5-2007

Biogeography and Genetic Diversity of Toxin Producing Cyanobacteria in a Laurentian Great Lake

Johanna Maaria Rinta-Kanto
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Johanna Maaria Rinta-Kanto entitled "Biogeography and Genetic Diversity of Toxin Producing Cyanobacteria in a Laurentian Great Lake." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Steven W. Wilhelm, Major Professor

We have read this dissertation and recommend its acceptance:

Todd Reynolds, Erik Zinser, Kurt Lamour, Alice Layton

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Linda Painter
Interim Dean of Graduate Studies

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BIOGEOGRAPHY AND GENETIC DIVERSITY OF TOXIN PRODUCING CYANOBACTERIA IN A LAURENTIAN GREAT LAKE

A Dissertation

Presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Johanna Maaria Rinta-Kanto

May, 2007

To Dan and my family for their love and constant support

ACKNOWLEDGEMENTS

Making it through a doctoral program is an ambitious endeavor which would be impossible to accomplish alone. Therefore I owe thanks to everyone who has helped me in so many ways and during my time in graduate school. Here are just few of the ones who deserve my deepest gratitude:

I want to thank my advisor Dr. Steven Wilhelm for initially sparking my interest in the field of aquatic microbial ecology and giving me the opportunity to work on this fascinating research project. His contagious enthusiasm towards science has been a source of inspiration while completing this project. Moreover, his advice has not only helped me to get this work done, but he has also given me the opportunity to mature as a scientist along the way.

I want to express gratitude to my committee members, Dr. Kurt Lamour, Dr. Todd Reynolds, Dr. Erik Zinser and courtesy member Dr. Alice Layton for their comments and insight for my research. I am also grateful to the co-authors of my papers as well as numerous collaborators who have assisted with sampling, analyses of the samples and by sharing their thoughts and comments to improve my manuscripts. I want to give special thanks to Dr. Anthony Ouellette; his guidance and insight has helped me to solve many research problems. Also, thanks to Lynn Ferguson for her help with processing of dozens of DNA samples. I owe big thanks to all my friends for their support and I thank all past and present members of the Wilhelmlab for their friendship and assistance with problems big and small.

I am thankful to my family for always encouraging me to reach my goals and for their love and support for my endeavors. Last, although he should be the first, I want to thank my husband Dan for his love, admirable patience, support, and companionship as well as for proofreading all parts of this dissertation.

I am grateful for the Helsingin Sanomat Centennial foundation for their generous scholarship as well as for International Association for Great Lakes Research and Science Alliance for providing financial support for my work.

ABSTRACT

The North American Great Lakes are a vital source on a global scale, as they hold ~18 % of the potable water resources on our planet. Cyanobacteria of the genus *Microcystis* are commonly found in fresh water environments around the world, and since the mid-1990s also in Lake Erie. The reasons for the success for these potentially toxic cyanobacteria in Lake Erie are not completely understood. In this study we have applied modern molecular tools to analyze field samples to provide an insight into the genotypic composition and diversity of the *Microcystis* community in the past and present day Lake Erie. We have also analyzed a three-year data set to identify specific environmental factors that contribute to the abundance of *Microcystis* genotypes and microcystin production. In addition, in a laboratory-based study we examined the effect of nutrients on transcriptional activity of the microcystin synthetase gene *mcyD*.

The results of this study suggest that, although toxic *Microcystis* form < 10 % of the total cyanobacterial population in Lake Erie, the toxin-producing *Microcystis* community in Lake Erie is diverse, and that these populations are stabile on a time scale of decades. Sediments acting as a reservoir of *Microcystis* are likely contributing to the persistence of the population. Although *Microcystis* is the dominant microcystin producer in the lake, other microcystin-producing cyanobacteria were also found in spatially isolated regions of the lake. While microcystin concentration in Lake Erie is correlated positively with total phosphorus ($P<0.001$) and surface reactive phosphorus ($P<0.001$), and negatively with the molar ratio total nitrogen to total phosphorus ($P<0.001$); toxic *Microcystis* abundance correlates negatively with NO_3 concentration ($P=0.04$) and positively with surface water temperatures (ranging from 20.8 °C to 27.4 °C) ($P=0.03$). These observations, along with findings from culture based experiments, suggest decoupling of the factors governing proliferation of toxic cells and toxin production. Culture based experiments also suggested that the chemical form of phosphorus may be an important factor in regulating microcystin biosynthesis in *Microcystis* based on monitoring relative transcriptional activity of the *mcyD* gene. The transcriptional activity of *mcyD* was higher ($P=0.118$) in cells grown in BG11-medium containing 2.3 µM organic phosphorus (glycerol 2-phosphate disodium salt hydrate) than in cells grown in BG11-medium containing 2.3 µM inorganic phosphorus (K_2HPO_4).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Part I

LITERATURE REVIEW

Background

Freshwater cyanobacteria

Cyanobacteria are prokaryotic photoautotrophs that thrive in a wide range of mostly aquatic habitats. The cellular organization and basic functions regarding growth and photosynthesis have been comprehensively described in the literature (e.g. Sandgren, 1988; Peschek et al., 1999) and since the late 1990s, the sequencing of entire genomes of or smaller specific regions of within a genome (e.g. gene cassettes involved in certain metabolic functions, such as toxin production) (Nishizawa et al., 2000; Tillett et al., 2000) has allowed more extensive understanding of their unique lifestyle and metabolic traits (Kaneko et al., 2001; Palenik et al., 2003; Rocap et al., 2003).

The importance of cyanobacteria in the global ecosystem is indisputable: marine cyanobacteria *Synechococcus* and *Prochlorococcus* are estimated to carry out 32 – 88 % of primary production in the oligotrophic ocean (Rocap et al., 2002). There are no estimates regarding the percent contribution of cyanobacteria to primary production in freshwater systems or in the Great Lakes, however, in Lake Erie picoplankton (0.2 – 2.0 μm size class), which include cyanobacteria, make up on the average 44 % (range 10 – 66 %) of the biomass based on size-fractionated chlorophyll *a* estimates (Twiss et al., 2005). Cyanobacteria are important as the first link in the food web, nitrogen fixers, succession pioneers, and sources or pharmaceutical compounds (Dietrich and Hoeger, 2005).

While cyanobacteria generally have relatively simple basic metabolic requirements consisting of carbon dioxide, light, water, and inorganic nutrients (Mur et al., 1999), some cyanobacteria have exhibited more complicated traits as they are auxotrophs for compounds such as vitamin B12 (Wilhelm and Trick, 1995). They also possess efficient uptake and retention mechanisms for phosphate, nitrate and bicarbonate (Ritchie et al., 1997; Herrero et al., 2001; Badger and Price, 2003). In addition to the above mechanisms, cyanobacteria are capable of producing high affinity iron chelators, known as siderophores, under iron deficient conditions to alleviate iron stress (Boyer et al., 1987; Wilhelm and Trick, 1994), and some filamentous cyanobacterial genera possess

heterocysts, cells specialized to fix atmospheric N_2 (Paerl, 1988). In total, cyanobacteria have unique traits that allow them to dominate many phytoplankton communities as a result of the interactions between their physiology and the physical and chemical characteristics of the aquatic system itself.

Numerous studies have been carried out that characterized the environmental conditions which allow cyanobacteria to proliferate (Dokulil and Teubner, 2000). The major mechanisms hypothesized to promote cyanobacterial success in freshwater systems include increased availability of nitrogen and phosphorus, low nitrogen to phosphorus ratio (N:P) (< 29:1 by weight), low ambient CO_2 concentration and high pH, elevated water temperature, water column stability, buoyancy regulation, ability to store nutrients, trace element competition and avoidance by grazers due to excretion of toxins and other organic compounds (Paerl, 1988; Dokulil and Teubner, 2000; Downing et al., 2001).

A range of bloom-forming freshwater cyanobacterial genera includes strains capable of producing toxins and other bioactive compounds (Carmichael, 2001; Welker and von Döhren, 2006). Currently known cyanobacterial toxins are classified into three broad groups based on their chemical structure: cyclic peptides (the hepatotoxins microcystin and nodularin), alkaloids (the neurotoxic anatoxins, saxitoxins, dermatotoxic aplysiatoxins and lyngbyatoxins) and lipopolysaccharides (irritant toxins) (Sivonen and Jones, 1999). The presence of cyanobacteria able to produce these toxins can compromise water quality leading to both economic and public health concerns. Due to this potential to cause damage, cyanobacterial blooms are commonly included within the larger designation of harmful algal blooms (HABs), a broad term that encompasses a broad class of events caused by marine or freshwater microalgae that have a negative impact on human activities (Zingone and Enevoldsen, 2000).

Harmful algal blooms in freshwater – an old phenomenon and an emerging threat

Although during the 20th century the occurrence of cyanobacterial blooms has been often linked to human-inflicted eutrophication of natural waters, humans have encountered cyanobacterial blooms well prior to the industrial revolution. The early reports of

cyanobacterial blooms were commonly descriptions of visible cyanobacterial masses formed in water bodies, or reports of adverse effects on animal or human health linked to consumption of water tainted with toxic cyanobacteria (Hayman, 1992; Codd et al., 1994). The earliest reference suggesting the presence of (presumably toxic) cyanobacterial blooms can be found in the Bible (Exodus 7:20-21). Moreover, approximately 1000 years ago, the troops of Chinese general Zhu Ge-Ling became ill from drinking green water upon crossing a stream. Australian aboriginals have been known to use sand filters to pretreat their potable water, indicating awareness of health effects of water contaminated by toxic cyanobacteria (Hayman, 1992; Codd et al., 1994) and 17th century Dutch painters have illustrated agricultural and urban landscapes including water bodies that are bright green in color (Paerl and Steppe, 2003). Altogether, based on historical documents and remarks found in the folklore, literature and art originating from every continent inhabited by humans, it is obvious that cyanobacterial blooms have been a global phenomenon for at least two millennia (Codd et al., 1994; Bartram et al., 1999; Codd et al., 1999; Paerl and Steppe, 2003). The first report in scientific literature documenting deaths of farm animals due to ingesting green scum from a bloom of toxic cyanobacteria (*Nodualria spumigena*) in Lake Alexandrina in Australia dates back to the late 19th century (Francis, 1878).

Harmful algal blooms in coastal marine waters have been intensively monitored due to significant economic impacts of bloom events, and recently cyanobacterial blooms in inland freshwaters have also been recognized as a serious threat to human health and fisheries resources (Boyer, 2006). Several reports in the literature have suggested that observations of harmful algal blooms around the world have increased in the post World War II era (Sellner et al., 2003). One suggested factor increasing the frequency of HABs is the (perhaps inaccurately named) process of "cultural eutrophication" (caused by land clearing, extensive use of fertilizers, large scale cattle farming and the discharge of sewage) mobilizing nutrients that are known to generate conditions where cyanobacterial blooms often develop (Cloern, 2001; Sellner et al., 2003). Modeling studies suggest that global climate warming has the potential to alter the overall functioning of aquatic ecosystems, also causing changes in the spatial and temporal distribution of bloom

forming cyanobacteria (Malmaeus et al., 2006). Besides environmental changes, the awareness of the devastating ecological, economic and health impacts of HABs has increased rapidly, which has led to development of improved HAB monitoring networks around the world. Therefore, it is somewhat debatable whether the observed increase in the frequency of harmful algal blooms is actually a result of increased frequency of the blooms or a reflection of improved monitoring and reporting of the bloom events (Sellner et al., 2003).

Microcystis and microcystin

Microcystis is one of the most well studied freshwater cyanobacterial genus due to its common occurrence in bodies of water around the world and its ability to form blooms and produce potent toxins that can threaten the health of humans and animals (Carmichael, 1996). *Microcystis* cells are 1-6 μm in diameter, characteristically coccoid-shape, forming microscopic to macroscopic colonies (Komarek, 2003). *Microcystis* cells have gas vacuoles which allow them to float on the surface of the water and form characteristic surface scums (Paerl, 1988). *Microcystis* does not form heterocysts or akinetes, nevertheless the cells can revert to resting stages during unfavorable growth conditions (Paerl, 1988). These resting stages may sink to the sediments (Reynolds et al., 1981) from where they can migrate back into the water column forming an inoculum for pelagic growth upon return of favorable growth conditions (Brunberg and Blomqvist, 2003; Ihle et al., 2005). A recent study demonstrated that toxigenic *Microcystis* strains have higher requirements for N and P availability than non-toxigenic *Microcystis* strains, indicating the importance of these major nutrients in biomass and toxin production (Vezie et al., 2002). Hence it was speculated that non-toxic strains of *Microcystis* may be able to outcompete toxic strains under low-nutrient conditions.

Cyanobacteria in the genus *Microcystis* produce an array of secondary metabolites classified as microcystins, aeruginosins, microginins, anabaenopeptins, cyanopeptolins, microviridins and cyclamides (Welker and von Döhren, 2006). Microcystins are potent hepatotoxins and are the most commonly found cyanobacterial toxins in brackish and freshwater environments (Sivonen and Jones, 1999; Welker and von Döhren, 2006). To

date microcystin production has been discovered in *Microcystis* sp., *Synechococcus* sp., *Anabaena* sp., *Anabaenopsis* sp, *Nostoc* sp., *Phormidium* sp., *Planktothrix* sp., and *Oscillatoria* sp., *Haphalosiphon* sp., and in *Radiocystis fernandoi*, *Croococcus dispersus,* (Vieira et al., 2003; Carmichael and Li, 2006; Jungblut and Neilan, 2006). Strains that are capable as well as strains that are incapable of producing toxin (referred to as toxic and non-toxic strains, respectively) are found in each genus. In natural bloom forming populations toxic and non-toxic strains of more than one genus can co-exist (Rantala et al., 2006).

Microcystins exist in over sixty chemical forms, making these toxins the largest and most diverse group of cyanotoxins (Sivonen and Jones, 1999; Kaebernick and Neilan, 2001). Microcystin molecules contain seven peptide-linked amino acids, all of which can undergo structural variations (Figure 1) (Sivonen and Jones, 1999). Based on LD_{50} (an abbreviation for "lethal dose, 50 %"; a dose of substance that causes 50 % mortality) values determined through mouse bioassays, the toxicity of chemical variants of microcystins varies, with microcystin-LR (L-arginine and L-leucine at positions Z and X, respectively; see Figure 1) suggested to be the most toxic (Sivonen and Jones, 1999). Microcystin-LR is also the best studied variant of this large group of toxins (Dietrich and Hoeger, 2005). Microcystins are generally water soluble with a molecular weight of 800-1000 Da (Sivonen and Jones, 1999). These toxins can be analyzed and quantified using several different methodologies. In the past the most widely applied assay was the mouse bioassay. This technique, however, has poor sensitivity and reproducibility and has been replaced by more sophisticated and reliable *in vitro* analytical methods, such as an enzyme-linked immuno sorbent assay (ELISA), the protein phosphatase inhibition assay (PPI), and high performance liquid chromatography (HPLC). All of these approaches have variable detection ranges and sensitivities (Harada et al., 1999; Dietrich and Hoeger,

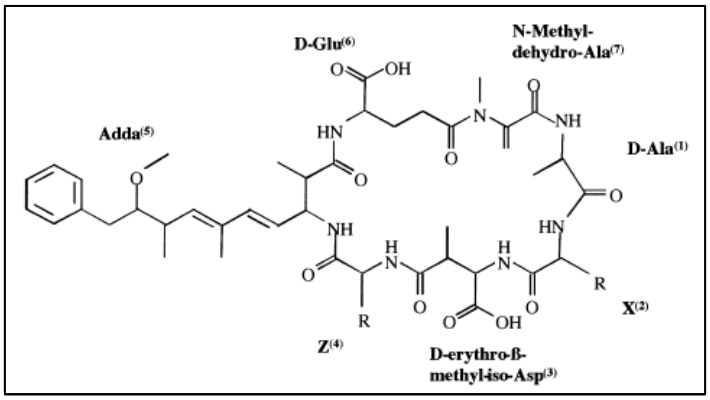

Figure 1. The general structure of microcystins. Cyclo(Adda-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z-) where X and Z are sites for variable L-amino acid substitutions, Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylaspartic acid and Mdha is N-methyl-dehydroalanine.

2005). Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) is commonly used for detection and identification of structural variants of microcystin produced by the cells in culture or in natural samples (Erhard et al., 2001).

Cellular biosynthesis of microcystins

Microcystins are synthesized in the cells by a non-ribosomal enzyme complex encoded by the microcystin synthetase (*mcy*) gene cluster (Kaebernick and Neilan, 2001). The biosynthetic pathway for cellular synthesis of microcystins and the gene clusters responsible for microcystin biosynthesis have been characterized in two strains of *Microcystis* (Nishizawa et al., 1999; Nishizawa et al., 2000; Tillett et al., 2000), in one strain of *Planktothrix* (Christiansen et al., 2003) and in one strain of *Anabaena* (Rouhiainen et al., 2004). In *Microcystis* spp. the gene cluster is located within the genome, it spans 55 kb and consists of two bi-directionally transcribed operons which contain 10 open reading frames (ORFs). The gene cluster is transcribed in two polycistronic transcripts (*mcyABC* and *mcyDEFGHIJ*). A transcriptional analysis revealed the presence of two alternate, light dependent transcription start sites for the two polycistronic transcripts located between *mcyA* and *mcyD* genes (Kaebernick et al., 2002). In the same study Kaebernick et al. (2002) demonstrated that the selection between the transcription start sites depends on the light intensity the cells are exposed to. Putative intercistronic transcription start sites have also been identified for individual *mcy* genes (*mcy E, F, G, H, I, J*), however the role of these sites in regulation of *mcy* gene expression is still unknown (Kaebernick et al., 2002).

The ORFs encode non-ribosomal peptide synthetase (NRPS) domains, polyketide synthase (PKS) domains and tailoring enzymes which catalyze the 48 sequential enzymatic reactions involved in microcystin biosynthesis (Tillett et al., 2000). The general arrangement and transcriptional orientation of the *mcy* gene cluster is different in the three microcystin-producing genera (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). The content of *mcy* genes which are not primarily involved in peptide assembly is also characteristic in each genus, whereas the modular arrangement

and structural organization of the genes is conserved throughout the genera (Welker and von Döhren, 2006). The presence of the gene cluster in the genome is currently thought to indicate whether the strain is considered to be toxic (Dittmann et al., 1997). Though this is generally shown to be the case, a few exceptions to the rule have been reported (Meissner et al., 1996). The central role of *mcy* genes in microcystin biosynthesis has been confirmed through gene disruption studies: mutations in genes *mcyA*, *B*, *D*, *E*, *F* and *H* in *Microcystis* have been shown to completely abolish the ability of the cells to synthesize the toxin (Dittmann et al., 1997; Nishizawa et al., 1999; Tillett et al., 2000; Pearson et al., 2004). Unlike other microbial NPRS systems, the *mcy* genes are generally though to be constitutively expressed (Welker and von Döhren, 2006). Nonetheless, new evidence suggests this may not be the case, as a ferric uptake regulator (fur) binding site has been found within the bidirectional promoter region (Martin-Luna et al., 2006a; Martin-Luna et al., 2006b) and recent laboratory studies suggest Fe-mediated transcriptional regulation of *mcy* gene cluster may occur (B. Neilan, unpublished). A strain producing *mcyA* and *mcyB* mRNA transcripts but no toxin was found in a previous study (Mikalsen et al., 2003) suggesting that the regulation of microcystin production may take place on a translational or posttranslational level. Genetic variation in *mcy* genes has been shown to be linked to production of different microcystin isoforms (Mikalsen et al., 2003).

Regulation of microcystin production

The effects of nutrients (nitrogen and phosphorus), trace metals (especially Fe), light intensity, pH and temperature on the toxicity of *Microcystis* have been investigated in several studies (See Table 1 for summary. Tables are in Appendix at the end of Part 1). The studies conducted so far have not been able to conclusively elucidate the major factors in the regulation of microcystin production in *Microcystis*. The lack of consensus can be, at least partly, explained by several methodological differences between individual studies. First, typically the individual studies have focused on analyzing the toxicity in a pure culture of some strain of *Microcystis*. This alone may be problematic since toxin production under identical conditions has been shown to vary between

Microcystis strains (Vezie et al., 2002). Secondly, cultures have been grown in different conditions (batch cultures, continuous cultures) and a variety of media has been used for growing the cells (Sivonen and Jones, 1999). Third, typically the effect of only a single factor has been investigated at a time. Fourth, a diversity of methods has been applied to determine the toxicity of the cells. And finally, some studies have quantified just select isoforms of microcystin, whereas others have quantified the combination of several isoforms as total microcystin. Studies quantifying just one isoform may give a biased view of the actual toxicity because microcystin producing strains can often produce more than one variant of microcystin and evidence from culture studies and environmental samples suggests that environmental conditions can affect the composition of microcystins produced by a single strain (Luukkainen et al., 1994; Vasconcelos et al., 1995; Böttcher et al., 2001; Tonk et al., 2005).

Microcystin in the environment and ecological significance of microcystin

Laboratory-based batch culture experiments on microcystin-producing cyanobacteria (*Microcystis, Anabaena* and *Aphanizomenon* sp.) have shown that approximately 80% of toxin stays within the intact cells (Codd et al., 1999). Toxins are released in the surrounding medium upon increased permeability or lysis of aging cells (Sivonen and Jones, 1999). Due to varying growth states of cells in natural systems, the division of microcystins between intra- and extracellular pools has been difficult to estimate; the fractionation has been found to vary from 100% extracellular to 100 % intracellular (Codd et al., 1999). Sequence analysis of the *mcy* gene cluster in *Microcystis* suggested the *mcyH* gene is encoding a putative ATP-binding cassette (ABC) transporter, predicted to be involved in microcystin export (Pearson et al., 2004). However in the current literature no information exists regarding active microcystin export from the cells.

Microcystins are remarkably resistant to chemical degradation; they are resistant to hydrolysis and oxidation at neutral pH range and can withstand even boiling temperatures. The chemical degradation process is slow, allowing toxins to persist for years. Full sunlight causes a slow photochemical breakdown of microcystins and the process is further accelerated by the presence of water soluble phycobiliproteins or humic

substances (Sivonen and Jones, 1999). In natural waters enzymatic degradation of microcystins by bacteria and algae accelerates the degradation process significantly (Bourne et al., 1996; Ou et al., 2005).

The ecological role of microcystins is not yet understood (Wiegand and Pflugmacher, 2005; Babica et al., 2006). It seems unlikely that production of microcystins is an indispensable trait for the cells since both toxic and non-toxic cells coexist in natural populations (Kaebernick and Neilan, 2001). Several hypotheses have been presented for the potential role of microcystin, (Wiegand and Pflugmacher, 2005). Utkilen & Gjølme (1995) proposed a role for microcystin as an intracellular iron chelator, although experiments conducted at the University of Tennessee and Dickinson College (Wilhelm and Witter, unpublished) have demonstrated that the toxin has no iron-chelating ability. It has also been suggested that microcystin may act in cell to cell signaling among phytoplankton (Babica et al., 2006) and as a grazing deterrent against zooplankton (Jang et al., 2003) or filter feeders (such as *Dreissena polymorpha*) (Vanderploeg et al., 2001). The changes in pigment content of mutant *Microcystis* with impaired ability to produce microcystin suggested that microcystins may play a role in the light adaptation process of the cells (Hesse et al., 2001). Contradictory results from studies investigating these hypotheses have prevented acceptance of any of these hypotheses as such. Furthermore, *Microcystis* and other cyanobacteria are capable of producing a host of other bioactive compounds along with microcystin, which may make studying the precise effects of microcystin in natural systems especially challenging (Welker and von Döhren, 2006).

Toxicity mechanism of microcystins

The mechanism of toxicity in vertebrate cells for microcystin-LR has been well studied and described (Sivonen and Jones, 1999; Wiegand and Pflugmacher, 2005). Water soluble microcystin-LR is sequestered into vertebrate liver cells through a highly expressed bile acid carrier transport system. Once inside the hepatocytes, the toxin will localize in the nucleus where the ADDA-moiety of a microcystin-LR molecule effectively blocks the active site of nuclear protein phosphatases 1 and 2A (Mackintosh et

al., 1990; Guzman et al., 2003; Wiegand and Pflugmacher, 2005). The decreased phosphatase activity seen upon exposure to the toxin contributes to the lethal and sublethal effects in cells, such as severe intrahepatic hemorrhage, rounding and dissociation of hepatocytes, and oxidative stress through a variety of mechanisms (Guzman et al., 2003). In humans, acute exposure to microcystins is known to have caused gastroenteritis and liver damage (Kuiper-Goodman et al., 1999) and, in the most severe case, death of 60 hemodialysis patients in Brazil in 1996 (Pouria et al., 1998). Some evidence of tumor-promoting activity of microcystins also exists (Falconer, 1991; Dietrich and Hoeger, 2005). Based mostly on animal studies, the tolerable daily intake (TDI) value for microcystin-LR has been determined as 0.04 μg kg^{-1} (Kuiper-Goodman et al., 1999). Based on the TDI, The World Health Organization (WHO) has determined a provisional guideline value of 1.0 μg L^{-1} for microcystin-LR in drinking water. Water containing less than 1.0 μg L^{-1} of microcystin LR does not pose a health risk and should be acceptable for lifelong consumption (Falconer et al., 1999). However, the effects of long-term, low-level exposure to microcystins have not been extensively studied.

Modern techniques to study cyanobacteria

Historically, a combination of microscopy, phytopigment analysis and chemical analysis to detect and quantify cyanotoxins has been the most common approach to the study toxic cyanobacteria in cultures and in natural samples (Chorus and Bartram, 1999). Cyanobacterial taxonomy has been established based on morphological features, such as the shape and dimensions of the cells, presence of structurally differentiated cells, and whether the cells grow as solitary cells or in colonies (Paerl, 1988; Watanabe, 1996; Komarek, 2003). As an example, the definition of "species" within the genus *Microcystis* was based on identification of different morphotypes, each one of which was originally considered equal to a "species" (Otsuka et al., 1999). Several studies have, however, found a disagreement between morphological taxonomy and molecular taxonomy of *Microcystis* (Neilan et al., 1995; Otsuka et al., 1998; Otsuka et al., 1999). These discrepancies can partially be explained by the morphological plasticity that takes place

during isolation of strains in different culture collections and the effects of long-term culture maintenance in laboratory conditions (Doers and Parker, 1988).

Quantification of cyanobacteria in samples forms the core data in many ecological studies of cyanobacteria. **L**ight microscopy is often the method of choice for enumerating cyanobacteria in samples. Knowing that the ability to produce toxin is a genotypic trait, enumeration of phenotypically identical cells can provide only some insight into the toxigenic potential of the cyanobacterial population. In genetically distinct cyanobacteria, generations of selection and genetic transfer have lead to multiple variations in the genetic systems associated with the production of these toxins (Rantala et al., 2004). The knowledge of genes involved in toxin synthesis in each genus provides researchers with the opportunity to develop molecular tools that can be used to identify general pathways for cyanotoxin production. The genes involved in toxin synthesis can be used for nucleic acid-based detection and identification of cyanobacteria employing these pathways (Fergusson and Saint, 2003; Dittmann and Börner, 2005; Jungblut and Neilan, 2006).

Currently most of the work on the development of molecular probes has focused on polymerase chain reaction (PCR)-based techniques that amplify specific products to indicate the presence or absence of the host organism (Ouellette and Wilhelm, 2003), and on developing phylogenetic markers to classify cyanobacteria and smaller subgroups (Dittmann and Börner, 2005). Moreover, a wide range of PCR-based "fingerprinting" techniques has been developed for profiling cyanobacterial populations, including randomly amplified polymorphic DNA (RAPD)-PCR, highly iterated palindromic sequence (HIP)-PCR, analysis of repetitive extragenomic palindromic (REP) elements (REP-PCR), enterobacterial repetitive intergenic consensus (ERIC)-PCR, (terminal) restriction fragment length polymorphism (T)RFLP and denaturing gradient gel electrophoresis (DGGE) (Neilan, 1995; Smith et al., 1998; Lyra et al., 2001; Kurmayer et al., 2002; Hisbergues et al., 2003; Janse et al., 2003).

In utilizing the quantitative aspect of PCR based techniques, it has become possible to specifically quantify cells belonging to a certain genus and furthermore to quantify the cells within the specific genus bearing the genes required for toxin production. To provide a quantitative aspect in freshwater cyanobacterial ecology, a

quantitative real-time PCR (qPCR) technique known as Taq-nuclease assay (or Taq-man PCR) was first introduced in a study of *Synechococcus* ecotypes in deep lakes (Becker et al., 2002). This technique, along with other established qPCR techniques, has been subsequently applied for detection and quantification of *Microcystis* and *Anabaena* specific *mcy* gene copies in cultures as well as in natural samples (Foulds et al., 2002; Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003) and for estimating the abundance of cells carrying the specific target genes in natural samples (Kurmayer and Kutzenberger, 2003).

Target genes which have been utilized in molecular detection and phylogenetic classification of potentially toxic freshwater cyanobacteria in published studies, are listed in Table 2. Sequences from small subunit ribosomal RNA (SSU rRNA) genes have been widely used for phylogenetic studies of cyanobacteria in marine and freshwater environments (Urbach et al., 1992; Neilan et al., 1997). Although 16S rRNA gene sequences are often considered to contain enough sequence information for phylogenetic studies among eubacteria, the slow evolution of these sequences can be disadvantageous when studying cyanobacterial populations beyond the genus level. For example in the case of *Microcystis*, 16S rRNA gene sequences can be reliably used for genus-specific identification of potentially toxic *Microcystis* (Neilan et al., 1997), but to attain better phylogenetic resolution beyond genus level, more polymorphic sequences have been utilized as phylogenetic markers, such as sequences of 16S-23S rRNA internal transcribed spacer (ITS), intergenic spacer (IGS) region between phycocyanin subunit genes *cpcB* and *cpcA* (Kim et al., 2006) and functional genes encoding cyanobacterial RNA polymerase subunits γ (*rpoC1*) and σ (*rpoD1*), and the subunit B protein of DNA gyrase (topoisomerase type II) (*gyrB*) (Seo and Yokota, 2003). Genes involved in toxin synthesis can also be used for phylogenetic characterization of smaller subgroups of cyanobacteria, such as microcystin-producers (Hisbergues et al., 2003) and nitrogen fixing cyanobacteria (Dyble et al., 2002). The most reliable way to date to detect and quantify microcystin-producing genotypes is to use *mcy* genes as a target for molecular probes. Strains carrying *mcy* genes appear to be erratically distributed in phylogenic trees based on conventional phylogenetic markers (such as phycocyanin intergenic spacer

region and ribosomal RNA genes) (Neilan et al., 1995; Neilan et al., 1997; Tillett et al., 2001; Mikalsen et al., 2003). This is not always the case among toxic cyanobacteria, for example, phylogenetic analysis of *Anabaena circinalis*-specific 16S rRNA sequences demonstrated division of paralytic shellfish poison-producing strains and non-toxic strains into monophyletic groups (Beltran and Neilan, 2000).

A major advantage of using molecular techniques instead of microscopic methods is the ability to enhance the taxonomic resolution from genus-level to genotype-level, which is not possible through other methods (Ouellette and Wilhelm, 2003). Quantitative techniques provide a wide detection range (seven orders of magnitude for target gene copies, 5 orders of magnitude for cells) and high sensitivity (less than 10 target gene copies can be detected in a single reaction), which is essential in the analysis of natural microbial communities (Becker et al., 2002; Kurmayer and Kutzenberger, 2003). The sensitivity of molecular techniques can also be a source of variable results, thus successful implementation of these techniques requires knowledge of both the technique itself as well as the potential pitfalls.

Historical perspective on the ecosystem of Lake Erie

The North American Great Lakes contain approximately one fifth of the Earth's potable water supply, which makes them a vital resource on a global scale. Lake Erie is the twelfth largest lake in the world and the shallowest, warmest and most productive of the five Laurentian Great Lakes, and maintains a position of socioeconomic importance in the region (Makarewicz and Bertram, 1991; Fuller, 1995; Munawar et al., 2002). Natural geological division divides the lake into three basins, western, central and eastern, each of which has unique ecological characteristics (Makarewicz and Bertram, 1991). The Lake Erie drainage basin is the most populated compared to the other Great Lakes; according to the 1990 census slightly over 11.5 million people lived in the area, which is roughly a third of the total Great Lakes basin population (Fuller, 1995). Consequently, the Lake Erie ecosystem has suffered from a range of anthropogenic impacts including agricultural runoff, sewage discharge and industrial pollution (Makarewicz and Bertram, 1991; Munawar et al., 2002).

Lake-wide eutrophication caused by excessive phosphorus loading was a major problem in Lake Erie in 1950s and 1960s. In the 1970s strict limits for phosphorus discharge were set in order to improve the water quality in the entire lake (Fuller, 1995). By the mid 1980s phosphorus loading had been cut significantly and this change was considered to have had a positive impact on the overall water quality, indicated by decreased phytoplankton biomass for all three basins (Makarewicz and Bertram, 1991; Makarewicz, 1993; Conroy et al., 2005). Besides the major changes in nutrient inputs, colonization of Lake Erie by the zebra (*Dreissena polymorpha*) and quagga mussels (*Dreissena bugensis*) (collectively referred to as dreissenids) in late 1980s remarkably altered the phytoplankton abundance and speciation in the lake (Nicholls and Hopkins, 1993; Makarewicz et al., 1999; Munawar et al., 2002; Conroy et al., 2005). The selective filter-feeding and nutrient remineralization by the dreissenid mussels has been suggested to cause a shift in nutrient ratios more towards conditions that stimulate phytoplankton growth and to make conditions favorable to cyanobacterial growth (Vanderploeg et al., 2001; Raikow et al., 2004; Sarnelle et al., 2005).

Microcystis in Lake Erie

Microcystis has been observed in the western and central basins of the lake during spring and summer phytoplankton surveys between 1983 – 1987 (Makarewicz, 1993), but there is no cyanotoxin data available from this period to assess the toxin production potential of these *Microcystis* populations. In October of 1995 a remarkably dense bloom of *Microcystis* appeared in the western basin of the lake and during the bloom *Microcystis* cell abundance varied from 3×10^6 to 3×10^9 cells liter^{-1} and total microcystin concentration in water samples collected from the already declining bloom reached >1 μg liter^{-1} (Brittain et al., 2000). The appearance of *Microcystis* blooms has been raising interest toward finding an explanation for the appearance of toxic cyanobacteria in the western basin of the Lake (Brittain et al., 2000; Vanderploeg et al., 2001). Recently, the phytoplankton (especially picoplankton size class) growth on Lake Erie was confirmed to be limited by the availability of phosphorus (Wilhelm et al., 2003; DeBruyn et al., 2004)

and periodically also by iron (Twiss et al., 2005), however the effect of these conditions on the dynamics of *Microcystis* has not been investigated.

Since 1970s, the phytoplankton and water quality of Lake Erie has been surveyed on a routine basis by governmental agencies based in Canada and in the United States. In previous studies chlorophyll *a* concentration has been used as the proxy for the phytoplankton biomass, and the species diversity and abundance has been assessed through microscopy. Xu and Tabita (1996) applied molecular tools to assess the diversity of active fixing CO_2 fixing microbes in Lake Erie through studying the expression and sequence diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase gene. While their study revealed the presence of a diverse community of CO_2 fixing microbes in southern part of western basin of Lake Erie, no *Micorcystis* was detected at the time the study was conducted. A lake-wide study carried out on Lake Erie in 2002 provided information about prokaryotic and cyanobacterial diversity in surface waters and about the distribution of toxic *Microcystis* derived from 16S rRNA gene sequences from natural samples (Ouellette et al., 2006). The results indicated that potentially toxic genotypes had throughout the lake implying that the potential for toxigenic *Microcystis* blooms is not only limited to the western basin of the lake (Ouellette et al., 2006).

OBJECTIVES OF THIS STUDY

The goals of this study are to address a series of hypotheses which have been set to characterize aspects of the diversity and function cyanobacterial community in Lake Erie and to gain a better understanding of the dynamics of toxic cyanobacteria which are an integral part of the Lake Erie ecosystem. The hypotheses to be tested are:

I. The microcystin producing cyanobacterial population in Lake Erie is genetically diverse and the diversity of microcystin producing cyanobacterial genera in Lake Erie is reflected by the diversity of DNA sequence polymorphisms of the *mcyA* gene fragments.

II. Cyanobacterial genera other than *Microcystis* contribute to microcystin production in Lake Erie.

III. Reservoirs of *Microcystis* exist within Lake Erie and help to support *Microcystis* communities in Lake Erie.

IV. Environmental factors (specifically N, P, Fe, pH and water temperature) influence the abundance of cyanobacteria, total *Microcystis* and toxic *Microcystis* as well as microcystin production by *Microcystis*.

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APPENDIX I

Table 1. Factors affecting toxicity of *Microcystis* sp.

Parameter	*Microcystis strain(s) studied*	*Effect on microcystin production in Microcystis*	*Method used to determine toxicity*	*Reference*
Culture pH (range 6.5-10.5)	*M. aeruginosa* UV-006, UV-010	Highest toxicity at pH 6.5 and pH 10.5	Mouse assay (LD_{50})	Van der Westhuizen and Eloff, 1983
Free Fe^{2+}	*M. aeruginosa* CYA 228/1	Free Fe^{2+} ↓, toxicity ↓	Microcystin* to cellular protein ratio, mg microcystin g^{-1} dry weight)	Utkilen and Gjølme, 1995
$FeCl_3$ (3.5 μM – 0.1 μM)	*M. aeruginosa* CYA 228/1	Fe ↓, toxicity ↑	ng microcystin-RR* μg^{-1} chlorophyll ratio and microcystin-RR* μg^{-1} protein	Lyck et al., 1996
Fe (removal of Fe from BG11 medium	*M. aeruginosa* PCC 7806	Fe ↓, toxin yield ↑	mg microcystin-LR* g^{-1} dry weight	Lukac and Aegerter, 1993
Fe	*M. aeruginosa* PCC 7806	Free Fe^{2+} ↓, toxicity ↑	Electrophoretic mobility shift assay (EMSA), ng microcystin-LR* ng^{-1} protein	Martin-Luna et al., 2006b
Trace metals (Al, Cd, Cr, Cu, Fe, Mn, Ni, Sn, Zn)	*M. aeruginosa* PCC 7806	Zn ↑ toxin yield ↑ Al, Cd, Cr, Cu, Mn, Ni, Sn: no significant effect	mg microcystin-LR* g^{-1} dry weight	Lukac and Aegerter, 1993
Light intensity (range 7.53, 30.1, 75.3 μmol photons m^{-2} s^{-1})	*M. aeruginosa* M288	Highest toxicity at 30.1 μmol photons m^{-2} s^{-1}	Mouse assay (LD_{50})	Watanabe and Oishi, 1985

Table 1 continued.

Parameter	*Microcystis strain(s) studied*	*Effect on microcystin production in Microcystis*	*Method used to determine toxicity*	*Reference*
Light intensity (range 21-205 µmol photons m^{-2} s^{-1})	*M. aeruginosa* UV-006	Highest toxicity at 145 µmol photons m^{-2} s^{-1}	Mouse assay (LD_{50})	Van der Westhuizen and Eloff, 1985
Light intensity (range 5 – 50 µmol photons m^{-2} s^{-1})	*M. aeruginosa* 7813	No effect on toxicity	Mouse assay (LD_{50})	Codd and Poon, 1988
Light intensity (range 20-75 µmol photons m^{-2} s^{-1})	*M. aeruginosa* CYA228/1	highest toxicity at 40 µmol photons m^{-2} s^{-1}	mg microcystin* g^{-1} dry weight	Utkilen and Gjølme, 1992
Light intensity (range 16-68 µmol photons m^{-2} s^{-1})	*M. aeruginosa* PCC 7806	*mcyB* transcription upregulated in high light during early and middle growth phase. No effect on toxin content $cell^{-1}$.	PP2A inhibition assay, RT-PCR (detection of *mcyB* mRNA transcripts)	Kaebernick et al., 2000
Irradiance (range 2.4-73 µmol photons m^{-2} s^{-1})	*M. aeruginosa* HUB5-2-4	Highest toxin cell quota at 73 µmol photons m^{-2} s^{-1}.	fg total microcystin* $cell^{-1}$	Böttcher et al., 2001
Photosynthetically active radiation (PAR) (range 10-403 µmol photons m^{-2} s^{-1})	*M. aeruginosa* PCC 7806	PAR ↑, cellular microcystin content ↑ (highest toxicity at 126 photons m^{-2} s^{-1}) under PAR limited conditions. No change in dissolved toxin concentration during light-dark cycle.	fg microcystin-LR* $cell^{-1}$	Wiedner et al., 2003

Table 1 continued.

Parameter	*Microcystis strain(s) studied*	*Effect on microcystin production in Microcystis*	*Method used to determine toxicity*	*Reference*
Light-dark cycle	*M. aeruginosa* PCC 7806	Intracellular toxin content ↑ during light period. No change in dissolved toxin concentration during light-dark cycle.	Intracellular toxin content: fg microcystin-LR* $cell^{-1}$. Extracellular toxin content: μg L^{-1} (PPIA)	Wiedner et al., 2003
Light	*M. aeruginosa* NIVA-CYA 228/1	Toxicity increased in darkness	fg microcystin* $cell^{-1}$	Lyck, 2004
Light quality	*M. aeruginosa* PCC 7806	Red light: *mcyB* and *mcyD* transcription upregulated.	RT-PCR (detection of *mcyB* mRNA transcripts)	Kaebernick et al., 2000
Inhibition of PS (1 mM MV) and chemical stress (250 mM NaCl)	*M. aeruginosa* PCC 7806	*mcyB* transcription downregulated	RT-PCR (detection of *mcyB* mRNA transcripts)	Kaebernick et al., 2000
Temperature (+16-36°C)	*M. aeruginosa* UV-006	Highest toxicity at +20°C	Mouse assay (LD_{50})	Van der Westhuizen and Eloff, 1985
Temperature (+18, 25, 32°C)	*M. aeruginosa* M288	Temperature ↓, toxicity ↑ (highest toxicity at +18°C)	Mouse assay (LD_{50})	Watanabe and Oishi, 1985
Temperature (+15, 25, 34°C)	*M. aeruginosa* 7813	Highest toxicity at +25°C	Mouse assay (LD_{50})	Codd and Poon, 1988
P (undiluted MA medium, 10 and 20 fold dilutions)	*M. aeruginosa* M288	P ↑, toxicity ↑	Mouse assay (LD_{50})	Watanabe and Oishi, 1985

Table 1 continued.

Parameter	*Microcystis strain(s) studied*	*Effect on microcystin production in Microcystis*	*Method used to determine toxicity*	*Reference*
P (14.4, 143.5 μM K_2HPO_4)	*M. aeruginosa* CYA228/1	P ↑, toxin content per dry weight ↑, toxin content to protein content ↓	ng microcystin-RR* μg^{-1} dry weight, ng microcystin-RR* ng^{-1} protein	Utkilen and Gjølme, 1995
Phosphorus limitation	*M. aeruginosa* UTEX 2388	P ↓, toxicity ↑	μg total microcystin* g^{-1} dry weight	Oh et al., 2000
P	*M. aeruginosa* UTEX 2388	N constant, total P ↓, toxicity ↑	μg microcystin* g^{-1} dry weight	Lee et al., 2000
Nitrogen (undiluted MA medium, 10 and 20 fold dilutions)	*M. aeruginosa* M288	N ↑, toxicity ↑	Mouse assay (LD_{50})	Watanabe and Oishi, 1985
N (0.35-5.8 mM $NaNO_3$)	*M. aeruginosa* CYA228/1	N ↑, toxin content per dry weight ↑, no effect on toxin content to protein content	ng microcystin-RR* μg^{-1} dry weight, ng microcystin-RR* ng^{-1} protein	Utkilen and Gjølme, 1995
N	*M. aeruginosa* UTEX 2388	P constant, N ↑, toxicity ↑. Highest toxicity at N:P ratios of 16:1 and 50:1.	μg microcystin* g^{-1} dry weight	Lee et al., 2000
N and P	M. aeruginosa 205 M. aeruginosa GL260735	Highest intracellular microcystin content: strain 205: 458<N:P<664. Strain GL260735: 237<N:P<735.	μg microcystin* mL^{-1}	Vezie et al., 2002

Table 1 continued.

Parameter	*Microcystis strain(s) studied*	*Effect on microcystin production in Microcystis*	*Method used to determine toxicity*	*Reference*
Removal of inorganic N, P and C from BG11 medium	*M. aeruginosa* 7813	- PO_4: no effect on toxicity - NO_3: toxicity ↓ - CO_2: toxicity ↓	Mouse assay (LD_{50})	Codd and Poon, 1988
Growth phase	*M. aeruginosa* UTEX 2388	Maximum toxicity at exponential growth phase	µg total microcystin* g^{-1} dry weight	Lee et al., 2000
Growth phase	*M. aeruginosa* UV-006, UV-010	Maximum toxicity at late log phase	Mouse assay (LD_{50})	Van der Westhuizen and Eloff, 1983
Cell division rate	*M. aeruginosa* MASH10	Microcystin production rate is equal to cell division rate	mg microcystin* g^{-1} dry weight, fg microcystin* $cell^{-1}$, µg microcystin* mL^{-1} culture	Orr and Jones, 1998
Cell cycle	*M. viridis* NIES102	Microcystin production is regulated by cell cycle rather than culture conditions	fg total microcystin* $cell^{-1}$	Kameyama et al., 2004
Culture doubling time (t_D)	*M. aeruginosa* UV-006	Doubling time ↓, toxicity ↑	Mouse assay (LD_{50})	Van der Westhuizen and Eloff, 1985
Cell division rate	*M. aeruginosa* NIVA-CYA 228/1	Inverse relationship between cell division rate and cell quota for microcystin	fg microcystin* $cell^{-1}$	Lyck, 2004

*)Microcystins analyzed using HPLC

Table 2. Oligonucleotide probes used in studies of potentially toxic cyanobacteria.

Target Group/Genus	*Target gene*	*Name of the Primer and Sequence (5'- 3')*	*Reference*
Cyanobacteria	PC[a] operon	PCβF: GGCTGCTTGTTTACGCGACA PCαR: CCAGTACCACCAGCAACTAA	Neilan et al., 1995
	16S-23S rRNA ITS[c]	16CITS: TGTAAAACGACGGCCAGTCCATGGAAG(C/T)TGGTCA(C/T)G 23CITS: CCTCTGTGTGCCTAGGTATCC	Neilan et al., 1997
	16S rRNA	PLG1.1: ACGGGTGAGTAACGCGTRA PLG2.1: CTTATGCAGGCGAGTTGCAGC	Urbach et al., 1992
	16S rRNA	CYA106F: CGGACGGGTGAGTAACGCGTGA CYA359F: GGGGAATYTTCCGCAATGGG CYA781R: GACTAC(T/A)GGGGTATCTAATCCC(A/T)TT	Nübel et al., 1997
	16S rRNA	CC: TGTAAAACGACGGCCAGTCCAGACTCCTACGGGAGGCAGC CD: CGCGTTAGCATCGGCACGGCTCGG	Rudi et al., 1997
	cpcBA IGS[b]	CPC1F: GGCKGCYTGYYTRCGYGACATGGA CPC1R: GCHGATWCYCAAGGNCGYTT	Kim et al., 2006
Microcystis, Anabaena, Planktothrix, Nostoc	*mcyA*	mcyA-Cd 1F: AAAATTAAAAGCCGTATCAAA mcyA-Cd 1R: AAAAGTGTTTTATTAGCGGCTCAT	Hisbergues et al., 2003
Microcystis, Anabaena, Planktothrix, Nodularia, Nostoc	*mcyD*	mcyDF: GATCCGATTGAATTAGAAAG mcyDR: GTATTCCCCAAGATTGCC	Rantala et al., 2004
	mcyE	mcyE-F2: GAAATTTGTGTAGAAGGTGC mcyE-R4: AATTCTAAAGCCCAAAGACG	Rantala et al., 2004

Table 2 continued.

Target Group/Genus	*Target gene*	*Name of the Primer and Sequence (5'- 3')*	*Reference*
Microcystis, Anabaena, Planktothrix, Nodularia, Nostoc, Phormidium	Amino transferase domain in *mcyE* and *ndaF*	HEPF: TTTGGGGTTAACTTTTTTGGGCATAGTC HEPR: AATTCTTGAGGCTGTAAATCGGGTTT	Jungblut and Neilan, 2006
Microcystis	16S rRNA	209F: ATGTGCCGCGAGGTGAAACCTAAT 409R: TTACAA(C/T)CCAA(G/A)(G/A)CCTTCCTCCC	Neilan et al., 1997
	16S rRNA	16S F1: CGCAATGGGCGAAAGCCTGACGGAGC 16S R1: GCGTGCGTACTCCCCAGGCGGGATAC	Nonneman and Zimba, 2002
	16S rRNA	CH: AGCCAAGTCTGCCGTCAAATCA CI: ACCGCTACACTGGGAATTCCTG	Rudi et al., 1997
	PC[a] operon intergenic spacer region	PcMafwd: GGTCTGCGCGAAACCTATGT PcMarev: GGTCAACACTTTAGCGGCG	Kurmayer et al., 2003
	mcyA NMT[d]	MSF: ATCCAGCAGTTGAGCAAGC MSR: TGCAGATAACTCCGCAGTTG	Tillett et al., 2001
	mcyB	MCY F1: TGGGAAGATGTTCTTCAGGTATCCAA MCY R1: AGAGTGGAAACAATATGATAAGCTAC	Nonneman and Zimba, 2002
	mcyB	McyBMafwd1: AATCAACGGTTAGTTGCTTATGT Tox4r: CACTAACCCCTATTTTGGATACC	Kurmayer et al., 2003
	mcyC	PSCF1: GCAACATCCCAAGAGCAAAG PSCR1: CCGACAACATCACAAAGGC	Ouahid et al., 2005
	mcyD	PKDF1: GACGCTCAAATGATGAAAC PKDR1: GCAACCGATAAAAACTCCC PKDF2: AGTTATTCTCCTCAAGCC PKDR2: CATTCGTTCCACTAAATCC	Ouahid et al., 2005

Table 2 continued.

Target Group/Genus	*Target gene*	*Name of the Primer and Sequence (5'- 3')*	*Reference*
Microcystis	*mcyE*	PKEF1: CGCAAACCCGATTTACAG PKER1: CCCCTACCATCTTCATCTTC	Ouahid et al., 2005
	mcyG	PKGF1: ACTCTCAAGTTATCCTCCCTC PKGR1: AATCGCTAAAACGCCACC	Ouahid et al., 2005
	mcyE	mcyE-F2: (Rantala et al., 2004) MicmcyE-R8: CAATGGGAGCATAACGAG	Vaitomaa et al., 2003
	mcyA	MISYf: CGACCGAGGAATTTCAAGCT MISYr: AGTATCCGACCAAGTTACCCAAAC TaqMan probe MISYTM: TTAAATCGGAAATTATCCCA-GAAAATGCCGT	Foulds et al., 2002
	mcyB	MIf: GCAGCGAACTCTTGAAGGGTTTATG MIr: GCGGATTCTGTGCAGCTTGTTCTTC	Foulds et al., 2002
	PC[a] operon	118F: GCTACTTCGACCGCGCC 245R: TCCTACGGTTTAATTGAGACTAGCC TaqMan probe: CCGCTGCTGTCGCCTAGTCCCTG	Kurmayer and Kutzenberger, 2003
	mcyB	30F: CCTACCGAGCGCTTGGG 108R: GAAAATCCCCTAAAGATTCCTGAGT TaqMan probe: CACCAAAGAAACACCCGAATCTGAGA-GG	Kurmayer and Kutzenberger, 2003
	mcyA-B	135-F: GACTTATAGCCATCTCATCT 676-R: TTGACGCTCTGTTTGTAA	Mikalsen et al., 2003
	mcyB	2156-F: ATCACTTCAATCTAACGACT 3111-R: AGTTGCTGCTGTAAGAAA	Mikalsen et al., 2003
Anabaena	*A. circinalis rpoC1*	Ana2: GATAGCATCCTCAATTTCTAGCCATTGG Ana4: CTCTGAAGCCAGAAATGGACGGC	Fergusson and Saint, 2003

Table 2 continued.

Target Group/Genus	*Target gene*	*Name of the Primer and Sequence (5'- 3')*	*Reference*
Anabaena	*mcyE*	mcyE-F2: (Rantala et al., 2004) AnamcyE-12R: CAATCTCGGTATAGCGGC	Vaitomaa et al., 2003
	A. circinalis 16S	27F1: AGAGTTTGATCCTGGCTCAG (Neilan et al., 1997) AC510R: CAATGCCACCTACGGACT	Beltran and Neilan, 2000
	Non-toxic *A. circinalis* 16S	ACB2F: AGGCTTCCTGCCCTGGG AC510R: CAATGCCACCTACGGACT	Beltran and Neilan, 2000
	Toxic *A. circinalis* 16S	ACB1F: GCTAGTTGGTGGTGTAAGA AC510R: CAATGCCACCTACGGACT	Beltran and Neilan, 2000
Nodularia	16S rDNA	NS2: GGCGAAGGCGCTCTACTA 1494R: (Neilan et al 1997)	Moffitt et al., 2001
	Toxic *Nodualria* spp. 16S rDNA	NTS: TGTGATGCAAATCTCA(C/A)A 1494R: (Neilan et al 1997)	Moffitt et al., 2001
Cylindrospermopsis	RNA polymerase γ subunit (*rpoC1*)	Cyl2: GGCATTCCTAGTTATATTGCCATACTA Cyl4: GCCCGTTTTTGTCCCTTTCGTGC	Wilson et al., 2000
	polyketide synthase (pks)	M4: GAAGCTCTGGAATCCGGTAA M5: AATCCTTACGGGATCCGGTGC	Schembri et al., 2001
	peptide synthetase (ps)	M13: GGCAAATTGTGATAGCCACGAGC M14: GATGGAACATCGCTCACTGGTG	Schembri et al., 2001
	polyketide synthase (pks)	K18: CCTCGCACATAGCCATTTGC M4: (Schembri et al. 2001)	Fergusson and Saint, 2003
	dinitrogenase reductase (*nifH*)	cylnif F: TAARGCTCAAACTACCGTAT cylnif R: ATTTAGACTTCGTTTCCTAC	Dyble et al., 2002
	PC[a] (*cpcBA*-IGS)	Cylcpc F: GGCTTACGCGAAACCTATATA PCαR: (Neilan et al. 1995)	Dyble et al., 2002

Table 2 continued.

Target Group/Genus	*Target gene*	*Name of the Primer and Sequence (5'- 3')*	*Reference*
Planktothrix	16s rRNA	CN: GGAAGGTTCTTGGATTGTCAACCC CO: TGCCTTTGCGAGGTTAAGCCT	Rudi et al., 1997
	mcyE	mcyE-F2: (Rantala et al., 2004) mcyE-plaR3: CTCAATCTGAGGATAACGAT	Rantala et al., 2006
	mcyA	mcyA.fw: ATGTCACCTATTGGGCTTGC mcyA.rev: TCGATTCCCCTAAGTGATGC	Mbedi et al., 2005
	mcyB	mcyB.fw: ATTACAGCAGAGAAAATCCAAGCA mcyB.rev: TCGCAATAGCGGGATCA	Mbedi et al., 2005
	mcyCJ	mcyCJ.fw: TTGGATACAAGCGACAAAAGG mcyCJ.rev: TCTCCAGCTTGAAGTTCTGC	(Mbedi et al., 2005)
	mcyE	mcyE.fw: TTACCTAATTATCCCTTTCAAAG mcyE.rev: CAATGGGTAAGGTTTGCTT	(Mbedi et al., 2005)
	mcyEG	mcyEG.fw: GAATTCATTTTTGTTGAGGAAGG mcyEG.rev: AGAAAACAAGCCCAGAGTGC	(Mbedi et al., 2005)
	mcyHA	mcyHA.fw: TTAGATGAAGCCACCAGTGC mcyHA.rev: GATTAAAAATTGAATAGCTGCTAGG	(Mbedi et al., 2005)
	mcyT	mcyT.fw: CCCAATCTAACCCCAACTGC mcyT.rev: CAATAGCGATTTTCCCAAGC	(Mbedi et al., 2005)
	mcyTD	mcyTD.fw: ATCCGCCCATACTGTGACC mcyTD.rev: GATTTTGCCCGGTTTACTCC	(Mbedi et al., 2005)
Nostoc	16s rRNA	CP: GGTCCGCAGGTGGCATTGT CQ: GCCCCGACCATACTCGATTTCTGT	(Rudi et al., 1997)

Part II

QUANTIFICATION OF TOXIC *MICROCYSTIS* SPP. DURING THE 2003 AND 2004 BLOOMS IN WESTERN LAKE ERIE USING QUANTITATIVE REAL-TIME PCR.

This part is a version of a paper published with same title in journal Environmental Science and Technology in 2005 by Johanna M. Rinta-Kanto, Anthony J.A. Ouellette, Gregory L. Boyer, Michael R. Twiss, Thomas B. Bridgeman, Steven W. Wilhelm.

Rinta-Kanto, J.M., Boyer, G.L., Twiss, M.R., Bridgeman, T., Wilhelm, S.W. 2005. Quantification of *Microcystis* spp. during the 2003 and 2004 blooms in Western Lake Erie using quantitative real-time PCR. Environmental Science and Technology, 39: 4198-4205.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions in this paper were 1) Assisting in the development of the real-time PCR method and validating the use of genomic DNA standards for quantifying toxic Microcystis cells, 2) collecting most of the environmental samples for this study, 3) analyzing the samples for the study and analyzing the data 4) gathering the background literature and, 5) Most of the writing of the manuscript and correspondence to the reviewer comments.

Introduction

Recurring blooms of toxic cyanobacteria in the western basin of Lake Erie have been a nuisance during the past decade. *Microcystis* spp. have been commonly found in samples collected from the bloom areas during summer since 1996 (Brittain et al., 2000). *Microcystis* blooms have often been associated with varying concentrations of the toxin microcystin measured in the surrounding water. It is common for microcystin concentrations in the western basin of Lake Erie to exceed the provisional guideline concentration of 1.0 μg L^{-1} set by the World Health Organization (Chorus and Bartram, 1999). Our goal in this study was to utilize quantitative PCR (qPCR) for a single step detection and quantification of target genes for assessment of the total abundance of cyanobacteria, *Microcystis* and potentially toxic *Microcystis* in natural water samples. Use of this method improves the resolution at which mixed natural populations consisting of toxigenic and nontoxic *Microcystis* can be analyzed. Use of qPCR also expedites the analysis of bloom samples, reducing the number of time consuming steps involved in the analysis of bloom samples using microscopy and chemical toxin analysis.

Microcystins are nonribosomally synthesized cyclic heptapeptides produced by *Microcystis* spp., as well as other species of cyanobacteria belonging to genera *Anabaena*, *Nostoc*, and *Oscillatoria* (Sivonen and Jones, 1999; Nishizawa et al., 2000). Microcystins are among the most wide spread cyanobacterial toxins to be found in lakes and in brackish water world wide (Sivonen and Jones, 1999). Blooms of toxin producing *Microcystis* cause severe aesthetic water quality problems (Jacoby et al., 2000) and pose a health risk to humans and animals upon ingestion of contaminated water (Codd, 1995; Murphy et al., 2000; Nonneman and Zimba, 2002). Trophic transfer of microcystins in the food web (from phytoplankton to planktivorous fish) has also been hypothesized (Brittain et al., 2000; Murphy et al., 2000). Cyanobacterial blooms may be influenced by numerous factors, such as lake morphometry, nutrient availability, and environmental factors affecting population size and dispersal (Sivonen and Jones, 1999; Jacoby et al., 2000). In field studies, the production of microcystin has been shown to be correlated to

concentrations of carbon, dissolved phosphorous and nitrogen, pH, chlorophyll *a*, and solar radiation (Vezie et al., 1998). However, the combination of environmental conditions responsible for inducing a toxin producing bloom remains unknown not only in the Lake Erie ecosystem, but also in other freshwater systems where toxic *Microcystis* blooms occur.

In the past, detection of *Microcystis* in water samples has commonly been based on microscopic techniques combined with the chemical detection of microcystin in the water samples (e.g. Brittain et al., 2000). However, discerning amongst cyanobacterial strains solely based on cellular morphology is difficult, or even impossible (Baker et al., 2002; Ouellette and Wilhelm, 2003). The PCR-based techniques allow for detection of specific DNA sequences, from which a distinction can be made between toxic and nontoxic strains of *Microcystis* spp. (Nishizawa et al., 2000). This approach subsequently facilitates an analysis of the distribution of genotypes based on the presence or absence of a combination of target genes in the samples (Ouellette and Wilhelm, 2003). In the current study an initial screening of samples was completed by multiplex PCR using a combination of previously published primer sets (Ouellette and Wilhelm, 2003). A quantitative real-time PCR technique, the Taq-nuclease assay (TNA) (also known as 5' nuclease assay) was used to quantify cells carrying specific target genes in our samples. The Taq-nuclease assay has been recently applied successfully to the analysis of microbial components of natural water samples (Becker et al., 2000; Bach et al., 2002; Becker et al., 2002; Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003).

In the initial screening, the distribution of cyanobacteria was studied using a cyanobacterial-specific 16S ribosomal RNA gene sequence as a target. At the same time, occurrence of toxigenic *Microcystis* spp. was studied through the detection of a *Microcystis* specific 16S rRNA gene sequence and toxin synthetase genes *mcy*B and *mcy*D in a multiplex PCR assay (Ouellette and Wilhelm, 2003). Subsequently, Taq-nuclease assays were employed for the quantification of cyanobacterial and *Microcystis* 16S rDNA and *mcy*D gene copies and the total abundance of cells carrying these target genes in all samples.

In this study we report observations concurrent with two toxic cyanobacterial blooms that occurred in the Western Basin of Lake Erie in late summer of 2003 and late summer 2004. The greenish biomass of a large algal bloom in summer 2003 at the mouth of Maumee River was visible in true-color LANDSAT images of this area (Figure 1). Whether these intense cyanobacterial blooms that developed in recent years are products of an indigenous *Microcystis* population or if *Microcystis* cells are transported into the system from outside sources is not clear. The aim of this study was to provide further information about the spatial distribution, abundance and possible origin of *Microcystis* in the western basin of Lake Erie. The results of this study provide more detailed information about the structure of the cyanobacterial community in Lake Erie than previous studies, while also raising questions about the presence of other microcystin-producing cyanobacteria in the western basin of Lake Erie.

Materials and Methods

Sample collection

Due to logistical constraints, water samples were collected by three independent groups who were concurrently working on Lake Erie at different locations in the western basin of the Lake on August 15th, 2003. Researchers on the R/V *Lake Guardian*, C.C.G.S. *Limnos* and the research support craft of the Lake Erie Center (Toledo, OH) collected samples and observations. In addition, samples were collected during field work in August 2004 onboard the C.C.G.S. *Limnos*. All sampling sites in 2003 and 2004 are indicated in the map in Figure 2. In all cases, water samples were collected from 1 m depth using the ship's surface water pump (C.C.G.S. *Limnos*) or Niskin bottles (R/V *Lake Guardian*). Cells used to extract DNA for PCR analysis were collected by filtering onto

Figure 1. LANDSAT 7 image taken August 18, 2003. The true color composite image demonstrates the presence of a significant bloom of phytoplankton in the surface waters (image from OhioLink LANDSAT 7 server).

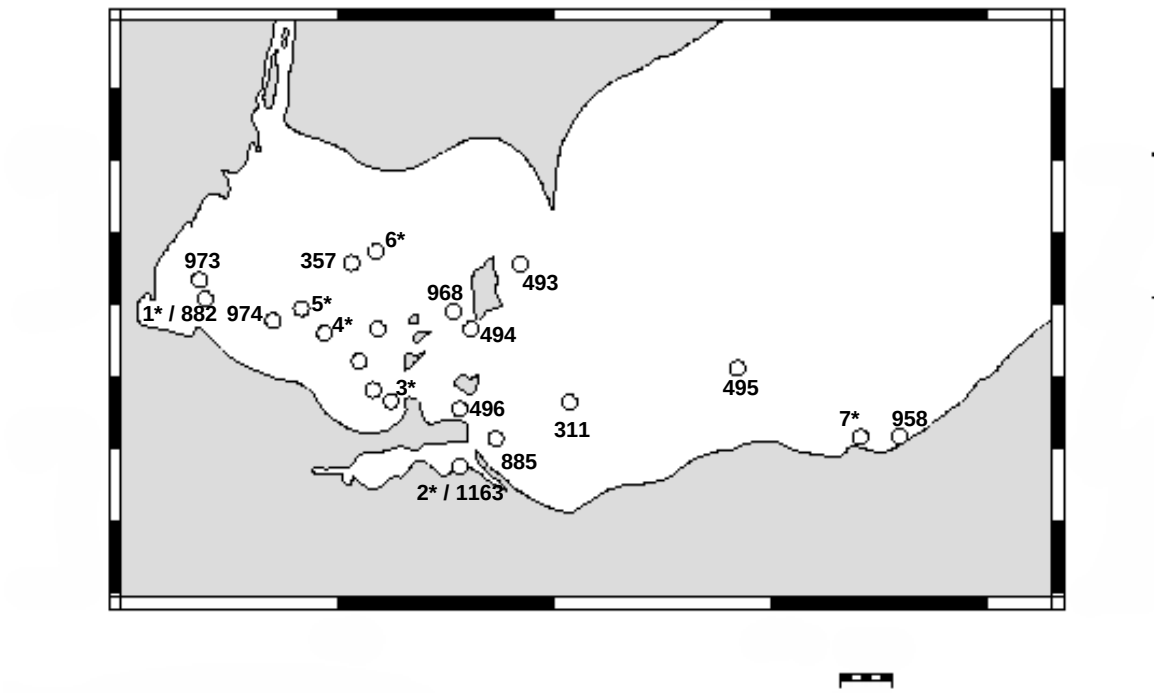

Figure 2. Sampling sites in 2003 and 2004. Sampling sites visited in 2003 are indicated with a number 1-7 and an asterisk. Stations were renumbered consecutively because different groups collecting samples used different coding system for the sampling sites. The sampling sites used in August 2004 are numbered according to Environment Canada station coding system consisting of 3 and 4 digit station numbers as indicated on the map.

47 mm diameter, 0.22-μm nominal pore-size polycarbonate membrane filters (Millipore), which were immediately frozen (-20°C) until processing.

Phytoplankton biomass

Chlorophyll *a* (a proxy for phytoplankton biomass) in water samples was determined using the non-acidification protocol (Welschmeyer, 1994). Samples were collected on 0.2-μm nominal pore-size polycarbonate filters (47-mm diam., Millipore) and Chlorophyll-*a* retained on the filters was extracted (*ca.* 24 h, 4 °C) in 90 % acetone. Chlorophyll *a* concentrations were quantified with either an AU-10 or TD-700 fluorometer (Turner Designs; Sunnyvale, CA).

Microcystin concentration

Microcystin in water samples was determined with protein phosphatase inhibition assays. Samples were collected on GF/F (Whatman) filters. The assays were run in 96-well plates containing 0.1 mU enzyme (recombinant protein phosphatase 1A, catalytic subunit, Roche Applied Science), 1.05 mg para-nitrophenyl phosphate (Sigma) and 10 μl of sample or microcystin-LR (Sigma Biochemical) using the method of Carmichael and An (Carmichael and An, 1999). The rate of phosphate hydrolysis was calculated from the change in absorbance at 405 nm over 1 hr and compared to the control (no added microcystin-LR) and standards containing between 6 and 40 μg L^{-1} of microcystin-LR. Blanks (no enzyme, no toxin), unknowns, standards, and controls were all run in duplicate.

Extraction of DNA from natural samples

High molecular weight nucleic acids were isolated using a modification of the protocol of Giovannoni et al. (Giovannoni et al., 1990). Cells collected onto filters were suspended from the filter in lysis buffer (40mM EDTA, 400 mM NaCl, 50mM Tris-hydrochloride, pH 9). Cells were lysed by adding lysozyme to a final concentration of 1 mg mL^{-1} followed by incubation at 37ºC for 20 min. After incubation, proteinase K was added to a final concentration of 50 μg mL^{-1} and sodium dodecyl sulfate to a final concentration of

0.5%. The cell suspension was then incubated at 50ºC for 2 h. DNA was extracted by first adding a phenol:chloroform:isoamyl alcohol (25:24:1) volume equal to the aqueous phase, with a subsequent extraction of the aqueous phase using an equal volume of chloroform isoamyl alcohol (24:1). DNA was precipitated overnight (-20 ºC) after the addition of absolute ethanol (2 × aqueous volume) and 10 M ammonium acetate (0.1 × the volume of the aqueous phase). DNA was collected the next day by centrifugation at 11,900 × g for 25 min (Beckman J2-21 centrifuge equipped with Fiber Lite™ F21B rotor, Piramoon Technologies, Santa Clara, CA, USA). DNA pellets were air-dried and subsequently resuspended in sterile 1 × TE buffer, pH 8. Concentration and purity of extracted DNA was measured spectrophotometrically (BioMate5, Thermo Spectronics) as previously described (Sambrook and Russell, 2001).

Multiplex PCR

Initial sample screening was carried out using a combination of the four primer sets described by Ouellette and Wilhelm (Ouellette and Wilhelm, 2003). All reactions were performed in 50 µL volumes in 96-well plates (Eppendorf). For each sample, two separate PCR reactions were set up; one reaction to detect cyanobacteria using primers CYAN 108F and 16S CYR (Table 1) and a second multiplex reaction to detect *Microcystis* specific 16S rDNA fragment and the microcystin toxin synthetase genes *mcy*B and *mcy*D using three primer sets: MICR 185F and MICR 431R, *mcy*B 2959F and *mcy*B 3278R, and, *mcy*D F2 and *mcy*D R2 (Table 1). All reactions contained 400 nM of each primer, 200 nM of dNTPs, 1×Mg-free PCR buffer (Promega, Madison, WI, USA), 2 mM $MgCl_2$, 300 ng μL^{-1} (final concentration) bovine serum albumin (Sigma cat # A-7030) (Kirchman et al., 2001), 0.04 U μL^{-1} (final concentration) Taq polymerase (Promega, Madison, WI, USA), and 20-200 ng DNA template. Bovine serum albumin was added into the reactions because it has been shown to enhance the sensitivity of the PCR-based detection of target genes in natural samples (Kirchman et al., 2001). The PCR protocol consisted of an initial denaturation step at 95°C for 5 min, 50 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 30 s, and a final single step of 72°C for 15 min. Each PCR reaction was subjected to electrophoresis in 6% polyacrylamide gels. DNA bands were

Table 1. PCR primer sets and Taq nuclease assay probes (Taq) used in this study.

Primer	Sequence (5'- 3')	Reference
*mcy*B 2959F	TGGGAAGATGTTCTTCAGGTATCCAA	(Nonneman and Zimba, 2002)
*mcy*B 3278R	AGAGTGGAAACAATATGATAAGCTAC	(Nonneman and Zimba, 2002)
*mcy*D F2	GGTTCGCCTGGTCAAAGTAA	(Kaebernick et al., 2000)
*mcy*D R2	CCTCGCTAAAGAAGGGTTGA	(Kaebernick et al., 2000)
*mcy*D F2 (Taq)	FAM[a] –ATGCTCTAATGCAGCAACGGCAAA-BHQ-1[b]	This study
MICR 184F	GCCGCRAGGTGAAAMCTAA	(Neilan et al., 1997)
MICR 431R	AATCCAAARACCTTCCTCCC	(Neilan et al., 1997)
MICR 228F (Taq)	FAM[a] –AAGAGCTTGCGTCTGATTAGCTAGT- BHQ-1[b]	This study
CYAN108F (PLG1.3)[c]	ACGGGTGAGTAACRCGTRA	(Urbach et al., 1992)
16SCYR[a]	CTTCAYGYAGGCGAGTTGCAGC	modified from Urbach et al. (1992)
CYAN 108F (PLG1.3)[d]	ACGGGTGAGTAACRCGTRA	(Urbach et al., 1992)
CYAN 377R[b]	CCATGGCGGAAAATTCCCC	(Nübel et al., 1997)
CYAN 328R (Taq)	FAM[a] –CTCAGTCCCAGTGTGGCTGNTC- BHQ-1[b]	This study

[a]6-carboxyfluorescein

[b]Black Hole Quencher-1™ (quenching range 480-580 nm) (Biosearch Technologies, Inc., Novato, CA, USA)

[c]CYAN 108F and 16S CYR are used in conventional PCR assay

[d]CYAN 108F and CYAN 377R are used in Taq-nuclease assay

visualized under UV illumination after staining the gel with 0.01% SYBR green I (Molecular Probes, Eugene, OR, USA) in TBE (90 mM Tris-borate, 1mM EDTA, pH 8.0).

Real time quantitative PCR (qPCR)

To provide quantitative information on cyanobacterial, specifically *Microcystis*, populations in Lake Erie, all samples were subjected to qPCR analysis to quantify copy numbers of cyanobacteria-specific 16S rDNA, *Microcystis*-specific 16S rDNA and *mcy*D genes in water samples, as well as the abundance of cells carrying these target genes in the original samples.

Dual labeled probes CYAN 328R, MICR 228F, mcyDF2 (Table 1) were designed to accompany each primer set in qPCR. Briefly, the probes were designed according to guidelines from Applied Biosystems (http://home.appliedbiosystems.com [Applied Biosystems, Foster City, CA]) and from Bustin et al., 2000 (Bustin, 2000). To confirm that probes will not form secondary structures, the probe sequences were checked using the mfold web server (Zuker, 2003). The functionality and sensitivity of the probe was confirmed by assaying different pure cyanobacterial cultures in the laboratory prior to analysis of natural samples.

Amplifications and quantifications were performed using the BioRad iCycler equipped with the iQ real time fluorescence detection system and software, version 3.0 (Bio-Rad, Hercules, CA, USA). Triplicate Taq-nuclease assays were performed to quantify the gene copies for each sample. All reactions were carried out in a total volume of 25 μL. Three separate assays were performed to detect and quantify cyanobacterial 16S rDNA, *Microcystis* 16S rDNA and *mcy*D in the samples. For cyanobacterial 16S and *Microcystis* 16S assays, each PCR reaction contained 10 μL of Eppendorf HotMasterMix (Brinkmann Instruments, Inc., Westbury, NY, USA). For *mcy*D assays, each PCR reaction contained 12.5 μL of Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). In addition, all 3 assays contained 10 μM of each primer (Sigma-Genosys, Inc., The Woodlands, TX, USA), 10 μM of Taq-probe (Biosearch Technologies, Inc., Novato, CA, USA), 300 ng μL^{-1} bovine serum albumin (Sigma cat # A-7030) (Kirchman et al., 2001)

and 5 µl of undiluted or ten fold diluted template DNA suspension. Each PCR reaction was run in triplicate on a 96-well plate (Bio-Rad, Hercules, CA, USA), sealed with optical quality sealing tape (Bio-Rad, Hercules, CA, USA). Two negative controls without DNA were included for each PCR run. The PCR program for cyanobacterial 16S rDNA and *Microcystis* specific 16S rDNA primers consisted of 1.5 min at 95°C, 55 cycles at 95°C for 30 s, 56°C for 1 min, and 65°C for 20 s. The PCR program for *mcy*D assay consisted of 3 min at 50°C, 10 min at 95°C followed by 45 cycles of 30 s at 95°C, 1 min at 61°C and 20 s at 72°C. High cycle numbers were required so that the most dilute standards (discussed below), as well as samples with low concentration of target DNA, could be quantified.

Threshold cycle (C_t) calculations were completed automatically for each real time PCR assay by the iCycler software using the maximum correlation coefficient approach. In this approach the threshold is automatically determined to obtain the highest possible correlation coefficient (r^2) for the standard curve (Table 2). Gene copies per sample were calculated using a standard curve (target gene copy number vs. C_t) determined for each assay. Cell abundance was inferred from a standard curve (cell abundance vs. C_t) determined in each assay. Real-time PCR data was analyzed using BioRad iCycler iQ™ real time detection system software version 3.0.

Standards for real-time PCR: Preparation of single copy plasmid standard

A cyanobacterial 16S rDNA fragment was amplified by PCR using primers CYAN 108F and 16S CYR (Table 1) from *Microcystis aeruginosa* LE-3 (Brittain et al., 2000) as described above. The DNA fragment was cloned into PCR 2.1 vector using a TOPO-TA

Table 2. Efficiencies and standard curve parameters of from real-time PCR analysis for the cyanobacterial, *Microcystis* and *mcyD* specific primer sets.

Target gene	Standard	Efficiency (%)	Slope	y-intercept	r^2
Cyan 16S	Plasmid DNA	92.1	-3.526	45.877	0.999
Cyan 16S	LE-3 genomic DNA	95.4	-3.437	37.628	0.997
Micr 16S	Plasmid DNA	97.9	-3.373	42.815	0.997
Micr 16S	LE-3 genomic DNA	104.7	-3.213	35.037	0.999
*mcy*D	Plasmid DNA	98.5	-3.359	41.138	0.997
*mcy*D	LE-3 genomic DNA	94.2	-3.470	36.864	0.998

cloning kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Plasmid DNA was purified using a Wizard Plus Minipreps kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Inserts in the clones were confirmed by PCR using primers CYAN 108F and 16S CYR and subsequent electrophoresis. The DNA concentration (ABS 260 nm) and purity (ABS 260 nm / ABS 280 nm) of the plasmid preparation was determined spectrophotometrically (BioMate5, Thermo Spectronics), as previously described (Sambrook and Russell, 2001). The molecular weight of the double stranded plasmid and the double stranded PCR product was determined using Biopolymer Calculator, version 4.4.1 (http://paris.chem.yale.edu/extinct.html). Using Avogadro's number, 6.022 × 1023 plasmid copies mol-1, the plasmid copy number of the stock was determined. A plasmid standard for mcyD-assays was prepared as described above, but the PCR amplicon was obtained using mcyDF2 and mcyDR2 primers (Table 1). Dilutions containing 1× 106 - 5 plasmid copies μL-1 were prepared to establish a linear standard curve for real-time PCR assays.

Microcystis aeruginosa LE-3 genomic DNA standard

Microcystis aeruginosa LE-3 (Brittain et al., 2000) were grown in batch cultures in BG11 medium (Kerry et al., 1988) at +25°C and *ca* 80 μmol photons m^{-2} s^{-1}. Cells from a known volume of LE-3 culture were harvested onto a GF/F filter (Whatman), lysed and DNA isolated as described above. A subsample (2 mL) of the cell culture was obtained immediately before the cells were harvested to determine the cell density of the culture by direct microscopic count. For microscopic enumeration of the *M. aeruginosa* LE-3 cultures, cells were harvested on a 0.22-μm nominal pore-size black polycarbonate membrane filter (Poretics). The filter was mounted on a glass slide (Fisher Scientific), then a drop of immersion oil (Type FF) (R.G. Chargille Laboratories, Inc., Cedar Grove, N.J. USA) was added on top of the filter and covered with a glass cover slip. Autofluorescent cyanobacterial cells were enumerated under 1000 x magnification with a *Leica DMRXA* epifluorescence microscope (Excitation filter λ = 530 – 595 nm; Dichroic mirror λ = 600 nm; Barrier filter λ = 615 nm) equipped with an ocular grid. Twenty fields or 200 cells were counted from each sample. The cell abundance of the original cell

culture was related to the total yield of extracted genomic DNA by dividing the total DNA yield (ng DNA) by the total number of cells contained in the original volume of liquid culture. This gave us a way to relate how much DNA corresponded to one cell in the *Microcystis* LE-3 sample. We used 100 fold dilutions of the DNA sample to establish the genomic DNA standard curve (see discussion below).

Quantitative PCR - Detection limits

Standard curves were established using four serial dilutions of standard plasmid DNA and genomic DNA isolated from *M. aeruginosa* LE-3 pure culture. For all real-time PCR assays the dilutions of the plasmid standard ranged from 5×10^6 to 25 plasmid copies per reaction. DNA concentrations for the 16S standards ranged from 2.6×10^6 to 1.3 ng plasmid DNA per reaction and for *mcy*D DNA concentrations of the plasmid standards ranged from 2.2×10^{-2} to 1.1×10^{-7} ng plasmid DNA per reaction. Using these standards, the lower detection limit of our assay was 25 target gene copies per reaction, which corresponds roughly to 5000 gene copies per liter of lake water. Genomic DNA from *M. aeruginosa* LE-3 culture was serially diluted to correspond to cell densities from 1.31×10^6 to 1.31 cells per reaction with corresponding DNA concentrations $308 - 3.1 \times 10^{-4}$ ng genomic DNA per reaction. Thus the lowest detection limit was 1.31 cells per 5 μL subsample, corresponding roughly to 262 cells per liter of lake water.

Results

Station descriptions

Chlorophyll *a* concentrations from individual sampling sites are summarized in Table 3. In 2003 chlorophyll *a* concentrations ranged from 4 to 40 μg L^{-1} in the western basin; in 2004 concentrations varied from 7 to 22 μg L^{-1}. In 2003 the highest chlorophyll concentrations were found at sites 1, 5 and 6 which are located in the proximity of the tip of the green algal mass visible in the LANDSAT image (Figure 1).

In 2003 and 2004, samples from all stations contained various concentrations of microcystins-LR (Table 3). The microcystin-concentration exceeded the safety limit (1.0

Table 3. Chlorophyll a and toxin concentrations in samples collected in the western basin of Lake Erie in August 2003 and 2004. August 2003 sample stations are numbered 1-7 and 2004 sampling stations numbered with 3 or 4 digits. Microcystin concentrations are expressed in microcystin-LR equivalents per liter.

Sampling station	Chlorophyll *a* (μg L^{-1})	Microcystin (μg LR eqv. L^{-1})
1	40.0	15.39
2	5.22	<0.25
3	6.40	0.33
4	4.00	<0.25
5	15.26	0.44
6	26.04	1.80
7	6.48	<0.25
493	8.83	0.072
311	14.10	0.292
1163	20.08	2.583
885	19.12	0.145
496	21.70	0.408
495	15.45	0.406
494	12.78	0.333
357	7.39	0.142
974	7.84	0.979
882	8.25	0.038

μg L^{-1}) set by the World Health Organization (Chorus and Bartram, 1999) at two sampling locations (sites 1 and 6) in 2003 and at one location in 2004 (site 1163). In 2003 the highest concentration of microcystin was detected at the mouth of Maumee River (sampling site 1) where the toxin concentration varied from 14.3 to 20.0 μg L^{-1}. At sampling site 6, 1.8 μg L^{-1} microcystin was found in the water column. In 2004, the highest concentrations of microcystins were found at stations 974 and 1163.

Multiplex PCR analysis

Results from the initial multiplex PCR analysis of 2003 and 2004 samples are presented in Table 4. PCR analysis indicated the presence of cyanobacteria and *Microcystis* spp. in all sampling sites. In 2003, toxigenic *Microcystis* spp. were present in six out of seven sampling sites, indicated by the presence of a *Microcystis*-specific 16S rRNA gene fragment and either one or both microcystin toxin synthetase genes *mcy*B and *mcy*D (Figure 3). Interestingly, at site 3 in 2003, neither of the toxin synthetase genes *mcy*B or *mcy*D were detected by PCR, despite detectable microcystin concentrations in the water. In 2004, all target genes were detected by PCR in all samples analyzed, indicating the presence of toxic *Microcystis* spp. at all sampling sites.

Gene copy numbers and cell abundance in lake water samples

The mean and the standard deviations of the triplicate real-time PCR assays are reported in Table 5 for all samples. In 2003 the highest abundance of cyanobacterial 16S rDNA target genes was detected at the mouth of Maumee River. At other sampling sites the abundance was 1-3 orders of magnitude lower. The abundance of *Microcystis* spp. 16S rDNA genes were highest at the mouth of Maumee River and the quantities decreased as distance increased from the mouth of the river. A similar trend was found in the abundance of *mcy*D copies, with abundances 2-3 orders of magnitude lower at sites 4 and 5 relative to site 1. At sites 3 and 6, abundances of *Microcystis* 16S genes and *mcy*D were below the quantifiable limit of our assay. In 2004 the quantities of all target genes in all samples were within quantifiable limits of the real-time PCR assay. Based on the percentages calculated using the quantities of cyanobacterial 16S and *Microcystis* 16S in

Table 4. Initial screening of water samples using multiplex PCR assays. The columns are labeled with the PCR primers used for the analysis. The presence or absence of a visible band in the gel after staining with SYBR green I is indicated by "+" or "-".

Sampling site	Cya 16S	Micr 16S	*mcy*B	*mcy*D
1	+	+	+	+
2	+	+	-	+
3	+	+	-	-
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
493	+	+	+	+
311	+	+	+	+
1163	+	+	+	+
885	+	+	+	+
496	+	+	+	+
495	+	+	+	+
494	+	+	+	+
357	+	+	+	+
974	+	+	+	+
882	+	+	+	+

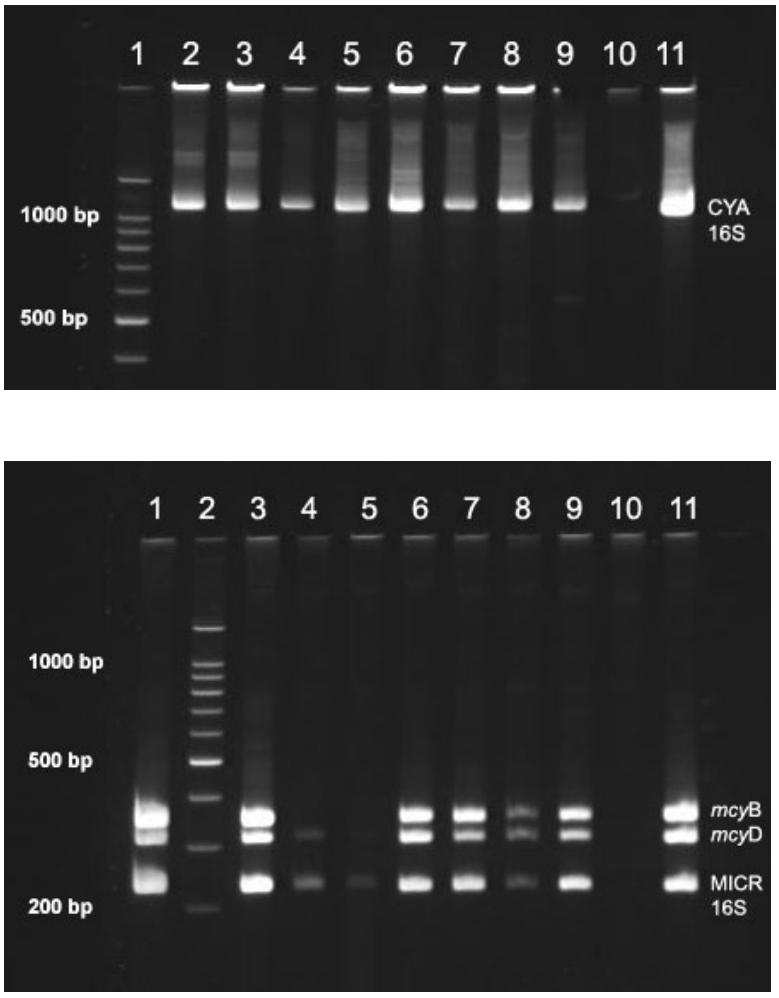

Figure 3. Gel image of multiplex PCR results. Gel A. Detection of cyanobacterial 16S rDNA fragment. Lane 1: 100 bp molecular weight marker; Lane 2: site 1a; Lane 3: site 1b; Lane 4: site 2; Lane 5: site 3; Lane 6: site 4; Lane 7: site 5; Lane 8: site 6; Lane 9: site 7; Lane 10: negative control, no template DNA; Lane 11: positive control (*M. aeruginosa* LE-3 genomic DNA). Gel B: detection of *Microcystis* spp. 16S rDNA fragment and microcystin synthetase genes *mcy*B and *mcy*D. Lane 1: site 1a; Lane 2: 100 bp molecular weight marker; Lane 3: site 1b; Lane 4: site 2; Lane 5: site 3; Lane 6: site 4; Lane 7: site 5; Lane 8: site 6; Lane 9: site 7; Lane 10: negative control, no template DNA; Lane 11: positive control (*M. aeruginosa* LE-3 genomic DNA).

the samples (Table 5), *Microcystis* dominated (>50%) the cyanobacterial population at site 1 in 2003 and at stations 357 and 882 in 2004. Using the *Microcystis* LE-3 genomic DNA standard, we estimated the abundance of cyanobacteria and *Microcystis* spp. cells at the mouth of Maumee River to be about 10^8 cells L^{-1}. The abundance of all cell types decreased towards the sampling sites 4 and 5, which are located approximately at the tip of the blooming mass originating from the mouth of Maumee River as seen on the LANDSAT image (Figure 1).

A low, but still detectable, abundance of *Microcystis* was found at sites 2 and 7. Although *Microcystis* was also found to be present at sites at sites 3 and 6, the abundances of *Microcystis* cells were too low to be quantified in our assay. The highest abundance of cells carrying *mcy*D gene were found at sites 1, 4 and 5. In 2004 the abundances of *Microcystis* and toxic *Microcystis* were within quantifiable limits at all sampling sites. The abundance of total Microcystis varied from 10^3 to 10^6 cells per liter. The highest abundances of total *Microcystis* and toxic *Microcystis* cells were found at station 496. At station 974 a notably high concentration of microcystin LR was detected (Table 3), however the abundance of toxic *Microcystis* was only approximately 800 cells per liter (Table 5).

Table 5. qPCR-based quantification of abundances of three target genes in water samples, and cell abundance of total cyanobacteria, total *Microcystis* and toxic *Microcystis* (cells carrying *mcyD* gene) in samples collected in August 2003 (sampling sites numbered 1-7) and August 2004. Description for all samples n= 3 (± SD) except, sampling site 1, where n=5 (±SD), sampling site 974 toxic *Microcystis*, n=2. ND=not detected, BQL= detected but below quantifiable limit.

Sampling site	Cyan 16S copies L^{-1}	Micr 16S copies L^{-1}	*mcy*D copies L^{-1}	Total cyanobacteria L^{-1}	Total *Microcystis* L^{-1}	Toxic *Microcystis* L^{-1}
1	$3.9 (\pm 3.8) \times 10^{10}$	$3.4 (\pm 0.5) \times 10^{10}$	$3.2 (\pm 0.6) \times 10^{8}$	$9.9 (\pm 1.1) \times 10^{8}$	$3.9 (\pm 1.1) \times 10^{8}$	$1.1 (\pm 0.3) \times 10^{6}$
2	$1.7 (\pm 0.5) \times 10^{7}$	$6.2 (\pm 1.8) \times 10^{4}$	BQL	$3.2 (\pm 0.6) \times 10^{5}$	$1.8 (\pm 0.5) \times 10^{3}$	BQL
3	$1.5 (\pm 0.1) \times 10^{9}$	BQL	ND	$3.1 (\pm 0.3) \times 10^{7}$	BQL	ND
4	$2.1 (\pm 0.6) \times 10^{8}$	$1.1 (\pm 0.7) \times 10^{7}$	$2.8 (\pm 0.6) \times 10^{6}$	$4.7 (\pm 0.5) \times 10^{6}$	$6.6 (\pm 4.3) \times 10^{4}$	$9.0 (\pm 4.0) \times 10^{4}$
5	$1.0 (\pm 0.1) \times 10^{8}$	$1.7 (\pm 0.0) \times 10^{7}$	$7.0 (\pm 4.2) \times 10^{5}$	$1.9 (\pm 0.1) \times 10^{6}$	$1.0 (\pm 0.0) \times 10^{5}$	$3.4 (\pm 2.0) \times 10^{4}$
6	$2.9 (\pm 0.3) \times 10^{8}$	BQL	BQL	$5.0 (\pm 0.6) \times 10^{6}$	BQL	$4.2 (\pm 1.2) \times 10^{3}$
7	$8.7 (\pm 0.2) \times 10^{7}$	$2.4 (\pm 6.9) \times 10^{5}$	$7.7 (\pm 2.7) \times 10^{4}$	$1.6 (\pm 0.1) \times 10^{6}$	$7.5 (\pm 0.5) \times 10^{3}$	$8.6 (\pm 2.8) \times 10^{3}$
493	$4.3 (\pm 1.0) \times 10^{8}$	$5.1 (\pm 0.8) \times 10^{7}$	$4.2 (\pm 2.8) \times 10^{5}$	$5.5 (\pm 1.4) \times 10^{6}$	$7.0 (\pm 1.1) \times 10^{5}$	$2.0 (\pm 1.3) \times 10^{4}$
311	$6.6 (\pm 0.3) \times 10^{8}$	$1.3 (\pm 0.7) \times 10^{8}$	$3.9 (\pm 0.5) \times 10^{6}$	$8.7 (\pm 0.4) \times 10^{5}$	$1.8 (\pm 1.0) \times 10^{6}$	$1.8 (\pm 0.2) \times 10^{5}$

Table 5 continued.

Sampling site	Cyan 16S copies L^{-1}	Micr 16S copies L^{-1}	*mcy*D copies L^{-1}	Total cyanobacteria L^{-1}	Total *Microcystis* L^{-1}	Toxic *Microcystis* L^{-1}
1163	$5.6 (\pm 0.4) \times 10^{9}$	$5.6 (\pm 0.4) \times 10^{7}$	$1.5 (\pm 1.0) \times 10^{6}$	$7.9 (\pm 0.2) \times 10^{7}$	$7.4 (\pm 0.6) \times 10^{5}$	$6.8 (\pm 4.5) \times 10^{4}$
885	$2.8 (\pm 0.3) \times 10^{8}$	$4.0 (\pm 0.8) \times 10^{7}$	$3.5 (\pm 0.3) \times 10^{6}$	$3.9 (\pm 0.4) \times 10^{6}$	$5.4 (\pm 1.1) \times 10^{5}$	$1.6 (\pm 1.6) \times 10^{5}$
496	$8.0 (\pm 2.2) \times 10^{8}$	$1.7 (\pm 0.07) \times 10^{8}$	$6.0 (\pm 0.3) \times 10^{6}$	$1.6 (\pm 7.8) \times 10^{7}$	$3.2 (\pm 1.5) \times 10^{6}$	$2.8 (\pm 0.1) \times 10^{5}$
495	$1.2 (\pm 0.4) \times 10^{8}$	$8.3 (\pm 0.3) \times 10^{6}$	$1.5 (\pm 0.7) \times 10^{5}$	$1.3 (\pm 0.8) \times 10^{6}$	$8.4 (\pm 2.9) \times 10^{4}$	$7.1 (\pm 3.4) \times 10^{3}$
357	$2.5 (\pm 1.3) \times 10^{7}$	$0.8 (\pm 1.1) \times 10^{7}$	$3.5 (\pm 0.3) \times 10^{4}$	$2.7 (\pm 1.9) \times 10^{5}$	$5.4 (\pm 6.4) \times 10^{4}$	$1.7 (\pm 0.2) \times 10^{3}$
974	$1.2 (\pm 0.05) \times 10^{7}$	$4.0 (\pm 1.3) \times 10^{5}$	$1.5 (\pm 1.0) \times 10^{4}$	$1.4 (\pm 0.06) \times 10^{5}$	$4.6 (\pm 1.5) \times 10^{3}$	$8.0 (\pm \text{ND}) \times 10^{2}$
882	$6.2 (\pm 1.0) \times 10^{7}$	$3.5 (\pm 0.1) \times 10^{7}$	$7.4 (\pm 1.5) \times 10^{5}$	$7.5 (\pm 1.3) \times 10^{5}$	$4.8 (\pm 0.2) \times 10^{5}$	$3.4 (\pm 0.7) \times 10^{4}$

Discussion

Three important implications arise from the results of this study. Firstly, a tiered response to a potential toxic cyanobacterial bloom was demonstrated to be a practical approach to monitoring these events. Combined use of satellite, standard and then quantitative PCR allowed us to rapidly and reliably detect and characterize this bloom event. Secondly, the results of this study suggest that not all strains of *Microcystis* are capable of producing toxin. Finally, the results demonstrate the limits of molecular approaches, as microcystin-producing cyanobacteria not detected by the probes used in this study (which were developed from our current knowledge of *Microcystis* gene system) appear to have persisted in some areas where we obtained our samples. As such, this work highlights the strengths of these tools as well as our continuing need for the development of a better understanding of the causative agents of freshwater cyanotoxin production.

At the time of sampling on August 15th 2003, a bloom of cyanobacteria was persisting in the western basin of Lake Erie. The bloom area was well visible as a green mass in the water column in the LANDSAT image (Figure 1) taken three days after sampling. In addition, ground level observations (by M.R. Twiss and T. Bridgeman) noted thick green slicks on the surface. At the time of sampling, another distinguishable toxigenic algal bloom was located at Sandusky Bay. *Microcystis* was abundant in the western basin of Lake Erie also in August 2004. Various concentrations of microcystins, determined using the protein phosphatase inhibition assay and expressed as microcystin-LR equivalents, were detected at all sampling sites, however it is notable that toxin producing *Microcystis* spp. were not present at every sampling location in 2003.

Based on qPCR analysis of the samples, the abundance of total *Microcystis* spp. cells varied from 4×10^8 to 2×10^3 cells per liter among the sites where the abundance of *Microcystis* spp. was within the quantifiable limits. These results agree with a previous survey of the abundance of *Microcystis* in the western basin of Lake Erie. In the summer months (June-August) of 1995, 1996 and 1997 the total *Microcystis* abundance was

reported to vary between 2×10^2 and 3×10^9 cells per liter (Brittain et al., 2000). Thus the real-time PCR based method used in this study provides data that are comparable to earlier results obtained through microscopic examination.

There are two possible explanations for finding microcystin in the water but no toxic *Microcystis*. One is that the abundance of *Microcystis* spp. producing microcystin was extremely low in these samples and it was not detectable through conventional PCR due to a collapsed or senescent bloom in the sampling area. The detection of microcystins in the absence of living cells has been documented previously and it is known that dissolved microcystin persists in the water column for several weeks (Sivonen and Jones, 1999), whereas naked DNA remains in PCR detectable form for only approximately 10 days in lake water (Deere et al., 1996). The other possible explanation for finding no toxigenic *Microcystis* but finding microcystin is the production of microcystins by other cyanobacteria. Other cyanobacterial species, in addition to *Microcystis* species, belonging to genera *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc* and *Anabaenopsis* are known producers of microcystins (Sivonen and Jones, 1999) and at least *Anabaena* and *Planktothrix* were observed during a visit to Sandusky Bay in July 2003 *via* light microscopy (Wilhelm and Boyer, unpublished data). As the molecular probes used in this study are highly specific for *Microcystis* spp. (Ouellette and Wilhelm, 2003), the presence of other microcystin producers in water samples could not be detected.

Estimates of the density of cells carrying the specific 16S rDNA target genes from *Microcystis* spp. are presented here using genomic DNA from a *Microcystis aeruginosa* isolate originally collected in Lake Erie. Implicit in this work is that variations in the copy number of 16S rDNA genes occur within the genomes of different prokaryotes; a brief survey of the literature suggest these copies can range from 1 to 4 or more per genome. As such, any estimates of cell density using this approach are built around the caveat that variations in this copy number per genome within the natural population will be a source of error. In the case of the *mcy*D gene, it appears that cells carry only one copy per genome (Kaebernick et al., 2002). Estimates of the percentage of the total *Microcystis* population that are toxic are therefore sensitive to this ratio: in the case of the current study this conversion alludes to more toxic *Microcystis* cells than total

Microcystis cells. Although this obviously cannot be the case, the results none the less provide a snapshot of the approximate dominance of the potentially toxigenic strains within the community.

The data presented here suggest that the composition of cyanobacterial communities varies spatially in the western basin of Lake Erie, and suggest that in some regions (e.g. Sandusky Bay) novel toxigenic organisms may persist. By employing a combination of satellite images, toxin data and qualitative and quantitative PCR data, we suggest a tier-wise approach that allows for a precise evaluation of the composition of cyanobacterial blooms within this lake. Moreover, the data presented here demonstrate that, at least for the August 2003 bloom, the Maumee River/Bay region appears to act as a potential source of *Microcystis* populations in this system. Ongoing studies, including sequence analysis of toxin genes from *Microcystis* isolated from different locations within this system will hopefully shed some light on this issue, and provide resource managers and researchers with information concerning regions of bloom initiation, which in the future could act as focus points for monitoring programs.

Acknowledgements

We thank Michael Satchwell for running the protein phosphatase inhibition assays and R. Michael McKay for assistance in obtaining the LANDSAT image and for helpful discussions. This article includes research supported by funds from the American Water Works Association (AWWA #2818), the National Oceanic and Atmospheric Administration Coastal Ocean Program (MERHAB-LGL, #NA 160 P2788) and the National Science Foundation (DEB-0129118), and the Lake Erie Trophic Transfer Study (United States Environmental Protection Agency - Great Lakes National Program Office).

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Part III

DIVERSITY OF MICROCYSTIN-PRODUCING CYANOBACTERIA IN SPATIALLY ISOLATED REGIONS OF LAKE ERIE

This part is an expanded version of the manuscript published as a short form paper with same title in journal Applied and Environmental Microbiology in 2006 by Johanna M. Rinta-Kanto and Steven W. Wilhelm.

Rinta-Kanto J.M. and Wilhelm, S.W. 2006. Diversity of microcystin-producing cyanobacteria in spatially isolated regions of Lake Erie. Applied and Environmental Microbiology, 72: 5083-5085.

My use of “we” in this chapter refers to my co-author and myself. My primary contributions in this paper were 1) Collecting the environmental samples for this study, 2) Analyzing the samples for the study and analyzing the data 3) Gathering the background literature and, 4) Most of the writing of the manuscript and correspondence to the reviewer comments.

Introduction

The North American Great Lakes contain approximately one fifth of the Earth's potable water supply, making them a vital resource on a global scale. Although it is volumetrically the smallest, Lake Erie is the most productive of the five Laurentian Great Lakes, and maintains a position of socioeconomic importance in the region (Fuller, 1995; Munawar et al., 2002). Since the mid-1990s, recurring blooms of *Microcystis* spp. have formed in the western basin of Lake Erie and now the annual reappearance of *Microcystis* is well documented in the literature (Brittain et al., 2000; Vanderploeg et al., 2001; Vincent et al., 2004; Rinta-Kanto et al., 2005). The presence of other microcystin producers in this system, however, has garnered less attention. Recently our group reported results from surveys on *Microcystis* abundance in the western basin of Lake Erie in August of 2003 and 2004 (Rinta-Kanto et al., 2005). The study also revealed in some locations the presence of significant quantities of cyanotoxins (1.3-2.6 μg L^{-1} microcystin-LR equivalents) coinciding with high densities of cyanobacteria (up to 0.8 - 1.0×10^{8} cells L^{-1}), but a relatively low abundance of toxic *Microcystis*. Microscopic observations of water samples collected in 2004 from Sandusky Bay (Station 1163) revealed the presence of a high abundance of *Planktothrix aghardii*, which are well-known microcystin producing cyanobacteria (Christiansen et al., 2003), as such suggesting that the microcystin-producing cyanobacterial community may not be entirely composed of *Microcystis* spp.

The hepatotoxic microcystins are the largest and most diverse group of cyanotoxins produced by cyanobacteria commonly belonging to genera *Microcystis*, *Planktothrix*, *Anabaena* (Sivonen and Jones, 1999; Kaebernick and Neilan, 2001). Microcystin-producing strains have also been identified in the genera *Nostoc* and *Anabaenopsis* (Sivonen and Jones, 1999). Microcystins are produced in the cells non-ribosomally via a peptide synthetase complex and there are over 60 chemical forms of microcystins of varying toxicities (Kaebernick and Neilan, 2001), with new information on different congeners appearing regularly. The *mcy* gene cluster encoding the

components of the microcystin synthetase and the biosynthetic pathway for microcystin biosynthesis have been characterized in *Microcystis*, *Planktothrix* and *Anabaena* (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004). The phenotypes of the toxin producing and non-toxin producing cyanobacteria are similar, thus the microcystin biosynthesis genes are commonly used as targets for molecular probes for the selective detection of potentially toxin-producing cyanobacterial genotypes in water samples (*e.g.* (Ouellette and Wilhelm, 2003; Dittmann and Börner, 2005).

The goal of the current study was to build on the previous observations and use molecular techniques to study the diversity of the microcystin-producing community in locations where the highest microcystin-LR concentrations in 2003 and 2004 were observed. Specifically, we wanted to determine whether *Planktothrix aghardii,* observed microscopically in 2004, could potentially produce microcystin in the western basin of the lake. The microcystin-producing cyanobacterial community was characterized by amplifying a region encoding the condensation domain from the *mcyA* genes carried by the microcystin-producing genotypes of *Anabaena*, *Planktothrix* and *Microcystis,* using a previously published primer set (Hisbergues et al., 2003). The *mcyA* gene is one of the genes encoding microcystin synthetase found in potentially toxic genotypes of microcystin-producing cyanobacteria. The sequences of *mcyA* gene amplicons obtained from natural samples were cloned and sequenced and the sequences were used to study the identities and phylogeny of microcystin-producers in the natural community.

Materials and Methods

Sample collection

Samples were collected from the western basin of Lake Erie in 2003 and 2004 onboard C.C.G.S *Limnos* (Figure 1. for sampling locations). Water samples were collected at 1 meter depth using a pump deployed over the board of the ship. Water was collected into clean plastic bottles. Chlorophyll *a* and toxin (microcystin-LR) equivalents were determined as previously described (Rinta-Kanto et al., 2005).

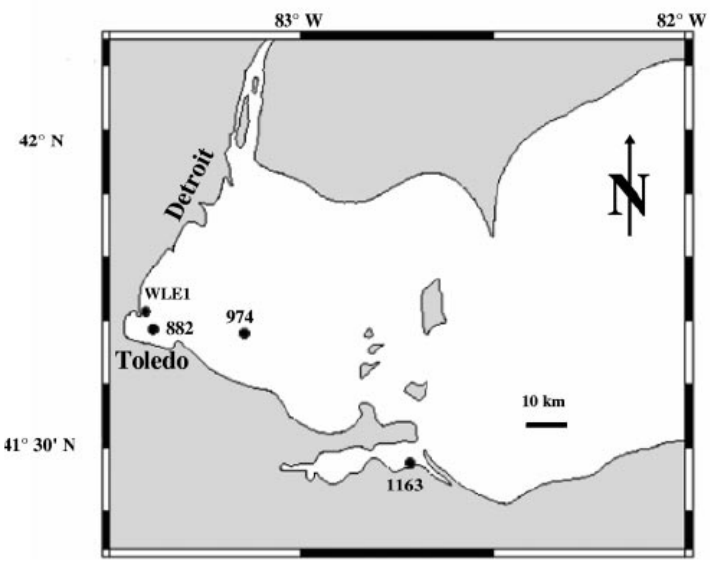

Figure 1. Map of station locations and corresponding station codes in the western basin of Lake Erie

Construction of the mcyA clonal library

Cells for DNA extraction were collected by filtering a known volume of lake water through 47 mm diameter, 0.22- μm nominal pore-size polycarbonate membrane filter (Millipore). DNA was extracted from cells collected on filters as described previously (Rinta-Kanto et al., 2005). The primer pair *mcyA*-Cd 1R and *mcyA*-Cd 1F (Hisbergues et al., 2003) was used to amplify 291-297 bp *mcyA* gene fragments from natural samples. Reactions were carried out in EasyStart tubes (Molecular BioProducts, San Diego, CA, USA) in a final volume of 50 μL containing 0.4 μM of each primer, 300 ng μL^{-1} bovine serum albumin (Sigma cat # A-7030; (Kirchman et al., 2001)), 2.5 U Taq polymerase (Promega), 0.1 % Triton X-100 (Molecular BioProducts), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1 × PCR buffer and 5 μL of undiluted or ten-fold diluted DNA extracted from natural samples. Thermal cycling was completed using an Eppendorf Mastercycler gradient-thermocycler using the following protocol: initial denaturation at 95ºC for 10 min, 40 cycles of 94ºC for 30 s, 53ºC for 30 s, 72ºC for 30 s, and a final extension step at 72ºC for 5 min. The PCR-amplified *mcyA* fragments were purified by running 10 μL of each PCR product along with 100 bp DNA ladder (Promega, Madison, WI, USA) on a 2 % agarose gel in 1 × Tris-borate-EDTA buffer (Sambrook and Russell, 2001). The presence of the correct size band was confirmed by ethidium bromide staining and visualizing the gel under UV transillumination. The *mcyA* DNA of the correct size was excised from the agarose gel and the DNA from the bands extracted using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). Clone libraries were generated using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cloned inserts of the correct size were verified by *Eco*RI digestion (Promega, Madison, WI, USA). Thirty eight clones were picked from each sample for sequencing. Plasmid DNA was isolated and high throughput sequencing of the *mcyA* gene inserts was completed at the Clemson University Genomics Institute, using the M13 forward primer site within the cloning vector as the sequencing start point (Invitrogen, Carlsbad, CA, USA).

mcyA sequences from pure culture of Microcystis aeruginosa LE-3

M. aeruginosa LE-3 (a Lake Erie isolate) was grown in modified BG11 medium (Kerry et al., 1988) at +25°C under continuous illumination at *ca* 80 μmol of photons m^{-2} s^{-1}. The cells were grown in a batch culture for 3 weeks, then pelleted by centrifuging and the genomic DNA was extracted using the xanthogenate method (Tillett and Neilan, 2000) as described in Ouellette et al. (Ouellette et al., 2006). The *mcyA* gene fragments from the extracted DNA samples were PCR amplified and cloned as described above. Sequencing of this fragment was completed at the Molecular Biology Resource Facility at the University of Tennessee.

Sequence analysis

Vector and primer sequences were removed from the DNA sequences and all sequences were manually checked and edited as required prior to the analysis using the Bio Edit software program (Hall, 1999). The *mcyA* sequences from natural samples were examined both at the nucleotide level and at the amino acid level. Individual DNA sequences from natural samples were queried against the GenBank database using BLAST and BLASTX searches (Altschul et al., 1997) in order to find highly similar DNA and amino acid sequences from the data base originating from cultured isolates of microcystin-producing cyanobacteria. Prior to phylogenetic analysis, all nucleotide sequences were translated into amino acid sequences. Identical McyA sequences were removed from the data set leaving only representative unique sequences in the data set. These remaining amino acid sequences were then aligned using Clustal W-software with McyA amino acid sequences retrieved from GenBank (Thompson et al., 1994). Phylogenetic reconstruction was performed using the Mega 3.0 software package (Kumar et al., 2004). Phylogenetic relationships between the translated sequences were inferred through a neighbor-joining analysis using a Poisson correction distance. Bootstrap values were determined using 2000 iterations. Dendograms were also created using the UPGMA and minimum evolution approaches which yielded comparable tree topologies (not shown).

Detection of potentially toxic Planktothrix in water samples

To confirm the presence of potentially toxic *Planktothrix* in the water samples analyzed in this study, the primer pair mcyE.fw and mcyE.rev (Mbedi et al., 2005) was used to amplify a 589 bp *Planktothrix*-specific fragment of the *mcyE* gene. PCR amplifications were carried out in EasyStart tubes (Molecular BioProducts, San Diego, CA, USA) in final volume of 50 µL containing 0.4 µM of each primer, 2.5 U Taq polymerase (Promega, Madison, WI, USA), 0.1 % Triton X-100 (Molecular BioProducts), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1 × PCR buffer and 5 µL of undiluted or ten-fold diluted DNA extracted from natural samples. Thermal cycling was carried out using an Eppendorf Mastercycler gradient-thermocycler and the thermal cycling protocol was modified from the protocol specified by Mbedi et al. (Mbedi et al., 2005) consisting of an initial denaturation step at 95ºC for 10 min, 40 cycles of 95ºC for 10 s, 50ºC for 10 s, 72ºC for 30 s, and final extension step at 72ºC for 10 min. PCR purification and visualization of the 589 bp *mcyE* fragments was performed as described above.

GenBank accession numbers for all *mcyA* and *mcyE* DNA sequences reported here are DQ379674-DQ379711.

Results

Sampling locations are indicated in the map in Figure 1. Table 1 shows a summary of sampling dates, the percentage which potentially toxic *Microcystis* represents of the entire cyanobacterial cell abundance at each station and the outcome of *Planktothrix* specific PCR.

Overall, the results from BLAST and BLASTX searches confirmed that all inserts in the clones sequenced originated from the *mcyA* gene of the microcystin synthetase complex. Sequences from station 1163 (Sandusky Bay) in 2003 and 2004 were identical

Table 1. Sampling stations and sampling dates, estimated % of potentially toxic *Microcystis* of the abundance of total cyanobacteria (cells L-1) and +/- indicating the presence or absence of toxic *Planktothrix* at the sampling locations based on detection of *Planktothrix* specific *mcyE* fragment by PCR.

Station	Date	Chl a ($\mu g\ L^{-1}$)	MC-LR ($\mu g\ L^{-1}$)	% of toxic *Microcystis* of all cyanobacteria	*Planktothrix* specific *mcyE* detected
1163	Aug. 15, 2003	25.1	1.3	0.03	+
WLE1	Aug. 15, 2003*	40.0	15.4	4.7	-
1163	Aug. 19, 2004*	20.1	2.46 (± .02)	0.1	+
974	Aug. 19, 2004*	7.8	0.99 (±.01)	0.4	-
882	Aug. 20, 2004*	8.3	0.04	4.5	-

* Toxin and chlorophyll data for marked stations from Rinta-Kanto et al., 2005

to sequences from *Planktothrix aghardii* isolates (99-100 % identity at the nucleotide level; and 100% identity and at the amino acid level). Secondly, the *mcyA* sequences from western basin stations WLE1, 882 and 974 were most similar with sequences from *Microcystis* isolates: 93-100 % identity at the nucleotide level, 93-100 % identity at the amino acid level.

Phylogenetic analysis was conducted among the McyA amino acid sequences (76-78 residues in length) translated from natural samples and McyA sequences of other microcystin producing cyanobacterial genera (*Planktothrix aghardii*, *Microcystis* spp., *Anabaena* spp. and *Nostoc* spp.) deposited in the GenBank data base. A translated McyA sequence of *M. aeruginosa* isolate LE-3 was also included in the analysis. The grouping of the different sequences in the neighbor-joining tree confirmed that 100% of the McyA sequences from station 1163 were located in the same clade with *Planktothrix aghardii isolates*, whereas 100% of the McyA sequences from stations WLE1, 882 and 974 were located in the same clade with *Microcystis* isolates (Figure 2). The sequences within the *Microcystis*-clade were remarkably diverse. Most of these sequences (including the McyA sequences from cultured isolates of *Microcystis*) contained a two amino acid residue deletion compared to the *Planktothrix*-like McyA sequences. However, a subset of the *Microcystis*-like sequences (20 sequences from station 974 and one sequence from station WLE1) did not have this deletion. Despite lacking the two amino acid deletion, these sequences were still most similar to *Microcystis* sequences (93-97% identity at the nucleotide level; 96-93% identity at the amino acid level). Despite the high % identity, the sequences containing the extra amino acids had no exact match in the GenBank data base at the time the searches were done (January 2005). The McyA sequence alignment revealed that the two residue insertion in the *Microcystin*-like sequences occurred at the same position where the two extra amino acids are located in *Planktothrix* sequences (Figure 3).

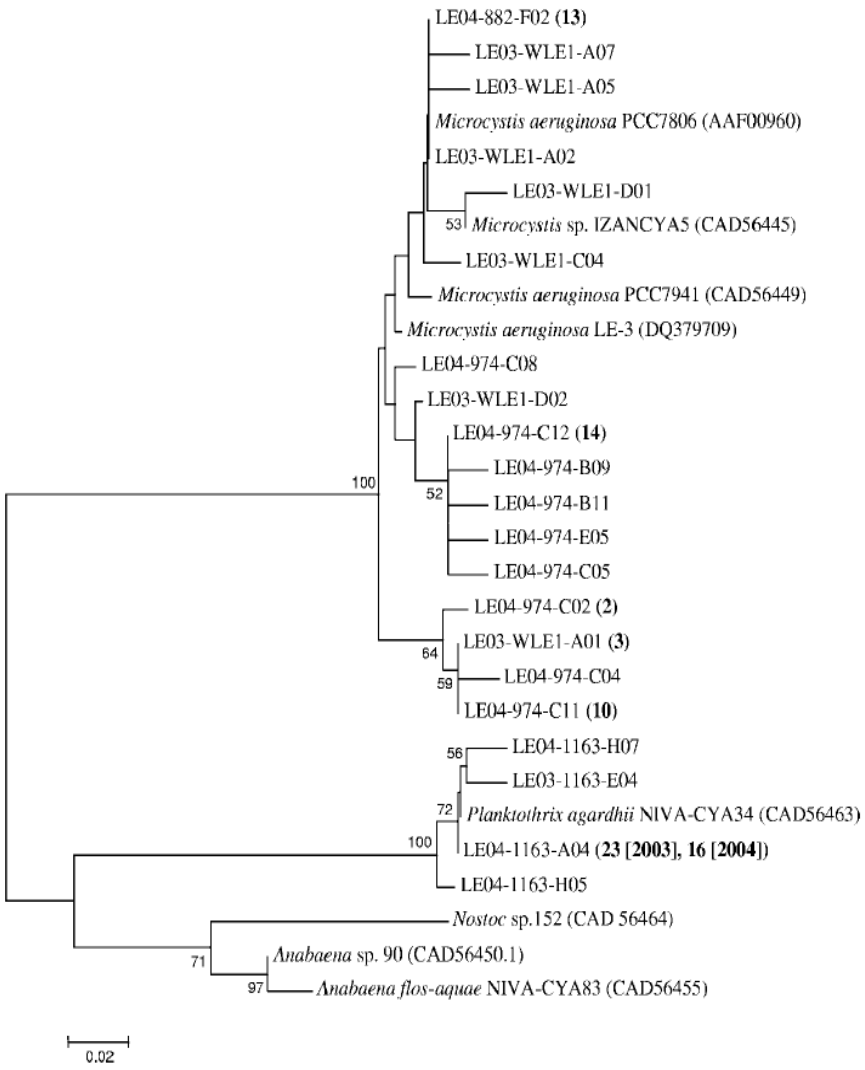

Figure 2. A neighbor-joining tree displaying the relationship between partial McyA sequences (76-78 amino acids). The coding system for the clones as follows: LE03-1163: Lake Erie, year 2003, station 1163; LE03-WLEI: Lake Erie, year 2003, station WLEI; LE04-1163: Lake Erie, year 2004, station 1163; LE04-974: Lake Erie, year 2004, station 974; LE04-882: Lake Erie, year 2004, station 882. The numbers in parentheses after the codes for the clones indicate the number of identical sequences represented by these sequences. Accession numbers are in parentheses after the names for reference sequences obtained from GenBank. Bootstrap values > 50% (for 2000 iterations) are displayed at the branch nodes. The scale bar represents substitutions per site.

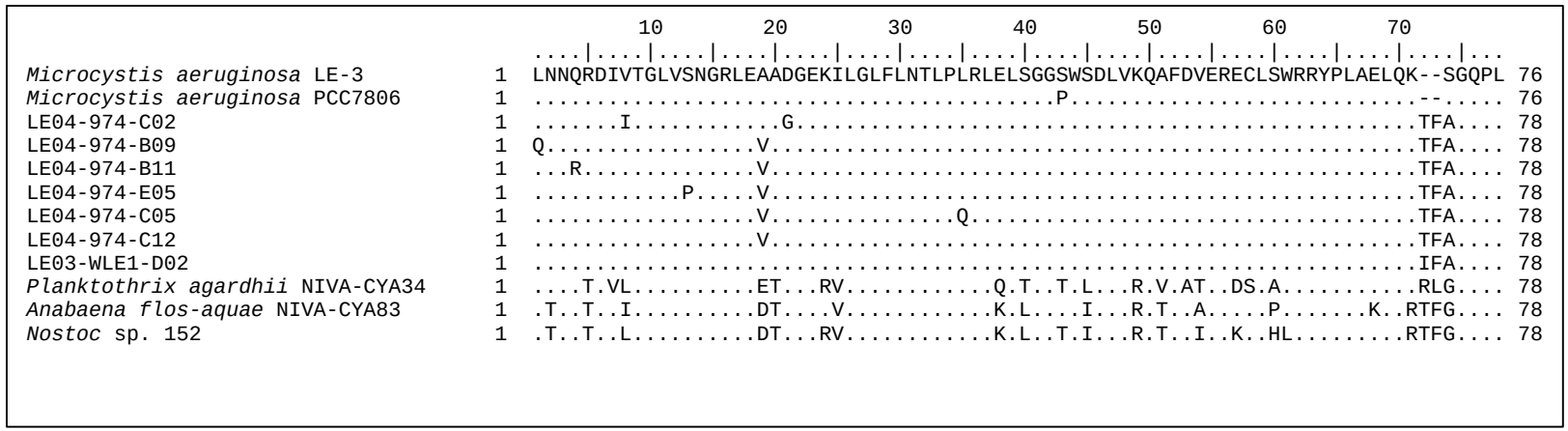

Figure 3. Multiple alignment of McyA amino acid sequences. Aligned sequences include McyA sequences of cultured isolates of *Microcystis aeruginosa* LE-3 (translated from the *mcyA* gene sequence DQ379709), *Microcystis aeruginosa* PCC7806 (AAF00960.1), *Planktothrix aghardii* NIVA-CYA34 (CAD56463.1), *Anabaena flos-aquae* NIVA-CYA83 (CAD56455.1) and *Nostoc* sp. 152 (CAD 5663.1) with novel *Microcystis*-like McyA sequences from natural samples that contain two extra amino acids unlike *Microcystis*-sequences from cultured isolates. GenBank accession numbers are in parentheses after the name of the cultured isolate.

Discussion

The application of molecular techniques to questions of cyanotoxin production is critical as it allows for a precise identification of the potentially toxigenic organism within a bloom event. On a larger scale it also provides principal insight into the global distribution of toxic cyanobacteria, and may ultimately lead to the development of a biogeographical model for bloom initiating populations. In the context of the current study, we have examined a conserved genetic element to determine which organism(s) within a population may be responsible for the production of the hepatotoxic microcystins. This approach has been useful in identifying the apparent toxin producers in spatially separated regions of the same lake. Moreover, the analysis of sequence data has identified a genetic fragment not associated with any currently known toxin producers, suggesting that still other potential toxin producers may exist. We couch these observations within the framework of developing a better understanding of the key factors that regulate cyanobacterial blooms in Lake Erie as well as other freshwater environments.

Microcystis, *Planktothrix* and *Anabaena* are the most common microcystin-producing cyanobacteria, and their potentially toxic genotypes can not only be detected but also identified based on unique sequences within the *mcyA* amplicons (Hisbergues et al., 2003). Phylogenetic analysis of McyA sequences from our natural samples along with sequences from cultured isolates shows that the sequences retrieved from the natural samples fall into two distinct clusters. All McyA sequences from station 1163 were most similar to *Planktothrix agardhii* sequences, whereas sequences from the other stations were most similar to *Microcystis*. No *Planktothrix*-like McyA sequences were found outside station 1163, and *Microcystis*-like sequences were found at all sampling location except station 1163. Based on the sequence analysis data, the populations of *Planktothrix* and *Microcystis* are clearly separated spatially. This, however, does not seem to be the case in the lake based on the analysis of lake water samples through other PCR-based methods. In our previous study (Rinta-Kanto et al., 2005), the abundance of potentially

toxic *Microcystis* determined by real-time PCR at station 1163 was 3.4 (±0.6) $\times 10^4$ cells L^{-1} in August of 2003 (J.M. Rinta-Kanto, unpublished data) and 6.8 (±4.5) $\times 10^4$ cells L^{-1} in August of 2004 (Rinta-Kanto et al., 2005). At those times cyanobacterial abundance was 1.2 (±0.3) $\times 10^8$ and 7.9 (±0.2) $\times 10^7$ cells L^{-1}, respectively (J.M. Rinta-Kanto, unpublished data and (Rinta-Kanto et al., 2005)). Thus, it is likely that *Planktothrix mcyA* fragments were preferentially amplified from the samples collected from station 1163 due to low relative abundance of potentially toxic *Microcystis*. This idea is corroborated by high abundance (2.3×10^8 cells L^{-1}) of *Planktothrix aghardii* in August 2004 determined through microscopic counts (Renhui Li, unpublished data). In fact, the abundance of *P. aghardii* exceeds the total abundance of cyanobacteria in August 2004 determined through real-time PCR using *Microcystis aeruginosa* LE-3 genomic DNA as a quantification standard for cell abundance (Rinta-Kanto et al., 2005). While this is an indication of high relative abundance of *Planktothrix* in the cyanobacterial population potentially causing preferential amplification of *Planktothrix mcyA* fragments, this discrepancy also highlights the problems that still exist when quantifying by real-time PCR cyanobacterial cells with variable genomic content in mixed populations. Moreover, PCR analysis confirmed that potentially toxic *Planktothrix* spp. were not present at the time of sampling in the other sampling sites. These findings also emphasize the importance of parallel analyses when using molecular probes to study mixed natural populations to better understand the complexity of the system.

Phylogenetic analysis indicated that the *Microcystis*-like *mcyA* sequences were more diverse than the *Planktothrix*-like sequences, as indicated by the number of unique sequences in the phylogenetic tree (Figure 2). Consistently with the observations from a previous study (Hisbergues et al., 2003), the majority of translated *Microcystis*-like *mcyA* gene sequences from natural samples contained a two residue deletion when aligned with *Planktothrix* sequences. In our data set, novel *Microcystis*-like sequences lacking the characteristic deletion were discovered. The effect of the additional two residues on microcystin-production in the host strains is unknown. We consider it unlikely that these additional residues in the *Microcystis*-like sequences are PCR artifacts, since 21 of our *Microcystin*-like sequences from natural samples contained the two residue insertion at

the same location as the *Planktothrix*-like sequences. This observation suggests that the Lake Erie toxigenic cyanobacterial populations (potentially *Microcystis)* contain uncultured diversity.

The conditions that support the *Microcystis* and *Planktothrix* populations in Lake Erie are not yet known and future research will be directed to elucidating these factors in more detail. *Planktothrix* spp. are known to thrive in low light environments, commonly outcompeting other cyanobacteria that have high light requirements (Wiedner et al., 2002), and the abundance of *Oscillatoriales* (including *Planktothrix*) has been shown to be significantly correlated with Secchi depth (measuring water clarity) (Scheffer et al., 1997). In Lake Erie Secchi depths < 1m are commonly observed in Sandusky Bay (the area of station 1163 on the map in Figure 1), whereas elsewhere in the western basin Secchi depths of > 1 m are common (SW Wilhelm, unpublished data). Thus, the light conditions in Sandusky Bay may be more favorable to *Planktothrix* spp. than to *Microcystis* spp. Filter feeding activity of Dreissenids may not specifically contribute to the local dominance of *Planktothrix*, since the mussels have not been shown to discriminate between filamentous or unicellular cyanobacteria as their food source (Pires et al., 2005). Overall, the conditions that support the dominance of toxigenic *Micorocystis* and *Planktothrix* populations in these two distinct locations have to be taken into account in the bloom management strategies.

The data presented here reveal a new aspect of the diversity of microcystin-producing cyanobacteria in Lake Erie. Although the results of this study do not give information about the toxin production at the time of sampling by the different cyanobacterial genera, the findings indicate that microcystin production during cyanobacterial blooms in the western basin of Lake Erie may not be solely carried out by *Microcystis* spp., as previously thought. Based on the observations from this study we can conclude that *Planktothrix* is a potential cyanotoxin producer in parts of the western basin.

Acknowledgements

We thank the Captain and crew of the CCGS *Limnos* as well as Bob Hess, Greg Boyer, George Bullerjahn, Gary LeCleir and Tom Bridgeman for assistance and for helpful discussions and Wayne Carmichael for providing the *M. aeruginosa* LE-3 isolate used in this study. We also thank Renhui Li for providing the enumerations of *Planktothrix* in Sandusky Bay. This article includes research supported by funds from the National Oceanic and Atmospheric Administration Coastal Ocean Program (MERHAB-LGL, #NA 160 P2788), an Oceans and Human Health external grant (NA05NOS4781251), the National Science Foundation (DEB-0129118), and Helsingin Sanomat Centennial Foundation.

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Part IV

MICROCYSTIN-PRODUCTION IN LAKE ERIE: INSIGHTS FROM ARCHIVED PELAGIC AND SEDIMENT SAMPLES

This part is a version of a manuscript with same title prepared to be submitted in journal Environmental Microbiology by Johanna M. Rinta-Kanto, Matthew A. Saxton, Jennifer M. DeBruyn, Christopher H. Marvin, Kenneth A. Krieger, and Steven W. Wilhelm.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions in this paper were 1) Collecting part of the environmental samples for this study, 2) Formulating the hypothesis 3) Gathering the raw data from different sources and analyzing the data 4) Gathering the background literature and, 5) Most of the writing of the manuscript.

Introduction

It is now well known that cyanobacterial blooms induced by eutrophication were a significant threat to the water quality of Lake Erie in the 1970s. Management efforts, focusing on radical reductions in external phosphorus loading to the lake, resulted in temporary mitigation of the problem until the mid 1990's (Makarewicz, 1993; Munawar et al., 2002; Conroy et al., 2005). After reaching a minimum in the early 1990s, the summer-season cyanobacterial biomass in Lake Erie again began showing an increasing trend, despite no significant increase in the external phosphorus loading (Conroy et al., 2005). Previously published data sets suggest that the most common cyanobacteria in the 1970s were *Aphanizomenon flos-aquae* and *Anabaena* spp. (Makarewicz, 1993). These data sets also indicate the presence of *Microcystis* among other members of the Cyanophyta in the phytoplankton assemblages of Lake Erie during 1970s (eastern basin) and from the mid to late 1980s (western basin), although there is no evidence of *Microcystis* bloom events at those times (Hopkins and Lea, 1982; Makarewicz, 1993). The first documented toxic *Microcystis* bloom event did not occur in the western basin of the lake until 1995 (Brittain et al., 2000). Since then, however, *Microcystis* has become a well documented phenomenon, with significant blooms (producing up to 15 $\mu g\ L^{-1}$ of the toxin microcystin) occurring in several years (Vincent et al., 2004; Conroy et al., 2005; Rinta-Kanto et al., 2005; Ouellette et al., 2006). Lake Erie also supports a large community of single cell picoplankton (e.g. *Synechococcus*) (Ouellette et al., 2006). These non-toxic cyanobacteria are usually disregarded in studies of cyanobacterial blooms because they tend to focus on the toxin producing species.

The seasonal appearance of *Microcystis* has induced public health concerns due to Lake Erie's important role in recreation, fisheries and municipal drinking water supply in the densely populated area surrounding this Great Lake (Fuller, 1995). Cyanobacteria belonging to the genus *Microcystis* are non- nitrogen fixing unicellular cyanobacteria, which are capable of producing a range of chemical variants of microcystin (Carmichael, 1996). Microcystins are potent hepatotoxins, which have been reported to induce

gastrointestinal symptoms, potentially promote liver tumors and can even cause death due to liver failure upon ingestion of toxin (Pouria et al., 1998; Falconer, 2005).

Recent studies in Lake Erie have attempted to develop a better understanding of why *Microcystis* spp. are now thriving in the lake. The seasonal distribution and abundance of *Microcystis* (Rinta-Kanto et al., 2005; Ouellette et al., 2006; Rinta-Kanto and Wilhelm, 2006) and potential effects of the activities of dreissenid mussels (*Dreissena polymorpha*, *Dreissena bugensis*) on nutrient balance and *Microcystis* spp. abundance have been investigated (Raikow et al., 2004; Conroy and Culver, 2005). While a number of studies conducted in the past decade have focused on investigating the present situation, less attention has been drawn to the historical record of *Microcystis* in Lake Erie and the potential internal reservoirs of *Microcystis* in the lake, such as within sediments. In other freshwater systems sediments have been suggested to play an integral role in the annual cycle of *Microcystis* (Preston et al., 1980).

The first goal of this study was to examine present day (2004) sediments to assess the relative abundance of cyanobacteria within the sediment microbial assemblage as well as to determine the genetic diversity and viability of the sediment-based *Microcystis* populations. The second goal was to characterize the microcystin producing cyanobacteria in archived pelagic phytoplankton samples and archived surface sediments which were collected from Lake Erie in 1970s and then to compare their diversity to microcystin-producers currently found in Lake Erie. The diversity of microcystin-producing cyanobacterial populations was studied using a *mcyA* gene fragment which has been useful in characterizing the phylogenetics of the microcystin producers in this system (Rinta-Kanto and Wilhelm, 2006). This gene has been shown to be conserved on the genus level in microcystin-producing cyanobacteria, allowing identification of microcystin-producers belonging to different genera based on the polymorphisms in the DNA sequence of this gene (Hisbergues et al., 2003).

Materials and Methods

Sample collection

Box cores were obtained from three locations on Lake Erie in July of 2004 onboard C.C.G.S. *Limnos* (Figure 1). A series of sub-cores were obtained from each box core using clean, graduated acrylic coring tubes (2.5 cm diameter). Cores were sectioned every two centimeters between the top of the sediment and 10 cm depth. The sections were stored in sterile plastic bags at -20°C until they could be processed in the laboratory. Surface water samples (1 m) were collected from the same locations where box cores were obtained using a submersible pump, and cells for DNA extraction were collected by filtering a known volume of lake water through 47-mm diameter, 0.22- μm nominal pore-size polycarbonate membrane filters (Millipore). The filtration apparatus was rinsed between the samples using a 10 % NaClO solution and distilled water to prevent the carryover of residual biomass between samples.

Archived samples of Lake Erie sediments were retrieved from controlled storage at the National Water Research Institute (Burlington, ON) where they have been maintained since their initial collection. In 1971 these sediments were collected as described previously (Frank et al., 1977) (see locations on the map), using a Shipek grab sampler and with subsampling of the top 3 cm of the sediment. After previous examinations, residual samples were freeze dried and stored under refrigeration.

Phytoplankton samples collected from Lake Erie in 1978 and in 1979 were taken from locations indicated on the map (Figure 1). The 1978 samples were collected by a vertical tow of a 243 μm-mesh, 0.5-m diameter plankton net from near the bottom to the surface. The 1979 samples were collected in a similar manner but with a 64 μm-mesh, 0.5-m diameter plankton net. These phytoplankton samples were preserved with formalin and stored at Heidelberg College (Tiffin, OH) until they were made available for this study.

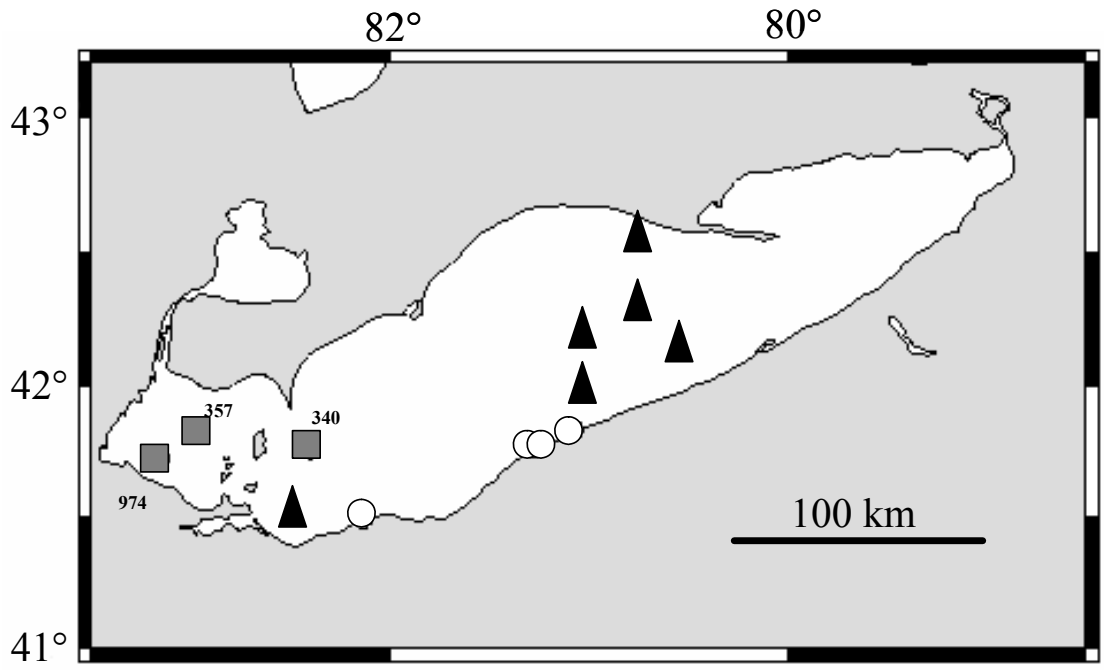


Figure 1. Map of sampling locations in Lake Erie. Squares: 2004 sediment cores. Triangles: 1971 sediment cores. Circles: 1978 and 1979 plankton net tow samples. Stations mentioned in the text by their respective codes are labeled in the map.

Culturing cell material from sediments

To collect sediment samples for culture work, a box core was obtained from Lake Erie from station 357 (see map on Figure 1) in August of 2006. Sub-cores were obtained from the box core using clean graduated acrylic coring tubes (2.5 cm diameter), which were sectioned into 0-5 cm and 5-10 cm fractions. Each section was placed in a sterile 1 L volumetric flask, which was covered with aluminum foil, except the top 5 cm of the neck. The volumetric flask was filled to the top with lake water filtered through a 0.2-μm nominal pore–size polycarbonate filter (Millipore). The bottles were capped with a sterile glass stopper and sealed with parafilm and maintained at room temperature in ambient light. After 8 weeks of incubation, green cellular material which had risen to the surface of the liquid in the bottles was collected with a sterile transfer pipette into a microcentrifuge tube, and centrifuged in a microcentifuge for 20 min at 10,000 × g. The resulting pellet was subjected to molecular analysis. Residual material in the culture vessels was also maintained for microscopic examination and in lab culture isolation (e.g., Figure 2).

DNA extraction

DNA from sediments collected in 2004 was extracted using FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad, CA). For each depth, two parallel extractions were completed (denoted as Set 1 and Set 2). For each extraction, 0.5 g (wet weight) of sediment was used. DNA from 1971 sediment samples was extracted using UltraClean Soil DNA Kit (Mo Bio Laboratories, Carlsbad, CA, USA) using 1.0 g of freeze dried material for each extraction. To dilute substances potentially inhibiting PCR amplification, one hundred and one thousand-fold dilutions of DNA extracts were used as templates for PCR reactions.

DNA from lake water samples was extracted from cells collected on filters using a method described in (Rinta-Kanto et al., 2005). DNA for quantitative real time PCR (qPCR) standards was extracted from *Microcystis aeruginosa* LE-3 (Brittain et al., 2000)

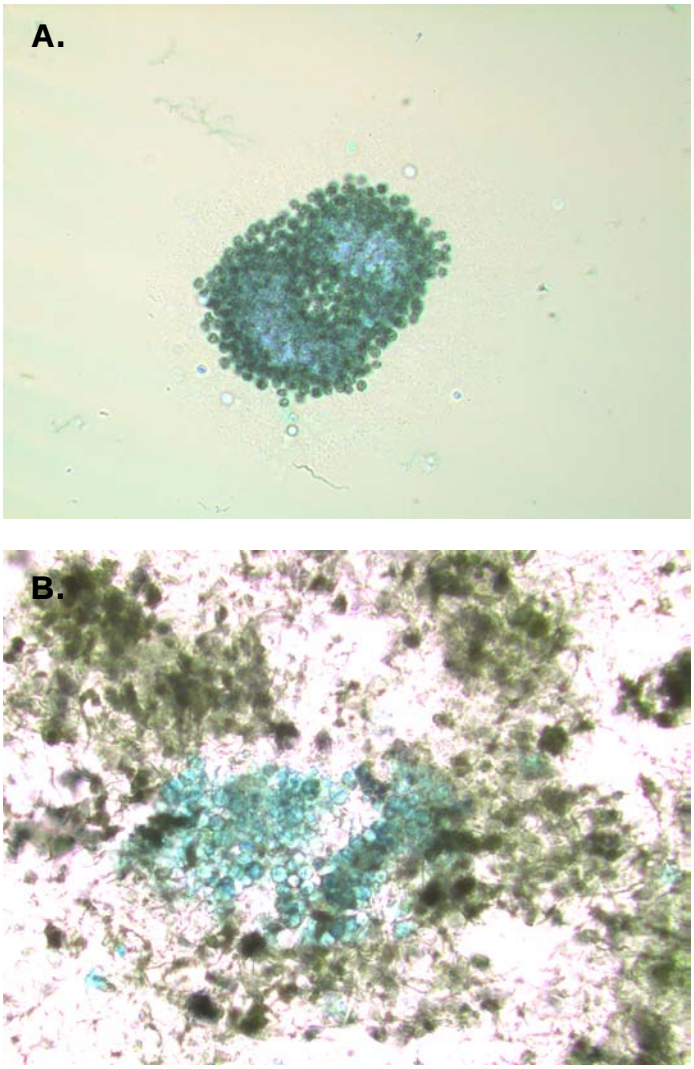


Figure 2. Light microscopy images of material cultured from Lake Erie sediment samples collected in 2006. Colonies with morphological characteristics consistent with Microcystis spp. were observed both in isolation (A.) and integrated amongst other cellular materials (B.).

grown in modified BG-11 medium, and the standards were prepared as previously described (Rinta-Kanto et al., 2005).

DNA from archived plankton samples was extracted from 1 mL aliquots of the 1978/9 formalin preserved plankton samples using a modification of the method described by Fiallo et al. (1992). Samples were centrifuged at 14,000 rpm for 5 min and the supernatant discarded. Samples were resuspended in one milliliter of Hank's Buffered Saline Solution (HBSS) and placed on a rocking platform for 30 min. After this, centrifugation was repeated and the supernatant was removed prior to a repeat of this washing step. After the final centrifugation and removal of the supernatant, the cells were resuspended in 100 μL of sterile Milli-Q (Millipore) water. The cells were lysed by subjecting the samples to a freeze thaw cycle (10 min at -80°C, 1 min at +65°C) three times and a final heating at 95°C for 10 min. Two microliters of undiluted or ten-fold diluted lysate was used as a template for PCR.

Cells within the material collected from sediment culture bottles were lysed using MicroLysis reagent (The Gel Company) according to the manufacturer's instructions. Two microliters of the undiluted and ten-fold diluted cell lysis suspension was used as a template for PCR.

PCR

The presence of cyanobacteria, all known *Microcystis* cell-types (toxic or non-toxic) and toxic *Microcystis* (defined by the presence of genes in the toxin biosynthetic pathway) in water and sediment samples were determined by multiplex PCR. The reactions were carried out using four primer sets (CYA, MICR, *mcyB* and *mcyD*) as described in detail previously (Ouellette and Wilhelm, 2003; Rinta-Kanto et al., 2005).

For phylogenetic studies, the primer pair *mcyA*-Cd 1R and *mcyA*-Cd 1F (Hisbergues et al., 2003) was used to amplify 291-297 bp fragments of the *mcyA* gene from one sediment sample collected in 2004 (station 357), from six sediment samples collected in 1971 and from four formalin-preserved samples collected in 1978-79. PCR reactions were carried out in EasyStart tubes (Molecular BioProducts, San Diego, CA, USA) in a final volume of 50 μL containing 0.4 μM of each primer, 300 ng μL^{-1} bovine

serum albumin (Sigma cat # A-7030) (Kirchman et al., 2001), 2.5 U Taq polymerase (Promega), 0.1 % Triton X-100 (Molecular BioProducts), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1 × PCR buffer and 2 µL of undiluted or ten-fold diluted DNA extracted from natural samples. Thermal cycling was completed using an Eppendorf Mastercycler gradient-thermocycler using the following protocol: initial denaturation at 95º C for 10 min, 40 cycles of 94º C for 30 s, 59º C for 30 s, 72º C for 30 s, and a final extension step at 72º C for 5 min. The PCR-amplified *mcyA* fragments were purified by running 10 µL of each PCR product along with 100 bp DNA ladder (Promega, Madison, WI, USA) on a 2 % agarose gel in 1 × Tris-borate-EDTA buffer (Sambrook and Russell, 2001). The presence of the correct-size amplicon was confirmed by ethidium bromide staining and visualizing the gel under UV transillumination.

Construction of the mcyA clonal library and sequencing of mcyA fragments

The *mcyA* DNA bands were excised from the agarose gel and the DNA from the bands extracted using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). Clone libraries were generated using a TOPO-TA Cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cloned inserts of the correct size were verified by *Eco*RI digestion (Promega, Madison, WI, USA).

For the *mcyA* clone library from 2004 sediment samples, 19 clones from each depth (except 20 clones from 6-8 cm depth) were picked for sequencing. Plasmid DNA extraction and high throughput sequencing of the *mcyA* gene inserts was completed at the Clemson University Genomics Institute, using the M13 forward primer site within the cloning vector as the sequencing start point (Invitrogen, Carlsbad, CA, USA).

The *mcyA* gene fragments from extracted DNA from the 1971 sediment samples and formalin-preserved phytoplankton samples were PCR amplified and cloned as described above. Plasmid DNA was extracted using Wizard Minipreps-kit (Promega). Two *mcyA* inserts were sequenced from each sediment sample and six inserts from net tow samples at the Molecular Biology Resource Facility at the University of Tennessee. All sequences from this study have been deposited in GenBank and are listed under accession numbers EF178215 - EF178270.

Sequence analysis

Vector and primer sequences were removed from the *mcyA* DNA sequences and all sequences were manually checked and edited as required prior to the analysis using the Bio Edit software program (Hall, 1999). The *mcyA* sequences from natural samples were examined both at the nucleotide level and at the amino acid level. Individual DNA sequences from natural samples were queried against the GenBank database using BLASTN and BLASTX searches (Altschul et al., 1997) to find similar amino acid sequences from the data base originating from other microcystin-producing cyanobacteria. Prior to phylogenetic analysis, all nucleotide sequences were translated into amino acid sequences. Identical McyA sequences were removed leaving only unique representative sequences in the data set. Clustal W-software (Thompson et al., 1994) was used to align these remaining amino acid sequences with sequences retrieved from GenBank. Phylogenetic reconstruction was performed using the Mega 3.1 software package (Kumar et al., 2004). Phylogenetic relationships between the translated sequences were inferred through a neighbor-joining analysis using a Poisson correction distance. Bootstrap values were determined using 2000 iterations.

qPCR

Eubacterial 16S rDNA copies, Cyanobacterial 16S rDNA copies, *Microcystis* specific 16S rDNA copies and *mcyD* gene copies in environmental samples were quantified using qPCR. The abundance of cells carrying these target genes was also determined through qPCR using a genomic DNA standard. The primers, probes, protocols and standards for cyanobacteria used in this study have been described in detail in Rinta-Kanto et al. (2005). Eubacterial 16S copies in the 2004 sediment samples were quantified as described (Dionisi et al., 2004).

Results

Multiplex PCR

Cyanobacterial 16S fragments were amplified from all three water samples collected in 2004 sediment core collection sites, whereas *Microcystis* specific 16S, *mcyB* and *mcyD* specific fragments were not amplified from any of these samples. Subsequent sampling in August of that year (data not shown, Rinta-Kanto et al. 2005) did reveal PCR positives at these stations for all products.

PCR products were generated with all four primer sets (Cyanobacterial 16S, *Microcystis* 16S, *mcyB* and *mcyD*) from cell material collected from 0-5 cm sediment (2006) sample incubation indicating that potentially toxic *Microcystis* had been released from the sediments and risen to the surface during incubation (Figure 2). Subsequent examination of samples from this material demonstrated cells that were morphologically consistent with *Microcystis* spp. (Figure 2). Cell material collected from the surface of the liquid in the bottle containing sediment from 5-10 cm depth yielded PCR products with cyanobacterial 16S primers and *Microcystis* 16S primers (Figure 3).

qPCR

Across all depths sampled in the 2004 sediments, eubacterial 16S rDNA copy abundance ranged from 9.4×10^9 to 6.3×10^8 copies and cyanobacterial 16S rDNA abundance ranged from 6.2×10^7 to 9.5×10^4 copies per gram wet weight of sediment (Figure 4). Cyanobacterial 16S rDNA copies formed 0.04 – 1.14 % of total eubacterial 16S rDNA copy abundance.

The profiles of cell abundance (reported as *Microcystis aeruginosa* LE-3 cell equivalents) of Cyanobacteria, *Microcystis* and toxic *Microcystis* in the 2004 core samples are presented in Figure 5. In samples from station 357, all cell types were detected at all depths. The cell abundances declined with increasing depth of the sediment and toxic *Microcystis* levels fell below the detection limit (approximately < 200 cells per gram of sediment in this analysis) below 8 cm (in set 1) and between 6-8 cm (in set 2). In

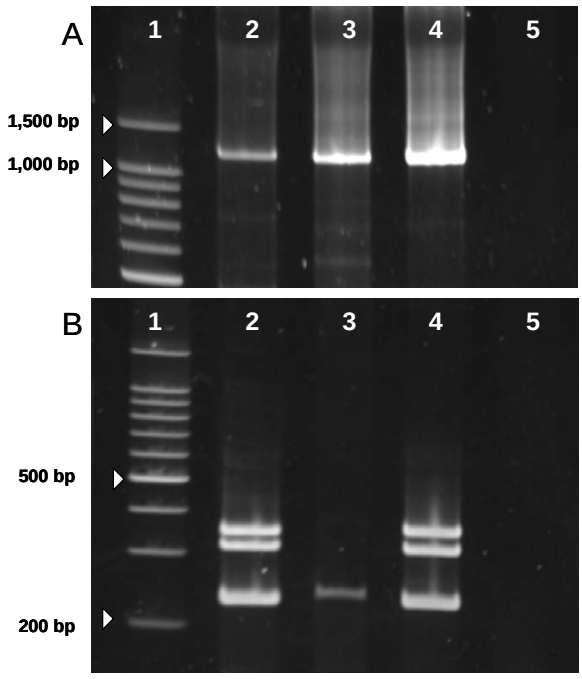


Figure 3. PCR products amplified from biomass that grew in sediment sample incubations. PCR products were amplified with primer sets specific to cyanobacterial 16S rDNA (gel A), *Microcystis* microcystin synthetase genes *mcyB*, *mcyD* and *Microcystis* 16S rDNA (gel B). Lanes (numbering and descriptions are identical for gels A and B) : 1. 100 bp molecular weight ladder; 2. biomass from 0-5 cm sediment; 3. biomass from 5-10 cm sediment; 4. positive control (*Microcystis aeruginosa* LE-3 genomic DNA used as a template); 5. negative control, no template DNA added.

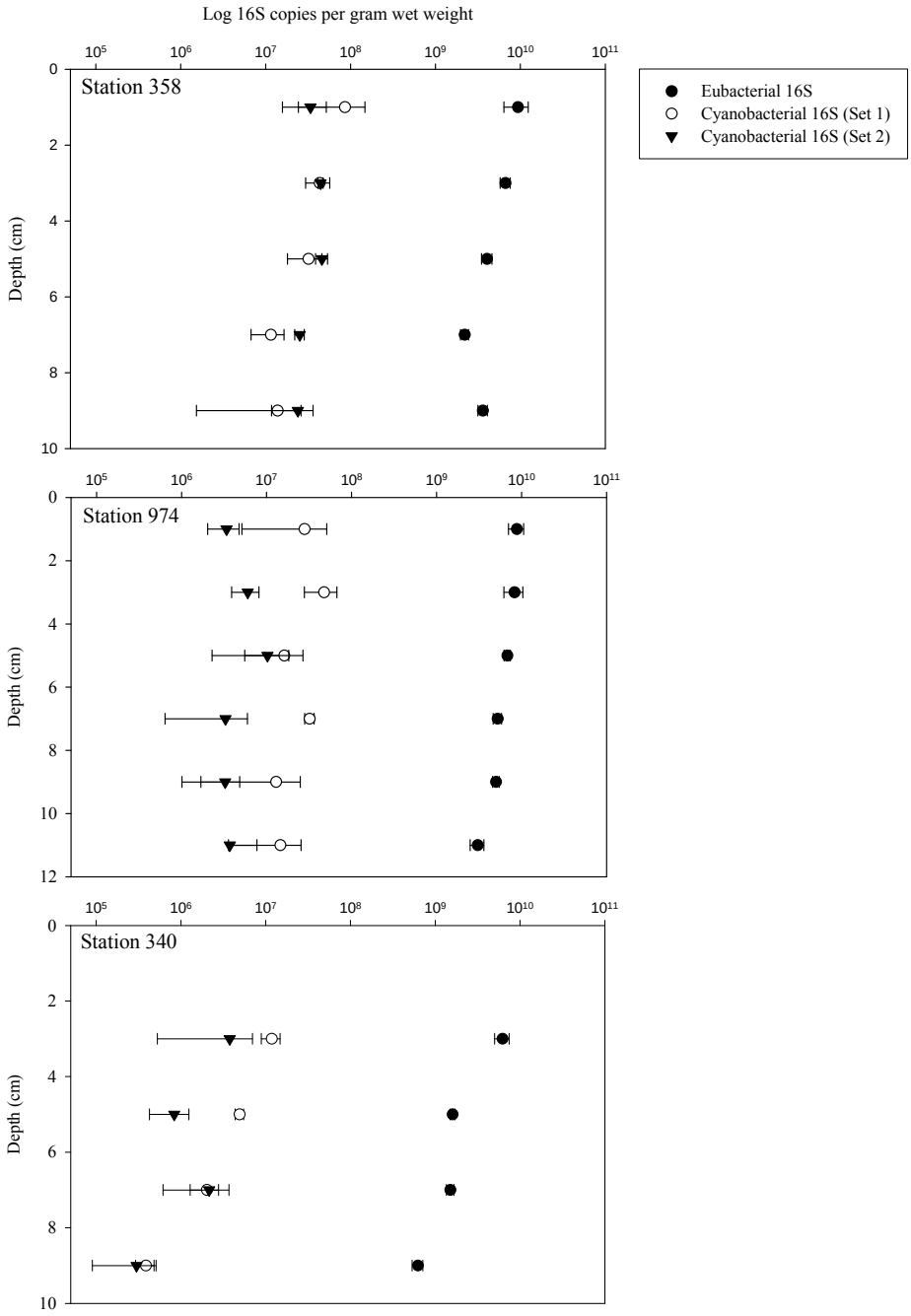
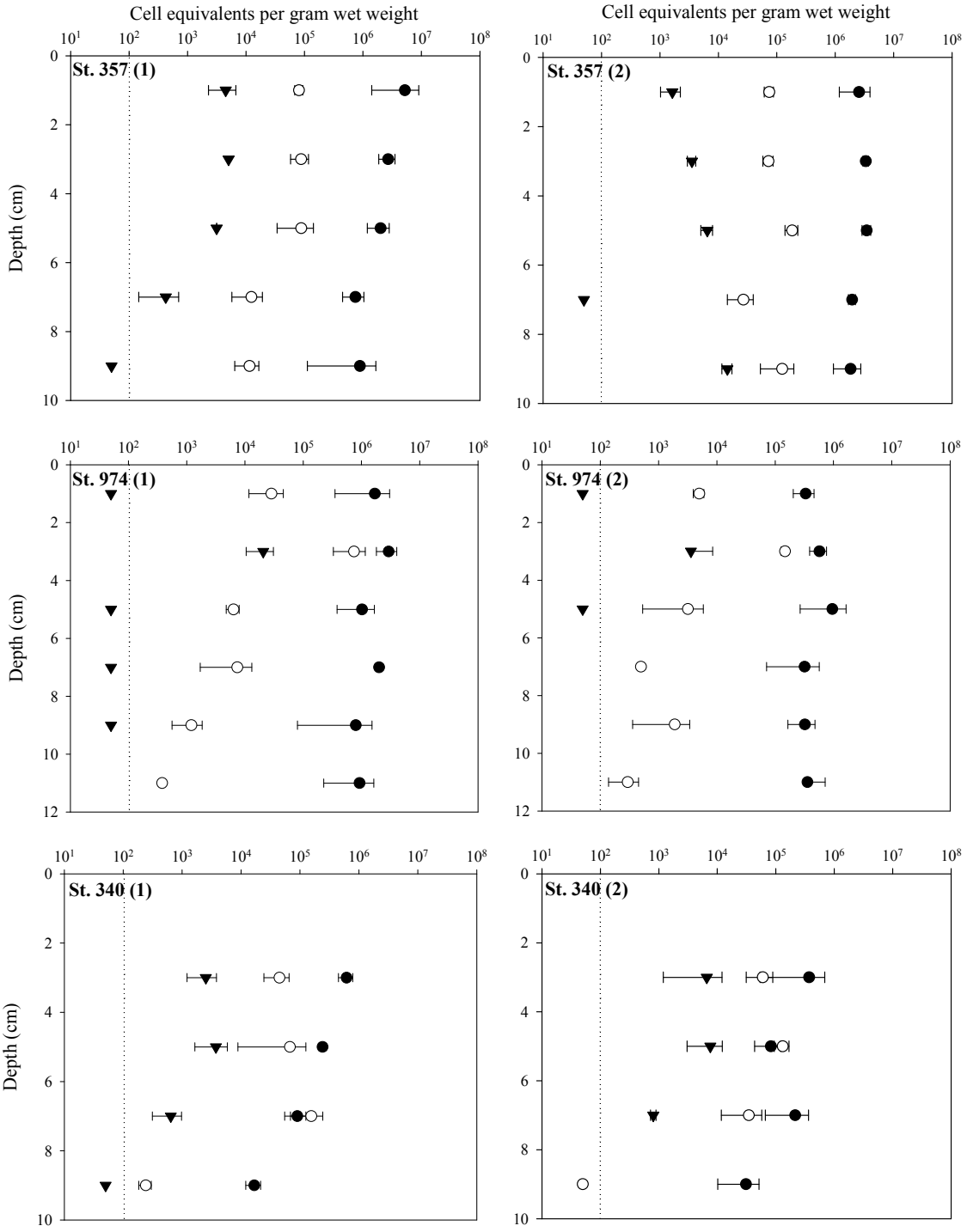


Figure 4. Vertical profiles of eubacterial 16S rDNA copy abundance and cyanobacterial 16S rDNA abundance in 2004 sediment core samples expressed as 16S copies per gram wet weight of sediment. Cyanobacterial 16S copies were determined from two DNA samples (Set 1 and Set 2) extracted from a single sample. Eubacterial 16S copies were determined from one of the DNA samples (Set 2). Error bars represent standard deviation among replicate assays (n=3).

Figure 5. Vertical profiles of the abundance of three cell types in 2004 sediment samples. Cell equivalents were quantified from duplicate DNA samples (labeled as (1) and (2) after the station code) extracted from each sediment sample. Cell abundance is expressed as *Microcystis aeruginosa* LE-3 cell equivalents. Black circles: cyanobacteria, open circles: *Microcystis* (total abundance), black triangles: toxic *Microcystis*. Dotted line represents the detection limit of the qPCR assay (200 cell equivalents per gram of sediment wet weight). Samples containing < 200 cell equivalents per gram of sediment wet weight were given a default value of 50 in order to make these data points visible in the graph. The error bars represent standard deviation among replicate assays (n=3).


Cell equivalents per gram wet weight
Cell equivalents per gram wet weight
St. 357 (1)
St. 357 (2)
St. 974 (1)
St. 974 (2)
St. 340 (1)
St. 340 (2)
Depth (cm)
Depth (cm)
Depth (cm)

the core sample from station 974, toxic *Microcystis* abundance was quantifiable (> 200 cells per gram of sediment wet weight) only at 2-4 cm depth of the sediment. No toxic *Microcystis* was detected in the 10-12 cm deep samples.

The relative abundance of *Microcystis* of total cyanobacteria based on 16S rDNA copies was highest in the sediment core from station 340. At 6-8 cm depth (set 1) and at 4-6 cm depth (set 2) the abundance of total *Microcystis* appears to exceed the abundance of Cyanobacteria.

Clone library and sequencing results

We obtained a total of 89 *mcyA* sequences from the 2004 sediment sample. The *mcyA* sequences from the top of the sediment to the depth of 10 cm were 92 – 100 % identical among themselves. Compared to nucleotide and amino acid sequences of cultured isolates deposited in GenBank the sequences from 2004 sediments were 92 – 100 % identical to *Microcystis*-sequences at the nucleotide level and over 92 % at the amino acid level, with identities between 69 – 81 % (nucleotide) for *Nostoc*, *Anabaena* and *Planktothrix*.

All *mcyA* sequences amplified from the 1971 sediment samples were 96 – 100 % identical with *Microcystis mcyA* nucleotide sequences and 94 – 100 % identical with *Microcystis* McyA amino acid sequences. Identities with other common microcystin producers (*Anabaena* spp., *Planktothrix aghardii*, *Nostoc* sp.) ranged from 69 – 76 % at nucleotide level. Three *mcyA* amplicons out of six that were sequenced from the 1978-1979 net tow samples had unique sequences and they showed 86 – 99 % (nucleotide and amino acid) identity to *Microcystis mcyA*.

Phylogenetic analysis

We performed a Neighbor-Joining analysis of the unique McyA sequences generated in this study to assess the phylogenetic relationship between these sequences and other published sequences. The resulting phylogenetic tree is presented in Figure 6. As references we retrieved McyA sequences of isolates of *Anabaena* spp., *Nostoc* sp.,

Figure 6. A Neighbor-Joining tree of McyA sequences translated from *mcyA* sequences from natural samples. The tree is based on 2000 iterations. Bootstrap values >50 % are presented in as black circles in the corresponding nodes of the tree. Light blue triangles: formalin-preserved samples. Red triangles: 1971 sediment samples. Blue squares: 2004 sediment, 0-2 cm depth; yellow squares: 2004 sediment, 2-4 cm depth; green squares: 2004 sediment, 4-6 cm depth; pink squares: 2004 sediment 6-8 cm depth; brown squares: 2004 sediment, 8-10 cm depth. Numbers in parenthesis on the right side of the colored symbols indicate the number of identical sequences found in the same sample. The scale bar indicates substitutions per site.


(9) (4) (4) (8)
Microcystis aeruginosa PCC7806 (AAF00960)
(3)
Microcystis aeruginosa PCC 7941 (MAE515460)
Microcystis aeruginosa LE-3 (DQ379709)
(5) (3) (3) (13)
(2)
Uncultured L. Erie cyanobacterium clone (DQ379638)
Uncultured L. Erie cyanobacterium clone (DQ379694)
(3)
Uncultured L. Erie cyanobacterium clone (DQ3790687)
Anabaena sp. 90 (CAD56450)
Anabaena flos-aquae NIVA-CYA83 (CAD56455)
Planktothrix sp. NIVA-CYA34 (PSP515474)
Nostoc sp. IO-102-I (AY566856)
0.02

Planktothrix aghardii, *Microcystis* spp. from GenBank data base. In addition we included McyA sequences from the Lake Erie water column generated in our previous study (Rinta-Kanto and Wilhelm, 2006) (GenBank accession numbers of these sequences are included in the phylogenetic tree). All McyA sequences from natural samples clustered together with *Microcystis* sequences in the neighbor joining tree as expected based on the % identity of the McyA sequences. Despite relatively high identity to *Microcystis* McyA sequences, the sequences from natural samples showed diversity among themselves which can be seen in the shallow branching of the phylogenetic tree in the *Microcystis* cluster.

Moreover we amplified unusual *Microcystis*-like *mcyA* sequences containing six extra nucleotides (which translate to two amino acids) from the DNA extracted from the 1971 and 2004 sediments. These sequences are 95 – 99 % identical (at the amino acid level) to unique sequences originating from DNA samples amplified from water samples collected elsewhere in the lake in 2004 (Rinta-Kanto and Wilhelm, 2006).

Discussion

The development of an understanding of the factors driving the reoccurrence of *Microcystis* blooms as well as the factors which control the diversity of cyanobacterial populations within Lake Erie ultimately relies on defining the source inocula for the pelagic cyanobacterial population within each season. Ouellette et al. (2006) demonstrated that *Microcystis* was well distributed throughout the surface waters of Lake Erie during summer months and that it was present in the water column even during non-bloom conditions. In the current study we have addressed the potential role of sediments as a reservoir for these populations. Moreover, we have also examined two sets of historical samples from the 1970s (archived sediments and formalin preserved net tow samples) to determine whether the current day population of microcystin producers has been present in the lake during the large blooms in the 1970s.

Using the eubacterial and cyanobacterial 16S rDNA copy abundance as a proxy for the abundance of cells from these populations, we can conclude that cyanobacteria

form only a fraction of the total bacterial population within Lake Erie sediments. This is not surprising in that it is doubtful that cyanobacteria could maintain high growth rates in this environment (as they are phototrophs), while hetertrophic bacteria would no doubt proliferate. The results presented here indicate however, that cyanobacteria, including toxic and non-toxic *Microcystis* spp. can be found as deep as 12 cm in the sediment. This is in concordance with studies from other freshwater bodies where *Microcystis* communities have been shown to exhibit pelagic-benthic oscillation as a part of their life cycle (Takamura et al., 1984). Preston et al. (1980) demonstrated using ^{15}N-labeled *Microcystis* that the populations over-wintering in the sediment can serve as inocula for succeeding pelagic populations. We are unable to estimate the time period which our 10 cm sediment core represents because sediment deposition rates in Western Lake Erie are extremely difficult to determine due to frequent sediment resuspension events in these shallow waters. The dormant, over-wintering colonies on the sediment surface may also be transported deeper in the sediments due to sediment resuspension and bioturbation, such as burrowing activities of invertebrates (Takamura et al., 1984; Ståhl-Delbanco and Hansson, 2002). Recruitment of *Microcystis* from sediments is thought to be induced by environmental stimuli (such as altered light levels, temperature, nutrient levels, oxygen availability), bioturbation due to activities of invertebrates burrowing into the sediments (Ståhl-Delbanco and Hansson, 2002), or through passive sediment resuspension or sediment resuspension facilitated by wind driven mixing (Schelske et al., 1995; Verspagen et al., 2004). At the time of sampling in 2004 no *Microcystis* were detected by PCR in the epilimnion of the lake at the sampling sites where the sediment cores were collected. Our finding could indicate that at the time of sampling in 2004 *Microcystis* in Lake Erie sediments had not yet been resuspended in the water column (at least not in detectable quantities), or that we failed to capture pelagic *Microcystis* at the time due to our sampling design. Regardless, we now know that it is possible for cells from the sediments in Lake Erie's western basin to grow pelagically if introduced into the water column. The culture experiment demonstrated that the *Microcystis* in the Lake Erie surface sediments are able to float to the surface, suggesting that at least a fraction of *Microcystis* residing in the sediment is viable and able to regain their buoyancy. This

further supports the hypothesis that the sediments can act as a dynamic reservoir of *Microcystis* in Lake Erie.

Given this information, as well as to gain some insight into the composition of the microcystin-producing community three decades ago, we analyzed surface sediments from samples collected in 1971. The amplification of *Microcystis*-like *mcyA* sequences from sediment and pelagic samples collected from around the lake in the 1970s clearly demonstrates that toxic cyanobacteria were present more than thirty years ago when these samples were taken. The presence of *Microcystis*-like sequences in the top 10-12 cm of the sediment also in 2004 provides a perspective on the diversity of microcystin-producers in Lake Erie, suggesting that a similar community of toxic *Microcystis* spp. has been the dominant microcystin producer in the lake for decades. This finding is in some contrast to reports suggesting that *Anabaena* spp. were the dominant cyanobacteria in Lake Erie in 1970s (Makarewicz, 1993). *Anabaena* strains are capable of producing a range of cyanotoxins, including microcystin (Carmichael, 2001). The *mcyA* primer set utilized in this study has been demonstrated to amplify *mcyA* fragments from microcystin-producing *Anabaena* spp. (Hisbergues et al., 2003). Therefore it is somewhat surprising we found no *Anabaena*-like *mcyA* sequences in our clone libraries, however it is also possible that the *Anabaena* strains present in Lake Erie in the 1970s were not producing microcystin, in which case we would not have been able to detect these cyanobacteria with the methods employed in this study. It is also possible that we did not detect the presence of other microcystin producers in 1970s samples due to low number of clones analyzed (two clones were sequenced from each 1970s sediment sample and two clones were sequenced from each 1970s formalin sample).

The phylogenetic analysis shows that although the natural assemblage of *Microcystis*-like McyA sequences is diverse, bearing from 1 to 10 mostly randomly distributed amino acid differences among themselves, there are also distinctive similarities among these samples even though they were collected three decades apart. In the previous study we discovered unique *Microcystis*-like *mcyA* sequences with six extra nucleotides (corresponding to two amino acids) in samples collected from the western basin of Lake Erie (Rinta-Kanto and Wilhelm, 2006). *Microcystis*-like *mcyA* sequences

containing the same 6 extra bases in an identical position in the sequence were also found in this study from the 2004 and 1971 sediments indicating the persistence of these unique sequences in Lake Erie. Even though the sequences with 6 extra bases (or two extra amino acids) are over 90 % similar to *Microcystis* sequences, based on the sequence information available in public databases, we can not tell if they truly belong to *Microcystis* spp. or whether they originate from a still unknown microcystin producer, as examinations of cultured representatives have not yet yielded similar sequences.

Although this study provides insight into the composition of the cyanobacterial community in the lake, it is not a comprehensive inventory of the past and present microcystin-producing community in the lake, since we did not include samples from embayments, which may generate suitable habitats for other *Microcystin*-producers (Rinta-Kanto and Wilhelm, 2006) as well as other toxic cyanobacteria. The relatively high similarity of the sequences found in the sediment and net tow samples analyzed here strongly suggests that the *Microcystis* community in the lake is persistent on the temporal scale of decades and as such is relatively stable. Because sediments of the lake may serve as a sink and a source of *Microcystis* in the water column, our observations suggest that management plans must address the persistence and stability of these populations.

Acknowledgments

We thank the Captains and crew of the CCGS *Limnos* as well as George Bullerjahn, Hunter Carrick, Dan Walsh and Greg Boyer for assistance and helpful discussions as well as Wayne Carmichael for providing the *M. aeruginosa* LE-3 isolate. This article includes research supported by funds from the National Oceanic and Atmospheric Administration Coastal Ocean Program (MERHAB-LGL, #NA 160 P2788), an Oceans and Human Health external grant (NA05NOS4781251), the National Science Foundation (DEB-0129118), and Helsingin Sanomat Centennial Foundation (to JMR-K).

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Part V

GENETIC POTENTIAL AND THE INFLUENCE OF ENVIRONMENTAL PARAMETERS ON TOXIN PRODUCTION IN LAKE ERIE *MICROCYSTIS* SPP.

This part is a version of a manuscript with same title prepared to be submitted for publication in journal Environmental Microbiology by Johanna M. Rinta-Kanto, Jennifer M. DeBruyn, Elizabeth Konopko, Richard A. Bourbonniere, Gregory L. Boyer, Steven W. Wilhelm

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions in this paper were 1) Collection of most of the environmental DNA and chlorophyll samples for this study, 2) Performing the DNA based analyses for the samples 3) Formulating hypotheses, 5) Gathering the raw data from for statistical analysis and analyzing the output from the statistical analysis 6) Gathering the background literature and, 7) Most of the writing of the manuscript.

Introduction

The occurrence of *Microcystis* in the lower Great Lakes of North America, especially in Lake Erie, has been observed annually over the past decade (Brittain et al., 2000; Vincent et al., 2004; Rinta-Kanto et al., 2005). In the 1960s cyanobacterial blooms were common in the severely eutrophied Lake Erie. Radical reductions in external phosphorus loading since 1970s were implemented to reduce the cyanobacterial biomass and to diminish the extent and duration of the anoxia in the central basin of the lake (Makarewicz and Bertram, 1991). By late 1980s water quality had improved substantially, and algal biomass had decreased up to 89 % in off-shore waters (Makarewicz, 1993). The increased frequency of cyanobacterial blooms since the mid-1990s has been considered as a sign of eutrophic conditions returning to the lake (Conroy et al., 2005). And although several theories have been put forth as to how changes in lake biology and geochemistry may influence the community structure of phytoplankton (Arnott and Vanni, 1996; Vanderploeg et al., 2001), the reasons for the seasonal proliferation of *Microcystis* in Lake Erie are unresolved.

In natural assemblages non-nitrogen fixing *Microcystis* cells exist in colonies consisting of single cells held together by a mucilaginous matrix which can float on the water surface with the help of intracellular gas vesicles (Komarek, 2003). The toxin-producing (toxic) genotypes of *Microcystis* carry a 55 kb microcystin synthetase (*mcy*) gene cluster required for production of the toxin microcystin, whereas non-toxic genotypes lack this gene cluster and the ability to produce the toxin (Kaebernick and Neilan, 2001). Microcystins are a chemically diverse group of cyanotoxins which in humans have been known to have caused gastroenteritis, liver damage and, in the most severe case, the death of 60 hemodialysis patients in Brazil in 1996 (Pouria et al., 1998; Kuiper-Goodman et al., 1999; Sivonen and Jones, 1999). Some evidence of tumor-promoting activity of microcystins also exists (Falconer, 1991; Dietrich and Hoeger, 2005).

Due to growing concerns regarding potentially toxic *Microcystis* blooms, several studies have addressed the factors which influence the proliferation and toxin production of *Microcystis*, including examinations of the relationships between limnological factors and the abundance of *Microcystis* in natural assemblages (Wicks and Thiel, 1990; Kotak et al., 2000; Oh et al., 2001; Graham et al., 2004; Giani et al., 2005). In complex aquatic systems the conditions that allow development of *Microcystis* blooms and induce toxin production are usually the sum of a variety of factors. Studies of *Microcystis* in natural populations have suggested some of the key factors affecting cell proliferation and toxin production including increased phosphorus loading, the stoichiometric ratio of nitrogen and phosphorus, solar radiation, pH and temperature of the water, primary production and oxygen saturation (Wicks and Thiel, 1990; Kotak et al., 2000). Relying on traditional methods for cell quantification, these studies have always addressed the total *Microcystis* population, which in natural assemblages is known to consist of phenotypically identical toxic and non-toxic genotypes (Vezie et al., 1998; Kurmayer and Kutzenberger, 2003). As such there is currently no information on the factors which may specifically affect the abundance of toxic genotypes of *Microcystis* in natural environments and how these environmental factors influence toxin production. This lack of quantitative information on specific genotypes has been partially caused by methodological constraints. To specifically address the toxin-producing *Microcystis*, quantitative molecular methods must be employed. Quantitative real-time PCR has been successfully applied in studies of the abundance of toxic and non-toxic genotypes of *Microcystis* and other cyanobacteria cultures and in natural samples (Kurmayer et al., 2003; Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003; Rinta-Kanto et al., 2005).

The present study is the first one to combine the use of quantitative PCR (qPCR) to quantify cyanobacteria, total *Microcystis* and toxic genotypes of *Microcystis* and to relate this data to environmental variables through correlation analysis across spatial and temporal scales. To accomplish this we have collected cell abundance data and environmental data during the summer seasons of 2003, 2004 and 2005. The first goal of this study was to determine the proportion of the natural *Microcystis* population which is potentially toxic (in this case as demonstrated by the presence of a gene in the

microcystin biosynthetic pathway). The second goal was to elucidate how environmental parameters influence the proliferation of toxic *Microcystis* relative to the total *Microcystis* and cyanobacterial communities. With an over-arching hypothesis that toxin production is linked to cell proliferation, our approach in this study may ultimately shed light on the (as of yet) unknown role of the toxin in *Microcystis* biochemistry and ecology.

Materials and Methods

Sample collection

Samples used to generate data for this study were collected from Lake Erie on board the *C.C.G.S. Limnos* and, additionally in August 2003, on board the *R/V Lake Guardian* and a research support craft from Lake Erie Center (Toledo, OH) as previously described (Rinta-Kanto et al., 2005). Samples from 1 m depth were collected using a surface water pump (on board *C.C.G.S. Limnos*) or Niskin bottles (*R/V Lake Guardian*). Samples from deeper than 1 m were collected using Niskin bottles mounted on a rosette sampler. Temperature was determined using the deckboard profiler on the *C.C.G.S. Limnos.* Water column pH was collected on deck of the ship after sample collection using a standard lab probe (Fisher Scientific).

Natural water samples for DNA extraction were collected by filtering known volumes of lake water on 0.22-µm nominal pore-size polycarbonate filters (Poretics). Filter funnels were rinsed with 10 % sodium hypochlorite solution and distilled water between samples to reduce the potential for cell carryover between samples. Filters were stored frozen (-20 °C) until DNA extraction. DNA extraction was carried out as described previously (Rinta-Kanto et al., 2005).

qPCR assay

The abundance of all cyanobacteria, all *Microcystis* and toxic *Microcystis* were independently determined using the qPCR assays described in detail in Rinta-Kanto et al. (2005). To standardize target gene quantities and cell quantities in samples, both single

copy insert plasmids as well as whole cell genomic standards were used to establish a standard curve as described in the previous study. In this study all cell densities in natural samples are reported as *Microcystis aeruginosa* LE-3 equivalents. Target gene quantities are not reported here because they are not equivalent to the abundance of the cells that carry these genes. To elucidate the relationship between target gene copies in cells of *Microcystis aeruginosa* LE-3, we quantified the abundance of cyanobacterial 16S copies and *mcyD* genes in genomic DNA extracted from a known quantity of *Microcystis aeruginosa* LE-3 cells. The DNA from M. aeruginosa LE-3 was prepared as described in Rinta-Kanto et al. (2005) and the qPCR analyses were completed as described in the previous study.

Pigment analyses

Water column chlorophyll-*a* concentrations were used as a proxy for phytoplankton biomass. Size-fractionated chlorophyll a was estimated in duplicate samples of lake water filtered on 0.2-µm, 2.0-µm and 20-µm nominal pore-size polycarbonate filters (47-mm diameter, Millipore) after extraction (*ca.* 24 h, 4 °C) in 90 % acetone. Chlorophyll-*a* retained on the filters was quantified with either an AU-10 or TD-700 fluorometer (Turner Designs; Sunnyvale, CA) using the non-acidification protocol (Welschmeyer, 1994).

Discrete water samples (1 L) were collected at 1 m depth and filtered onto 47-mm Whatman 934-AH glass fiber filters for phycocyanin determinations. Phycocyanin concentrations were estimated fluorometrically using a modification of the method of Abalde et. al. (1998) and Siegelman and Kycia (1978). Phycocyanin was extracted from these filters by freezing the samples at -21°C and thawing at 4°C three times in 10 mM phosphate buffer (pH 6.8) under dim light. The extract was clarified by centrifugation at 22,000 x g for 15 minutes and the phycocyanin concentration in the supernatant determined by fluorescence using a Turner Designs 10-AU fluorometer equipped with a 577 nm band pass excitation filter and 660 nm cutoff emission filter with a cool white light source.

Microcystin quantification

Microcystin in water samples was determined with protein phosphatase inhibition assays. Samples were collected on GF/F (Whatman) filters. The assays were run in 96-well plates containing 0.1 mU enzyme (recombinant protein phosphatase 1A, catalytic subunit, Roche Applied Science), 1.05 mg para-nitrophenyl phosphate (Sigma) and 10 µl of sample or microcystin-LR (Sigma Biochemical) using the method of Carmichael and An (1999). The rate of phosphate hydrolysis was calculated from the change in absorbance at 405 nm over 1 h, and then compared to the control (no added microcystin-LR) and to standards containing between 6 and 40 µg L^{-1} microcystin-LR. Blanks (no enzyme, no toxin), unknowns, standards, and controls were all run in duplicate.

Nutrients

Total phosphorus (TP) (filtered and unfiltered), surface reactive phosphorus (SRP), total nitrogen (TN) (filtered and unfiltered), NO_3, NH_3 and SiO_2 were determined from lake water samples in 2004 and in 2005 as described in DeBruyn et al. (2004). Nutrient concentrations in the filtered fraction refer to the dissolved nutrients passing through 0.22-µm nominal pore-size filter, whereas the unfiltered concentrations were measured directly from the lake water.

Environmental variables used for statistical analysis

The abundance of cyanobacteria, total *Microcystis* and toxic *Microcystis* cells for each sampling location was the arithmetic mean of triplicate PCR reactions run for each sample. Total chlorophyll a concentration (chlorophyll a retained on a 0.2-µm nominal pore-size filter), and size fractionated chlorophyll concentrations (size classes 0.2 – 2 µm, 2 – 2 µm, and >20 µm) were calculated the as arithmetic mean of single measurements from duplicate samples; microcystin-LR concentration was an arithmetic mean of duplicate measurements from single samples; and phycocyanin concentration, pH, TN (dissolved and dissolved + particulate), NO_3, NH_3, SiO_2, SRP, TP (dissolved and dissolved + particulate) were results from single measurements. Molar TN:TP ratio was calculated using the molar concentrations of dissolved TN and TP.

Statistical analysis

Since assumptions of normality were not met in all variables, the nonparametric Spearman Rank Correlation coefficient was calculated as a measure of correlation between all possible pairs of variables. Analysis was completed using the NCSS statistical analysis software package. Correlations warranting $P < 0.05$ were considered significant in this analysis.

Results

The stations which were occupied in Lake Erie during the 2003 – 2005 sampling are indicated on the map (Figure 1). Environmental data collected from the stations are summarized in Table 1 and 2 (All tables in appendix at the end of part). Cell abundances quantified in samples collected from different locations in western basin in the summer seasons of 2003-2005 are presented in Figure 2 and discussed further below.

The relationship between the abundance of target genes and cells

Linear regressions between *Microcystis aeruginosa* LE-3 cells and the abundance of cyanobacterial 16S copies and *mcyD* genes are presented in Figure 3a and 3b. The results suggest that one cell carries as many as 90 16S rRNA genes and 35 *mcyD* genes. Based on this data in can be inferred that there are up to three 16S rRNA copies per genome, while a cell may carry 35 copies of its genome.

Community composition

The cell abundance data presented in Figure 2 was used to determine the relative abundances of total *Microcystis* and toxic *Microcystis* as well as the relative abundance of toxic genotypes within the *Microcystis* population (Table 3). The relative abundance of *Microcystis* in the cyanobacterial population shows seasonal variability between months and years. Of the three years studied here, the relative abundance of total *Microcystis* was lowest through the sampling season in 2005. The relative abundance of toxic *Microcystis* did not exceed 8 % of the total cyanobacterial abundance in any of the western basin

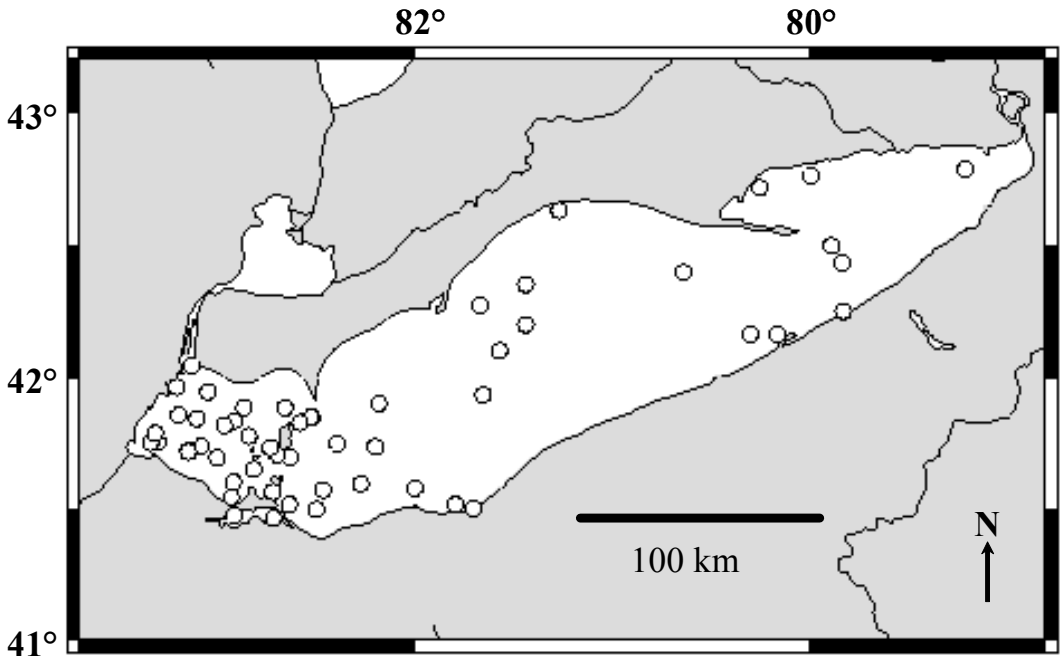

Figure 1. Sampling locations 2003-2005.

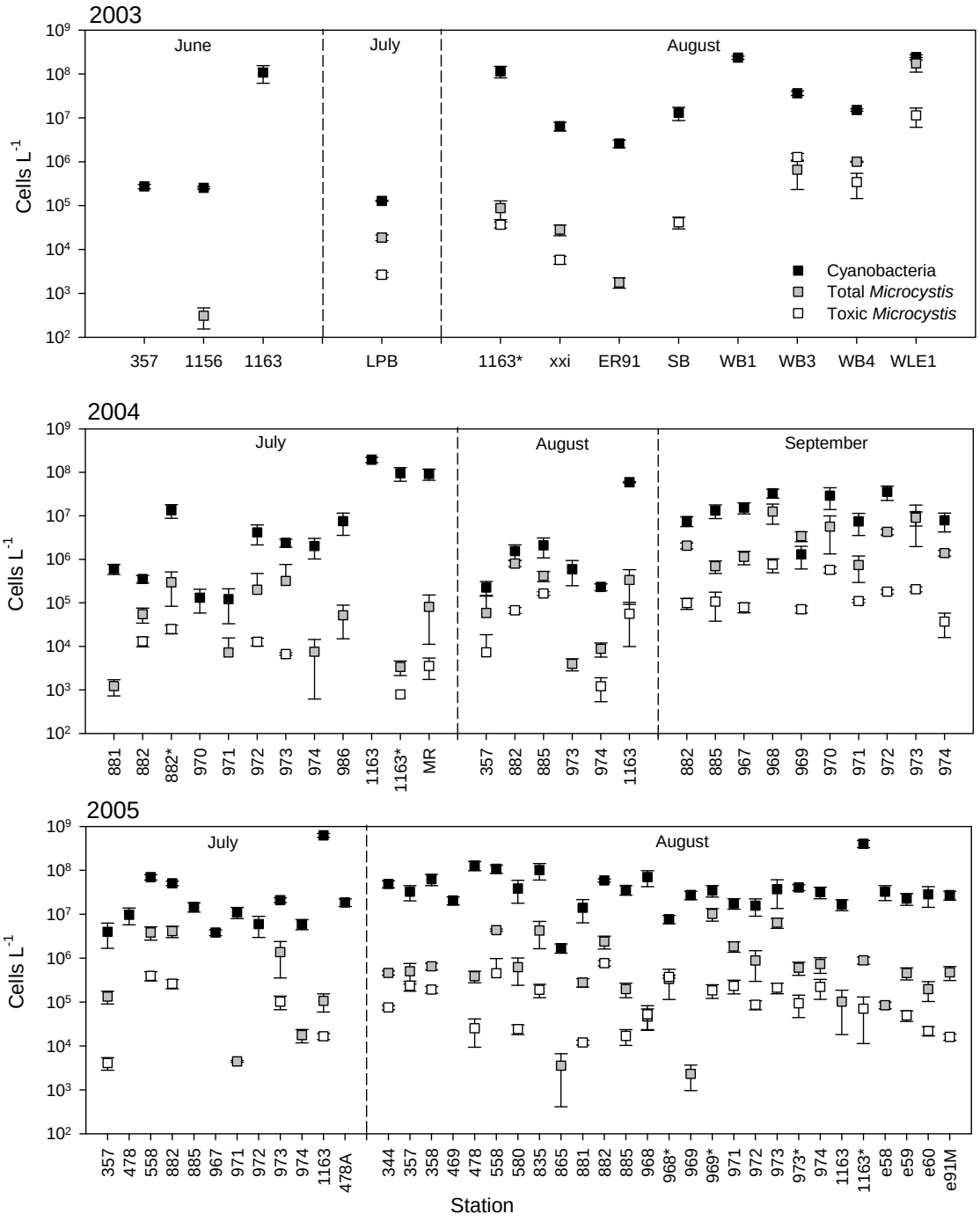

Figure 2. Cell abundances on western basin sampling locations in 2003-2005. Station numbers are organized according to their codes for each month. An asterisk indicates that same station was visited twice at separate times during one month. Error bars represent standard deviation among replicate assays (n=3).

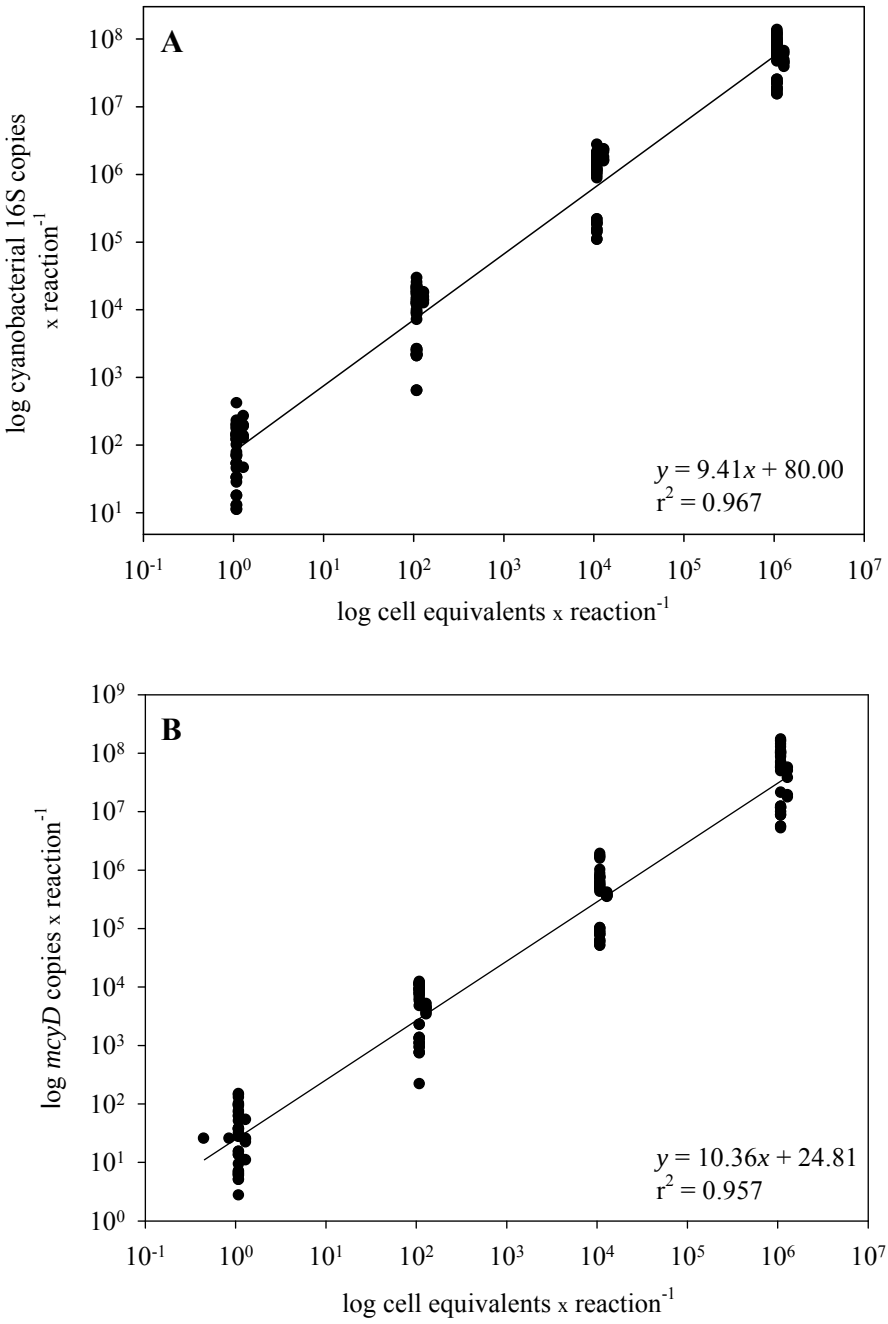

Figure 3. Linear regression between target gene copy abundance and *Microcystis aeruginosa* LE-3 cell equivalents. Graph A: Target gene cyanobacterial 16S rRNA gene. Graph B: target gene *mcyD*. Cell equivalents correspond to a quantity of DNA that was extracted from a known quantity of *Microcystis aeruginosa* LE-3 cells.

samples. Toxic *Microcystis* showed the greatest relative abundance in the total cyanobacterial population and in the total *Microcystis* population in August of each sampling year.

Abundance of Cyanobacteria, total Microcystis and toxic Microcystis

The Spearman Rank Correlation coefficient (r_s), number of samples (n) and probability values (P) for each correlation are presented in Table 4 (entire lake data set) and 5 (western basin data set).

In the entire lake data set the total abundance of cyanobacteria showed significant positive correlation with total *Microcystis* as well as toxic *Microcystis* cell abundance. Also, phycocyanin concentration was positively correlated with the abundances of total cyanobacteria and total *Microcystis*. Surface temperature and the temperature at sampling depth (temperatures at surface and other depths) showed a positive correlation with the abundance of total cyanobacteria, while the abundance of toxic *Microcystis* correlated positively with just surface temperature. Total cyanobacteria and total *Microcystis* cell abundance were negatively correlated with NO_3, NH_3, TN and TN:TP ratio. Toxic *Microcystis* cell abundance yielded a significant negative correlation with NO_3. TN showed a negative correlation with the relative abundance of toxic genotypes in the *Microcystis* population ($r_s = 0.6326$, n = 36, P = 0.0309). TP (dissolved and dissolved + particulate fractions) showed a significant positive correlation with cyanobacteria and total *Microcystis* cell abundance, while SRP was positively correlated ($P < 0.05$) only with total abundance of cyanobacteria. The relative abundance of total *Microcystis* in the cyanobacterial population showed a significant positive correlation with dissolved TP ($r_s = 0.3118$, n = 52, P = 0.0244), whereas the relationship was not significantly correlated with the relative abundance of toxic *Microcystis* ($r_s = 0.0952$, n = 39, P = 0.5645).

In the western basin data set the total abundance of cyanobacteria showed significant positive correlation to total *Microcystis* as well as toxic *Microcystis* cell abundance. Phytopigments, chlorophyll-*a* and phycocyanin correlated with the cell abundance of cyanobacteria and total *Microcystis*. Surface temperature was significantly correlated only to total cyanobacterial abundance. SiO_2 concentration yielded a

significant positive correlation with total cyanobacterial abundance. In the entire lake dataset and in the western basin data set, no correlation was seen between pH and cell abundance.

Relationship of microcystin with environmental variables

The r_s, n and P values for each correlation are presented in Table 6. In the entire lake data set microcystin-LR concentration (reported as microcystin-LR equivalents) yielded a significant positive correlation with the cell abundance of cyanobacteria, total *Microcystis* and toxic *Microcystis*. A significant positive correlation was also found between microcystin concentration and the relative abundance of *Microcystis* (r_s = 0.4233, n = 69, P = 0.0003) and toxic *Microcystis* (r_s=0.3378, n=58, P=0.0095) in the cyanobacterial population. Chlorophyll *a* concentrations in > 0.2 μm, 2.0 – 20 μm and > 20 μm size classes were positively correlated (P < 0.0001) with microcystin. Of nutrients, SiO_2, SRP and TP showed significant positive correlation with toxin, whereas the TN:TP ratio yielded a significant negative correlation.

In the western basin microcystin concentration yielded a positive correlation with the abundance of total *Microcystis*. Microcystin also yielded significant positive correlation with the relative abundance of total *Microcystis* (r_s = 0.6960, n = 35, P < 0.0001) and toxic *Microcystis* (r_s = 0.5907, n = 29, P = 0.0007) in the cyanobacterial population. Total chlorophyll-*a* concentration (> 0.2 μm size class) and phycocyanin yielded significant positive correlations with toxin. Microcystin concentrations were positively correlated with TP (P < 0.05), while the TN:TP ratio yielded a significant negative correlation.

Discussion

Lake Erie is geographically divided into three basins: eastern, central and western. The western basin of the lake is the shallowest of the three basins and due to the vicinity of urban centers such as Detroit and Toledo it is highly influenced by human activities (Makarewicz and Bertram, 1991). Although toxic *Microcystis* is now known to have

spread throughout the lake, the largest occurrences of *Microcystis* blooms in the past decade have been within the western basin (Brittain et al., 2000; Rinta-Kanto et al., 2005; Ouellette et al., 2006). As such, we have analyzed the data covering the entire lake but also separately for the western basin so that any anomalies may become apparent.

Toxic *Microcystis* genotypes form less than 10 % of the total cyanobacteria in the western basin of Lake Erie and generally coexist with non-toxic genotypes in the *Microcystis* populations. In a previous study the proportion of toxic genotypes of the total *Microcystis* population was estimated to be 1.7 – 71% (Kurmayer and Kutzenberger, 2003) from colonies collected from Lake Wannsee (Germany). Most of our estimates fall within this range, which seems reasonable considering that the analysis was carried out independently, using different target genes and in a different location. It should be noted that in our data set some data points yielded relative abundances for *Microcystis* > 100 % of the total cyanobacterial abundance. This likely results from using the 16S rRNA gene as a target gene for quantification of both cyanobacteria and *Microcystis* and the variable copy number of 16S rRNA operons in cells in natural populations. The 16S rRNA operon copy number in cyanobacteria is commonly more than one due to rRNA operon multiplicity (Acinas et al., 2004) and polyploidy. For example, *Synechococcus* and *Synechocystis* strains can carry 6-10 identical copies of their genomes (Castenholz et al., 1992). In the case of *Microcystis aeruginosa*, estimates based on the results of this study suggest that there are three copies of the 16S rDNA gene per genome, but as many as 35 genomes per cell. As such it is anticipated that some variation in the results will occur due to this variance in genetic composition.

In the entire lake data set, the cell abundances of all three cell types correlated among themselves as well as with microcystin concentration, but in the western basin only total *Microcystis* abundance correlated with microcystin. In the western basin the maximum cyanobacterial abundance did not coincide with maximum microcystin concentration, which can at least partly be explained by the presence of a multitude of other cyanobacteria in the western basin of Lake Erie in the summer months (Wilhelm, unpublished data). Secondly, although the abundance of toxic *Microcystis* was strongly correlated to the abundance of cyanobacteria and total *Microcystis*, the abundance of

toxic genotypes of *Microcystis* did not correlate with microcystin concentration in the western basin. The reason for this may be that conditions ideal for cell growth alone are not enough to trigger toxin production. Our data from the natural population does not directly agree with previous culture based studies, which have suggested a link between cell cycle and toxin production, suggesting maximal toxin production takes place at periods of maximal growth (Orr and Jones, 1998; Lee et al., 2000). Indeed, the complete lack of understanding of the role of microcystin in cell physiology leaves us unable to hypothesize about how such mechanisms may even function. The potential presence of other microcystin-producing cyanobacteria (*e.g. Planktothrix* sp., *Anabaena* sp.) may also be reflected in the weak positive correlation between microcystin and the cell abundance of toxic *Microcystis*. As well, the lack of strong correlation may be also affected by the rather small sample size (n=29) used in the correlation analysis for the western basin data set.

In the whole lake data set, the water temperature was strongly correlated with total cyanobacterial abundance and the abundance of toxic *Microcystis*, although the water temperature showed no correlation with microcystin concentration. Temperature has been shown to have a positive effect on both growth rates and cell toxicity in culture-based studies (Codd and Poon, 1988; Wicks and Thiel, 1990). Observations from natural populations supporting our findings suggest that temperature can affect the biomass of the cells but not toxin production; water temperature showed positive correlation with *Microcystis* abundance and yielded no correlation with microcystin in two previous studies addressing environmental factors and the abundance of *Microcystis* and concentrations of microcystin in small Canadian lakes (Kotak et al., 1995; Kotak et al., 2000).

Surprisingly, no correlation was seen between the pH and any of the cell types quantified in this study. In Lake Erie, *Microcystis* proliferation began to occur annually in 1995, coincidental to a net drop in the total alkalinity of the western basin (Barbiero et al., 2006) and seems to annually reoccur late in the season after blooms of other phytoplankton would have presumably decreased dissolved CO_2 concentrations. Cyanobacteria are generally thought to be able to thrive in an environment with low CO_2

and high pH with help of efficient carbon concentrating mechanisms (Badger and Price, 2003) which allow them to outcompete many eukaryotes. Positive correlation between pH and the biomass of total *Microcystis* and cellular microcystin content in natural populations has been suggested previously (Wicks and Thiel, 1990; Kotak et al., 2000; Rantala et al., 2006). Previously studies on Lake Erie have suggested that *Microcystis* proliferation may be linked to zebra mussel filter feeding (Vanderploeg et al., 2001). However, while we agree that this linkage may exist, we hypothesize that the proliferation of zebra mussels led to the decrease in water column alkalinity and thus may have given *Microcystis* a further advantage due to the presence of these carbon concentrating components. As such it may be in our study that total pH is a poor proxy for alkalinity and that a more refined measure of this parameter is needed prior to the rejection of this apparent linkage.

In the entire lake data set, the biomass of total cyanobacteria and total *Microcystis* showed a strong negative correlation with NO_3, NH_3 and TN. The abundance of toxic *Microcystis* showed a negative correlation only with NO_3. In both data sets TN yielded a significant negative correlation with the relative abundance of toxic genotypes within the *Microcystis* population. Nitrogen is an essential nutrient for cyanobacteria and because *Microcystis* is not known to be able to fix dinitrogen these cyanobacteria are generally thought to respond to increasing N concentration with increasing biomass (Watanabe and Oishi, 1985; Utkilen and Gjølme, 1995; Giani et al., 2005; Rantala et al., 2006). Several studies have however observed the opposite: a negative correlation was observed between TN and *Microcystis* abundance in a survey conducted in eutrophic to hypereutrophic lakes in Alberta, Canada (Kotak et al., 2000). To explain the negative correlation it was speculated that microcystin could serve as an intracellular storage form of N, allowing toxic cells to survive in a low N environment, however no more evidence has been provided to prove or disprove this hypothesis. Blomqvist et al. (1994) proposed that due to their low assimilation rates of nitrate, cyanobacteria are not able to compete with other phytoplankton in a nitrate rich environment whereas efficient uptake systems for ammonium allows non-nitrogen fixing cyanobacteria to outcompete other phytoplankton in an ammonium-rich environment. The theory was used to explain why

the onset of non-nitrogen fixing cyanobacteria often correlates with depletion of nitrate. This theory could, at least partly, explain the strong negative correlations seen between total- and toxic *Microcystis* and nitrate.

In this study total cyanobacteria and total *Microcystis* biomass were also positively correlated with TP in the entire lake data set. Interestingly, though, in our study the cell abundance of toxic *Microcystis* was not correlated with TP, whereas microcystin showed a positive correlation with TP and SRP in the entire lake data set and with only TP in the western basin. Increasing phosphorus concentrations have been shown to elicit increasing toxicity in cultures of *Microcystis* (Watanabe and Oishi, 1985; Utkilen and Gjølme, 1995), but in natural populations correlation of phosphorus and microcystin concentrations have yielded variable results. A previous study conducted in southern Quebec lakes found a positive correlation between phosphorus concentration and the relative biomass of *Microcystis* among other phytoplankton species but no correlation was found between phosphorus and microcystin (Giani et al., 2005), whereas Kotak et al. (2000) found a positive correlation with phosphorus and microcystin in a study of small Canadian lakes. The different responses of toxic *Microcystis* biomass and microcystin to TP could indicate that phosphorus has a differential effect on cell growth and toxin production.

Besides the availability of nitrogen and phosphorus, the ratio of these nutrients has been shown to influence the abundance of cyanobacteria in general, as well as growth and toxin production of *Microcystis* (Vezie et al., 2002; Downing et al., 2005). In general, low nitrogen-to phosphorus ratios have been considered to favor the development of cyanobacterial blooms (Smith, 1983), although the exact ratio is not always considered to be the best predictor for cyanobacterial dominance (Downing et al., 2001). In the present study the data collected from the entire lake showed a negative correlation between the TN:TP ratio and cell abundances of cyanobacteria and total *Microcystis*. In the western basin only the relative abundance of toxic *Microcystis* genotypes yielded a negative correlation with the TN:TP ratio. Microcystin also showed a negative correlation with TN:TP in both data sets analyzed. Negative correlation with TN:TP and positive

correlation with P (discussed above) could indicate that an increased P input in a low N environment may induce toxin production in the Lake Erie *Microcystis* population.

One surprising result was that both the cyanobacteria and total *Microcystis* correlated positively with silica. Silica is typically thought to be a component of (and potential growth limiting element for) diatoms. In this system it was a surprise to see this relationship within our data. Although not born out of our observations, it appears that silica in this case is acting as a marker of water column geochemistry, either released into the water column from sediments or from external sources. As such, its distribution in the water column may be tightly linked to external nutrient inputs in the system.

Microcystis spp. are not the only microcystin producers in the western basin of Lake Erie (Rinta-Kanto and Wilhelm, 2006), which may partly explain the non-significant correlation between toxic *Microcystis* cell abundance and microcystin-LR concentrations we have observed. Microcystin production by other microcystin producers (*e.g.,* potentially toxigenic *Planktothrix* spp. found in Sandusky Bay) may respond differently to environmental conditions. *Anabaena* spp., *Aphanizomenon* spp., and a variety of other cyanobacteria are commonly seen in microscopic examinations of the water column, and in recent years many have been shown to be heterocyst bearing (unpublished data). As such, it is anticipated that altered TN:TP ratios would lead to very different cellular physiologies in these genera relative to *Microcystis* spp., and to differential growth and toxin production potentials. Moreover, these cells are implicated in toxin production in several locations within the Lake where microcystin concentrations are significant yet *Microcystis* cells are not detectable (see Figure 4 and discussion to follow).

In this study cell abundance was estimated using a quantitative PCR-method, which is only capable of quantifying target gene copies within the limits of the standard curve established for the analysis. In our method, samples which yield less than 25 target gene copies in the final PCR reaction (the amount corresponds to cell abundance of roughly 200 cells L^{-1}) can not be reliably quantified in the assay even though the target genes were detected at low levels. These samples have been excluded from the correlation analysis, which was based on the available quantitative data. Therefore we

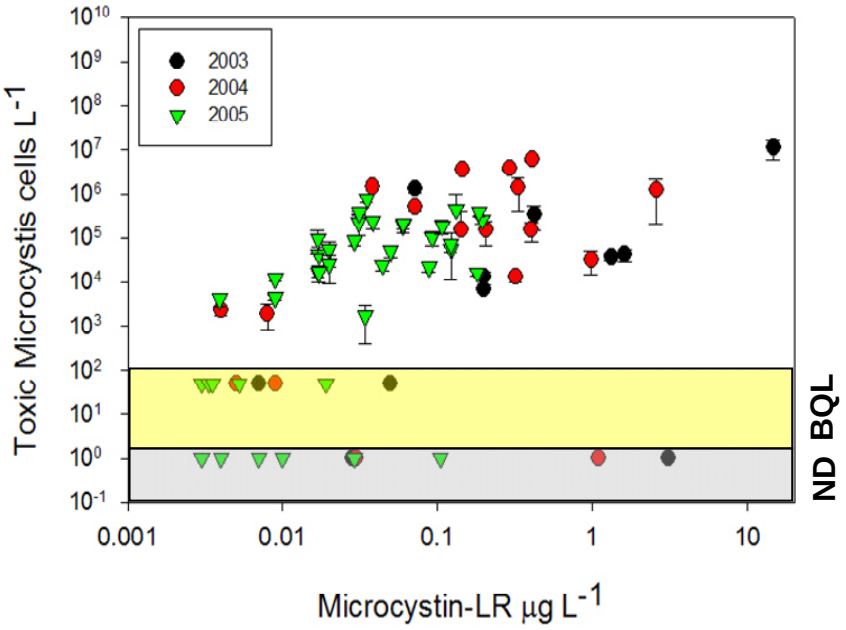

Figure 4. The relationship between the abundance of toxic *Microcystis* cells and microcystin concentration (reported as microcystin-LR equivalents) at corresponding locations in Lake Erie. The box shaded with yellow indicates samples where the quantity of *Micorcystis* cells carrying the target gene (*mcyD*) was below quantifiable limit (BQL) of our assay (approximately 200 cells L^{-1}). The box shaded with grey indicates samples in which *Micorcystis* cells carrying the target gene (*mcyD*) were not detected (ND). To be able to include these samples in this plot, these data points were given a default value of 1.

have not been able to include samples in the analysis which contained very low quantities of *Microcystis* or cyanobacteria in general, causing us to lose some fine scale resolution in the correlations involving these variables. As well, microcystin was quantified using protein phosphatase inhibition assay which provides a measure of toxin activity, and as such is reported as microcystin-LR equivalents (the standard used for comparison). Different chemical variants of microcystin have been shown to have different levels of activity (Sivonen and Jones, 1999) and therefore the finite concentrations of the toxin may also vary.

Conclusions

In this study we have examined three summer seasons where Lake Erie *Microcystis* formed a variable proportion of the total cyanobacterial community each year. In two of the seasons (2003 and 2004) significant blooms occurred resulting in relatively high toxin concentrations (up to 15 μg L^{-1}), while in the third season (2005) toxin concentrations were generally low (< 1 μg L^{-1}). As such this data set is unique in that it spans a possible range of *Microcystis* bloom events for Lake Erie. Based on monthly mean values, total *Microcystis* formed up to ~50 % of the cyanobacterial community whereas toxic *Microcystis* forms on the average less than 10 % of the total cyanobacterial abundance. Among total *Microcystis*, on the average up to ~60 % of the cells were toxic genotypes.

In terms of water column chemistry, the data support previous observations in that the abundance of *Microcystis* and toxic *Microcystis* correlated with low N:P ratios. Water temperature also correlated with the abundance of toxic cells, but surprisingly not with toxin production, suggesting (at least in this case) a possible disconnect between cell proliferation and toxin production by these cells. In our data sets toxin concentration was linked to nutrient concentrations (N:P ratios and phosphorus), re-emphasizing the decoupling between factors affecting the abundance of toxic *Microcystis* cells and their toxicity.

This study is the first to correlate the effects of environmental factors on the abundance of *Microcystis* genotypes in Lake Erie, giving insight into differences between factors influencing the total *Microcystin* population vs. the toxic *Microcystis* population.

The outcome of this study shows clearly that responses of *Microcystis* in culture conditions can be very different from what is observed in natural systems and that environmental dynamics of *Microcystis* can not be understood by simply combining data from culture based studies, in spite of the difficulties of interpreting complex interactions in nature. In the recent years the Lake Erie ecosystem has once again entered a new phase, signified by the return of cyanobacterial blooms (Conroy et al., 2005). The current study represents an important first-step in the development of our understanding of how different cyanobacterial populations and specifically *Microcystis* in Lake Erie are responding to the present "state" of the Lake, which is very different from the eutrophic period which supported algal blooms at the end of 1960s and early 1970s.

Acknowledgments

We thank the Captains and crews of the CCGS *Limnos* and RV Lake Guardian as well as Bob Hess, George Bullerjahn, Michael Twiss, R. Michael McKay and Tom Bridgeman and Lynn Ferguson for assistance and helpful discussions as well as Wayne Carmichael for providing the *M. aeruginosa* LE-3 isolate. This article includes research supported by funds from the National Oceanic and Atmospheric Administration Coastal Ocean Program (MERHAB-LGL, #NA 160 P2788), an Oceans and Human Health external grant (NA05NOS4781251), the National Science Foundation (DEB-0129118), and Helsingin Sanomat Centennial Foundation (to JMR-K).

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APPENDIX V

Table 1. Raw data summary. n= total stations sampled. n′ = stations where results were positive. Where only one data point is available it is shown as the minimum only.

Date		Cyanobacteria (cells L^{-1})	*Microcystis* (cells L^{-1})	Toxic *Microcystis* (cells L^{-1})	Toxin (µg L^{-1})	Surface temp.
2003						
June	MIN	2.6 (±0.2) × 10^5	3.1 (±1.6) × 10^2	Not detected	3.1	16.0
	MAX	1.1 (±0.5) × 10^8	-		-	19.6
		n=4	n′=1		n′=1	n=3
July	MIN	1.3 (±0.0) × 10^5	1.3 (±0.0) × 10^5	2.7 (±0.4) × 10^3	0.007	22.5
	MAX	7.0 (±0.8) × 10^8	7.0 (±0.8) × 10^8	6.2 (±0.1) × 10^6	-	23.2
		n=4	n′=4	n′=3	n′=1	(n=2)
Aug.	MIN	4.8 (±1.5) × 10^5	1.8 (±0.5) × 10^3	8.8 (±1.6) × 10^2	0.02	24.3
	MAX	2.4 (±0.3) × 10^8	1.7 (±0.6) × 10^8	1.1 (±0.5) × 10^7	14.0	25.1
		n=17	n′=15	n′=15	n′=10	n=5
2004						
July	MIN	4.0 (±7.0) × 10^3	4.4 (±2.3) × 10^5	7.9 (±0.0) × 10^2	0.004	20.4
	MAX	2.0 (±0.3) × 10^8	7.2 (±8.5) × 10^5	2.8 (±0.9) × 10^4	1.86	26.0
		n=28	n′=22	n′=15	n′=6	n=21

Table 1 continued.

Date		Cyanobacteria (cells L^{-1})	*Microcystis* (cells L^{-1})	Toxic *Microcystis* (cells L^{-1})	Toxin (µg L^{-1})	Surface temp.
2004						
Aug.	MIN	2.3 (±0.8) × 10^5	1.0 (±0.2) × 10^3	4.6 (±0.4) × 10^2	1.2	20.8
	MAX	7.0 (±0.9) × 10^6	2.5 (±0.8) × 10^6	2.8 (±0.1) × 10^5	21.7	22.2
		n=14	n´=17	n´=13	n´=20	n=15
Sept.	MIN	1.3 (±0.7) × 10^6	7.0 (±2.2) × 10^5	3.7 (±2.1) × 10^4	n/a	n/a
	MAX	3.6 (±1.3) × 10^7	1.3 (±0.6) × 10^7	5.6 (±5.4) × 10^5,		
		n=13	n´=13	n´=13		
2005						
July	MIN	8.9 (±3.4) × 10^2	3.6 (±3.1) × 10^3	4.1 (±1.3) × 10^3	0.003	24.8
	MAX	6.3 (0.7) × 10^8	4.1 (±1.2) × 10^6	4.0 (±0.9) × 10^5	0.2	27.4
		n=34	n´=9	n´=5	n´=20	n=12
Aug.	MIN	5.9 (±9.4) × 10^5	3.6 (±3.1) × 10^3	1.7 (±1.3) × 10^3	0.0004	23.1
	MAX	4.0 (±0.8) × 10^8	1.0 (±0.3) × 10^7	7.7 (±1.1) × 10^5	0.1	27.1
		n=55	n´=50	n´=37	n´= 38	n=38

Table 2. Nutrient data summary.

Year, month		NO_3 (mg L^{-1})	NH_3 (mg L^{-1})	SiO_2 (mg L^{-1})	SRP (mg L^{-1})	TP (F) (mg L^{-1})	TP (UF) (mg L^{-1})	TN (F) (mg L^{-1})	N:P (molar ratio)
July 2004	MIN	0.2	0.1	0.1	0.0005	0.008	0.02	0.3	0.92
(n = 9)	MAX	2.4	0.3	0.9	0.03	2.5	2.4	2.7	134.4
July 2005	MIN	0.009	0.005	0.1	0.001	0.004	0.006	0.4	19.3
(n = 31)	MAX	0.5	0.3	3.7	0.06	0.09	0.2	0.9	388.9
Aug 2005	MIN	0.004	0.005	0.1	0.0007	0.005	0.01	0.02	2.78
(n = 37 – 41)	MAX	1.4	0.1	4.2	0.1	0.1	0.2	2.4	179.5

Table 3. Relative abundances of total *Microcystis* and toxic *Microcystis* in western basin of Lake Erie. Results are given as averages of all samples collect for that time along with the range of minimum and maximum estimates.

Year	**month**	**% total *Microcystis* of total cyanobacteria**	**% toxic *Microcystis* of total cyanobacteria**	**% toxic genotypes of total *Microcystis***
2003	June	0.1 (0)	0	0
	July	14.6 (0)	2.1 (0)	14.2 (0)
	August	13.4 (0.07 – 71.4)	1.8 (0.03 – 4.7)	59.5 (6.6 – ~100)
2004	July	4.3 (0.004 – 15.5)	0.7 (0.001 – 3.7)	11.4 (2.1 – 23.7)
	August	17.1 (0.6 – 51.7)	3.2 (0.1 – 7.8)	18.2 (8.4 – 39.3)
	September	49.1 (5.3 – ~100)	1.7 (0.5 – 5.5)	6.9 (2.1 – 15.3)
2005	July	3.4 (0.02 – 6.5)	0.3 (0.003 – 0.6)	8.5 (3.1 – 15.6)
	August	3.7 (0.008 – 29.5)	0.6 (0.02 – 4.9)	22.5 (1.8 – ~100)

Table 4. Spearman correlation coefficients, n and P values for cell abundances in the whole lake data set. P-values < 0.05 are bolded.

	Total Cyanobacteria			Total *Microcystis*			Toxic *Microcystis*		
	r_s	n	P	r_s	n	P	r_s	n	P
Total *Microcystis* cells L^{-1}	0.6161	128	**0.0000**						
Toxic *Microcystis* cells L^{-1}	0.4238	100	**0.0000**	0.7774	98	**0.0000**			
Total chl *a* µg L^{-1}	0.5906	146	**0.0000**	0.4872	111	**0.0000**	0.4731	84	**0.0000**
Phycocyanin	0.7771	46	**0.0000**	0.4071	33	**0.0187**	-0.0052	29	0.9788
Microcystin-LR	0.3266	88	**0.0019**	0.4016	70	**0.0006**	0.3080	58	**0.0187**
pH	0.1321	57	0.3271	-0.1519	38	0.3626	-0.2946	32	0.1017
Surface temperature	0.3324	112	**0.0003**	0.1550	96	0.1315	0.2546	71	**0.0321**
Temperature at sampling depth	0.3926	116	**0.0000**	0.0527	86	0.6297	0.1687	66	0.1757
NO_3 mg L^{-1}	-0.3359	78	**0.0026**	-0.3835	49	**0.0065**	-0.3473	36	**0.0380**
NH_3 (dissolved) mg L^{-1}	-0.3497	65	**0.0043**	-0.3261	45	**0.0288**	-0.2948	36	0.0809
SIO_2 (dissolved) mg L^{-1}	0.3184	81	**0.0038**	0.2748	52	**0.0487**	0.2564	39	0.1151
SRP (dissolved) mg L^{-1}	0.2622	80	**0.0188**	0.0877	51	0.5408	0.2786	38	0.0904
TP (dissolved) mg L^{-1}	0.4706	81	**0.0000**	0.4152	52	**0.0022**	0.2443	39	0.1340
TP (particulate) mg L^{-1}	0.5706	81	**0.0000**	0.3766	52	**0.0059**	0.2407	39	0.1399
TN (dissolved) mg L^{-1}	-0.3143	80	**0.0045**	-0.2990	51	**0.0330**	-0.3089	38	0.0591
N:P ratio	-0.4799	80	**0.0000**	-0.3493	51	**0.0120**	-0.2159	38	0.1930

Table 5. Spearman correlation coefficients, n and P values for cell abundances in the western basin data set. P-values < 0.05 are bolded.

	Total Cyanobacteria			Total *Microcystis*			Toxic *Microcystis*		
	r_s	n	P	r_s	n	P	r_s	n	P
Total *Microcystis* cells L^{-1}	0.5138	50	**0.0001**						
Toxic *Microcystis* cells L^{-1}	0.4200	42	**0.0056**	0.6757	42	**0.0000**			
Total chl *a* µg L^{-1}	0.5379	45	**0.0001**	0.4299	38	**0.0071**	0.2641	31	0.1511
Phycocyanin	0.7731	25	**0.0000**	0.5444	20	**0.0131**	0.2198	18	0.3808
pH	-0.2917	27	0.1398	-0.1215	22	0.5901	-0.0860	19	0.7262
Surface temperature	0.4933	41	**0.0010**	0.2472	35	0.1522	0.1864	27	0.3519
Temperature at sampling depth	0.2564	41	0.1057	0.0589	35	0.7370	0.0678	27	0.7367
NO_3 mg L^{-1}	-0.3897	25	0.0542	-0.3294	20	0.1561	-0.1339	16	0.6210
NH_3 (dissolved) mg L^{-1}	0.0397	22	0.8608	-0.2720	20	0.2460	-0.0813	17	0.7563
SIO_2 (dissolved) mg L^{-1}	0.4036	28	**0.0332**	0.2866	23	0.1850	0.0000	19	1.0000
SRP (dissolved) mg L^{-1}	0.2655	27	0.1807	0.0017	22	0.9940	0.0870	18	0.7315
TP (dissolved) mg L^{-1}	0.2389	28	0.2207	0.1038	23	0.6375	0.1027	19	0.6757
TP (particulate) mg L^{-1}	0.2272	28	0.2450	0.1310	23	0.5514	0.0570	19	0.8166
TN (dissolved) mg L^{-1}	-0.2425	28	0.2138	-0.1166	23	0.5962	-0.0930	19	0.7050
N:P ratio	-0.2140	28	0.2742	0.0257	23	0.9074	-0.1491	19	0.5423

Table 6. Spearman correlation coefficients, n and P values for microcystin-LR concentrations in the whole Lake Erie data set as well as for the western basin only. P-values < 0.05 are bolded.

	Lake Erie			Western Basin only		
	r_s	n	P	r_s	n	P
Total cyanobacteria	0.3266	88	**0.0019**	0.1423	41	0.3749
Total *Microcystis*	0.4016	70	**0.0006**	0.5046	35	**0.0020**
Toxic *Microcytis*	0.3080	58	**0.0187**	0.3412	29	0.0701
% cyanobacteria *Microcystis*	0.4233	69	**0.0003**	0.6960	35	**0.0000**
% cyanobacteria toxic *Microcystis*	0.3378	58	**0.0095**	0.5907	29	**0.0007**
% *Microcystis* toxic	0.1159	57	0.3907	0.0318	29	0.8700
Temp. at sampling depth	-0.0579	73	0.6266	0.0656	34	0.7125
Surface temp. (°C)	-0.0296	67	0.8119	0.0182	34	0.9187
Chl *a* > 0.2 μm size class	0.6608	85	**0.0000**	0.3831	40	**0.0147**
Chl *a* 0.2 - 2.0 μm size class	0.0212	66	0.8660	0.0328	33	0.8563
Chl *a* 2.0 - 20 μm size class	0.6220	66	**0.0000**	0.3309	33	**0.0600**
Chl *a* > 20 μm size class	0.6485	66	**0.0000**	0.3112	33	**0.0779**
Phycocyanin	0.7633	45	**0.0000**	0.6274	25	**0.0008**
pH	-0.1566	46	0.2986	-0.3086	27	0.1173
NO_3 (dissolved) mg L^{-1}	-0.1778	46	0.2370	-0.0958	25	0.6487
NH_3 (dissolved) mg L^{-1}	-0.1548	38	0.3534	0.0856	22	0.7049
SiO_2 (dissolved) mg L^{-1}	0.3526	49	**0.0130**	-0.0079	28	0.9680
SRP (dissolved) mg L^{-1}	0.4241	48	**0.0027**	0.1915	27	0.3386
TP (dissolved) mg L^{-1}	0.6907	49	**0.0000**	0.5475	28	**0.0026**
TP (part.+diss.) mg L^{-1}	0.7505	49	**0.0000**	0.5929	28	**0.0009**
TN (dissolved) mg L^{-1}	-0.0725	48	0.6242	0.1202	28	0.5425
N:P	-0.6363	48	**0.0000**	-0.4048	28	**0.0326**

Part VI

THE EFFECT OF IRON AND PHOSPHORUS DEPLETION ON GROWTH AND TRANSCRIPTION OF MICROCYSTIN SYNTHETASE GENE *MCYD* IN *MICROCYSTIS AERUGINOSA*

This part is a version of a manuscript prepared to be submitted for publication in journal FEMS Microbiology Letters by Johanna M. Rinta-Kanto, Dinielle B. Truitt, Shannon Efteland, R. Michael L. McKay and Steven W. Wilhelm

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions in this paper were 1) Co design of the experiments for the study, 2) Collection and preparation of the DNA and RNA samples for analysis 2) Performing the DNA based analyses for the samples 3) Gathering the background literature and, 4) Most of the writing of the manuscript.

Introduction

Microcystis spp. are non-nitrogen fixing freshwater cyanobacteria that can form large accumulations of biomass (blooms) on the surface of inland waters. These bloom forming populations often consist of multiple strains of *Microcystis*, some of which are capable of producing toxin microcystin (Rinta-Kanto et al., 2005). Microcystins are potent hepatotoxins produced by *Microcystis* as well as other genera of cyanobacteria including *Anabaena* and *Planktothrix* (Jungblut and Neilan, 2006). Currently there are over sixty known chemical variants of this toxin (Sivonen and Jones, 1999), with as many as 80 possible variants thought to exist (GL Boyer, pers comm.). In *Microcystis*, a 55-kb microcystin synthetase (*mcy*) gene cluster in the genome makes the toxin-producing strains genotypically distinct from the non-toxic strains of *Microcystis* (Meissner et al., 1996; Dittmann et al., 1997; Nishizawa et al., 1999; Nishizawa et al., 2000; Tillett et al., 2000).

The effects of environmental factors on microcystin production by *Microcystis* have been studied in field and laboratory conditions; however these studies have given contradictory results. Phosphorus, for example, which is generally regarded as one of the key factors inducing cyanobacterial proliferation in often phosphorus-limited freshwater systems (Guildford and Hecky, 2000), has been shown in independent studies to both increase and decrease toxicity of *Microcystis* (Watanabe and Oishi, 1985; Lee et al., 1994; Utkilen and Gjølme, 1995; Oh et al., 2000). Iron has also been shown to affect the toxicity of *Microcystis* both negatively and positively in culture based studies (Lukac and Aegerter, 1993; Utkilen and Gjølme, 1995; Lyck et al., 1996; Martin-Luna et al., 2006b).

Despite interest in the regulation of toxicity of *Microcystis*, relatively little is still known about the influence of environmental factors on transcriptional regulation of the *mcy* gene cluster. The *mcy* gene cluster in *Microcystis* is transcribed as two polycistronic transcripts (*mcyABC* and *mcyDEFGHIJ*). For both transcripts, two alternate transcription start sites have been located between genes *mcyA* and *mcyD*, and the use of the two start sites has been shown to depend on ambient light intensity (Kaebernick et al., 2002). In a

previous study, light intensity was semi-quantitatively shown to affect the accumulation of *mcyB* transcripts: a higher quantity of *mcyB* mRNA transcripts accumulated in cells grown under high light intensities (68 μmol photons m^{-2} s^{-1}) than in cells grown under low light (16 μmol photons m^{-2} s^{-1}) (Kaebernick et al., 2000). Currently there is no published literature about the effects of any other factors on transcriptional activity of *mcy* genes.

The involvement of iron in transcriptional regulation has been recently suspected due to the presence of a ferric uptake regulator (Fur) protein binding site (known as Fur box or iron box, (Escolar et al., 1999)) within the bidirectional promoter region between *mcyA* and *mcyD* as well as in the promoter regions for *mcyE, G, H,* and *J* in *Microcystis aeruginosa* (Martin-Luna et al., 2006a; Martin-Luna et al., 2006b). Furthermore, it has been shown *in vitro* using gel mobility shift assays, that in the presence of ferric iron, Fur protein from *Microcystis aeruginosa* binds to Fur boxes located in promoter regions of microcystin synthetase genes (Martin-Luna et al., 2006b). In the same study Martin-Luna et al. (2006b) reported a 125% increase in microcystin-LR (relative to total protein content) after incubating *M. aeruginosa* with 50 μM 2,2'-bipyridyl for 30 minutes. Although these experiments suggest that Fe availability potentially affects microcystin production, there is no direct evidence of the effect(s) on the transcriptional activity of *mcy* genes.

Primary productivity in Lake Erie is known to be influenced by the availability of phosphorus and trace metals (including Fe) to phytoplankton (Wilhelm et al., 2003; Twiss et al., 2005). Because these factors may also influence *Microcystis* proliferation and on its toxin production, we chose to study the effects of iron and phosphorus on the transcriptional regulation of the *mcyD* gene. In the present study our goal was to empirically determine the effects of decreasing the availability of iron or phosphorus on transcription of the *mcyD* gene. We hypothesized that phosphorus-limitation would affect the biomass of *Microcystis* as well as the expression of *mcyD* gene. Furthermore, assuming the Fur protein and previously described binding site play a role in *mcy* gene expression, we hypothesized *mcyD* mRNA transcripts would increase in *Microcystis aeruginosa* cells grown under induced Fe-deficiency.

Materials and Methods

Cultures – phosphorus experiment

To test the effect of altered phosphorus concentrations on *mcyD* transcription, unialgal *Microcystis aeruginosa* PCC 7806 was grown in modified BG-11 medium (Kerry et al., 1988) in 50 mL glass tubes (Kimax). BG-11 for the decreased phosphorus cultures was prepared by reducing the amount of phosphorus relative to the original concentration within the BG-11 medium (230 μM, 100 % P). Treatment cultures were grown in phosphorus concentrations of 80% (184 μM P), 60% (138 μM P), 40% (94 μM P), 20% (46 μM P), 10% (23 μM P) and 1% (2.3 μM P) of the original phosphorus concentration. BG11 medium was also prepared containing 23 and 2.3 μM organic phosphorus; in these media the original inorganic phosphorus source ($K_2HPO_4 \cdot 3H_2O$) was omitted and replaced with an organic phosphorus source (glycerol 2-phosphate disodium salt hydrate). Triplicate cultures were grown in each phosphorus concentration. Prior to use, all culture tubes and media bottles had been soaked in 1% HCl and multiply rinsed with Milli-Q water.

Cells for DNA and RNA extractions were harvested from cultures at t=136 h by filtering approximately 10 mL (exact volume was recorded for each replicate) onto 25 mm diameter, 0.2-μm nominal pore-size polycarbonate filters (Poretics). Filters were placed in sterile 2 mL microcentrifuge tubes and stored frozen at -80°C until processed.

Cultures – Fe chelator addition

Unialgal *Microcystis aeruginosa* PCC 7806 were grown in BG11 medium (Kerry et al., 1988) in 40 mL polycarbonate Oakridge tubes. Cultures (27 mL total volume) were inoculated with 2 mL *M. aeruginosa* PCC 7806 late exponential phase stock culture, and triplicate cultures were amended with fungal siderophore desferrioxamine B (DFB) (Sigma) (Wells, 1999) to final concentrations of 50 nM and 100 nM DFB. A triplicate set of unamended cultures was maintained as controls. To reduce external Fe contamination, nutrient stocks and water used to prepare the medium were treated with Chelex-100 resin (Price et al., 1989). All culture vessels and media bottles were soaked in 1% HCl and

rinsed with Chelex-100 treated Milli-Q water before use. All culture manipulations were performed in class-100 clean room conditions to maintain aseptic conditions and to avoid trace metal contamination. Culture medium was prepared in sterile 1 L polycarbonate flasks, and sterilized by bringing the liquid to boil three times in a microwave oven (Keller et al., 1988) The liquid was allowed to cool down back to room temperature between the heating steps.

Cells were harvested from cultures at t = 96 h by centrifugation at 10,000 × g for 20 min. The culture medium was removed from the tubes and the cells were resuspended in 500 µL sterile culture medium. The cell suspension was divided in half by volume and the aliquots were transferred into sterile microcentrifuge tubes. These samples were centrifuged as above, the supernatant was removed and cells were stored frozen at -80°C until processed.

All Cultures

Biomass accumulation in all cultures was measured as *in vivo* fluorescence using a Turner Designs TD-700 bench top fluorometer equipped with the manufacturer's *in vivo* chlorophyll-*a* filter set. Growth rates in cultures were estimated by plotting the natural logarithm of fluorescence units against time (days) and determining the slope of the growth curve. Each culture was grown until the late logarithmic growth phase before transfer. The cultures were grown and transferred twice in their respective media before growing the third generation of cells that were harvested for DNA and RNA extraction. All cultures were grown at +25ºC under illumination of *ca* 10 µmols photons m^{-2} s^{-1}.

DNA extraction

Prior to DNA extraction, cells were resuspended in 500 µL sterile lysis buffer (Rinta-Kanto et al., 2005) and ~200 µL sterile zirconia-silica beads were added to each sample. Cells were lysed by bead beating in a Mini-Beadbeater (BioSpec Products) for 2 min at 4800 rpm. DNA was extracted from the lysate using phenol-chloroform extraction and precipitated using a standard ethanol-ammonium acetate precipitation protocol (Sambrook and Russell, 2001). DNA was collected by centrifuging the sample tubes in a

refrigerated bench top centrifuge at 16 000 rpm for 30 min. The supernatant was removed, the DNA pellet was washed with ice cold 70% ethanol and the centrifugation step was repeated. After centrifugation the ethanol was removed and the DNA was air dried at room temperature. DNA was resuspended in 100 µL sterile 1 × TE buffer and the samples were stored frozen at -80ºC.

RNA extraction

To extract RNA, cells were first resuspended in lysis solution supplied with RNAqueous kit (Ambion) and ~200 µL sterile zirconia-silica beads, which had been treated with 0.1% diethyl pyrocarbonate (DEPC) prior to use to remove RNAse contamination, were added into each sample tube. The cells were lysed through bead beating in a Mini-Beadbeater (BioSpec Products) for 2 min at 4800 rpm. RNA was extracted from the lysate using RNAqueous kit (Ambion) and in the final step of extraction the RNA was eluted in 60 µL elution buffer. Carry-over DNA was eliminated from RNA extracts using Turbo DNA Free kit (Ambion). DNA-free RNA was quantified spectrophotometrically (Sambrook and Russell, 2001) and stored frozen at -80ºC.

qPCR and RT-qPCR

The primers, Taq-man probe and plasmid standards used in quantitative PCR (qPCR) and RT-qPCR assays have been described in detail previously (Rinta-Kanto et al., 2005). The reactions for qPCR assay for quantification of *mcyD* gene copies consisted of 12.5 µL of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.4 µM (final concentration) of primers mcyD F2, mcyD R2 (Sigma-Genosys, Inc.), 0.4 µM (final concentration) of Taq-probe (Biosearch Technologies, Inc.), 5 µL of ten-fold or hundred-fold diluted template DNA and sterile Milli-Q water to adjust the reaction volume to 25 µL. Triplicate reactions were run for each template dilution and standard on a 96-well plate using Opticon 2 instrument (BioRad). Negative controls with no DNA template added were included for each PCR run.

The reaction mixtures for reverse transcription quantitative PCR (RT-qPCR) assays for RNA samples consisted of 12.5 µL of 2 × QuantiTect Probe mastermix (Qiagen, Novato, CA, USA), 0.25 µL of QuantiTect RT Mix (Qiagen), 0.4 µM (final

concentration) of primers mcyD-F2 and mcyD-R2 (Sigma-Genosys, Inc.), 0.4 μM (final concentration) of mcyD-F2 Taq-probe (Biosearch Technologies, Inc.), and 5 μl of two-fold or twenty-fold diluted template RNA. The reaction volume was adjusted to 25 μL with DEPC treated sterile Milli-Q water. QuantiTect RT Mix was omitted from the PCR reactions for plasmid standards and DEPC treated sterile Milli-Q water was added to adjust the reaction volume accordingly to 25 μL. Each reaction was run in triplicate on a 96-well plate. The thermal cycling protocol for RT-qPCR consisted of an initial reverse transcription step at 30 min at 50°C, then 15 min at 95°C, followed by 45 cycles of 30 s at 95°C, 1 min at 61°C and 20 s at 72°C. Control reactions with no template were included in the assay to ensure there was no RNA contamination in the PCR reagents. Control reactions containing template RNA with no reverse transcriptase were also included in the assay to ensure there was no DNA contamination in the reagents. The primers, probe and the dilutions of the RNA extract were prepared using DEPC treated sterile Milli-Q water.

Analysis

In both qPCR assays, *mcyD* DNA or cDNA copies were quantified against a standard curve (target gene copy number vs. C_t) generated using a serial dilution of plasmids containing one copy of the target *mcyD* gene. Threshold cycle (C_t) calculations were completed automatically for each qPCR assay by the Opticon Monitor analysis software, using the maximum correlation coefficient approach. In this approach the threshold is automatically determined to obtain the highest possible correlation coefficient (r^2) for the standard curve.

The relative expression of the *mcyD* gene in the cultured cells was calculated by normalizing the quantity of *mcyD* cDNA copies mL^{-1} to the quantity of *mcyD* gene copies mL^{-1} for each replicate culture. The statistical significance of differences between the relative abundance of cDNA copies per gene copy in each culture was tested using the independent-samples Student's t-test.

Results

The effect of phosphorus

Growth curves based on *in vivo* chlorophyll fluorescence show that all cultures (except cultures grown in 138 μM P) had reached exponential growth phase by the time cells were harvested (data not shown). The growth rates showed significant differences between phosphorus treatments, however the reduction of phosphorus in the media did not result in a linear response in reduction of growth rates (Figure 1). The 138 μM P treatment was excluded from the analysis because all three cultures died in the beginning of the experiment for an unknown reason.

The relative quantity of *mcyD* cDNA copies per *mcyD* gene copy did not show significant changes across the inorganic phosphorus concentrations (Figure 2). Cultures grown with 23 μM or 2.3 μM organic P showed an increase in the relative *mcyD* cDNA copy number, and the increase of relative expression in 2.3 μM organic P was statistically significant compared to cultures grown with 2.3 μM inorganic P (t-test, P = 0.118).

The effect of Fe chelator

Growth curves based on *in vivo* chlorophyll fluorescence showed that all cultures had reached exponential growth phase by the time of harvesting the cells (data not shown). The growth rate in culture amended with 50 nM DFB was significantly higher than the unamended control (Figure 3). The relative number of *mcyD* cDNA copies per *mcyD* gene copy decreased in cultures amended with DFB compared to the unamended cultures. The relative abundance of *mcyD* cDNA copies was significantly lower in cultures grown with 100 nM DFB than in cultures with no added Fe chelator (t-test, one tailed P=0.045) (Figure 4).

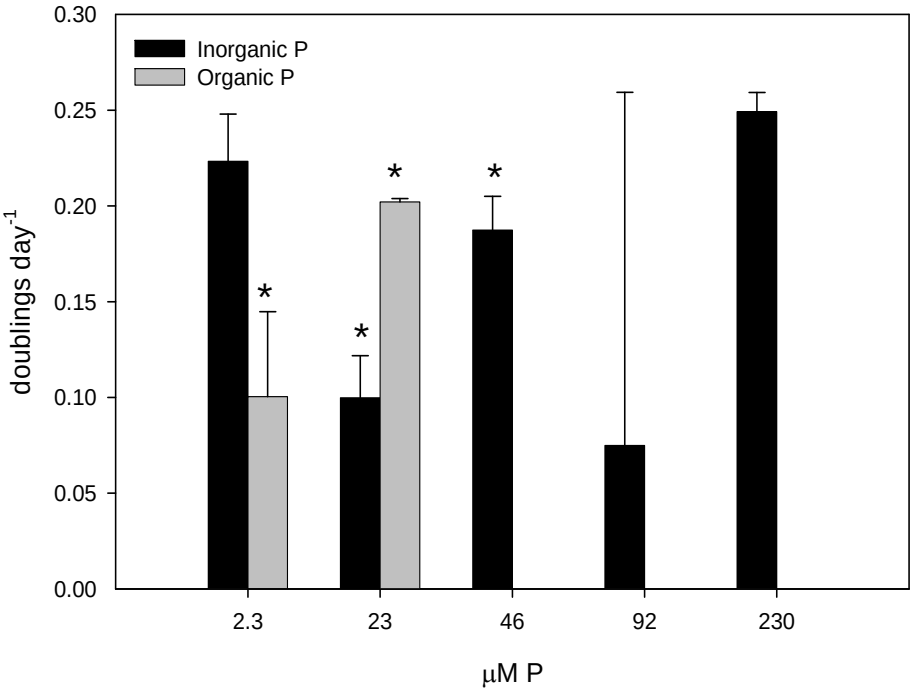

Figure 1. Growth rates (ln *in vivo* chlorophyll fluorescence over time) for *M. aeruginosa* PCC 7806 cultures grown in different P-concentrations. The error bars represent standard deviation. Asterisk indicates significant difference (t-test, $P < 0.05$) in growth rate compared to culture grown in 230 μM inorganic P (regular BG11). The error bars represent standard deviation (n = 3).

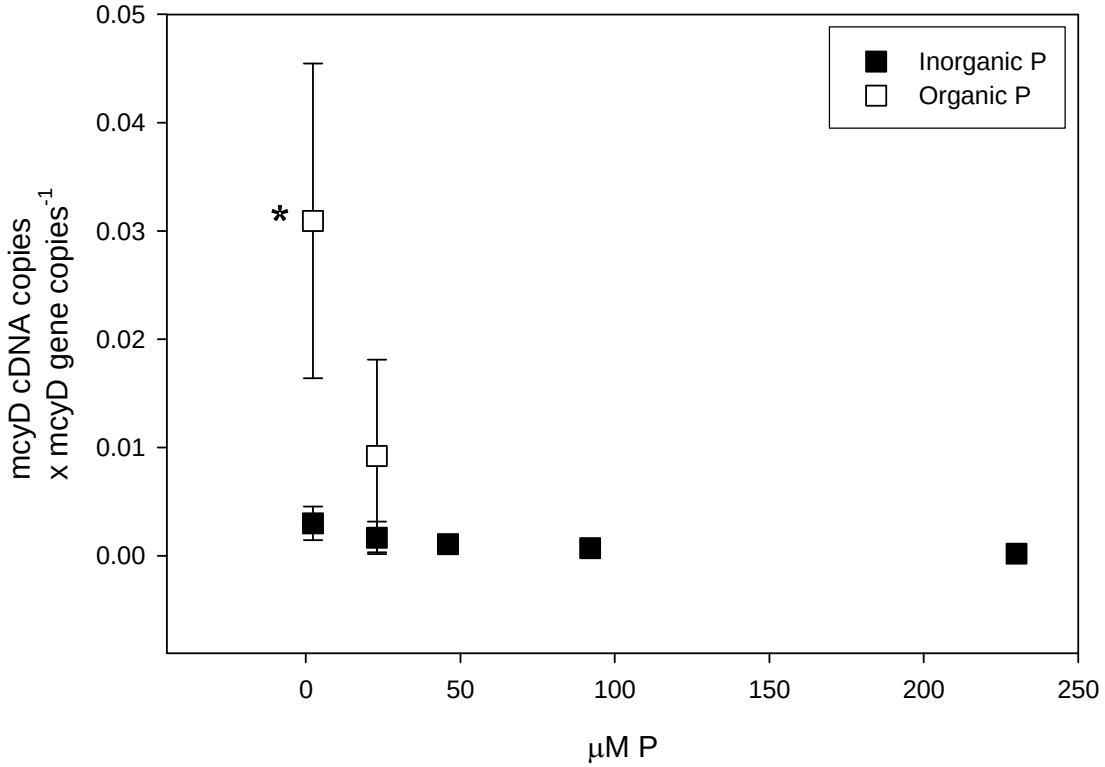

Figure 2. Relative expression of *mcyD* expressed as relative number of *mcyD* cDNA copies per *mcyD* gene copy in cultures grown under a series of phosphorus concentrations. Difference in relative expression between cells grown in 2.3 µM organic phosphorus (*) and 2.3 µM inorganic phosphorus is significant (t-test, P = 0.118). The error bars represent standard error (n = 3).

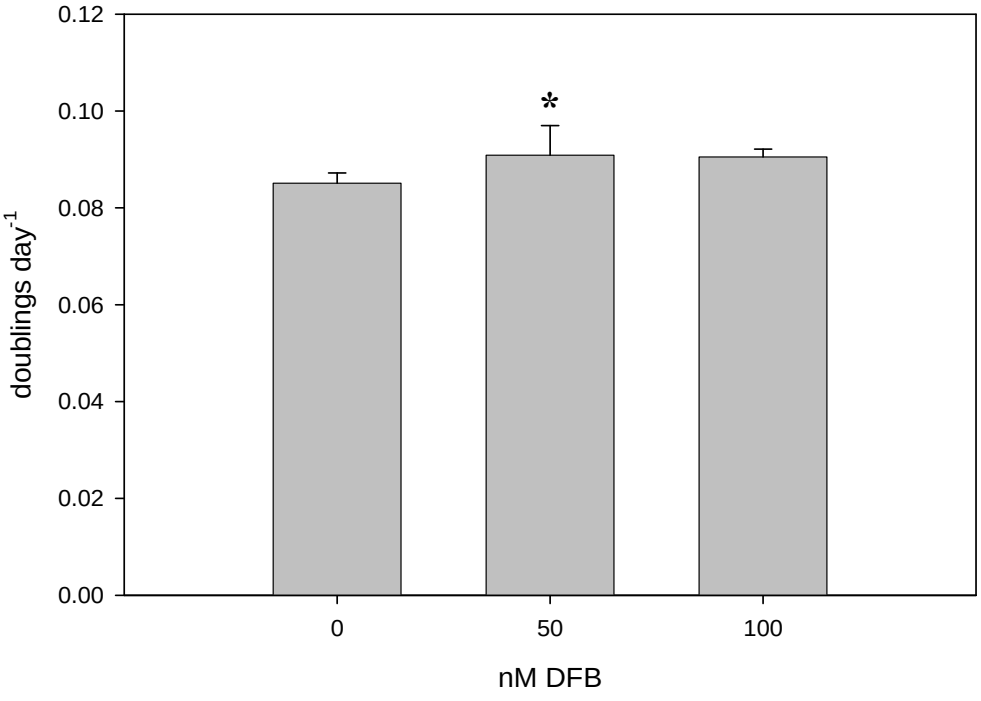

Figure 3. Growth rates (log *in vivo* chlorophyll fluorescence over time) for ***M. aeruginosa*** PCC 7806 cultures grown with 0 nM, 50 nM and 100 nM DFB. Asterisk mark indicates significant difference in growth rate compared to culture grown in 0 nM DFB (t-test, $P < 0.05$). The error bars represent standard deviation (n = 3).

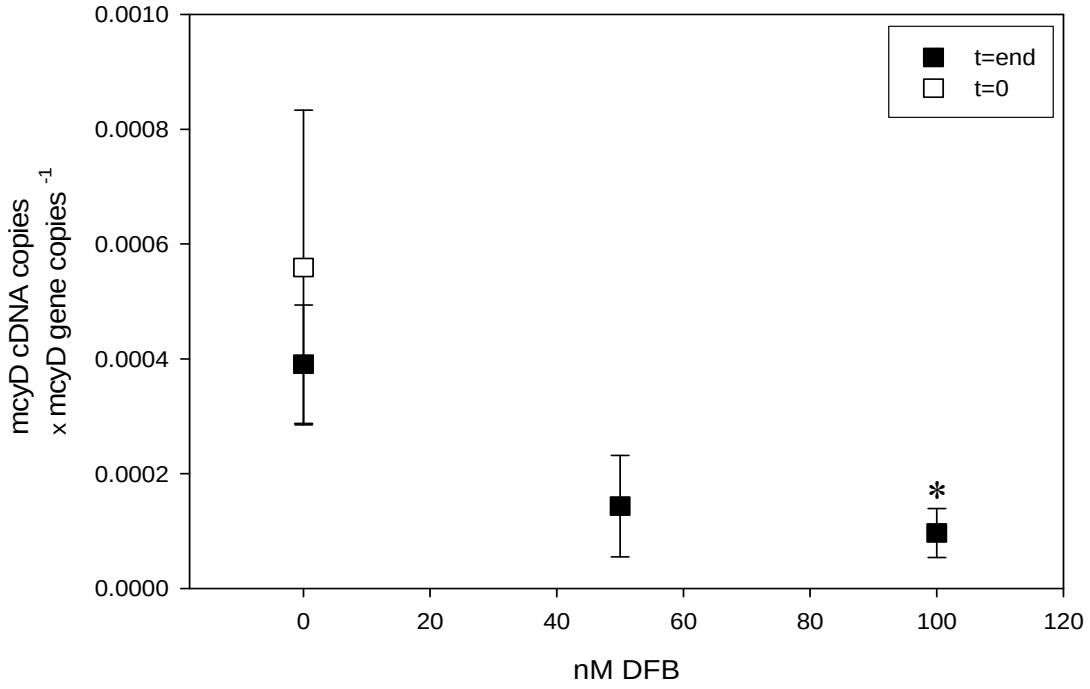

Figure 4. Relative expression of *mcyD* gene (*mcyD* cDNA copies per *mcyD* gene copy) in cultures of *M. aeruginosa* PCC 7806 as a response to increasing concentration of DFB. Asterisk indicates significant difference in relative expression compared to unamended control culture at t = end (t-test, one tailed P = 0.045). The error bars represent standard error (n = 3).

Discussion

BG11 medium is rich in nitrogen (17.6 mM N) and it is typically used for the generic growth of cyanobacteria (including *Microcystis*). In our attempts to exacerbate the P-limited growth of cells, we established conditions with molar nitrogen to phosphorus ratios (N:P) ranging from 77 to 7625. Studies in natural systems with TN:TP ratios of >50 have suggested that these conditions generally result in P-deficient growth (Guildford and Hecky, 2000). In our experiments decreasing the inorganic P concentration did not show a significant effect on the relative *mcyD* expression, however it seems that organic phosphorus had a stronger effect on the expression than the corresponding concentrations of inorganic phosphorus. It has been shown that culture media with different phosphorus sources bring forth different responses in *Microcystis* cultures (growth, macromolecular composition, toxicity) (Rao et al., 1996). Although Microcystis grown in MA medium (Watanabe and Oishi, 1985) was shown to be more toxic than cells grown in CB (Shirai et al., 1989), A_3M_7 (Carmichael et al., 1988) or BG11 (Warterbury and Stainer, 1981) media, the differences may not only be resulting from differential P sources and Microcystis cultures may be responding to the differential composition of the culture media investigated.

Adding the DFB in the cultures is known to reduce the availability of Fe to the cells but render the cells in Fe-limited conditions where *Microcystis* is still known to be able to maintain growth (Efteland, 2004). The increase in the growth rate in cultures amended with DFB is likely seen due to activation of the high affinity Fe-uptake system in Fe-deplete conditions. This is characteristic of several cyanobacterial species and evidence exists that *Microcystis* also utilizes this mechanism to enhance its Fe uptake (Wilhelm, 1995; Efteland, 2004). The Fe-deplete conditions are also known to induce the Fur-protein to leave its binding site allowing the Fur-regulated genes to be expressed (Escolar et al., 1999). In our experiment the relative expression levels were generally low, compared to the relative expression in the cells grown in different concentrations of phosphorus. However the reduced availability of Fe to *M. aeruginosa* PCC7806 appeared

to repress the relative *mcyD* expression, which is not expected should Fur have a role in regulation of *mcyD* expression as was suggested recently by Martin-Luna et al. (2006b).

Cultures were grown under relatively low light intensities because in brighter light the cultures had a tendency to bleach and die or accumulate biomass very slowly. We suspect the nutrient manipulations may have made the cultures more light sensitive. It is possible that the low light conditions may have suppressed the overall level of expression of the *mcyD* gene, as light intensity has been shown to reduce the transcription of the *mcyB* gene (Kaebernick et al., 2000). However, if the light intensity was an issue, it should have affected all cultures systematically because all cultures were maintained in same location.

In conclusion, our experiments indicated relatively weak effects of phosphorus and iron on the relative transcriptional activity of the *mcyD* gene. On the other hand, these experiments do not exclude the possibility of nutrients influencing the transcriptional activity of microcystin synthetase gene cluster. While these experiments have assessed the effects of single nutrients in transcriptional regulation of the *mcy* gene cluster, synergistic action of different factors (*e.g.* nutrients, light) in regulating the transcriptional activity in the environment can not be ruled out.

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Part VII

CONCLUSIONS

In this series of studies I have examined the ecology of cyanobacteria, specifically those of *Microcystis* spp. in Lake Erie. *Microcystis* are common cyanobacteria in freshwater environments worldwide, but prior to the mid-1990s these organisms were not dominant members of the phytoplankton assemblage in Lake Erie. Prior to this study, one report of a major *Microcystis* bloom event in 1995 had been published (Brittain et al., 2000) but concerns regarding increased abundance of *Microcystis* in Lake Erie had been raised in the context of other studies (Vanderploeg et al., 2001; Murphy et al., 2003). There was only one recent report concerning cell abundances of *Microcystis* and microcystin concentrations in Lake Erie (Brittain et al., 2000). And although *Microcystis* had already been extensively characterized in cultures and several studies had addressed *Microcystis* populations in natural assemblages, before I began studying this topic, there were no published studies that had applied quantitative PCR (qPCR) for quantification of *Microcystis* genotypes. During the course of my research I have employed the qPCR technique for quantification of cyanobacteria, total *Microcystis* and toxic *Microcystis*. I have used the technique along with other molecular tools in a series of studies designed to expand the knowledge of *Microcystis* in Lake Erie and to investigate factors affecting the toxicity of *Microcystis*. Through my investigations I have demonstrated the following:

i. The natural diversity of *Microcystis* in Lake Erie is greater than what is currently represented in culture collections and in public databases. As an example of the previously undiscovered diversity, *mcyA* sequences containing six extra bases were found, which appear to be characteristic of Lake Erie populations, but no identical matches to the sequences are deposited in public data bases.
ii. Cyanobacterial genera other than *Microcystis* contribute to microcystin production in Lake Erie, contrary to previous assumption. Based on identification of microcystin-producing cyanobacteria by their characteristic sequences in the microcystin synthetase gene *mcyA*, we have shown that *Planktothrix* spp. are dominant microcystin producers in spatially isolated regions of the lake.

iii. Reservoirs of *Microcystis* exist within Lake Erie. Sediments were shown to harbor significant quantities of cyanobacteria ($5 \times 10^3 - 4 \times 10^6$ cell equivalents per gram of sediment wet weight) and *Microcystis* ($2 \times 10^2 - 7 \times 10^5$ cells per gram of sediment wet weight) in the top 10-12 cm layer. It was shown that the cyanobacteria, including toxic *Microcystis*, deposited in the top 5 cm of the sediment can return to the pelagic community.

iv. The reservoirs of *Microcystis* may help to support *Microcystis* communities in Lake Erie. Sequence analysis of *mcyA* gene fragments from sediment and phytoplankton samples collected in the 1970s and in 2004 suggests that the toxic *Microcystis* community in the lake has persisted on the temporal scale of decades and, as such, is relatively stabile.

v. Environmental factors (specifically N, P, Fe, pH and water temperature) influence the abundance of cyanobacteria, total *Microcystis* and toxic *Microcystis* as well as microcystin production by *Microcystis*. Of the factors tested in this study, primarily the concentration of phosphorus and the ratio of total nitrogen and phosphorus correlated with microcystin concentrations in Lake Erie whereas the abundance of toxic *Microcystis* cells was correlated with NO_3 and water temperature. Moreover, *Microcystis* forms a variable proportion of the total cyanobacterial community in Lake Erie each year. In a study spanning three summer seasons, monthly means of cell abundances estimated through qPCR quantification indicated that total *Microcystis* formed up to ~50 % of the cyanobacterial community, whereas toxic *Microcystis* formed on the average less than 10 % of the total cyanobacterial abundance. Among total *Microcystis*, on the average up to ~60 % of the cells were toxic genotypes.

vi. Essential nutrients, such as iron and phosphorus influence the expression of toxin synthetase gene *mcyD*. Furthermore, the source of phosphorus (organic vs. inorganic) causes differential effects in *mcyD* expression. Induced Fe depletion decreased relative *mcyD* expression significantly, indicating a lack of Fe-mediated regulation of microcystin production.

The conclusions listed above demonstrate that *Microcystis* spp. are well established cyanobacteria in Lake Erie. The similarities between samples collected 30 years apart in the lake show that some of the sequence diversity has been preserved there over decades. It is remarkable that although the toxic genotypes of *Microcystis* currently form only less than 10 % of the cyanobacterial community in Lake Erie, the population is persistent in the lake. Because its sediment can act as a sink and a source of *Microcystis*, this likely contributes to the persistence of *Microcystis* populations in the lake. In Lake Erie the abundance of *Microcystis* and microcystin production are correlated strongly with nutrient availability. Toxic *Microcystis* abundance is additionally correlated with water temperature, suggesting a decoupling of factors governing proliferation of toxic cells and toxin production. Decoupling the cell growth and relative expression of the *mcyD* gene was demonstrated in a culture based study testing the effect of Fe depletion. The natural diversity of microcystin producing cyanobacteria which my research has demonstrated leads me to believe that the diversity in sequences of functional genes may reflect functional plasticity required in the environment, which can also contribute to the persistence of *Microcystis* in this system. The diversity can also be a basis of a much greater functional potential that can affect toxin production and cell proliferation than can be anticipated from studying a limited number of cultured strains in the laboratory, which warrants further studies on the complex natural populations. Overall, this study implies, firstly, that monitoring cyanobacterial population abundance, diversity and function has allowed a better perception of the persistent nature and composition of the *Microcystis* population in Lake Erie; and, secondly, that molecular level observations have improved our understanding of the relationship between toxic cell abundance, toxin production and the role of environmental factors in regulating these phenomena.

It is apparent that the management plans tailored to reduce the harmful algal blooms in Lake Erie in the late 1970s resulted in the desired effects until the early 1990s, but have since become ineffective as the ecosystem is changing (Matisoff and Ciborowski, 2005). Altogether, our studies provide insight into the present day *Microcystis* population and we hope that the findings will be of significant value in the future revision of lake management plans.

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VITA

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