., Sahlsten, E., 1998. Inorganic and organic nitrogen cycling in Chesapeake Bay: autotrophic versus heterotrophic processes and relationships to carbon flux, Aquat. Microb. Ecol. 15: 177–189.

Bronk D. A., 2002. Dynamics of DON, in: Biogeochemistry of Marine Dissolved Organic Matter, edited by: Hansell, D. A. and Carlson, C. A., Academic Press, San Diego, pp. 153–249.

Bronk, D. A., See, J. H., Bradley, P., Killburg, L., 2007. DON as a source of bioavaliable nitrogen for phytoplankton. Biogeosciences 4: 283-296.

Burkholder, J. M., Glasgow, H. B., 1997. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: behavior, impacts and environmental controls. Limnol. Oceanogr. 42: 1052–1075.

Burkholder, J. M., 1998. Implications of harmful microalgae and heterotrophic dinoflagellates in management of sustainable marine fisheries. Ecol. Appl. 8: S37–S62.

Calbet, A., Landry, M. R., 2004. Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. Limnol. Oceanogr. 49: 51-57.

Camacho, F. A., Thacker R. W., 2006. Amphipod herbivory on the freshwater cyanobacterium *Lyngbya wollei*: Chemical stimulants and morphological defenses. Limnol. Oceanogr. 51: 1870-1875.

Canale, R. P., Vogel, A. H., 1974. Effects of temperature on phytoplankton growth. Journal of the environmental engineering division, American Society of Civil Engineers 100: 229-241.

Carlson, C. A., Ducklow, H. W., 1995. Dissolved organic carbon in the upper ocean of the central equatorial Pacific Ocean, 1992: Daily and fine scale vertical variations, Deep-Sea Res. II, 42: 639–656.

Carmichael,W. W., 1992. A status report on planktonic cyanobacteria (blue-green algae) and their toxins. EPA/600/R-92/079, United States Environmental Protection Agency, Cincinnati, Ohio.

Carmichael ,W. W., Falconer, I. R., 1993. Diseases related to freshwater blue green algal toxins, and control measures. In: Falconer, I. R., ed. Algal Toxins in Seafood and Drinking Water. Academic Press, pg 187-209.

Carmichael W. W. 1994. The toxins of cyanobacteria. Scientific Am Jan 270: 78-86.

Carmichael, W. W., An, J. S., 1999. Using an enzyme linked immunosorbent assay (ELISA) and a protein phosphatase inhibition assay (PPIA) for the detection of microcystins and nodularins. Nat. Toxins 7: 377–385.

Carmichael, W. W., 2001. Health effects of toxin producing cyanobacteria: "The CyanoHABs". Human and Eco. Risk Ass. 7: 1393-1407.

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R.W., Sharpley, A. N., Smith, V. H., 1998. Nonpoint pollution of surface waters with phosphorous and nitrogen, Ecological Applications 8: 559-568.

Chan, F., Pace, M. L., Howarth, R. W., Marino, R. M., 2004. Bloom formation in heterocystic nitrogen-fixing cyanobacteria: The dependence on colony size and zooplankton grazing. Limnol. Oceanog. 49: 2171–2178.

Cho, B. C., Park M. G., Shim, J. H., Azam, F., 1996. Significance of bacteria in urea dynamics in coastal surface waters. Marine Ecology Progress Series. 142: 19-26.

Chorus, I., Bartram, J., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. World Health Organization. E&FN Spon, Routledge, London.

Christiansen, G., Fastner, J., Erhard, M., Börner, T., Dittmann, E., 2003. Microcystin biosynthesis in *Planktothrix*: Genes, Evolution, and Manipulation. J. Bacteriol. 185: 564-572.

Cochlan, W. P., Herndon, J., Kudela, R.M., 2008. Inorganic and organic nitrogen uptake by the toxigenic diatom *Pseudo-nitzschia australis* (Bacillariophyceae). Harmful Algae 8: 111–118.

Codd, G. A., Poon, G. K., 1988. Cyanobacterial toxins. In: Rogers, L.J., Gallon, J.R. (Eds.), Biochemistry of the Algae and Cyanobacteria. Clarendon Press, Oxford, England, pp. 283–296.

Codd, G. A., 1995. Cyanobacterial toxins: Occurrence, properties and biological significance. Wat. Sci. Technol. 32: 149-156.

Coffin, R. B., 1989. Bacterial uptake of dissolved free and combined amino acids in estuarine waters. Limnology and Oceanography. 34: 531–542.

Cohen, P., 1989. The structure and regulation of protein phosphatases. Annu. Rev. Biochem. 58: 453–508.

Cole, G. T., Wynne, M. J., 1974. Endocytosis of *Microcystis aeruginosa* by *Ochromonas danica*. J Phycol 10: 397–410.

Cowie, G. L., Hedges, J. I., 1992. Improved amino acid quantification in environmental samples: chraged-matched recovery standards and reduced analysis time. Marine Chemistry 37: 223-238.

Cox, P. A., Banack, S. A., Susan, J., Murch, S. J., Rasmussen, U., Tien, G., Bidigare, R. R., Metcalf, J. S., Morrison, L. F., Codd, G. A., Bergman, B., 2005. Diverse taxa of cyanobacteria produce-N-methylamino-L-alanine, a neurotoxic amino acid. PNAS 102: 5074–5078.

Coyne, K. J., Hutchins, D. A., Hare, C. E., Cary S. C., 2001. Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. Aquat. Microb. Ecol. 24: 275-285.

Coyne, K. J., Handy, S. M., Demir, E., Whereat, E. B., Hutchins, D. A., Portune, K. J., Doblin, M. A., Cary, S. C., 2005. Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. Limnol. Oceanogr.: Methods 3: 381–391.

Dai, R., Liu, H., Qu, J., Zhao, X., Hou, Y., 2009. Effects of amino acids on microcystin production of the *Microcystis aeruginosa*. J. Hazard. Mater. 161: 730-736.

Davis, T. W., Berry, D. L., Boyer, G. L., Gobler, C. J., 2009. The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. Harmful Algae 8: 715–725.

Davidson, F. F., 1959. Poisoning of wild and domestic animals by a toxic waterbloom of *Nostoc rivulare* Kutz. J. Am. Water Works Ass., 51: 1277-1287.

de Bernardi, R., Giussani, G., 1990. Are blue green algae a suitable food for zooplankton? A review. Hydrobiologia 200/201: 29–41.

Demott, W. R., 1989. The role of competition in zooplankton succession, p. 195–252. In U. Sommer [ed.], Plankton ecology: Succession in plankton communities. Springer-Verlag.

DeMott, W. R., Moxter, F. 1991. Foriging cyanobacteria by copepods: Responses to chemical defense and resource abundance. Ecology 72: 1820-1834.

DeMott, W. R., Zhang, Q.-X., Carmichael, W.W., 2001. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. Limnol. Oceanogr. 36: 1346–1357.

Dempster, E. L., Pryor, K. V., Francis, D., Young, J. E., Rogers, H. J., 1999. Rapid DNA extraction from ferns for PCR-based analyses. Biotechniques 27: 66–68.

Deonarine, S. N., Gobler, C. J., Lonsdale, D. J., Caron, D. A., 2006. Role of zooplankton in the onset and demise of harmful brown tide blooms (*Aureococcus anophagefferens*) in US mid-Atlantic estuaries. *Aquat*. Microb. Ecol. 44: 181-195.

Dittmann, E., Erhard, M., Kaebernick, M., Scheler, C., Neilan, B. A., von Dohren, H., Borner, T., 2001. Altered expression of two light-dependent genes in a microcystin-lacking mutant of *Microcystis aeruginosa* PCC 7806. Microbiol.-SGM 147: 3113–3119.

Downing, J. A., Watson, S. B., McCauley, E., 2001. Predicting cyanobacteria dominance in lakes. Can. J. Fish. Aquat. Sci. 58: 1905–1908.

Dryden, R. C., Wright, S. J. L., 1987. Predation of cyanobacteria by Protozoa. Can. J. Microbiol. 33: 471–482.

Duy T. N., Lam P. K. S., Shaw G. R., Connell D. W., 2000. Toxicology and Risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water, Rev. Environ. Contam. Tox- icol. 163, 113–186.

Edmondson, W. T., Litt, A. H., 1982. *Daphnia* in Lake Washington. Limnol. Oceanogr. 27: 272-293.

Eppley, R. W., 1972. Temperature and phytoplankton growth in the sea. Fish. Bull. 70: 1063–1085.

Falconer, I. R., Smith, J. V., Jackson, A. R. B., Jones, A., Runnegar, M. T. C., 1988. Oral toxicity of a bloom of the cyanobacterium *Microcystis aeruginosa* administered to mice over periods up to 1 year. Journal of Toxicology and Environmental Health 24: 291–305.

Falconer, I. R., 1999. An overview of problems caused by toxic blue-green algae (cyanobacteria) in drinking and recreational water. Environ. Toxicol. 14: 5-12.

Falconer, I. R., 2007. Cyanobacterial toxins present in *Microcystis aeruginosa* extracts-More than microcystins! Toxicon 50: 585-588.

Fan, C., Glibert, P., Burkholder, J., 2003. Characterization of the affinity for nitrogen, uptake kinetics, and environmental relationships for *Prorocentrum minimum* in natural blooms and laboratory cultures, Harmful Algae, 2: 283–299.

Fastner, J., Erhard, M., von Döhren, H., 2001. Determination of oligopeptide diversity within a natural population of *Microcystis* spp. (Cyanobacteria) by typing single colonies by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appli. Eviron. Microbio. 67: 5069-5076.

Fischer, W. J., Garthwaite, I., Miles, C. O., Ross, K. M., Aggen, J. B., Chamberlin, A. R., Towers, N. R., Dietrich, D. R., 2001. Congener-independent immunoassay for Microcystins and nodularians. Eviron. Sci. Technol. 35: 4849-4856.

Fitzgeorge, R. B., Clark, S. A., Keevil, C. W., 1994. Routes of intoxication. In: G.A. Codd, T.M. Jeffries, C.W. Keevil, and E. Potter [Eds.] 1st international symposium on detection methods for cyanobacterial (blue-green algal) toxins, royal society of chemistry, Cambridge, UK, 69-74.

Fleming, L. E., Rivero, C., Burns, J., Williams, C., Bean, J. A., Shea, K. A., Stinn, J., 2002. Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. Harmful Algae 1: 157-168.

Fogel, G. B., Collins, C. R., Brunk, C. F., 1999. Prokaryotic genome size and SSU rDNA copy number: Estimation of microbial relative abundance from a mixed population. Microb. Ecol. 38: 93-113.

Fogg, G. E., 1969. The physiology of an algal nuisance. Proc. Roy. Soc. Lond. [Biol.], 173: 175-189.

Francis, G., 1878 poisonous Australian lake. Nature, 18: 11-12.

Frangeul, L., Quillardet, P., Castets, A.-M., Humbert, F., Matthijs, H. CP., Cortez, D., et al., 2008. Highly plastic genome of *Microcystis aeruginosa* PCC 7806, a ubiquitous toxic freshwater cyanobacterium. BMC Genomics. 9: 274–294.

Frost, B. W., 1972. Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod *Calanus pacificus*. Limnol. Oceanogr. 17: 805–815.

Fu, F.-X., Warner, M. E., Zhang, Y., Feng, Y., Hutchins, D. A., 2007. Effects of increased temperature and CO_2 on photosynthesis, growth, and elemental ratios in marine *Synechococcus* and *Prochlorococcus* (cyanobacteria). J. Phycol. 43: 485-496.

Fuhrman, J., 1987. Close coupling between release and uptake of dissolved free amino acids in seawater studied by an isotope dilution approach, Mar. Ecol. Prog. Ser. 37: 45–52.

Fujiki, H., Suganuma, M., 1993. Tumor promotion by inhibitors of protein phosphatases and 1 and 2A: the okadaic acid class of compounds. Adv. Cancer Res. 61: 143-194.

Fulton, R. S. III, Paerl H. W., 1987a. Effects of colonial morphology on zooplankton utilization of algal resources during blue-green algal (*Microcystis aeruginosa*) blooms. Limnol. Oceanogr. 32: 634-644.

Fulton, R. S. III, Paerl H. W., 1987b. Toxic and inhibitory effects of the blue-green alga *Microcystis aeruginosa* on herbivorous zooplankton. J. Plankton Res. 9: 837-855.

Fulton, R. S. III, Paerl H. W., 1988a. Effects of blue-green alga *Microcystis seruginosa* on zooplankton competitive relations. Oceologia. 76: 383-389.

Fulton, R. S. III, Paerl H. W., 1988b. Zooplankton feeding selectivity for unicellular and colonial *Microcystis aeruginosa*. Bull. Mar. Sci. 43: 500-508.

Fujimoto, N., Sudo, R., Sugiura, N., Inamori, Y., 1997. Nutrient-limited growth of *Microcystis aeruginosa* and *Phormidium tenue* and competition under various N:P supply ratios and temperature. Limnol. Oceanogr. 42: 250–256.

Fulton, R. S. III, Paerl H. W., 1987a. Effects of colonial morphology on zooplankton utilization of algal resources during blue-green algal (*Microcystis aeruginosa*) blooms. Limnol. Oceanogr. 32: 634-644.

Fulton, R. S. III, Paerl H. W., 1987b. Toxic and inhibitory effects of the blue-green alga *Microcystis aeruginosa* on herbivorous zooplankton. J. Plankton Res. 9: 837-855.

Fulton, R. S. III, Paerl H. W., 1988a. Effects of blue-green alga *Microcystis seruginosa* on zooplankton competitive relations. Oceologia. 76: 383-389.

Fulton, R. S. III, Paerl H. W., 1988b. Zooplankton feeding selectivity for unicellular and colonial *Microcystis aeruginosa*. Bull. Mar. Sci. 43: 500-508.

Gaines, G., Elbrächter, M., 1987. Heterotrophic nutrition. In Taylor, F. J. R. (ed.) The Biology of Dinoftagetlates, Botanical Monographs 21, Blackwell Scientific Publications, Oxford, pp. 224-268.

GEOHAB (Global Ecology and Oceanography of Harmful Algal Blooms Programme), 2001. Science Plan. Glibert, P., Pitcher, G. (eds.). SCOR and IOC, Baltimore, MD, and Paris, France.

George, D. G., Edwards, R. W., 1974. Population dynamics and production of *Daphnia hyalina* in a eutrophic reservoir. Freshwater Biol. 4: 445-465.

Gilwicz, Z. M., 1977. Food size selection and seasonal succession of filter feeding zooplankton in a eutrophic lake. Ekol. Pol. 25: 179-225.

Gilwicz, Z. M., Seidlar, E., 1980. Food size limitation and algae interfering with food collection in *Daphnia*. Arch. Hydrobiol. 88: 155-177.

Glibert, P. M., Magnien, R., Lomas, M. W., Alexander, J., Fan, C., Haramoto, E., Trice, T. M., Kana, T. M., 2001. Harmful algal blooms in the Chesapeake and Coastal Bays of Maryland, USA: comparisons of 1997, 1998, and 1999 events. Estuaries 24: 875–883.

Glibert, P. M., Anderson, D. A., Gentien, P., Grane´ li, E., Sellner, K. G., 2005a. The global, complex phenomena of harmful algal blooms. Oceanography 18: 136–147.

Glibert, P. M., Harrison, J., Heil, C., Seitzinger, S., 2006a. Escalating worldwide use of urea—a global change contributing to coastal eutrophication. Biogeochemistry 77: 441–463.

Glibert, P. M., Burkholder, J. M., Parrow, M. W., Lewitus, A. J., Gustafson, D.E., 2006b. Direct uptake of nitrogen by *Pfiesteria piscicida* and *Pfiesteria shumwayae*, and nitrogen nutritional preferences. Harmful Algae 5: 380–394.

Glibert, P. M., Burkholder, J. M., 2006. The complex relationships between increasing fertilization of the Earth, coastal eutrophication, and HAB proliferation. In: Grane´ li, E., Turner, J. (Eds.), The Ecology of Harmful Algae. Springer-Verlag, New York, pp. 341–354.

Glibert, P. M., Legrand, C., 2006. The diverse nutrient strategies of HABs: focus on osmotrophy. In: Grane´ li, E., Turner, J. (Eds.), Ecology of Harmful Algae. Springer, pp. 163–176.

Glibert, P. M., Wazniak, C. E., Hall, M., Sturgis, B., 2007. Seasonal and interannual trends in nitrogen and brown tide in Maryland's Coastal Bays. Ecol. Appl. 17: S79–S87.

Gobler, C. J., Sãnudo-Wilhelmy, S. A., 2001a. Effects of organic carbon, organic nitrogen, inorganic nutrients, and iron additions on the growth of phytoplankton and bacteria during a brown tide bloom. Mar. Ecol. Prog. Ser. 219: 19–34.

Gobler, C. J., Sañudo-Wilhelmy, S. A., 2001b. Temporal variability of groundwater seepage andBrown Tide bloom in a Long Island embayment. Mar. Ecol. Prog. Ser. 217: 299-309.

Gobler, C. J., Renaghan, M., Buck, N., 2002. Impacts of nutrients and grazing mortality on the abundance of *Aureococcus anophagefferens* during a New York Brown Tide bloom. Limnol. Oceanogr. 47: 129–141.

Gobler, C. J., Lonsdale, D. J., Boyer, G. L., 2005. A synthesis and review of causes and impact of harmful brown tide blooms caused by the alga, *Aureococcus anophagefferens*. Estuaries 28, 726–749.

Gobler, C. J., Davis, T. W., Coyne, K. J., Boyer, G. L., 2007. The interactive influences of nutrient loading and zooplankton grazing on the growth and toxicity of cyanobacteria blooms in a eutrophic lake. *Harmful Algae* 6: 119-133.

Goldman, J. C., Carpenter, E. J., 1974. A kinetic approach to the effect of temperature on algal growth. Limnol. Oceanogr. 19: 756–766.

Granéli, E., Anderson, D. M., Carlsson, P., Maestrini, S. Y., 1997. Light and dark carbon uptake by *Dinophysis* species in comparison to other photosynthetic and heterotrophic dinoflagellates. Aquat. Microb. Ecol. 13: 177–186.

Granéli, E., Carlsson, P., Legrand, C., 1999. The role of C, N and P in dissolved and particulate matter as a nutritional source for phytoplankton growth, including toxic species. Aquat. Ecol. 33: 17–27.

Guo, N., Xie, P., 2006. Development of tolerance against toxic *Microcystis aeruginosa* in three cladocerans and the ecological implications. Environ. Pollut. 143: 513–518.

Gustafsson, S., Rengefors, K. E., Hansson, L. A., 2005. Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. Ecology 86: 2561–2567.

Ha, J. H., Hidaka, T., Tsuno, H., 2009. Quantification of toxic *Microcystis* and evaluation of its dominance ratio in blooms using Real-Time PCR. Environ. Sci. Technol. 43: 812–818.

Hairston, N. G. Jr, Lampert, W., Cáceres, C., Holtmeier, C., Weider, L., Gaedke, U., Fischer, J., Fox, J. A., Post D. M., 1999. Rapid evolution revealed by dormant eggs. Nature 401: 446.

Hairston, N. G. Jr, Holtmeier, C. L., Lampert, W., Weider, L. J., Post, D. M., Fischer, J. M. Cáceres, C. E., Fox, J. A., Gaedke, U., 2001. Natural selection for grazer resistance to toxic cyanobacteria: Evolution of phenotypic plasticity? Evolution 55: 2203–2214.

Hallegraeff, G. M., 1993. A review of harmful algal blooms and their apparent global increase. Pycologia 32: 79–99.

Hansson, L.-A., Gyllström, M., Ståhl-Delbanco, A., Svensson, M., 2004. Responses to nutrient and predation gradients in aquatic systems. A mesocosm experiment in a Swedish lake. Freshwater Biology 49: 1538–1550

Hansson, L.-A., Gustafsson, S., Rengefors, K., Bomark, L., 2007. Cyanobacterial chemical warfare affects zooplankton community composition. Freshwater Biology 52: 1290-1301.

Harris, R., Wiebe, P., Lenz, J., Skjoldal, H.R., Huntley, M., 2000. ICES Zooplankton Methodology Manual, Acaemic Press, California, 87–94.

HARRNESS (Harmful Algal Research and Response: A National Environmental Science Strategy), 2005. Ramsdell, J., Anderson, D., P. Glibert (eds.). Ecological Society of America, Washington DC.

Hartmann, H. J., 1985. Feeding of *Daphnia pulicaria* and *Diaptomus ashlandi* on mixtures of unicellular and filamentous algae. Verh. Int. Verein. Limnol. 22: 3178–3183.

Hasle, G. R., 1978. The inverted microscope method. Monogr. Oceanogr. Methodol. 6, 88–96.

Hecky, R. E., Kilman, P., 1988. Nutrient limitation of phytoplankton in freshwater and marine environments. Limnol. Oceanogr. 33: 796–822.

Heisler, J. P., Gilbert, J., Burkholder, J., Anderson, D., Cochlan, W., Dennison, W., Dortch, Q., Gobler, C. J., Heil, C., Humphries, E., Lewitus, A., Magnien, R., Marshall, H., Sellner, K., Stockwell, D., Stoecker, D., Suddleson, M., 2008. Eutrophication and Harmful Algal Blooms: A Scientific Consensus. Harmful Algae 8: 3-13.

Herndon, J., Cochlan, W. P., 2007. Nitrogen utilization by the raphidophyte *Heterosigma akashiwo*: growth and uptake kinetics in laboratory cultures. Harmful Algae 6: 260–270.

Hesse, K., Kohl, J. G., 2001. Effects of light and nutrient supply on growth and microcystin content of different strains of *Microcystis aeruginosa*. In: Cyanotoxins: Occurrence, Causes, Consequences. pp. 104-115. (Chorus, I., Ed.), Springer Verlag, Heidelberg.

Hesse, K., Dittmann, E., Börner, T., 2001. Consequences of impaired microcystin production for light-dependent growth and pigmentation of *Microcystis aeruginosa* PCC 7806. FEMS Microbiol Ecol 37: 39-43.

Hoagland, P., Anderson, D. M., Kaoru, Y., White, A. W., 2002. The economic effects of harmful algal blooms in the United States: Estimates, assessment issues and information needs. Estuaries 25: 819–837.

Hoch, M. P., Kirchman, D. L., 1995. Ammonium uptake by heterotrophic bacteria in the Delaware estuary and adjacent coastal waters. Limnol. Oceanogr. 40: 886-897.

Holm, N. P., Shapiro, J. S., 1984. An examination of lipid reserves and the nutritional status of *Daphnia pulex* fed *Aphanizomenon flos-aquae*. Limnol. Oceanogr. 29: 1137-1140.

Honkanen, R. E., Zwiller, J., Moore, R. E., Daily, S. L., Kahatra, B. S., Dukelow, M., Boynton, A. L., 1990. Characterization of Microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. J. Biol. Chem. 265: 19401-19404.

Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., Van der Lin-den, P. J., Dai, X., Maskell, K., Johnson, C. A., 2001. Climate Change 2001: The Scientific Basis. Cambridge University Press, Cambridge, 881 pp.

Howarth, R., Paerl, H. W., 2008. Coastal marine eutrophication: Control of both nitrogen and phosphorus is necessary. PNAS Letter vol. 105: no. 49.

Hudnell, K. H., Dortch, Q., 2008. A synopsis of research needs identified at the interagency, international symposium on cyanobacterial harmful algal blooms (ISOC-HAB). In: *Cyanobacterial Harmful Algal Blooms: state of the Science and Research Needs* Series: Advances in Experimental Medicine and Biology, Vol. 619 Hudnell, K.H. (Ed.), XXIV, 950 p.

Ibelings, B. W., Bruning, K., Jonge, J. D., K. Wolfstein, K., Pires, L. M. D., Postma, J., Burger, T., 2005. Distribution of microcystins in a lake food web: no evidence for biomagnification, Microbiol. Ecol. **49:** 487–500.

Infante, A., Riehl, W., 1984. The effect of Cyanophyta upon zooplankton in a eutrophic tropical lake. Hydrobiologia 113: 293-298.

IPCC, 2001. A report of working group I of the Intergovernmental Panel on Climate Change. Summary for Policymakers and Technical Summary.

Ito, E., Kondo, F., Terao, K., Harada, K-I., 1997. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. Toxicon 35: 1453-1457.

Ivlev, V. S., 1961. Experimental Ecology of the Feeding Fishes. Yale University Press, New Haven, CT, 302 pp.

Jang, M. H., Ha, K., Joo, G. J., Takamura, N., 2003. Toxin production of cyanobacteria is increased by exposure to zooplankton. Freshwater Biol. 48: 1540–1550.

Jang, M. H., Jong, M. J., Takamura, N., 2007. Changes in microcystin production in cyanobacteria exposed to zooplankton at different population densities and infochemical concentrations. Limnol. Oceanogr. 52: 1454-1466.

Jeppesen, E., Jensen, J. P., Søndergaard, M., Lauridsen, T., 2000. Trophic structure, species richness and biodiversity in Danish lakes: changes along a phosphorus gradient. Freshwater Biol. 45: 201–218.

Jiang, Y., Ji, B., Wong, R. N. S., Wong, M. H., 2008. Statistical study on the effects of environmental factors on the growth and microcystins production of bloom-forming cyanobacterium-*Microcystis aeruginosa*. Harmful Algae 7: 127-136.

Jensen, J. P., Jeppesen, E., Olrik, K., Kristensen, P., 1994. Impact of nutrients and physical factors on the shift from cyanobacterial to chlorophyte dominance in shallow Danish lakes. Can. J. Fish. Aquat. Sci. 51: 1692–1699.

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

Jones, M. N., 1984. Nitrate reduction by shaking with cadmium: Alternative to cadmium columns. Water Res. 18: 643-646.

Jørgensen, N. O. G., Kroer, N., Coffin, R. B., Yang, X.-H., Lee, C., 1993. Dissolved free amino acids, combined amino acids, and DNA as sources of carbon and nitrogen to marine bacteria, Mar. Ecol. Prog. Ser. 98: 135–148.

Jungmann, D., 1992. Toxic compounds isolated from *Microcystis* PCC7806 that are more active against *Daphnia* than two microcystins. Limnol. Oceanog. 37: 1777–1783.

Jungmann, D., Benndorf, J., 1994. Toxicity to *Daphnia* of a compound extracted from laboratory and natural *Microcystis* spp., and the role of microcystins. Freshwater Biol. 32: 13-20.

Jungmann, D., 1995. Isolation, purification, and characterization of new Daphnia-toxic compound from axenic *Microcystis flos-aquae* strain PCC7806. J Chem. Ecol. 21: 1665-1676.

Kaebernick, M., Neilan, B. A., Borner, T., Dittmann, E., 2000. Light and the transcriptional response of the microcystin biosynthesis gene cluster. Appl. Eviron. Microbiol. 66: 3387–3392.

Kaebernick, M., Neilan. B. A., 2001. Ecological and molecular investigations of cyanotoxin production. FEMS Microbiol. Ecol. 35:1-9.

Kaebernick, M., Rohrlack, T., Christoffersen, K., Neilan, B. A., 2001. A spontaneous mutant of microcystin biosynthesis: Genetic characterization and effect on Daphnia. Environ. Microbiol. 3: 669–679.

Kaneko, T., Narajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M., et al., 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Res. 14: 1-10.

Kardinaal, W. E. A., Visser, P. M., 2005. Dynamics of cyanobacterial toxins: sources of variability in microcystin concentrations, p. 41–63. *In* J. Huisman, H. C. P. Matthijs, and P. M. Visser (ed.), Harmful cyanobacteria. Springer, Dordrecht, The Netherlands.

Kardinaal, W. E. A., Tonk, L., Janse, I., Hol, S., Slot, P., Huisman, J., Visser, P. M., 2007. Competition for light between toxic and non-toxic strains of the harmful cyanobacterium *Microcystis*. Appl. Environ. Microbiol. 73: 2939–2946.

Kim, H. R., Kim, C. K., Ahn, T. S., Yoo, S. A., Lee, D. H., 2005. Effects of temperature and light on microcystin synthetase gene transcription in *Microcystis aeruginosa*. Key Engineering Materials 277–279, 606–611.

Kim, B.-R., Nakano, S., Kim, B.-H., Han, M.-S., 2006. Grazing and growth of the heterotrophic flagellate *Diphylleia rotans* on the cyanobacterium *Microcystis aeruginosa*. Aquat. Microb. Ecol. 45: 163-170.

Klaveness, D., 1995. *Collodictyon triciliatum* H. J. Carter (1865)—a common but fixation-sensitive algivorous flagellate from the limnopelagial. Nordic. J. Freshw. Res. 70: 3–11.

Konopka, A., Brock, T. D. 1978. Effect of temperature on blue-green algae (cyanobacteria) in Lake Mendota. Appl. Environ. Microbiol. 36: 572-576.

Koon, M. S., 1960. On the cause of Sazan disease in the Volga estuary. Zool. Zh. 39: 1531–1537.

Kotak, B. G., Lam, A. K.-Y., Prepas, E. E., Kenefick, S. L., Hrudey, S. E., 1995. Variability of the hepatotoxin microcystin-LR in hypereutrophic drinking water lakes. J. Phycol. 31: 248–263.

Kudela, R. M., Cochlan, W. P., 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off Southern California. Aquat. Microb. Ecol. 21: 31–47.

Kudela, R. M., Lane, J. Q., Cochlan, W. P., 2008. The potential role of anthropogenically derived nitrogen in the growth of harmful algae in California, USA. Harmful Algae 8: 103–110.

Kurmayer, R., Dittmann, E., Fastner, J., Chorus, I., 2002. Diversity of microcystin genes within a population of the toxic cyanobacterium Microcystis spp. in Lake Wannsee (Berlin, Germany), Microb. Ecol. 43: 107–118.

Kurmayer, R., Kutzenberger, T., 2003. Application of real time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis sp*. Appl. Environ. Microbiol. 69: 6723–6730.

Lacouture, R. V., Sniezek, J. H., Sellner, K. G., 1993. Level I Report: Maryland Chesapeake Bay Water Quality Monitoring Program-Phytoplankton and Microzooplankton Component. Academy of Natural Sciences, Philadelphia, Benedict Estuarine Research Laboratory, Benedict, Maryland.

Lahti, K., Rapala, J., Fardig, M., Niemela, M., Sivonen, K., 1997. Persistence of cyanobacterial hepatotoxin, microcystin-LR in particulate material and dissolved in lake water. Water Res. 31: 1005–1012.

Lampert, W., 1981. Inhibitory and toxic effects of blue-green algae on *Daphnia*. Int. Rev. Gesamten Hydrobiol. 66: 285-298.

Lampert, W., 1982. Further studies on the inhibitory effect of the toxic blue-green algae *Microcystis aeruginosa* on the filtering rate of *Daphnia*. Int. Rev. Gesamten Hydrobiol. 95: 207-220.

Lampert, W., 1987. Laboratory studies on zooplankton-cyanobacteria interactions. N. Z. J. Mar. Freshw. Res. 21: 483–490.

Landry, M. R., Kirshtein, J., Constantinou, J., 1995. A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental test in the central equatorial Pacific. Mar. Ecol. Prog. Ser. 120: 53–63.

Lechowicz, M. J., 1982. The sampling characteristics of electivity indexes. Oecologia 52: 22-30.

Lee, T., Tsuzuki, M., Takeuchi, T., Yokoyama, K., Karube, I., 1994. *In vivo* fluorometric method for early detection of cyanobacterial waterblooms. Journal of App. Phycology 6: 489-495.

Lee, S. J., Jang, M.-H., Kim, H.-S., Yoon, B.-D., Oh, H. M., 2000. Variation of microcystin content of *Microcystis aeruginosa* relative to medium N:P ratio and growth stage. J. Appl.. Microbiol. 89: 323-329.

Lewitus, A. J., Burkholder, J. M., Glasgow Jr., H. B., Glibert, P. M., Willis, B. M., Hayes, K C., 1999. Mixotrophy and nitrogen uptake by *Pfiesteria piscicida* (Dinophyceae). J. Phycol. 35: 1430–1437.

Lewitus, A. J., 2006. Osmotrophy in marine microalgae. In: Subba-Rao, D.V. (Ed.), Algal cultures, Analogues of blooms and applications. Science Publishers, Enfield, NH, USA, pp. 343–383.

Likens, G. E., 1972. Eutrophication and aquatic ecosystems. In: Nutrients and Eutrophication: The Limiting-Nutrient Controversy. Limnol. Oceanogr. Special Symposium. 1: 3–13.

Lindroth, P., Mopper, K., 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with ophthaldialdehyde. Anal. Chem. 51: 1667–1674.

Livak, K. J., Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 25: 402–408.

Lomas, M. W., Glibert, P. M., Berg, G. M., Burford, M., 1996. Characterization of nitrogen uptake by natural populations of *Aureococcus anophagefferens* (Chrysophyceae) as a function of incubation duration, substrate concentration, light and temperature. J. Phycol. 32: 907–916.

Lomas, M. W., Glibert, P. M., Clougherty, D. A., Huber, D. R., Jones, J., Alexander, J., Haramoto, E., 2001. Elevated organic nutrient ratios associated with brown tide blooms of *Aureococcus anophagefferens* (Pelagophyceae). J. Plank. Res. 23: 1339–1344.

Lomas, M. W., Kana, T. M., MacIntyre, H. L., Cornwell, J. C., Nuzzi, R., Waters, R., 2004. Interannual variability of *Aureococcus anophagefferens* in Quantuck Bay, Long Island: natural test of the DON hypothesis. Harmful Algae 3: 389–402.

Lukač, M., Aegerter, R., 1993. Influence of trace metals on growth and toxin production of *Microcystis aeruginosa. Toxicon* 31:293-305.

Lűrling, M., 2003. *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis aeruginosa* in different mixtures with the green alga *Scenedemsus obliquus*. Limnol. Oceanog. 48: 2214–2220.

Maryland Department of the Environment, 2000. Total maximum daily loads of nitrogen and phosphorus for the Transquaking River Dorchester, Maryland. Submitted to: Watershed Protection Division U.S. Environmental Protection Agency, Region III, Philadelphia, PA, USA.

Mitamura, O., Saijo, Y., Hino, K., Barbosa, F. A. R., 1995. The significance of regenerated nitrogen for phytoplankton productivity in the Rio Doce Valley Lakes, Brazil. Archiv. für Hydrobiologie, 134: 179–194.

Moisander, P. H., Ochiai, M., Lincoff, A., 2009. Nutrient Limitation of Microcystis aeruginosa in northern California Klamath River reservoirs. Harmful Algae 8: 889-897.

Moore, R. E., Chen, J. L., Moore, B. S., Patterson, G. M. L., Carmichael, W. W., 1991. Biosynthesis of microcystin-LR. Origin of the carbons in the Adda and Masp units. J. Am. Chem. Soc. 113: 5083–5084.

Mueller, D. K., Helsel, D. R., 1996. Nutrients in the Nation's waters—Too much of a good thing? U.S. Geological Survey Circular 1136, 24 p.

Mulholland, M. R., Gobler, C. J., Lee, C., 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnol. Oceanogr. 47: 1094–1108.

Mulholland, M. R., Heil, C. A., Bronk, D. A., O'Neil, J. M., Bernhardt, P., 2004. Does nitrogen regeneration from the N2 fixing cyanobacteria *Trichodesmium* spp. fuel *Karenia brevis* blooms in the Gulf of Mexico? In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography and Intergovernmental Oceanogrpahic Commission of UNESCO, pp. 47–49.

Myer, G. E., Gruendling, G. K., 1979. Limnology of Lake Champlain, Lake Champlain Basin Study, New England River Basins Commission, Burlington, VT.

Nagata, S., Tsutsumi, T., Hasegawa, A., Yoshida, F., Ueno, Y., Watanabe, M.F., 1997. Enzyme immunoassay for the direst determination of microcystins in environmental water. J. AOAC Int. 80: 408-417.

Namikoshi, M., Rinehart, K. L., 1996. Bioactive compounds produced by cyanobacteria. J Ind. Microbiol. Biot. 17: 373–384.

Neilan, B. A., Jacobs, D., DelDot, T., Blackall, L. L., Hawkins, P. R., Cox, P. T., Goodman, A. E., 1997. rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. Int. J. Syst. Bacteriol. 47: 693–697.

Nishibe, Y., Kawabata, Z., Nakano, S., 2002. Grazing on *Microcystis aeruginosa* by the heterotrophic flagellate *Collodictyon triciliatum* in a hypertrophic pond. Aquatic Microb. Ecol. 29: 173–179.

Nishibe, Y., Manage, P. M., Kawabata, Z., Nakano, S., 2004. Trophic coupling of a testate amoeba and *Microcystis* species in a hypertrophic pond. Limnology 5: 71–76.

Nishizawa, T., Asayama, M. M., Fujii, K., Harada, K., Ochi, K.,Shirai, M., 1999. Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. J. Biochem. 126: 520-529.

Nishizawa, T., Ueda, A., Asayama, M., Fujii, K., Harada, K., Ochi, K.,Shirai, M., 2000. Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. J. Biochem. 127: 779-789.

Nizan, S., Dimentman, C., Shilo, M., 1986. Acute toxic effects of the cyanobacterium *Microcystis aeruginosa* on *Daphnia magna*. Limnol. Oceanogr. 31: 497-502.

Oberholster, P. J., Botha, A.-M., Cloete, T. E., 2006. Toxic cyanobacterial blooms in a shallow, artificially mixed urban lake in Colorado, USA. Lakes& Reservoirs: Research and Management 11: 111-123.

O'Brian, W. J., DeNoyelles, Jr., F., 1974. Filtering rate of *Ceriodaphnia reticulata* in pond waters of varying phytoplankton concentrations. Am. Midl. Nat. 91: 509-512.

Ochumba, P. B. O., 1990, Massive fish kills within Nyanza Gulf of Lake Victoria. Hydrobiol. 208, 93-99.

Oh, H.-M., Lee, S. J., Jang, M.-H., Yoon, B.-D., 2000. Microcystin production by *Microcystis aeruginosa* in a phosphorous-limited chemostat. Appl. Environ. Microbiol. 66: 176–179.

Ohtake, A., Shirai, M., Aida, T., Mori, N., Harada, K-I., Matsuura, K., Nakano, M., 1989. Toxicity of Microcystis species isolated from natural blooms and purification of the toxin. Appl. Environ. Microbiol. 55: 3202-3207.

Oliver, R. L., Ganf , G. G., 2000. Freshwater Blooms. In: "The Ecology of Cyanobacteria, Their diversity in time and space" B. A. Whitton, and M. Potts, Eds., Kluwer Acadenuc, Dordrecht, The Netherlands., pp. 149-194.

Orcutt, J. D., Pace, M. L., 1984. Seasonal dynamics of rotifer and crustacean zooplankton populations in a eutrophic monomictic lake with a note on rotifer sampling techniques. Hydrobiologia 119: 73-80.

Orr, P. T., Jones, G. J., 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnol. Oceanogr. 43: 1604–1614.

Otsuka, S., Suda, S., Li, R., Watanabe, M., Oyaizu, H., Matsumoto, S., Watanabe, M. M., 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus Microcystis based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiol. Lett. 172: 15–21.

Ou, D., Song, L., Gan, N., Chen, W., 2005. Effects of microcystins on and toxin degradation by *Poterioochromonas* sp. Environ. Toxicol. 20: 373–380.

Paerl, H. W. 1982. Environmental factors promoting and regulating N,-fixing blue-green algal blooms in the Chowan River, N.C. Univ. North Carolina Water Resour. Res. Inst. Rep. 176.

Paerl, H. W., 1988. Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. Limnol. Oceanogr. 33: 823-847

Paerl, H. W., 1988b. Growth and reproductive strategies of freshwater blue-green algae (cyanobacteria), In: Growth and Reproductive Strategies of Freshwater Phytoplankton. C. D. Sandgren, ed. Cambridge University Press, Cambridge, pp. 261-315.

Paerl, H. W., Millie, D. F., 1996. Physiological ecology of toxic cyanobacteria. Phycologia 35: 160-167.

Paerl, H. W., 1997. Coastal eutrophication and harmful algal blooms: Importance of anthropogenic deposition and groundwater as “new” nitrogen and other nitrogen sources. Limnol. Oceanogr. 42: 1154-1165.

Paerl, H. W., Fulton, R. S., Moisander, P. H., Dyble, J., 2001. Harmful freshwater algal blooms with an emphasis on cyanobacteria. The Scientific World 1: 76-113.

Paerl, H. W., Huisman, J., 2008. Blooms like it hot. Science 320: 57-58.

Paerl, H. W., 2008. Nutrient and other environmental controls of harmful cyanobacterial blooms along the freshwater-marine continuum. In: *Cyanobacterial Harmful Algal Blooms: state of the Science and Research Needs* Series: Advances in Experimental Medicine and Biology, Vol. 619 Hudnell, K.H. (Ed.), 2008, XXIV, 950 p.

Paerl, H. W., 2009. Controlling eutrophication along the Freshwater-marine continuum: Duel nutrient (N and P) reductions are essential. Estuaries and Coasts 32: 593-601.

Parsons, T. R., Maita, Y., Lalli, C. M., 1984. A Manuel of chemical and biological methods for seawater analysis. Pergomon Press, Oxford.

Paul, V. J. 2008. Global warming and cyanobacterial harmful algal booms. In: Cyanobacterial Harmful Algal Blooms: state of the Science and Research Needs Series: Advances in Experimental Medicine and Biology, Vol. 619. Hudnell, K.H. (Ed.), 2008, XXIV, 950 p.

Peperzak, L., 2003. Climate change and harmful algal blooms in the North Sea. Acta. Oecol. 24: 139-144.

Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K. A., Krause, E., Codd, G. A., Steinberg, C. E. W., 1998. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. Biochimica et Biophysica 1425: 527-533.

Pflugmacher, S., 2002. Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. Environ. Toxicol. 17: 407–413.

Philipp, R., Rowland, M. G. M., Baxter, P. J., McKenzie, C., Bell, R. H., 1991. Health risks from exposure to algae. CDR (London: England Rev) 1: R67-68.

Pinckney, J. L., Millie, D. F., Vinyard, B. T., Paerl, H. W., 1997. Environmental controls of phytoplankton bloom dynamics in the Neuse River Estuary (North Carolina, USA). Can. J. Fish. Aquat. Sci. 54: 2491-2501.

Pinckney, J. L., Paerl, H. W., Harrington, M. B., Howe, K. E., 1998. Annual cycles of phytoplankton community-structure and bloom dynamics in the Neuse River estuary, North Carolina. Mar. Biol. 131: 371-381.

Porter, K. G., Orcutt, J. D., 1980. Nutritional adequacy, manageability, and toxicity as factors that determine the food quality of green and blue-green algae for *Daphnia*,

p. 268–281. In W. C. Kerfoot [ed.], Evolution and ecology of zooplankton communities. Univ. of New England Press.

Présing, M., Preston, T., Takátsy, A., Sprőber, P., Kováca, A. W., Vörös, L., Kenesi, G., Kóbor, I., 2008. Phytoplankton nitrogen demand and the significance of internal and external nitrogen sources in a large shallow lake (Lake Balaton, Hungary). Hydrobiologia 599: 87-95.

Price, N. M, Harrison, P. J., 1987. Comparison of methods for the analysis of dissolved urea in seawater. Marine Biology 94: 307–317.

Puckett, L. J., 1994. Nonpoint and point sources of nitrogen in major watersheds of the United States: U.S. Geological Survey Water-Resources Investigations Report 94–4001, 9 p.

Rapala, J., Sivonen, K., Lyra, C., Niemela, S. I., 1997. Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimulation. App. Env. Microbiol. 63: 2206-2212.

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

Renyolds, C. S., Walsby, A. E., 1975. Waterblooms. Biol. Rev. 50: 437-481.

Reshef, V., Carmeli, S., 2001. Protease inhibitors from a water bloom of the cyanobacterium *Microcystis aeruginosa*. Tetrahedron 57: 2885-2894.

Reynolds, C. S., 1987. The response of phytoplankton communities to changing lake environments. Aquat. Sci. 49: 220-236.

Reynolds, C. S., 1997. Successional Development, Energetics, and Diversity in Planktonic Communities. In: Biodiversity: An ecological perspective. Abe, T., Levin, S.A., and Higashi, M. (Eds.), Springer, 247 p.

Reynolds, C. S., 2006. Ecology of Phytoplankton. Cambridge Univ. Press, Cambridge.

Richman, S., Heinle, D. R., Huff, R., 1977. Grazing by adult estuarine copepods of the Chesapeake Bay. Mar. Biol. 42: 69-84.

Richman, S., Dodson, S. I., 1983. The effect of food quality on feeding and respiration by *Daphnia* and *Diaptomus*. Limnol. Oceanogr. 28: 948-956.

Rinta-Kanto, J. M., Ouellette, A. J. A., Boyer, G. L., Twiss, M. R., Bridgeman, T. B., Wilhelm, S. W., 2005. Quantification of Toxic *Microcystis spp*. during the 2003

and 2004 Blooms in Western Lake Erie using Quantitative Real-Time PCR. Environ. Sci. Technol. 39: 4198–4205.

Rinta-Kanto, J. M., Wilhelm, S. W., 2006. Diversity of microcystin-producing cyanobacteria in spatially isolated regions of Lake Erie. Appl. Eviron. Micorbiol. 72: 5083–5085.

Rinta-Kanto, J. M., Konopko, E. A., DeBruyn, J. M., Boubonniere, R. A., Boyer, G. L., Wilhelm, S. W., 2009. Lake Erie *Microcystis*: relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. Harmful Algae 8: 665-673.

Robarts, R. D., Zohary, T., 1987. Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. New Zeal. J. Mar. Freshw. Res. 21: 391-399.

Rodger, H. D., Turnbull, T., Edwards, C., Codd, G. A., 1994. Cyanobacterial (blue-green-algal) bloom associated pathology in brown trout, *Salmo trutta* L., in Loch Leven, Scotland, J. Fish Dis. **17:** 177–181.

Rohrlack, T., Dittmann, E., Henning, M., Borner, T., Kohl, J.G., 1999. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Appl. Environ. Microbiol. 65: 737–739.

Rohrlack, T., Christoffersen, K., Hansen, P. E., Zhang, W., Czarnecki, O., Henning, M., Fastner, J., Erhard, M., Neilan, B., Kaebernick, M., 2003. Isolation, characterization, and quantitative analysis of microviridin J, a new *Microcystis* metabolite toxic to *Daphnia*. J Chem. Ecol. 29: 1757–1770.

Rohrlack, T., Christoffersen, K., Dittman, E., Noguiera, I., Vasconcelos, V., Börner, T., 2005. Ingestion of microcystin by *Daphnia*: intestinal uptake and toxic effects. Limnol. Oceanogr. 50: 440–448.

Rolland, A., Bird, D. F., Giani, A., 2005. Seasonal changes in composition of the cyanobacterial community and the occurrence of hepatotoxic blooms in the eastern townships, Québec, Canada. J. Plank. Res. 27: 683-694.

Rosen, B. H., Shambaugh, A. D., Watzin, M. C., Boyer, G., Smith, F., Ferber, L., Eliopoulous, C., Stangel, P., 2001. Evaluation of potential blue-green algal toxins in Lake Champlain. Lake Champlain Basin Program, Grand Isle, Vermont.

Rouhiainen, L., Vakkilainen, T., Siemer, B. L., Buikema, W., Haselkorn, R, Sivonen, K., 2004. Genes Coding for Hepatotoxic Heptapeptides (Microcystins) in the Cyanobacterium *Anabaena* Strain. 90. Appl. Environ. Microbiol. 70: 686-692.

Sarnelle, O., Wilson, A. E., 2005. Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. Limnol. Oceanogr. 50: 1565–1570.

Sarnelle, O., 2007. Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. Limno. Oceanogr., 52: 2120-2127.

Schatz, D., Keren, Y., Vardi, A., Sukenik, A., Carmeil, S., Börner, T., Dittmann, E., Kaplan, A., 2007. Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins. Eviron. Microbiol. 9: 965-970.

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

Seitzinger, S., Sanders, R., 1997. Contribution of dissolved organic nitrogen from rivers to estuarine eutrophication, Mar. Ecol. Prog. Ser. 159: 1–12.

Sellner, K. G., Brownlee, D. C., Buundy, M. H., Brownlkee, S. G., Braun, K. R. 1993. Zooplankton grazing in a Potomac River cyanobacteria bloom. Estuaries 16: 859-872.

Sellner, K. G., Olson, M. M., Kononen, K., 1994. Copepod grazing in a summer cyanobacteria bloom in the Gulf of Finland. Hydrobiologia 292/293: 249-254.

Sevrin-Reyssac, J., Pletikosic, M., 1990. Cyanobacteria in fish ponds. Aquaculture 88: 1–20.

Shambaugh, A., Duchovany, A., McIntosh, A., 1999. A survey of Lake Champlain's plankton, in: Lake Champlain in Transition: From research toward restoration, Water Science Application 1, American Geophysical Union, Washington, 323-340.

Sivonen, K., 1990. Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by Oscillatoria agardhii strains. Appl. Environ. Microbiol. 56: 2658–2666.

Smith, V. H., 1983. Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. Science 221: 669–671.

Smith, V. H., 1986. Light and nutrient effects on the relative biomass of blue-green algae in lake phytoplankton. Can. J. Fish. Aquat. Sci. 43: 148–153.

Smith, A., Gilbert, J., 1995. Relative susceptibilities of rotifers and cladocerans to *Microcystis aeruginosa*. Archiv. für Hydrobiologie. 132: 309–336.

Spero, H., Morée, M. D., 1981. Phagotrophic feeding and its importance to the life cycle of the holozoic dinoflagellate *Gymnodinium fungiforme*. J. Phycot. 17: 43-51.

Stoecker, D. K., Gifford, D. J., Putt, M., 1994. Preservation of marine planktonic ciliates: Losses and cell shrinkage during fixation. Mar. Ecol. Prog. Ser. 110: 293–299.

Stoecker, D., 1999. Mixotrophy among dinoflagellates. J. Eukaryot. Microbiol. 46: 397–401.

Sugiura, N., Inamori, Y., Ouchiyama, T., Sudo, R., 1992. Degradation of cyanobacteria, *Microcystis* by microflagellate, *Monas guttula*. Water Sci. Technol. 26: 2173–2176.

Takamura, N., Iwakuma, T., Yasuno, M., 1985. Photosynthesis and primary production of *Microcystis aeruginosa* Ktitz. in Lake Kasumigaura. J. Plankton Res. 7: 303-312.

Takamura, N., Iwakuma, T., Yasuno, M., 1987. Uptake of ^{13}C and ^{15}N (ammonium, nitrate, and urea) by *Microcystis* in Lake Kasumigaura. J. Plankton Res. 9: 151-165.

Tango, P. J., Butler, W., 2008. Cyanotoxins in tidal waters of Chesapeake Bay. Northeast. Nat. 15: 403-416.

Taş, S., Okuş, E., Aslan-Yilmaz, A., 2006. A bloom of a cyanobacterium *Microcystis* cf. *aeruginosa* in a severly polluted estuary, the Golden Horn, Turkey. Estuar. Coast. Shelf Sci. 68: 593-599.

Thompson, J.M., Ferguson, A.J.D., Reynolds, C.S., 1982. Natural filtration-rates of zooplankton in a closed system-the derivation of a community grazing index. J. Plankton Res. 4: 545-560.

Threlkeld, T., 1979. The midsummer dynamics of two *Daphnia* species in Wintergreen Lake, Michigan. Ecology 60: 165-179.

Tillett, D., Dittmann, E., Erhard, M., von Dohren, H., Borner, T., Neilan, B.A., 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide–polyketide synthetase system. Chem. Biol. 7: 753–764.

Tilman, D., Kiesling, R. L., 1984. Freshwater algal ecology: taxonomic trade-offs in the temperature dependence of nutrient competitive abilities. In: Klug, M. J.; Reddy, C. A. ed., Current perspectives in microbial ecology, pp. 314-319. Washington D.C. American Society for Microbiology.

Tonk, L., Boch, K., Visser, P., Huisman, J., 2007. Salt tolerance of the harmful cyanobacterium *Microcystis aeruginosa*. Aquat. Microb. Ecol. 46: 117-123.

Trimbee, A. M., Prepas, E. E., 1987. Evaluation of total phosphorus as a predictor of relative biomass of blue-green algae with an emphasis on Alberta lakes. Can. J. Fish. Aquat. Sci. 44: 1337–1342.

Turner, P. C., Gammie, A. J., et al. 1990. Pneumonia Associated with Contact with Cyanobacteria. Brit. Med. J. 300: 1440-1441.

Utkilen, H., Gjølme, N., 1995. Iron-stimulatedtoxin production in *Microcystis aeruginosa*. Appl. Eviron. Microbiol. 61: 797-800.

Valderrama, J. C., 1981. The simultaneous analysis of total N and total P in natural waters. Mar. Chem. 10**:** 109–122.

Van der Westhuizen, A. J.; Eloff, J. N., 1985: Effect of temperature and light on the toxicity and growth of the blue-green alga *Microcystis aeruginosa* (UV-006) Planta 163: 55-59.

Van Dolah, F. M., Roelke, D., Greene, R. M., 2001. Health and ecological impacts of harmful algal blooms: risk assessment needs. Human Ecol. Risk Assess. 7: 1329–1345.

Vanderploeg, H. A., Scavia, D., 1979a. Two electivity indices for feeding with special reference to zooplankton grazing. J Fish. Res. Board. Can. 36: 362-365.

Vanderploeg, H. A., Scavia, D., 1979b. Calculation and use of selectivity coefficients of feeding: zooplankton grazing. Ecological Modelling 7: 135-149.

Vardi, A., Schatz, D., Beeri, K., Motro, U., Sukenik, A., Levine, A., Kaplan, A., 2002. Dinoflagellate-cyanobacterium communication may determine the composition of phytoplankton assemblage in a mesotrophic lake. Curr. Biol. 12: 1767–1772.

Vargo, G. A., Heil, C. A., Ault, D. N., Neely, M. B., Murasko, S., Havens, J., Lester, K. M., Dixon, L. K., Merkt, R., Walsh, J., Weisberg, R., Steidinger, K. A., 2004. Four *Karenia brevis* blooms: a comparative analysis. In: Steidinger, K.A., Landsberg, J.A., Tomas, C.R., Vargo, G.A. (Eds.), Harmful Algae 2002 Proceedings of the Xth International Conference on Harmful Algae, Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and the Intergovernmental Oceanographic Commission of UNESCO, St. Petersburg (FL), pp. 14–16.

Vézie, C., Brient, L., Sivonen, K., Bertru, G., Lefeuvre, J-C.,Salkinoja-Salonen, M., 1998. Variation of microcystin contentof cyanobacterial blooms and isolated strains in Lake Grand-Lieu (France). Microb. Ecol. 35: 126-135.

Vézie, C., Rapala, J., Vaitomaa, J., Seitsonen, J., Sivonen, K., 2002. Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microb. Ecol. 43: 443-454.

Vollenweider, R. A., Kerekes, J. J., 1982. Eutrophication of waters: Monitoring, assessment, and control. OCED, Paris.

Von Elert, E., Oberer, L., Merkel, P., Huhn, T., Blom, J. F., 2005. Cyanopeptolin 954, a chlorine containing chymotrypsin inhibitor of *Microcystis aeruginosa* NIVA Cya 43. J. Nat. Prod. 68: 1324-1327.

Watanabe, M. F., Oishi, S., 1985. Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. Appl. Environ. Microbiol. 49: 1342–1344.

Watras, C. J., Baker, A.L., 1988. Detection of Planktonic Cyanobacteria by Tandem in vivo Fluorometry. Hydrobiologia. 169: 77-84.

Watson, S. B., McCauley, E., Downing, J. A., 1997. Patterns in phytoplankton taxonomic composition across temperate lakes of differing nutrient status. Limnol. Oceanogr. 42: 487–495.

Watzin, M. C., Miller, E. B., Shambaugh, A. D., Kreider, M. A., 2006. Application of the WHO alert level framework to cyanobacterial monitoring of Lake Champlain, Vermont. Environ. Tox. 21: 278-288.

Welker, M., von Döhren, H., Tauscher, H., Steinberg, C. E. W., Erhard, M., 2003. Toxic *Microcystis* in shallow Lake Müggelsee (Germany): dynamics, distribution, diversity. Arch. Hydrobiol. 157: 227-248.

Welker, M., Šejnohová, L., Némethová, D., von Döhren, H., Jarkovský, J., Marŝálek, B., 2007. Seasonal shifts in chemotype composition of *Microcystis* sp. communities in the pelagial and the sediment of a shallow reservoir. Limnol. Oceanogr. 52: 609-619.

Weissing, F. J., Huisman, J., 1994. Growth and competition in a light gradient. J. Theor. Biol. 168: 323–336.

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

Wiedner, C., Visser, P. M., Fastner, J., Metcalf, J. S., Codd, G. A., Mur1, L. R., 2003. Effects of light on the microcystin content of *Microcystis* strain PCC 7806, Appl. Environ. Microbiol. 69: 1475–1481.

Wilson, A. E., Sarnelle, O., Tillmanns, A. R., 2006. The effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: Meta-analysis of laboratory experiments. Limnol. Oceanogr. 51: 1915-1924.

Yoshida, T., Takashima, Y., Tomaru, Y., Shirai, Y., Takao, Y., Hiroisha, S., Nagasaki, K., 2006. Isolation and characterization of a cyanophage infecting the toxic cyanobacterium *Microcystis aeruginosa*. Appl. Environ. Microbiol. 72: 1239–1247.

Yu, S.-Z., 1995. Primary prevention of hepatocellular carcinoma. J. Gastroenterol. Hepatol. 10: 674-682.

Zegura, B., Sedmak, B., Filipic, M., 2003. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon 41: 41-48.

Zhang, X., Watanabe, M. M., Inouye, I., 1996. Light and electron microscopy of grazing by *Poterioochromonas malhamensis* (Chrysophyceae) on a range of phytoplankton taxa. J Phycol 32: 37–46.

Zurawell, R. W., Chen, H., Burke J. M., Prepas E. E., 2005. Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. J Tox Eviron Heal, Part B 8: 1-37.