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Ph.D. (Biology)

GRADE / DEGREE

Department of Biology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Microcystin Production and the Dominance of Toxigenic Strains of Cyanobacteria in Lake and Culture Studies

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Microcystin production and the dominance of toxigenic strains of cyanobacteria in lake and culture studies

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
In partial fulfillment of the requirements for the
PhD degree in the
Ottawa-Carleton Institute of Biology
Specialization in Chemical and Environmental Toxicology

Thèse soumise à la
Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention du doctorat
L'Institut de biologie d'Ottawa-Carleton

Your file Votre référence
ISBN: 978-0-494-59506-0
Our file Notre référence
ISBN: 978-0-494-59506-0

To Mom, Dad, and
Justin

Abstract

Cyanobacteria produce toxic compounds with the hepatotoxic microcystins being the most commonly encountered in freshwater. Microcystins are produced by several cyanobacterial genera and show large spatial and temporal variation in aquatic ecosystems as both toxigenic and non-toxigenic strains may co-exist and reach bloom levels. The regulation of microcystin production, the ecological or physiological role(s) of microcystins, and the factors that specifically promote toxic cyanobacterial blooms are not yet well understood. The first part of this thesis tested the role of light intensity in regulating the production and composition of microcystin congeners using laboratory culture experiments. The second part, using field observations and a lake enclosure experiment, examined the effects of physical, chemical and biological variables on microcystin concentrations and the growth of toxigenic cyanobacteria in a shallow, mesotrophic lake. Throughout, chemical and molecular based methods were utilized along with traditional culture and limnological methods in order to measure microcystin production and the presence of toxigenic cyanobacteria.

Light intensity had a significant effect on microcystin concentrations and congener composition. However, there were no clear advantages of either toxigenic or non-toxigenic strains in terms of their ability to grow, compete for light resources, or dominate in a mixed-culture setting. The response of each strain of *Microcystis aeruginosa* was unique such that generalizations could not be made between toxigenic and non-toxigenic strains in terms of their competitive ability in nature; microcystin production did not appear to impair the competitive ability of toxigenic strains. In field studies of a shallow mesotrophic lake over two years, the number of microcystin gene copy numbers (*mcy*D) did not correlate well with microcystin concentrations. Both within the lake and across a nutrient gradient established in *in situ* enclosures, the best predictor of the presence of toxigenic strains was the actual biomass of potentially toxigenic genera (mainly *Anabaena* and *Microcystis*), rather than any particular chemical or physical variable such as light. However, both environmental and biological variables were significant in predicting actual microcystin concentrations. The combination of molecular, chemical, and taxonomic data appears necessary to understand and predict toxic cyanobacterial blooms.

Résumé

Certaines cyanobactéries produisent des composés toxiques tels que les microcystines hépatotoxiques qui sont le plus fréquemment retrouvées en eau douce. Les microcystines sont produites par plusieurs genres de cyanobactéries et leur concentration est très variable dans les écosystèmes aquatiques du point de vue temporel et spatial, puisque des souches toxicogènes et non toxicogènes peuvent coexister pour former des fleurs d'eau (blooms) importantes. Les facteurs influençant la production des microcystines, leurs rôles écologiques et physiologiques, ainsi que les facteurs qui stimulent spécifiquement le foisonnement de cyanobactéries toxicogènes sont largement inconnus. En premier lieu, cette thèse avait pour objectif de tester le rôle de l'intensité de la lumière à titre de facteur important dans le contrôle de la production et de la composition en congénères de microcystines, et ce dans le cadre d'expériences de culture en laboratoire. En second lieu, grâce à des observations sur le terrain et une expérience utilisant des enclos dans un lac, cette thèse a aussi étudié les effets de variables physiques, chimiques et biologiques sur les concentrations de microcystines et sur le foisonnement de cyanobactéries toxicogènes dans un lac peu profond et mésotrophique. Tout au long de ces recherches, diverses méthodes de nature chimique et moléculaire ont été utilisées, ainsi que des méthodes plus traditionnelles de culture et de limnologie, afin de mesurer la production de microcystines et la présence de cyanobactéries toxicogènes.

L'intensité de la lumière a exercé un effet considérable sur les concentrations de microcystines et sur la composition de congénères. Cependant, les souches toxicogènes ou non toxicogènes n'avaient aucun avantage précis quant à leur capacité à foisonner, à entrer en compétition, ou à dominer dans un environnement à cultures mixtes. La réponse de chaque souche de *Microcystis aeruginosa* était unique, de sorte qu'il était impossible de généraliser sur les souches toxicogènes et non toxicogènes quant à leur capacité de compétition dans les milieux aquatiques ; la production de microcystines ne semble pas être désavantageuse pour les souches toxicogènes. Sur une période de deux ans, des études sur le terrain ont démontré que le nombre de copies de gènes de microcystines (*mcy*D) n'était pas bien corrélé avec les concentrations de microcystines. Dans le lac même ainsi que sur un gradient de nutriments établi dans des enclos *in situ*, le meilleur indice de la présence de souches toxicogènes était la biomasse de genres potentiellement toxicogènes (surtout

Anabaena et *Microcystis*), plutôt qu'une variable chimique ou physique spécifique telle que la lumière. Cependant, les variables environnementales et biologiques ont joué un rôle important dans la prédiction des concentrations de microcystines. La combinaison de données moléculaires, chimiques et taxonomiques semble nécessaire afin de comprendre et de prédire les fleurs de cyanobactéries toxicogènes.

Acknowledgements

A doctoral thesis is not simply the work of one individual but rather the result of years of support, dedication, love, guidance, and encouragement from a group of people without whom I would not have been able to complete this journey.

I would first like to acknowledge Dr. Frances Pick my thesis supervisor for her kindness, positive attitude, and unwavering support in both my professional and personal endeavours. Frances, you are an amazing woman and I have been honoured and privileged to be a part of your lab group.

I would also like to thank my committee members Dr. J.T. Arnason at the University of Ottawa and Dr. David Miller of Carleton University. Over the course of this project they have been a great source of ideas, support, and feedback throughout my research progress. I have great respect for their advice and opinions along with the research each has carried out in their distinguished careers.

In the past four and a half years I have been fortunate to work in a number of different laboratories to complete my research including at the University of Ottawa, Health Canada, the National Research Council (NRC) Biotechnology Research Institute (BRI) in Montréal and the NRC Institute for Marine Biosciences (IMB) in Halifax. I am greatly indebted to the knowledge, technical assistance, equipment, and expertise of the following people I would like to recognize. First, at the University of Ottawa I would like to recognize Lynda Kimpe and Dr. Ammar Saleem for their amazing assistance and great patience. Lynda thank you for letting me invade your lab space on countless occasions. Ammar, thank you for being such a patient and outstanding teacher. Our lab group truly appreciates the time and knowledge both of you have given to us.

From Health Canada at Tunney's Pasture in Ottawa I would like to thank Dr. Rocio Aranda-Rodriguez and her group including Jeromy Harvey. From NRC-BRI I would like to thank Dr. Charles Greer and his lab group for welcoming me on several occasions. In particular I must pay special tribute to my 'molecular guru' Nathalie Fortin who with great patience and skill taught me how to do a QPCR with success. I would also like to acknowledge Dr. David Bird from UQAM who helped to get our collaboration off the ground with Health Canada and NRC-BRI in order to study cyanobacterial blooms in Canada and to develop molecular and analytical tools. It was an absolute pleasure to be a part of

this group. I would also like to thank Dr. Michael Quilliam at NRC-IMB in Halifax and his lab group, especially Krista Thomas and Pearse McCarron, for their assistance with LC-MS work and graciously letting me into their lab for a visit. Other special thanks to Serge Parent, Linda Ley, Philip Pelletier, and all the staff in the Biology Department at the University of Ottawa. Funding for this doctoral research was provided by an NSERC CGS, University of Ottawa Excellence Scholarship, and an NSERC Strategic Grant.

Throughout my time at the University of Ottawa I have been fortunate to meet some exceptional people who have made this journey all the more worthwhile. First, to my Pick lab mates past and present, especially Marc Demers, Angeline Tillmanns, Jacinthe Contant, Lieserl Woods, Andrew Waye, Christine Lavoie, Medina Sarak, Justin Meyer, Rebecca Dalton, Stacey Saucier, Muriel Mérette, and Arthur Zastepa. All of you have been a part of my journey and I am happy to have been in such great company in the lab. To my friends spanning from Ottawa, Sault Ste. Marie, Kapuskasing and beyond- thank you for your support, words of encouragement, and utmost patience when I tried to explain what exactly I was studying. In particular I would like to thank the lovely Dominique McMahon- thank you for explaining to me what a PCR was and helping me with my molecular homework, for standing next to me on one of the most important days of my life, and for being your wonderful self. A special thank you as well to Karen Foster for many 'coffee breaks' and being a sounding board for all things thesis related.

To my family I would like to simply say I love you. You have stood by me and cheered me on every step of the way. To my Renaud family I cannot say enough about the incredible way that all of you have contributed to my success. In particular, Mireille you are absolutely wonderful and thank you for letting me be your roommate. Lise and Yvon, I cannot express enough gratitude for your kindness, generosity, and support. Un grand merci!

To my brothers Luke, Jeff, and Pat. I know sometimes you didn't know exactly what I've been up to for the past while in studying this green stuff that grows in lakes, but thank you for always being interested, supportive, and kind to your geeky sister.

To Mom and Dad, Laura and Allen LeBlanc. You have been my greatest fans from day one and have never wavered in your belief in me and what I am capable of doing. You gave me the groundwork I needed to tackle any challenge that may come my way and I will forever be proud to be your daughter and that I have both of you as my guideposts in life.

To conclude I would like to thank my partner in crime, my buddy, my husband, my best friend, Justin. For over ten years you have always been supportive, made me laugh, and loved me completely as I am quirks and all. As I finish this adventure I have a happy and fulfilled heart knowing you are there with me to start the next.

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Chapter 1
Introduction to Toxic Cyanobacteria

1.1 Introduction

Cyanobacterial blooms are a major problem worldwide with impacts on both ecosystem and human health (Sivonen & Jones 1999). Cyanobacterial blooms can cause taste and odour problems, are aesthetically displeasing, and can lead to fish and benthic invertebrate mortality from declines in oxygen. In addition, several genera of cyanobacteria produce toxic compounds termed 'cyanotoxins' (Carmichael 1994, Zurawell *et al.* 2005).

Cyanobacteria are photosynthetic prokaryotes and thus are bacteria which contain chlorophyll a, phycobilins, and other pigments which allow them to carry out photosynthesis. There are approximately 150 genera of cyanobacteria found in freshwater, marine, and terrestrial environments, comprising approximately 2000 species, of which at least 40 species are known to produce cyanotoxins (Haider *et al.* 2003).

The first scholarly report of a toxic bloom occurred in Australia in 1878 (Zurawell *et al.* 2005). Since that time there have been hundreds of recorded cases of livestock and animal deaths (cattle, sheep, dogs, and birds) resulting from ingestion of cyanobacterial blooms (Carmichael1994). One of the most dramatic cases of human poisoning attributed to cyanobacterial toxins occurred in 1996 when dozens of hemo-dialysis patients died in Caruaru, Brazil, after direct exposure to a water supply contaminated by a cyanobacterial bloom. Toxic compounds (presumably the cyanotoxins) had caused acute lethal toxicity in the liver (Dawson 1998). A growing number of communities in Canada are now being confronted by recreational and drinking water affected by toxic cyanobacterial blooms including major freshwater resources such as Lakes Erie, Ontario, Winnipeg, Champlain, and numerous smaller lakes and rivers across the country (Boyer *et al.* 2004, Rinta-Kanto *et al.* 2005).

While the biological mode of action and toxicity of most cyanotoxins is well understood, the reasons why cyanobacteria produce toxins have not been clearly established (Gupta *et al.* 2001). From an evolutionary perspective the production of toxins must benefit the host cyanobacterium in some way (Codd 1995) as they are too unique and structurally complex to be considered as 'waste' compounds, or excretion products (Paerl & Millie 1996, Leflaive & Ten-Hage 2007). Several potential ecological roles for cyanotoxins have been proposed. These include defense against grazers, symbiotic relationships, metal acquisition and storage (i.e. iron chelators), and reserve pools of metabolites (Codd 1995). It is also

hypothesized that cyanotoxins have an actual function in cyanobacterial physiology or ecology (Leflaive & Ten-Hage 2007).

Cyanotoxins are considered secondary metabolites and are grouped according to the physiological systems, organs, tissues, or cells which they primarily affect i.e. neurotoxins, hepatotoxins, cytotoxins, irritants, and gastrointestinal toxins (Codd *et al.* 2005). The two most studied groups are the hepatotoxins (i.e. microcystins, nodularin, cylindrospermopsin) and the neurotoxins (i.e. anatoxins, saxitoxins). This thesis focuses on the most common toxin of human health concern, the hepatotoxic microcystins produced by several cyanobacterial genera including *Microcystis, Anabaena, Planktothrix, Gloeotrichia, Nostoc, Aphanocapsa* (a picocyanobacterium), *Arthrospira,* and even a terrestrial strain of *Nostoc* (Domingos *et al.* 1999, Oksanen *et al.* 2004, Ballot *et al.* 2005, Zurawell *et al.* 2005, Carey *et al.* 2007).

1.2 Microcystins

Microcystins are cyclic-septapeptides (Fig. 1). They contain two variable L-amino acids plus three D-amino acids and the Adda group which is essential for the expression of biological activity (An & Carmichael 1994). The two variable amino acids allow for different combinations of the molecule to be synthesized. There are currently more than 80 known variants or congeners of microcystin (Dittmann & Wiegand 2006). In general a single strain of *Microcystis aeruginosa* produces multiple congeners (Codd 1995). Toxic strains can also vary both in the quantity and composition of microcystin congeners produced.

Microcystins can be measured using a variety of analytical techniques including mouse bioassays, protein phosphatase inhibition assays (PPIA), ELISA (enzyme-linked immunosorbent assay), HPLC-UV with PDA (UltraViolet with Photo Diode Array detection), and HPLC-MS (HPLC with Mass Spectrometry). In the earliest stages of microcystin detection mouse bioassays were used in order to assess the toxicity of the (at the time) unknown toxic compounds being produced by some cyanobacterial blooms. Microcystin concentrations were represented as LD_{50} values in terms of mouse body weight (i.e. $\mu g \cdot kg^{-1}$). When the structure of the microcystin molecule was better understood analytical methods were then developed that were targeted more specifically to detect

microcystins using immunochemical/enzyme (i.e. PPIA or ELISA) and/or chemical techniques (i.e. HPLC-UV, HPLC-MS). However, no one method has thus far proven to be the definitive technique for measuring microcystins.

The PPIA and ELISA are both immunochemical/enzyme based assays. In an ELISA assay antibodies are used that recognise the Adda component of the microcystin molecule and can measure total concentration of microcystins, while the PPIA reacts to the inhibitory action of microcystins to protein phostphatase and thus can give an indication of the total toxicity of a sample. Both of these methods are more sensitive than HPLC, and with their lower limits of detection they do not necessarily require a pre-concentration step. They are also relatively quick to perform, are inexpensive, and require minimum sample processing. However, while they can give an indication of the toxicity of a sample, these methods do not identify which microcystin congeners are present, and have also been shown in the past to have occasional problems with cross-reactivity resulting in an overestimation of microcystin concentrations (Mountfort *et al.* 2005, Sangolkar *et al.* 2006, Tillmanns *et al.* 2007).

Chemical methods such as HPLC or LC-MS are more specific than the PPIA or ELISA. They allow one to determine the actual identity of microcystin congeners present in a sample. The identification of microcystins is based on the detection of the main chromophore at 238 nm, which is a diene in the Adda component of the microcystin molecule (Fig. 1). While one can achieve greater specificity in terms of the actual microcystins present in a sample there are several disadvantages to an HPLC or HPLC-MS approach. The process is time consuming as environmental samples need to be pre-concentrated and extracted prior to analysis. The use of HPLC can also be technically demanding and expensive. The biggest drawback however is the lack of certified reference materials. There are more than 80 known microcystins yet less than a dozen are readily available as standards. Therefore while HPLC using UV-PDA detection is often considered the 'gold-standard' in terms of microcystin detection, the method still has several drawbacks.

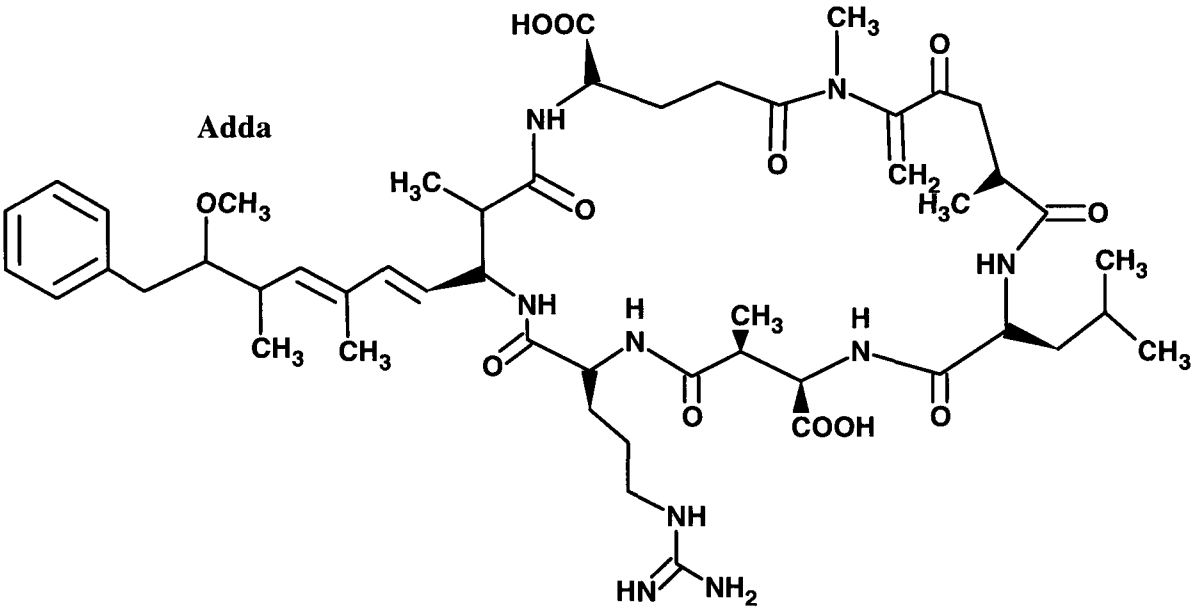


Figure 1. Structure of the hepatotoxic microcystins (microcystin-LR). The molecule is a cyclic septapeptide, with two variable amino acid positions. The side Adda moiety is essential for the compound to induce a biological effect.

The use of mass spectrometry can help to further identify microcystin congeners beyond the positive identification of a UV peak at 238 nm. However, once again due to the lack of available standards, it is difficult to be completely certain of the true identity of a suspected microcystin peak. There is a growing consensus that using a combined approach of both an immunological/enzyme method such as PPIA or ELISA in concurrence with HPLC coupled with mass spectrometry represents the ideal way of analyzing microcystins from both field and culture samples(Mountfort *et al.* 2005, Sangolkar *et al.* 2006, Tillmanns *et al.* 2007).

Microcystins exert their toxic effects in vertebrates by inhibiting enzymatic pathways principally the serine-threonine protein phosphatase enzyme. It is the introduction of the Adda tail portion of the microcystin molecule into the hydrophobic groove at the catalytic site of protein phosphatase that renders the enzyme inactive. Structural changes to Adda, including even a stereochemical change or synthetic microcystin without Adda, are consequently non-toxic (Wiegand & Pflugmacher 2005, Stewart & Falconer 2008). When this pathway is inhibited the cytoskeletal proteins that normally would not be phosphorylated can be, resulting in a disruption of the cytoskeleton (Dawson 1998). LD_{50} estimates for microcystins are in the range of 25 to 600 $\mu g \cdot kg^{-1}$ (based on toxicity to rodents) (Dawson 1998, Dittmann & Wiegand 2006, Stewart & Falconer 2008). Therefore LD_{50} estimates for some microcystins are lower than the LD_{50} for compounds such as potassium cyanide (Sakai *et al.* 2007). Microcystins are potent tumor promoters and while there are not as many cases of acute poisonings, the concern lies with chronic doses of microcystins over long periods of time. Epidemiological studies have shown that in places like China, where water supplies are often contaminated, there are sharp increases in cancers of the liver (Falconer 2005a). The World Health Organization (WHO) has established a drinking water guideline for microcystins of 1 $\mu g \cdot L^{-1}$ daily intake which is based on the toxicity of the most common microcystin congener of microcystin-LR (Falconer & Humpage 2005). In Canada a guideline has been set at 1.5 $\mu g \cdot L^{-1}$(Health Canada 2002).

Overall microcystins appear to have adverse effects on a number of different types of aquatic organisms; however the wide range of toxicological studies completed show significant variation in the severity of microcystin toxicity. For example, microcystins may have some allelopathic effects on the growth and function of other algae and higher aquatic plants including species such as *Nostoc muscorum, Anabaena, Ceratophyllum demersum,*

and *Spirodela oligorrhiza* (Pflugmacher *et al.* 1999, 2002, Singh *et al.* 2001, Rowmanowska-Duda & Tarcyzynska 2002). However, other studies have shown contradictory results with no adverse effects of microcystins on aquatic macrophytes detected (LeBlanc *et al.* 2005, Babica *et al.* 2006). The toxic effects of cyanotoxins on zooplankton have also been studied extensively (Lampert 1982, DeMott *et al.* 1991, Hawser *et al.* 1992, Rohrlack *et al.* 1999, Jang *et al.* 2003, Lurling & van der Grinten 2003). However, it is still not completely clear whether or not it is the toxins that are adversely affecting zooplankton, or whether the observed negative effects are the result of the poor nutritional quality of cyanobacteria, or of feeding inhibition due to the size and shape of cyanobacterial cells (Sivonen & Jones 1999, Tillmanns *et al.* 2008).

Other aquatic animals have been tested as well for effects of microcystins including Australian Black Tiger Prawns, zebrafish, trout, and several types of amphibians (including axolotl, smooth newt, and marsh frogs) (Oberemm *et al.* 1999, Wang *et al.* 2005, Pflugmacher *et al.* 2005). In terms of fish it is not only the production of cyanotoxins that may adversely affect fish populations but the physical changes in a water body due to the growth of a cyanobacterial bloom. A high pH and low oxygen environment, which can result from excessive cyanobacterial growth, may be more of a problem for fish populations than the potential toxic effects of microcystins (Wiegand & Pflugmacher 2005).

1.3 Regulation of Microcystin Production

Microcystins are produced non-ribosomally and are considered secondary metabolites as no primary role for the microcystins in cyanobacterial growth or reproduction has been determined to date. A literature review reports that anywhere from 25 to 75% of cyanobacterial blooms produce cyanotoxins (Zurawell *et al.* 2005). However, it is not completely known why toxigenic strains tend to dominate and whether the production of microcystins might confer a potential competitive advantage to toxigenic strains. As a result it has not been possible to predict with consistency and accuracy the toxicity of blooms. Unfortunately the regulation of the entire microcystin gene complex is not well understood (Tonk *et al.* 2005). If the factors responsible for regulating the expression of microcystin genes were known this might explain why and how toxigenic species seem to dominate water blooms.

It is thought that the genes responsible for microcystin production were present in the last common ancestor of a large number of cyanobacteria due to the fact that microcystins can be produced by a wide variety of distantly related cyanobacterial genera (Rantala *et al.* 2004, Dittmann & Borner 2005). It is believed that the ability to produce microcystins has been repeatedly lost during evolution, i.e. that the production of microcystins and nodularins are an ancestral relict that may have been lost in non-toxigenic strains (Rantala *et al.* 2004, Dittmann & Borner 2005, Jungblut & Neilan 2006). Evidence for this lies in phylogenetic analyses which show a co-evolution of both housekeeping and microcystin synthetase genes (Rantala *et al.* 2004). While both toxigenic and non-toxigenic strains of *Microcystis aeruginosa* contain DNA sequences for homologous peptide synthetase genes, they differ in the presence of the specific genes encoding for microcystin production (Dittmann *et al.* 1997).

Microcystin synthesis is directed by a 55 kb gene cluster divided into two operons *mcy*ABC and *mcy*DEFGHIJ (Fig. 2, Tillett *et al.* 2000). Each operon is responsible for one cycle of polyketide or polypeptide chain elongation (Nishizawa *et al.* 1999). The smaller operon (*mcy*ABC) is thought to be involved in the growth of the septapeptide chain that eventually forms a cyclical structure in the microcystin molecule. The larger operon (*mcy*D-J) is thought to encode enzymes that catalyze the formation of the Adda side chain (Tillett *et al.* 2000). Upon further examination the specific roles of all the *mcy* genes have been determined. *Mcy*A-C, E, and G are involved in peptide synthesis; *mcy*D, and E are involved in polyketide synthesis; *mcy*J-methylation; *mcy*F- epimerization; *mcy*I- dehydration; and *mcy*H- localization (Tillett *et al.* 2000). It is thought that perhaps the *mcy*B gene may be involved with the activation of the 'variable L-amino acids' which result in the great variety (>80) of microcystin congeners (Bittencourt-Oliveira 2003). There is a strong correlation between the microcystin isoforms produced and the genetic variants of the *mcy*B module and it is believed that the genetic variants are the result of recombinations between modules (i.e. between *mcy*B and *mcy*C) (Nishizawa *et al.* 2000, Mikalsen *et al.* 2003, Kurmayer *et al.* 2005).

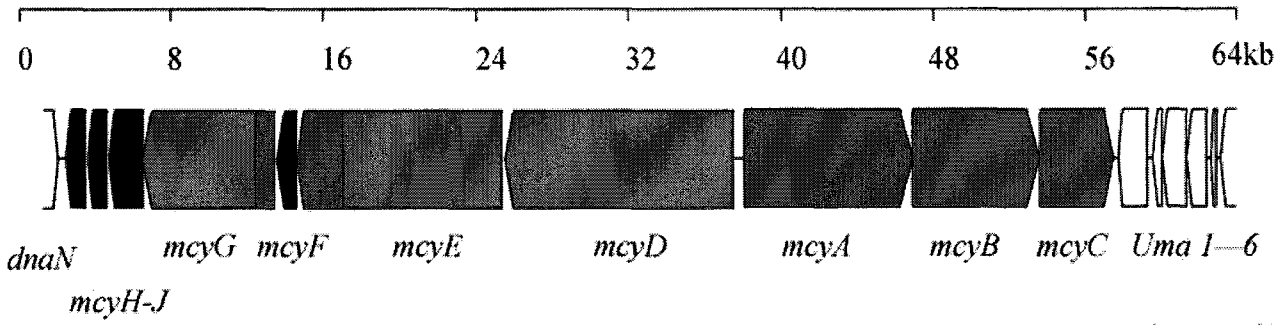


Figure 2. Organization of the gene cluster for microcystin biosynthesis. The direction of transcription and the relative sizes of the open reading frames are indicated. (Tillett *et al.* 2000).

There are numerous factors that can affect the expression of a gene. In the context of the microcystin synthetase gene cluster, the production of microcystins can be considered at three levels: genetic, population, and cellular (Fig. 3).The first level at which microcystin production can be regulated is at the level of the gene. It is now known that if a strain of *Microcystis aeruginosa* has the microcystin synthetase gene cluster it has the capacity to produce microcystins, while if it does not have the gene cluster, or is missing a portion of the gene cluster, the strain will not produce microcystins and thus would be deemed 'non-toxigenic'. If a strain does have the gene cluster, however, then the expression of the genes can be affected in number of ways. Cyanobacteria live in aquatic environments therefore a number of physical, chemical, and biological variables from their surroundings may affect all cell processes (growth, division, etc.) which may result in the up and/or down regulation of certain genes. Several variables such as light intensity, temperature, nutrients and trace metals have been examined under laboratory conditions and investigated with respect to their effect on microcystin production (Kaebernick & Neilan 2001). At the population level the overall concentrations of microcystins in a given environment may be affected by both the genetic and cellular regulation of microcystin production. First, if there are a greater number of non-toxigenic genotypes present the amount of microcystin produced by the toxin producers will be 'diluted'. Recent studies, including the present thesis, have begun to incorporate quantitative-PCR (QPCR) techniques in order to quantify the toxigenic versus non-toxigenic genotypes in a water body (Rinta-Kanto *et al.* 2005, Rueckert *et al.* 2007, Yoshida *et al.* 2007, Briand *et al.* 2008). This type of QPCR analysis becomes even more critical when trying to determine if any external factors promote the growth of toxin producing strains over non-toxin producers, which may help us to better predict when a bloom may or may not be toxic.

Several studies have examined the factors which affect total microcystin production at the cellular level such as temperature, nutrients (iron, phosphorus, nitrogen) and light. Temperature is thought to have an effect (if minor) on microcystin production based on culture experiments. Temperature changes from 16-36°C resulted in the greatest microcystin production at 20°C in *Microcystis*, with decreased rates of production occurring as temperature increased (van der Westhuizen *et al.* 1986). Temperature was also shown to affect the production of

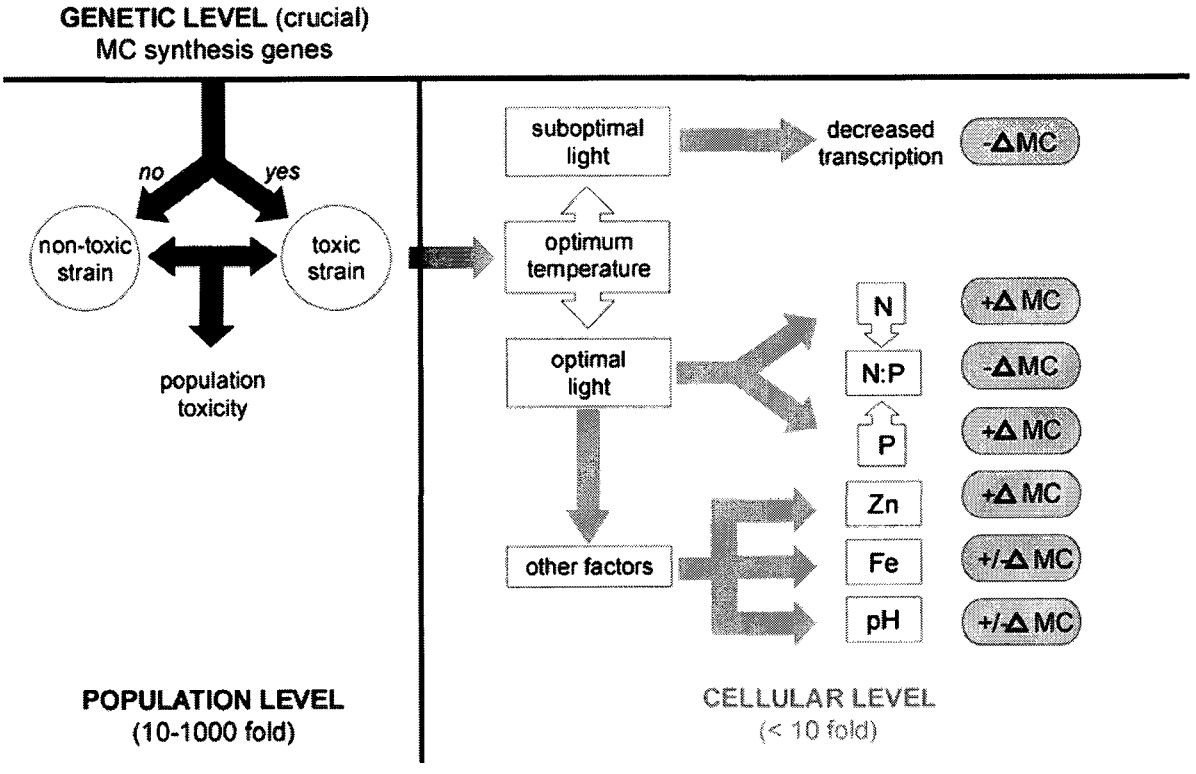


Figure 3. Schematic representing the different levels at which microcystin production can be regulated. (Zurawell *et al.* 2005).

nodularin in *Nodularia spumigena,* with the optimum temperature for nodularin production being lower than the optimum temperature for growth (Hobson & Fallowfield 2003).

Iron has an effect on cyanobacterial competition with other organisms as cyanobacteria can dominate other algal species through the release of specific iron chelators (Murphy *et al.* 1976). Lyck *et al.* (1996) compared the growth of toxigenic and non-toxigenic strains of *Microcystis aeruginosa* under iron-replete and iron-depleted conditions. Iron starvation resulted in an increase in toxicity and the toxigenic strain was sustained much longer than the non-toxigenic during iron depletion. Utkilen & Gjolme (1995) found that a microcystin-producing *M. aeruginosa* strain had a more efficient iron uptake system than a non-toxin producing strain and believed that microcystins were produced to act as intracellular chelators of iron. In contrast, Amé & Wunderlin (2005) demonstrated that increases in iron concentration significantly increased total microcystin concentrations.

Nutrients such as nitrogen (N) and phosphorus (P), and the ratio of N:P, have been shown to affect microcystin production. Vézie *et al.* (2002) determined that non-toxigenic strains of *Microcystis* required fewer nutrients for their growth at low concentrations, but that at high nutrient concentrations the toxigenic strains grew better. Therefore under conditions of nutrient limitation non-toxigenic strains might be favoured because they do not have the added cost of producing microcystins which, being produced via a multi-enzyme complex, may require a lot of energy and nutrients such as nitrogen (Vézie *et al.* 2002). In contrast, under eutrophic conditions higher levels of nutrients may favour the development of toxigenic strains over non-toxigenic *Microcystis aeruginosa* strains.

Downing *et al.* (2005a) varied N:P ratios in batch cultures of *Microcystis aeruginosa* and determined that the maximum growth rates were obtained with N:P ratios greater than 18 which also correlated with cellular protein content. Studies using N:P ratios provide interesting insights into the effects of nutrients on microcystin content because often microcystin production levels correlate to nutrient ratios irrespective of the actual concentrations of nutrients used (Lee *et al.* 2000, Downing *et al.* 2005a). However, these relationships can also be difficult to fully interpret if N or P is a limiting nutrient which then in turn would affect growth rate and thus microcystin production. It is thought that nitrogen may be a more critical nutrient for microcystin production because it is needed for protein

synthesis and that in N-limiting conditions reductions in microcystin content would occur (Downing *et al.* 2005a).

1.4 Light

Light intensity may also affect microcystin production. Utkilen & Gjolme (1992) grew the *Microcystis aeruginosa* strain CYA 229/1 in continuous cultures under different light intensities (25-75 $\mu M \cdot m^{-2} \cdot s^{-1}$) and varying light qualities (white, green, and red light). Toxin production increased up to 40 $\mu M \cdot m^{-2} \cdot s^{-1}$ after which toxin production decreased. Toxin production was also slightly enhanced by both red and green light (Utkilen & Gjolme 1992). Wiedner *et al.* (2003) also found similar patterns, with microcystin production increasing until an irradiance of 80 $\mu M \cdot m^{-2} \cdot s^{-1}$, after which increasing irradiance resulted in a decline of microcystin production.

There is also some evidence to suggest that light may have noticeable effects on the activity of the microcystin synthetase gene cluster. Kaebernick *et al.* (2000) demonstrated that under high light intensities (and especially light in the red spectrum) there were increases in microcystin peptide synthetase gene transcription. They proposed that the microcystin synthetase gene cluster is regulated by light intensity, and that the toxin is produced constitutively under low and medium light intensities, but is increased under higher light intensities. They also suggested that there were different thresholds of light intensity for initiation and upregulation of toxin production. Kaebernick *et al.* (2002) did a follow-up study in which they used RT-PCR to detect mRNA transcripts in the *mcy*A-J cluster. They performed a complete transcriptional analysis of the *mcy*A-J cluster, and also examined transcription under different light conditions. As in their earlier work, they confirmed that light dependent transcription start sites existed for *mcy*A and *mcy*D. This indicates that the *mcy* gene cluster may be constitutively transcribed under low light conditions and that under high light conditions an activator is involved (Kaebernick *et al.* 2002).

Most studies examining the effects of light and other environmental variables used toxigenic strains alone, or both toxigenic and non-toxigenic strains. However, to truly understand the role that microcystins play in toxin producing strains, Hesse *et al.* (2001) created a mutant strain of a toxigenic *Microcystis aeruginosa*, knocking out the gene for microcystin production to determine if the loss of microcystin would have phenotypic

consequences for the cyanobacterium, especially in light-dependent processes. The growth rates of both wild-type and mutant strains did not differ, however there were differences in biovolume between strains. This suggested that there was a change in internal structure in the mutant cells as differences in light refraction (scattering) would contribute to different biovolume measurements between strains. Also, cellular concentrations of pigments were 20% lower in the mutant cells compared to the wild-type cells. Microcystins may play a role in light adaptation processes because the transcription of microcystin synthetase genes appears to be light-regulated, and that microcystins themselves are concentrated around the thylakoid membranes of cyanobacterial cells (Young *et al.* 2005, 2008).

1.5 Thesis Rationale and Objectives

It is inherently difficult to study toxic cyanobacterial blooms as the overall toxicity of a cyanobacterial bloom can be highly variable and thus difficult to predict. First, not only do several cyanobacterial genera have the potential to produce the same cyanotoxin (microcystins are known to be produced by more than four different genera) there are some species that have the ability to produce more than one type of toxin. For example, *Anabaena flos-aquae* produces both the hepatotoxin microcystin and the neurotoxin anatoxin-a (Kearns & Hunter 2000). Also, most toxin producing genera have both toxigenic and non-toxigenic strains. In the past this has made it extremely difficult to determine which species or strain was responsible for toxin production in the environment.

Second, toxicity can vary temporally within and between years, diurnally, and also spatially throughout a water body (Lambert *et al.* 1994, Kotak *et al.* 1995). In extreme cases microcystin concentrations can vary by several orders of magnitude which is linked to both the abundance of cyanobacteria present and the composition of strains, in particular the relative amounts of those that can and those that cannot produce microcystins (Graham *et al.* 2006). This makes an accurate risk assessment of a particular water body difficult to undertake.

Third, the actual physiological and ecological bases for toxin production are still unclear. Most of the organisms that may potentially be affected by cyanobacterial toxins (fish, humans, livestock etc.) are not natural enemies or consumers of cyanobacteria (Paerl & Millie 1996). It is unlikely that cyanotoxins are produced to be released into the

environment as inhibitors or toxins. In fact microcystins remain largely intracellular with little active release into the environment until cell death or lysis occurs which explains why the majority of microcystins are detected from a particulate rather than a dissolved fraction (Rohrlack & Hyenstrand 2007). Thus while the environmental factors that lead to cyanobacterial bloom formation are fairly well known, the factors that specifically promote the growth of toxigenic microcystin bloom events are not well understood. Field and laboratory studies are now focusing on examining variables such as nutrients (nitrogen, phosphorous, iron, N:P ratio), temperature, and light, to determine their roles (if any) in promoting the growth of toxic cyanobacterial blooms. This growing area of research also includes the development of molecular tools specific to studying toxic cyanobacterial blooms *in situ*.

In this research I tested the role of light in regulating the production of microcystins in terms of both the quantity and composition of microcystin congeners produced. The overall goal was to determine if light intensity was a significant factor in toxic bloom development and in the dominance of toxigenic strains of cyanobacteria. Field and enclosure studies were also conducted to determine not only the effect of light, but also a range of environmental and physical variables, on both microcystin concentrations and the growth of toxigenic cyanobacteria. The research is divided into four chapters encompassing laboratory experiments (Chapters 2 and 3) and field studies (Chapters 4 and 5).

Chapter 2

The effect of light intensity on growth and production of microcystin congeners in *Microcystis aeruginosa* and *Microcystis flos-aquae*

Abstract

There are currently more than 80 known congeners of the hepatotoxin microcystin produced by several different cyanobacterial genera. The effects of environmental factors on total microcystin production have been examined including the effect of nutrients, light, and temperature. However, few studies have examined the effect of environmental variables on the production of specific microcystin congeners which may vary in their toxicity. Whether the toxin profile is a constant, stable characteristic of a given strain or is under environmental control is not known. This study measured growth and microcystin production in seven strains of *Microcystis* including six strains of *M. aeruginosa* and one of *M. flos aquae* under three different light intensity levels: low (10 $\mu M \cdot m^{-2} \cdot s^{-1}$), medium (60 $\mu M \cdot m^{-2} \cdot s^{-1}$), and high (150 $\mu M \cdot m^{-2} \cdot s^{-1}$). Three strains of *Microcystis* were microcystin producers while four were not. Growth rates of all strains were significantly affected by the light intensity with most strains having maximal growth rates when grown under medium or high light intensities. There was no pattern, however, in terms of toxigenic or non-toxigenic strains having higher or lower growth rates than one another when grown under different light intensities. Total microcystin production was also significantly affected by light intensity with the greatest microcystin production occurring under medium light intensities. Light intensity also affected the proportions of microcystin congeners produced by each toxigenic strain. While the same congeners were produced within a strain regardless of the light intensity, the proportion of a congener produced by a strain was significantly affected by the light level under which it was grown. Strains UTCC 299 and 300 each produced two congeners while UTCC 464 had six congeners, one of which may be a novel compound.

2.1 Introduction

Both light quality and light intensity have been shown to affect the growth of cyanobacteria along with the total production of cyanotoxins (Kaebernick *et al.* 2000). Light has also been shown to affect the production of microcystins at the genetic level (Kaebernick *et al.* 2000, 2002). However, very few studies have determined whether environmental variables such as light affect the specific microcystin congeners produced by toxigenic cyanobacteria. Not all congeners of microcystin have the same toxicity (in terms of intraperitoneal mouse LD_{50} values). For example microcystin-LR has an LD_{50} of 50 $\mu g \cdot kg^{-1}$ whereas other microcystin variants have LD_{50} values of up to 600 $\mu g \cdot kg^{-1}$ (Dittmann & Wiegand 2006, Stewart & Falconer 2008). Therefore in terms of assessing the risk of a toxic cyanobacterial bloom to humans, livestock, and other organisms, the identity and conditions under which different microcystin congeners are produced are important factors to understand.

Early studies showed that changes in both temperature and light could affect the composition of toxins produced. Before the availability of rapid and reliable HPLC technology to detect the specific microcystin congeners produced, van der Westhuizen *et al.* (1986) demonstrated that at different temperatures different peptide compositions of microcystins were obtained with three peptides detected for cultures of *Microcystis aeruginosa* grown at 16 and 20°C, while only two peptides were detected at 28 and 36°C. They also observed an increase in toxicity (determined using mouse bioassays) with an increase in irradiance from 21 to 35 $\mu M \cdot m^{-2} \cdot s^{-1}$.

Rapala *et al.* (1997) examined the effect of temperature, light, and nutrients (phosphorus and nitrogen) on growth and microcystin production in two strains of *Anabaena.* This was one of the first studies to show the importance of external growth stimuli in regulating levels and proportions of microcystin congeners in cyanobacteria. Their results showed that increases in phosphorus resulted in increased rates of growth and of microcystin production, while very high or very low temperatures decreased microcystin concentrations. Light had varying effects on both *Anabaena* strains. The microcystin content of cells grown at four different light intensities (2-100 $\mu M \cdot m^{-2} \cdot s^{-1}$) did not correlate positively with growth: microcystins remained fairly constant between lower light levels while at higher levels of irradiance microcystin content decreased (Rapala *et al.* 1997). As for the specific

microcystin congeners examined it was found that light affected the production of microcystin-LR, microcystin-RR, [D-Asp^3] microcystin-LR, and [D-Asp^3] microcystin-RR differently between the two strains of *Anabaena* tested. For one strain of *Anabaena* increasing irradiance did not affect the levels of microcystin congener production, while for the second strain microcystin-LR and microcystin-RR significantly increased from low to medium light levels, while the other two congeners did not change in concentration (Rapala *et al.* 1997).

More recently, Tonk *et al.* (2005) examined the effect of light intensity on the *mcy*A transcript, total microcystin production, and on the production of specific microcystin congeners in the filamentous cyanobacterium *Planktothrix agardhii*. Two microcystin congeners were produced: microcystin-DeRR and microcystin-DeLR. When cultures were transferred from low to high light or vice versa, the concentrations of both microcystin congeners subsequently changed with the level of irradiance. With increased photon irradiance the concentration of microcystin-DeRR decreased while the concentration of microcystin-DeLR increased. However, at light levels greater than 100 $\mu M \cdot m^{-2} \cdot s^{-1}$, both the transcription rate of *mcy*A and total microcystin production decreased significantly. A potential explanation of this shift in microcystin congener production may be that different light conditions result in changes in the composition of available amino acids. It is of note that microcystin-DeLR is at least four times as toxic as is microcystin-DeRR, therefore there was a shift towards the production of a more toxic microcystin congener as photon irradiance increased (Tonk *et al.* 2005).

The goal of the present study was to determine the effect of light intensity on the production of different microcystin congeners in the cyanobacterium *Microcystis aeruginosa* and to determine if light intensity affected the rate of growth of toxigenic and non-toxigenic strains differently. I hypothesized that the intensity of light would affect not only the total amount of microcystin produced, but also the composition of microcystin congeners produced (i.e. LR, RR, YR, LA, etc.). I predicted that a difference would be seen in the response of the toxigenic versus non-toxigenic strains to changing light intensities: at low light levels the non-toxigenic strains would maintain a higher growth rate than toxigenic strains because of the energetic costs associated with microcystin production.

2.2 Methods

2.2.1 Culturing Techniques

Six strains of *Microcystis aeruginosa* and one strain of *Microcystis flos-aquae* were obtained from the University of Toronto Culture Collection (UTCC) including the microcystin-producing strains UTCC 299, 300, and 464, and the non-microcystin producing strains UTCC 124, 632, 633 and 460. Table 1 provides information regarding the history of each strain. Cultures were grown in 500 ml Erlenmeyer flasks containing 250 ml of BG-11 (Sigma C3061), a growth medium comprised of nutrients and trace metals necessary for cyanobacterial growth (Stein 1973).

Each strain was initially grown at 25°C in a Conviron growth chamber at a light intensity of 60 $\mu M \cdot m^{-2} \cdot s^{-1}$and a 12 hour photoperiod. However, before the start of the experiment, strains were transferred in replicates to fresh BG-11 growth medium and allowed to acclimate for at least one week at three different light levels: low (10 $\mu M \cdot m^{-2} \cdot s^{-1}$), medium (60 $\mu M \cdot m^{-2} \cdot s^{-1}$), and high (150 $\mu M \cdot m^{-2} \cdot s^{-1}$).

2.2.2 Growth Experiments

On day 'zero' cultures acclimated at each light level were transferred to fresh BG-11 growth medium, with three replicates of each strain grown at each light intensity. For a period of fourteen days the two main dependent variables measured for each replicate were 1) growth, and 2) level of microcystin production, including the production of different microcystin congeners. This type of batch culture condition simulates the development of a cyanobacterial bloom and the beginning of its collapse in nature (Lyck 2004).

2.2.3 Growth Rate Measurements

Growth of each strain was monitored using two techniques. First, the optical density (O.D.) of each strain was measured daily by recording the absorbance of each strain at 750 nm using a Pye-Unicam spectrophotometer (Stein 1973). Optical density was plotted versus time to obtain the growth curve for each replicate of each *Microcystis* strain, and the growth rate of each replicate was estimated from the slope of the exponential growth phase.

Table 1. Origins of six strains of *Microcystis aeruginosa*, and one strain of *M. flos-aquae* (*) used in growth experiments in which each strain was grown at three different light intensities.

Strain	*Strain Characteristics*
UTCC 299	Isolated from Pretzlaff Pond, Alberta, Canada, August 7, 1990. Deposited by E. Prepas/A. Lam as #45-2A.
UTCC 300	Isolated from Pretzlaff Pond, Alberta, Canada, August 7, 1990. Deposited by E. Prepas/A. Lam as #45-4A.
UTCC 464	Isolated from Trampling Lake, Saskatchewan, Canada, July 1998. Deposited by D. Parker as UWOCC#E7.
UTCC 124	Isolated from Heart Lake Ontario, July 1987 by J. Acreman.
UTCC 632	Received March 2005 from UTEX as UTEX LB 2061. Isolation: 9/48 by G.C. Gerloff from Lake Mendota; Madison, WI-USA; deposited in UTEX as Patterson's 1036AX (Gerloff *et al.* 1950); RELATIVES: CCAP 1450/1 a.k.a. *Diplocystis aeruginosa*, *Anacystis cyanea*; PCC 7005 *Microcystis sp.*; UTCC LB 73.
UTCC 633	UTCC 633 *Microcystis aeruginosa* Received March 2005 from UTEX as UTEX LB 2386. Collection: 9/54 by A. Zehnder; Little Rideau Lake, Ontario, Canada; Isolation: W. Carmichael; deposition in UTEX: 4/84 by P. Gorham as NRC-1(SS-17) non-toxic; RELATIVES: CCAP 1450/4; SAG 14.85; PCC 7941 *Microcystis aeruginosa* TYPE CULTURE; UTCC 468; NIVA CYA 31; NOTES: (Allen & Gorham 1981).
UTCC 460*	Isolated from Fox River, Station 2, Oshkosh, Wisconsin, USA. August 30, 1975. Deposited by D. Parker July 1998 as UWOCC#C3(3X).

Growth was also measured by preserving a subsample of culture every second day in 10% paraformaldehyde and subsequently counting cells using flow-cytometry. The flow cytometer used was a Beckman Coulter FC500. A solution of 20 µl of YG (yellow-green) fluorescent 1 µm beads (Polysciences 18660 Fluoresbrite® YG Microspheres, Calibration Grade 1.00 µm) was added to a 2 ml sub-sample of each replicate for a final bead concentration of 1 x 10^6 beads·ml^{-1}. Beads were added to each sample prior to analysis via flow cytometry so that cyanobacterial cell concentrations could be determined. Each sample was counted for a maximum of 3 minutes or until 100 000 events had been reached. The beads fluoresce in FL1 (green fluorescence, 515-545 nm) while the cyanobacterial cells have a strong fluorescence signal in FL4 (phycocyanin red-orange fluorescence, 653-669 nm). Cell concentrations were calculated by using the ratio of gated beads per known concentration of beads added to the ratio of gated cells per unknown concentration of cells. With the concentration of cells determined cell density could be plotted versus time to obtain a growth curve (as with the spectrophotometric analysis). The growth rate of each replicate was calculated by measuring the slope of the exponential growth phase.

2.2.4 Microcystin Analysis

Microcystins were measured on days two, six, ten, and fourteen of the experiment to determine how toxin levels changed between strains and light intensities. For microcystin analysis 20 ml of each strain was filtered onto a pre-ashed (500°C for 2 hours), pre-weighed Whatman GF/C filter (1822-047). Filters were oven-dried overnight at 60°C, re-weighed to determine net dry biomass, and frozen at -20°C for subsequent analysis. Before extraction of microcystins, all filters were re-hydrated with distilled water and re-frozen at -20°C (Tillmanns *et al.* 2007). This was done to prepare the cells to burst during the extraction procedure, thus ensuring a more efficient total extraction of all toxins. The freezing process ruptures cell walls and releases intracellular toxin (Lehman 2007).

Microcystins were extracted using an Accelerated Solvent Extractor (ASE) under high temperature and pressure to burst the cells and elute the microcystins using 75% methanol (Aranda-Rodriguez *et al.* 2005). Before samples were extracted an internal standard was added. Nodularin is a hepatotoxin similar in structure to microcystin yet sufficiently different for HPLC analysis (as it gives its own distinct peak at a consistent

retention time) and thus was used as an internal standard to calculate percent recoveries on each sample (Aranda-Rodriguez *et al.* 2005). Each filter was spiked with 50 µl of 10 ppm nodularin (Alexis Biochemicals ALX 350-061-C250) so that the percent of nodularin lost during extraction and evaporation could be calculated. In most cases a greater than 90% recovery of nodularin was achieved using the ASE extraction method for all experimental samples.

For ASE extractions each spiked filter was placed in a metal cell, surrounded by the sorbent Hydromatrix (Varian 0019-8004). Each sample was then subjected to a cycle of high temperature and pressure to burst the cells, releasing any microcystins present. The cycle was to fill the cell with the solvent (75% methanol, MeOH) and heat for five minutes to a temperature of 80°C; remain at a steady temperature of 80°C and a pressure of 2000 psi for another five minutes; and last to purge the cell of all solvent into a collection vial. This cycle was repeated twice for all samples. MeOH (75%) is suggested to be the most suitable solvent for microcystin extractions as it can extract microcystins with a wide range of polarities (Sangolkar *et al.* 2006). Approximately 23 ml of extract was obtained after each ASE extraction. Samples were then evaporated down to dryness under nitrogen using a Turbovap Evaporator at 60°C and 16 psi. Samples were re-suspended in 1 ml of 50% MeOH in water and transferred to an HPLC vial and kept at 4°C until analysis by HPLC could be carried out.

Microcystin extracts were analyzed using HPLC with UV-PDA detection (Agilent Technologies 1100 Series).The following microcystin congeners were commercially available as standards: microcystin-LR, YR, RR, and LA (Sigma M2912, M4069, M1537, and M4194). Standard curves were created for each congener using concentrations of 10, 5, 2.5, 1, 0.5, 0.1, and 0.05 ppm. HPLC analysis was conducted using a Zorbax SB-C18 column (150 x 3 x 5 µm). A two-step mobile phase was used consisting of 0.05% (v/v) trifluoroacetic acid (TFA) in water and 0.05% (v/v) TFA in acetonitrile. UV spectra were collected between 200 and 300 nm using a PD40 photodiode array detector and concentrations were calculated from peak area. The absorbance spectrum of each suspected microcystin peak was examined at a wavelength of 238 nm to determine if it had the characteristic shape of a microcystin peak thus confirming its identity as a microcystin. Microcystins have characteristic spectra attributed to the main chromophore, a diene in the

Adda residue (Sangolkar *et al.* 2006). Examples of chromatograms and UV spectra can be seen in Appendix I.

Microcystin concentrations were calculated by comparing the peak area from each sample to the standard curve of MC-LR, YR, RR, and LA. The closest standard to 'unknown' peaks in terms of retention time was used as the standard curve for these congeners. Concentrations were then adjusted to account for percent recovery which was calculated for each nodularin peak in each chromatogram (as the initial amount of nodularin was known-10 ppm). Microcystin concentrations were then standardized across three parameters: 1) dry weight, 2) volume of water filtered, and 3) cell density.

In some cases peaks appeared that had UV spectra with maxima at 238 nm characteristic of microcystins but that did not correspond to the retention times of any of the four microcystin standards available. In this case select samples were re-run using an LC-MS approach. Mass spectrometry was carried out at the NRC-IMB in Halifax, Nova Scotia, using an API4000 Q-Trap LC/MS/MS. Analysis was carried out using a Zorbax SB-C18 RRHT 2.1x50 mm 1.8 μm column with a flow rate of 0.3 $ml \cdot min^{-1}$. The mobile phase consisted of deionized water with 50 mM formic acid, 2 mM ammonium formate, and 95% acetonitrile with 50 mM formic acid, 2 mM ammonium formate. When specific peaks were found via UV detection that did not correspond to one of the four microcystin standards a general scan searching for compounds with masses ranging from 100-1200 Da was carried out. Microcystin congeners exhibit molecular weights ranging from 900-1120 Da (Howard & Boyer 2007). If peaks were found that had masses and fragmentation patterns corresponding to microcystins a second run on the LC-MS was carried out using the selected ion scan mode in order to specifically scan for those particular compounds. Based on the mass of the parent ion and the fragmentation pattern the identity of possible microcystin congeners could be determined. This type of mass spectrometry produces mainly protonated (M+H+) or divalent ions (M+2H+) and information provided by mass spectrometry provides a fingerprint for the individual toxins and can be used to confirm the presence of various microcystin congeners (McElhiney & Lawton 2005, Phelan & Downing 2007).

2.2.5 Statistical Analyses

Growth rates for all culture strains were calculated (whether measured by spectrophotometry or flow cytometry) using a linear regression on 'ln' transformed data. The slope of the growth curve in the exponential phase of growth was calculated as the growth rate per day (d^{-1}).

One-way ANOVA's were carried out to determine the effect of light intensity on 1) the growth of each strain of *Microcystis*, and 2) the growth of all strains under a given light intensity. A two-way ANOVA was carried out to determine the effect of both strain and light intensity on growth rates. Lastly a one-way ANOVA was carried out to compare the growth rates of toxigenic versus non-toxigenic strains of *Microcystis*. For all statistical analyses a significance level of $p<0.05$ was used.

Toxin congener composition was compared among and between strains and light levels in a similar way. One-way ANOVA's were carried out to determine the effect of light intensity on overall toxin production in the three microcystin producing strains (UTCC 299, 300, and 464), along with the effect of light intensity on the production of different microcystin congeners within each toxigenic strain (both the total amount of each congener and the proportion of each congener). In all statistical analyses the assumptions of normality, homoscedasticity, and independence were verified using the residuals from each analysis and in general significance was taken at $p<0.05$. All statistics were carried out using Systat 11.0.

2.3 Results

2.3.1 Growth Rates

Growth rates for each strain of *Microcystis* grown under three light intensities are shown in Table 2 (data for individual replicates in Appendix II). Overall, light intensity had a significant effect on the growth rate of each individual strain ($p<0.001$). In general most strains had the lowest growth rate when grown under the lowest light intensity (Table 3). However, some strains had maximal growth rates under medium light intensity (300, 632, 633, 460) while others had maximal growth rates when grown under the highest light intensity (299, 124, 464). The ranking of the growth rates of each strain varied slightly depending on the method used to estimate growth rate (optical density O.D. or changes in cell density C.D.) (Table 3). For example, for UTCC 299 using O.D. measurements the highest growth rate was found under high light, followed by medium and low light intensities. However, using C.D. measurements, the highest growth rate for UTCC 299 was found under medium light intensity followed by high and then low light intensity.

Overall light intensity was a significant variable when all strains were pooled together (Table 4, $p<0.001$). The highest growth rates were attained under medium light intensity, followed by high and low light intensities. There was significant variation in terms of the ranking of strains when comparing between light levels and also between methods of measuring growth rates. For example, under low light intensity UTCC 632 had the highest growth rate when measured by O.D., but was ranked third when measured by C.D. Also, while UTCC 632 had a higher growth rate when grown under low light intensity, it fell slightly in the rankings when grown under medium and high light intensities. However, even with changes in rankings of each strain between light intensities, there was no obvious trend or pattern suggesting that toxigenic strains were consistently growing faster or slower than non-toxigenic strains. Under any given light intensity, toxigenic strains UTCC 299, 300, and 464 never ranked in a cluster together and neither did non-toxigenic strains UTCC 124, 632, 633 or 460.

Table 2. Growth rates (d^{-1}) for all seven strains (means ± S.D.) as calculated by changes in optical density (O.D.) or cell density (C.D.) (n=3).

	Light Condition					
	Low (10 μM)		*Medium (60 μM)*		*High (150 μM)*	
Strain	**O.D.**	**C.D.**	**O.D.**	**C.D.**	**O.D.**	**C.D.**
Toxigenic:						
299	0.002 ± 0.002	0.099 ±0.055	0.018 ± 0.002	0.424 ± 0.013	0.016 ± 0.003	0.294 ± 0.017
300	0.004 ± 0.0002	0.162 ± 0.067	0.081 ± 0.001	0.409 ± 0.029	0.063 ± 0.001	0.116 ± 0.010
464	0.003 ± 0.0003	0.109 ± 0.008	0.045 ±0.003	0.121 ± 0.009	0.045 ± 0.0007	0.131 ± 0.021
Non-Toxigenic:						
124	0.0003 ± 0.0003	0.086 ± 0.021	0.006 ± 0.006	0.279 ± 0.008	0.015 ± 0.016	0.380 ± 0.182
632	0.008 ± 0.002	0.146 ± 0.019	0.043 ± 0.015	0.224 ±0.019	0.034 ± 0.008	0.292 ± 0.074
633	0.004 ± 0.0005	0.180 ±0.031	0.047 ±0.005	0.220 ± 0.008	0.044 ± 0.004	0.244 ± 0.014
460	0.007 ± 0.0002	0.084 ± 0.037	0.027 ± 0.003	0.042 ± 0.029	0.024 ± 0.001	0.048 ±0.019

Table 3. Effect of light intensity on growth rates of each strain of *Microcystis* as determined by changes in optical density (O.D.) or cell density (C.D.). Light intensities are ranked in order of the highest to the lowest observed growth rate. P-values in brackets are from one-way ANOVA analyses with light intensity as the grouping factor.

Strain	***Growth rate rankings under different light treatments***	
299	O.D.	High ≥ Medium > Low (p=0.061)
	C.D.	Medium > High > Low (p<0.0001)
300	O.D.	Medium > High > Low (p=0.027)
	C.D.	Medium > Low ≥ High (p<0.0001)
464	O.D.	High > Medium > Low (p<0.0001)
	C.D.	High = Medium = Low (p=0.266)
124	O.D.	High > Medium >Low (p<0.0001)
	C.D.	High ≥ Medium > Low (p=0.026)
632	O.D.	Medium > High > Low (p=0.013)
	C.D.	High > Medium > Low (p=0.022)
633	O.D.	Medium > High > Low (p<0.0001)
	C.D.	High ≥ Medium ≥ Low (p=0.022)
460	O.D.	Medium > High > Low (p<0.0001)
	C.D.	High = Medium = Low (p=0.243).

Table 4. Overall and individual effects of light levels on growth rates of the seven strains of *Microcystis*. Strains are ranked in order from highest to lowest growth rate. Strains indicated in bold are those that produce microcystins. P-values in brackets are from one-way ANOVA analyses with strain as the grouping factor.

		Ranking
Overall Light	O.D.	medium>high>low (p<0.0001)
	C.D.	medium>high>low (p=0.002)
Low Light	O.D.	632>460>**300**>633>**464**>**299**>124 (p<0.0001)
	C.D.	633>**300**>632>**464**>**299**>124>460 (p=0.049)
Medium Light	O.D.	**300**>633>**464**>632>460>**299**>124 (p<0.0001)
	C.D.	**299**>**300**>124>632>633>**464**>460 (p<0.0001)
High Light	O.D.	**300**>**464**>633>632>460>**299**>124 (p<0.0001)
	C.D.	124>**299**>632>633>**464**>**300**>460 (p<0.0001)

2.3.2 Total Microcystin Analysis

All statistical analyses of microcystin data were done for day 14 of the experiment as at this point there was sufficient microcystin present in the cultures to be consistently and reliably detected (Figs. 4-6). UTCC 124, 632, 633 (*Microcystis aeruginosa*), and 460 (*M. flos-aquae*) did not produce any microcystins. Strain UTCC 464 produced the largest total amount of microcystin followed by UTCC 300, and 299. UTCC 464 also produced the largest number of microcystin congeners (six), while UTCC 299 and UTCC 300 produced two microcystin congeners each.

Figure 4 shows total microcystin production in micrograms per gram of dry weight for each strain grown under the three light intensities. Overall, when pooling data from all three strains across light levels, there were no significant effects of light intensity ($p>0.05$). However, when examining the strains individually, UTCC 464 had the greatest concentration of microcystin per gram of dry weight followed by UTCC 300 and UTCC 299 ($p<0.001$). Within each light level UTCC 464 also had the highest concentration of microcystins (high light $p<0.01$, medium light $p=0.05$, low light $p<0.05$). For UTCC 464 and UTCC 300 there were no significant differences in total microcystin production per gram of dry weight when these strains were grown under different light intensities ($p>0.05$ for UTCC 300, and UTCC 464). However, UTCC 299 had significantly higher microcystin per gram dry weight when grown under medium light intensity compared to either high or low light intensities ($p<0.01$).

Total microcystin for each toxigenic strain was also calculated per unit volume in micrograms per litre (Fig. 5). Unlike when microcystins were reported as $\mu g \cdot g^{-1}$, when standardizing per unit volume there was a significant effect of light on microcystin production when pooling data from all three strains across light levels. However, when strains were examined individually, UTCC 464 had the highest concentration of microcystin followed by UTCC 300 and UTCC 299 ($p<0.001$). This trend also held true when examining the strains across light levels with UTCC 464 having the highest concentration of microcystins followed by UTCC 300 and UTCC 299 (high, medium, and low light $p<0.01$). When all three toxigenic strains were examined individually there was a significant difference in total microcystin production per litre when these strains were grown under different light intensities. All three strains had the highest microcystin concentration per litre

when grown under medium light intensity, followed by high light, and low light intensity (UTCC 299 $p<0.01$, UTCC 300 $p<0.05$, UTCC 464 $p<0.001$).

The third way in which total microcystin concentrations were standardized was as a microcystin cell quota, or micrograms per cell (Fig. 6). Overall light did not have a significant effect on the microcystin cell quota ($p>0.05$). UTCC 464 produced more microcystin per cell than did UTCC 299 or UTCC 300 ($p<0.001$). This also held true when each light level was examined individually with UTCC 464 producing more microcystin per cell than UTCC 299 or UTCC 300 under low, medium, and high light intensities ($p<0.05$). For each strain examined individually, however, the results differ. UTCC 299 and UTCC 300 did not produce significantly different amounts of microcystin per cell under different light intensities (UTCC 299 and UTCC 300 $p>0.05$), whereas UTCC 464 produced significantly more microcystin per cell when grown under low light intensity compared to when it was grown under medium or high light intensities ($p<0.001$).

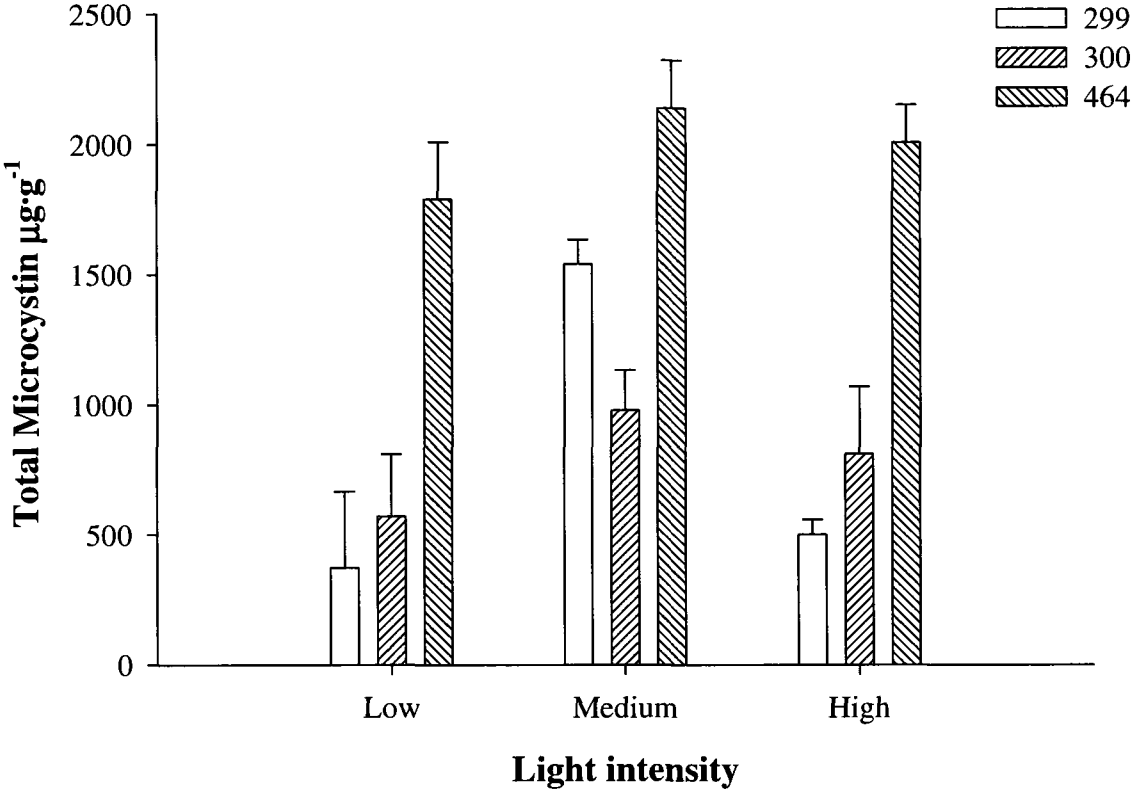


Figure 4. Total microcystin concentration ($\mu g \cdot g^{-1}$ dry weight ± S.E., n=3) on day 14 of growth for *Microcystis aeruginosa* strains UTCC 299, UTCC 300, and UTCC 464.

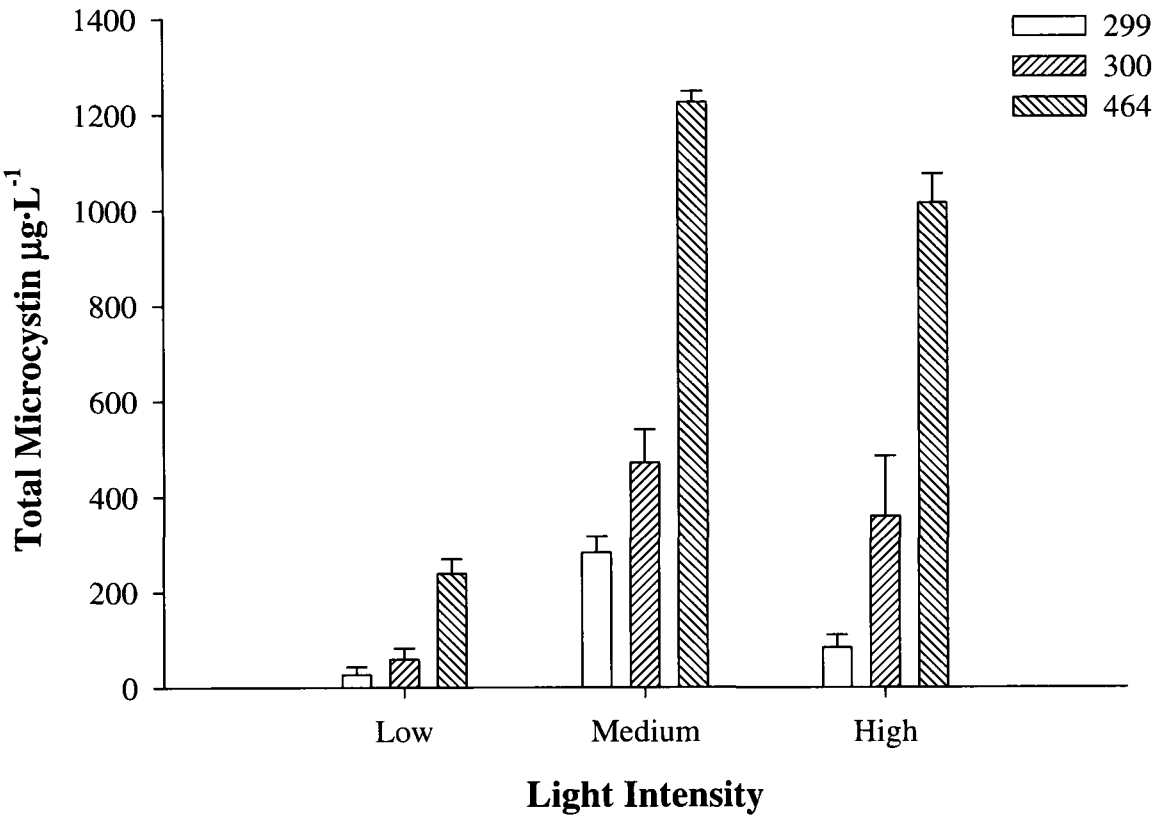


Figure 5. Total microcystin concentration ($\mu g \cdot L^{-1}$ ± S.E., n=3) on day 14 of growth for *Microcystis aeruginosa* strains UTCC 299, UTCC 300, and UTCC 464.

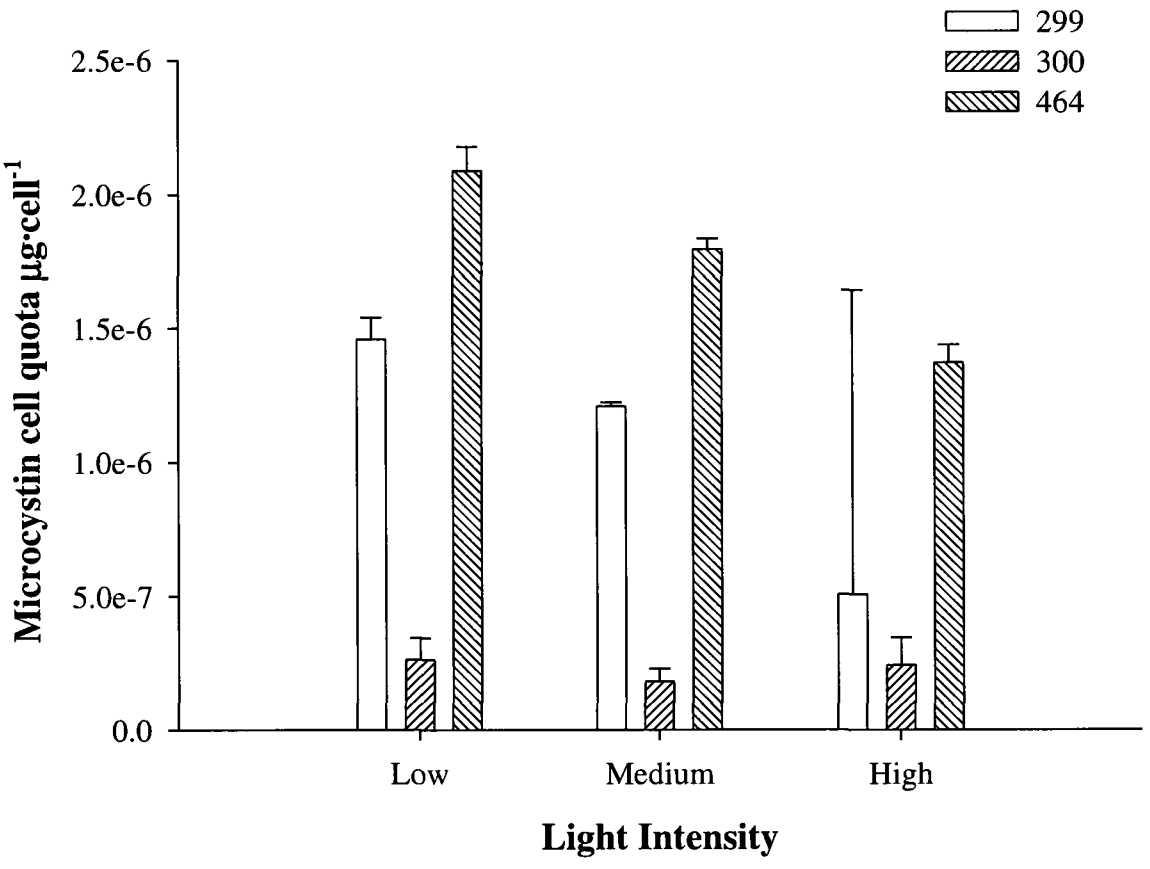


Figure 6. Microcystin cell quota (µg·cell^{-1} ± S.E, n=3) on day 14 of growth for *Microcystis aeruginosa* strains UTCC 299, UTCC 300, and UTCC 464.

2.3.3 Microcystin Congener Analysis

For all three toxigenic strains the confirmation of any HPLC peaks considered to be microcystins was carried out using mass spectrometry. The fragmentation pattern for any peak considered to be a microcystin was analyzed and the mass compared to a compiled list of more than 90 different microcystin congeners and their respective masses. In particular, the characteristic fragment with a mass of 135 Da was searched for as it corresponds to the Adda portion of the microcystin molecule. In some cases, however, while the mass of the parent ion fragment was in the range for microcystins, and while the 135 fragment was also present, the total mass for a particular peak did not correspond exactly to one of the known microcystins. Or, in some cases, more than one microcystin may have the same mass and therefore it was not possible to decipher which congener was actually being produced. Further analytical analyses to identify these peaks are being carried out in co-operation with the NRC Institute for Marine Biosciences in Halifax, Nova Scotia, in collaboration with Dr. Michael Quilliam. While we believe these peaks to be microcystins, in the interim the identities of these microcystin congeners are referred to as 'unknowns' until the issue of conferring an identity to the congeners can be resolved. Examples of mass spectrometry analysis can be found in Appendix III.

The proportions of microcystin congeners for each toxic strain grown under three light intensities are shown in Figures 7-9. For UTCC 299 two main microcystin congeners were produced: microcystin-LR and unknown congener 1 (Fig. 7). The proportion of each congener did change significantly depending on the light level under which the strain was grown. The proportion of microcystin-LR was greater under high light compared to medium and low light intensities ($p<0.05$). Conversely the proportion of unknown congener 1 was greater under low light intensity compared to medium or high light intensities ($p<0.05$). Under low light there was roughly an equal amount of each congener being produced, however as the light intensity increased so did the proportion of microcystin-LR in the total microcystin pool.

For UTCC 300, two microcystin congeners were detected corresponding to microcystin-LR, and [Dha7]desmethylmicrocystin-LR. For microcystin-LR there was a greater proportion of this congener under low light intensity than under high or medium light (Fig. 8, $p<0.05$). Conversely, there was greater [Dha7]desmethylmicrocystin-LR produced

under high and medium light intensities than under low light ($p<0.05$). Therefore, while under high and medium light intensities these two congeners were produced in almost equal proportions, under low light there was significantly more microcystin-LR produced than [Dha7]desmethylmicrocystin-LR.

UTCC 464 was a very interesting strain as while it not only produced the highest concentrations of microcystin under all light intensities it also produced the greatest number of microcystin congeners. Six different congeners were detected in UTCC 464, including microcystin-YR, and unknown congeners 3 through 7 (Fig. 9). The congener of most interest was unknown 4 which comprised the greatest fraction of total microcystin produced by UTCC 464 under all three light levels. The greatest proportion of unknown 4 was produced under low light, followed by medium and high light intensities. The proportions of other congeners also varied with changing light intensities such as microcystin-YR ($p=0.05$), unknown congener 5 ($p<0.01$), unknown congener 6 ($p<0.001$), and unknown congener 7 ($p<0.001$). Only the proportion of unknown congener 3 did not vary significantly with light level ($p>0.05$).

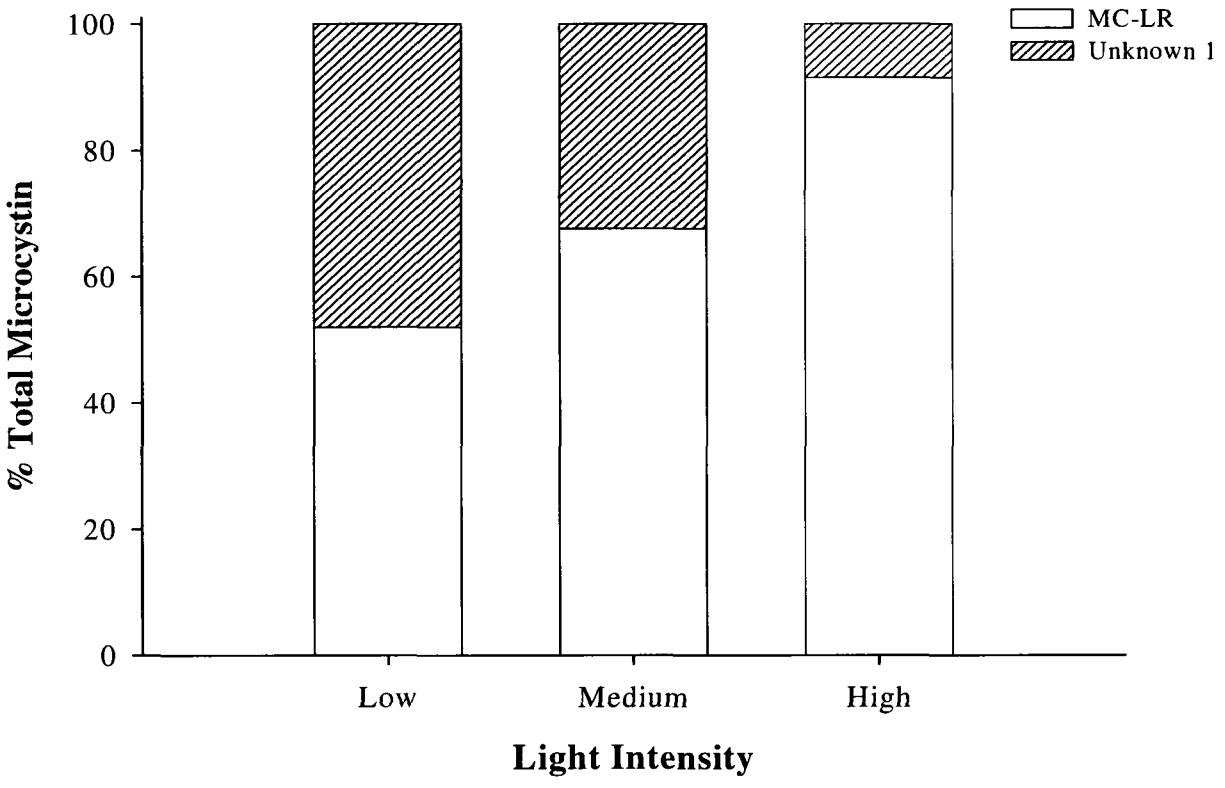


Figure 7. Proportions of the two microcystin congeners produced by UTCC 299.

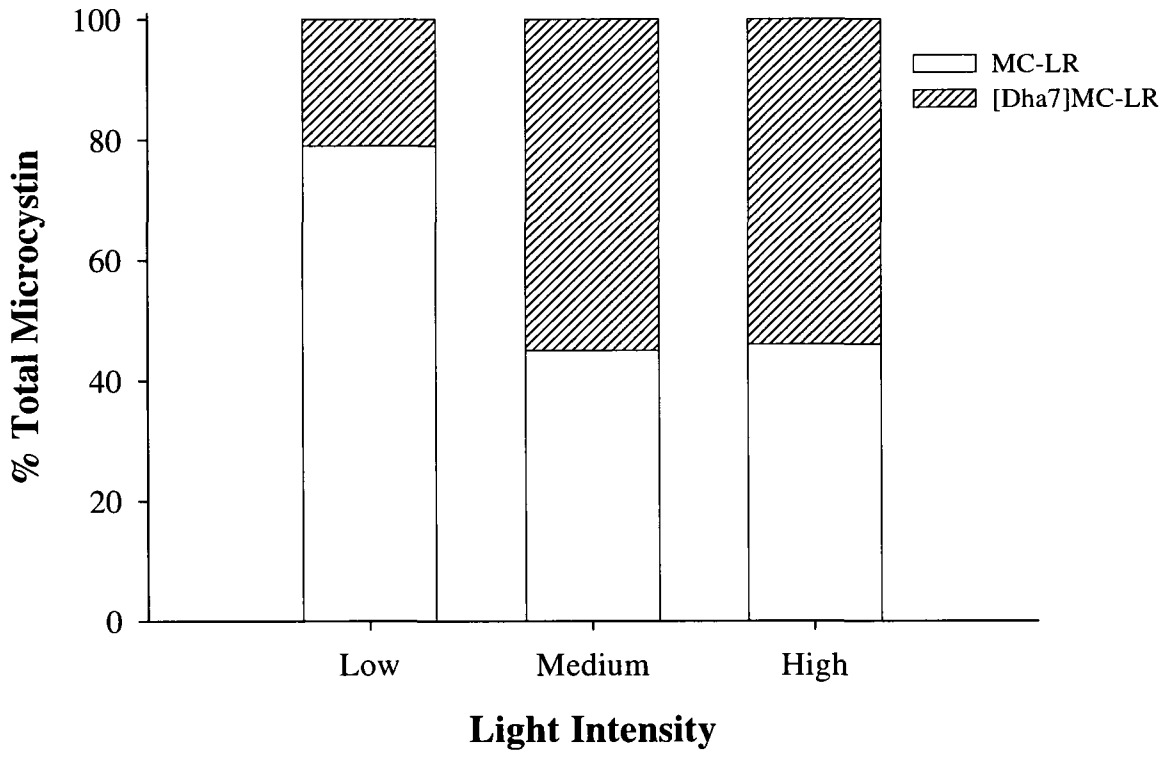


Figure 8. Proportions of the two microcystin congeners produced by UTCC 300.

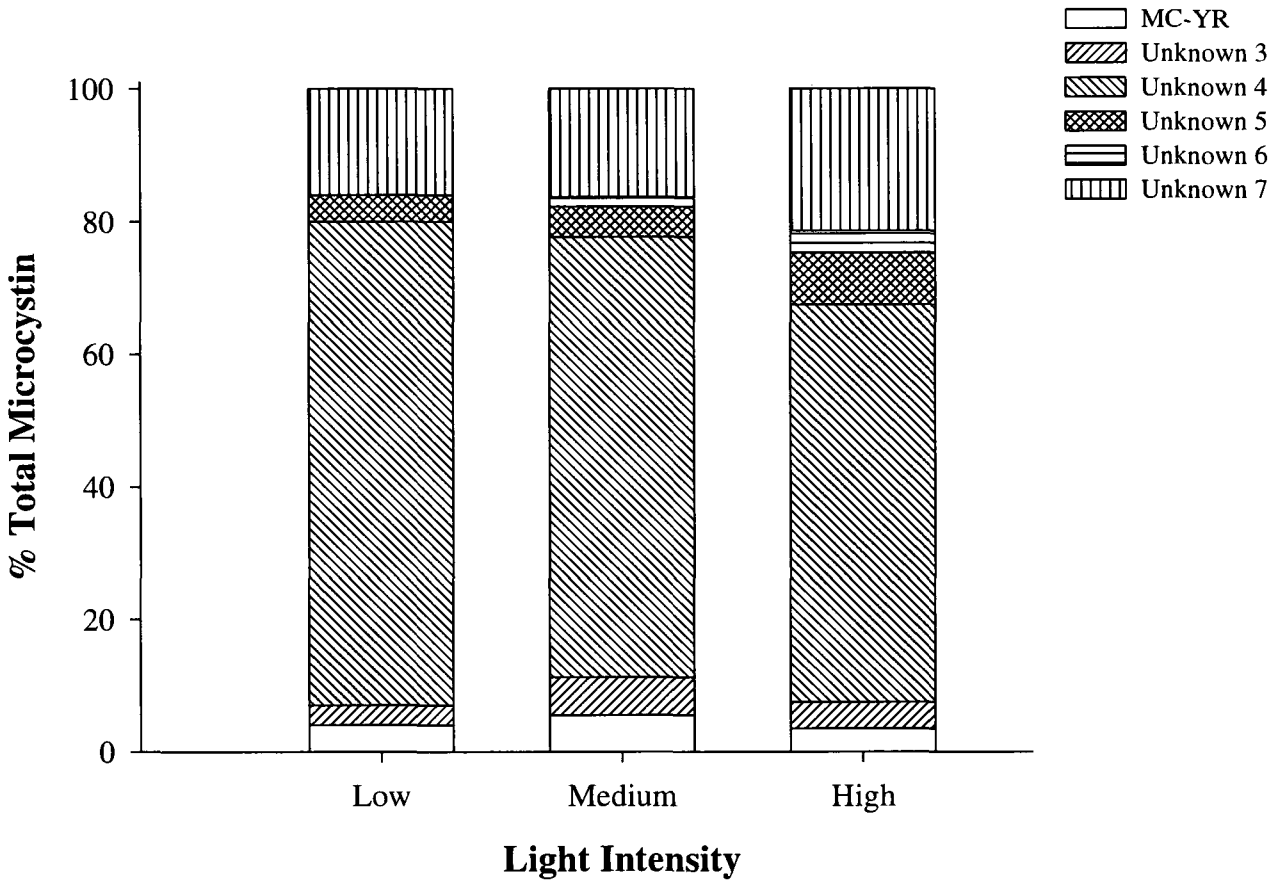


Figure 9. Proportions of the six microcystin congeners produced by UTCC 464.

2.4 Discussion

2.4.1 Comparison of Growth Rates

Light intensity had a significant effect on the growth rate of each strain of *Microcystis*. However, results were strain dependent. For example, toxigenic strain UTCC 299 grew best under medium light intensity, whereas toxigenic strain UTCC 464 grew best under a high light intensity. It has previously been suggested that microcystins might be involved in light adaptation which may confer an advantage to toxigenic strains under limiting light conditions (Hesse *et al.* 2001). However, based on this study, the production of microcystins did not appear to confer a growth advantage to toxigenic strains. Overall, there were no significant differences in growth rates between toxigenic and non-toxigenic strains of *M. aeruginosa*. Therefore, toxigenic strains do not appear to have any particular advantage in terms of growth when grown individually under different light intensities.

There have been few culture studies that have included growth rate in their analysis of the effect of different environmental factors on both toxigenic and non-toxigenic strains of cyanobacteria including species of *Microcystis*. Recently, Tonk *et al.* (2008a) examined how light, phosphorus availability, and temperature, affected the peptide content and peptide production in strains of *Anabaena* and *Microcystis*. Cultures were grown under light saturated and limiting conditions. As in the present study they found that growth rates were not consistently different between toxigenic and non-toxigenic strains with respect to light saturating or light limiting conditions. Tonk *et al.* (2008a) also found a relationship between growth rate and peptide production (including the production of microcystins), however this relationship depended on the parameter being varied. For example, under phosphorus limitation they found that the production of microcystin increased with increases in growth rates. However, while increases in light and temperature resulted in a higher growth rate for *Microcystis*, the production of peptides, including microcystins, decreased. The strains examined in the present study did not all follow this pattern. The toxigenic strains UTCC 299, 300 and 464 had their highest growth rates under medium (UTCC 299, 300) or high light intensity (UTCC 464) which did not correspond with the light intensity under which the highest concentrations of microcystins were measured. For UTCC 299 and UTCC 300 growth rates were the highest under medium light intensities and so were microcystin concentrations when expressed per mass or volume. However, when microcystin

concentrations were expressed as $\mu g \cdot cell^{-1}$, there was no significant difference in microcystin production between light intensity levels. For UTCC 464 the highest growth rate was achieved under high light, however the highest microcystin concentrations were detected under medium ($\mu g \cdot g^{-1}$ or $\mu g \cdot L^{-1}$) or low ($\mu g \cdot cell^{-1}$) light intensity. Clearly microcystin content cannot generally be predicted by growth rate.

Other studies have also examined how nutrient concentrations affect the growth of toxigenic and non-toxigenic strains of *Microcystis.* It has been suggested that non-toxigenic strains require fewer nutrients for their growth at low nutrient concentrations than do toxigenic strains (Vézie *et al.* 2002). Conversely, with high nutrient concentrations toxigenic strains appear to grow better than non-toxigenic strains (Vézie *et al.* 2002). Therefore, in the case of nutrient limitation non-toxigenic strains might be superior competitors over toxigenic strains. This trend of non-toxigenic strains growing better than toxigenic strains was not seen in the UTCC strains examined in this study when cultures were grown under light-limiting conditions. There was no clear evidence that pointed to one strain growing better than another based on its ability to produce microcystins. While light did have a significant effect on the growth rates of each strain, toxicity, or lack thereof, did not confer any advantage under light limiting or saturating conditions. Many more strains will need to be examined in order to determine whether this is universally the case for toxigenic cyanobacteria.

2.4.2 Total Microcystin Production

Microcystin production was examined in all seven strains of *Microcystis* grown in this study. In the toxigenic strains UTCC 299, 300, and 464 total microcystin production varied significantly between strains and between light levels exhibiting a four to five fold difference when expressed per gram of dry weight. Toxin production was greatest in UTCC 464, followed by 299 and 300. Light had a significant effect on total microcystin when standardized as $\mu g \cdot L^{-1}$ ($p<0.001$) but not as $\mu g \cdot g^{-1}$ ($p>0.05$), or as $\mu g \cdot cell^{-1}$ ($p>0.05$) suggesting that while light affects cell abundance (and hence volumetric microcystin concentrations) the microcystin content of cells appears to remain constant.

As previously mentioned, several studies have found that microcystin production is closely tied to the growth rate of the strain. It has been suggested that microcystin

production is controlled by various environmental parameters on the rate of cell division, and not through any direct effect on the metabolic pathways of toxin production (Orr & Jones 1998). Therefore difficulties may arise when attempting to express the concentration of microcystin as a ratio to another cell component (such as biomass, chlorophyll, protein content) when the cell component itself may be affected by the experimental treatment (Long *et al.* 2001). However, as mentioned earlier, the results from this study do not show a definitive correlation between growth rate and microcystin production, as found recently by Tonk *et al.* (2008a). Currently there are no definitive standard methods by which microcystins are analyzed across laboratories including the choice of a standardizing parameter (i.e. volume, dry weight, per cell, chlorophyll). Therefore, depending on the type of study and the endpoint of the analyses, standardizing per unit biomass, unit volume, or per cell may be appropriate. For example, for water quality managers the choice of expressing microcystins per unit volume ($\mu g \cdot L^{-1}$) may be the best option as concentrations can then be readily compared to World Health Organization and other microcystin drinking water standards (Tillmanns *et al.* 2007). For the purpose of this study microcystin concentrations standardized per unit biomass, volume, or per cell were plotted against each other for comparison (see Appendix IV). A stronger correlation was found between microcystin concentrations expressed as $\mu g \cdot g^{-1}$and $\mu g \cdot L^{-1}$ ($r^2 = 0.61$), and $\mu g \cdot g^{-1}$ and $\mu g \cdot cell^{-1}$($r^2 = 0.74$), but not between $\mu g \cdot cell^{-1}$ and $\mu g \cdot L^{-1}$ ($r^2 = 0.103$). In this study, microcystin concentrations were reported using all three standardizing parameters and will be discussed so in terms of discrepancies in relationships between toxin production and light intensity.

Light intensity has been shown in previous studies to have a significant effect on total microcystin production in toxigenic strains of cyanobacteria. Wiedner *et al.* (2003) showed that microcystin concentration and irradiance were positively correlated under light-limited growth and negatively correlated under light saturation. Using continuous cultures, growth was limited by light intensities up to 80 $\mu M \cdot m^{-2} \cdot s^{-1}$ and higher light intensities had no effect on growth rate. Concurrently, with increasing irradiance, the total microcystin per cell increased steeply but with any further increases in irradiance microcystin content began to decrease (Wiedner *et al.* 2003).

In this study, depending on how microcystin concentrations were standardized, there was in some cases a significant effect of light intensity on total microcystin production. For

example, when represented in $\mu g \cdot g^{-1}$, only UTCC 299 produced relatively more microcystin under medium light intensity compared to low or high light. UTCC 300 and UTCC 464 did not show any significant response to light intensity when microcystin concentration was expressed in this way (Fig. 4). However, if microcystin concentrations were expressed as $\mu g \cdot L^{-1}$ there was a significant overall effect of light on each strain with all three toxigenic strains producing the highest amount of total microcystins under medium light intensities. This is in agreement with Wiedner *et al.* (2003) who reported that the light intensity beyond which no further increases in microcystin occurred was 80 $\mu M \cdot m^{-2} \cdot s^{-1}$, slightly higher than the 60 $\mu M \cdot m^{-2} \cdot s^{-1}$used as the medium light intensity level in the present study. Lastly, when microcystins were expressed as $\mu g \cdot cell^{-1}$ there was no significant effect of light intensity on toxin production in both UTCC 299 and 300, however UTCC 464 produced significantly more microcystins under the lowest light intensity.

The results from this study, when examined along with previous culture studies, show that while light intensity does have a significant effect on microcystin production there is considerable variation between strains of the same species. Molecular tools and techniques are now being developed alongside analytical techniques; not only can total microcystin concentrations be measured, but the presence and expression of the microcystin synthetase genes as well. The effect of light and other environmental parameters can be examined at different levels to determine if and how effects at the genetic level influence the phenotypic expression of the gene and the actual concentrations of gene products. Kaebernick *et al.* (2000) grew batch cultures of *M. aeruginosa* under three light intensities (low 16 μM, medium 31 μM, and high 68 μM) and showed a significant effect of light on gene activity involved in microcystin biosynthesis (*mcy*B and *mcy*D) and suggested that the light intensities influencing the toxicity of *M. aeruginosa* were those below 40 $\mu M \cdot m^{-2} \cdot s^{-1}$. At higher light intensities they saw a lack of correlation between an increase in *mcy*B and *mcy*D transcription and cellular toxin content which may suggest microcystin release from the cell under higher light conditions.

2.4.3 Microcystin Congener Profiles

The three toxigenic strains studied in this experiment all produced not only different total microcystin concentrations but each strain also had a different pattern of microcystin congener production indicating each strain represents a different microcystin chemotype. UTCC 299 and UTCC 300 produced two different microcystin congeners each while UTCC 464 produced six different congeners. Of particular note are UTCC 299 and UTCC 300 which were isolated from the same pond on the same date, yet are two distinct chemotypes based on their different microcystin congener profiles.

The analysis of microcystin congeners has not been conducted for many strains or species of toxigenic cyanobacteria and it is not generally known if toxin composition changes with environmental conditions or whether the toxin profile of a species or strain is a fixed trait. In this study, I found that not only did the absolute concentration of each toxin congener vary with changes in light intensity, but that the proportions of each congener were also affected by light intensity (Fig. 7-9). UTCC 299 produced microcystin-LR, and an unknown microcystin congener (Unknown 1); microcystin-LR comprised more than 90% of the total microcystin concentration under high light, however under medium and low light this proportion decreased to approximately 70% and 50%. UTCC 300 also produced microcystin-LR however unlike UTCC 299, UTCC 300 had a greater proportion of microcystin-LR when growth under low light (~80%) as compared to high or medium light intensity (~40%). Recently, Rohrlack & Hyenstrand (2007) found that during periods when cultures were exposed to high light (140 $\mu M \cdot m^{-2} \cdot s^{-1}$) *Microcystis aeruginosa* strain PCC 7806 produced mostly (D-Asp3) microcystin-LR which lacks the usual methylation of the amino acid Asp, however the cells switched to the production of microcystin-LR when they were subjected to darkness. This is consistent with the results here for UTCC 300 which also switched to producing more microcystin-LR when grown under light-limiting conditions.

In an earlier study, Tonk *et al.* (2005) detected two main microcystin congeners (microcystin-DeRR and microcystin-DeLR) produced by *Planktothrix agardhii,* a filamentous cyanobacterium, exposed to different light intensities. While the total concentration of microcystin did not change significantly, with increases in light intensity past a threshold of 60 $\mu M \cdot m^{-2} \cdot s^{-1}$, there was an observed shift in the proportions of the two microcystin congeners produced. When the cultures of *P. agardhii* were grown at higher

light intensities (>100 $\mu M \cdot m^{-2} \cdot s^{-1}$) the more toxic congener microcystin-DeLR was produced. They concluded that light intensity exerts some control over the transcription of the microcystin synthetase gene complex which in turn results in a change in the proportions of microcystin congeners produced. Tonk *et al.* (2005) proposed several reasons why the composition of microcystin congeners would change with light conditions including postulating a conformational change in the substrate-binding pocket of the first module of *mcy*B enzyme which could lead to a change in substrate specificity. Another possibility was a change in amino acid availability depending on the light level. For example, in their experiment an increase in photosynthesis at higher light intensity may raise the carbon/nitrogen ratio of the cells which would favour leucine over arginine and thus result in a greater production of microcystin-DeLR. In a follow-up study Tonk *et al.* (2008b) grew *Planktothrix agardhiii* with and without the external addition of the amino acids leucine and arginine. They predicted that environmental changes which affect cell metabolism may cause differences in the intracellular availability of leucine and arginine which could then affect the production of different microcystin congeners. Since leucine has one nitrogen while arginine contains four nitrogens, low nitrogen availability may shift the amino acid availability composition in favour of leucine. Indeed the addition of leucine to the growth medium resulted in a strong increase in the leucine rich [Asp^3] microcystin-LR congener compared to the arginine rich [Asp^3] microcystin-RR congener, while adding arginine resulted in the opposite trend. However, they also found that nitrogen limitation did not change the ratio of these two congeners in their strain of *Planktothrix agardhii* studied. Overall they concluded that the availability of amino acids during microcystin polyketide synthesis is a major determinant of the composition of microcystin congeners in their test strain, whereas the availability of nitrogen was not. Assembling different microcystin congeners might be a strategy of cyanobacteria to sustain continued production of microcystins under different environmental conditions. However, Yan *et al.* (2004) showed in an earlier study of *Microcystis aeruginosa* that the production of microcystin-LR was actually inhibited when leucine and arginine were added to cultures (Yan *et al.* 2004), which contradicts the findings of Tonk *et al.* (2008b).

Light is clearly but one variable among many that may have an impact on cyanobacterial growth, microcystin production and composition. For example, Oh *et al.* (2000) examined

the effect of nutrients on the production of microcystins in *Microcystis aeruginosa.* In their study when growth of *M. aeruginosa* was reduced under phosphorus limitation, the microcystin content was higher (as also seen in Tonk *et al.* 2008a). In these phosphorus-limited cells there was an increasing tendency not only for increases in total microcystin content (2-3x greater), but also a shift towards production of the more toxic microcystin-LR compared to microcystin-RR. They speculated that phosphorus-limited conditions may have a protective function by increasing toxicity towards grazing by zooplankton. In another study, Kamayema *et al.* (2002) examined the effects of nitrate and phosphate on the production of microcystin-LR, -RR, and YR in batch cultures of *Microcystis viridis.* They found that microcystin-RR and total microcystin production was greatly affected by the concentration of nitrate, however the production of microcystins decreased with increasing phosphate concentration. Even with changes in total microcystin production, overall they found that the ratio of microcystin congeners obtained from cultures in the exponential phase of growth was a near constant. The microcystin profile of their one strain was constant regardless of the nitrate and phosphate concentrations. This contradicts the results obtained here with respect to light intensity as the proportions of each congener did not remain constant for UTCC 299, 300, and 464.

2.4.4 Molecular Considerations

Based on the results of this and previous studies it is clear that environmental variables such as light and nutrients have a significant impact on microcystin production in terms of the congeners produced. While some studies have attempted to explain this phenomenon in terms of amino acid availability (i.e. Tonk *et al.* 2008b), it must have a molecular basis. The structure of the microcystin synthetase cluster is now well known and the roles for each domain within this complex are more or less understood. Studies can now incorporate this knowledge into an understanding of the genetic basis for the production of such a wide variety of microcystin congeners.

The first point to note is that there may be differences in the adenylation domain in the first module of the *mcy*B operon in the microcystin synthetase gene cluster (Mikalsen *et al.* 2003). It has been shown that there is a strong correlation between the microcystin congeners produced and the genetic variants of the *mcy*B module (Mikalsen *et al.* 2003).

The genetic variants may be the result of recombination events, perhaps between modules within the gene cluster i.e. between *mcy*B and *mcy*C. Mikalsen *et al.* (2003) speculated that theoretically each genetic variant resulting from such a recombination event could be correlated to the production of a particular microcystin congener.

Recombination in *mcy*B and *mcy*C has been shown to result in a greater genetic diversity of the microcystin synthetase gene cluster (Kurmayer *et al.* 2005). Fewer *et al.* (2007) found a significant correlation between certain genotypes and the occurrence of certain amino acids into specific positions of the microcystin molecule. They too suggested that recombination seems to be a general feature in *mcy* genes and that DNA polymorphisms within specific regions of *mcy*B and *mcy*C are associated with the synthesis of specific microcystin congeners. In fact *mcy*B and *mcy*C are thought to be recombination hotspots within the microcystin synthetase cluster (Fewer *et al.* 2007).

2.4.5 Conclusions

The goal of this study was to determine the effect of light intensity on the production of different microcystin congeners in the cyanobacterium *Microcystis aeruginosa* and secondly to determine if light intensity affects the rate of growth of toxigenic and non-toxigenic strains differently. Overall, there was an effect of light intensity on growth of all strains of *Microcystis* and on the production of different microcystin congeners in the three toxigenic strains studied. While all three toxigenic strains produced microcystins, each strain had a different microcystin congener profile suggesting each strain represents a different chemotype. Further studies with these strains might incorporate molecular techniques to attempt to distinguish the genetic differences between these chemotypes in order to understand how different strains of the same species have such varying toxin profiles.

I hypothesized that changes in light intensity would affect toxigenic and non-toxigenic strains differently. Theoretically, because microcystins require energy to be produced, under growth-limiting conditions such as light-limitation, non-toxigenic strains would have a competitive advantage. However, while the light intensity at which a culture was grown did significantly affect its growth rate, there was no trend in terms of microcystin or non-microcystin producing strains having lower or higher growth rates overall when grown under light-limiting or light-saturating conditions. In addition, while microcystins

have been implicated in light adaptation processes in this regard there was also no clear advantage of the toxigenic strains.

By studying cyanobacteria in a simple culture setting it was hoped that one might gain insights into the mechanisms of growth and toxin production that occur in nature. The results from this study demonstrate the ability of different strains to produce a range of microcystin congeners and highlight the biochemical and physiological differences between strains of the same species. However, this type of single organism experiment that helps define the physiological (or fundamental) niche of an individual strain or species may not necessarily predict its performance in nature, that is when grown with others in competition for the same resources (i.e. the realized or ecological niche). Mixed culture experiments combining both toxigenic and non-toxigenic strains of cyanobacteria in competition may provide a better understanding of the ecological role(s) of cyanotoxins such as the microcystins.

Chapter 3

The effect of light intensity on the relative dominance of toxigenic and non-toxigenic strains of *Microcystis aeruginosa*- The use of Quantitative PCR in mixed cyanobacterial growth experiments

Abstract

The production of microcystins by toxigenic cyanobacterial species appears to be strain and not species specific. In nature the factors that regulate the dominance of toxigenic strains over non-toxigenic strains or vice versa during a cyanobacterial bloom are largely unknown. In this study I tested the effect of light intensity on the performance of a microcystin producing strain of *Microcystis aeruginosa* when grown in mixed cultures with non-microcystin producing strains. Under light limitation, due to the potential energetic cost of producing microcystins, it was predicted that the non-toxigenic strains would dominate and the converse would arise when light was not limiting growth. The toxigenic strain UTCC 300 (University of Toronto Culture Collection) was grown with each of two non-toxigenic strains (UTCC 632 and UTCC 633) under low (20 $\mu M \cdot m^{-2} \cdot s^{-1}$) and high (80 $\mu M \cdot m^{-2} \cdot s^{-1}$) light intensities, for fourteen days. The variables measured were growth rate, total microcystin production, and *mcy*D gene copy number using QPCR. In contrast to the theoretical prediction, under low light intensity the toxigenic strain UTCC 300 became dominant in both of the mixed cultures of UTCC 300+632 and UTCC 300+633. When grown under high light intensity the toxigenic UTCC 300 still appeared to dominate over the non-toxigenic strain UTCC 632, however less so over UTCC 633 and this despite its lower intrinsic growth rate compared to UTCC 300 when grown alone. This study indicates that environmental factors such as light availability have significant yet variable effects on individual strains of *M. aeruginosa* which in turn may result in a change in strain dominance.

3.1 Introduction

Competition for common resources is an important mechanism structuring biological communities, and particularly in algal communities (Tilman 1977, 1981). For example, cyanobacteria tend to be favoured in aquatic environments during eutrophication for a number of reasons including the ability of some species to fix nitrogen, their ability to regulate their position in the water column due to the presence of gas vacuoles (giving them an advantage in terms of light acquisition), and their ability to defend themselves against grazers. The latter may be due to morphological traits (Porter & McDonough 1984) or to the production of cyanobacterial toxins which may also result in the suppression of the growth of other phytoplankton species (Codd *et al.* 1985, Sterner 1989).

Several competition studies have been carried out to determine the effects that cyanobacteria have on each other and on other types of phytoplankton. Many studies have utilized mixed culture experiments to examine the effects of changing nutrient, light, temperature, and dilution rates on growth parameters of algal species. For example, Hyenstrand *et al.* (2000) examined the role of nitrogen in determining the outcome of competition between the green alga *Scenedesmus* and the cyanobacterium *Synechococcus*. They determined that the cyanobacterium was favoured by a small-pulse, high-frequency supply mode of inorganic nitrogen, whereas the green alga could assimilate ammonium rapidly and was favoured when there were large, less frequent pulses of nitrogen supplied. Competition has been examined between *Microcystis* sp. and other algal species including *Phormidium tenue*, a filamentous cyanobacterium (Fujimoto *et al.* 1997), *Scenedesmus quadricauda*, a green alga (Takeya *et al.* 2004), and *Asterionella formosa*, a diatom (Holm & Armstrong 1981). *Microcystis aeruginosa* has been shown to dominate under culture conditions of low N:P ratios and high temperatures (Fujimoto *et al.* 1997). Yamamoto *et al.* (2005) grew *Microcystis aeruginosa* in mixed culture with *Staurastrum dorsidentiferum* and *Synedra ulna*, which always resulted in the dominance of *M. aeruginosa* regardless of pH or irradiance level.

Studies have also made use of purified extracts of microcystins, or have directly used toxigenic strains of cyanobacteria in mixed culture experiments. Sedmak & Elersek (2005) examined the effects of environmentally relevant concentrations of microcystin when added to cultures of non-microcystin producing *Microcystis aeruginosa*, and of the green alga

Scenedesmus quadricauda. Cells of the unicellular non-toxin producing *M. aeruginosa* tended to aggregate and form pseudo-colonies when exposed to microcystins, as did the cells of *S. quadricauda*, while control cultures of both remained more dispersed. Photosynthetic pigment production was also affected in the microcystin treatments with chlorophyll content significantly increased in *S. quadricauda* and phycocyanin significantly increased in *M. aeruginosa*. Kearns & Hunter (2001) grew a toxigenic strain of the cyanobacterium *Anabaena flos-aquae* in mixed culture with the green alga *Chlamydomonas reinhardtii* and determined that the concentration of the cyanotoxin anatoxin-a increased in the presence of the green alga, whereas the concentration of microcystin did not. In fact, microcystin production was slightly suppressed by extracellular products of *C. reinhardtii*. Jang *et al.* (2006) examined changes in microcystin production by *M. aeruginosa* in response to indirect exposure to two non-microcystin producing strains of *A. flos-aquae* and *P. agardhii*. They saw a significant increase in the intracellular microcystin concentration when the toxigenic strain was exposed to the medium filtrate of the non-toxin producing strains.

While variations in a number of environmental and biological parameters (i.e. nutrients, grazers etc.) may affect the outcome of algal competition, light in particular is a critical resource for all algal species in the aquatic environment and can be the key factor in determining which species become dominant. Understanding the ability of a species or specific strain to adapt and be competitive for light is essential for predicting the outcome of algal succession, including the possibility of a toxigenic or non-toxigenic strain dominating during a cyanobacterial bloom. Huisman *et al.* (1999) examined competition for light between two green algae (*Chlorella vulgaris* and *Scenedesmus protuberans*) and two cyanobacterial species (*Aphanizomenon flos-aquae* and *Microcystis aeruginosa*) in various combinations in mixed cultures. The species with the lowest critical light intensity (the point at which the culture is so dense that not enough light is available for continued growth and hence the species abundance will decrease) were superior competitors independent of the type of algae or cyanobacteria used and their maximum growth rates. Therefore, while cyanobacteria can be favoured by light-limiting conditions due to their competitive advantage from buoyancy regulation (Reynolds *et al.* 1987), Huisman *et al.* (1999) found this was not always the case, and that another factor(s) may explain dominance of cyanobacteria in eutrophic waters (Huisman *et al.* 1999).

The studies presented thus far have been examples of experiments which used either two non-toxigenic, or one toxigenic and one non-toxigenic strain of different cyanobacterial or algal species. While there are several examples of mixed culture experiments using *Microcystis aeruginosa* grown with other types of algae or non-toxigenic cyanobacteria, there are few studies that have grown toxigenic and non-toxigenic strains of *M. aeruginosa* simultaneously. The difficulty with such experiments is that toxigenic and non-toxigenic strains of *M. aeruginosa* cannot be separated morphologically using traditional microscopy techniques.

One recent study that attempted to determine the outcome of competition in *M. aeruginosa* grew two toxigenic and two non-toxigenic strains of *M. aeruginosa* in light limited chemostats (Kardinaal *et al.* 2007a). The population dynamics of the four strains was measured using both light absorbance spectra and molecular techniques including DGGE of the ITS (internal transcribed spacer) region gene. In all experiments the toxigenic strain lost in competition for light and was completely replaced by the non-toxigenic strain within approximately two weeks. Even when the toxigenic strains were given a significant advantage in terms of the number of cells at the start of the experiment the non-toxigenic strain still dominated. These results suggest that non-toxigenic strains are better competitors for light than are toxigenic strains and that consequently strain composition within *Microcystis* populations may determine the overall microcystin concentration.

Based on these results, Kardinaal *et al.* (2007a) proposed that toxigenic strains of *Microcystis aeruginosa* are less competitive than non-toxigenic at low light intensities. However, based on the seven *M. aeruginosa* strains studied in Chapter 2, in general the toxigenic strains did not necessarily grow more slowly under low light than the non-toxigenic strains. Furthermore, previous studies have suggested that microcystins may play a role in light adaptation processes (Kaebernick *et al.* 2000, Hesse *et al.* 2001, Kaebernick *et al.* 2002, Young *et al.* 2005, Jahnichen *et al.* 2007). Therefore if microcystins do confer some sort of advantage to toxin-producing strains under light-limiting conditions then we would expect the toxigenic strains to dominate the non-toxigenic strains when grown under light-limiting conditions. Conversely, if microcystin synthesis proves to be a costly process for toxigenic strains when grown under sub-optimal conditions such as light-limitation, it would be expected that non-toxigenic strains would be expected to dominate.

The goal of this study was to determine whether a toxigenic strain of *Microcystis aeruginosa* would dominate over non-toxigenic strains when in competition for light. In lakes toxigenic strains often dominate cyanobacterial blooms with estimates suggesting that more than 50% of cyanobacterial blooms produce cyanotoxins (Graham *et al.* 2004). Usually by the time a bloom develops nutrient resources are quickly used up and increased cell densities cause reduced light availability. This change in light regime during the development of a bloom may affect the outcome of competition between cyanobacterial strains. In the following experiment batch cultures of *Microcystis aeruginosa* were grown under low (20 $\mu M \cdot m^{-2} \cdot s^{-1}$), and high (80 $\mu M \cdot m^{-2} \cdot s^{-1}$) light intensity to determine how well a toxigenic strain grew in mixed cultures with non-toxigenic strains and to determine which strain dominated under each light condition. Toxigenic genotypes were distinguished from non-toxigenic using QPCR using primers of the *mcy*D gene of the microcystin synthetase gene complex.

3.2 Methods

3.2.1 Culturing Techniques

Three strains of *Microcystis aeruginosa* from the University of Toronto Culture Collection (UTCC) were grown: the microcystin producing UTCC 300 and the non-microcystin producers UTCC 632 and UTCC 633. UTCC 300 was chosen as the toxigenic strain as it had a simple microcystin congener profile producing only two microcystin congeners (confirmed using HPLC-MS), and a more similar growth rate to that of UTCC 632 or UTCC 633 in comparison to other toxigenic strains tested. UTCC 632 and 633 were chosen as the non-toxigenic strains as they both showed no evidence of microcystin production as confirmed by chemical and molecular techniques. All cultures were initially grown at 25°C in a Conviron growth chamber under a light intensity of 60 $\mu M \cdot m^{-2} \cdot s^{-1}$ and a 12 hour day/night cycle. Cultures were grown in 500 ml Erlenmeyer flasks containing 250 ml of BG-11 growth medium (Sigma C3061) (Stein 1973). However, before the start of the experiment strains were transferred in replicates to fresh BG-11 growth medium and allowed to acclimate for at least one week at two different light levels: low (20 $\mu M \cdot m^{-2} \cdot s^{-1}$) and high (80 $\mu M \cdot m^{-2} \cdot s^{-1}$).

3.2.2 Mixed Culture Experiments

On day zero of the experiment the cell density of all three strains was determined using flow cytometry. With cell densities calculated, five cultures were initiated in fresh growth medium for growth under both low and high light intensities. Each of UTCC 300, 632, and 633 were grown as single cultures, along with two mixed cultures of UTCC 300+632, and UTCC 300+633. Each mixed culture was established by adding the same cell density of each individual culture to a flask containing new growth medium so that both strains would start with the same number of cells. Three replicates for each type of culture were grown under both light intensities. For fourteen days the three main variables measured were: 1) growth rate, 2) microcystin production, and 3) *mcy*D gene copy number to determine the proportion of toxigenic cells in the mixed cultures. Batch cultures were grown for a period of two weeks during which they would progress to a stationary phase of growth. This type of culture simulates the growth of a cyanobacterial bloom and the beginning of its collapse in nature (Lyck 2004).

3.2.3 Growth Rate Measurements

The growth of each strain was monitored using two techniques. First, the optical density (O.D.) was measured daily by recording the absorbance of each strain at 750 nm using a Pye-Unicam spectrophotometer (Stein 1973). Growth was also measured by preserving a subsample of culture every second day in 10% paraformaldehyde and subsequently counting cells using flow-cytometry. All growth rate analyses were carried out as in section 2.2.3.

3.2.4 Microcystin Analysis

Analysis for microcystins was carried out on days 2, 6, 10, and 14. For microcystin analysis 20 ml of each strain was filtered onto a pre-ashed (500°C for 2 hours), pre-weighed Whatman GF/C filter (1822-047). Filters were oven-dried overnight at 60°C, re-weighed to determine net dry biomass, and frozen at -20°C for subsequent analysis. Before extraction of microcystins, all filters were re-hydrated with distilled water and re-frozen at -20°C. This is done to prepare the cells to burst during the extraction procedure, thus ensuring a more efficient total extraction of all toxins. The freezing process ruptures cell walls and releases intracellular toxin (Lehman 2007). All microcystin extractions, HPLC-PDA, and HPLC-MS analyses were carried out as in section 2.2.4. In all samples containing UTCC 300 (UTCC 300, 300+632, and 300+633) more than a 90% recovery of nodularin was achieved.

3.2.5 DNA Extraction

Molecular techniques were used to differentiate between strains of *Microcystis aeruginosa* with the *mcy*D gene from those without. Therefore, along with microcystin analysis, each replicate of each strain was additionally filtered on days 2, 6, 10, and 14 for molecular analysis. A subsample of 10-20 ml of each of UTCC 300, 632, 633, 300+632, and 300+633 was filtered onto a Millipore 0.22 μm membrane filter and immediately placed in a 50 ml Falcon tube and frozen at -20°C pending subsequent analysis.

DNA was extracted from each sample using a procedure adapted from Hisbergues *et al.* (2003). Each filter was thawed and then subject to the following processes: an initial incubation with lysozyme in TES (Tris-EDTA-NaCl) (37°C, 1 hour, incubator with rotation); a second incubation with the addition of proteinase K (37°C, 1 hour, incubator with

rotation); a third incubation with the addition of 20% SDS (50°C, 2 hours, water bath); followed by a series of chloroform-isoamyl alcohol (24 :1), and phenol-chloroform-isoamyl alcohol extractions (25:24:1). DNA was also treated with RNAse A, precipitated overnight in cold iso-propanol, and subsequently spun down and re-constituted in TE (Tris-EDTA). DNA was stored at -20°C until further analysis. This standard phenol-chloroform extraction procedure has been found to yield quite accurate results (Schober & Kurmayer 2006, Schober *et al.* 2007). All reagents used were of a high purity molecular grade.

3.2.6 Quantitative PCR

With DNA extracted, initial PCR reactions were carried out using primers based on the *mcy*D gene of the microcystin gene-cluster to determine the presence/absence of the toxin gene. A PCR was also carried out with the primers for both *Microcystis* (the MIC primers) and for cyanobacteria in general (the CYA primers) (Table 5). The MIC primers were designed to detect *Microcystis* as a genus, while CYA primers were designed to recognize cyanobacteria based on the 16S rRNA gene. The MIC and CYA primers were used in initial PCR reactions to ensure that the DNA extracted from both toxigenic and non-toxigenic cultures would properly amplify in a PCR reaction.

The *mcy*D primers were not taken directly from the literature, but were re-designed based on sequences previously entered into GenBank. Sequences for *mcy*D were obtained from GenBank that had been entered up to March 2006 for *mcy*D in *Microcystis*, *Anabaena*, and *Planktothrix*. These sequences were aligned by N. Fortin (NRC-BRI, Montreal) and the *mcy*D 1 and 2 primers were designed based on two highly conserved regions of the *mcy*D gene (Table 5), with the provisional patent title: "DETECTION OF MICROCYSTIN-PRODUCING CYANOBACTERIA" Nathalie Fortin and Charles W. Greer (NRC). The first primer, *mcy*D1 is within the polyketide (KS) region of the *mcy*D gene while the second primer designed, *mcy*D2, is based on the first dehydratase (DH) domain of *mcy*D. *Mcy*D is believed to be a highly conserved region of the microcystin synthetase complex and compared to some other potential microcystin primers in general it has worked well and has shown little evidence for recombination (Tanabe *et al.* 2004). For example, Hotto *et al.* (2007) found that compared to *mcy*B, their *mcy*D primer set detected the gene more often than the *mcy*B primer set and was more efficient at detecting low concentrations of the gene.

Both *mcy*D1 and *mcy*D2 were used in exploratory PCR reactions and after several repeat experiments it was determined that *mcy*D1 yielded a more consistent PCR result with the cultures and a better QPCR reaction efficiency and thus was used in the remainder of all PCR and QPCR analyses.

Before QPCR could be carried out all DNA extracts were quantified using a Molecular Probe kit (Invitrogen P7589) based on PicoGreen, an ultra sensitive fluorescent nucleic acid stain which can be used to quantify double-stranded DNA. A microplate reader was used to measure the fluorescence at 480 nm and based on the results obtained from a standard curve the concentration of DNA in each sample was calculated. When DNA samples were quantified they were then diluted to a concentration of 10 $ng \cdot \mu l^{-1}$. All samples were then re-quantified using the same PicoGreen method to confirm the final concentration of DNA in this dilution. This was then labeled as DNA dilution 10^0. This DNA dilution of 10^0 was then further diluted to 10^{-1}, 10^{-2}, and 10^{-3}.

For QPCR analysis three dilutions for each sample were run in duplicate on a RotorGene 6000 QPCR cycler. QPCR reagents included RNAse free water (Qiagen), Quantitect SYBR Green PCR kit (Qiagen 204145), $MgCl_2$ (25 mM), *mcy*D1 forward primer (20 µM) and *mcy*D1 reverse primer (20 µM), BSA (bovine sera albumin) in a total reaction volume of 20 µl (which included 5 µl DNA extract). The BSA aids in binding PCR inhibitors in the samples (Ouellette *et al.* 2006). Positive and negative controls were run in every QPCR run. This included the UTCC 299 standard curve plasmid at a concentration of 10^{-6} as a positive control, and non-toxigenic strain UTCC 632 and 5 mM Tris-Cl as negative controls.

The standard curve was created by N. Fortin (NRC-BRI, Montréal) by creating a plasmid that had only one copy of the *mcy*D gene. First, the strain UTCC 299 was run in a PCR reaction to amplify *mcy*D1. The PCR product was then purified and put into a p-drive vector and transformed into *E.coli*. The culture was grown and the size of the *mcy*D insert was verified. The plasmid was cut once to make it linear and then subsequently quantified.

Table 5. Primers used to identify the *mcy*D gene in the microcystin synthetase gene cluster, *Microcystis* as a genus (MIC), and cyanobacteria in general (CYA). *Mcy*D primers were designed in conjunction with N. Fortin at the NRC-BRI (A provisional patent filed with the title: "DETECTION OF MICROCYSTIN-PRODUCING CYANOBACTERIA" by Nathalie Fortin and Charles W. Greer (NRC))

Primer Name	*Amplicon Size*	*Primer Sequence 5'-3'*	*Target Gene*	*Reference*
MCYDF1	~130bp	TGGGGATGGACTCTCTCACTTC	*mcy*D (KS)	N.Fortin NRC-BRI
MCYDR1		GGCTTCAACATTCGGAAAACG		
MCYDF2	~130bp	TACGGGAGTAACTTTCGGCTCA	*mcy*D (DH)	N.Fortin NRC-BRI
MCYDR2		ACAAGCATCTAACATAGCGGGA		
MIC184F	~230bp	GCCGCRAGGTGAAAMCTAA	*Microcystis* 16s rRNA	Ouellette *et al.* 2006
MIC431R		AATCCAAARACCTTCCTCCC		
CYA108F	~1200bp	ACGGGTGAGTAACRCGTRA	Cyanobacteria 16s rRNA	Ouellette *et al.* 2006
CYA165R		CTTCAYGYAGGCGAGTTGCAGC		

F- forward primer, R- reverse primer, R- A or G, M- A or C, Y- C or T

This plasmid then contained only one copy of *mcy*D1 and was diluted from 10^{-3} to 10^{-8}. These dilutions were run in QPCR reactions using different concentrations of $MgCl_2$ and primers so that a high reaction efficiency was obtained (QPCR standard curve reaction efficiency =0.95). QPCR reaction conditions were as follows: 1) hold temperature at 95°C for 15 minutes; 2) cycle (45 repeats) of 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 29 seconds; 3) Melt 65-95°C, holding for 45 seconds on the first step and for five seconds on the next steps. An example of a QPCR run form can be seen in Appendix V.

In order to determine the number of *mcy*D gene copies per ml of culture or lake water the number of gene copies detected via QPCR was adjusted based on the total quantity of DNA extracted for each sample. The number of *mcy*D gene copies detected was first divided by quantity of DNA run in each reaction (approximately 10 $ng \cdot \mu l^{-1}$) so that the number of gene copies per nanogram of DNA was obtained. This value was then multiplied by total quantity of DNA extracted to get the number of *mcy*D copies per total DNA. When divided by the volume of water filtered for a given sample the number of *mcy*D gene copes per ml of water was obtained.

3.2.7 Statistical Analyses

One-way ANOVA's were carried out to determine the effect of light intensity on: 1) the growth of each strain of *Microcystis*; and 2) the growth of all strains under a given light intensity. A two-way ANOVA was carried out to determine the effect of both strain and light intensity on growth rates. Lastly, a one-way ANOVA was carried out to compare the growth rates of toxigenic versus non-toxigenic strains of *Microcystis*.

Microcystin concentrations were compared among and between cultures and light levels in a similar way. One-way ANOVA's were carried out to determine the effect of light intensity on overall toxin production in the three cultures containing the toxigenic strain (300, 300+632, 300+633), along with the effect of light intensity on the production of microcystin congeners within each culture.

For molecular analyses the number of *mcy*D gene copies was determined for days 2, 6, 10 and 14 of the experiment. The number of *mcy*D gene copies was compared between UTCC 300, 300+632, and 300+633 under both low and high light intensities using a one-way ANOVA with culture as the grouping factor. Each culture was also analyzed individually on each day sampled using a one-way ANOVA with light as the grouping factor to determine if light intensity had a significant effect on *mcy*D copy numbers.

In all statistical analyses the assumptions of normality, homoscedasticity, and independence were verified using the residuals from each analysis. All statistics were carried out using the statistics software Systat 11.0.

3.3 Results

3.3.1 Growth Rates

Mean growth rates for the microcystin-producing strain UTCC 300, the non-microcystin strains UTCC 632 and UTCC 633, along with the mixed cultures of UTCC 300+632 and UTCC 300+633 are shown in Table 6. Original growth curves for replicates of each strain are found in Appendix VI.

For each individual strain the effect of light on growth rate differed. The toxigenic strain UTCC 300 did not have a significantly different growth rate when grown under high or low light intensity when growth was measured using changes in cell density ($p>0.05$). However, when changes in optical density were examined, UTCC 300 grew significantly faster under high light ($p<0.001$). Both non-toxigenic strains had higher growth rates under low light intensity when calculating growth rate from changes in cell density ($p<0.05$), however when calculating growth rates from changes in optical density both non-toxigenic strains had greater growth rates when grown under high light (UTCC 632 $p<0.05$, UTCC 633 $p<0.01$).

When comparing the growth rates of each uni-algal culture, whether using cell density or optical density, there were no significant differences between UTCC 300, UTCC 632, and UTCC 633 under low light intensity ($p>0.05$). However, when each strain was grown under high light, level there was a significant difference with UTCC 300 having the highest growth rate, followed by UTCC 632 and UTCC 633 ($p<0.05$).

For the mixed cultures UTCC 300+632 and UTCC 300+633, both had higher overall growth rates when grown under low than high light ($p<0.05$) when growth rates were calculated using cell density. Using changes in optical density, as with the uni-algal cultures, growth rate was higher under the high light intensity for UTCC 300+632 ($p<0.05$). However, for UTCC 300+633 there was no significant difference in growth rates between low and high light intensities ($p>0.05$).

Table 6. Growth rates (d^{-1}) for UTCC 300, 632, and 633 grown individually in batch cultures and in combination as 300+632, and 300+633 (means ± S.D., n=3) as calculated by changes in optical density (O.D.) or cell density (C.D.).

Microcystis aeruginosa Culture	*Light Intensity*			
	Low *20 $\mu M \cdot m^{-2} \cdot s^{-1}$*		***High*** *80 $\mu M \cdot m^{-2} \cdot s^{-1}$*	
	O.D.	C.D.	O.D.	C.D.
300	0.045 ±0.005	0.372 ±0.019	0.074 ±0.004	0.376 ±0.015
632	0.041 ±0.009	0.374 ±0.010	0.052 ±0.002	0.331 ±0.008
633	0.030 ±0.003	0.392 ±0.004	0.040 ±0.002	0.225 ±0.013
300+632	0.053 ±0.005	0.328 ±0.006	0.065 ± 0.003	0.306 ± 0.004
300+633	0.053 ±0.004	0.329 ±0.003	0.055 ±0.002	0.259 ±0.004

3.3.2 Microcystin Production

The two major microcystin congeners produced by UTCC 300 as determined by LC-MS were *microcystin-LR* and *[Dha7]desmethylmicrocystin-LR*. The concentrations of these two congeners were added to obtain a value for total microcystin. Total microcystin for UTCC 300, UTCC 300+632 and UTCC 300+633 at day 14 are shown in Figure 10. For UTCC 300 there was no difference in total microcystin production when the strain was grown at low and high light levels when total microcystin was expressed as $\mu g \cdot g^{-1}$ ($p>0.05$) or $\mu g \cdot cell^{-1}$ ($p>0.05$). However, when expressed on a volumetric basis there was more microcystin produced by UTCC 300 when grown under high than low light ($p<0.01$).

For UTCC 300+632 there were no significant differences between high and low light in terms of total microcystin production when expressed as $\mu g \cdot g^{-1}$ ($p>0.05$), $\mu g \cdot cell^{-1}$ ($p>0.05$) or $\mu g \cdot L^{-1}$ ($p>0.05$). For UTCC 300+633 there was slightly more microcystin per gram produced under low than high light ($p<0.01$). However when expressed per cell or per unit volume there was no significant difference between light levels ($\mu g \cdot cell^{-1}$and $\mu g \cdot L^{-1}$ $p>0.05$).

Total microcystin production was also compared between cultures grown under each light intensity. Under high light UTCC 300 produced more microcystin than UTCC 300+632 and UTCC 300+633 independent of how total microcystin was standardized ($\mu g \cdot g^{-1}$, $\mu g \cdot L^{-1}$, and $\mu g \cdot cell^{-1}$ $p<0.001$). When cultures were grown under low light UTCC 300 produced significantly more microcystin when expressed as $\mu g \cdot g^{-1}$ ($p<0.05$) or $\mu g \cdot cell^{-1}$ ($p<0.05$), but there was no significant difference with the mixed cultures when expressed as $\mu g \cdot L^{-1}$ ($p>0.05$).

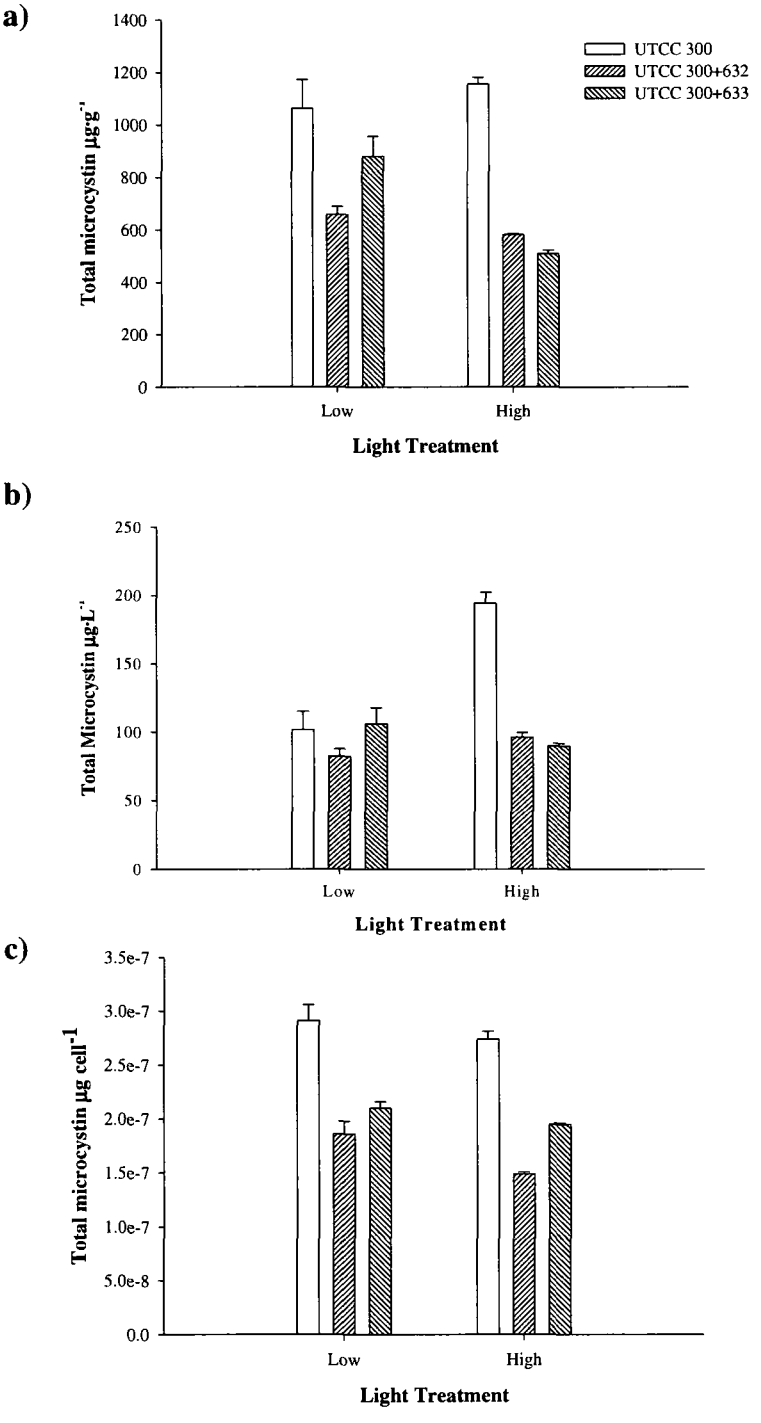


Figure 10. Total microcystin production on day 14 for UTCC 300, 300+632, and 300+633 expressed as **a)** $\mu g \cdot g^{-1}$ (± S.E., n=3), **b)** $\mu g \cdot L^{-1}$ (± S.E., n=3), and **c)** $\mu g \cdot cell^{-1}$ (± S.E., n=3).

3.3.3 Microcystin Congener Analysis

The proportions of the two microcystin congeners detected in each culture under both low and high light intensity are shown in Figure 11. For UTCC 300 there was a higher proportion of microcystin-LR under high than low light ($p<0.001$). Conversely, [Dha7] desmethylmicrocystin-LR had a slightly greater proportion under low than under high light ($p<0.001$). For UTCC 300+632 (Fig. 11b) and UTCC 300+633 (Fig. 11c) there was no significant difference in the proportion of either microcystin congener when grown under high or low light levels (UTCC 300+632 and UTCC 300+633 $p>0.05$).

3.3.4 PCR Reactions with CYA, MIC, and *mcy*D primers

Before QPCR reactions were carried out all DNA extractions were first used in simple PCR reactions. The PCR amplification products from both UTCC culture and field DNA extracts when using the CYA and MIC primers are shown in Figure 12. All of the cultures used in this experiment (300, 632, 633, 300+632, and 300+633) amplified a fragment using the CYA primers and when using the MIC primers (Fig. 12). These same primers worked with DNA extracted from a lake containing cyanobacteria including *Microcystis,* while amplification patterns were obtained with a positive control (UTCC 299-*Microcystis aeruginosa*) and no amplification with negative controls (5 mM Tris-Cl).

The next sets of primers to be tested were the *mcy*D1 primers. While two sets of *mcy*D primers were designed (*mcy*D1 and *mcy*D2) based on two different regions of the *mcy*D gene, *mcy*D1 (KS) was chosen as the best primer to use in the QPCR reactions. Figure 13 shows the results of a PCR reaction using DNA extracts from each culture amplifed with the *mcy*D1 primers. There is clearly an amplification product of approximately 130 base pairs (size based on the DNA ladder in the first lane of the gel) indicating that the *mcy*D1 primers amplified the *mcy*D gene.

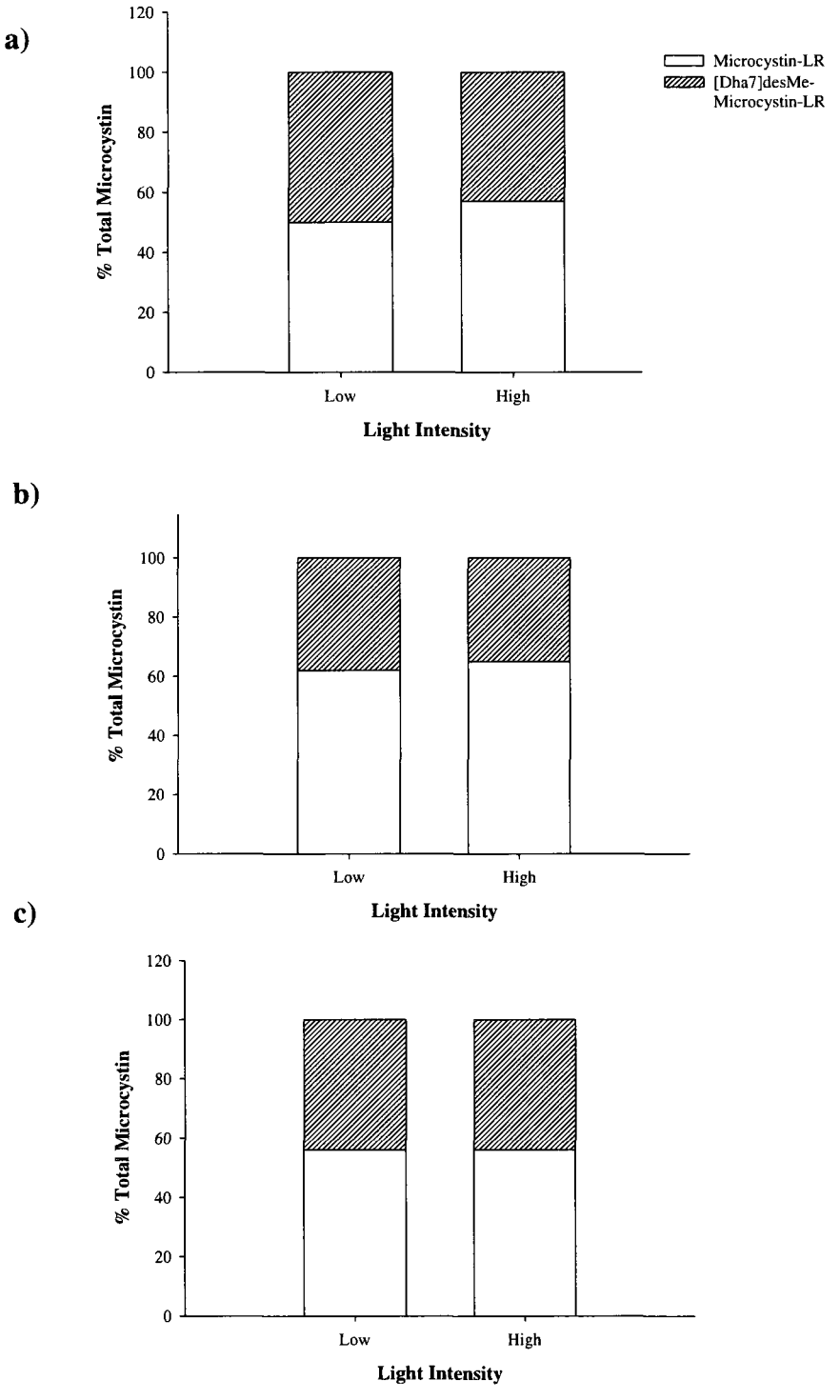
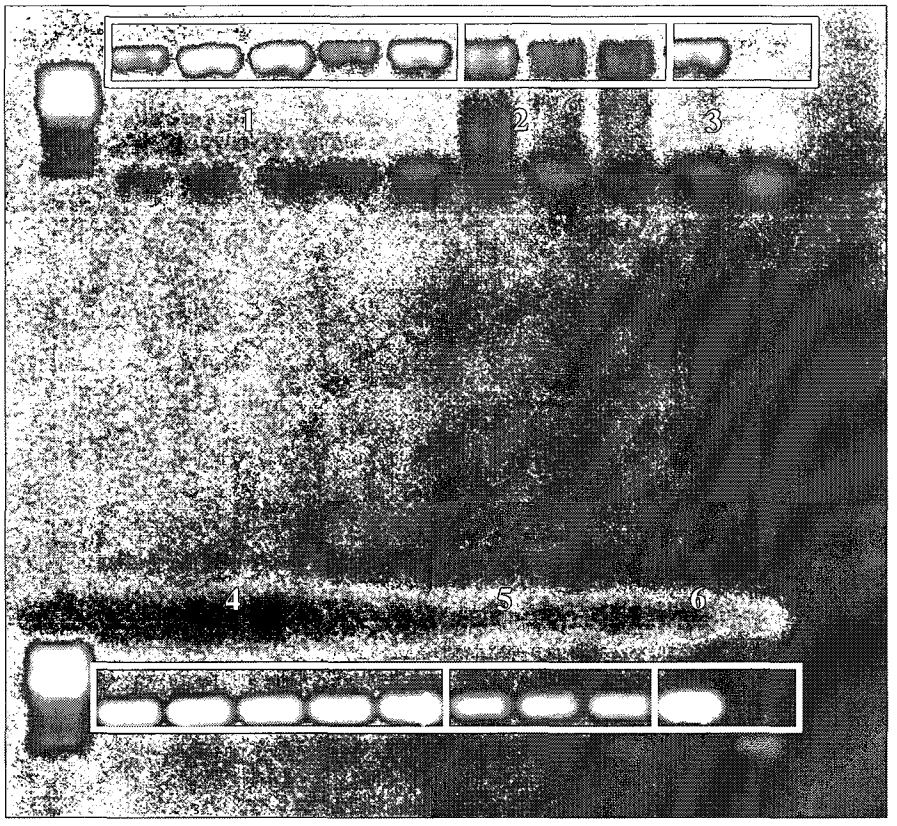
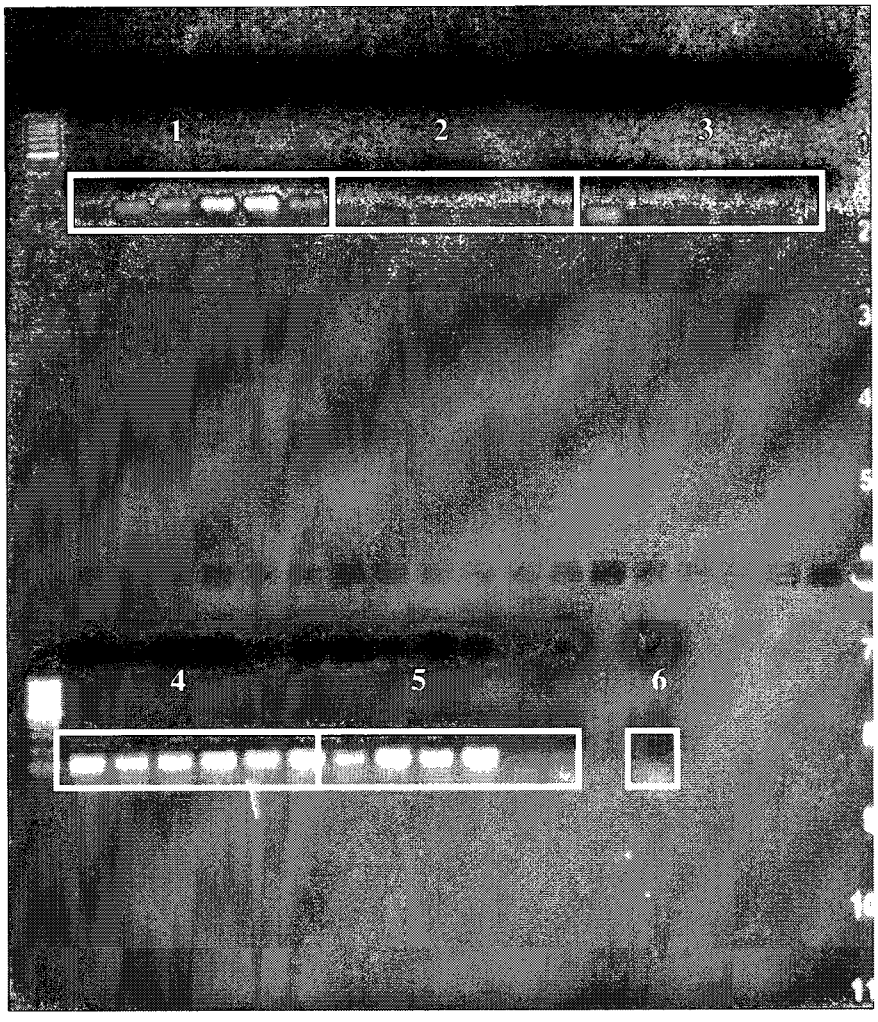


Figure 11. Proportions of two microcystin congeners produced by **a)** UTCC 300, **b)** UTCC 300+632, and **c)** UTCC 300+633.



Left to right :
1 – CYA Primers: UTCC 300, 632, 633, 300+632, 300+633
2 – CYA primers : Constance Lake 2006
3 – CYA Primers: positive (UTCC 299) and negative control (5mM Tris-Cl)
4 – MIC primers: UTCC 300, 632, 633, 300+632, 300+633
5 – MIC Primers: Constance Lake 2006
6 – MIC Primers: positive (UTCC 299) and negative control (5mM Tris-Cl)

Figure 12. 1.4% Agarose gel showing results from a PCR reaction with CYA and MIC primers and DNA extracts from UTCC 300, 632, 633, 300+632, 300+633 (day 10), Constance Lake 2006, and positive and negative controls.



Left to right:
1 – UTCC 300 H1, H2, H3, L1, L2, L3
2 – UTCC 632 H1, H2, H3, L1, L2, L3
3 – UTCC 633 H1, H2, H3, L1, L2, L3
4 – UTCC 300 + 632 H1, H2, H3, L1, L2, L3
5 – UTCC 300 + 633 H1, H2, H3, L1, L2, L3
6 - negative control (5mM Tris-Cl)

Figure 13. 1.4% Agarose gel showing results from a PCR reaction with *mcy*D1 primers and DNA extracts from UTCC 300 (1), UTCC 632 (2), UTCC 633 (3), UTCC 300+632 (4), UTCC 300+633 (5), and negative control (6) (Day 10). H refers to high light intensity, L refers to low light intensity, and numbers to corresponding replicates.

There were clearly no amplification products obtained when using the *mcy*D primers on non-toxigenic strains UTCC 632 and 633. As expected, due to the presence of UTCC 300 in the mixed cultures, the *mcy*D1 primers clearly amplified in the mixed cultures.

3.3.5 QPCR

From QPCR analyses the number of copies of *mcy*D per ml could be determined based on the cycle threshold (Ct) values. The Ct is the cycle number at which fluorescence reaches a set threshold. Appendix VII shows an example of a fluorescence versus Ct figure along with a melting point analysis. All *mcy*D amplified products were expected to melt at the same temperature which indicates that the same QPCR product was amplified in each reaction run.

Under low light *mcy*D copies·ml^{-1} increased in UTCC 300, UTCC 300+632 and UTCC 300+633 over time (Fig. 14a). However, under low light there were no significant differences in *mcy*D copies·ml^{-1} across the three different cultures on any of the four days on which they were sampled (days 2, 6, 10, 14, $p>0.05$). This suggests that there were a similar number of toxigenic UTCC 300 cells in each of these cultures throughout the experiment. Under high light (Fig. 14b) there appeared to be fewer *mcy*D copies·ml^{-1} at day 14 yet cell counts indicated that there were higher cell densities under high than low light intensities. On day 2 under high light there were no significant differences in the copies of *mcy*D between the three cultures ($p>0.05$). However by day 6 there was a significant difference in the copies of *mcy*D between the three cultures ($p<0.01$) with UTCC 300 having the highest copy number followed by UTCC 300+632 and UTCC 300+633. This trend continued under high light through days 10 and day 14.

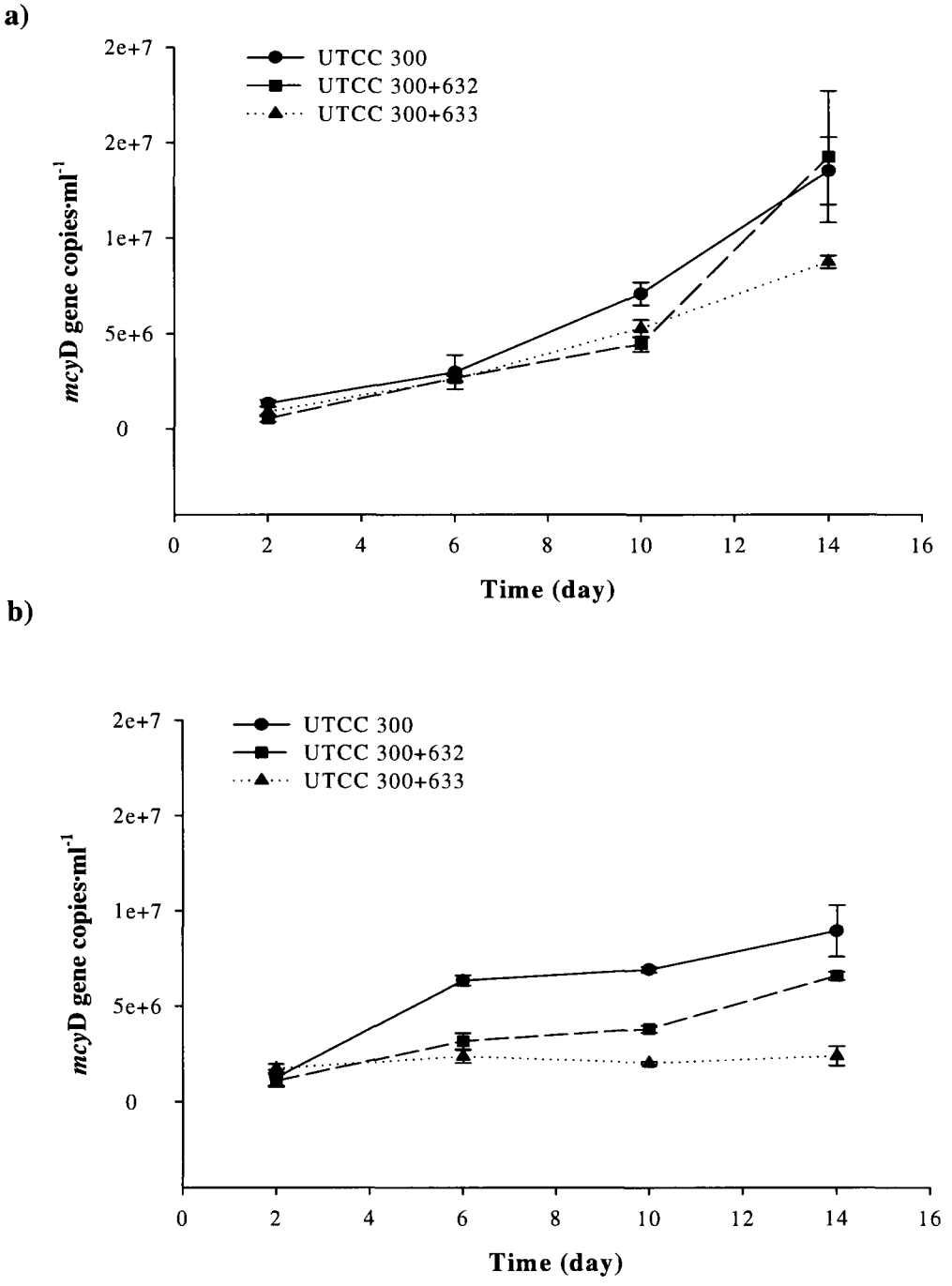


Figure 14. *mcy*D1 gene copy numbers per ml (± S.E., n=3) over time (day) for UTCC 300, 300+632, and 300+633 grown under two light intensities **a)** low, and **b)** high.

The proportion of toxigenic cells in each mixed culture of UTCC 300+632 and UTCC 300+633 are represented in Figure 15. The proportion was determined by dividing the *mcy*D copies·ml^{-1} for each of the mixed cultures by the *mcy*D copies·ml^{-1} for UTCC 300 on a given sampling day. UTCC 300 is the toxigenic strain and therefore all cells in the UTCC 300 culture should be toxigenic genotypes (i.e. 100%). By dividing the *mcy*D copies·ml^{-1} of the mixed cultures by the *mcy*D copies·ml^{-1}of UTCC 300 the proportion of toxigenic genotypes in the mixed cultures could be estimated. Under low light the mixed cultures had >60% *mcy*D on days 6, 10 and 14. Under high light UTCC 300+632 was also found to be comprised of 50% or more of UTCC 300 *mcy*D with the percentage appearing to increase with time. However, for the UTCC 300+633 culture less than 40% was UTCC 300.

These results suggest that the toxigenic strain UTCC 300 was dominating both non-toxigenic strains under low light conditions, whereas under high light conditions the non-toxigenic strain UTCC 633 appeared to be out-competing the toxigenic strain (Fig. 15). However, the results must also be examined in the context of cell density. In order to account for cell density scatter plots were examined of cell density vs. *mcy*D copies·ml^{-1} (Appendix VIII). Cell density in this case was considered a surrogate for time in this situation as cell density increased during the 14 day experiment. A linear regression was fit to each scatter plot for each culture grown under high and low light (Table 7). When the three cultures of UTCC 300, 300+632, and 300+633 were considered under low light intensity there was a strong relationship between *mcy*D copies·ml^{-1} and cell density for each culture ($p<0.05$). As in Figure 16a there was a similar number of *mcy*D gene copies·ml^{-1} in all cultures on any of the four sampling days.

Under high light intensity while *mcy*D gene copies were increasing over time in UTCC 300 and in UTCC 300+632, there appeared to be a flatter relationship in UTCC 300+633 (Fig. 14b). The results obtained by plotting *mcy*D copies·ml^{-1} versus cell density also indicate this to be the case. The slopes of the regression equations of cell density vs. *mcy*D for UTCC 300 and UTCC 300+632 were very similar (0.23 compared with 0.24), however the slope of the regression for UTCC 300+633 was flat (slope of 0.02). From the results in Figure 14b, Appendix VIII, and Table 7, UTCC 300 was clearly not increasing over time in the mixed culture of 300+633 when grown under high light intensity, and UTCC

633 appeared to outcompete UTCC 300 based on the lack of increase in *mcy*D gene copies as both time and cell density increased.

3.3.6 Microcystin concentration as a function of *mcy*D copies·ml^{-1}

In order to assess how microcystin concentrations could be predicted from *mcy*D gene copy numbers total microcystin concentrations from all replicates containing UTCC 300 were plotted with *mcy*D copies·ml^{-1} for day 14 of the experiment (Figure 16). There was a strong correlation between *mcy*D copies·ml^{-1} and total microcystin (μg·g^{-1}) ($R^2 = 0.92$) indicating that toxin concentrations could be reliably predicted using the molecular identification of *mcy*D gene copy numbers for UTCC 300.

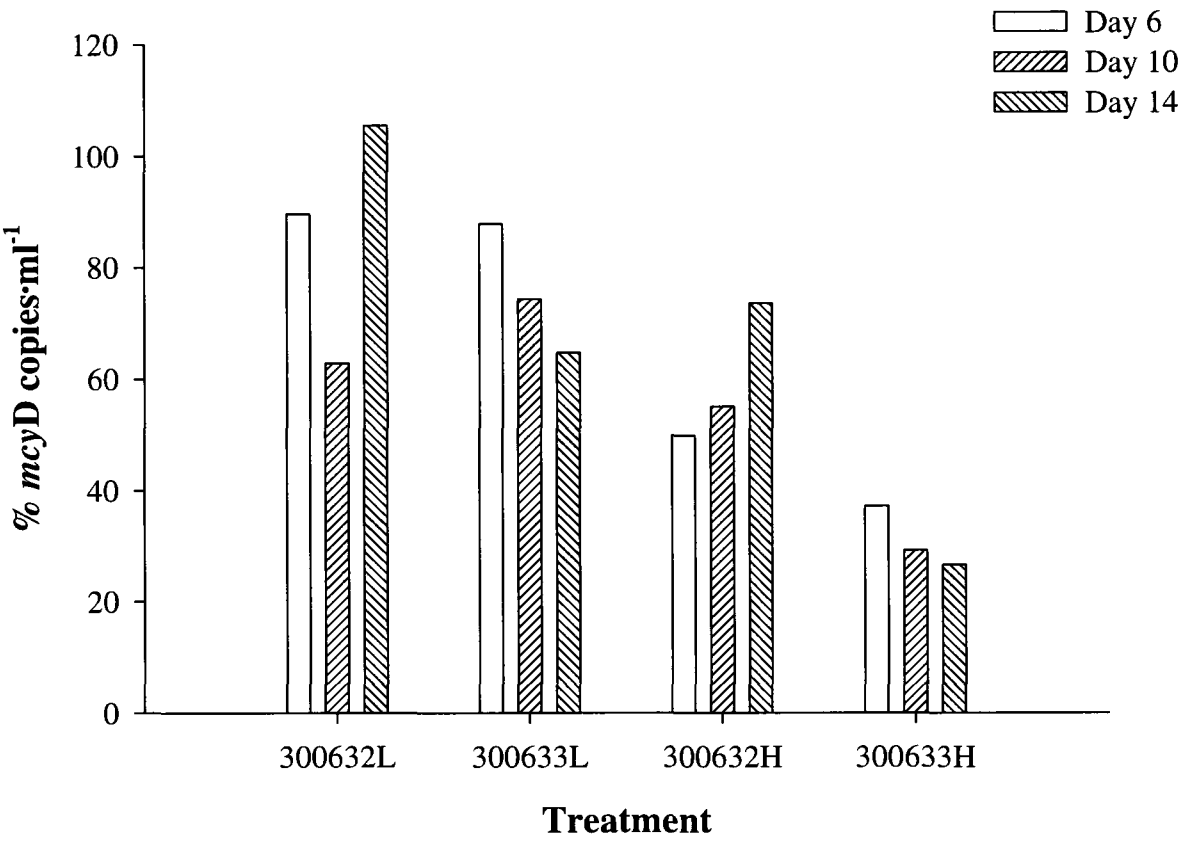


Figure 15. Percent of *mcy*D1 copies·ml^{-1}relative to pure culture of UTCC 300 for 300+632, and 300+633 grown under the two light intensities, low (L), high (H).

Table 7. Results from linear regression analysis of cell density (x) vs. *mcy*D copy numbers (y) for UTCC 300, 300+632, and 300+633 grown under low and high light intensities.

Culture	*Equation*	R^2
Low Light		
UTCC 300	$y= 0.691x + 2.0 \times 10^6$	$R^2= 0.631$
UTCC 300 + 632	$y= 0.472x + 9.8 \times 10^5$	$R^2= 0.926$
UTCC 300 + 633	$y= 0.416x + 1.0 \times 10^6$	$R^2= 0.930$
High Light		
UTCC 300	$y= 0.226x + 3.0 \times 10^6$	$R^2= 0.433$
UTCC 300 + 632	$y= 0.239x + 2.0 \times 10^6$	$R^2= 0.756$
UTCC 300 + 633	$y= 0.022x + 2.0 \times 10^6$	$R^2= 0.0226$

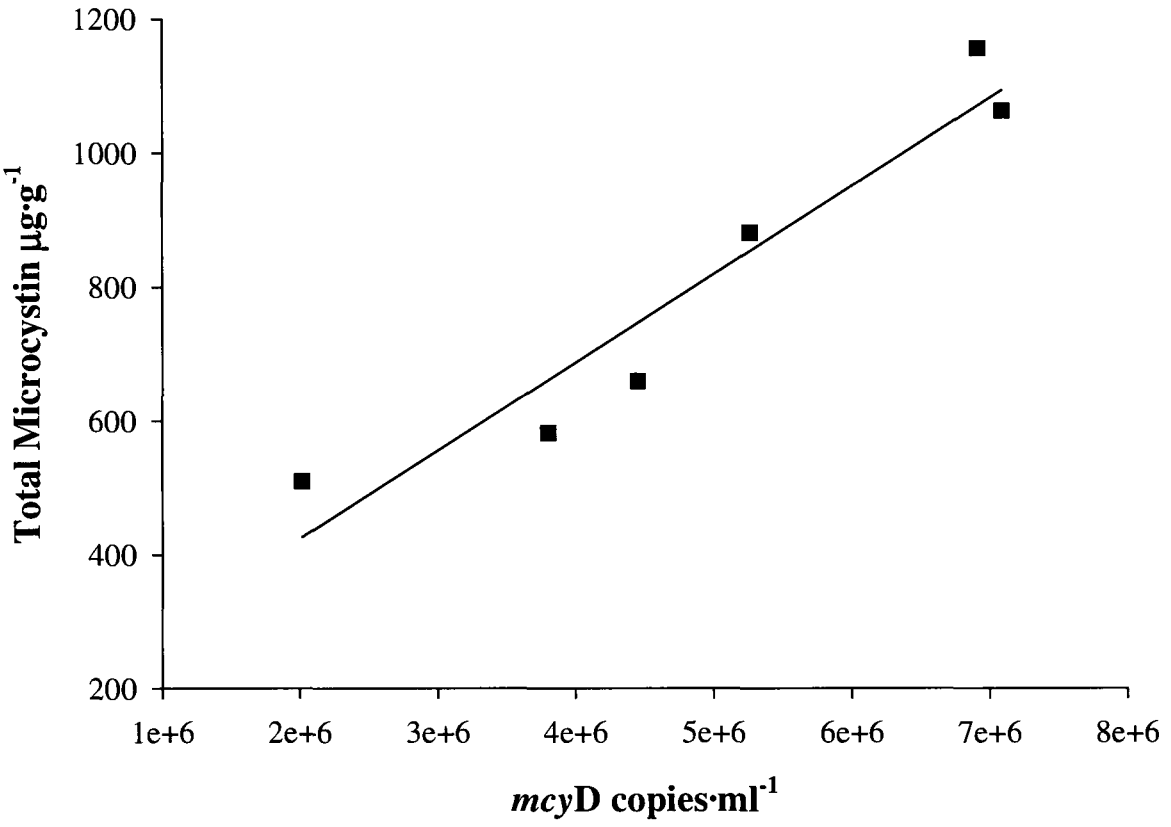


Figure 16. Total microcystin ($\mu g \cdot g^{-1}$) as a function of *mcy*D1 copies·ml^{-1} in cultures of *Microcystis aeruginosa* (UTCC 300, 300+632, 300+633 grown under high and low light)(n=6, y=160 + 1.3x, r^2=0.92).

3.4 Discussion

This study aimed to determine whether a toxigenic strain of *Microcystis aeruginosa* would dominate over non-toxigenic strains of the same species when in competition for light resources. Quantitative PCR (QPCR) and a primer designed based on the *mcy*D region of the microcystin synthetase gene complex were used to determine the proportion of toxigenic genotypes in a given sample. Mixed culture experiments are useful in explaining the patterns of genotype succession in nature as both toxigenic and non-toxigenic strains of cyanobacteria can be detected in natural water bodies (Kurmayer *et al.* 2004). Two studies have suggested that non-toxigenic strains tend to dominate later in the summer (Janse et al. 2004, Yoshida *et al.* 2008). In contrast another study found that in some cases non-toxigenic strains dominate while in others both toxigenic and non-toxigenic strains co-exist (Kardinaal *et al.* 2007b). Due to such temporal variation in strain dominance, the toxicity of blooms is currently difficult to predict. A better understanding of the factors that lead to the dominance of toxigenic strains is clearly needed for the management and public health reporting of toxic blooms.

In this study a toxigenic strain of *Microcystis aeruginosa* dominated the non-toxigenic strains under conditions of light limitation but not when light resources were abundant. In theory, should neither the toxigenic strain UTCC 300 nor non-toxigenic strains UTCC 632 and 633 have a particular advantage, the expected result in a mixed culture would be a 50/50 mix of each strain assuming similar intrinsic growth rates. Growth rates, microcystin production (including microcystin congener analysis), and molecular tools including PCR and QPCR reactions using primers based on *mcy*D were analyzed in order to determine under both low and high light intensities which strain, toxigenic or non-toxigenic, would dominate.

3.4.1 Comparison of Growth Rates

It is important to consider the growth rate of each uni-algal strain when carrying out a mixed culture experiment because if one strain grows significantly faster than another it may simply result in the dominance of the faster growing strain. In this experiment UTCC 300, 632 and 633 did not have significantly different growth rates when grown under low light (Table 6). However, based on the QPCR results, under low light UTCC 300 grew better than

both UTCC 632 and 633 in each of the mixed cultures. Because UTCC 300 was not growing significantly faster at this light intensity the resulting dominance of the toxigenic strain cannot be attributed to a faster growth rate per se.

The growth rates for *Microcystis aeruginosa* strains were also compared when grown under high light intensity. In this case there was a significant difference in growth rate between the three strains with the toxigenic UTCC 300 having a higher growth rate than either UTCC 632 or 633. However the non-toxigenic UTCC 633 out-competed toxigenic UTCC 300 when grown under high light, even though UTCC 633 had a significantly lower intrinsic growth rate than UTCC 300 at this light level.

3.4.2 Microcystin Production

For UTCC 300 there was no difference between high and low light in terms of toxin production when expressed per gram or per cell, but when expressed per litre more microcystin was produced under high compared to low light. Therefore, the production of microcystins does not appear to be a driving factor in explaining the dominance of UTCC 300 under low light conditions. Microcystin production also does not appear to be a hindrance to the toxigenic strain and in contrast to previous assumptions does not appear to be energetically costly (Vézie *et al.* 2002).

For UTCC 300+632 and 300+633 there were also no differences in microcystin concentrations detected at high and low light when expressed per gram or per cell. Two main microcystin congeners were detected, microcystin-LR and [Dha7] desmethylmicrocystin-LR. Based on total microcystin production and the production of different microcystin congeners the growth of UTCC 300 in contact and in competition with a non-toxigenic strain did not significantly change its patterns of microcystin production.

3.4.3 CYA, MIC and *mcy*D

The results from the PCR analyses with the three primer sets used in this experiment give confidence in the choice of DNA extraction methods and the choice of PCR and QPCR methods used with the *mcy*D primers. The *mcy*D primers used in this experiment were re-designed based on sequence data submitted to GenBank rather than using pre-existing primers from the literature. New sequence information is continually submitted to GenBank

and therefore it is prudent to ensure that the primers used are the best fit for the analyses. It was imperative to ensure that DNA from UTCC 300 and the mixed cultures 300+632 and 300+633 would amplify using the *mcy*D primers and that UTCC 632 and UTCC 633 alone would not. However in no case, either when carrying out a PCR or QPCR reaction, did either of UTCC 632 or 633 result in an amplification product. While it was expected that neither of UTCC 632 or 633 had the microcystin synthetase gene complex within their genome, these results confirmed that neither of UTCC 632 or 633 have the *mcy*D gene. This is in contrast to another strain of *Microcystis aeruginosa* UTCC124 that while it does not produce any microcystins (Chapter 2), it appears to carry the microcystin gene yielding amplification products with *mcy*B and *mcy*D (Ouellette *et al.* 2006). In this study, all copies of *mcy*D detected using QPCR analyses in the mixed cultures came from UTCC 300.

The QPCR analyses clearly showed an increase in *mcy*D copies over time in all cultures under both low and high light intensity with the exception of UTCC 300+633 grown under high light (Fig. 14). Under low light the mixed cultures 300+632 and 300+633 had greater than 50% of the *mcy*D copies·ml^{-1} that UTCC 300 had on days 6, 10 and 14 (Fig. 14, 15). This indicates that UTCC 300 grew better than UTCC 632 or 633 and this despite no difference in intrinsic growth rates among the strains grown individually. Under high light UTCC 632 and especially UTCC 633 did better in competition with the toxigenic strain than when grown under low light. While one might have predicted that UTCC 300 would dominate since it had a significantly higher intrinsic growth rate under high light, this was not observed.

3.4.4 Competition between Toxigenic and Non-toxigenic Strains

In aquatic environments there are two main factors that generate low light conditions- the depth of the mixed layer or water column (epilimnion) and its turbidity. Turbidity increases as biomass increases and thus may favour those organisms adapted to lower light intensities (Sedmak & Kosi 1998). In this study not only was light manipulated by physically growing the cultures at two different light intensities, but over time as biomass increased within each culture light resources would also be reduced. It has previously been demonstrated that the species or strain with the lowest 'critical light intensity' will be the superior competitor independent of the type of algae or cyanobacteria and their maximum

growth rates (Huisman *et al.* 1999). In this study under low light conditions there was no significant difference in terms of growth rates between UTCC 300, 632, and 633, yet UTCC 300 dominated both mixed cultures. Conversely, under high light UTCC 300 had a higher growth rate than 632 or 633 yet UTCC 633 dominated UTCC 300.

Microcystin biosynthesis occurs via a multi-enzyme complex and requires a lot of energy which may favour those non-toxigenic strains that need not perform this energy-consuming biosynthetic process. Under conditions of resource limitation non-toxigenic strains should in theory be superior competitors over toxigenic strains (Vézie *et al.* 2002). Should this assumption be correct then under conditions of light limitation toxin producing strains should be at a disadvantage. However, based on the work presented in the previous and current chapters, the toxigenic strains used did not appear to be inhibited in terms of their ability to grow in batch cultures under low light conditions and the non-toxigenic strains were not superior under conditions of light limitation. These results are in contrast to those of a recent quite analogous mixed culture study (Kardinaal *et al.* 2007a).

In a mixed culture competition experiment Kardinaal *et al.* (2007a) examined how competition for light would affect the dominance of toxigenic or non-toxigenic strains of *Microcystis aeruginosa*. In all cases the toxigenic strains lost in competition for light from the non-toxigenic strains with the toxigenic strain being completely replaced by the non-toxigenic strain within approximately two weeks. Even when the toxigenic strain was given a significant advantage in terms of the number of cells at the start of the experiment the non-toxigenic strain still grew to out-compete it. They suggested that non-toxigenic strains are better competitors for light than are toxigenic strains. In contrast, the results presented here do not support the hypothesis that non-toxigenic strains are better competitors for light than are toxigenic strains. When light was limiting the toxigenic strain UTCC 300 dominated both of the non-toxigenic strains. Only when light resources were abundant did both non-toxigenic strains fair better.

Microcystins have been the peptides most commonly studied from cyanobacteria due to the seriousness of their toxic effects. It is thought that the genes responsible for microcystin production were present in the last common ancestor of a large number of cyanobacteria due to the fact that microcystins can be produced by a wide variety of cyanobacterial genera (Rantala *et al.* 2004, Dittmann & Borner 2005). It is believed that

non-toxigenic strains arose through the repeated loss of one or several regions of the microcystin synthetase gene complex over time (Kurmayer *et al.* 2004, Dittmann & Borner 2005, Jungblut & Neilan 2006). However, it has also been observed that many strains have retained the genes required for microcystin synthesis which points to an important though still unknown function for microcystins (Kurmayer *et al.* 2004).

From the results of the present study it is unlikely that microcystins are responsible for explaining the dominance of one cyanobacterial strain over the other. Therefore, other traits need to be examined in order to determine why one strain may have a competitive advantage over another and if this is related to the production of various compounds that give one strain an edge in the aquatic environment. Studies are now considering the idea that non-toxigenic strains may produce other compounds that play a similar role in cell processes and functions as do the microcystins in toxigenic strains (Tonk *et al.* 2008a). For example many cyanobacteria are a source of a wide range of peptide like compounds including aeruginosins, microginins, anabaenopeptins, cyanopeptilins, and microviridins (Repka *et al.* 2004, Welker & Van Doren 2006). Further studies examining how toxigenic and non-toxigenic strains respond to varying environmental conditions should take into consideration not only microcystins, but these other types of compounds as well.

3.4.5 Conclusions

Previous studies have suggested that microcystins may play a role in light adaptation processes (Kaebernick *et al.* 2000, Hesse *et al.* 2001, Kaebernick *et al.* 2002, Young *et al.* 2005, Jahnichen *et al.* 2007). In this study, there was no significant increase in microcystin production at low light intensity that might indicate that the toxigenic strain was increasing microcystin production to help adapt to light limitation. Furthermore, there was no significant growth inhibition of the toxigenic strain under light limiting conditions when in competition with non-toxigenic strains suggesting that the production of microcystins did not result in a significant "cost" in terms of growth and competitive ability.

Overall these results do not support the hypothesis that microcystins are agents of adaptation or that microcystins are costly to produce and a hindrance in competition for limiting resources. However, very few studies of this type including both toxigenic and non-toxigenic cyanobacteria have been conducted; in fact there is only one other study (Kardinaal

et al. 2007a) which came to an opposite conclusion. Additional experiments are needed to test a larger number of strains and more combinations of toxigenic and non-toxigenic strains of not only *Microcystis aeruginosa* but other microcystin-producing species. This will also require the development and improvement of primers and PCR techniques including the use of multiplex PCR (Rinta-Kanto *et al.* 2005).

Chapter 4

The seasonal distribution of microcystin genes versus microcystins in a shallow mesotrophic lake

Abstract

The factors regulating the concentrations of microcystin genes, microcystins and toxigenic cyanobacteria in a shallow temperate lake were examined using analytical chemistry and molecular approaches. Constance Lake, a mesotrophic lake located within the limits of the City of Ottawa, Ontario, was sampled throughout the summers of 2006 and 2007. Along with standard limnological variables, water samples were also analyzed for total microcystins using HPLC and ELISA methods and the *mcy*D gene of the microcystin synthetase gene cluster to quantify (using QPCR) toxigenic cyanobacterial genotypes. While a surface cyanobacterial bloom did not occur in either year, both microcystins and *mcy*D gene copies·ml^{-1} were detected in both field seasons. Overall, higher microcystin concentrations and *mcy*D gene copies·ml^{-1} were detected in the summer of 2006 compared to 2007 and this despite no obvious differences in environmental variables such as temperature, light, and nutrient availability between years. There was also an inconsistent relationship between *mcy*D gene copies·ml^{-1} and total microcystin concentrations suggesting that molecular methods may not be as useful in predicting the toxicity of surface waters and should not be used in place of chemical analyses when public health is a consideration. In general, biotic variables such as total cyanobacterial biomass, and specifically the biomass of the common microcystin-producing cyanobacterium *Anabaena* and to a lesser extent *Microcystis* were more significantly correlated with *mcy*D gene copy numbers, while a combination of both biotic and abiotic variables (i.e. dissolved oxygen, pH, conductivity) were predictors of total microcystin concentrations.

4.1 Introduction

Cyanobacterial blooms can be defined as the excessive growth of usually one or several dominant cyanobacterial species (Sivonen & Jones 1999). Factors that promote cyanobacterial blooms include ample nutrients and light, calm waters and usually relatively warm water temperatures (Codd *et al.* 1985). Bloom events are increasing due to the continued eutrophication of water bodies from human activities (e.g. agriculture, sewage treatment) that result in large nutrient inputs into aquatic environments (Wiegand & Pflugmacher 2005). As mentioned previously (Chapter 1) there are several consequences of blooms including taste and odour problems and declines in the water quality and recreational value of lakes. Also, severe reductions in oxygen levels in aquatic ecosystems can occur following cyanobacterial blooms as oxygen is quickly used up by microbes during decomposition. Lastly, a more serious consequence affecting public health is the production of toxic secondary metabolites by several cyanobacterial genera. In Canada, a growing number of communities are now being confronted by recreational and drinking water tainted by toxic cyanobacterial blooms including major freshwater resources such as Lakes Erie, Ontario, Winnipeg, Champlain, and numerous smaller lakes and rivers across the country (Boyer *et al.* 2004, Rinta-Kanto *et al.* 2005).

Cyanobacteria tend to be favoured during eutrophication for a number of reasons including the ability of some species to fix nitrogen, to regulate their position in the water column due to the presence of gas vacuoles, and to defend themselves against grazers (Hyenstrand *et al.* 1998). The latter may be due to morphological features or the production of cyanobacterial toxins (cyanotoxins) which may also suppress the growth of other phytoplankton species (Codd *et al.* 1985). The study of cyanotoxins has increased significantly in the last two decades and toxins such as microcystins appear to be widespread in lakes. In a large survey of US lakes more than 50% of lakes sampled had detectable levels of microcystins (Graham *et al.* 2004).

It is inherently difficult to study toxic cyanobacterial blooms as the overall toxicity of a cyanobacterial bloom is highly variable both spatially and temporally (Lambert *et al.* 1994, Kotak *et al.* 1995, Graham *et al.* 2006). Wide variation in microcystin concentration is likely due to a number of factors including the composition of cyanobacterial species and strains that are present (i.e. the simultaneous presence of both toxigenic and non-toxigenic strains)

and the environmental variables which affect the growth of various species/strains (Graham *et al.* 2004). Several genera of cyanobacteria can produce the same type of cyanotoxin: microcystins can be produced by *Microcystis, Planktothrix,* and *Anabaena.* Most of the organisms that may be affected by cyanobacterial toxins (fish, humans, livestock etc.) are not natural enemies or consumers of cyanobacteria (Paerl & Millie 1996). Thus the actual physiological and ecological bases for toxin production remain unclear. Furthermore, while the factors that lead to cyanobacterial bloom formation are fairly well known, the factors that specifically promote the growth of toxin-producing species whether it be nutrients (actual concentrations and stoichiometry), temperature, light, or other environmental variables are not well understood.

Since the sequence and structure of the microcystin synthetase gene cluster was determined (Tillett *et al.* 2000) molecular techniques have been used to study toxic cyanobacterial blooms. Using molecular techniques one can theoretically follow the succession of microcystin producing genera in lakes over a growing season (Ouellette & Wilhelm 2003).With the structure of the microcystin synthetase gene cluster established, Tillett *et al.* (2001) designed a PCR primer for the N-methyl transferase domain of the *mcy*A gene to probe both culture and field samples. All 18 toxin producing species examined amplified a 1.3kb fragment and the production of toxins was verified. Pan *et al.* (2002) carried out a similar study, however their primers were designed to detect and characterize microcystin and non-microcystin producing *Microcystis* strains by specifically amplifying fragments of the *mcy*B gene. Thirty *Microcystis* strains were tested with 18 giving positive PCR signals while the remaining 12 strains did not. All 18 strains were toxin producing strains as verified by HPLC, ELISA, and mouse bioassay; the remaining 12 strains that did not amplify a PCR fragment were all determined to be non-toxigenic. Pan *et al.* (2002) tested both culture and field samples to determine how potential inhibitory compounds in field samples may affect PCR results. Based on these initial studies it appears that amplification of *mcy* genes by PCR from DNA isolated from both axenic cultures and field samples may be a sensitive means to differentiate between toxigenic and non-toxigenic *Microcystis* strains.

The development of these molecular techniques would allow earlier detection of toxigenic blooms, identification of the dominant organism within the blooms, and an

estimation of the level of toxic substances that are accumulating (Schatz *et al.* 2000). While there are several methods that can detect the actual toxin levels such as HPLC, mass spectrometry, ELISA, and protein phosphatase inhibition assays (PPIA), these are quite time consuming (Foulds *et al.* 2002). Incorporation of molecular techniques into monitoring of aquatic systems is the next step towards successful management of algal and cyanobacterial blooms. Once we fully understand the conditions under which toxigenic genotypes are favoured it may be possible to implement management techniques to curb the dominance of toxin-producing strains.

This study examined the distribution of toxigenic cyanobacteria throughout a growing season and how it relates to major environmental variables. Constance Lake, located within the limits of the City of Ottawa, was sampled throughout May-October of 2006 and 2007 for physical, chemical and biological variables along with microcystins and the *mcy*D gene required for microcystin production which is an indicator for toxigenic genotypes. The goal was to determine which environmental variables (both abiotic and biotic) best explained the variation in microcystin concentrations and *mcy*D gene copy numbers. An additional important goal was to verify whether these two measures of the presence and activity of toxigenic cyanobacteria were well correlated and consequently whether molecular methods could provide a more rapid and sensitive warning system for toxic bloom events.

4.2 Methods

Constance Lake (45°24'N, 75°59'W) is located within the limits of the City of Ottawa, just west of the suburb of Kanata. It is a shallow, mesotrophic lake (average depth of approximately 3 m), accessible, and has been studied for several summers. In the past the lake has had populations of cyanobacteria at low to moderate levels, with occasional blooms of a sub-tropical invasive species *Cylindrospermopsis raciborskii* (Hamilton *et al.* 2005). Toxigenic species such as *Microcystis aeruginosa* and *Anabaena flos-aquae* are found in Constance Lake, including toxigenic strains as microcystins have been detected previously throughout the growing season (Tillmanns 2009).

4.2.1 Field Sampling

Water samples were collected using an integrated tube sampler to a depth of 2 meters and stored in 2 litre Nalgene bottles previously triple-rinsed with lake water. A Hydrolab MiniSonde was used to measure temperature (°C), dissolved oxygen (DO, both in $mg \cdot L^{-1}$ and percent saturation), pH, redox potential (ORP), and specific conductivity (SPC) at intervals of 0.25 m through the water column Light penetration was determined using a Licor 190 PAR light meter taking readings at similar intervals. A 20 cm Secchi disk was also used to estimate water transparency. Two times the depth of the Secchi disk reading gives an approximation of the euphotic zone (or the depth 1% of the surface radiation) (Wetzel 2001).

Water samples were kept in coolers and taken to the Robert O. Pickard Environmental Centre Laboratory of the City of Ottawa for water chemistry analysis. Two replicate aliquots of collected water were sent for analysis on each sampling date. Analyses were performed for ammonia and ammonium, nitrate and nitrite, reactive phosphorus, total Kjeldahl nitrogen (TKN), and total phosphorus (TP). Occasional analyses were conducted for the major cations (Ca^{2+}, Mg^{+}, K^{+}, Na^{+}) and silica. Silica was of interest because at very low concentrations diatom growth may be limited and the growth of cyanobacteria favoured (Tilman *et al.* 1986).

Collected water samples were used to measure levels of algal pigments. One-litre subsamples were filtered on Whatman 934-AH filters for subsequent chlorophyll and accessory pigment analysis. Filters were frozen until chlorophylls and carotenoids could be

extracted and measured using a Pye-Unicam spectrophotometer. A standard method for pigment extraction was followed, using DMSO and acetone as solvents (Burnison 1980) and applying the trichromatic equation of Jeffrey and Humphrey (1975). Subsamples of 100 ml were also preserved in Boston round bottles with 1 ml of Lugol's iodine solution. Serge Parent of l'Université du Québec à Montréal (UQAM) assisted in the taxonomic verification of phytoplankton species.

4.2.2 Microcystin Analysis

In 2006 microcystin samples were filtered in duplicate and in 2007 samples were filtered in triplicate. 1-2 litres of integrated lake water was filtered onto a pre-ashed (500°C for 2 hours), pre-weighed Whatman GF/C filter (1822-047). Filters were oven-dried overnight at 60°C, re-weighed to determine net dry biomass, and frozen at -20°C for subsequent analysis. Before extraction of microcystins all filters were re-hydrated with distilled water and re-frozen at -20°C. This was done to prepare the cells to burst during the extraction procedure, thus ensuring a more efficient total extraction of all toxins (Tillmanns *et al.* 2007). Extraction methods for microcystins were carried out as described in section 2.2.4.

Microcystin extracts were analyzed using HPLC with PDA detection (Agilent Technologies 1100 Series).The following microcystin congeners were commercially available as standards: microcystin-LR, YR, RR, and LA (Sigma M2912, M4069, M1537, and M4194). Standard curves were created for each congener using concentrations of 10, 5, 2.5, 1, 0.5, 0.1, and 0.05 ppm. HPLC analysis was conducted using a Zorbax SB-C18 column (150 x 3 x 5μm). A two-step mobile phase was used consisting of 0.05% (v/v) trifluoroacetic acid (TFA) in water and 0.05% (v/v) TFA in acetonitrile. UV spectra were collected between 200 and 300 nm using a PD40 photodiode array detector; the absorbance spectrum of each suspected microcystin peak was examined at a wavelength of 238 nm to determine if it had the characteristic shape of a microcystin peak thus confirming its identity as a microcystin. Microcystins have characteristic spectra attributed to the main chromophore, a diene in the Adda residue (Sangolkar *et al.* 2006). Microcystin concentrations were calculated by comparing peak area from each sample to the standard curve of MC-LR. Concentrations were then adjusted to account for percent recovery that was calculated for

each nodularin peak in each chromatogram. Microcystin concentrations were then standardized across dry weight, and volume of water filtered.

An ELISA based method of microcystin quantitation was also used for samples collected in the summer of 2007. An ELISA is an Enzyme-Linked Immunosorbent Assay in which microcystins in a test sample compete with an enzyme-labeled microcystin for a limited number of antibody binding sites in a test well. The result of the reaction is a colour change from which the total amount of microcystin can be calculated (based on a standard curve). The darker the colour the lower the concentration of microcystin in a sample and vice versa. Samples to be run on an ELISA were extracted using the same procedure as that for HPLC analysis (ASE and 75% MeOH), however nodularin was not added as an internal standard as it cross-reacts with the antibodies in the ELISA.

4.2.3 DNA extraction, PCR, and QPCR

On each sampling date 0.5-1 L of lake water (integrated water column sample) was filtered on a Whatman GF/C filter (1822-047). The same filters were used for both microcystin and molecular analyses so that the same fraction of phytoplankton (> ~ 1.7 μm in diameter) was analyzed. DNA was extracted from each sample using a procedure adapted from Hisbergues *et al.* (2003) and the methods used were the same as those presented in section 3.2.5. After extraction DNA was stored at -20°C until further analysis. All reagents used were of a high purity molecular grade.

With DNA extracted, simple PCR reactions were carried out using a primer based on the *mcy*D gene of the microcystin gene-cluster to determine the presence/absence of the toxin gene. The *mcy*D primers were designed based on sequences previously entered into GenBank. *Mcy*D is believed to be a highly conserved region of the microcystin synthetase complex and compared to some other regions of this complex it has worked well and has shown little evidence for recombination (Tanabe *et al.* 2004).

Before QPCR could be carried out all DNA extracts were first quantified, using a Molecular Probe kit (Invitrogen P7589) based on PicoGreen, an ultra sensitive fluorescent nucleic acid stain which can be used to quantify double-stranded DNA. A microplate reader was used to measure the fluorescence at 480 nm and based on the results obtained from a standard curve the concentration of DNA in each sample was calculated.

When DNA samples were quantified they were then diluted to a concentration of 10 $ng \cdot \mu l^{-1}$. All samples were then re-quantified using the same PicoGreen method to confirm the final concentration of DNA in this dilution. This was then called DNA dilution 10^0. This DNA dilution of 10^0 was then further diluted to 10^{-1}, 10^{-2}, and 10^{-3}. For QPCR analysis three dilutions for each sample were run in duplicate on a RotorGene 6000 QPCR cycler and QPCR conditions were the same as those described in section 3.2.6.

4.2.4 Statistical Analyses

A two-step statistical approach was taken to determine the effect of biotic and abiotic variables on total microcystin, *mcy*D gene copy numbers, and cyanobacteria biomass. First, a series of simple linear regressions were carried out in order to determine which independent variables had a significant relationship with the six dependent variables of total microcystin $\mu g \cdot g^{-1}$, total microcystin $\mu g \cdot L^{-1}$, *mcy*D copies$\cdot ml^{-1}$, total cyanobacteria biomass, *Anabaena* biomass, and *Microcystis* biomass. The relationship between *mcy*D gene copies and total microcystin was also examined using simple linear regression. Second, a multiple regression approach was taken to determine which combination of independent variables resulted in the best overall model explaining the greatest proportion of the variance in each of the six dependent variables. All statistics were carried out on $\log_{10}$-transformed data so that the assumptions of regression analyses were met (independence, linearity, normal distribution, homoscedasticity) and in general significance was taken at $p<0.05$. Analyses were carried out using Systat 11.0.

4.3 Results

4.3.1 Physical, Chemical, and Biological Parameters

In general both sampling seasons (2006-2007) showed similar ranges for most variables including Secchi depth, temperature, dissolved oxygen, pH, total and reactive phosphorus, total nitrogen, chlorophyll a, and carotenoids (Table 8). Temperature was greater than 20°C by late June in both years and remained above this level into September, with both years having a maximum temperature of almost 27°C. Nutrient levels were typical for a mesotrophic lake. In Constance Lake the total Kjeldahl nitrogen fraction represents essentially total nitrogen as nitrate levels are very low and close to the analytical detection limit for most of the year. In 2006, the redox potential and specific conductivity range was slightly higher, while in 2007 ammonia plus ammonium concentrations were slightly higher than in 2006.

Total algal biomass based on chlorophyll a was similar between years but, in terms of algal biovolume based estimates, biomass reached higher levels in 2006. In terms of phytoplankton biomass, while both years saw significant growth of various phytoplankton groups (Figs. 17-18) a surface cyanobacterial bloom did not arise in either 2006 or 2007. Total biomass reached higher levels in 2006 than 2007, however 2007 had a higher peak of cyanobacteria biomass than did 2006 (Table 8). The three main cyanobacteria genera present in terms of biomass were *Anabaena* (10 species), *Microcystis* (3 species), and *Aphanizomenon* (3 species) with *Anabaena* being the dominant genus both years. A table of cyanobacterial species found in the lake during both field seasons can be found in Appendix IX along with the physical and chemical data.

In both 2006 and 2007 cyanobacteria were present throughout the entire summer with a peak occurring in late August 2006 in which they comprised almost 40% of total phytoplankton biomass (Fig. 17). In 2007 cyanobacteria biomass fluctuated at low levels early in the summer, yet reached higher levels than in 2006 comprising 40-50% total phytoplankton biomass by late August- early September (Fig. 18).

Table 8. Minimum and maximum values for variables measured in Constance Lake from May-October of 2006 (n=11) and 2007 (n=16).

Variable	*2006*	*2007*
Temperature (°C)	12.2-26.9	13.4-26.7
Dissolved Oxygen ($mg \cdot L^{-1}$)	7.2-10.5	6.1-9.9
pH	7.9-8.9	7.8-9.2
Oxidative-Reductive Potential (ORP) (mV)	317-607	327-482
Specific Conductivity (SPC) ($\mu S \cdot cm^{-1}$)	330-436	304-403
Secchi (m)	0.7-2.2	1.3-2.5
Light Extinction Coefficient	0.89-1.2	0.66-1.2
Total Phosphorus (TP) ($\mu g \cdot L^{-1}$)	18.5-36	19-34
Reactive Phosphorus (RP) ($\mu g \cdot L^{-1}$)	5.5-13	2.5-11
Total Kjeldahl Nitrogen (TKN) ($\mu g \cdot L^{-1}$)	600-740	420-870
TN:TP Ratio	17.7-35.6	19.3-34.0
NH_3 & NH_4 ($\mu g \cdot L^{-1}$)	4-19	2-50
Nitrate ($mg \cdot L^{-1}$)	0-0.01	0-0.03
Chlorophyll a ($\mu g \cdot L^{-1}$)	5.5-12.2	3.0-12.7
Chlorophyll b ($\mu g \cdot L^{-1}$)	2.0-3.7	1.5-10.8
Chlorophyll c ($\mu g \cdot L^{-1}$)	4.2-10.0	2.9-7.9
Carotenoids ($\mu g \cdot L^{-1}$)	3.6-20.1	1.6-19.4
Total Algal Biomass ($mg \cdot L^{-1}$)	1.29-8.55	1.11-5.49
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	23-1230	43-2373
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	0-736	0-1899
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	0-93	0-134
***Aphanizomenon* Biomass ($\mu g \cdot L^{-1}$)**	0-304	0-142
Total Microcystin ($\mu g \cdot g^{-1}$)	0-93.8	0.02-43.9
Total Microcystin ($\mu g \cdot L^{-1}$)	0-0.37	0.001-0.12
***mcy*D copies$\cdot ml^{-1}$**	0-1862	16-683

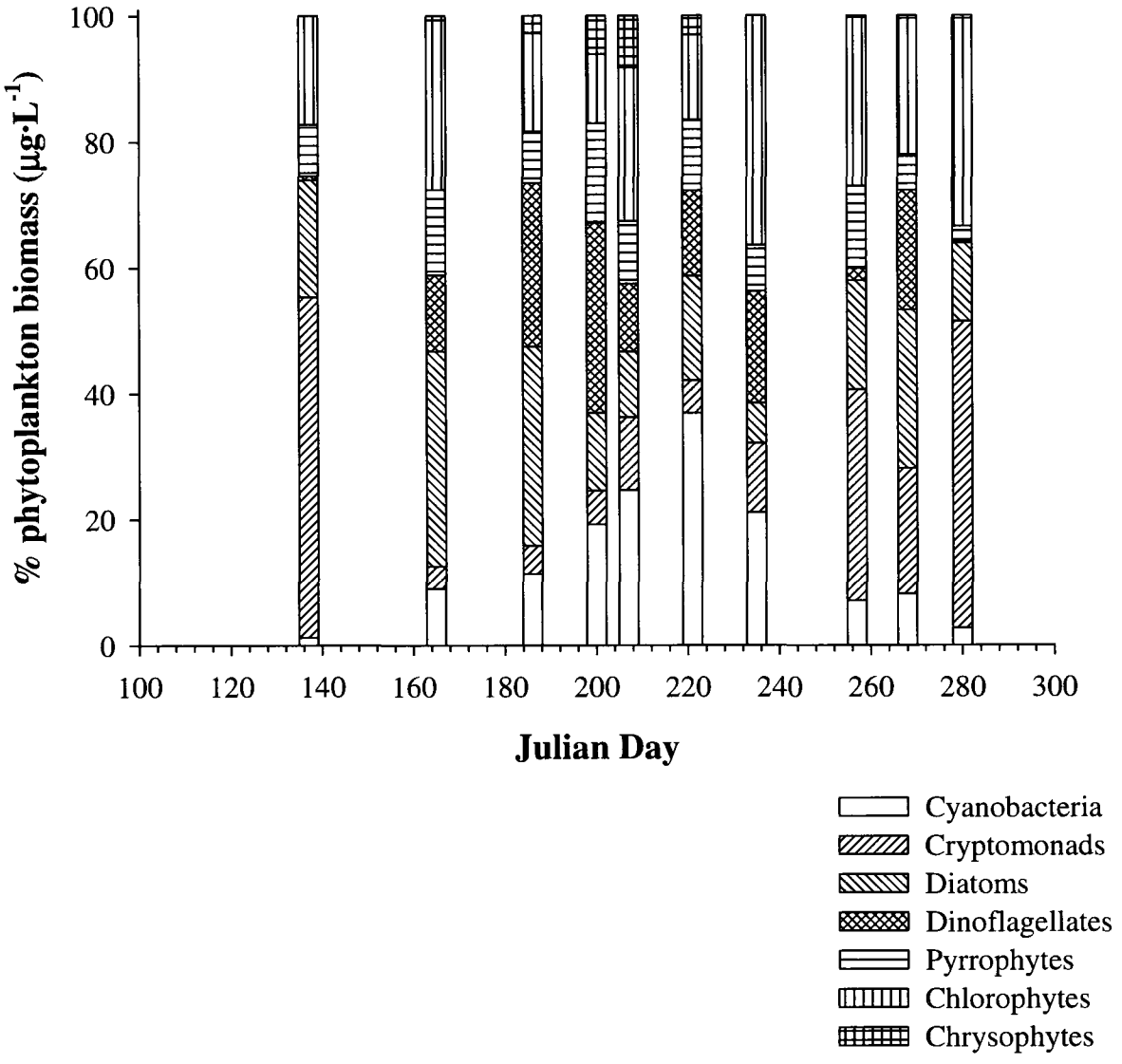


Figure 17. Percent phytoplankton biomass from May 17th to October 30th for Constance Lake 2006.

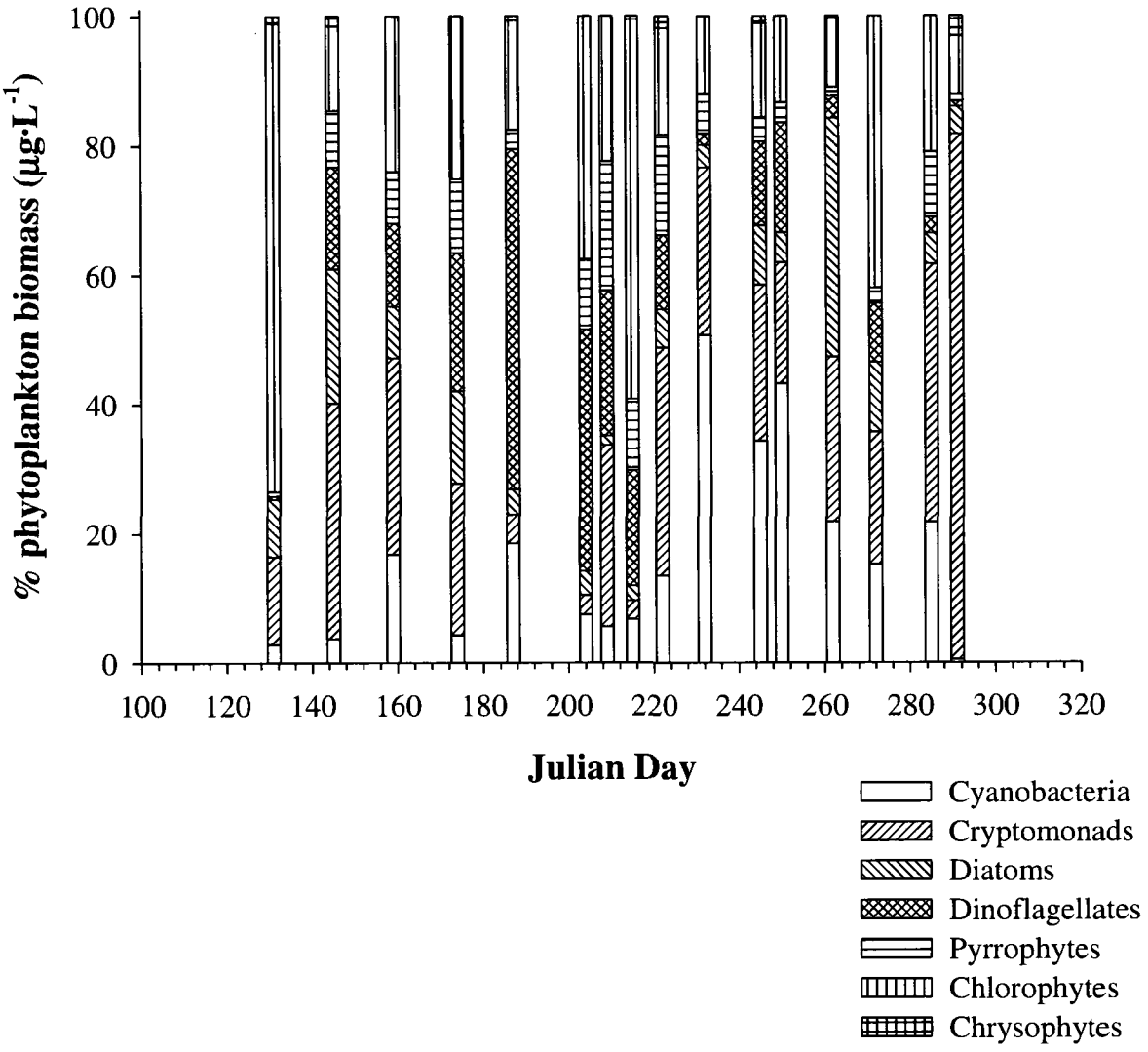


Figure 18. Percent phytoplankton biomass from May 11th to October 18th for Constance Lake 2007.

4.3.2 Microcystin Analysis

Concentrations of total microcystin were significantly higher in 2006 than 2007 (Table 8). However, in both summers, concentrations high enough to warrant concern based on the World Health Organization (WHO) or Canadian guidelines of 1 $\mu g \cdot L^{-1}$ and 1.5 $\mu g \cdot L^{-1}$ for drinking water were never reached. In 2006, microcystin levels were detected as early as May and reached a peak in early August before declining later in the summer (Fig. 19). In 2007, however, microcystin levels were lower than in 2006 throughout the entire summer with the exception of one spike in early August. Microcystin-LR and microcystin-LA were detected on several sampling dates in both summers, and the number of HPLC peaks identified as a microcystin based on retention time and characteristics of the UV spectrum ranged from 2-4 on any given sampling date.

4.3.3 QPCR Analysis of *mcy*D

The *mcy*D1 gene was detected throughout both summers, however *mcy*D gene copy numbers were much higher in 2006 than 2007 (Table 8). Figure 20 is an example of a gel from a regular PCR run with primers designed for *mcy*D1 and DNA extracted from the 11 sampling dates in 2006: the arrows indicate the sampling dates in 2006 where the 132 kb amplified fragment representing *mcy*D1 was detected. Quantitative PCR techniques allowed for the measurement of *mcy*D gene copy numbers (Fig. 21). In 2006, *mcy*D was detected throughout the summer with a peak in gene copy numbers occurring in late August. In 2007, *mcy*D gene copy numbers were much lower for most of the summer, however one peak occurred in late August-early September.

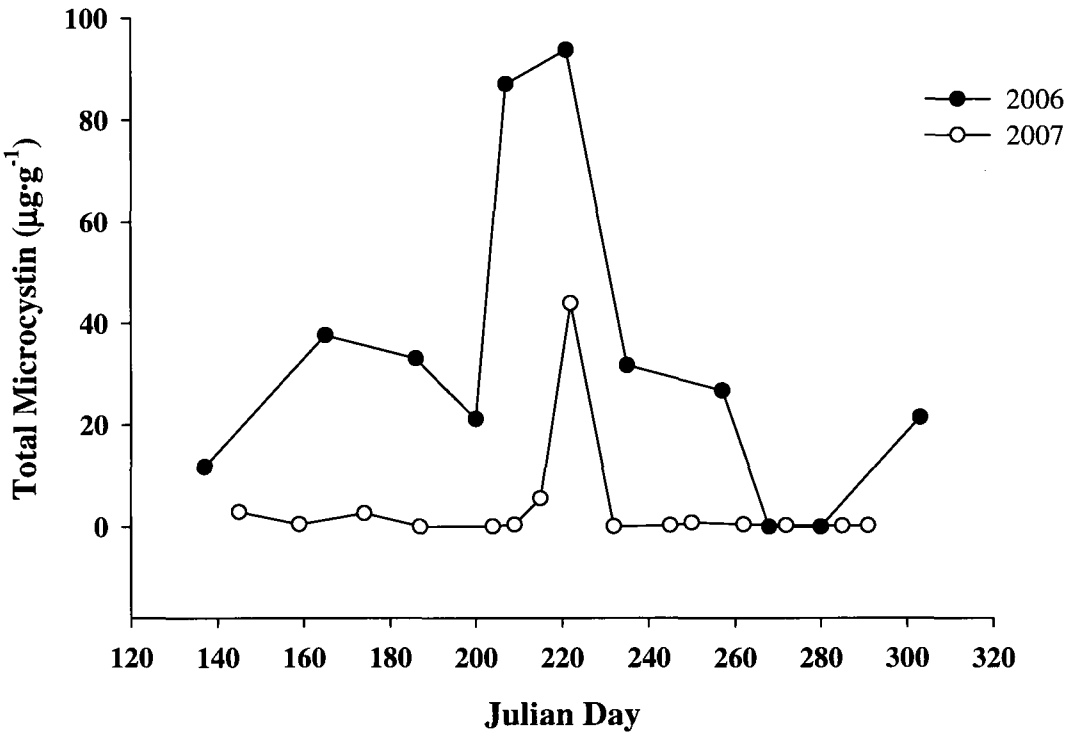
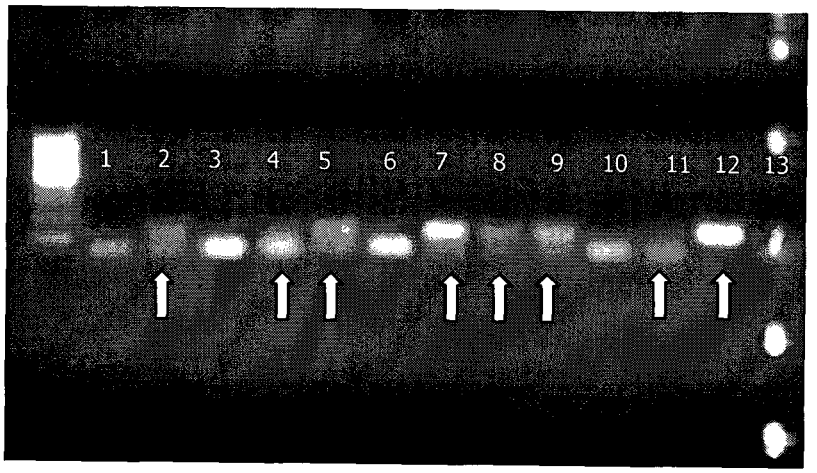


Figure 19. Total microcystin ($\mu g \cdot g^{-1}$) in Constance Lake for May to October of 2006 and 2007 as determined by HPLC (2006) and ELISA (2007). Limit of detection for HPLC was 1 ppm, and for ELISA 0.16 ppb.



1 Constance May 17
2 June 14
3 July 5
4 July 19
5 July 26
6 August 9
7 August 23
8 September 14
9 September 25
10 October 7
11 October 30
12 '+' control (UTCC 299)
13 '-' control (5mM Tris-Cl)

Figure 20. An example of a PCR gel from a reaction run with *mcy*D1 primers and DNA extracted from 11 sampling dates in Constance Lake in 2006. Arrows indicate lanes in which the *mcy*D1 gene was detected.

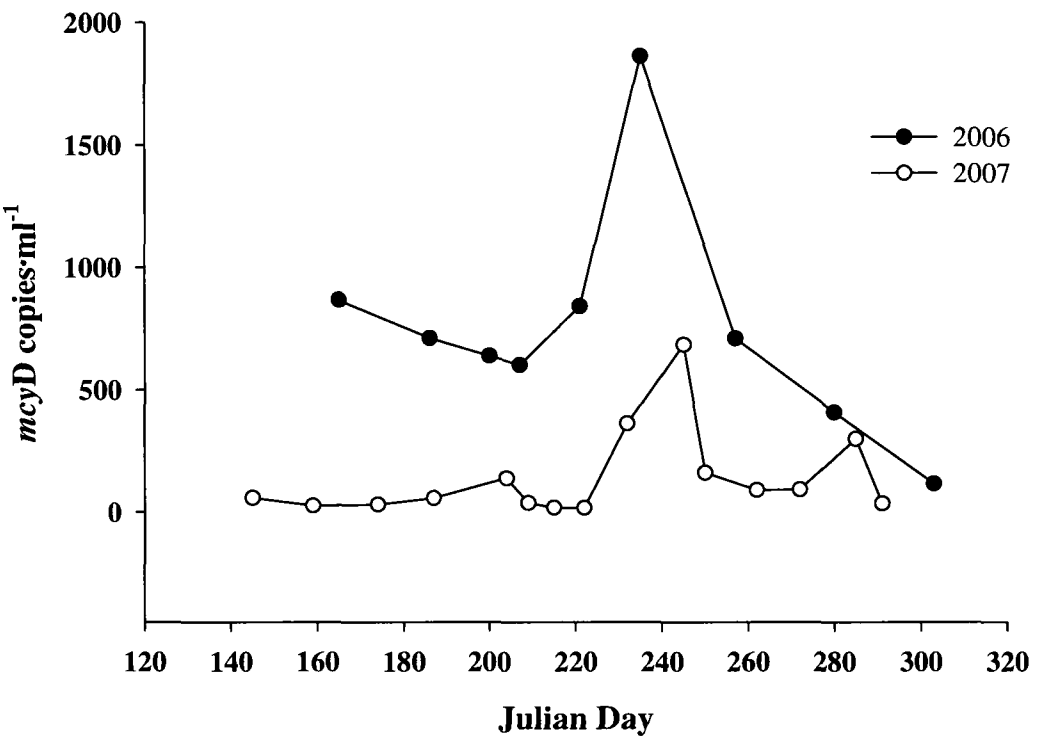


Figure 21. *mcy*D copies·ml^{-1} lake water in Constance Lake from May to October of 2006 and 2007 as determined by QPCR. QPCR detection limits were 33 copies·ml^{-1}.

4.3.4 Relationships between Total Microcystin, *mcy*D copies·ml^{-1}, and Cyanobacteria

The rationale for measuring both microcystins and gene copy number was to determine how well the microcystin gene copy number predicted the level of microcystin detected in the lake on a given sampling date. It was expected that the detection of maximum gene copy number would occur slightly before or at the same time as the detection of the maximum microcystin levels. However in both summers a peak in microcystin concentration was detected approximately 20 days before the peak in *mcy*D gene copy numbers occurred (Fig. 22).

In 2006 there was a close association between the pattern of cyanobacteria biomass throughout the summer and the resulting levels of microcystin detected in the lake (Fig. 23). This relationship was also observed with the individual cyanobacterial genera *Anabaena* and *Microcystis* where both of these genera had similar biomass trends in relationship to microcystin concentrations in the summer of 2006 (Appendix IX). In 2007, however, there was no clear association between cyanobacterial biomass and total microcystin concentrations.

The peak in cyanobacteria biomass occurred several weeks before the maximum *mcy*D gene copy number was detected in 2006 (Fig. 24). However in 2007 the pattern was reversed with the maximum *mcy*D copies·ml^{-1} detected slightly before the maximum peak in cyanobacteria biomass. This pattern was also observed when plotting *Anabaena* and *Microcystis* biomass and *mcy*D gene copy numbers over time in both 2006 and 2007 (Appendix IX).

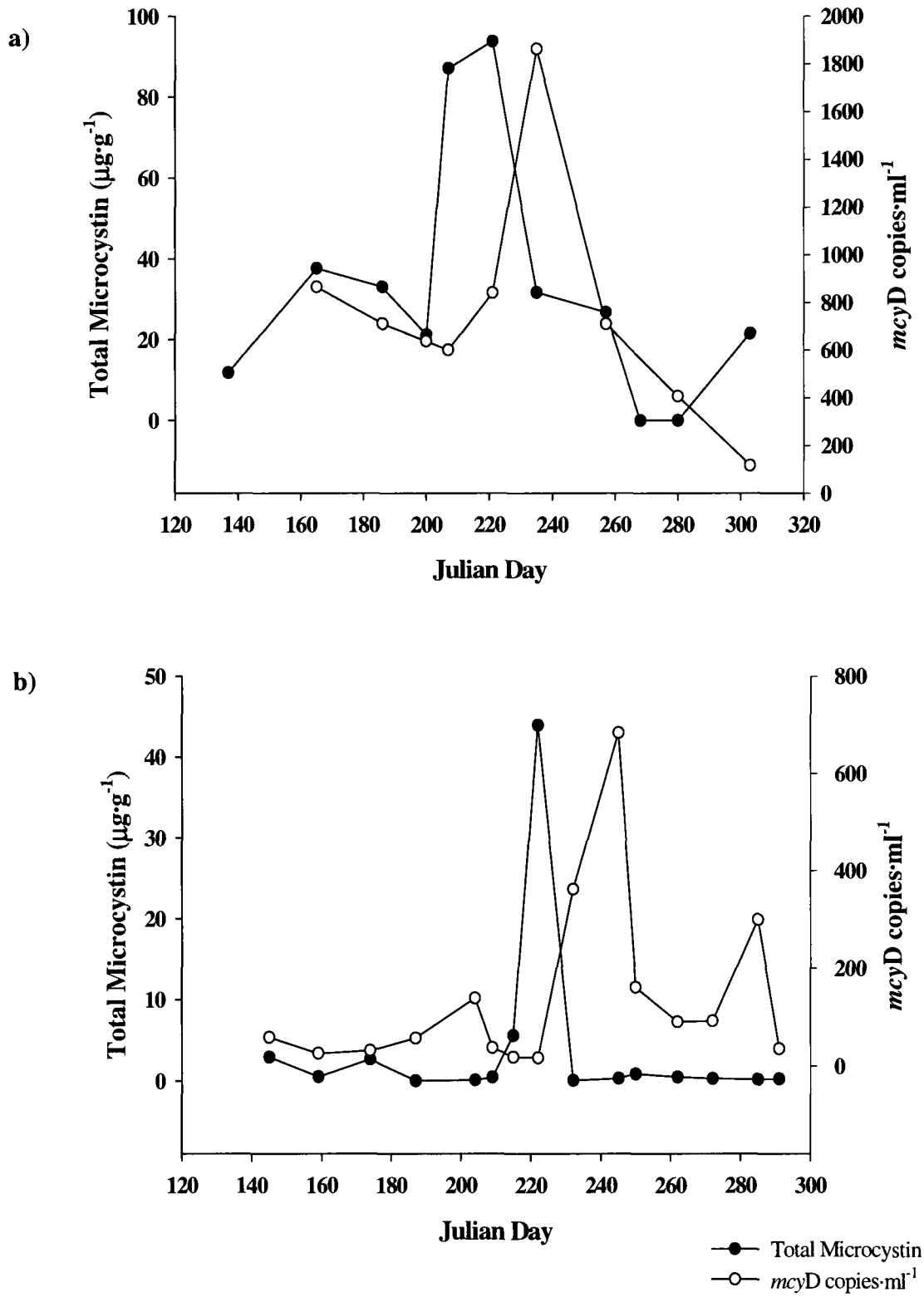


Figure 22. Total Microcystin ($\mu g \cdot g^{-1}$) and *mcy*D copies·ml^{-1} lake water for Constance Lake from May to October of **a)** 2006, and **b)** 2007.

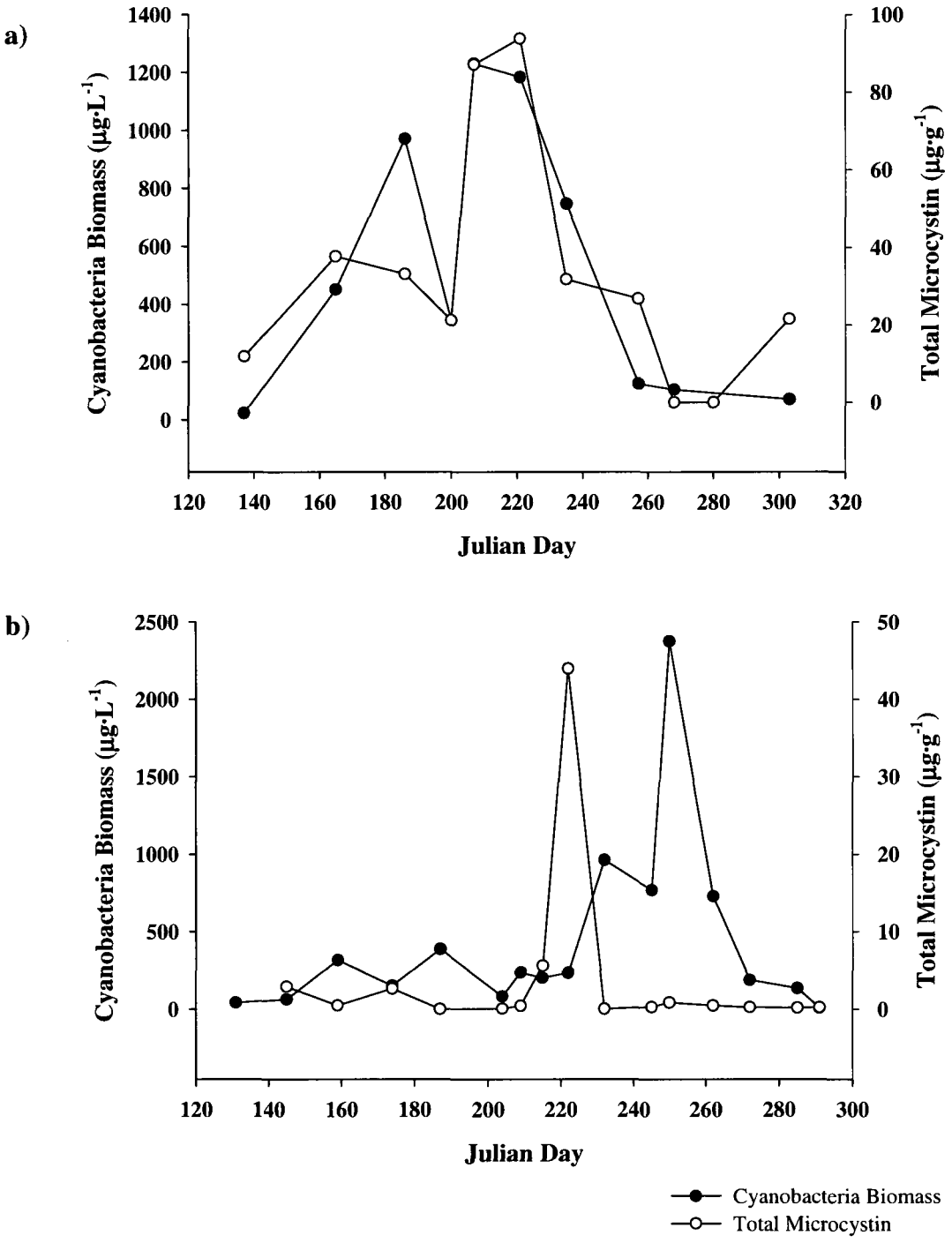


Figure 23. Cyanobacteria Biomass ($\mu g \cdot L^{-1}$) and Total Microcystin ($\mu g \cdot g^{-1}$) for Constance Lake from May to October of **a)** 2006, and **b)** 2007.

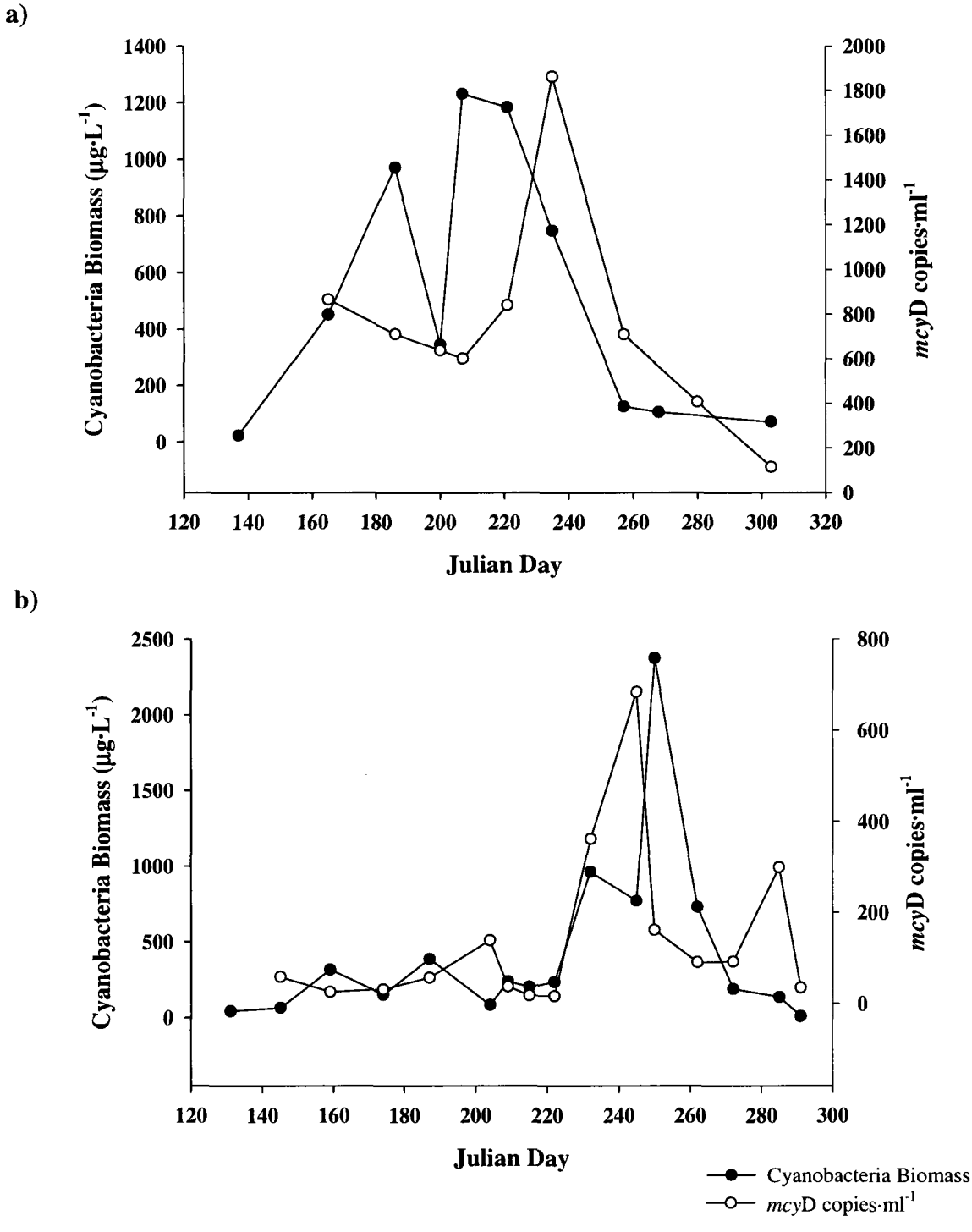


Figure 24. Cyanobacteria Biomass ($\mu g \cdot L^{-1}$) and *mcy*D copies·ml^{-1} for Constance Lake from May to October of **a)** 2006, and **b)** 2007.

The goal of the regression analysis was to determine how both abiotic and biotic factors affected microcystin production and gene copy number throughout the summers of 2006 and 2007 individually and when data from both summers were combined. It was necessary to examine not only total microcystin and *mcy*D copies·ml^{-1} as dependent variables, but cyanobacteria (including the two main toxigenic genera found in the lake in both summers- *Anabaena* and *Microcystis*) biomass as well in order to determine if abiotic factors had a significant relationship with microcystin production directly or rather if abiotic variables were affecting the biomass of cyanobacteria, including the presence of toxigenic cyanobacteria, which in turn would influence microcystin levels.

In 2006 total microcystin seston content (μg·g^{-1}) was not significantly related to any individual abiotic factor (Table 9). However it was strongly related to the biomass of cyanobacteria present in the lake over the summer. Not only was total cyanobacteria biomass significantly related to total microcystin content (μg·g^{-1}) (p=0.005), but so was *Anabaena* biomass alone (p=0.006) and to a much lesser extent *Microcystis* biomass (p=0.06). While total microcystin expressed as μg·g^{-1} had a significant relationship with cyanobacterial biomass, total microcystin expressed as μg·L^{-1} was not significantly related to any of the abiotic or biotic independent variables.

Gene copy numbers of *mcy*D in 2006 were significantly and positively related to two abiotic factors: water column temperature (p=0.001) and the light extinction coefficient (LEC) (p=0.023). Temperature and LEC themselves were strongly and positively correlated with one another (r=0.88, Appendix IX, Table 20). As with total microcystin content μg·g^{-1}, *mcy*D copies·ml^{-1} were also significantly related to total cyanobacterial biomass (p=0.006) and to a lesser extent *Anabaena* (p=0.072), and *Microcystis* (p=0.089) biomass.

Several variables were significantly related to total cyanobacterial biomass in 2006, as well as the biomass of *Anabaena* and *Microcystis* species (Table 9). Two main abiotic variables, namely temperature and pH, resulted in significant regression models with biomass. Temperature was positively correlated with pH (r=0.80, Appendix IX, Table 20).

In 2007 total microcystin concentrations were significantly lower than in 2006. Total microcystin seston content (μg·g^{-1}) did not show a significant relationship with any individual abiotic factors (Table 10). However, total microcystin content showed a negative relationship with chlorophyll c (p=0.027). As with total microcystin expressed as μg·g^{-1},

total microcystin expressed as $\mu g \cdot L^{-1}$also had a negative, significant relationship with *mcy*D copies$\cdot ml^{-1}$ (p=0.001). Gene copy numbers of *mcy*D in 2007 had significant regressions with two abiotic factors including conductivity (p=0.020) and TKN (p=0.010). Conductivity and TKN (or TN) themselves were significantly negatively correlated (r=-0.83, Appendix IX, Table 21). As in 2006, in 2007 *mcy*D copies$\cdot ml^{-1}$ were significantly related to cyanobacteria (p=0.050) and more specifically *Anabaena* (p=0.007) biomass.

While several variables were significantly related to total cyanobacterial biomass in 2006, in 2007 only temperature showed a significant relationship with cyanobacteria biomass (p=0.039). For *Anabaena* biomass in 2007, total Kjeldahl nitrogen (p=0.05) was the only environmental variable that showed a marginally significant relationship.

The third set of simple linear regressions carried out incorporated both 2006 and 2007 data combined (Table 11). Total microcystin ($\mu g \cdot g^{-1}$and $\mu g \cdot L^{-1}$) were both significantly related to two environmental variables: dissolved oxygen (negative relationship) and specific conductivity (positive relationship) (p-values <0.002). DO and SPC themselves were strongly and negatively correlated in the combined data set(r=-0.77). There was also a significant positive relationship between total microcystin ($\mu g \cdot L^{-1}$) and *mcy*D copies$\cdot ml^{-1}$ (p=0.050). For total microcystin ($\mu g \cdot g^{-1}$ or $\mu g \cdot L^{-1}$) there were no significant relationships with cyanobacterial biomass or with individual cyanobacterial genera, however a significant relationship was detected with total phytoplankton biomass (p<0.05). *Mcy*D gene copy number was also significantly and negatively related to dissolved oxygen (p<0.0001) and had a positive relationship with cyanobacteria biomass (p=0.013) for 2006-2007 combined data.

The 2006-2007 combined data, as with the separate yearly data, indicated that temperature remained a significant factor related to total cyanobacterial biomass, as well as *Anabaena* and *Microcystis* biomass (p-values <0.05). Total cyanobacterial biomass was also significantly related to TKN (p=0.005). *Anabaena* biomass for 2006-2007 was significantly related to pH (p=0.033) and ammonia and ammonium (p=0.056), while *Microcystis* biomass with light extinction (p=0.05) and reactive phosphorus (p=0.029). Positive correlations were found between temperature and pH (r=0.53) and light extinction (r=0.783), while a negative correlation was found between temperature and RP (r=-0.60, Appendix IX, Table 22).

Table 9. Results from linear regressions carried out with the dependent variables listed below and the independent variables listed in Table 8 for the summer of 2006. R^2, p-values and regression coefficients (RC) are noted (n=11).

Dependent Variable	*2006*
Total Microcystin ($\mu g \cdot g^{-1}$)	• Cyanobacteria Biomass (R^2=0.695, p=0.005, RC=0.396) • *Anabaena* Biomass (R^2=0.680, p=0.006, RC=0.212) • *Microcystis* Biomass (R^2=0.410, p=0.063, RC=0.263)
Total Microcystin ($\mu g \cdot L^{-1}$)	• *N/A*
***mcy*D copies$\cdot ml^{-1}$**	• Temperature (R^2=0.854, p=0.001, RC=1.12) • Light Extinction Coefficient (R^2=0.608, p=0.023, RC=1.52) • Cyanobacteria Biomass (R^2=0.680, p=0.006, RC=0.487) • *Anabaena* Biomass (R^2=0.442, p=0.072, RC=0.253) • *Microcystis* Biomass (R^2=0.406, p=0.089, RC=0.304)
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	• Temperature (R^2=0.394, p=0.050, RC=1.65) • pH (R^2=0.652, p=0.005, RC=32.52) • Total Biomass (R^2=0.486, p=0.025, RC=1.61) • *Anabaena* Biomass (R^2=0.929, p<0.0001, RC=0.522) • *Microcystis* Biomass (R^2=0.725, p=0.004, RC=0.738)
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	• Temperature (R^2=0.476, p=0.027, RC=3.37) • pH (R^2=0.662, p=0.004, RC=60.49) • Reactive Phosphorus (R^2=0.535, p=0.025, RC= -3.89) • Total Biomass (R^2=0.556, p=0.013, RC=3.18) • Cyanobacteria Biomass (R^2=0.929, p<0.0001, RC=1.78) • *Microcystis* Biomass (R^2=0.667, p=0.007, RC=1.31)
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	• Temperature (R^2=0.733, p=0.003, RC=2.52) • pH (R^2=0.729, p=0.003, RC=39.31) • *Anabaena* Biomass (R^2=0.667, p=0.007, RC=0.511)

Table 10. Results from linear regressions carried out with the dependent variables listed below and the independent variables listed in Table 8 for the summer of 2007. R^2, p-values and regression coefficients (RC) are noted (n=16).

Dependent Variable	*2007*
Total Microcystin ($\mu g \cdot g^{-1}$)	• Total Microcystin ($\mu g \cdot L^{-1}$) (R^2=0.610, p<0.0001, RC=0.599) • Chlorophyll c (R^2=0.303, p=0.027, RC= -1.28) • *mcy*D copies·ml^{-1}(R^2=0.221, p=0.066, RC= -0.589)
Total Microcystin ($\mu g \cdot L^{-1}$)	• Specific Conductivity (SPC) (R^2=0.268, p=0.048, RC= 14.97) • Total Microcystin ($\mu g \cdot g^{-1}$) (R^2=0.610, p<0.0001, RC=1.02) • *mcy*D copies·ml^{-1}(R^2=0.572, p=0.001, RC= -1.24)
***mcy*D copies·ml^{-1}**	• Specific Conductivity (SPC) (R^2=0.350, p=0.020, RC=-0.033) • Total Kjeldahl Nitrogen (R^2=0.385, p=0.010, RC=3.94) • N:P Ratio (R^2=0.253, p=0.047, RC=3.89) • Cyanobacteria Biomass (R^2=0.241, p=0.05, RC=0.561) • *Anabaena* Biomass (R^2=0.494, p=0.007, RC=0.558)
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	• Temperature (R^2=0.289, p=0.039, RC=3.30) • Carotenoids (R^2=0.247, p=0.050, RC=0.952) • *Anabaena* Biomass (R^2=0.857, p<0.0001, RC=0.481)
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	• Total Kjeldahl Nitrogen (R^2=0.303, p=0.050, RC=5.50) • Total Biomass (R^2=0.312, p=0.047, RC=2.39) • Cyanobacteria Biomass (R^2=0.857, p=<0.0001, RC=1.78)
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	•*N/A*

Table 11. Results from linear regressions carried out with the dependent variables listed below and the independent variables listed in Table 8 for the summers of 2006 and 2007 combined. R^2, p-values and regression coefficients (RC) are noted (n=27).

Dependent Variable	*2006-2007*
Total Microcystin ($\mu g \cdot g^{-1}$)	• Dissolved Oxygen (R^2=0.591, p<0.0001, RC=-1.67) • Specific Conductivity (SPC) (R^2=0.372, p=0.002, RC=14.53) • Total Microcystin ($\mu g \cdot L^{-1}$) (R^2=0.955, p<0.0001, RC=0.897) • Total Biomass (R^2=0.168, p=0.047, RC=1.83)
Total Microcystin ($\mu g \cdot L^{-1}$)	• Dissolved Oxygen (R^2=0.682, p<0.0001, RC= -1.96) • Specific Conductivity (SPC) (R^2=0.484, p<0.0001, RC=18.05) • Total Microcystin ($\mu g \cdot g^{-1}$) (R^2=0.955, p<0.0001, RC=1.06) • *mcy*D copies$\cdot ml^{-1}$(R^2=0.169, p=0.050, RC=0.801) • Total Biomass (R^2=0.169, p=0.046, RC=1.99)
***mcy*D copies$\cdot ml^{-1}$**	• Dissolved Oxygen (R^2=0.529, p<0.0001, RC= -0.850) • Carotenoids (R^2=0.212, p=0.027, RC=0.923) • Cyanobacteria Biomass (R^2=0.259, p=0.013, RC=0.679)
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	• Temperature (R^2=0.163, p=0.05, RC=1.31) • Total Kjeldahl Nitrogen (R^2=0.304, p=0.005, RC=3.29) • Total Biomass (R^2=0.295, p=0.004, RC=1.26) • *Anabaena* Biomass (R^2=0.876, p<0.0001, RC=0.508) • *Aphanizomenon* Biomass (R^2=0.325, p=0.007, RC=0.412)
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	• Temperature (R^2=0.306, p=0.009, RC=3.10) • pH (R^2=0.218, p=0.033, RC=27.17) • NH_3& NH_4 (R^2=0.170, p=0.056, RC= -0.640) • Total Biomass (R^2=0.350, p=0.004, RC=2.31) • Cyanobacteria Biomass (R^2=0.876, p<0.0001, RC=1.72) • *Aphanizomenon* Biomass (R^2=0.252, p=0.024, RC=0.594)
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	• Temperature (R^2=0.490, p=0.002, RC=2.58) • Light Extinction Coefficient (R^2=0.282, p=0.050, RC=2.22) • Reactive Phosphorus (R^2=0.297, p=0.029, RC= -1.73)

4.35 Multiple Regression Analysis

In 2006 for total microcystin seston content ($\mu g \cdot g^{-1}$) there were no significant multiple regression models that included abiotic variables alone. However, when *Anabaena* biomass and *mcy*D copies$\cdot ml^{-1}$were included a significant model resulted which incorporated light extinction and total phosphorus (Table 12). *Anabaena* biomass was by far the most significant contributor to this model and it was also a significant variable in the simple linear regression analysis with total microcystin ($\mu g \cdot g^{-1}$) (Table 9). For total microcystin ($\mu g \cdot L^{-1}$) only one significant model resulted, which included the environmental variables of dissolved oxygen, temperature, and total Kjeldahl nitrogen (TKN) (R^2=0.908, p=0.015).

Two significant regression models were obtained for *mcy*D copies$\cdot ml^{-1}$with the 2006 data. The first includes three environmental variables: temperature, light extinction and redox, while the second model includes *Anabaena* biomass, light extinction and redox potential. There was a high degree of collinearity between temperature and *Anabaena* biomass (Table 9): temperature was significantly related to *Anabaena* biomass on its own and both individually were significantly related to *mcy*D copies$\cdot ml^{-1}$. Temperature may significantly affect *Anabaena* biomass which in turn could increase the *mcy*D gene copies.

With respect to cyanobacteria biomass in 2006 there were no significant models that explained total cyanobacteria biomass. However, significant models were obtained for both *Anabaena* (with DO and TKN) and *Microcystis* biomass (with temperature, light extinction, and TP).

In 2007, for total microcystin ($\mu g \cdot g^{-1}$) only one quite marginally significant model resulted which contained abiotic variables alone (R^2=0.458, p=0.071) (Table 13). For total microcystin as $\mu g \cdot L^{-1}$, one model was highly significant and included the variables of conductivity, light extinction, DO, and *mcy*D copies$\cdot ml^{-1}$(R^2=0.887, p=0.001). In either case including a biotic parameter such as cyanobacterial biomass did not improve the resulting model. With respect to *mcy*D copies$\cdot ml^{-1}$ in 2007, there was no significant model that included abiotic factors alone and the best resulting model included *Microcystis* and *Anabaena* biomass (R^2=0.878, p=0.015) as predictors. For 2007, there were no significant models that explained *Microcystis* biomass, however both total cyanobacteria and *Anabaena* biomass led to models that included reactive phosphorus and light extinction. Reactive phosphorus was a significant variable in simple linear regression analysis for *Anabaena*

biomass, while light extinction was not found to be significant on its own for either cyanobacteria or *Anabaena* biomass in 2007 (Table 10).

For both years combined multiple regression results differed from those obtained for each individual year (Table 14). Two significant models resulted for total microcystin content ($\mu g \cdot g^{-1}$). The first model contained only the abiotic variables DO, pH, and TP (R^2=0.688, p<0.0001). In the second model, when *mcy*D copies·$ml^{-1}$was included, TP was no longer significant and the model included dissolved oxygen, pH, temperature and *mcy*D copies·ml^{-1}(R^2=0.757, p<0.0001). Dissolved oxygen was the most important variable in both regressions in terms of explaining the most variance, and overall the second model explained more of the variance in total microcystin ($\mu g \cdot g^{-1}$). For total microcystin ($\mu g \cdot L^{-1}$) two significant models also resulted. The first model included DO, pH, and TP (R^2=0.706, p<0.0001), while the second model included DO, pH, conductivity, light extinction, TKN, TP, and *mcy*D copies·ml^{-1} (R^2=0.898, p<0.0001). While the second model explained more variance it included four more variables than the first making interpretation more difficult. As with total microcystin content ($\mu g \cdot g^{-1}$), for total microcystin concentration ($\mu g \cdot L^{-1}$) dissolved oxygen was the most important variable in both models in terms of explaining the most variance. Dissolved oxygen was a significant variable in simple linear regressions for total microcystin (both $\mu g \cdot g^{-1}$and $\mu g \cdot L^{-1}$) (Table 11).

One significant multiple regression model was produced for *mcy*D copies·ml^{-1} for Constance Lake 2006-2007 data. The model produced included abiotic factors alone- DO, TKN, and NH_3NH_4 (R^2=0.713, p<0.0001). This differed from the multiple regression results for 2006 (Table 12) and 2007 (Table 13) where *Anabaena* biomass was the dominant variable in terms of explaining the most variation in *mcy*D copies·ml^{-1}.

The effect of the abiotic variables on cyanobacteria biomass was also examined using multiple regressions for Constance Lake 2006-2007. Two different models were produced for total cyanobacterial biomass. The first model contained temperature, total Kjeldahl nitrogen, and conductivity (R^2=0.600, p=<0.0001), while the second contained temperature, TKN, and ammonia+ammonium (R^2=0.603, p<0.001). The same situation also arose for *Anabaena* biomass with two resulting comparable models: the first contains temperature and TKN (R^2=0.426, p=0.009), while the second contains temperature and ammonia and ammonium (R^2=0.423, p=0.007). TKN includes ammonia and ammonium so these models

likely represent the same relationship. Only one model was produced for *Microcystis* biomass which included temperature, light extinction, TP, and TKN (R^2=0.923, p=<0.0001). For all three biomass variables temperature was a key factor in all the regression models. Temperature was also a significant variable in predicting cyanobacteria, *Anabaena*, and *Microcystis* biomass in simple linear regression analyses for 2006, 2007, and for all the data combined.

Table 12. Multiple regression results for significant models predicting the six dependent variables listed below for Constance Lake 2006. For Total Microcystin ($\mu g \cdot g^{-1}$and $\mu g \cdot L^{-1}$) and *mcy*D copies$\cdot ml^{-1}$ both abiotic and biotic independent variables were included, while Cyanobacteria, *Anabaena*, and *Microcystis* biomass were regressed with abiotic independent variables. R^2, p-values, constants, and regression coefficients are noted (n=11).

Dependent Variable	*2006*
Total Microcystin ($\mu g \cdot g^{-1}$)	• Total Microcystin= -1.55 + 0.38(*Anabaena* Biomass) – 0.97(LEC) – 2.16(TP) – 0.372(*mcy*D) (R^2=0.983, p=0.033)
Total Microcystin ($\mu g \cdot L^{-1}$)	• Total Microcystin= 7.03 - 4.44(DO) - 1.77(Temperature) + 9.27(TKN) (R^2=0.908, p=0.015)
***mcy*D copies$\cdot ml^{-1}$**	• *mcy*D copies$\cdot ml^{-1}$ = -2.43 + 1.77(Temperature) – 0.62(LEC) + 1.15(ORP) R^2=0.853, p=0.038)
	• *mcy*D copies$\cdot ml^{-1}$ = -2.84 + 0.25(*Anabaena* Biomass) + 0.89(LEC) + 2.02(ORP) R^2=0.922, p=0.036)
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	• *N/A*
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	• *Anabaena* Biomass= 12.552 – 8.12(DO) + 18.39(TKN) (R^2=0.845, p=0.004)
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	• *Microcystis* Biomass= -1.58 + 5.56(Temperature) - 4.18(LEC) + 2.85(TP) (R^2=0.986, p=0.003)

Table 13. Multiple regression results for significant models predicting the six dependent variables listed below for Constance Lake 2007. For Total Microcystin ($\mu g \cdot g^{-1}$and $\mu g \cdot L^{-1}$) and *mcy*D copies$\cdot ml^{-1}$ both abiotic and biotic independent variables were included, while Cyanobacteria, *Anabaena*, and *Microcystis* biomass were regressed with abiotic independent variables. R^2, p-values, constants, and regression coefficients are noted (n=16).

Dependent Variable	*2007*
Total Microcystin ($\mu g \cdot g^{-1}$)	• Total Microcystin= -7.35 + 5.58(Temperature) – 38.17(pH) + SPC(13.92)(R^2=0.458, p=0.071)
Total Microcystin ($\mu g \cdot L^{-1}$)	• Total Microcystin = -50.48 + 15.87(SPC) + 7.2(LEC) + 4.79(DO) – 0.689(*mcy*D)(R^2=0.887, p=0.001)
***mcy*D copies$\cdot ml^{-1}$**	• *mcy*D copies$\cdot ml^{-1}$ = 0.027+ 0.51(*Anabaena* Biomass) + 0.58(*Microcystis* Biomass) (R^2=0.878, p=0.015)
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	• Cyanobacteria Biomass= 6.84 + 3.33(LEC) + 1.89(RP) (R^2=0.639, p=0.006)
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	• *Anabaena* Biomass= 10.701 + 6.77(LEC) + 3.82(RP) (R^2=0.484, p=0.050)
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	• *N/A*

Table 14. Multiple regression results for significant models predicting the six dependent variables listed below for Constance Lake 2006-2007. For Total Microcystin ($\mu g \cdot g^{-1}$and $\mu g \cdot L^{-1}$) and *mcy*D copies·ml^{-1} both abiotic and biotic independent variables were included, while Cyanobacteria, *Anabaena*, and *Microcystis* biomass were regressed with abiotic independent variables. R^2, p-values, constants, and regression coefficients are noted (n=27).

Dependent Variable	*2006-2007*
Total Microcystin ($\mu g \cdot g^{-1}$)	• Total Microcystin= 17.59 – 2.42(DO) – 5.58 (pH) – 24.02(TP) (R^2=0.688, p<0.0001)
	• Total Microcystin= 31.35 – 2.88(DO) – 29.79(pH) + 2.56(Temperature) – 0.944(*mcy*D) (R^2=0.757, p<0.0001)
Total Microcystin ($\mu g \cdot L^{-1}$)	•Total Microcystin= 17.73 – 2.59(DO) – 25.33(pH) – 4.82(TP) (R^2=0.706, p<0.0001)
	• Total Microcystin= -15.34 - 1.98(DO) – 41.54(pH) + 19.39(SPC) + 3.07(LEC) + 6.11(TKN) – 5.43(TP) – 0.74(*mcy*D) (R^2=0.898, p<0.0001)
***mcy*D copies·ml^{-1}**	• *mcy*D copies·ml^{-1} = 3.31 – 0.75(DO) + 3.49(TKN) – 0.375(NH_3 & NH_4) (R^2=0.713, p<0.0001)
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	• Cyanobacteria Biomass= -6.69 + 2.63(Temperature) + 1.44(TKN) + 3.10(SPC) (R^2=0.600, p<0.0001)
	• Cyanobacteria Biomass= 0.901 + 1.37(Temperature) + 4.29(TKN) – 0.298(NH_3 & NH_4) (R^2=0.603, p<0.0001)
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	• *Anabaena* Biomass= -1.05 + 2.99(Temperature) + 4.89(TKN) (R^2=0.426, p=0.009)
	• *Anabaena* Biomass= -2.79 + 2.86(Temperature) – 0.54(NH_3 & NH_4) (R^2=0.423, p=0.007)
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	• *Microcystis* Biomass= -1.77 + 5.30(Temperature) – 3.74(LEC) + 2.71(TP) – 1.75(TKN) (R^2=0.923, p<0.0001)

4.4 Discussion

4.4.1 Cyanobacterial Growth in Constance Lake 2006-2007

In both summers temperature, nutrient concentrations, and light levels indicated that conditions were sufficient to support diverse phytoplankton growth, including the development of cyanobacterial populations in Constance Lake. During both summers phosphorus levels were consistently greater than 20 $\mu g \cdot L^{-1}$ indicating mesotrophic nutrient levels. The total nitrogen to phosphorus ratios in the lake suggest phosphorus rather than nitrogen limitation of algal growth, although the shallow nature of the lake would tend to promote high rates of denitrification. According to Downing *et al.* (2001), the risk of a cyanobacterial bloom increases significantly in lakes with phosphorus levels greater than 10 $\mu g \cdot L^{-1}$. Constance Lake lies within this threshold or change point. Secchi depth readings along with the shallow depth indicate that light was penetrating to very near or to the bottom of Constance Lake throughout both years such that light was not a limiting factor for phytoplankton growth. While conditions were adequate for cyanobacterial growth, and while cyanobacteria were present in both field seasons, in neither year did a surface cyanobacterial bloom occur. Several potentially toxigenic species were present in the lake in both years including those from the genera of *Microcystis, Anabaena, Aphanizomenon*, and *Cylindrospermopsis* (Appendix IX, Table 18).

Overall there were significant differences in terms of the detection of microcystins and of the *mcy*D gene of toxigenic strains between both sampling years which were not clearly explained by differences in cyanobacterial abundances between field seasons. The following discussion first examines microcystin concentrations in relation to both the biotic and abiotic variables measured in the lake over both field seasons, followed by a similar examination of *mcy*D gene copy numbers.

4.4.2 Relationship between microcystin concentrations and environmental variables across years in Constance Lake

Overall, both total microcystin and *mcy*D gene copy numbers were greater in 2006 than 2007. This is interesting considering there were no major differences in terms of nutrient or light availability between sampling years. Statistical comparisons of environmental parameters such as water temperature, dissolved oxygen, pH, Secchi depth,

total phosphorus, and total nitrogen revealed no significant differences between sampling years which might have explained the presence of more toxigenic genotypes and higher microcystin concentrations in 2006 than in 2007. This pattern has also been found in a few studies where consecutive field seasons at a study lake with similar limnological characteristics between years have resulted in very different patterns in microcystin concentrations. Izydorczyk *et al.* (2008) found significantly lower microcystin concentrations in the Sulejow Reservoir, Poland, in the summer of 2003 than in 2004, this despite having similar levels of cyanobacterial biomass in both summers. Also, Jacoby *et al.* (2000) found a pronounced and prolonged bloom of toxigenic *Microcystis aeruginosa* in their study lake in 1994 but not in 1995. The year of the bloom had higher levels of TP, decreased water transparency, high water column stability, and high surface water temperatures; however microcystin concentrations were unrelated to environmental factors and *M. aeruginosa* biomass. In Constance Lake there was higher total phytoplankton biomass and higher microcystin concentrations in 2006 than in 2007, however in 2007 cyanobacteria biomass eventually reached levels significantly higher than in 2006, including the biomass of several potentially toxigenic genera such as *Anabaena* and *Microcystis.*

In Constance Lake simple linear regression analyses showed that the abiotic variables measured (see Table 8) were not strongly related to total microcystins (seston content or concentration) in either field season (Tables 9-11). In 2006 total microcystin was positively and significantly related to total cyanobacteria, *Anabaena* and *Microcystis* biomass (Table 9). In 2007, total microcystin had a significant negative relationship with *mcy*D copies·ml^{-1} and a positive relationship with conductivity (Table 10), however overall there was little to no microcystin detected during most of the sampling season with the exception of one spike in early August. When the data from both field seasons were combined the regression results were quite different than when examined individually indicating that models derived from a single year's worth of data may not be useful in predicting concentrations in other years. For the 2006-2007 combined data total microcystin concentration exhibited a negative relationship with dissolved oxygen and a positive significant relationship with conductivity and *mcy*D copies·ml^{-1} indicating the importance of the presence of toxigenic genotypes in predicting total microcystin concentrations.

Several studies of lakes around the world have found significant relationships between microcystin concentrations and a wide range of parameters measured. However, there does not appear to be any general consensus in terms of which environmental variables influence microcystin production. For example, in one study low water residence time and high temperature were strongly correlated with the seasonal variation in total concentration of microcystin (Lehman *et al.* 2008), while in another it was nitrogen loading which enhanced both cyanobacterial growth and microcystin concentrations (Gobler *et al.* 2007). Microcystin concentrations may also have a significant relationship with chlorophyll a (Vézie *et al.* 1998), total phosphorus (Fahnenstiel *et al.* 2008), and total nitrogen (Graham *et al.* 2006).

Overall, based on simple linear regression analyses, the environmental variables measured in Constance Lake were not strong predictors of total microcystin ($\mu g \cdot g^{-1}$or $\mu g \cdot L^{1}$)in both field seasons, while biotic variables such as total cyanobacteria biomass, or biomass of species from the genera *Anabaena* and to a lesser extent *Microcystis* showed stronger relationships. In general biotic variables such as cyanobacterial biomass tended to overshadow any effect of environmental variables which may be a common pattern based on the present study and those published to date. For example, Albay *et al.* (2005) studied a lagoon in Istanbul, Turkey where increases in microcystin concentration were related to environmental variables including temperature, high concentrations of dissolved nutrients and high light intensity. However, there was also a strong positive correlation between total microcystin and *Microcystis* biomass and the same environmental variables were also strongly correlated with *Microcystis* biomass. The most likely scenario was that these environmental variables were affecting the dynamics of toxigenic cyanobacteria which were then determining the microcystin concentrations observed. For example, Fahnenstiel *et al.* (2008) found that *Microcystis aeruginosa* abundance explained over 50% of the variation in microcystin concentrations in Lake Huron, and in turn *M. aeruginosa* abundance was strongly correlated with total phosphorus. Similarly, Kotak *et al.* (1995, 2000) were able to explain a similar amount of variation for the same reason in a number of Canadian prairie lakes. Therefore, in many cases the biomass of toxigenic cyanobacteria remains the best predictor of microcystin concentrations.

While the results from the present study concur with those described above, there are also cases in which the relationships between cyanobacterial biomass and toxin concentrations are not as consistent. Ozawa *et al.* (2005) studied Lake Biwa, Japan, for three field seasons. As in the previously mentioned studies, microcystin concentrations were mostly a result of variation in the relative amount of *Microcystis aeruginosa.* However, the changes in microcystin concentration were not always consistent with the changes in *Microcystis* cell density. Rogalus & Watzin (2008) also found a significant relationship between potentially toxic cyanobacteria cell density and the concentration of microcystin, yet only 36% of the variation in microcystin content was explained and the highest cell densities and highest microcystin concentration did not always coincide. While Arnaud *et al.* (2008) found that 49% of the variability in microcystin concentration could be explained by *Planktothrix agardhii* biomass, the highest concentration of microcystin was actually observed when *Planktothrix agardhii* biomass was the lowest. Lastly, while Yéprémian *et al.* (2007) found a weak relationship between *Planktothrix agardhii* biomass and microcystin concentrations in a one-year study of their lake, if one outlying value is removed from their data set then the relationship between biomass and microcystin concentration is no longer significant. Therefore, while the results for Constance Lake are consistent with those from several other lake systems studied in terms of the significance of cyanobacterial biomass (including the biomass of potentially toxigenic genera), this is not necessarily a consistently observed trend.

In most of the published studies only one lake is studied, occasionally as in the case with Constance Lake for more than one field season. However, even when multiple lakes have been studied in larger surveys, there are still no clear patterns in microcystin production. Kotak *et al.* (1993) studied three lakes in the Canadian prairies and while one lake showed a good correlation between *M. aeruginosa* biomass and microcystin-LR, in the other two lakes no significant relationship was found. Graham *et al.* (2004) sampled 241 lakes across a latitudinal gradient where 98% of all samples collected had detectable microcystin concentrations and 78% of the lakes were positive for microcystins. Particulate microcystin was significantly correlated with latitude, nutrients, N:P ratio, chlorophyll, Secchi depth, temperature, TN and TP. However, despite the large sample size and number of variables considered, less than 50% of the variation in microcystin values was explained

and overall the relationship between microcystin and environmental factors was not significant or consistent.

In most of these field studies a correlation or simple linear regression analysis was conducted. In the present study a multiple regression analysis was also carried out to determine if there were significant general models combining multiple variables that may better explain the variation in total microcystin concentrations than single regressions alone. For Constance Lake in 2006 the best model for total microcystin expressed as $\mu g \cdot g^{-1}$ indicated that both *Anabaena* biomass and *mcy*D copies$\cdot ml^{-1}$ were significant variables with *Anabaena* biomass being the most important. However, when total microcystin was expressed as $\mu g \cdot L^{-1}$, the environmental variables of dissolved oxygen, temperature, and total Kjeldahl nitrogen were significant terms. In 2007 the multiple regression models indicated that environmental variables were playing a stronger role, including positive terms for temperature, conductivity, and light extinction, and negative terms for pH and *mcy*Dcopies$\cdot ml^{-1}$. It is important to remember that the microcystin concentrations in 2007 were much lower than in 2006 and any conclusions regarding this year should be taken with caution. When data from both years were combined dissolved oxygen, pH, total phosphorus, and *mcy*D copies$\cdot ml^{-1}$ were the variables which best predicted total microcystin concentrations ($\mu g \cdot g^{-1}$ or $\mu g \cdot L^{-1}$). Therefore, while the simple linear regression analyses indicated that biological variables such as cyanobacterial biomass and *mcy*D copies$\cdot ml^{-1}$ were better predictors of total microcystins, the multiple regression analyses indicated that several environmental parameters may have been important as well. In these multiple regressions environmental variables may simply have been better statistical predictors and may themselves be correlated with biotic variables less statistically significant and thus eliminated in the stepwise procedure. Simple or multiple regressions do not necessarily imply causation but can be used to indicate potential causal agents that would need to be tested through experimentation. A similar approach was taken by Rolland *et al.* (2005) in a study of four lakes in Québec. As in the present study, their goal was to separate out variations in microcystin concentrations due entirely to the presence or absence of specific cyanobacterial genera known to be toxigenic from variation explained due to changes in environmental conditions. Initially microcystin concentrations were most strongly correlated with chlorophyll a, total nitrogen, and alkalinity. However, when their analyses were repeated

including not only environmental variables but also the biomass of potentially toxigenic cyanobacteria (including *Anabaena* and *Microcystis* biomass), all environmental variables became non-significant except for TN. Overall, variations in microcystin concentrations could be attributed to the abundance of the genera *Microcystis* and *Anabaena*. A multiple regression model explained 75% of the variation in microcystin concentrations (MC-LR equivalents, $\mu g \cdot L^{-1}$) based on TN and the biomass of *Microcystis* and *Anabaena* (Rolland *et al.* 2005). In Constance Lake, only 2006 provided a model somewhat consistent with the latter finding with *Anabaena* being the most important toxigenic genus.

4.43 Microcystin gene copy numbers in Constance Lake

Theoretically using molecular techniques one can follow the development of toxigenic genotypes of cyanobacteria in lakes. For example, by using molecular techniques including PCR, Saker *et al.* (2007) determined that 71% of their samples from lakes, reservoirs, and rivers in Portugal contained potentially microcystin-producing cyanobacteria; while Kurmayer *et al.* (2002) found that 73% of their samples from German lakes contained potential toxin producing cyanobacteria. By measuring a suite of physical and chemical parameters, attempts can then be made to determine under which conditions these toxigenic genotypes might be favoured. The more toxigenic genotypes detected would then predict an increase in the microcystin concentrations measured as in theory the more copies of the gene the more gene product one would expect to detect. In laboratory studies, such as the results shown in Chapter 3, a significant correlation can be observed between the gene copy numbers detected and the microcystin concentrations measured (Fig. 16). In some field studies a good correlation has also been found between toxin concentrations and the detection of the genes required for toxin production. One such study of nodularin production in the Baltic Sea showed a highly significant correlation (r=0.89) between the gene copy numbers and the concentration of nodularin (Koskenniemi *et al.* 2007).

In Constance Lake, however, there was a mismatch between the point at which toxigenic genotypes were detected and when the maximum microcystin concentration was detected. In both 2006 and 2007 the peak in microcystin concentration was observed almost three weeks before the peak in *mcy*D copies$\cdot ml^{-1}$ (Fig. 22). Some studies have found distinct patterns in terms of when toxigenic strains were detected. For example, Janse *et al.* (2004)

found dominance of non-toxigenic strains mainly later in the growing season and in surface scums while the most toxigenic strains were found earlier in June. Vaitomaa *et al.* (2003) using *mcy*E primers to detect toxigenic strains of *Microcystis* and *Anabaena*, found a positive correlation between microcystins and *mcy*E gene copy numbers in one of their study lakes yet in another study lake the highest concentration of microcystin occurred almost 2 months prior to the highest *mcy*E gene copy number. Theoretically one would expect the detection of the maximum gene copy number to occur slightly before or near the same point in time as the detection of the maximum microcystin levels as the presence of the gene most likely indicates the presence of the resulting gene product, in this case being microcystins. The strong correlation found in the Baltic Sea between gene product and gene copy number (Koskenniemi *et al.* (2007) may be an exception: the situation in the Baltic may be somewhat unique because nodularin is only produced by one species of cyanobacterium (*Nodularia*), and therefore there was little to no error in correctly identifying the gene sequence and organism responsible for nodularin production.

In 2006 *mcy*D copies·ml^{-1} were significantly related to temperature and light extinction in simple linear regression models. However, it should be noted that temperature also had a significant relationship with total cyanobacteria, *Anabaena*, and *Microcystis* biomass, therefore temperature likely affected the growth of toxigenic cyanobacteria which in turn would have affected the *mcy*D copies·ml^{-1} detected. In 2006 and 2007 *Anabaena* biomass was a significant predictor of *mcy*D copies·ml^{-1} in both simple and multiple regression models, suggesting that certain species and strains of *Anabaena* were the main microcystin producers in Constance Lake.

During the course of this study, three studies were published that also examined the effect of both biotic and abiotic variables on the presence of toxigenic genotypes. Yoshida *et al.* (2007) measured the *mcy*A gene from *Microcystis aeruginosa* in Lake Mikata, Japan, along with genes to identify all strains, whether toxigenic or not, of the same species. As in the present study, their overall goal was to determine if there were environmental factors controlling temporal changes in microcystin producing and non-producing *M. aeruginosa* populations in Lake Mikata using QPCR. They found that a *mcy*A subpopulation of the total *M. aeruginosa* population varied from 0.5-35% and that there was a positive correlation

between *mcy*A gene copy numbers and nitrate concentrations but not with variables such as temperature and phosphorus.

While the Yoshida *et al.* (2007) study did find significant effects of environmental variables on *mcy*A genotypes, other studies have found a stronger effect of cyanobacterial biomass on *mcy* gene copy numbers such as found for Constance Lake. Briand *et al.* (2008) found that the most important factor having an impact on the proportion of the *mcy*A genotypes was variation in *Planktothrix agardhii* cell density. However, only 54% of the variation in microcystin concentration was explained by the dynamics of the density of cells with the microcystin producing genotype. They concluded that there is a need for experimental studies to elucidate how microcystin production is controlled in a cyanobacterial population composed of a mixture of microcystin-producing and non-microcystin producing cells. In their study neither the density of *P. agardhii* cells nor the density of cells with the *mcy*A genotype could predict the level of toxicity correctly over two years of sampling (Briand *et al.* 2008). This can also be said for Constance Lake as the *mcy*D copies·ml^{-1} was not consistent in predicting microcystin concentrations and in turn while cyanobacterial biomass was often significant in predicting *mcy*D copies·ml^{-1}, biomass was not consistent in its relationship with microcystin concentrations. In the third study, Kardinaal *et al.* (2007) examined the succession of toxigenic and non-toxigenic genotypes of *Microcystis* and found that microcystin concentrations developed roughly parallel to the biomass of toxigenic *Microcystis* or the gene copy numbers. However, fluctuations in microcystin concentration did not completely coincide with fluctuations in biomass.
In order to better understand the dynamics of toxigenic strains and to determine which factors are affecting these strains, a number of improvements can be made to field studies. First is the importance of primer design. Primers should be designed to detect all possible toxigenic genera capable of producing microcystins. In this study the *mcy*D primers were designed to detect strains of only *Microcystis* and *Anabaena* that contained this gene and thus had the potential to produce microcystins. However, as molecular techniques continue to improve, better primers can be designed to ensure that all toxigenic genotypes are detected.

An alternative explanation for the disconnect between gene copy numbers and toxin concentrations is the occurrence of false positives in detecting toxin genes. There is always

the possibility there are 'non-toxic' strains that still retain a small portion of the microcystin synthetase gene complex which result in an amplification product during PCR reactions with various *mcy* primers even though these strains lack the complete gene complex and thus cannot produce microcystins. This would lead to an overestimation of toxigenic genotypes in the environment. This phenomenon was observed in the UTCC culture 124 where a faint PCR amplification band was observed indicating that the *mcy*D primer had been amplified (Ouellette *et al.* 2006), yet repeated chemical analyses including both HPLC and ELISA have failed to detect microcystins in this strain (Chapter 2).

Another important point that may explain the poor correlation between microcystins and gene copy number is the question of persistence of microcystins which may last upwards of two weeks following the decline of blooms (Lahti *et al.* 1997). In Constance Lake microcystins can be detected in the spring time in 2006 (Fig. 19) when toxigenic cyanobacteria are rare and gene copy number is low. Given that all data were included in regression analyses persistence of microcystins could in part explain the poor correlation with gene copy numbers.

Other techniques that may be useful to incorporate include multiplex PCR and DGGE. Multiplex PCR allows one to determine in one PCR reaction the gene copy numbers of a particular genus or species as a whole (for example *Microcystis aeruginosa*), and the gene copy numbers of toxigenic genotypes alone. In one reaction a direct measurement of the proportion of toxigenic strains can be determined. DGGE allows one to separate out bands from a PCR reaction to determine from which genus or species the amplified *mcy* gene came from (when compared to pure cultures of various strains). For Constance Lake this would indicate which of the two prominent toxigenic genera, either *Anabaena* or *Microcystis* or others, was most responsible for microcystin production.

4.44 Conclusions

Overall significantly more *mcy*D gene copies and greater microcystin concentrations were measured in Constance Lake in the summer of 2006 than 2007. The differences between the seasons could not be explained by differences in major abiotic variables. Furthermore, 2007 had similar overall algal biomass levels to 2006, including the biomass of the most common potentially toxic genera of *Anabaena* and *Microcystis,* yet the presence of

toxin-producing genotypes and microcystin concentrations were much lower. Regression analyses indicate that while biotic variables such as cyanobacteria biomass (and in particular toxigenic taxa such as *Anabaena* and *Microcystis*) may be better predictors of *mcy*D gene copy numbers for both field seasons they are not necessarily the best predictors of total microcystin. In multiple regression analyses abiotic variables were more important in predicting total microcystins for 2006, 2007, and both years combined.

The results from this 2-year study of Constance Lake are consistent with trends found in other field studies recently conducted measuring both *mcy* genes and microcystin concentrations. However, the other study lakes were much more eutrophic systems compared to Constance Lake. Constance Lake has much lower nutrient concentrations and lower levels of cyanobacteria biomass whereas other study lakes had prolonged cyanobacterial blooms and much higher levels of toxin genes and total microcystins detected. While Constance Lake did not have a bloom or even particularly high levels of cyanobacteria in either year, the analytical and molecular methods were still able to detect toxigenic genotypes and microcystins.

Perhaps the major conclusion of this study is that molecular methods may not always be useful or reliable as a means of predicting toxin concentrations in the environment. In contrast to laboratory culture conditions, microcystin concentrations in the lake were not well correlated with microcystin gene copy numbers. While molecular methods can provide an earlier indication as to the presence of potential microcystin-producers in a lake, at this point they cannot be used as a reliable surrogate for actual toxin measurements. Ultimately molecular methods may not replace the traditional chemical and taxonomic methods typically used in aquatic ecology.

Chapter 5

Growth and dominance of toxigenic cyanobacteria along a nutrient gradient in lake enclosures

Abstract

An enclosure experiment was conducted in a shallow mesotrophic lake (Constance Lake, Ontario) to determine the effects of light and nutrients on the growth of toxigenic cyanobacteria. Three replicates of four treatments were established in a 2x2 factorial design including control enclosures, shaded ones without nutrient additions, shaded with nutrient additions, and un-shaded enclosures with nutrient additions. Due to the unforeseen additional input of nutrients from Canada Geese the effect of light could not be separated from that of nutrients and each enclosure was ultimately considered a separate ecosystem. A regression analysis approach was used to examine the effects of the nutrient gradient established on toxigenic cyanobacteria. For a period of fourteen days physical, chemical, and biological variables were measured including total microcystin concentrations and *mcy*D gene copy numbers. It was predicted that those enclosures with the highest light and nutrient resources would show increases in toxigenic cyanobacteria and thus higher *mcy*D gene copy numbers and total microcystins. Under conditions of low resource availability toxigenic strains were not expected to grow as well due to the added cost of producing microcystins. Overall, there was a significant increase in nutrient concentrations, chlorophyll a, and cyanobacteria biomass (including biomass of the potentially toxigenic genera *Anabaena* and *Microcystis*) in the enclosures compared to the same variables measured in the lake throughout the experiment. However, there were large variations in microcystin concentrations and *mcy*D gene copy numbers within and between enclosures and overall no significant effects of nutrients were found on either total microcystin concentrations or on *mcy*D gene copy numbers. Total microcystin seston content ($\mu g \cdot g^{-1}$) was best predicted by temperature, *mcy*D gene copy numbers, and *Aphanizomenon* biomass, while *mcy*D copies$\cdot ml^{-1}$ were best predicted by temperature and dissolved oxygen. While *mcy*D gene copy numbers were correlated with total microcystin concentrations, gene copy number only explained a small amount of variation in the toxin levels.

5.1 Introduction

In situ experiments provide insight into the ecological relevance of laboratory studies and can help in the interpretation of field observations. In aquatic systems, several types of *in situ* experiments can be carried out ranging from the use of large ponds, mesocosms and limnocorrals, to artificial streams (Graney *et al.* 1994). While lake enclosures have been used frequently to study phytoplankton community structure, there are both advantages and disadvantages to these types of experiments. The two most important advantages are that replication of treatments is possible, and that a control treatment can be established (Kalff 2002). In traditional field studies often only one lake or ecosystem is observed therefore replication is never really be achieved (Graney *et al.* 1994). Disadvantages to *in situ* experiments may include the cost and logistics of setting up such experiments. However the major disadvantage is that not all of the elements of the ecosystem are included because of spatial and temporal considerations. Therefore an extrapolation of results to the surrounding natural ecosystem may be difficult (Kalff 2002).

Planktonic organisms, because of their small size, rapid turnover and high taxonomic diversity, are particularly well suited for in situ experimentation using enclosures. *In situ* enclosures have been used widely to study the effects of environmental variables on zebra mussels (Heath *et al.* 1995), fish removal (Tuzun & Mason 1996), TN:TP ratios (Levine & Schindler 1999, Xie *et al.* 2003), solar UV radiation (Bergeron & Vincent 1997), and silica (Egge *et al.* 1992).

While there have been numerous enclosure (limnocorrals or mesocosms) studies carried out in lakes, few have focused specifically on the production of algal toxins. One study examined the relationship between cell division rate, microcystin production rate, and the cellular content of microcystin of *Microcystis aeruginosa* in field microcosms inoculated with a pre-cultured strain of the cyanobacterium (Lyck & Christoffersen 2003). Microcosms were monitored for 17 days during which time the density of natural phytoplankton in microcosms with added *Microcystis* remained constant, whereas the density of natural phytoplankton increased continually in control microcosms. This suggested that the natural phytoplankton was being suppressed by the addition of the pre-cultured strain of *Microcystis* (Lyck & Christoffersen 2003). In another short term study at a much smaller scale (72 hours in 9 L cubitainers), the effect of light and nutrients on microcystin production

was examined (Graham *et al.* (2006). When light was limiting microcystin concentrations decreased significantly which was correlated with decreases in chlorophyll concentrations. Graham *et al.* (2006) concluded that microcystins never responded independently of chlorophyll levels. This confirms earlier results from laboratory studies indicating that environmental variables such as light and nutrients influence microcystin indirectly by influencing changes in cyanobacterial biomass (Orr & Jones 1998).

To date, no studies appear to have examined experimentally the *in situ* dynamics of toxigenic versus non-toxigenic taxa. Experimental studies at realistic scales and *in situ* are required in order to determine the factors that lead to toxigenic cyanobacterial blooms in aquatic systems. Here I describe an enclosure experiment initially designed to determine how light quantity and the addition of nutrients (phosphorus and nitrogen) affect the growth and dominance of toxigenic cyanobacteria *in situ*. These are the major environmental variables linked to cyanobacterial growth in lakes (Pick& Lean 1987). The aim was also to determine how these factors affect both the levels of *mcy*D gene copy number and total microcystin production. Twelve enclosures were established on Constance Lake in the summer of 2007: half the enclosures were shaded to reduce light availability by almost 50%, and half the enclosures were enriched with nutrients (nitrogen and phosphorus). It was expected that light intensity would have a significant effect on the dominance of toxigenic and non-toxigenic strains of cyanobacteria, specifically within the common microcystin producing genera *Microcystis* and *Anabaena* found in the lake. Under reduced light intensity, due to the cost of producing microcystins, it was expected that both *mcy*D gene copy numbers and consequently total microcystin concentrations would be lower in shaded enclosures than those exposed to full light. Nutrient additions of nitrogen and phosphorus were expected to promote the growth of cyanobacteria and when combined with optimal light conditions provide the best scenario for the growth and proliferation of toxigenic cyanobacterial strains.

5.2 Methods

An *in situ* experiment was carried out in Constance Lake (45°24'N, 75°59'W) situated in the west end of the City of Ottawa, Ontario, during late July to early August of 2007. This is when water temperatures are highest and cyanobacteria become a significant fraction of the total phytoplankton biomass (Chapter 4). Constance Lake is shallow with a maximum depth of 3.3 m, a mean depth of 2 m, and surface area of 132 ha. Based on previous total phosphorus concentrations and chlorophyll *a* measurements (Hamilton *et al.* 2005), Constance Lake can be considered mesotrophic to eutrophic. The cyanobacterium *Cylindrospermopsis raciborskii*, a subtropical and potential toxin-producing cyanobacterium, was recorded for the first time in Canada in this lake (Hamilton *et al.* 2005). Constance Lake was chosen for this study because of its high algal diversity and abundance of cyanobacteria (including potential toxin producers), and a history of sampling since 1998.

Contrasting light and nutrient regimes were established to determine their effect on phytoplankton, focusing on the growth of toxigenic cyanobacteria. Three replicates for each of the four treatments were established including Control (un-shaded, no nutrients added), Light + Nutrients (un-shaded, with nutrients added), Shaded (shaded, no nutrients added), and Shaded + Nutrients (shaded with nutrients added). Weather over the 14 days of the experiment was favourable with full sun on most days.

5.2.1 Enclosure Construction and Set-up

The individual enclosures for the experiment consisted of plastic bags supported by floating wooden frames (Fig. 25). The wooden frames were built with 2" x 6" non-pressure treated lumber and divided into four separate sub-enclosures. The dimensions of the frames were one meter in width by four meters in length. There were three replicates of each of the treatments consisting of three separate wooden frames that each contained four enclosures of one meter by one meter. The first wooden frame included enclosures 1-4, the second included enclosures 5-8, and the third included enclosures 9-12.

In each set of four enclosures one of the four treatments was established. The first enclosure was the control, the second was shaded with no nutrients added, the third was also shaded but nutrients were added, and the fourth enclosure was not shaded but nutrients were added. The enclosures were shaded using a black fiberglass mesh which reduced light

penetration by approximately half. The mesh was stapled to a wooden frame and rested on top of each shaded enclosure. The mesh frames were attached to the enclosure with hinges which allowed for easy lifting. The nutrients added consisted of phosphorus (P) and nitrogen (N). Based on the data collected for Constance Lake during the summer of 2007, it was determined that the average ratio of total nitrogen to total phosphorus was 20:1 (TN:TP ratio). The same ratio of TN:TP was maintained when adding nutrients to the enclosures. Therefore, 50 mL of KPO_4 and 50 mL of $NaNO_3$ were added to increase the nutrient concentrations in nutrient addition treatments by approximately 5 $\mu g \cdot L^{-1}$ of phosphorus and 100 $\mu g \cdot L^{-1}$ of nitrogen every three days.

The enclosures were installed in Constance Lake on July 18th, 2007. Dock floats were added to increase the buoyancy of the wooden frames and to allow the bags to ride with the waves (Fig. 25). Solar lights were placed in one corner of each enclosure in order to make the enclosures visible at night. The 6mm heavy duty clear polyethylene bags chosen for this experiment were one and a half meters in length and one meter wide. Each enclosure bag was reinforced at the bottom and on each side using Tuck Tape ®. The sides at the opening of the bags were wrapped around strapping and stapled on all four inside panels of each individual enclosure.

When the enclosure setup was complete they were placed in shallow water and each bag was rinsed with 20 liters of water. The enclosures were then towed by boat to the designated area on the lake where one anchor was tied per frame. The three enclosure frames floated naturally according to the wind direction and aligned parallel to the wind riding the waves. The enclosures were established in a low-traffic area in order to minimize disturbance (i.e. float planes landing). The enclosures were also set up where the abundance of macrophytes was minimal and the water was approximately two meters in depth. Each enclosure bag was filled with 800L of lake water using a pump (10L per minute), and manually with buckets.

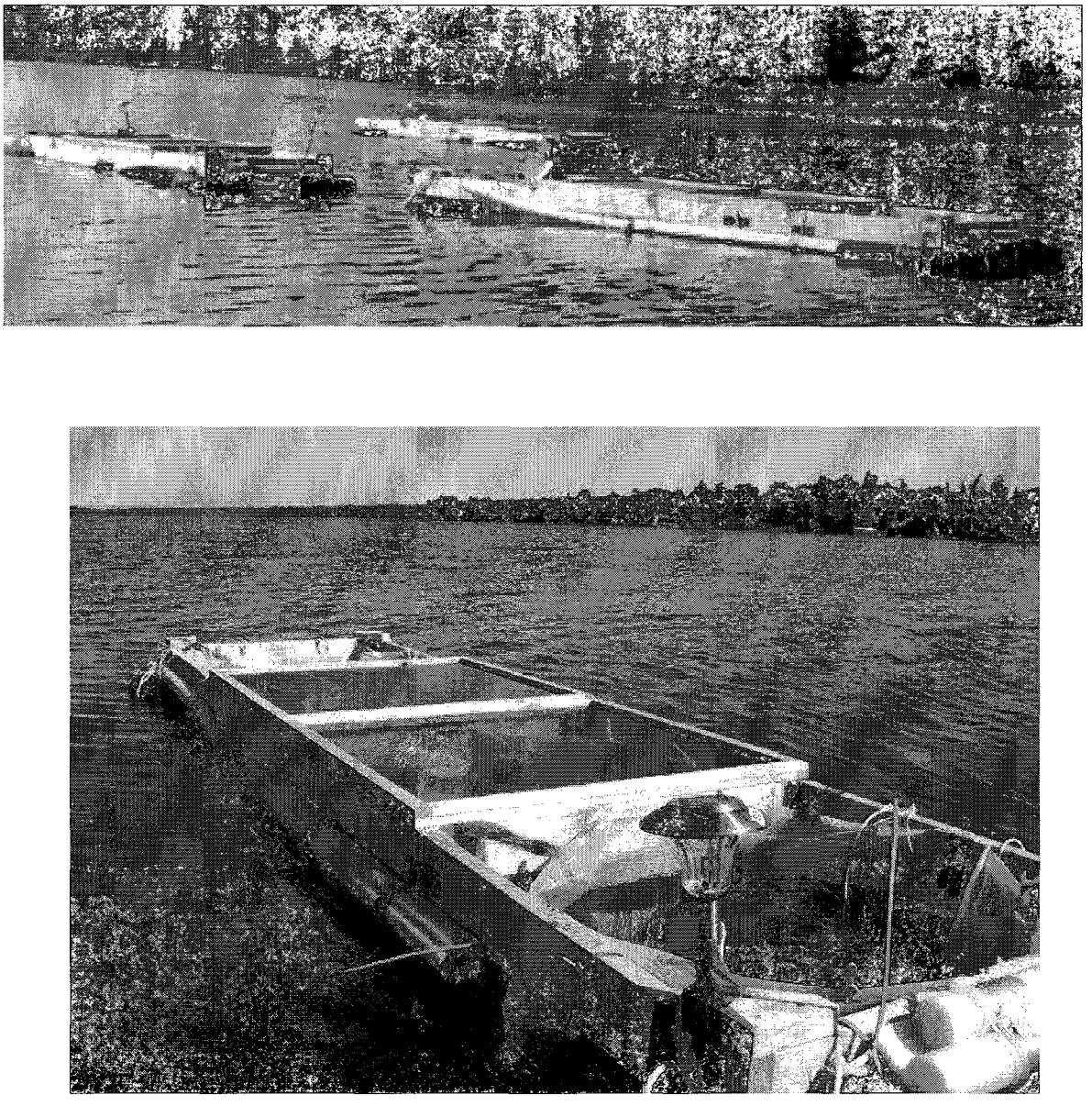


Figure 25. Enclosures at Constance Lake in the summer of 2007.
(Photos by S. LeBlanc Renaud.)

5.2.2 Sampling of Enclosures

The experiment began on July 22nd, 2007 (Day 0). On this day, water samples were collected and measurements of temperature, dissolved oxygen, pH, oxidative-reductive potential (ORP), and conductivity (SPC) were taken using a Hydrolab Minisonde Multiprobe 4a (Campbell Scientific). In addition, PAR (photosynthetic available radiation) was measured with depth using a LI-COR light meter.

Water samples were collected in the morning (between 10-11 AM) in Nalgene bottles. Two Nalgene bottles were assigned to individual enclosures in order to avoid cross contamination of the different treatments. The bottles were triple rinsed with lake water before collecting the samples. For chlorophyll analysis, 500mL of water was filtered using Whatman 934-AH 47 mm filters. Chlorophyll pigments were extracted using the standard DMSO/Acetone method (Burnison 1980) and readings analyzed with a Pye Unicam spectrophotometer. Water samples were also brought to the City of Ottawa's Robert O. Pickard Environmental Centre for nutrient analysis. Additional water was collected for microcystin and molecular analyses. Samples for phytoplankton taxonomic identification and enumeration were preserved in Lugol's on days 3 and 10. Samples were counted by Linda Ley (Canadian Museum of Nature, Ottawa) using the Utermöhl technique. A subsample of water from each enclosure was also preserved in a 10% paraformaldehyde solution to maintain, for approximately one month, the naturally occurring fluorescence in picocyanobacteria (Stockner *et al.* 2000). Picocyanobacterial abundance was examined using epifluorescence microscopy (Pick 1991) because of a previous report of a microcystin-producing strain among these bacterial size cyanobacteria (Domingos *et al.* 1999).

Following *in situ* measurements and water sampling, nutrients were added in the afternoon on day 0 (and subsequently on days 3, 7, and 10). On each of the 14 days of the experiment the individual enclosures were stirred completely at least once a day using a canoe paddle. The paddle was rinsed in the lake after each stir, once again, to avoid cross contamination of the different treatments. A sieve was also used to remove any debris (i.e. macrophytes) found on the surface of each enclosure.

5.2.3 Microcystin Analysis

Analysis for microcystins was carried out on days 0, 3, 7, 10 and 14 of the *in situ* experiment to determine how toxin levels changed between treatments of nutrients and light levels. For microcystin analysis duplicates of one litre were filtered onto a pre-ashed (500°C for 2 hours), pre-weighed Whatman GF/C filter (1822-047). Filters were oven-dried overnight at 60°C, re-weighed to determine net dry biomass, and frozen at -20°C for subsequent analysis. Before extraction of microcystins, all filters were re-hydrated with distilled water and re-frozen at -20°C. Microcystin extractions and analyses using ELISA were carried out in the same manner as in the described in sections 2.2.4 and 4.2.2.

5.2.4 DNA extraction and QPCR analysis

Water from each enclosure was also filtered on days 0, 3, 7, 10 and 14 for molecular analysis. Duplicate samples of 500 ml from each enclosure were filtered onto a Whatman GF/C filter and immediately placed in a sterile 50 ml falcon tube and frozen at -20°C pending further analysis. DNA extractions and QPCR were analyses carried out using the same techniques as described in sections 3.2.5 and 3.2.6.

5.2.5 Statistical Analyses

The experiment was set up as a 2 x 2 factorial design (the two factors being nutrients and light) through time. However, the presence of a flock of Canada Geese became problematic. The geese made nightly visits to the enclosures and used the sides and the mesh as a resting area. In so doing, they would often leave behind feces. The defecations were often found on the mesh screens, thus consequently falling into the enclosures. The mesh screens were thoroughly cleaned on a daily basis. Attempts were made to discourage the birds from aggregating on the enclosures, such as adding aluminum plates and small ropes around the enclosures. However, such attempts were either not effective or potentially dangerous for the geese; therefore extra cleaning efforts were conducted instead. Bird feces have been demonstrated to elevate nutrient concentrations in aquatic environments (Manny et al. 1994, Scherer et al. 1995). Since nutrients were added in the same ratio to each enclosure, the ones particularly impacted by geese would be those with very different TN:TP ratios than the lake (which averaged 26:1 during the experiment). Feces from Canada Geese

tend to have a TN:TP ratio of roughly 8:1 (Post *et al.* 1998). Figure 26 shows large variations in the ratios between enclosures over time compared to day 0 of the experiment. High ratios were most often observed in the control enclosures while low ratios were found particularly in the shaded enclosures. These results indicate an external influence on nutrient concentrations since the TN:TP ratio should not have changed in these enclosures as they were not manipulated for nutrients.

To test if the nutrient and light treatments were effective, t-tests with Bonferroni corrections were performed to compare treated versus non-treated enclosures for each sampling date. The results indicated that the treated enclosures were not significantly different from the non-treated enclosures ($p>0.05$) with respect to both nutrient concentrations and light penetration (based on light extinction coefficients). Screening did not significantly reduce light penetration such that the light treatments were not effective. The presence of geese furthermore meant that the effect of light could not be separated from that of nutrients. Consequently, regression analyses were used to determine the relationships between enclosure environmental conditions (in particular nutrient concentration and the nitrogen to phosphorus ratio), microcystin production, and *mcy*D gene copies·ml^{-1}.All statistics were carried out on $\log_{10}$-transformed data so that the assumptions of regression analyses were met (independence, linearity, normal distribution, homoscedasticity) and in general significance was taken at $p<0.05$. Analyses were carried out using Systat 11.0.

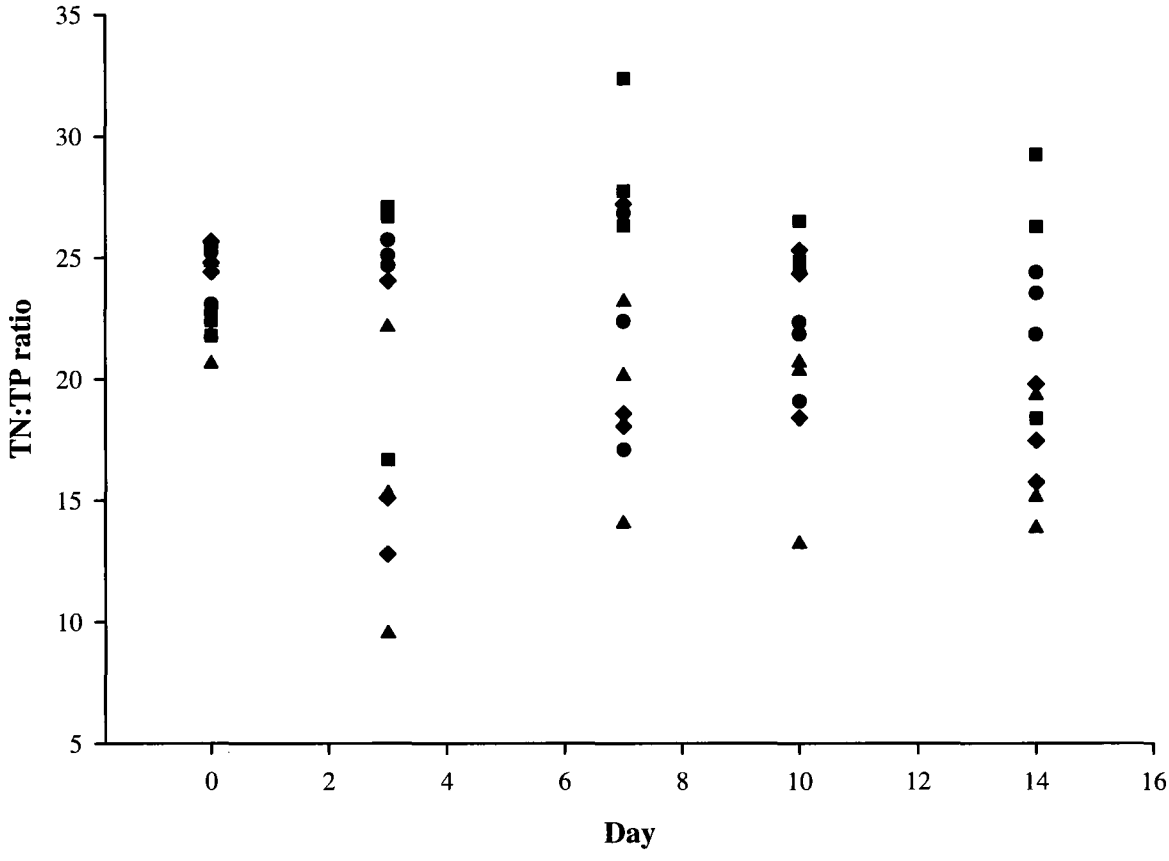


Figure 26. TN:TP ratios for all 12 enclosures on days 0, 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Circles represent control enclosures (un-shaded, no nutrients added); squares represent enclosures that are shaded with no nutrients added; triangles represent enclosures that are shaded with nutrients; and diamonds represent enclosures that are un-shaded with nutrients added.

5.3 Results

5.3.1 Physical, Chemical, and Biological Parameters

Table 15 shows the variables measured in all 12 enclosures as well as the lake itself during the same time period. Certain variables remained similar between the enclosures and lake throughout the duration of the experiment including temperature, pH, ORP, and SPC. In contrast, while temperature increased overall throughout the experiment followed by a slight decrease on day 14, all enclosures had similar temperatures on any particular day (Appendix X). Due to the input of nutrients to the enclosures by the addition of solutions of nitrogen and phosphorus in chemical additions as well as via the contribution from Canada Geese, nutrient levels in many enclosures were much greater. This large influx of nutrients thus resulted in significantly higher chlorophyll a values than the lake, along with increases in cyanobacterial biomass including the three main toxigenic genera of *Anabaena*, *Microcystis*, and *Aphanizomenon*.

As described in the methods section the nutrient additions to the selected enclosures was designed to increase the phosphorus concentration step-wise by 5 $\mu g \cdot L^{-1}$ and nitrogen concentration by 100 $\mu g \cdot L^{-1}$ in order to stimulate cyanobacterial growth. However, based on the range of TP and TKN indicated in Table 15 it is clear that nutrient inputs to certain enclosures far exceeded the levels that were added manually. If phosphorus was added to an enclosure at four time points during the experiment (Days 0, 3, 7, and 10) then in total 20 $\mu g \cdot L^{-1}$ would have been added to those treatments. Therefore, when added to the TP values present at the start of the experiment then the expected TP in the nutrient treatment enclosures would be 40-45 $\mu g \cdot L^{-1}$. However based on the range of TP values observed (Table 15, Fig. 27), the nutrient levels in many enclosures far exceeded the concentrations that should have resulted based on the inorganic nutrient inputs alone.

Table 15. Minimum and maximum values for variables measured in Constance Lake enclosures 1-12 and in the lake itself during the 14-day experiment, July 22nd- August 5th, 2007. Measurements and water samples were collected on days 0, 3, 7, 10, and 14.

Variable	*Enclosures*	*Lake*
Temperature (°C)	22.1-26.9	21.5-26.7
Dissolved Oxygen ($mg \cdot L^{-1}$)	6.9-14.1	6.8-9.9
pH	7.7-9.6	8.2-9.2
Oxidative-Reductive Potential (ORP) (mV)	320-386	328-343
Specific Conductivity (SPC) ($\mu S \cdot cm^{-1}$)	286-349	304-346
Light Extinction Coefficient (m^{-1})	0.40-1.73	0.75-1.24
Total Phosphorus (TP) ($\mu g \cdot L^{-1}$)	19-152	21-26
Reactive Phosphorus (RP) ($\mu g \cdot L^{-1}$)	3-108	2.5-7.5
Total Kjeldahl Nitrogen (TKN) ($\mu g \cdot L^{-1}$)	530-1635	540-655
NH_3 & NH_4 ($\mu g \cdot L^{-1}$)	5-669	2-44
TN:TP Ratio	9.5-32.4	22-30
Nitrate ($mg \cdot L^{-1}$)	0-0.15	n.d.
Chlorophyll *a* ($\mu g \cdot L^{-1}$)	2.2-46.5	5.5-9.8
Chlorophyll b ($\mu g \cdot L^{-1}$)	1.8-19.2	2.6-10.8
Chlorophyll c ($\mu g \cdot L^{-1}$)	0.19-9.1	5.5-6.9
Carotenoids ($\mu g \cdot L^{-1}$)	0-149.6	1.9-6.2
Total Algal Biomass ($mg \cdot L^{-1}$)	0.142-2.68	1.11-4.24
Cyanobacteria Biomass ($\mu g \cdot L^{-1}$)	4.4-1429	82-386
***Anabaena* Biomass ($\mu g \cdot L^{-1}$)**	0-112	25-92
***Microcystis* Biomass ($\mu g \cdot L^{-1}$)**	0-187	0-52
***Aphanizomenon* Biomass ($\mu g \cdot L^{-1}$)**	0-171	38-142
Total Microcystin ($\mu g \cdot g^{-1}$)	0-35.1	0.018-43.0
Total Microcystin ($\mu g \cdot L^{-1}$)	0-0.10	0-0.125
***mcy*D copies$\cdot ml^{-1}$**	8.4-1934	17-139

The manual nutrient additions were done in such a way that the nitrogen to phosphorus (TN:TP) ratio would be similar to the TN:TP ratio in the lake (approximately 20). The TN:TP ratios of all 12 enclosures were very similar at the beginning of the experiment (Fig. 26). However, as the experiment progressed and the presence of geese altered nutrient inputs, the TN:TP ratios began to vary. Other variables such as ammonia and ammonium, dissolved oxygen, light extinction coefficient, chlorophyll *a*, total microcystin, and *mcy*D copies·ml^{-1} showed considerable variation between enclosures and sampling dates.

Phytoplankton biomass responded significantly to nutrient inputs in the enclosures: the chlorophyll a increased up to 46.5 μg·L^{-1} compared to maximum of 9.8 μg·L^{-1} in the lake itself over the duration of the enclosure experiment (Table 15). On day 0 all enclosures had similar chlorophyll a values, however as the experiment progressed several enclosures had large increases in chlorophyll a values, including enclosures that were not nutrient addition treatments (Fig. 28). Not only were chlorophyll a values significantly affected by nutrients (Fig. 29), but total cyanobacterial biomass increased in the enclosures compared to the lake as well (Fig. 30). By contrast, the relationship between cyanobacterial biomass and nutrient concentrations (including total phosphorus) was not statistically significant ($p>0.05$).

Phytoplankton communities were identified for each enclosure on days 3 and 10 of the experiment (Fig. 30). Enclosures 1 and 7 were also counted on day 0 as a reference point to the starting conditions. On day 3 of the experiment the percent cyanobacterial biomass was less than 20% in all enclosures, however, by day 10 enclosures 1, 7, 8, 9, and 11, showed a noticeable increase in the contribution of cyanobacteria to the overall phytoplankton community. In general, the groups that responded positively to nutrient additions were largely Chlorophyta and diatoms. The remaining enclosures maintained a similar community structure or experienced lower proportions of cyanobacteria.

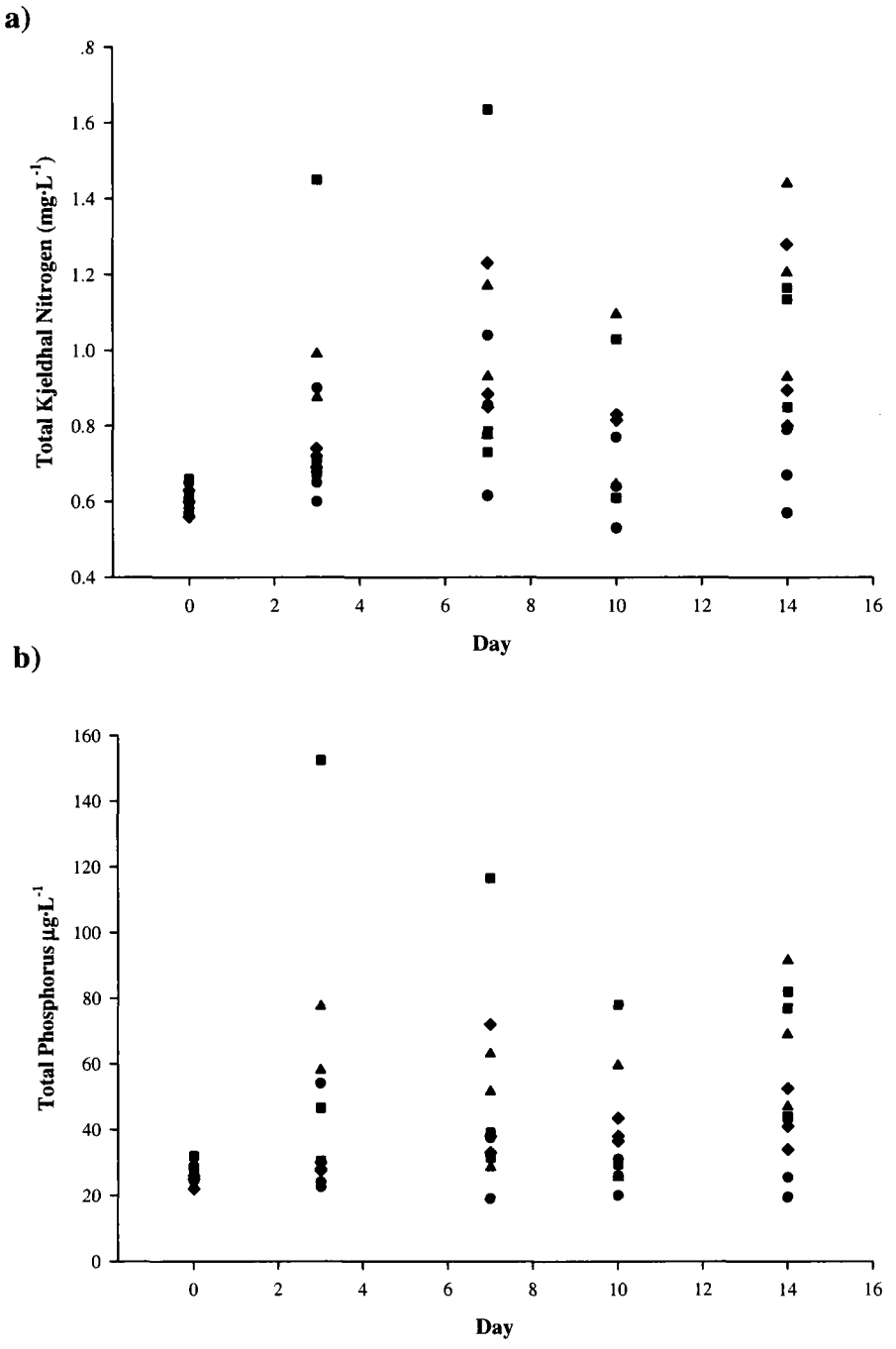


Figure 27. a) Total Kjeldahl Nitrogen, and **b)** Total Phosphorus for all 12 enclosures on days 0, 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Circles represent control enclosures (un-shaded, no nutrients added); squares represent enclosures that are shaded with no nutrients added; triangles represent enclosures that are shaded with nutrients; and diamonds represent enclosures that are un-shaded with nutrients added.

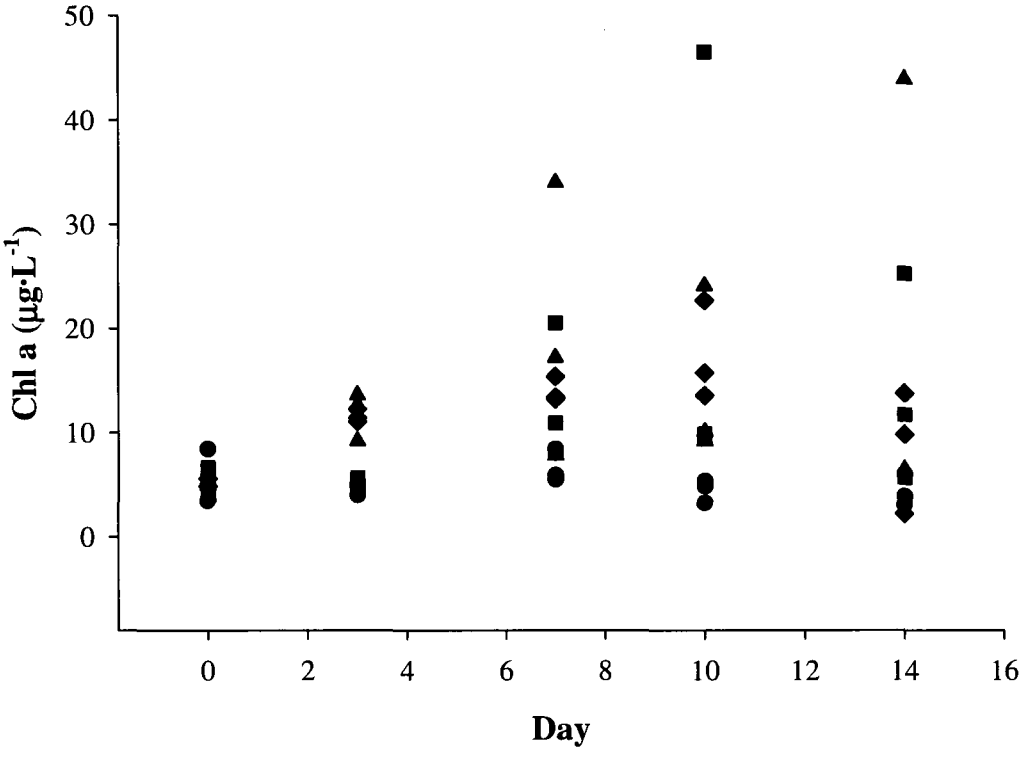


Figure 28. Chl *a* for all 12 enclosures on days 0, 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Circles represent control enclosures (un-shaded, no nutrients added); squares represent enclosures that are shaded with no nutrients added; triangles represent enclosures that are shaded with nutrients; and diamonds represent enclosures that are un-shaded with nutrients added.

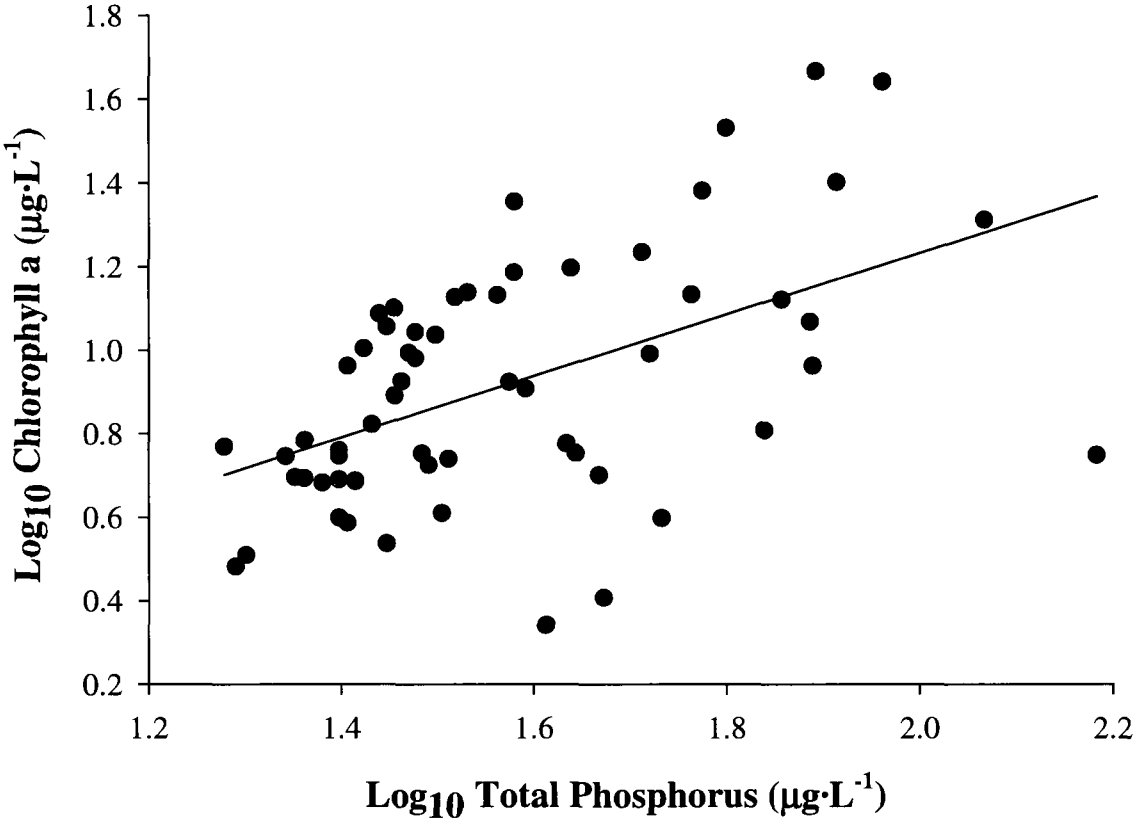


Figure 29. Log_{10}-transformed relationship between total phosphorus ($\mu g \cdot L^{-1}$) and chlorophyll a(R^2=0.174, p<0.0001).

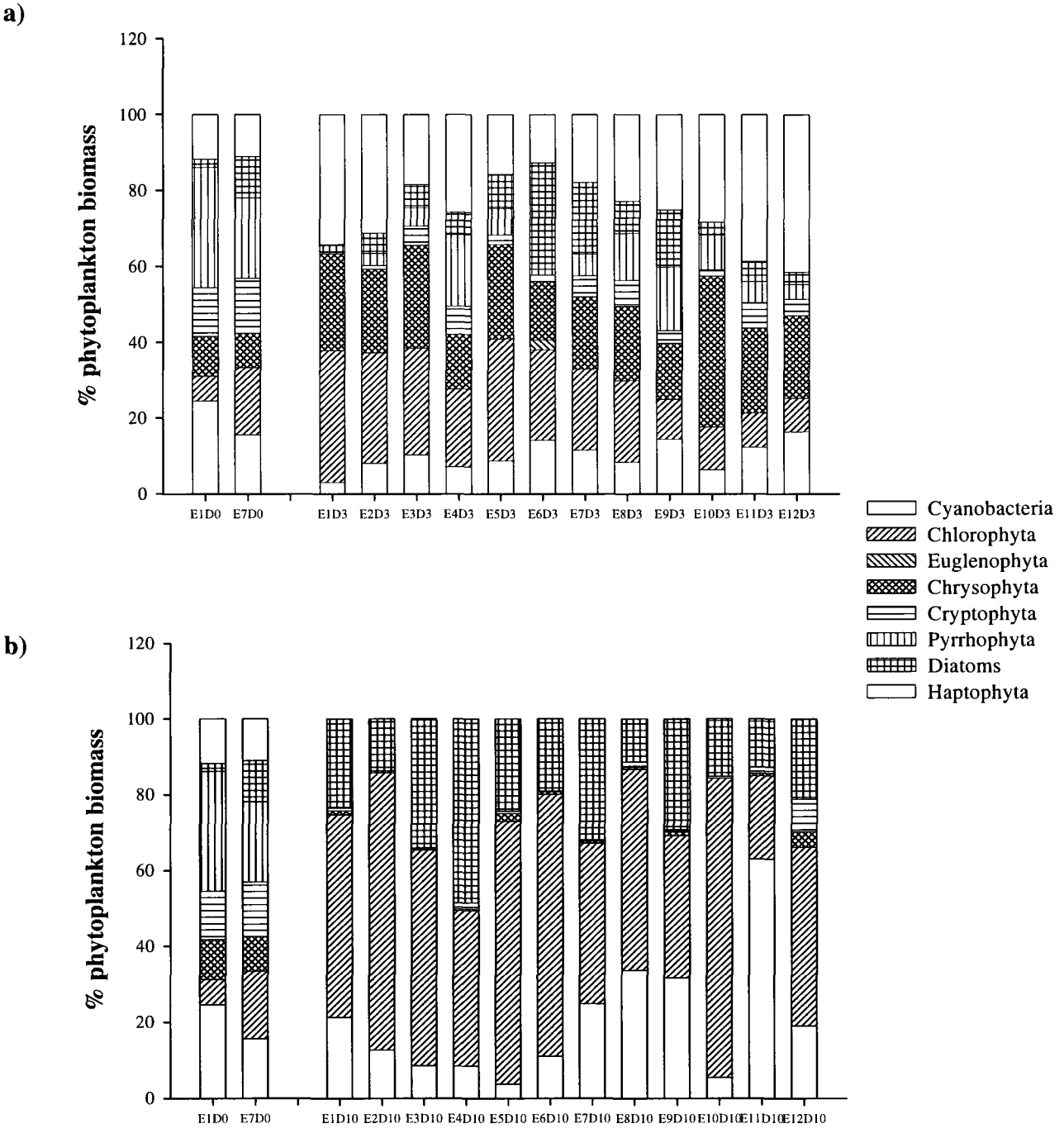


Figure 30. Percent phytoplankton biomass for Constance Lake Enclosures on **a)** day 3, and **b)** day 10 of the enclosure experiment. The percent cyanobacteria for each enclosure is represented by the white bar at the bottom of each column. Biomass was determined as biovolumes ($\mu g \cdot L^{-1}$). Day 0 for enclosures 1 and 7 are shown in each figure as an indication of starting conditions for each enclosure at the beginning of the experiment.

5.3.2 Microcystin Analysis

Overall total microcystin (both in $\mu g \cdot g^{-1}$ and $\mu g \cdot L^{-1}$) remained low and were comparable to concentrations measured in the lake itself (Table 15). Figure 31 shows large variations within enclosures through time, as well as significant variations between enclosures.

5.3.3 QPCR Analysis of *mcy*D

The *mcy*D1 gene was detected throughout the experiment and in all enclosures (Fig. 32). The maximum *mcy*D1 gene copy numbers per ml found in Constance lake during those 14 days in the summer of 2007 was 138, while in the enclosures a maximum *mcy*D1 gene copy number per ml of 1933 was recorded with several enclosures (1, 3, 4, 5, 7, 8, 10) having greater than 500 *mcy*D copies$\cdot ml^{-1}$.

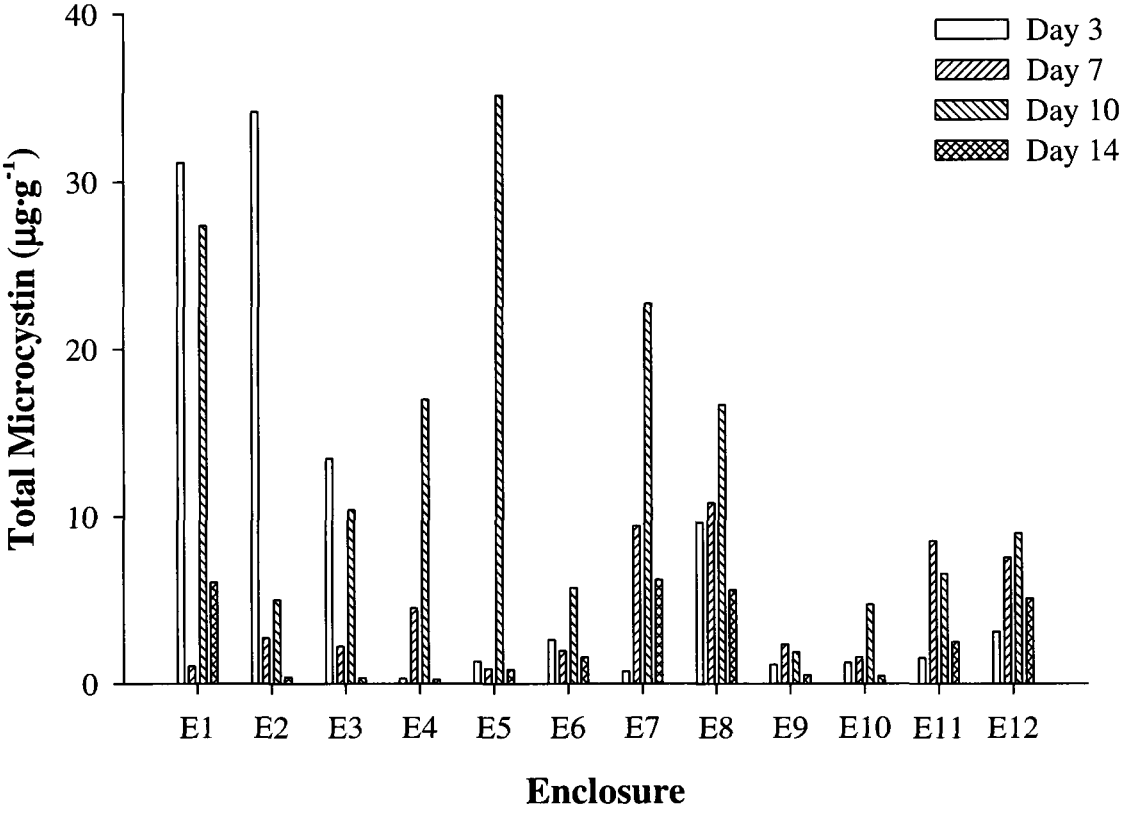


Figure 31. Total microcystin ($\mu g \cdot g^{-1}$) for enclosures 1-12 on days 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Control enclosures were 1, 5, and 9 (un-shaded, no nutrients added); Enclosures 2, 6, and 10 were shaded with no nutrients added; Enclosures 3, 7, and 11 were shaded with nutrients; and enclosures 4, 8 and 12 were un-shaded with nutrients added.

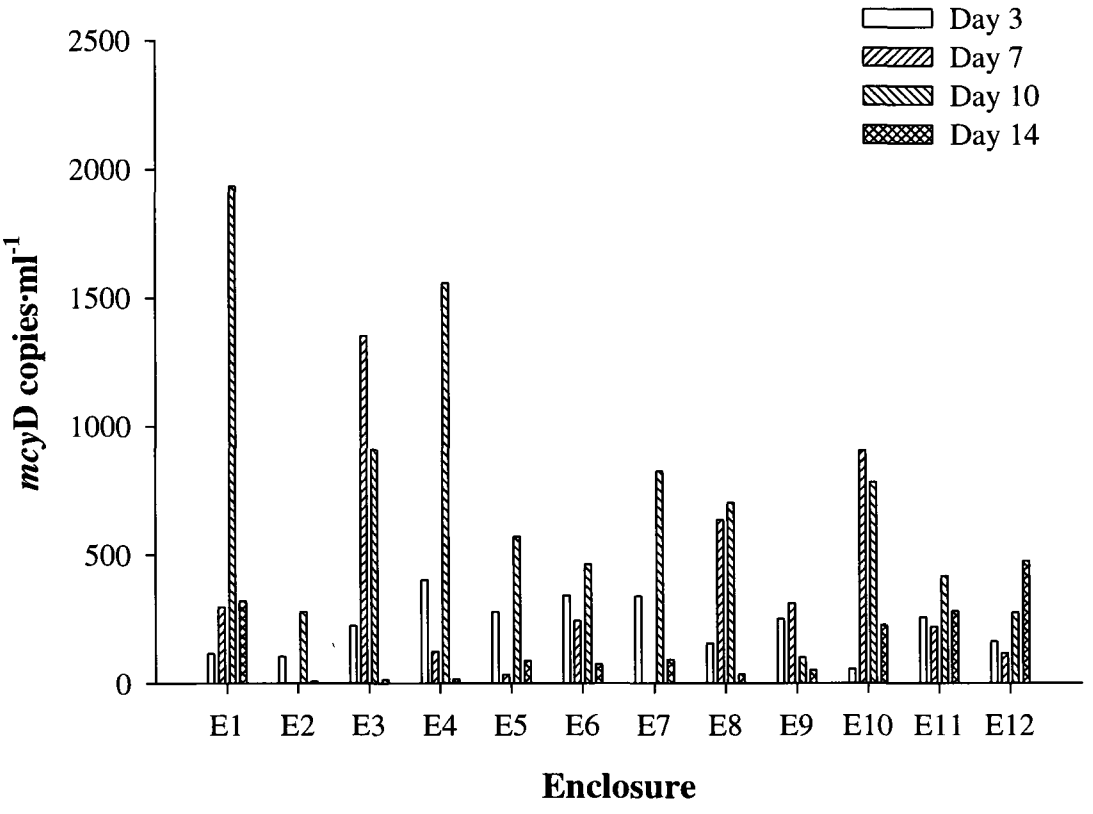


Figure 32. *mcy*D copies·ml^{-1} for enclosures 1-12 on days 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Control enclosures were 1, 5, and 9 (un-shaded, no nutrients added); Enclosures 2, 6, and 10 were shaded with no nutrients added; Enclosures 3, 7, and 11 were shaded with nutrients; and enclosures 4, 8 and 12 were un-shaded with nutrients added.

5.3.4 Simple Linear Regression Analysis

A two-step statistical approach was taken to determine which independent variables were significantly related to cyanobacteria biomass, total microcystin and *mcy*D copies·ml^{-1}. First, a simple linear regression approach was taken by which all independent variables (Table 15) were examined in a regression analysis one at a time with the dependent variables. Second, multiple regressions were carried out in order to determine the best predictive best model for each dependent variable. All data points from all twelve enclosures on each of the five sampling days were combined. The dependent variables of interest were: total microcystin (μg·g^{-1} and μg·L^{-1}), *mcy*D copies·ml^{-1}, and cyanobacterial biomass.

The first dependent variable examined was total microcystins. Despite the large variation in nutrient concentrations across the enclosures, total microcystins were not correlated with nutrient levels. Total microcystin seston content (μg·g^{-1}) was significantly related to only a single abiotic factor, oxidative reductive potential (ORP) (Table 16), but only a very small amount of variation was explained by ORP (8.4%). Total microcystin (μg·g^{-1}) was also positively correlated with *mcy*D copies·ml^{-1} and *Aphanizomenon* biomass. While the relationship between microcystin and *mcy*D copies·ml^{-1} (Fig. 33) had a lower p-value, *Aphanizomenon* biomass explained the greatest variation in total microcystins μg·g^{-1} at 29.5%. When total microcystin was expressed as μg·L^{-1} only dissolved oxygen had a significant relationship. However, while the regression was significant, only a small percent (9.5%) of the variation in total microcystin (μg·L^{-1}) was explained by changes in dissolved oxygen. As with the total microcystin expressed as μg·g^{-1}, both *mcy*D copies·ml^{-1} and *Aphanizomenon* biomass had significant, positive relationships with total microcystin μg·L^{-1} with *Aphanizomenon* biomass being the most significant and explaining over half the variation (Table 16). Total algal biomass was also marginally related to total microcystin μg·L^{-1} (Table 16).

Two abiotic factors resulted in positive significant regressions with *mcy*D copies·ml^{-1} including temperature ($p=0.006$, $R^2=0.149$), and dissolved oxygen ($p=0.001$, $R^2=0.219$). Also, as with total microcystin, both *Aphanizomenon* ($p=0.002$, $R^2=0.517$) and total algal biomass ($p=0.017$, $R^2=0.222$) showed a significant relationship with *mcy*D gene copy numbers throughout the experiment.

The fourth dependent variable examined was total cyanobacterial biomass. In the previous chapter examining the distribution of toxigenic cyanobacteria in Constance Lake individual toxigenic cyanobacterial genera were also added as independent variables. However, in this experiment phytoplankton community structure was analyzed on days 3 and 10 and often one or several of the potentially toxigenic cyanobacterial genera was not present in a particular enclosure on one of these two sampling dates. Therefore, due to a decrease in the number of data points available when the genera were examined individually, only total cyanobacterial biomass was considered. Five abiotic variables were related to cyanobacterial biomass including pH, dissolved oxygen, conductivity, temperature and NH_3&NH_4, with pH and dissolved oxygen resulting in the strongest individual relationships. Also of note is that there was no significant relationship between picocyanobacterial cell abundance and total microcystin or *mcy*D copies·ml^{-1}, although there was a significant positive relationship between picocyanobacterial cell abundance and total phosphorus among the enclosures.

Table 16. Results from linear regressions carried out with the dependent variables listed below and all independent variables measured for Enclosures 1-12 on days 0, 3, 7, 10, and 14 of the enclosure experiment. R^2, p-values and regression coefficients are noted (n=52).

Dependent Variable	
Total Microcystin µg·g^{-1}	• Oxidative-Reductive Potential (R^2=0.084, p=0.037, RC=9.1) • *mcy*D copies·ml^{-1} (R^2=0.161, p=0.004, RC=0.44) • *Aphanizomenon* Biomass (R^2=0.285, p=0.033, RC=0.56)
Total Microcystin µg·L^{-1}	• Dissolved Oxygen (R^2=0.095, p=0.026, RC=3.05) • *mcy*D copies·ml^{-1} (R^2=0.174, p=0.003, RC=0.48) • *Aphanizomenon* Biomass (R^2=0.551, p=0.001, RC=0.90) • Total Biomass (R^2=0.146, p=0.059, RC=0.70)
***mcy*D copies·ml^{-1}**	• Temperature (R^2=0.149, p=0.006, RC=8.74) • Dissolved Oxygen (R^2=0.219, p=0.001, RC=4.03) • *Aphanizomenon* Biomass (R^2=0.517, p=0.002, RC=0.65) • Total Biomass (R^2=0.222, p=0.017, RC=0.62)
Cyanobacteria Biomass µg·L^{-1}	• Temperature (R^2=0.215, p=0.017, RC=7.77) • Dissolved Oxygen (R^2=0.320, p=0.003, RC=4.54) • pH (R^2=0.437, p=0.000, RC=20.69) • Conductivity (R^2=0.241, p=0.011, RC=56.20) • NH_3NH_4 (R^2=0.147, p=0.053, RC= -0.45) • *Anabaena* Biomass (R^2=0.529, p=0.011, RC=0.54) • *Aphanizomenon* Biomass (R^2=0.362, p=0.014, RC=0.49) • Total Biomass (R^2=0.685, p<0.0001, RC=1.23)

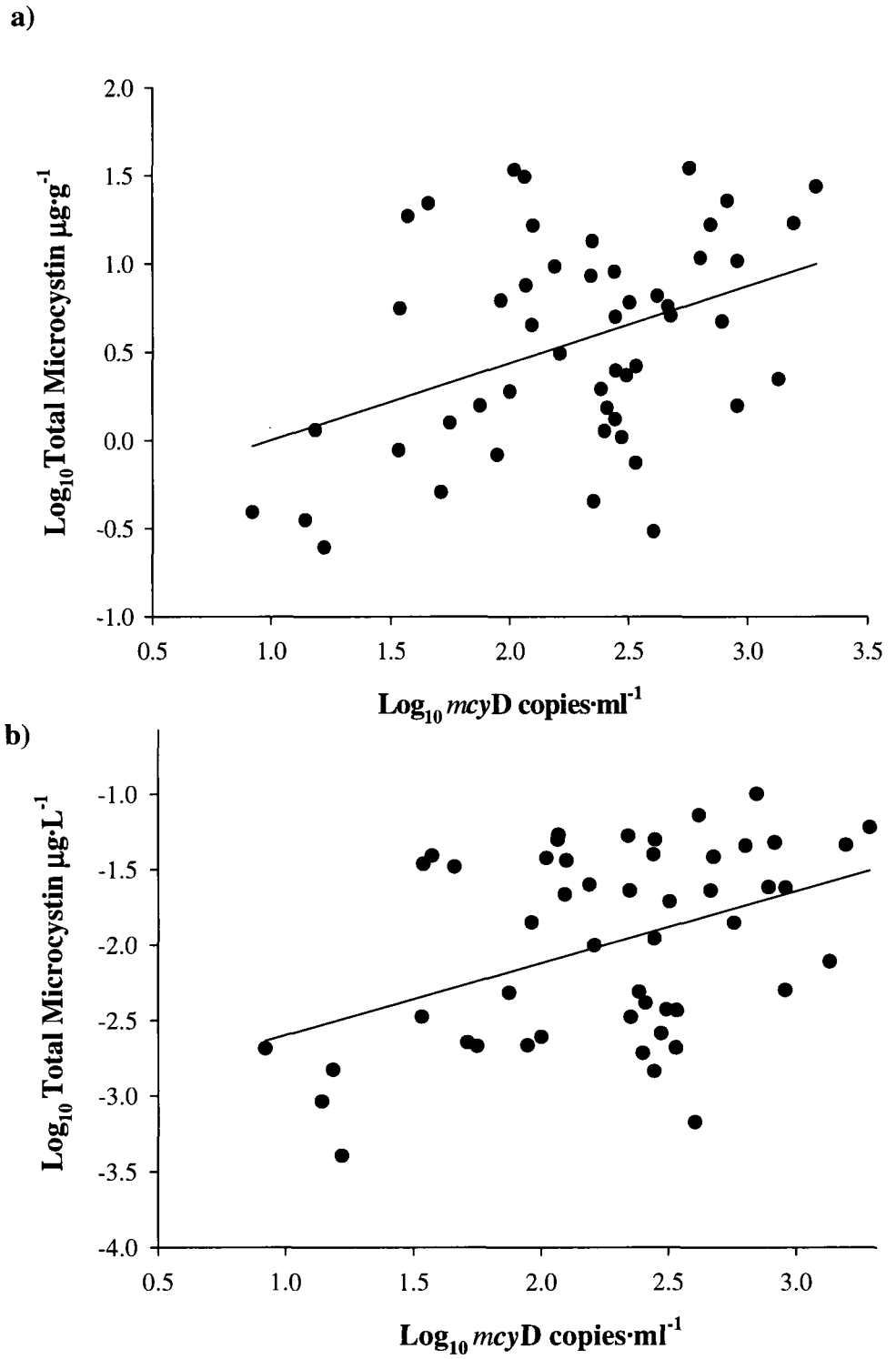


Figure 33. Log_{10}-transformed relationship between *mcy*D copies·ml^{-1} and **a)** total microcystin µg·g^{-1} (R^2=0.161, p=0.004, Regression Coefficient=0.436) and **b)** total microcystin µg·L^{-1} (R^2=0.174, p=0.003, Regression Coefficient=0.478).

5.3.5 Multiple Regression Analysis

A multiple regression approach was used to determine which variables gave the best predictive model for each of the dependent variables. Two significant multiple regression models were obtained for total microcystin $\mu g \cdot g^{-1}$ (Table 17). The first model incorporated the abiotic variables of temperature, dissolved oxygen and oxidative-reductive potential (ORP) (R^2=0.222, p=0.007), whereas the second model included temperature and *Anabaena* biomass (R^2=0.593, p=0.027). While *Aphanizomenon* biomass was a significant positive factor in simple regression models for total microcystin $\mu g \cdot g^{-1}$, the other main Nostocales genus *Anabaena* was a significant negative factor in multiple regression analysis. Two significant multiple regression models were also obtained for total microcystin $\mu g \cdot L^{-1}$. The first included the abiotic factors of temperature, light extinction, ORP, and dissolved oxygen (R^2=0.282, p=0.003). This was improved with the addition of *Anabaena* biomass which produced a significant model when both temperature and light extinction were included (R^2=0.888, p=0.001). Therefore for total microcystin ($\mu g \cdot g^{-1}$and $\mu g \cdot L^{-1}$) several common variables appeared between both simple and multiple regression analyses for both of these dependent variables,

The third dependent variable was *mcy*D copies$\cdot ml^{-1}$. One model was obtained which included only the abiotic factors temperature, dissolved oxygen, and pH (R^2=0.501, p<0.0001). While both *Aphanizomenon* and total biomass were significant independent variables in the simple linear regression analysis with *mcy*D copies$\cdot ml^{-1}$, neither of these parameters made strong improvements to a multiple regression model.

With respect to total cyanobacteria biomass, light extinction, dissolved oxygen, pH and NH_3NH_4 were the four main significant independent variables in the multiple regression model (R^2=0.714, p=0.000). All of these variables, with the exception of light extinction also resulted in a significant simple linear regression with cyanobacterial biomass (Table 16).

Table 17. Multiple regression models predicting the four dependent variables listed below for the Constance Lake 2007 enclosure experiment. For total microcystin ($\mu g \cdot g^{-1}$ and $\mu g \cdot L^{-1}$) and *mcy*D copies$\cdot ml^{-1}$ both abiotic and biotic independent variables were included, while cyanobacteria biomass was regressed with abiotic independent variables only. R^2, p-values and regression coefficients (RC) are noted (n=52).

Dependent Variable	
Total Microcystin $\mu g \cdot g^{-1}$	•Total Microcystin= -49.6 + 6.3(Temperature) + 2.9(DO) + 15.2(ORP) (R^2=0.222, p=0.007). • Total Microcystin= -30.3 + 22.5(Temperature) – 0.54(*Anabaena* Biomass) (R^2=0.593, p=0.027).
Total Microcystin $\mu g \cdot L^{-1}$	• Total Microcystin= -52.2 -1.4(LEC) + 8.0(Temperature) + 3.7(DO) + 13.9(ORP) (R^2=0.282, p=0.003). •Total Microcystin= -37.9 + 26.1(Temperature) – 3.0 (LEC) – 0.57(*Anabaena* Biomass) (R^2=0.888, p=0.001).
***mcy*D copies$\cdot ml^{-1}$**	•*mcy*D copies$\cdot ml^{-1}$= -3.7 + 10.2(Temperature) + 6.5(DO) – 15.6(pH)(R^2=0.501, p=0.000).
Cyanobacteria Biomass $\mu g \cdot L^{-1}$	• Cyanobacteria Biomass= -24.9 – 1.8(LEC) -4.8(DO) + 32.9(pH) – 0.59(NH_3NH_4) (R^2=0.714, p=0.000).

5.4 Discussion

5.4.1 Comparison of Physical, Chemical, and Biological Parameters between Constance Lake and the Enclosures

Several physical, chemical and biological parameters were measured for all twelve enclosures and the lake itself throughout the duration of the experiment. In general, the physical variables (temperature, pH, ORP, and SPC) remained similar between the enclosures and the lake (Table 15). However, nutrient concentrations were significantly different not only because of the manual input of potassium phosphate and sodium nitrate, but also because of the extra inputs from Canada Geese resting on the enclosure frames. These nutrient increases resulted in a much higher range of chlorophyll a in the enclosures than in the lake. Several studies have shown that inputs from allochthonous vectors such as geese are an important source of nutrients to aquatic environments. For example, Kitchell *et al.* (1999) and Olson *et al.* (2005) determined that more than 70% of the phosphorus and 30% of the nitrogen loaded into their study lakes were due to the presence of snow geese. The transfer of nutrients from agricultural and other terrestrial habitats to aquatic ecosystems by migratory waterfowl can also contribute to bottom-up control of trophic dynamics (Post *et al.* 1998, Jeffries 2000, Vanni 2002). Nutrient enrichment of this kind can stimulate aquatic plant and algae growth, which then reduce water quality and accelerate processes that fill lakes and reservoirs with sediments (Manny *et al.* 1994).

While significant increases in nutrients resulted in higher overall algal biomass for several enclosures compared to the lake, total phytoplankton biomass estimated from microscopic enumerations reached higher overall levels in the lake than in the enclosures (Table 15). However, when different phytoplankton groups were examined it was clear that cyanobacteria increased significantly in several enclosures, including the biomass of several potentially toxigenic genera such as *Anabaena* and *Microcystis* (Table 15). Cyanobacterial growth was clearly stimulated in many of the enclosures (1, 7, 8, 9, and 11, Fig. 30). While the factorial design of the experiment was not entirely successful, a strong gradient in nutrients was established which led to significant changes in chlorophyll a and cyanobacterial biomass.

The environmental variables that were most significantly related to total cyanobacterial biomass included temperature, dissolved oxygen, pH, conductivity, and

NH_3+NH_4. A multiple regression model including these same variables and light extinction, explained 71% of the variation in cyanobacterial biomass. With the exception of NH_3+NH_4, there were no other significant relationships between nutrients such as nitrogen and phosphorus or the TN:TP ratio with cyanobacterial biomass. This is interesting considering the long history of studies suggesting the importance of nutrient concentrations for overall phytoplankton growth and specifically for the development of cyanobacterial populations (Pick & Lean 1987). However, it is possible that the effect of light was simply a reflection of parallel changes in nutrients. But in Constance Lake itself over two years there was also no relationship between cyanobacterial biomass and nutrients and some of the variables such as temperature and pH that emerged as significant in both simple and multiple regression models.

5.4.2 Microcystin concentrations across a Nutrient Gradient

While cyanobacterial biomass was significantly different between the enclosures and Constance Lake during the experiment, total microcystin concentrations in the enclosures was similar to the range in the lake itself during the same time. While total cyanobacterial biomass, including the common microcystin producers *Anabaena* and *Microcystis,* increased in several enclosures this was not reflected in terms of increases in microcystin concentrations. This might indicate that the increased cyanobacterial growth in the enclosures may have been due to increases in non-toxigenic strains. However microcystin concentrations appeared to increase steadily in several enclosures (such as enclosures 4, 8, and 10) and in most enclosures there were large fluctuations in terms of microcystin concentrations (i.e. enclosures 1, 2, 3, 5). Regression analyses showed that total microcystin was significantly related to ORP and dissolved oxygen, but only a very small amount of variation in total microcystin concentrations was explained (8.4% and 9.5%). *Mcy*D copies·ml^{-1} and *Aphanizomenon* biomass also resulted in significant positive relationships with total microcystin, with *Aphanizomenon* biomass explaining the greatest amount of variation. However, this still explained only 50% or less of the variation while *mcy*D copies·ml^{-1} on its own explained roughly 16% of the variation in microcystin concentrations. As with the seasonal study of Constance Lake itself over two years, the correlation between gene copy number and microcystin product was not strong across the enclosures.

Of interest from these results was that microcystins showed a significant relationship with *Aphanizomenon* biomass. Species from the Nostocales genus *Aphanizomenon* are not known as microcystin producers and the most common microcystin producing genera are usually *Microcystis, Anabaena,* and *Planktothrix* (Zurawell *et al.* 2005). *Aphanizomenon* is more known for its capacity to produce neurotoxins such as the anatoxins and saxitoxins (paralytic shellfish poisons- PSPs), and the cyanotoxin cylindrospermopsin (Banker *et al.* 1997, Sivonen & Jones 1999, Ferreira *et al.* 2001, Falconer 2005, Wood *et al.* 2007). Thus far there have been no reports indicating that species of *Aphanizomenon* produce microcystins or contain any of the genes required for microcystin production (C. Wiedner, Berlin, personal communication). However, new species and strains of cyanobacteria that produce known or novel toxins are still being identified. Without the isolation of specific *Aphanizomenon* strains from Constance Lake to test for microcystin production and/or the presence of *mcy* genes, the relationship between microcystins and *Aphanizomenon* biomass found in the enclosures must be considered tentative. It should also be stressed that the trends described are based on statistical relationships that may or may not reflect a direct causal relationship.

Several studies have attempted to determine which environmental factors have a significant relationship with microcystin production in both field and culture studies. As described in the previous chapter there does not appear to be any general consensus in terms of which environmental variables influence microcystin production. For example, in one study low water residence time and high temperature were strongly correlated with the seasonal variation in total concentration of microcystin (Lehman *et al.* 2008), while in another it was nitrogen loading which enhanced both cyanobacterial growth and microcystin concentrations (Gobler *et al.* 2007). Microcystin concentrations have also been found to have significant relationships with chlorophyll a (Vézie *et al.* 1998), total phosphorus (Fahnenstiel *et al.* 2008), and nitrogen (Graham *et al.* 2006).

Some studies have found that microcystin production is indirectly affected by environmental factors in that variables such as nutrients and light usually affect the rate of cell division which in turn affects the rate of microcystin production (Orr & Jones 1998). Other studies have shown that while these environmental effects on total microcystin concentrations may be indirect, they still play an important role in determining microcystin

production. For example, Downing *et al.* (2005a) found a significant relationship between microcystin production and the TN:TP ratio in two strains of *Microcystis aeruginosa.* Cellular microcystin content correlated strongly with cellular protein content which was highest at TN:TP ratios greater than 18. In this enclosure study TN:TP ratios ranged from 9.5-32.4, however no significant relationship was found between these ratios and microcystin concentrations. In a second culture study, Downing *et al.* (2005b) grew a continuous culture of *Microcystis aeruginosa* PCC 7806 under varying nitrogen conditions. Microcystin concentrations were positively correlated with nitrate uptake and cellular nitrogen content, indicating that nitrogen may be a significant player in terms of promoting the growth and production of microcystins by toxigenic strains. However, in the enclosures, even with a massive increase in nitrogen concentration due to both manual and goose feces inputs, no significant relationships were observed between nitrogen levels and microcystin concentrations.

Other studies have shown that while nitrogen may be important for microcystin production the chemical form of nitrogen investigated must also be taken into consideration (Yan *et al.* 2004). In a culture study of *Microcystis aeruginosa,* Amé *et al.* (2005) found that the addition of ammonium resulted in a decrease in the total microcystin cell content. They then suggested that ammonium could have an inhibitory effect on microcystin production by *M. aeruginosa.* In the enclosures ammonia and ammonium concentrations were increased dramatically compared to the lake (Table 15) which was most likely due to inputs from the geese as the manual chemical additions of nitrogen were in the form of sodium nitrate and the conditions in the enclosures would not have led to much denitrification. While there were no significant negative correlations between microcystin and NH_3 and NH_4 concentrations, the results from Amé *et al.* (2005) do leave room for speculation as to the potential inhibitory impact that such high nutrient increases in the enclosures may have had on certain strains of cyanobacteria.

5.4.3 Gene Copies of *mcy*D across a Nutrient Gradient

While total microcystin concentrations were not significantly different between the enclosures and Constance Lake, there were large differences in terms of *mcy*D copies·ml^{-1}. Because there were no differences in microcystin concentrations the increase in

cyanobacterial biomass in the enclosures initially appeared to be due to increased growth of non-toxigenic strains yet the increased levels of *mcy*D copies·ml^{-1} suggest otherwise. However, as with total microcystin concentrations, *mcy*D copies·ml^{-1} were also quite variable between enclosures. For example, the control enclosure 1 had the highest recorded *mcy*D copies·ml^{-1} with 1933 copies·ml^{-1}. Similar levels were also found in enclosures 3 and 4 which both received nutrient additions, however enclosure 3 was shaded while enclosure 4 was not. Several enclosures had similar ranges of *mcy*D copies·ml^{-1} throughout the experiment including enclosures 6, 7, 8, 10, and 9, 11, and 12. Also, as was found in the field study of Constance Lake in both 2006 and 2007, there was often a disconnect between the highest microcystin concentrations and the highest *mcy*D gene copies observed. While enclosures 1 and 2 had high microcystin concentrations on day 3 of the experiment this was not reflected in their gene copy number. Another example is enclosure 10 which had low microcystin concentrations but higher *mcy*D copies·ml^{-1} than other enclosures with higher microcystin concentrations. While there was a significant relationship between *mcy*D copies·ml^{-1} and total microcystin in the enclosures, only a small proportion of the variance in total microcystin (<20%) was explained by changes in *mcy*D copies·ml^{-1}.

For the enclosure experiment the regression analyses indicate that *mcy*D copies·ml^{-1} had significant relationships with temperature, dissolved oxygen, total algal biomass, and *Aphanizomenon* biomass, while the multiple regression model showed that a model including only the abiotic factors of temperature, dissolved oxygen and pH was significant. It is interesting that *Aphanizomenon* biomass once again appeared significant. In the seasonal study of Constance Lake it was total cyanobacterial biomass along with the biomasses of *Anabaena* and *Microcystis* rather than *Aphanizomenon* that were significant predictors of *mcy*D copies·ml^{-1}.

Based on the regression analyses there were no significant relationships found in the enclosures between the nutrient gradient established and toxigenic cyanobacteria. However, oxygen levels appeared in several models and higher oxygen would be indicative of high levels of productivity which would stem from higher nutrient availability or a change in biomass turnover. It is unlikely that oxygen directly caused any increases in microcystin concentrations.

Other studies have demonstrated that nutrients such as nitrogen and phosphorus may have a strain specific effect on toxigenic and non-toxigenic strains of cyanobacteria. For example, Vézie *et al.* (2002) grew toxigenic and non-toxigenic strains of *Microcystis* in both low and high nutrient concentrations and found that the non-toxigenic strains required fewer nutrients for optimal growth. Their results suggested that the demands of different cyanobacterial strains to nutrients might vary to a significant extent within the same genus and therefore nutrient concentrations may be contributing to strain dominance. Yoshida *et al.* (2007) also found a significant relationship between nitrate concentrations and the presence of toxigenic strains of *Microcystis aeruginosa*. When surface nitrate concentrations increased there was a rise in the relative abundance of the *mcy*A *Microcystis aeruginosa* subpopulation. They suggested that high nitrate loading may be a significant factor promoting the growth of the microcystin subpopulations within *M. aeruginosa* communities in Lake Mikata, Japan. Nitrate did not have this effect in the present study.

Several improvements can be suggested for this type of experiment as the original ideas and intentions behind this type of *in situ* experiment may still prove useful in providing a better understanding of the development of toxigenic blooms in nature. The first improvement would be to carry out the experiment at a study site that may have more controlled conditions in terms of bird populations. A second major improvement would be to use a different type of enclosure bag to improve the difference in light conditions between shaded and un-shaded enclosures. While shading the enclosures did reduce light penetration from directly above the enclosures, the bags themselves under the surface of the water were too clear and allowed a significant amount of light to penetrate from the sides. The result was an ineffective light treatment and the effect of light could not be determined independently from the effect of nutrients.

5.4.4 Conclusions

The initial goals of this enclosure experiment were to determine how, in a factorial design, light and nutrients (phosphorus and nitrogen) affected the growth and dominance of toxigenic cyanobacteria *in situ* and to determine how light quantity may influence both the levels of *mcy*D gene copy number and total microcystin production. However, due to the ineffectiveness of the shading of the enclosures in the light treatments, and the extra nutrient

inputs from geese feces, a regression approach was taken to determine the effects of a nutrient gradient on total microcystin concentrations and *mcy*D gene copy numbers. The addition of nutrients was expected to promote the growth of cyanobacteria and based on previous laboratory studies specifically increase the growth of toxigenic strains (Vézie *et al.* 2002).

In general, nutrient concentrations, total cyanobacteria biomass (including the biomass of *Anabaena, Microcystis,* and *Aphanizomenon*), and *mcy*D copies·ml^{-1} were enhanced in the enclosures compared to the same variables measured in the lake itself during the same time period. However, regression analyses did not reveal clear significant relationships between the increased nutrient concentrations in the enclosures and the resulting microcystin concentrations and *mcy*D gene copy numbers. One possible reason for this may be that while the chemical nutrient inputs of potassium phosphate and sodium nitrate were added at regular time intervals (every three days), the extra nutrient inputs from the geese were added sporadically throughout the experiment. Due to the inconsistency of the nutrient additions it is perhaps not surprising that large variations were observed in terms of the responses of the phytoplankton communities in individual enclosures, along with total microcystin concentrations or *mcy*D copies·ml^{-1}. Very few *in situ* studies of this type have been attempted specifically measuring toxigenic cyanobacteria and microcystin concentrations. However they are necessary in order to bridge culture studies with field observations and to provide guidance in the management of cyanobacterial blooms.

Chapter 6
General Conclusion

Cyanobacterial blooms appear to be on the rise around the world, a consequence of increased anthropogenic nutrient loadings (Sivonen & Jones 1999) and possibly climate change (LeBlanc *et al.* 2007, Paerl & Huisman 2008). With any bloom there is always the risk of cyanotoxins which in turn can affect the health and welfare of those using affected waters for recreational, commercial, and household uses. Cyanobacterial toxins are a real and ongoing concern for water quality and lake and coastal managers around the globe. The advancement of research and technologies in this field has led to several methods that remove microcystins from drinking water, the most common human exposure route (e.g. UV irradiation, activated carbon adsorption, ozone oxidation, gamma irradiation etc.) (Rapala *et al.* 2006, Sakai *et al.* 2007, Zhang *et al.* 2007, Hudder *et al.* 2007). However, even with extensive monitoring and treatment of drinking water supplies, there are several other potential human exposures such as inadvertent ingestion of contaminated waters during recreational use and microcystins can be quite persistent, still detectable at low levels several weeks following a bloom (Lahti *et al.* 1997). Another alternative exposure route is through the consumption of plant or animal food products that have been exposed to cyanotoxins. For example, microcystins have been shown to accumulate on crops such as wheat and lettuce due to the use of contaminated water in spray irrigation practices (Codd *et al.* 1999, Pflugmacher *et al.* 2007).

Due mainly to human health risks, important advances have been made over the past two decades in measuring toxin concentrations and identifying toxin congeners. However, several questions remain, namely why some blooms are toxic and others are not. This is where the development of molecular tools may prove useful in predicting cyanotoxin production and the causes of toxic blooms. Molecular probes may allow earlier detection of toxic blooms, identification of the dominant organism within the blooms, and an estimation of the level of toxic substances that are accumulating (Schatz *et al.* 2000).

In this research I utilized both chemical and molecular techniques to examine the role of environmental and biological factors in regulating the production of microcystins. The overall goal of the culture studies was to determine if light intensity is a significant factor in toxin production and in the dominance of toxigenic strains over those that are not. Field and enclosure studies were designed to examine not only the effect of light, but also the effect of

nutrients on both microcystin concentrations and growth of toxigenic species and strains of cyanobacteria.

The results from the culture studies showed the very specific responses that individual cyanobacterial strains have to changing light. While the growth rates of all seven strains of *Microcystis* examined were significantly affected by light intensity, there were no patterns in terms of toxigenic or non-toxigenic strains having overall higher or lower growth rates when grown under different light intensities. Microcystin production did not appear to be a particularly costly process for toxigenic strains when grown under light-limiting conditions, nor did it appear to give toxigenic strains any sort of advantage in terms of growth under changing light intensities in contrast to previous culture work (Vézie *et al.* 2002). However, total microcystin production and the proportions of different microcystin congeners produced by each strain were significantly affected by light intensity. The production of microcystins by toxigenic cyanobacterial species appeared to be quite strain specific as each of the three toxigenic strains studied had a unique pattern of microcystin congener production.

The mixed culture experiments were carried out to determine how toxigenic and non-toxigenic strains of *Microcystis aeruginosa* interacted with one another when grown under light-saturating and light-limiting conditions. The toxigenic strain became dominant under low light conditions, while under high light intensity the toxigenic strain was less dominant. This finding was opposite to the theoretical predictions stating that microcystins are produced at a cost, and also opposite to the conclusions of a study with strains of *Microcystis* in competition for light (Kardinaal *et al.* 2007a).

The field studies were designed to determine which variables (whether physical, chemical, or biological) were correlated with microcystin production and the presence of toxigenic cyanobacteria. In Constance Lake, higher microcystin concentrations and *mcy*D gene copies·ml^{-1} were detected in the summer of 2006 compared to 2007, and this despite no obvious differences in environmental variables between years. Biological variables such as total cyanobacterial biomass, including the common microcystin-producing genera of *Anabaena* and *Microcystis*, had a greater impact on the *mcy*D gene copy numbers than did environmental variables alone, while both biotic and abiotic variables helped to explain the variation in total microcystin concentrations. There was also an inconsistent relationship

between *mcy*D gene copies·ml^{-1} and total microcystin concentrations. It is possible that the low levels of microcystins and microcystin producers in Constance Lake compared to more enriched systems was responsible for the lack of a significant relationship.

In the enclosure experiment the presence of specific cyanobacterial taxa was also, in general, more important for the detection of toxigenic genotypes than were environmental variables. There was a significant increase in nutrient concentrations, chl a, and cyanobacterial biomass (including *Anabaena, Microcystis*, and *Aphanizomenon*) in the enclosures compared to the same variables measured in Constance Lake throughout the experiment. However, there were large variations in terms of microcystin concentrations and *mcy*D gene copy numbers within and between enclosures and no significant effects of nutrients were found on either total microcystin concentrations or *mcy*D gene copy numbers.

While light intensity clearly has a significant effect on the growth of cyanobacterial strains and microcystin production in toxigenic strains, the response of each strain appears unique such that any generalizations cannot be made between toxigenic and non-toxigenic strains. There was no clear advantage or disadvantage in carrying the toxin gene in terms of the ability to grow and compete for light resources. A total of seven strains of *Microcystis* were used in the culture experiments which is more than the usual number of strains examined in several studies (i.e. Utkilen & Gjolme 1992, Rapala *et* al. 1997, Hesse *et al.* 2001, Kardinaal *et al.* 2007a). However, in nature the number of individual species, let alone strains of cyanobacteria that are present in an aquatic ecosystem are much higher and quite variable over time.

Toxigenic cyanobacteria remain a challenging research area as in a given bloom genetically different yet morphologically similar populations coexist with their own individual tolerances to environmental factors and with distinct toxicity (Bittencourt-Oliveira 2003, Wilson *et al.* 2005). Despite the potential of molecular biology, it is likely that traditional approaches based on taxonomy and chemical analyses of toxins will still be required. Continued research on toxic cyanobacterial blooms is clearly necessary particularly in the face of climate change which is a potential catalyst for the further expansion of cyanobacterial blooms (Paerl & Huisman 2008). Increased temperatures, high nutrient loading, enhanced stratification, longer water residence time, and salinization, all favour cyanobacterial dominance in many aquatic ecosystems and these conditions may all

be enhanced due to global warming. Cyanobacteria are ancient life forms which occupy every conceivable ecological niche. With an increase in global temperatures cyanobacterial populations are likely to increase due to an increase in human populations demanding drinking water (Falconer & Humpage 2005). It is anticipated that increased incidences of cyanobacterial blooms will most likely result in greater occurrences of toxic bloom events around the globe, including in Canadian waters (LeBlanc *et al.* 2008).

7.0 References

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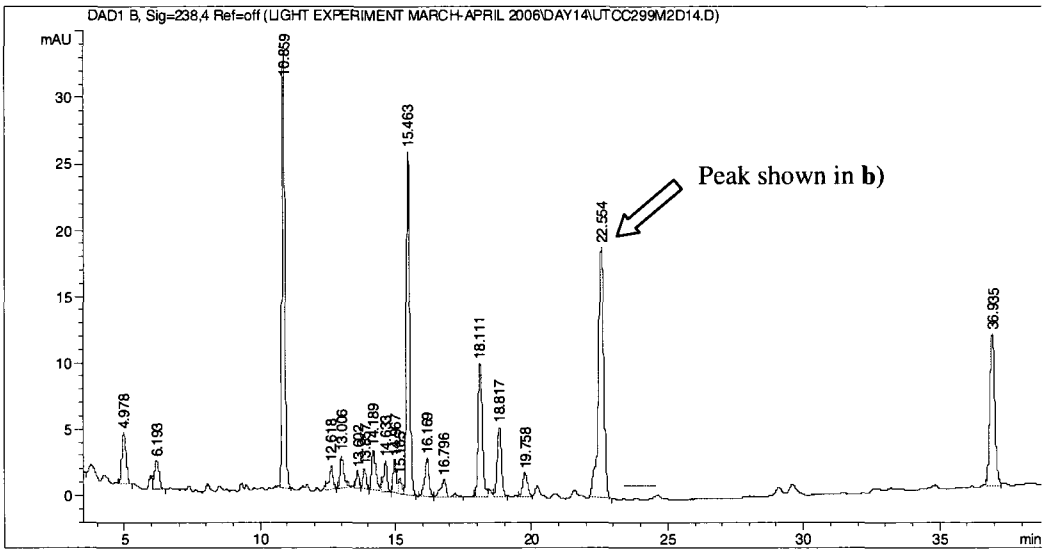
8.0 Appendices

APPENDIX I

a)



b) **c)**

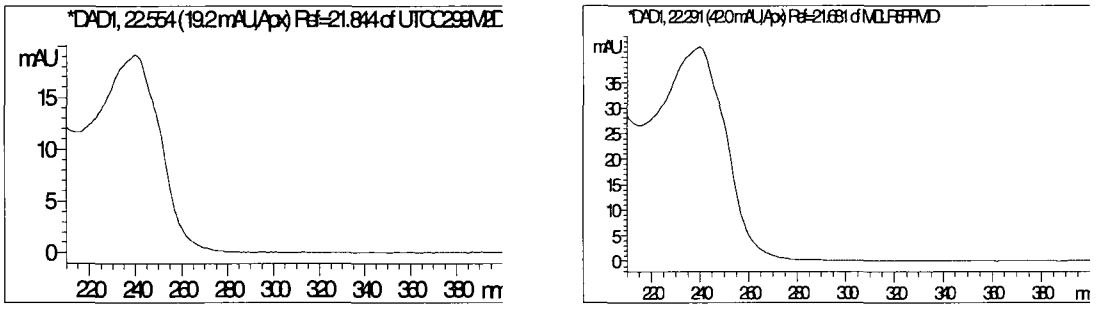


Figure 34. Examples of chromatograms and UV spectra obtained from both standards and samples. **a)** a chromatogram obtained from UTCC 299 on day 14 of the experiment grown under medium light intensity. **b)** UV spectrum of a peak from the chromatogram in a) at a retention time of 21.844 minutes. **c)** UV spectrum of a standard peak of microcystin-LR at a retention time of 21.881 minutes. Due to the similarities in retention times and UV peak shape at 238 nm this allows the identification of the peak in UTCC 299 at 21.844 minutes to be microcystin-LR.

APPENDIX II

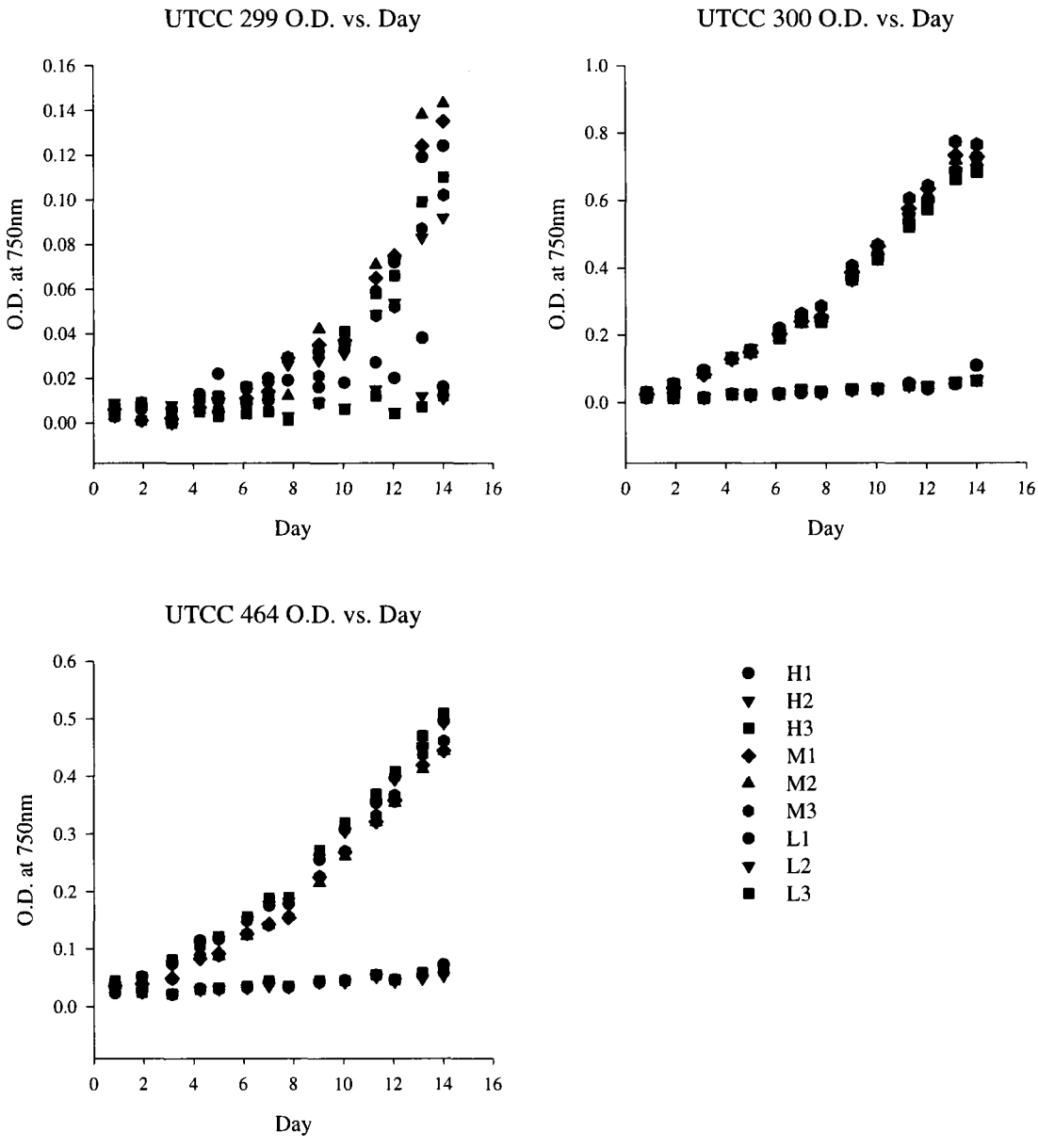


Figure 35. Original growth curves for replicates of each toxigenic strain (UTCC 299, 300, and 464) grown under three light intensities, Low (L), Medium (M), and High (H). Curves were plotted using changes in optical density (O.D.) over time (day).

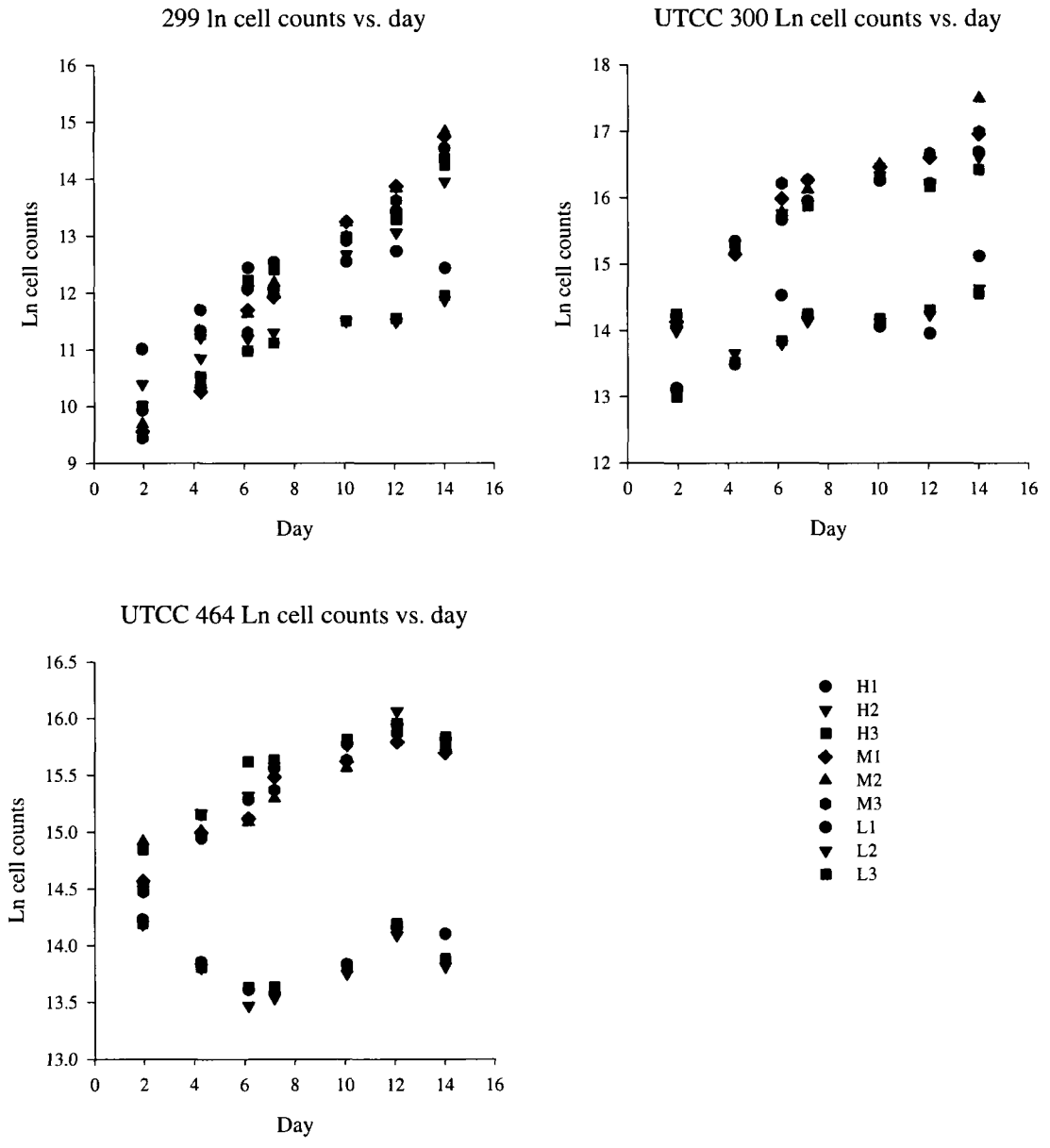


Figure 36. Original growth curves for replicates of each toxigenic strain (UTCC 299, 300, and 464) grown under three light intensities, Low (L), Medium (M), and High (H). Curves were plotted using changes in cell density over time (day).

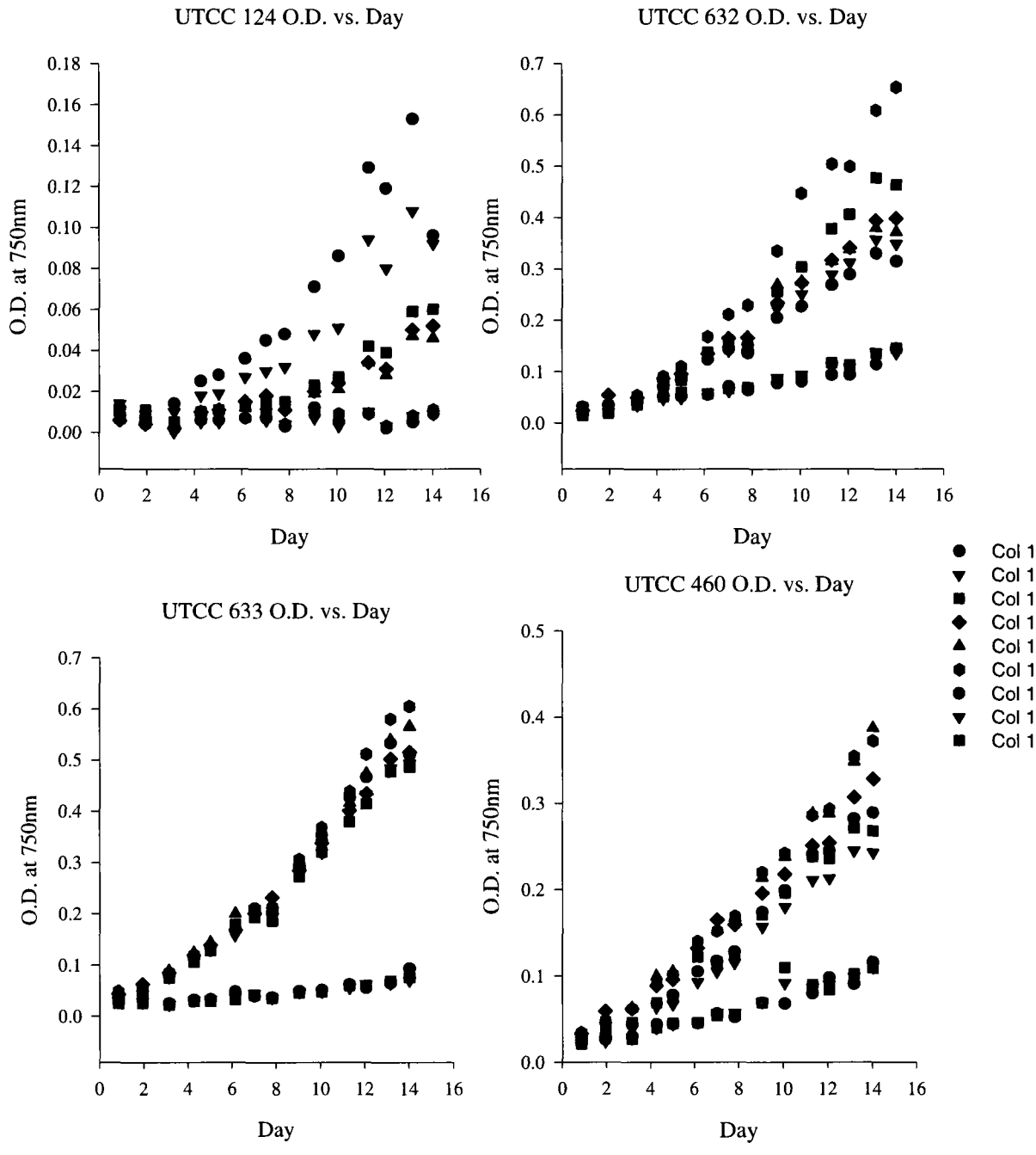


Figure 37. Original growth curves for replicates of each non-toxigenic strain (UTCC 124, 632, 633, and 460) grown under three light intensities, Low (L), Medium (M), and High (H). Curves were plotted using changes in optical density (O.D.) over time (day).

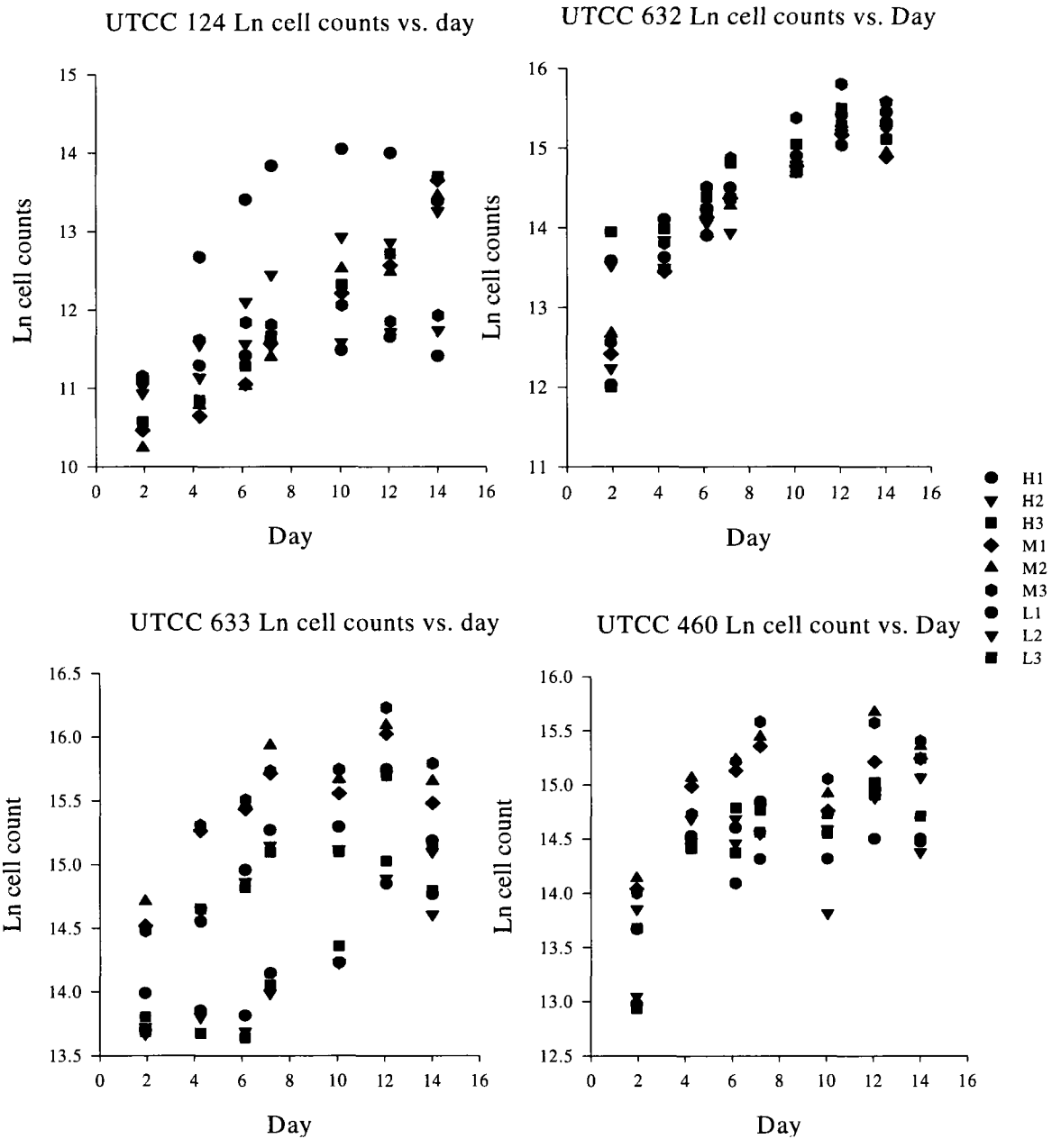


Figure 38. Original growth curves for replicates of each non-toxigenic strain (UTCC 124, 632, 633, and 460) grown under three light intensities, Low (L), Medium (M), and High (H). Curves were plotted using changes in cell density over time (day).

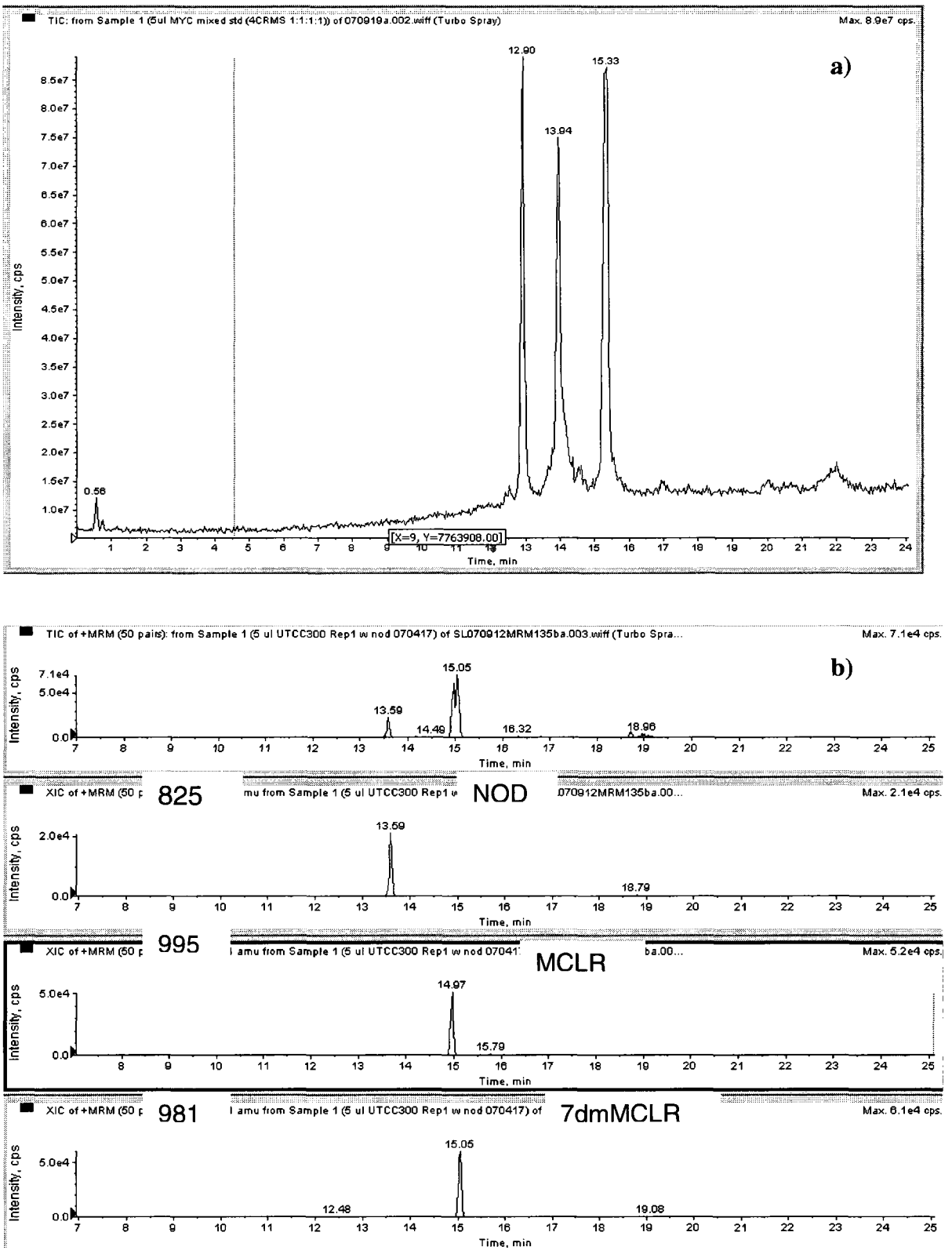
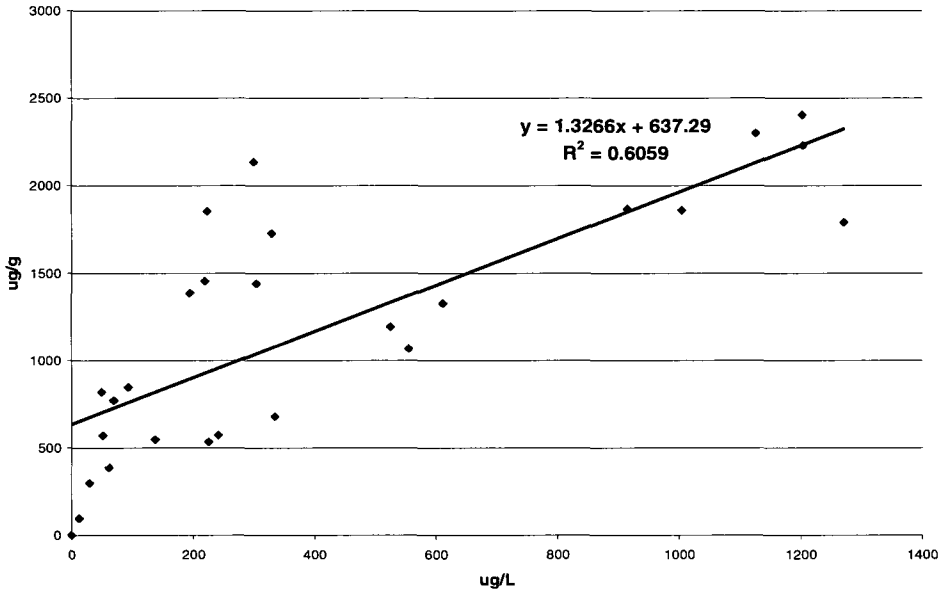


Figure 39. Examples of mass spectrometry analysis from **a)** a mixture of standard microcystins including MC-RR, -LR, 7desmethyl-LR, and nodularin (Nod), and **b)** An example of peaks found in the culture UTCC 300 spiked with nodularin.

APPENDIX IV

a)



b)

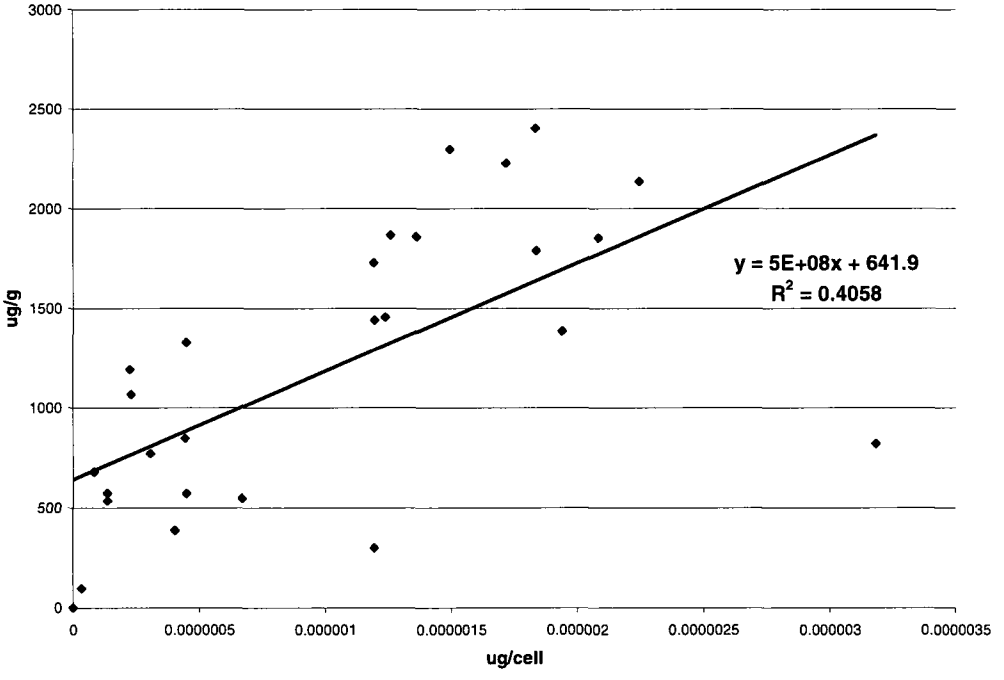


Figure 40. Relationship between total microcystin production when expressed as a) $\mu g \cdot g^{-1}$ versus $\mu g \cdot L^{-1}$, and as b) $\mu g \cdot g^{-1}$ versus $\mu g \cdot cell^{-1}$.

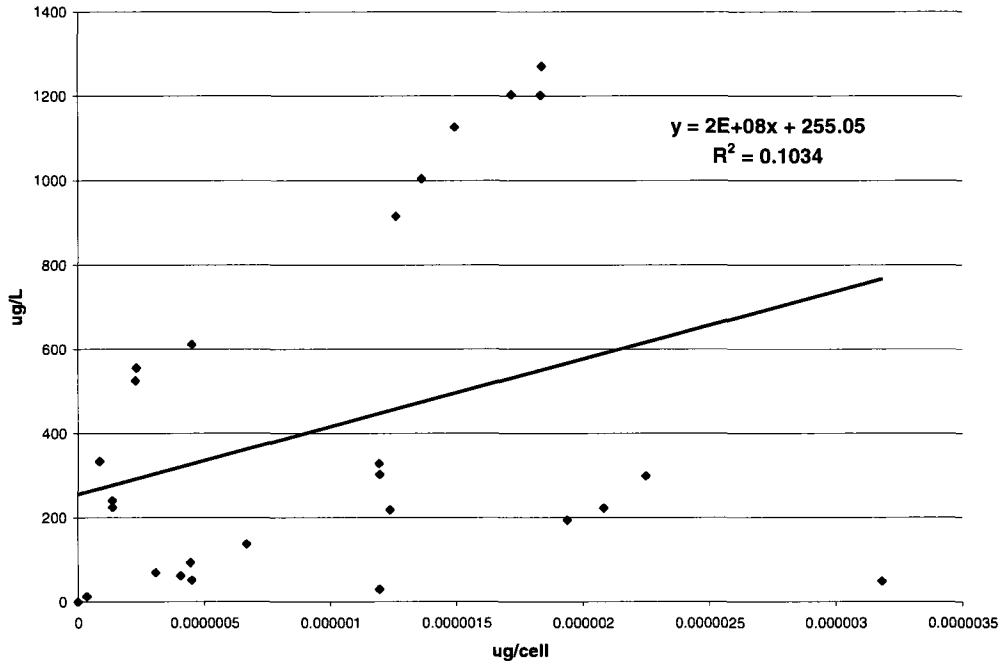


Figure 41. Relationship between total microcystin production when expressed as $\mu g \cdot L^{-1}$ versus $\mu g \cdot cell^{-1}$.

APPENDIX V

	Date : March 11 2008								
	Gene :	mcyD1			Pos. control :	pmcyD1-UTCC299 10-6			
	FRWD Primer:	F1*			Conc. Sample :	10-1 à 10-3			
	REV Primer:	R1*			Primer conc. :	0.8 µM			
	Ann. T°:				MgCl2 conc. :	4.0 mM			
Tube Id	UTCC 300M1D14	Final vol. (µl)	RNAse free water (Qiagen) (µl)	2x QuantiTect SYBR Green PCR (µL)	MgCl2 25 mM (µl)	FRWD Primer 20 µM (µl)	REV Primer 20 µM (µl)	Master mix Vol (µl)	DNA Ext. or Cont. (µl)
1	1 x 10-1	20	2.2	10	1.2	0.8	0.8	15	5
2	1 x 10-2	20	2.2	10	1.2	0.8	0.8	15	5
3	1 x 10-3	20	2.2	10	1.2	0.8	0.8	15	5
4	1 x 10-1	20	2.2	10	1.2	0.8	0.8	15	5
5	1 x 10-2	20	2.2	10	1.2	0.8	0.8	15	5
6	1 x 10-3	20	2.2	10	1.2	0.8	0.8	15	5
Mix 1	Total volume of Master Mix 1		13.86	63	7.56	5.04	5.04		
	UTCC 300M2D14								
7	1 x 10-1	20	2.2	10	1.2	0.8	0.8	15	5
8	1 x 10-2	20	2.2	10	1.2	0.8	0.8	15	5
9	1 x 10-3	20	2.2	10	1.2	0.8	0.8	15	5
10	1 x 10-1	20	2.2	10	1.2	0.8	0.8	15	5
11	1 x 10-2	20	2.2	10	1.2	0.8	0.8	15	5
12	1 x 10-3	20	2.2	10	1.2	0.8	0.8	15	5
Mix 1	Total volume of Master Mix 1		13.86	63	7.56	5.04	5.04		
	UTCC300M3D14								
13	1 x 10-1	20	2.2	10	1.2	0.8	0.8	15	5
14	1 x 10-2	20	2.2	10	1.2	0.8	0.8	15	5
15	1 x 10-3	20	2.2	10	1.2	0.8	0.8	15	5
16	1 x 10-1	20	2.2	10	1.2	0.8	0.8	15	5
17	1 x 10-2	20	2.2	10	1.2	0.8	0.8	15	5
18	1 x 10-3	20	2.2	10	1.2	0.8	0.8	15	5
Mix 1	Total volume of Master Mix 1		13.86	63	7.56	5.04	5.04		

Figure 42. An example of a QPCR run form indicating the samples, the primers and reagents used, and the volumes of each reagent used in a reaction.

APPENDIX VI

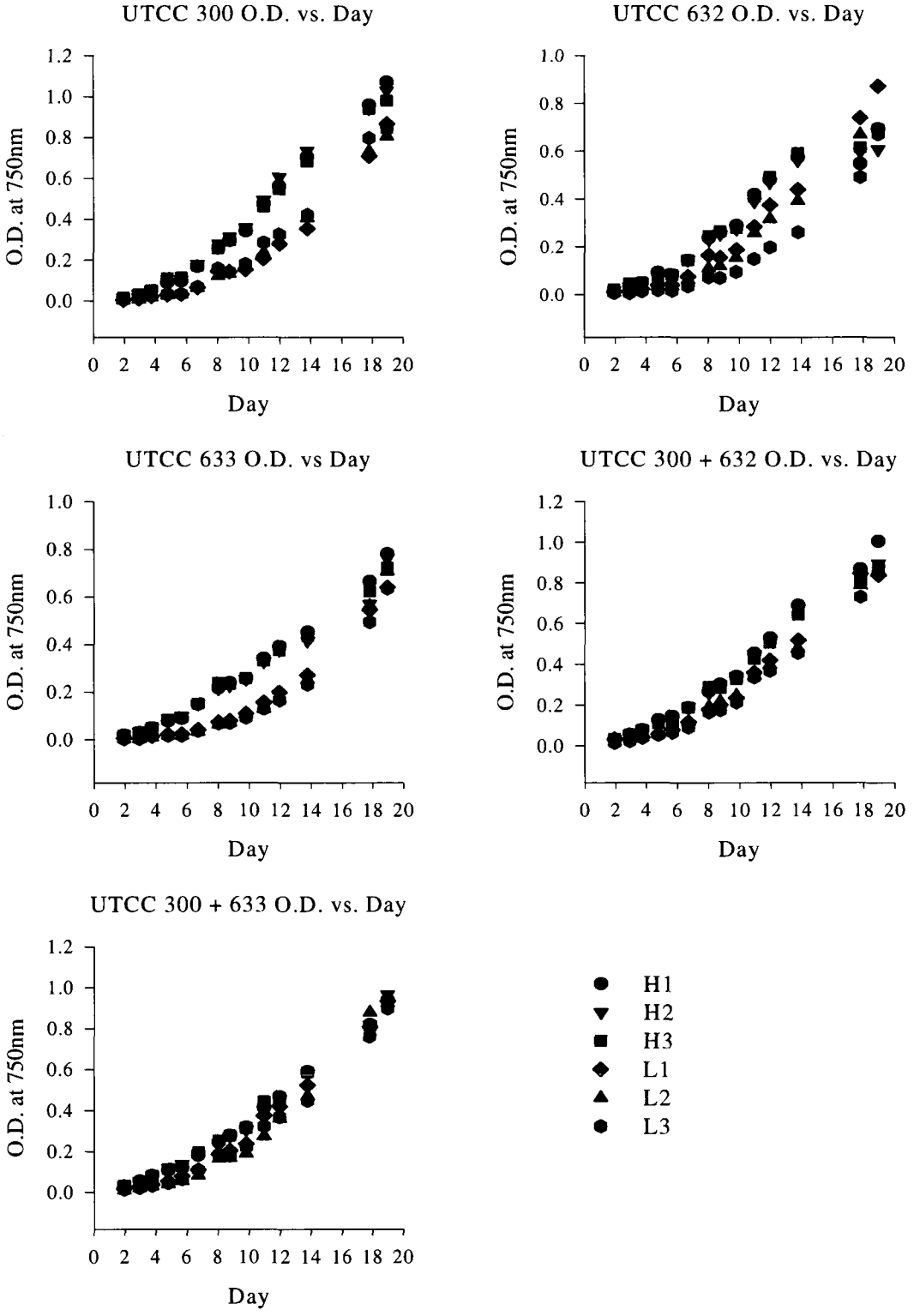

UTCC 300 O.D. vs. Day
UTCC 632 O.D. vs. Day
UTCC 633 O.D. vs Day
UTCC 300 + 632 O.D. vs. Day
UTCC 300 + 633 O.D. vs. Day
O.D. at 750nm
Day
H1
H2
H3
L1
L2
L3

Figure 43. Original growth curves for replicates of the three unialgal (UTCC 300, 632 and 633) and mixed cultures (300+632 and 300+633) grown under two light intensities, Low (L), and High (H). Curves were plotted using changes in optical density (O.D.) over time (day).

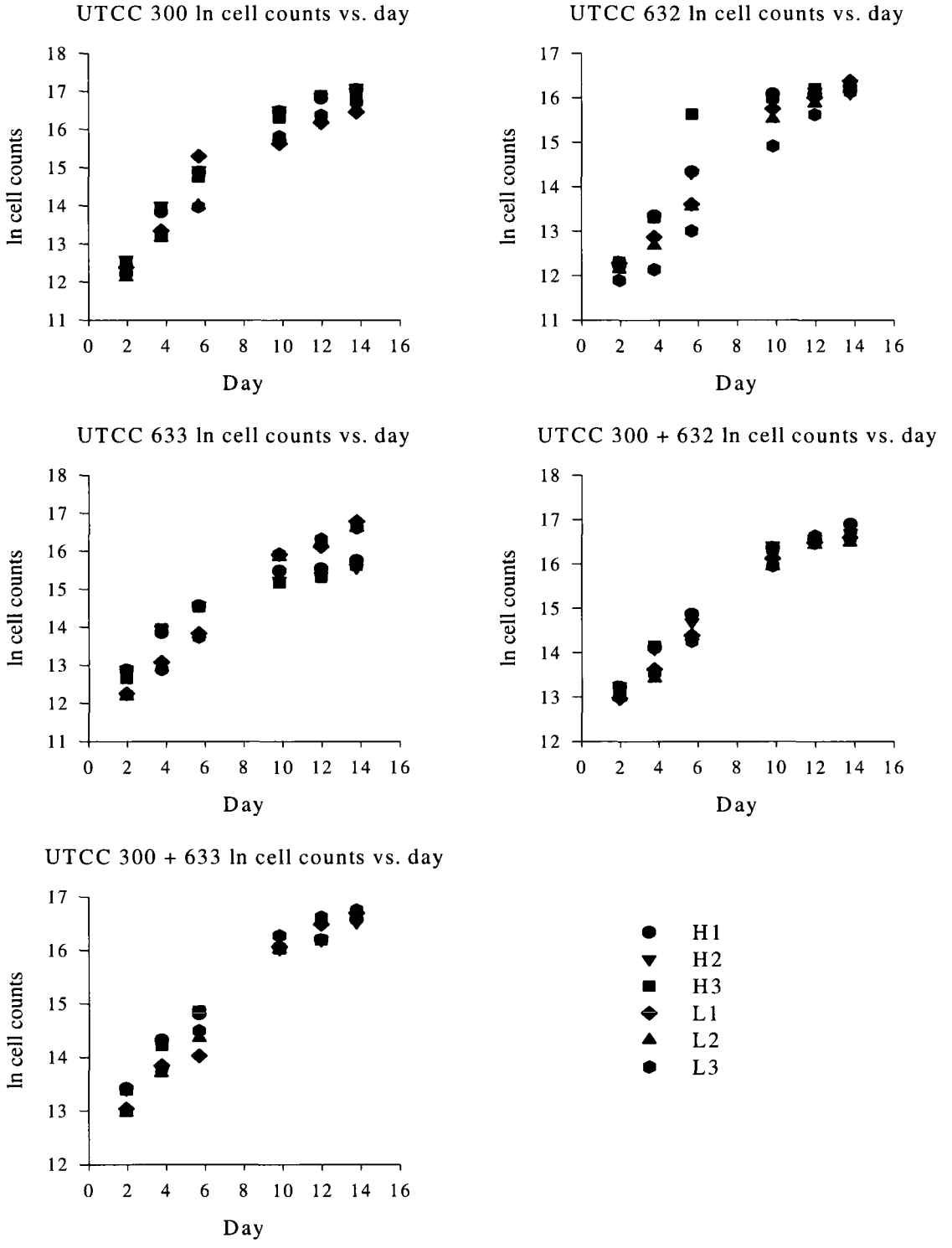
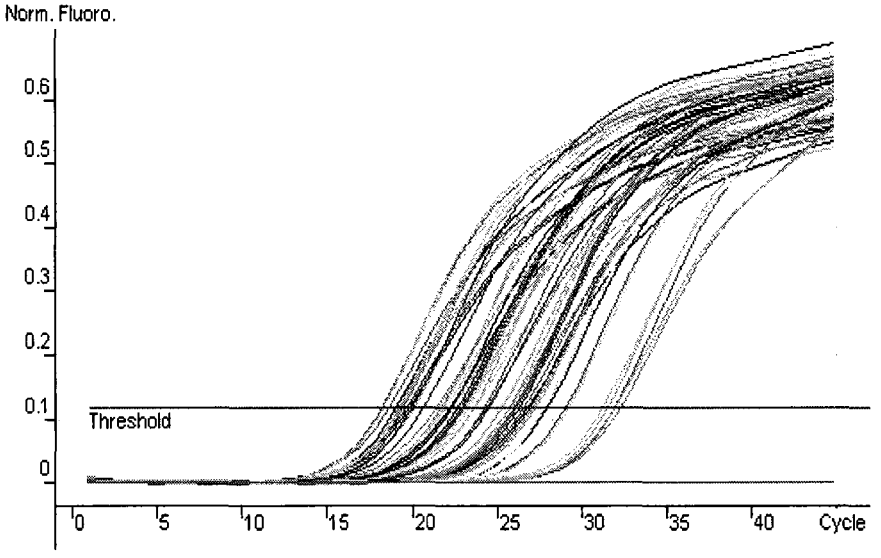


Figure 44. Original growth curves for replicates of the three unialgal (UTCC 300, 632 and 633) and mixed cultures (300+632 and 300+633) grown under two light intensities, Low (L), and High (H). Curves were plotted using changes in cell density over time (day).

APPENDIX VII

a)



b)

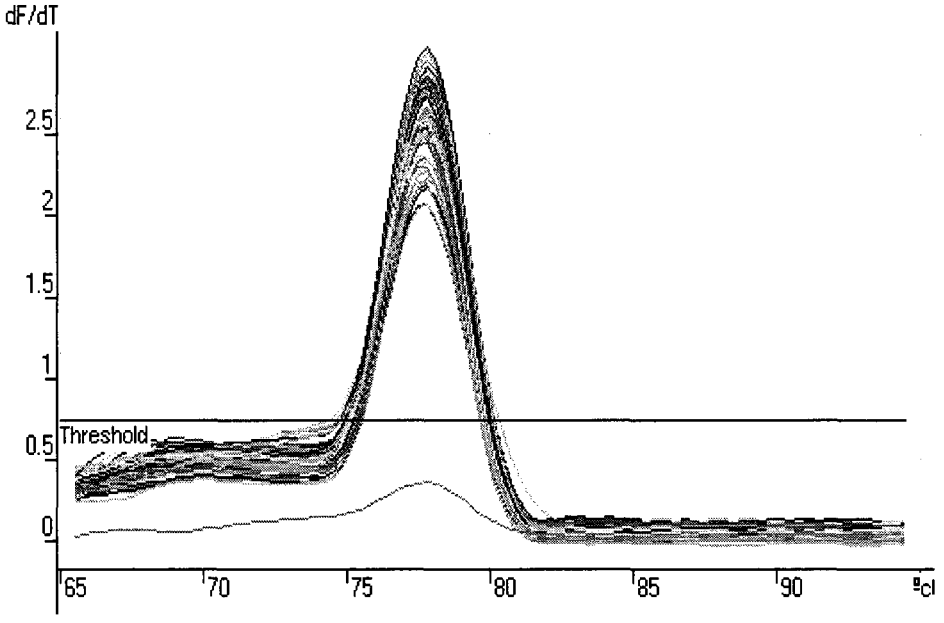
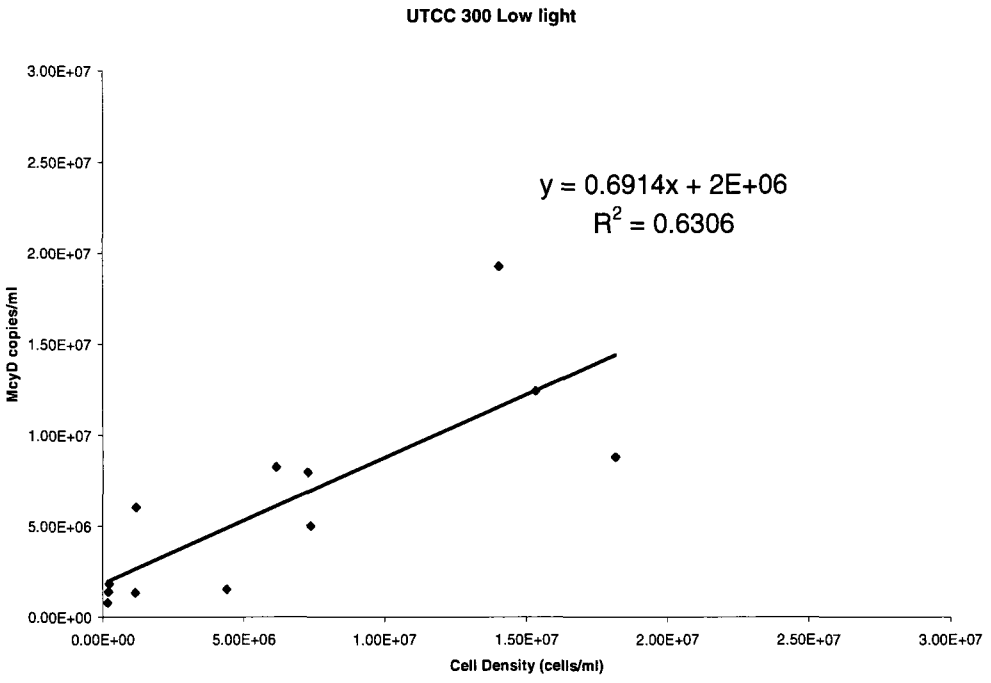


Figure 45. a) Example of a QPCR Critical threshold (Ct) curves with fluorescence increasing over time (represented as 'cycle'). The threshold level is set so as to determine at

which cycle a sample reaches this fluorescence intensity. Ct values can then be compared between samples. **b)** Example of a melting curve analysis. If all amplified products are the same then they should be melting at the same temperature as in this figure.

APPENDIX VIII

a)



b)

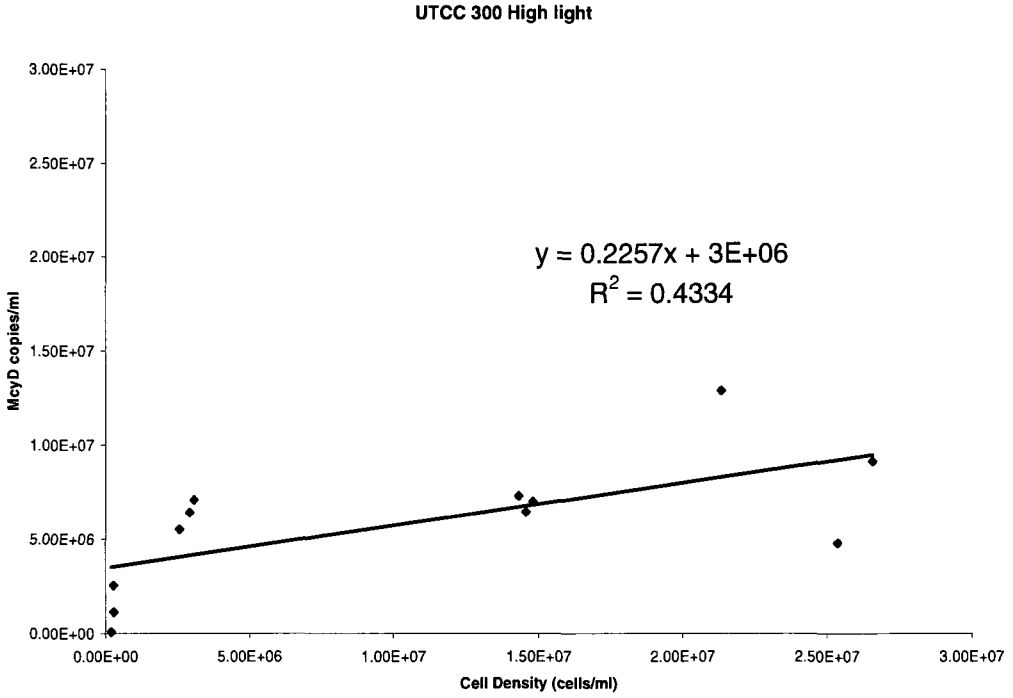
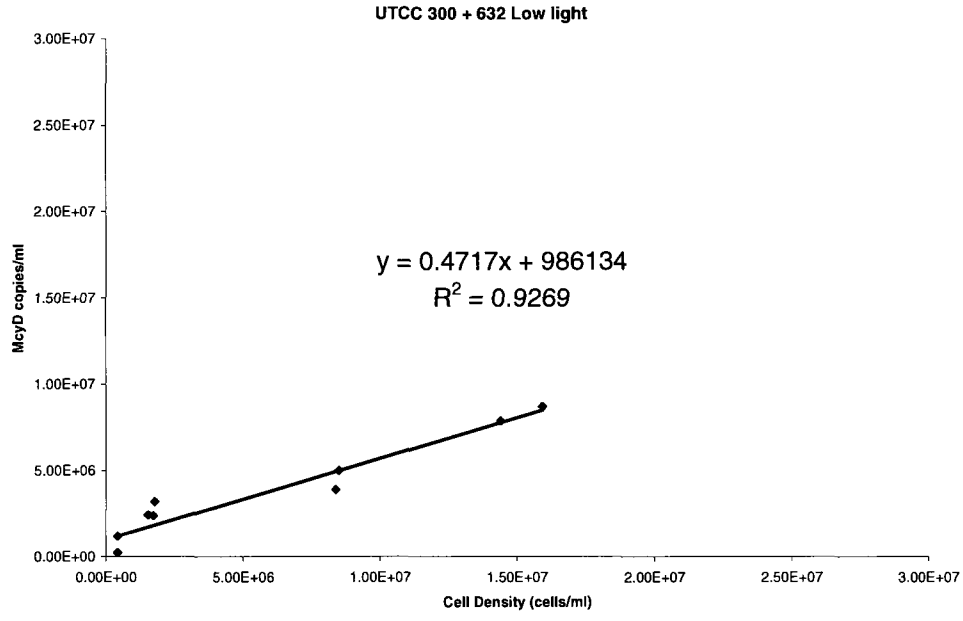


Figure 46. Scatter plots of *mcy*D copies·ml^{-1} versus cell density for UTCC 300 grown under **a)** Low light, and **b)** High light.

a)



b)

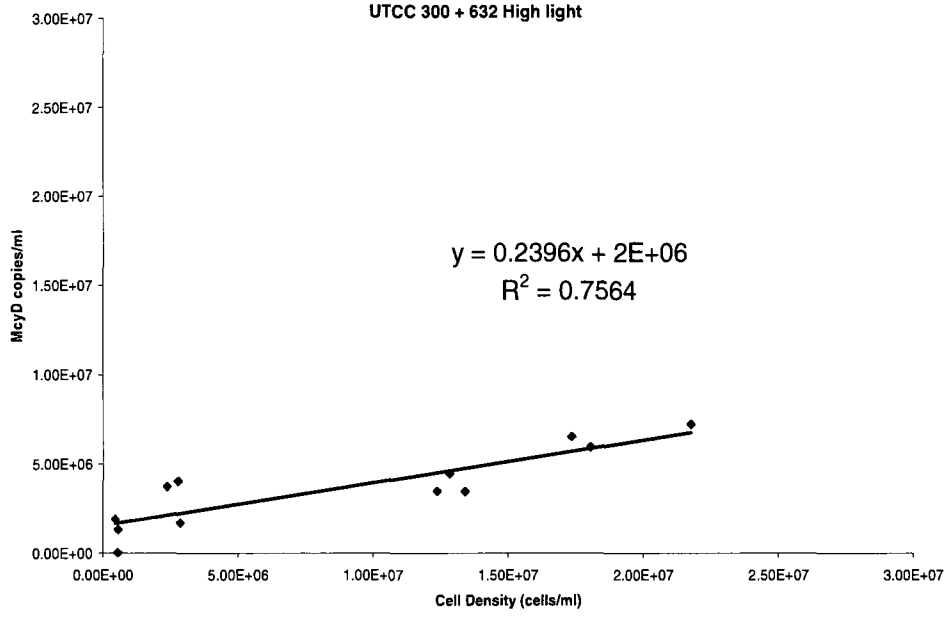


Figure 47. Scatter plots of *mcy*D copies·ml^{-1} versus cell density for UTCC 300+632 grown under **a)** Low light, and **b)** High light.

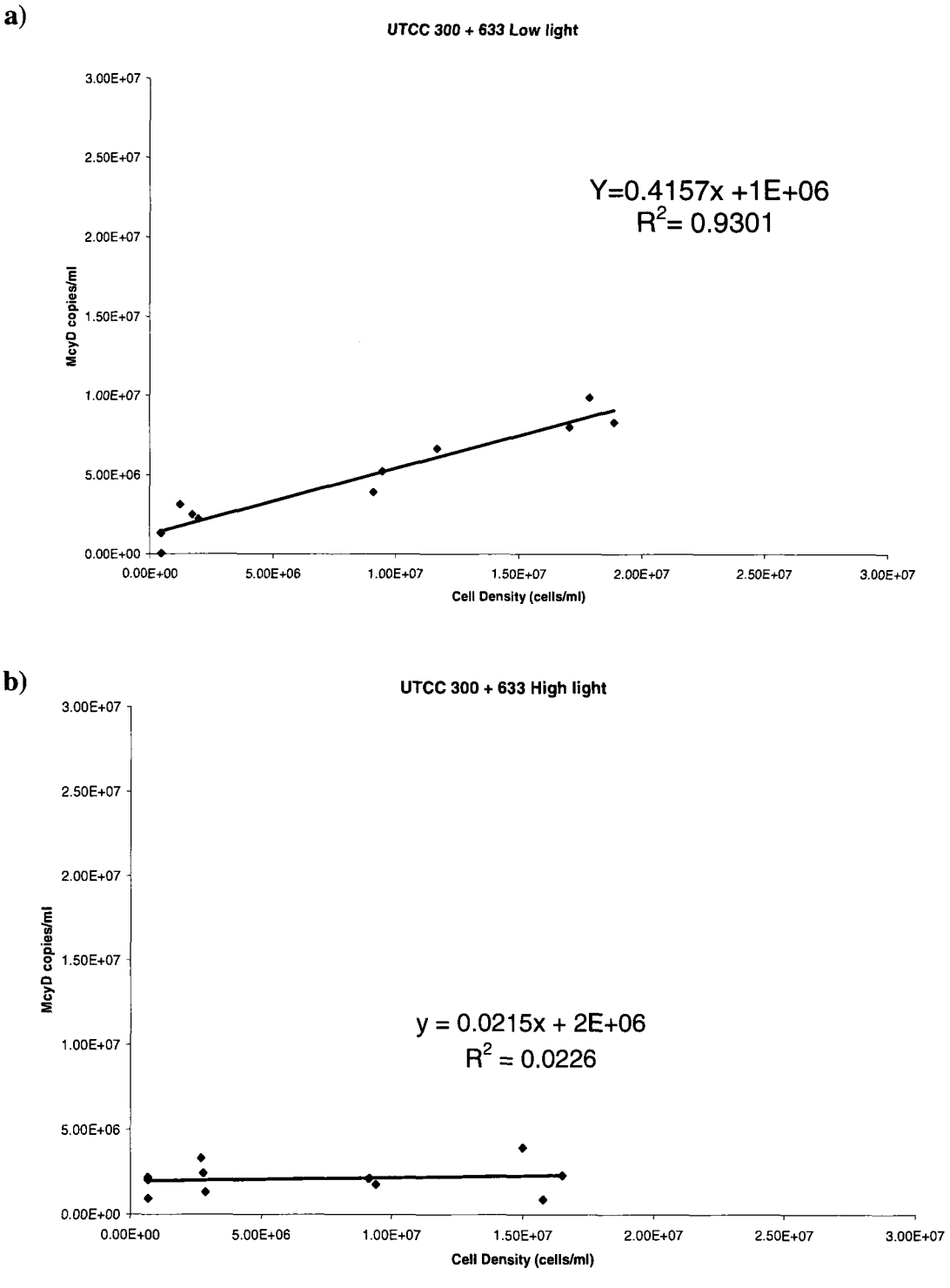


Figure 48. Scatter plots of *mcy*D copies·ml^{-1} versus cell density for UTCC 300+633 grown under **a)** Low light, and **b)** High light.

APPENDIX IX

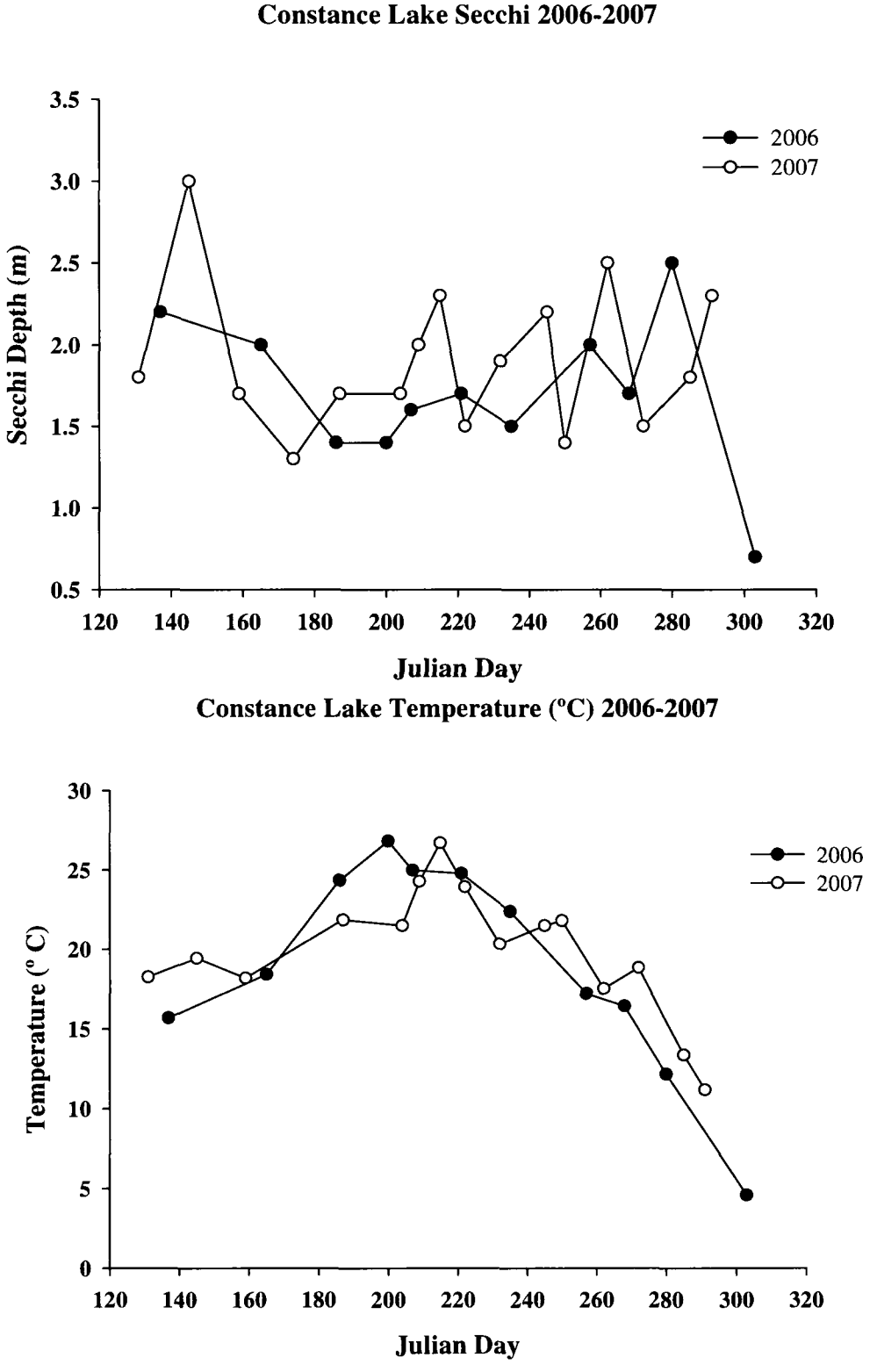


Figure 49. Secchi Depth and Temperature for Constance Lake during May-October of 2006 and 2007.

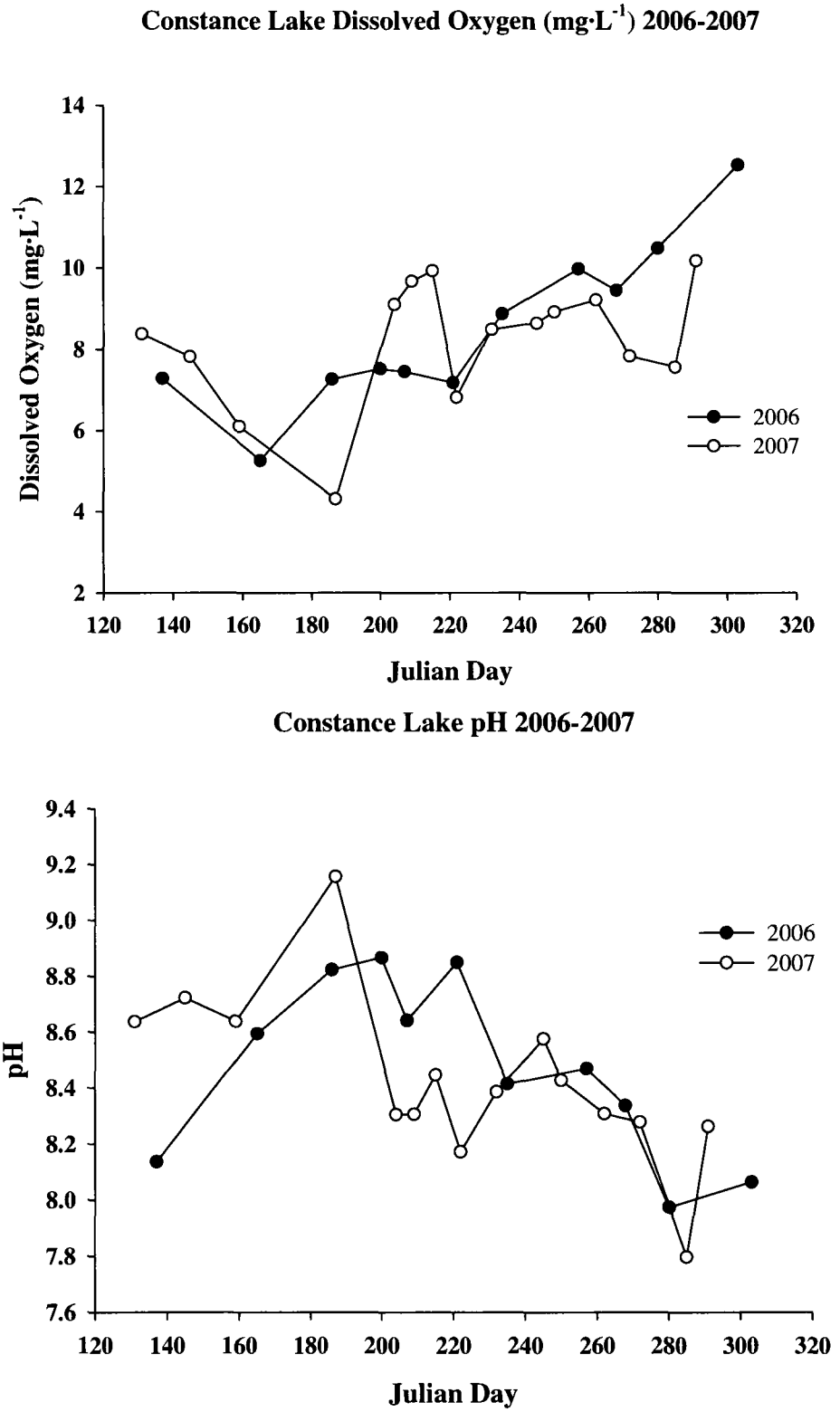
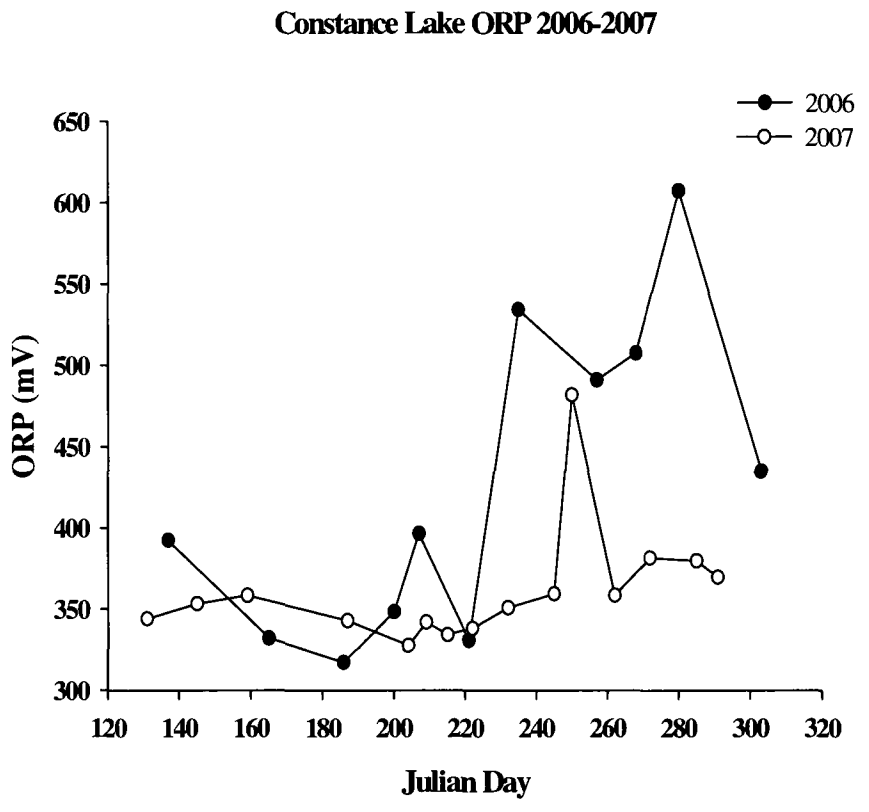


Figure 50. Dissolved Oxygen and pH for Constance Lake during May-October of 2006 and 2007.



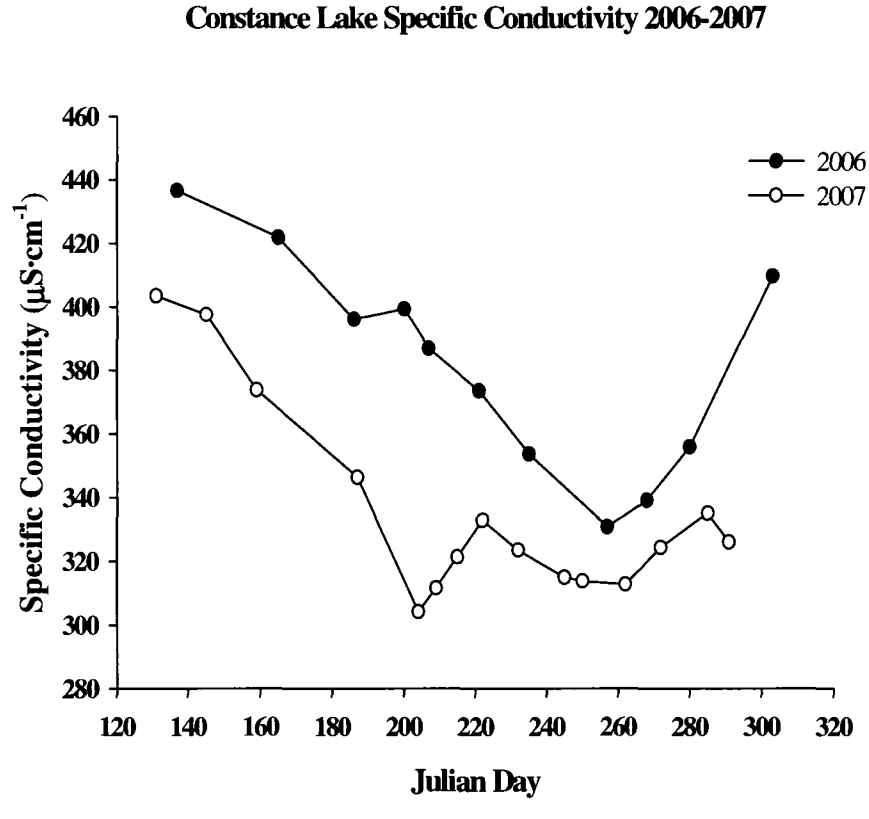


Figure 51. Oxidative-Reductive Potential (ORP) and Specific Conductivity (SPC) for Constance Lake during May-October of 2006 and 2007.

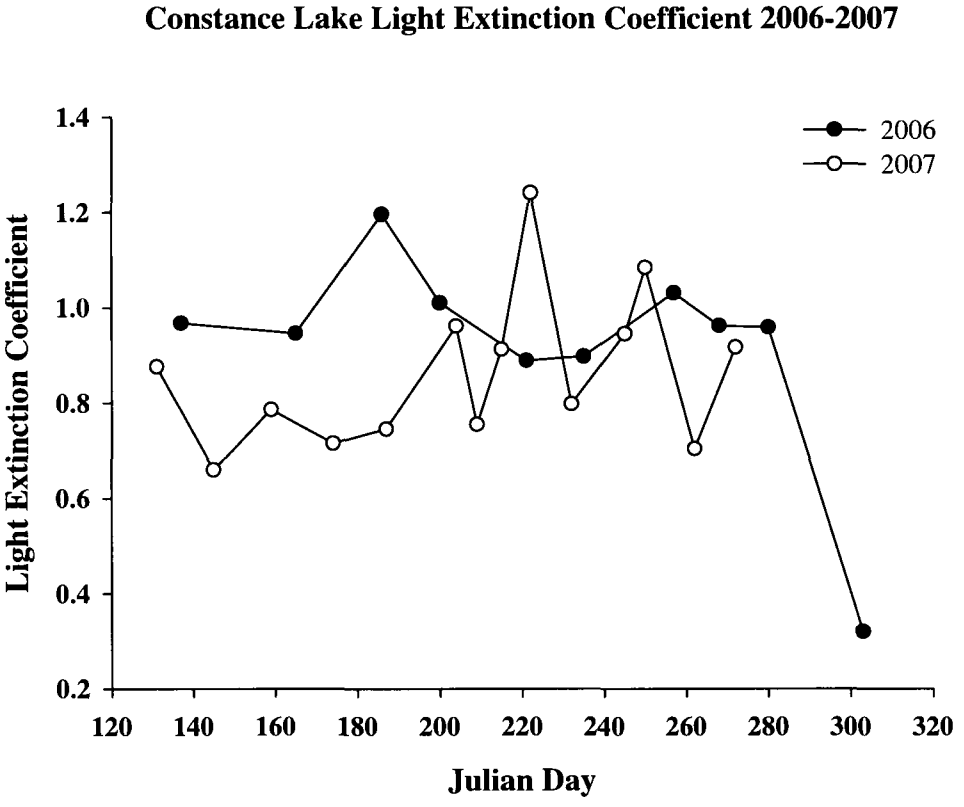


Figure 52. Light Extinction Coefficients for Constance Lake during May-October of 2006 and 2007.

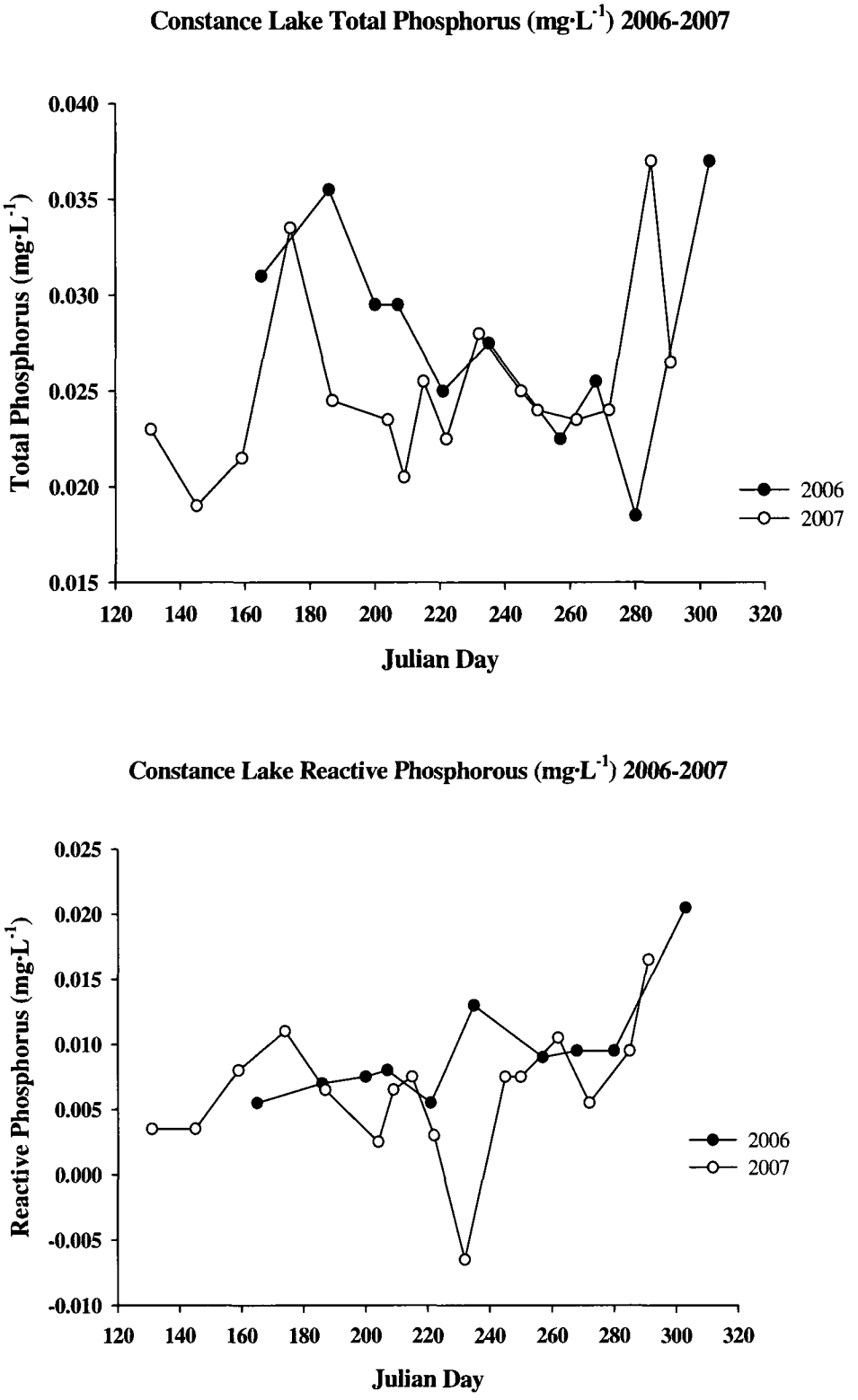


Figure 53. Total Phosphorus and Reactive Phosphorus for Constance Lake during May-October of 2006 and 2007.

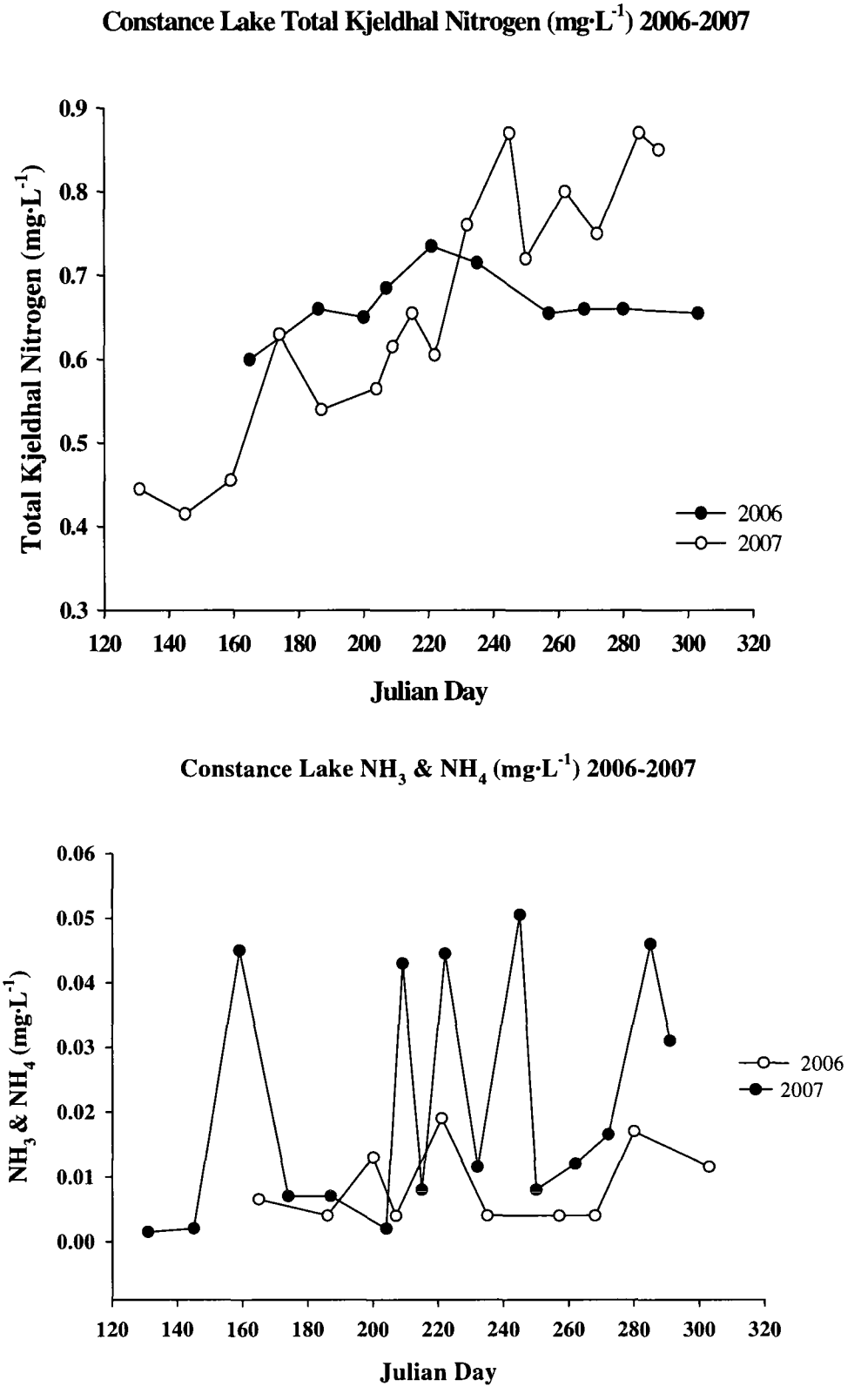


Figure 54. Total Kjeldahl Nitrogen and Ammonia and Ammonium for Constance Lake during May-October of 2006 and 2007.

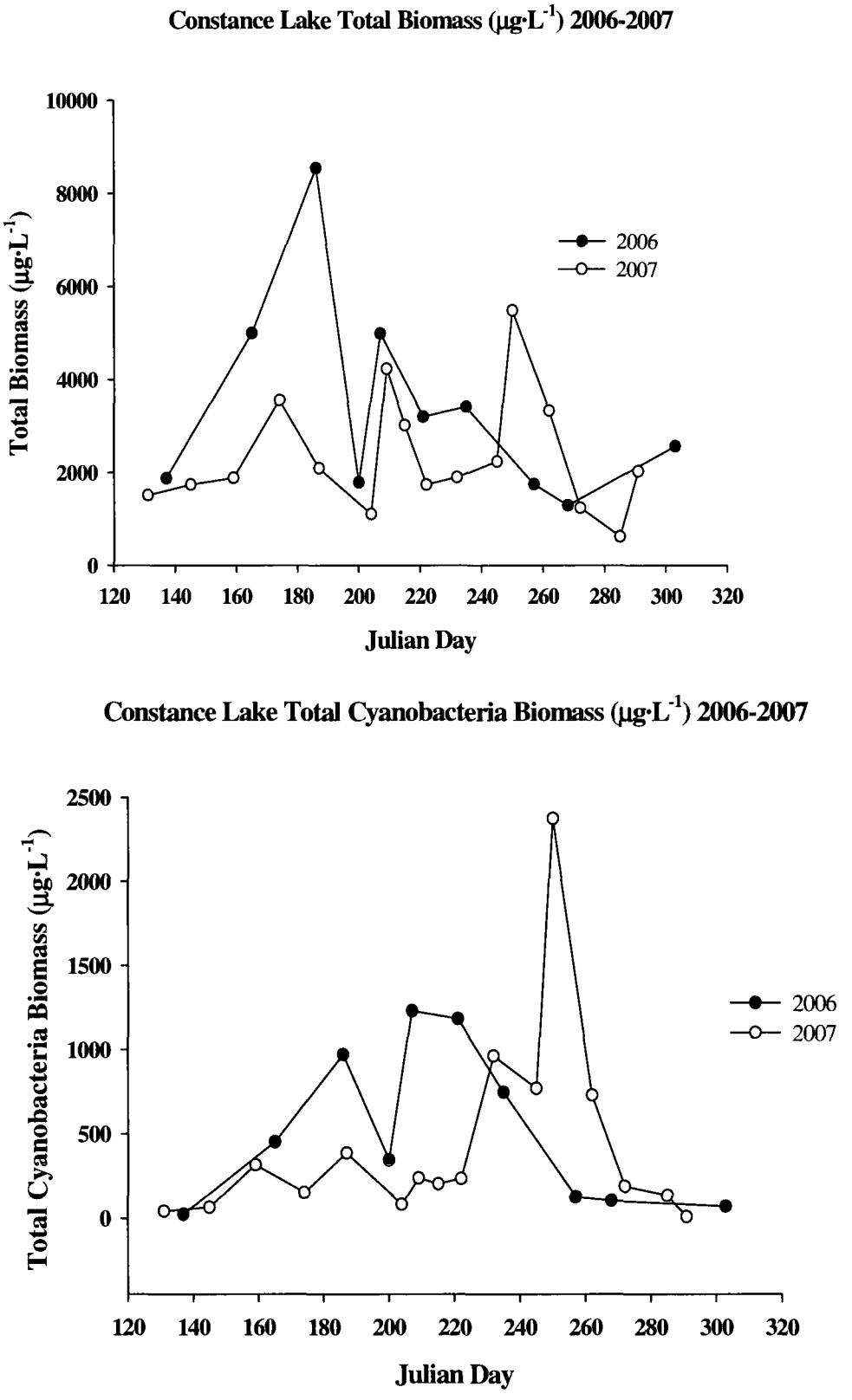


Figure 55. Total Biomass and Total Cyanobacteria Biomass for Constance Lake during May-October of 2006 and 2007.

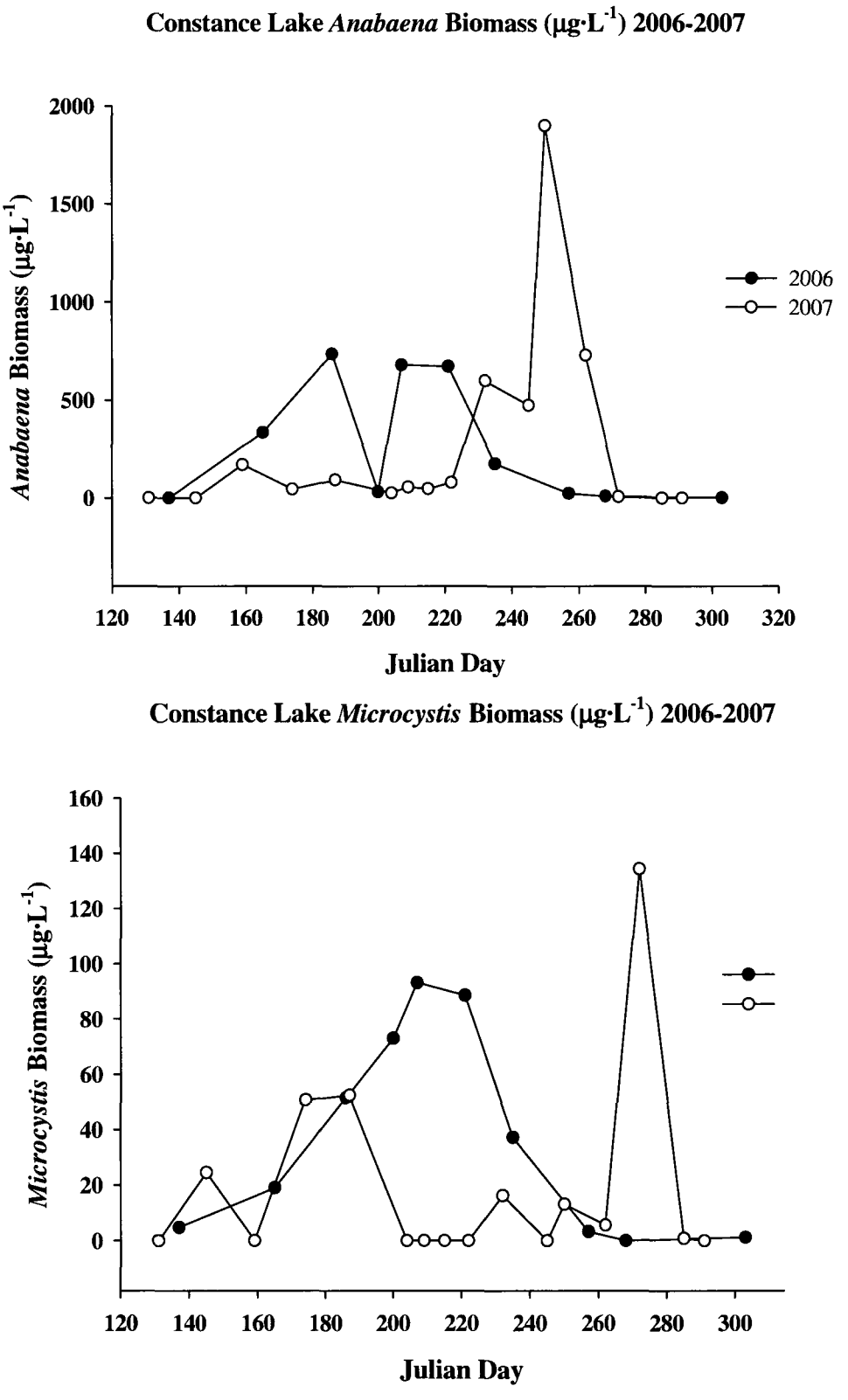
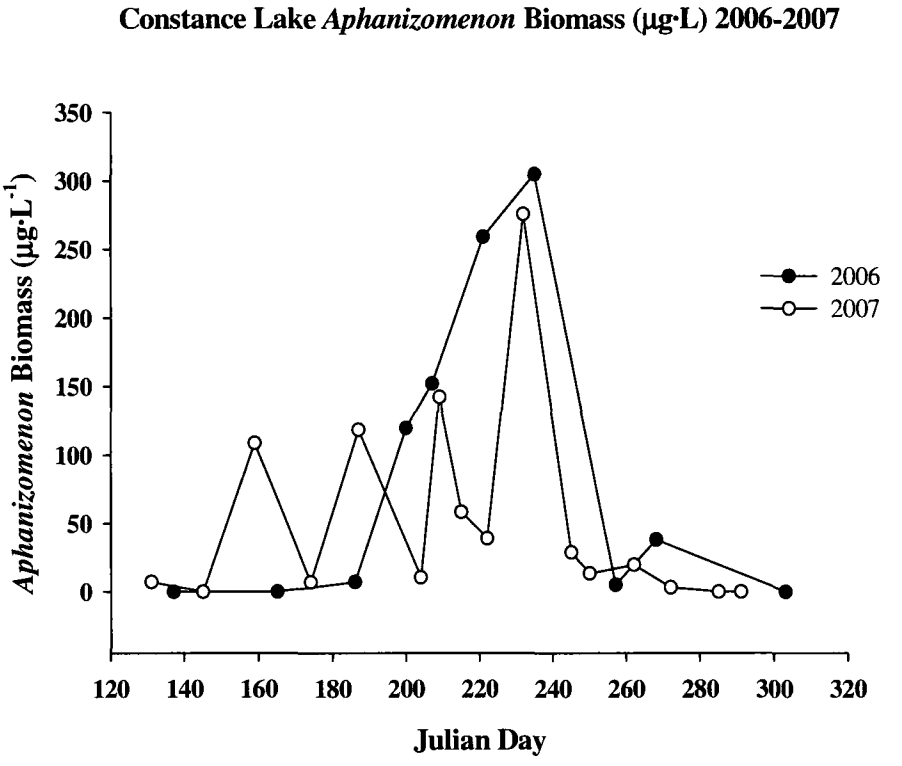


Figure 56. *Anabaena* and *Microcystis* Biomass for Constance Lake during May-October of 2006 and 2007.



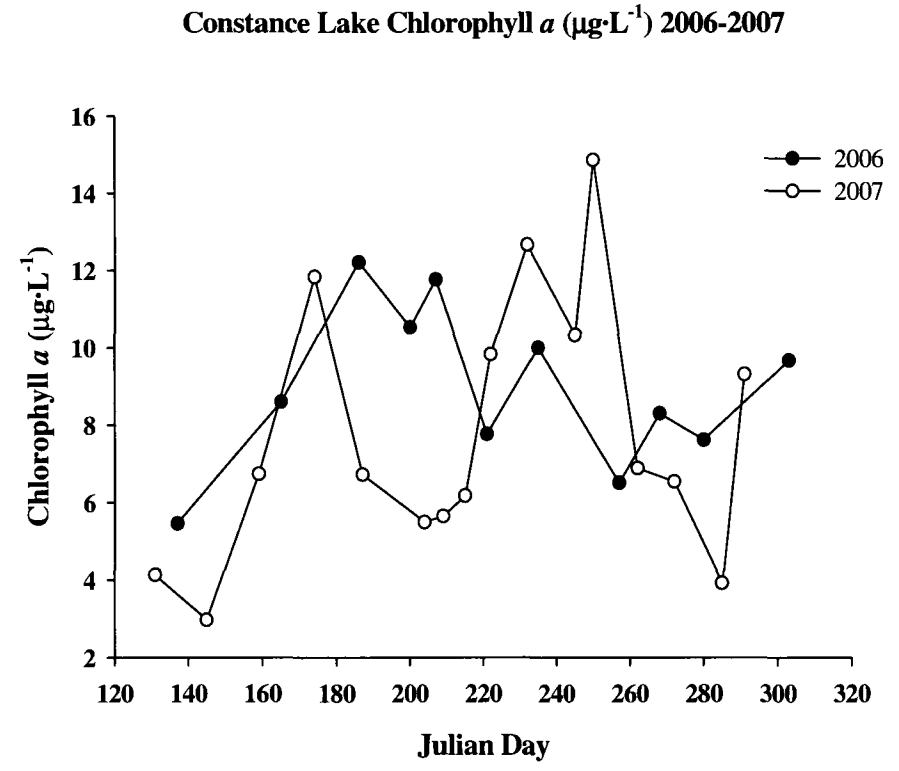


Figure 57. *Aphanizomenon* Biomass and Chlorophyll *a* for Constance Lake during May-October of 2006 and 2007.

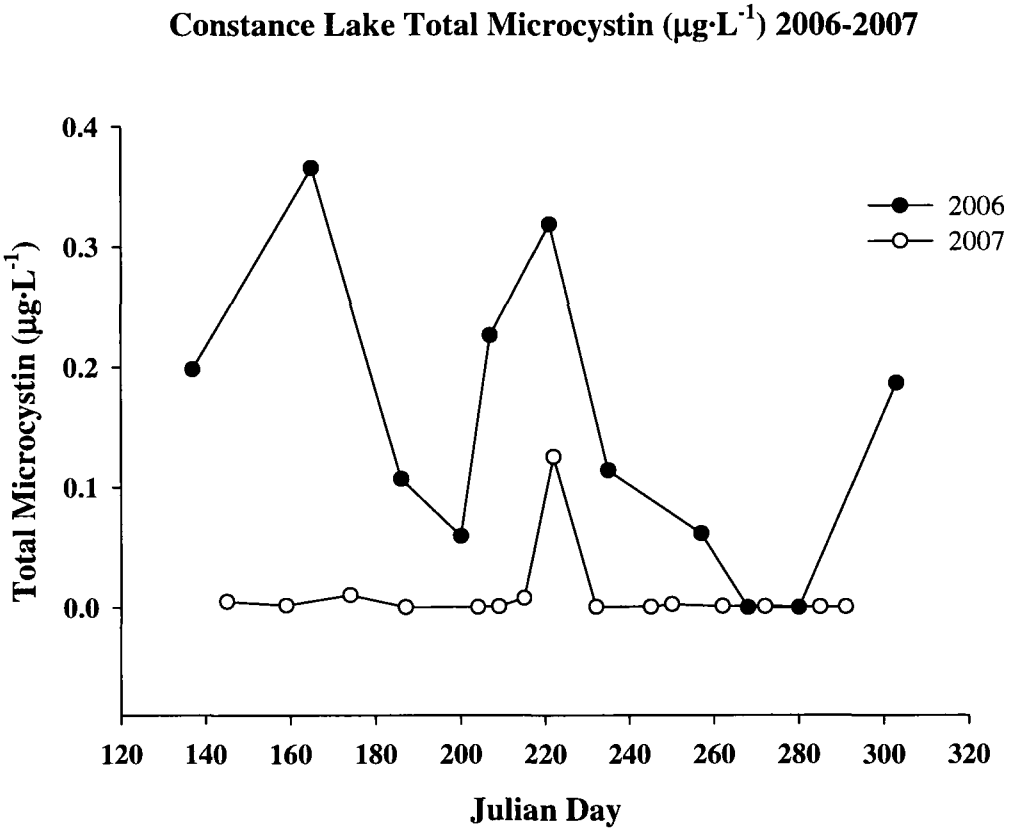


Figure 58. Total Microcystin ($\mu g \cdot L^{-1}$) for Constance Lake during May-October of 2006 and 2007.

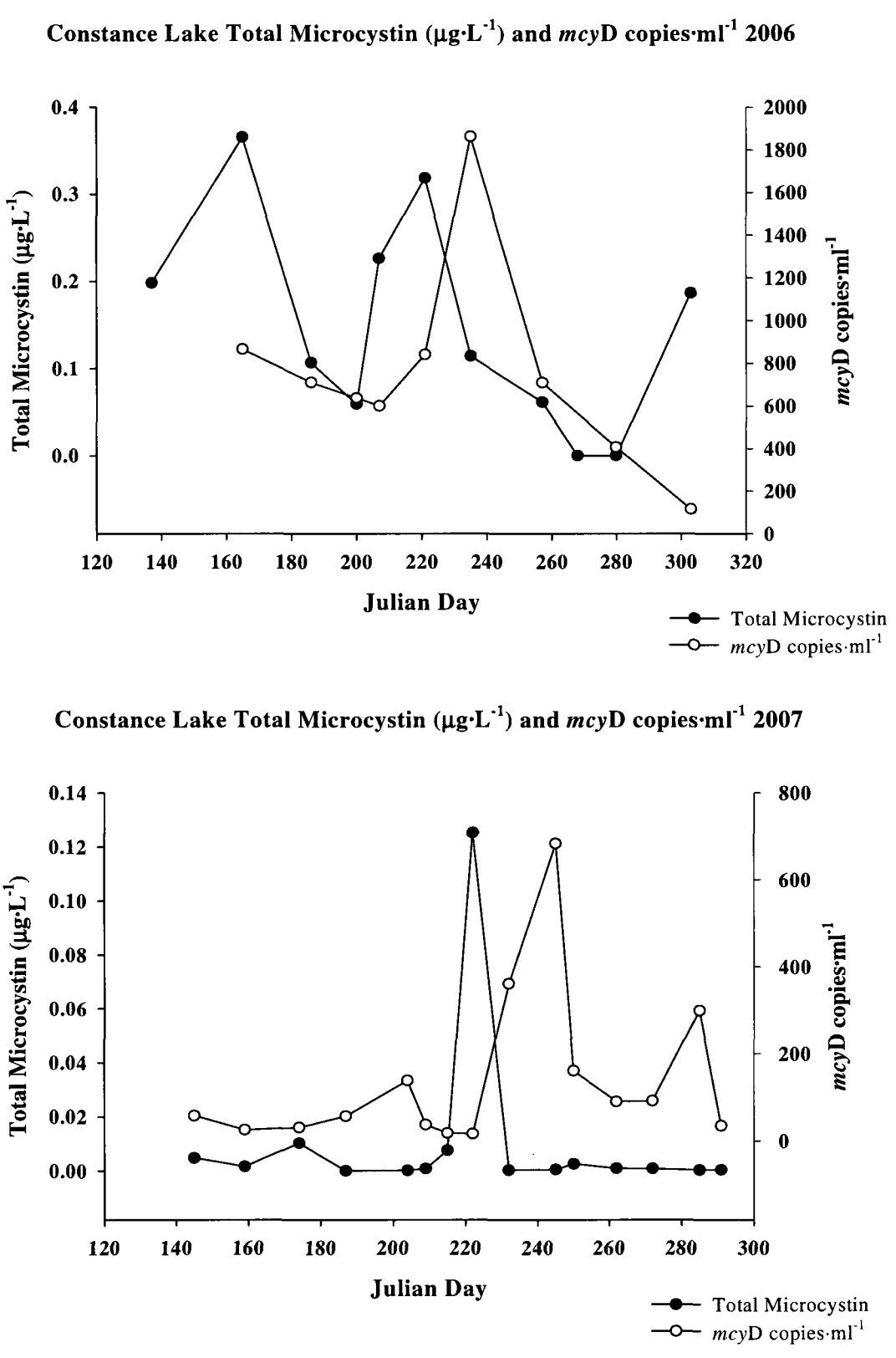
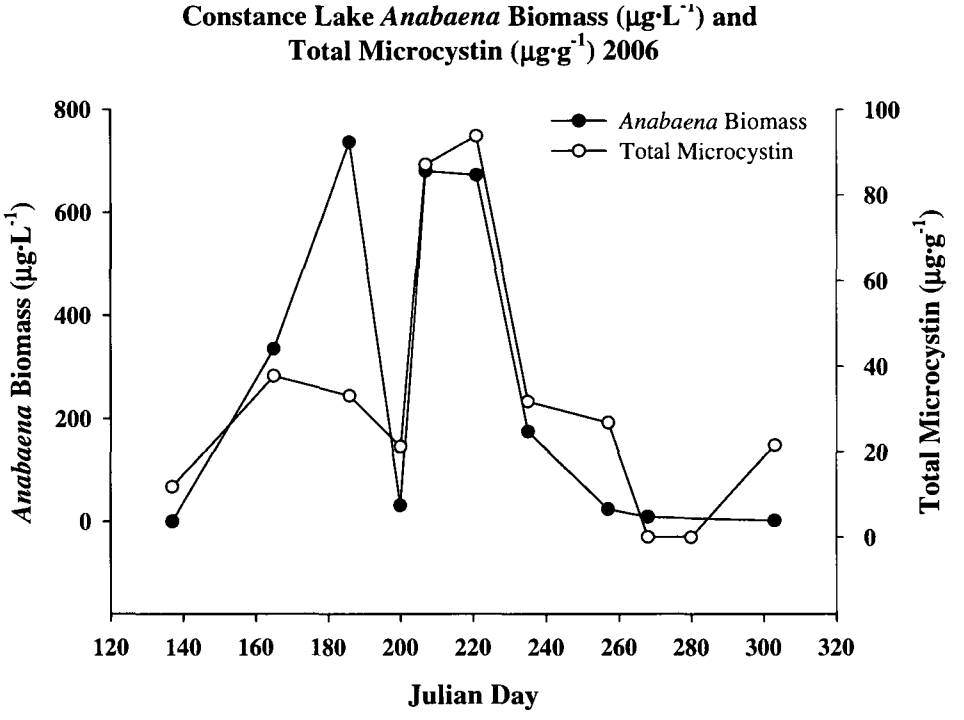


Figure 59. Total Microcystin (µg·L^{-1}) plotted with *mcy*D copies·ml^{-1} for Constance Lake during May-October of 2006 and 2007.



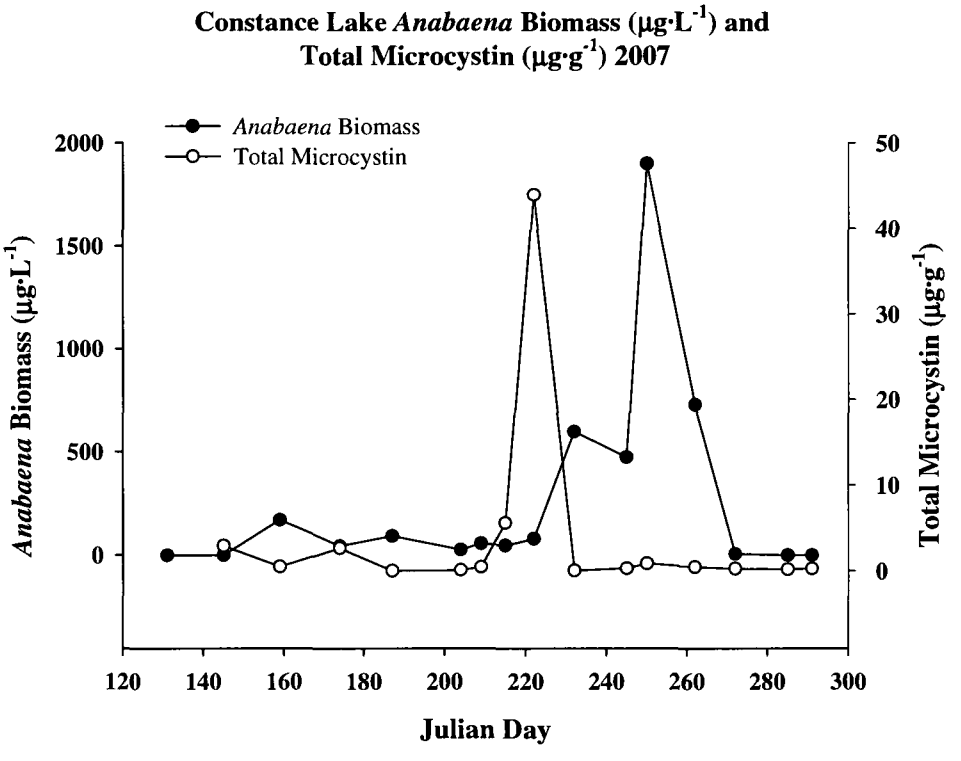


Figure 60. Total Microcystin (µg·g^{-1}) plotted with *Anabaena* biomass (µg·L^{-1}) for Constance Lake during May-October of 2006 and 2007.

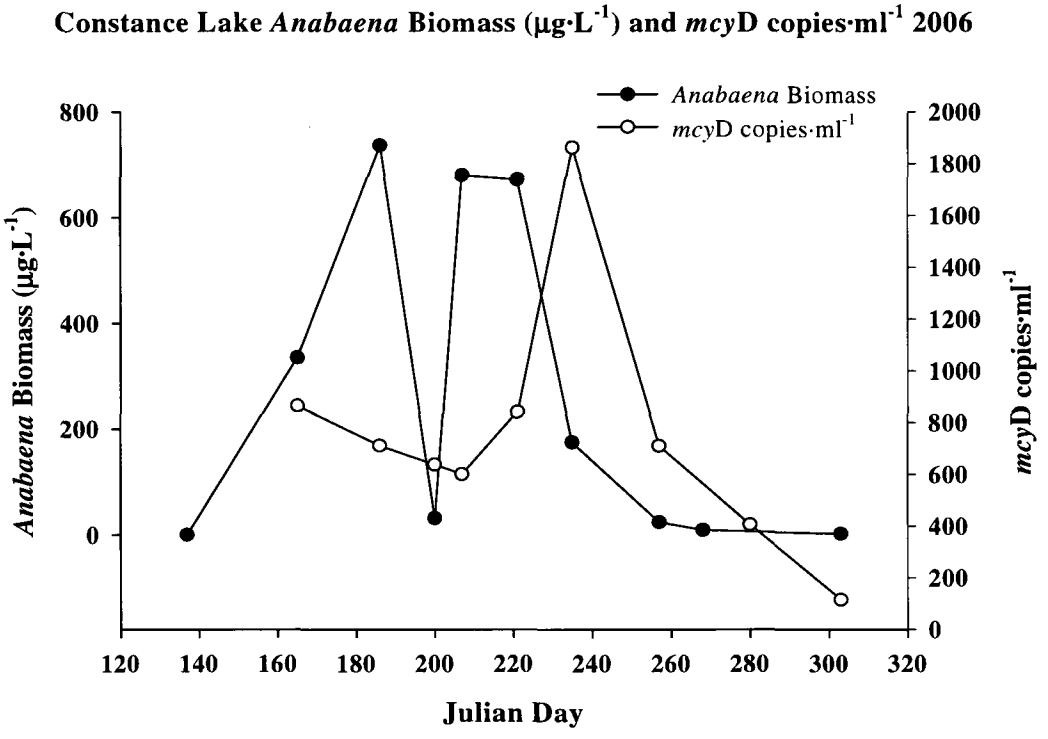


Figure 61. *mcy*D copes· ml^{-1} plotted with *Anabaena* biomass for Constance Lake during May-October of 2006 and 2007.

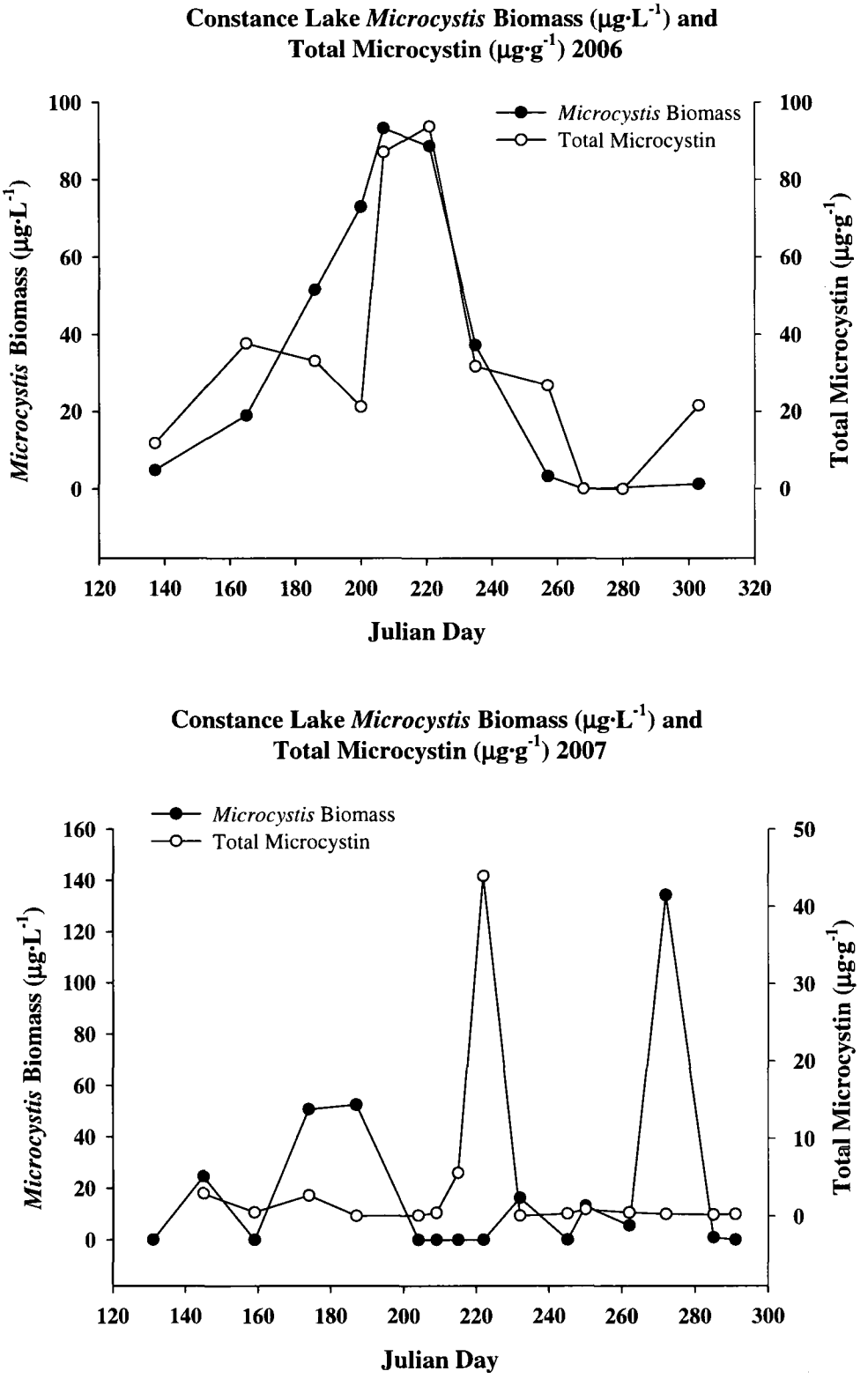


Figure 62. Total Microcystin (µg·g^{-1}) plotted with *Microcystis* biomass (µg·L^{-1}) for Constance Lake during May-October of 2006 and 2007.

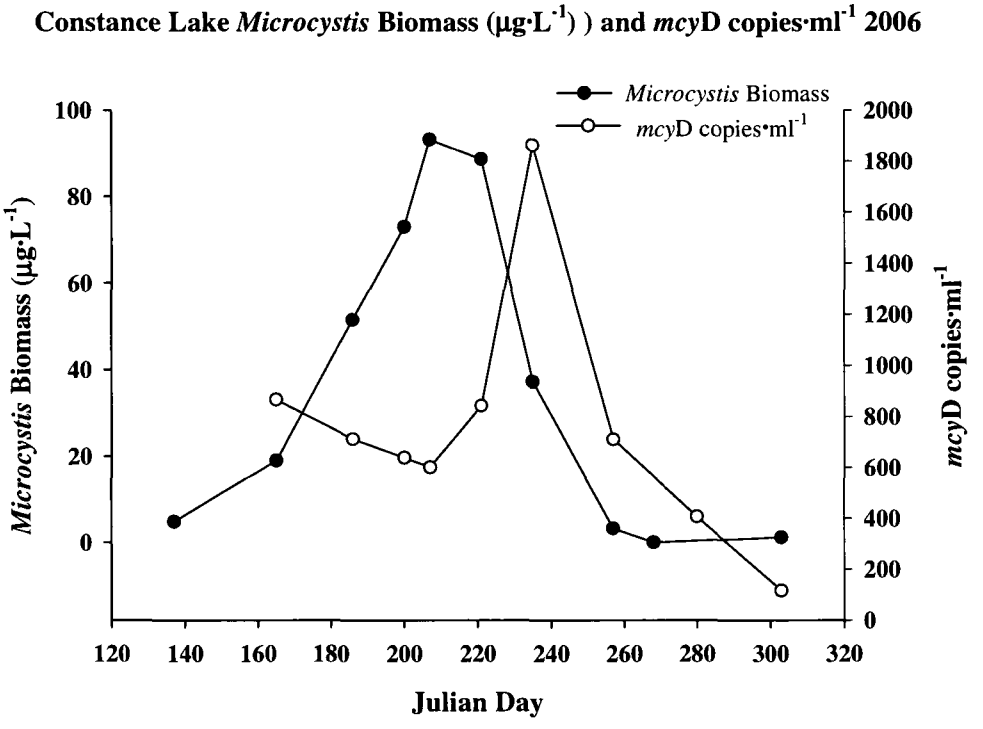
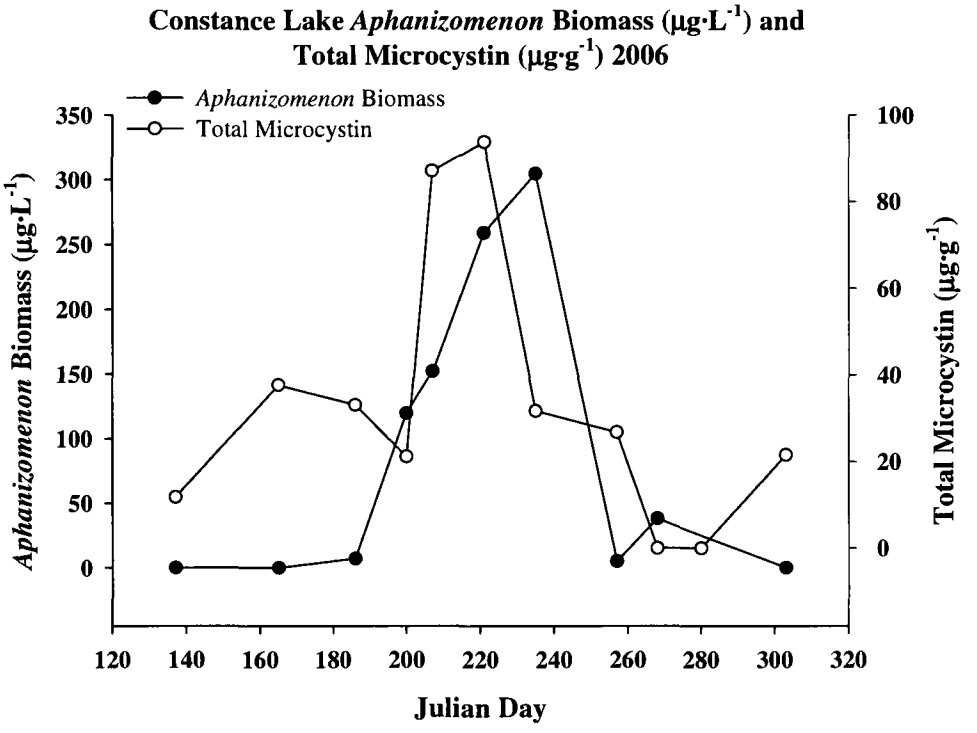


Figure 63. *Microcystis* biomass (µg·L^{-1}) plotted with *mcy*D copies·ml^{-1} for Constance Lake during May-October of 2006 and 2007.



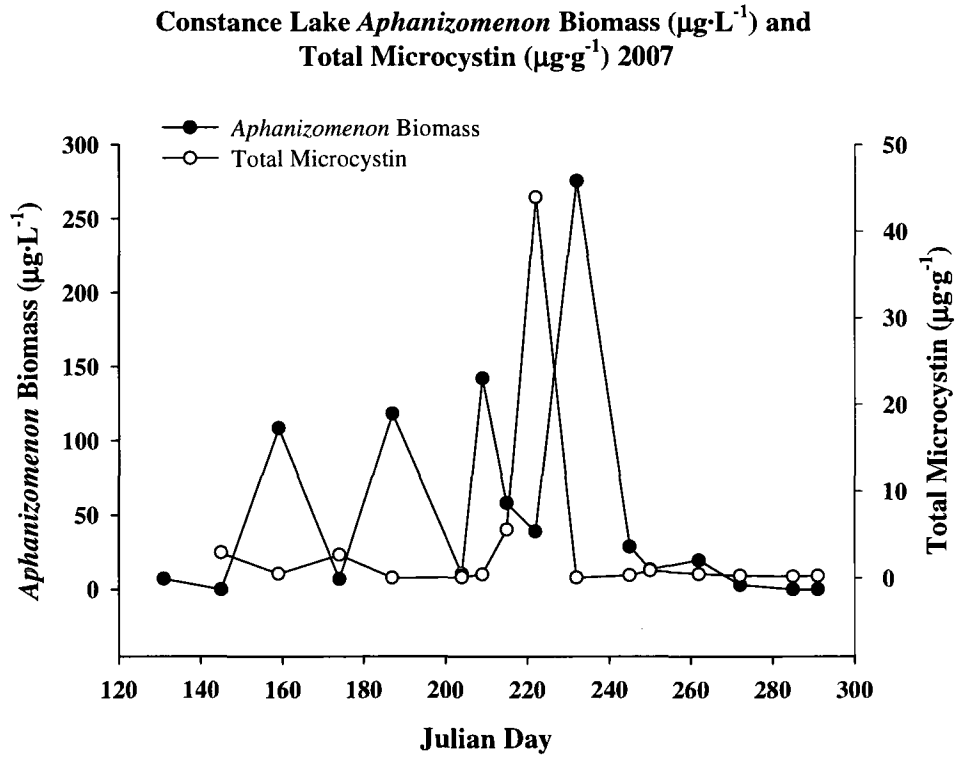
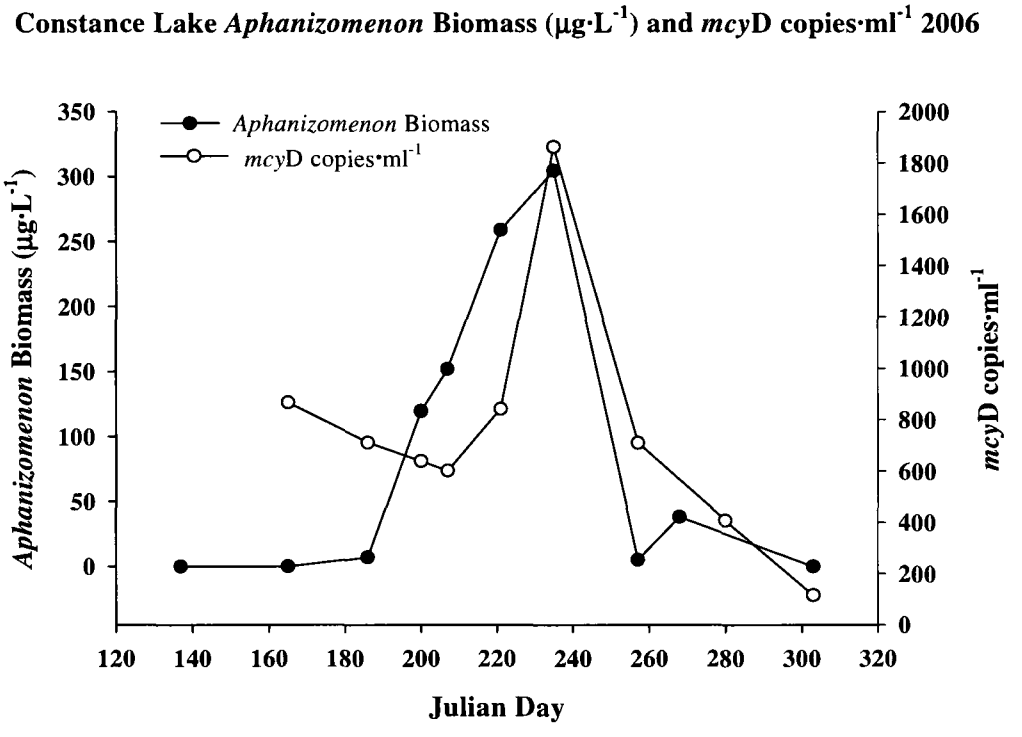


Figure 64. *Aphanizomenon* biomass plotted with Total Microcystin (µg·g^{-1}) for Constance Lake during May-October of 2006 and 2007.



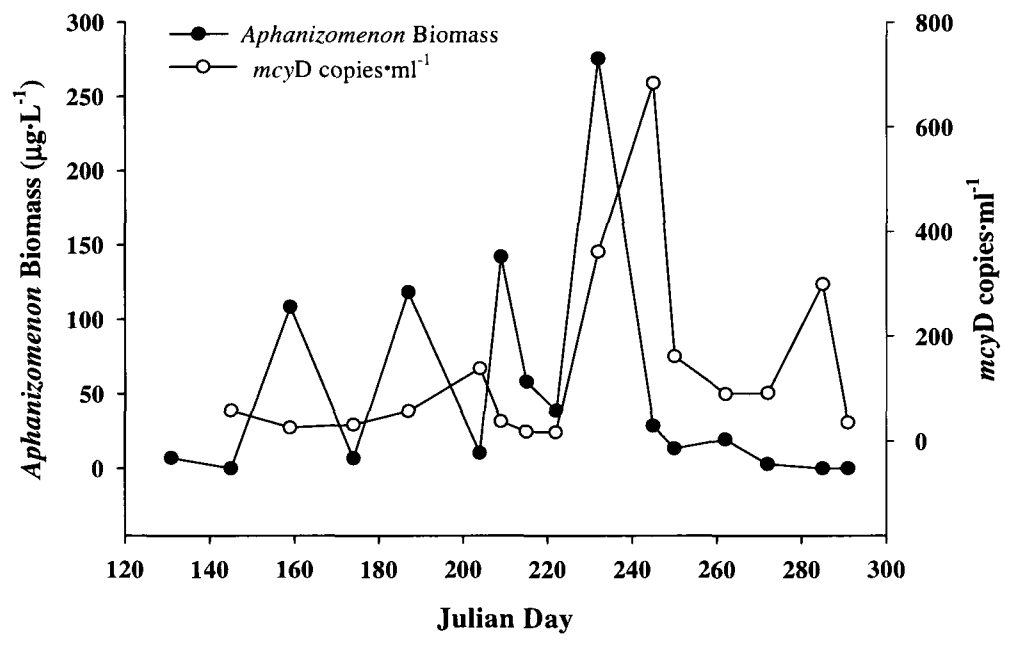


Figure 65. *Aphanizomenon* biomass (µg·L^{-1}) plotted with *mcy*D copies·ml^{-1} for Constance Lake during May-October of 2006 and 2007.

Table 18. Cyanobacterial species present in Constance Lake in the summers of 2006 and 2007.

Order	*2006*	*2007*
Nostocales	*Anabaena circinalis* *Anabaena flos-aquae* *Anabaena solitaria solitaria* *Anabaena spiroides* *Anabaena wisconsinense* *Anabaena planctonica* *Anabaena spiroides crassa* *Aphanizomenon flos-aquae* *Aphanizomenon gracile* *Aphanizomenon issatschenkoi* *Cylindrospermopsis raciborskii*	*Anabaena catenula solitaria* *Anabaena flos-aquae* *Anabaena mucosum* *Anabaena solitaria planctonica* *Anabaena spiroides* *Anabaena spiroides crassa* *Aphanizomenon flexuosum* *Aphanizomenon flos-aquae* *Aphanizomenon gracile* *Aphanizomenon issatschenkoi*
Chroococcales	*Microcystis aeruginosa* *Microcystis flos-aquae* *Microcystis weisenbergei* *Aphanocapsa holsatica* *Aphanocapsa elachista planctonica* *Aphanocapsa pulchra* *Merismopedia minima* *Merismopedia tenuissima* *Chroococcus minutus* *Woronichinia naegeliana* *Aphanothece clathrata brevis* *Coelosphaerium kuetzingianum*	*Microcystis aeruginosa* *Microcystis flos-aquae* *Aphanocapsa delicatissima* *Aphanocapsa holsatica* *Aphanothece clathrata* *Aphanothece clathrata brevis* *Chroococcus dispersus* *Chroococcus minutus* *Chroococcus prescottii* *Coelosphaerium kuetzingianum* *Merismopedia glauca* *Merismopedia minima* *Merismopedia tenuissima* *Rhabdoderma lineare* *Woronichinia naegeliana*
Oscillatoriales	*Lyngbya limnetica* *Lyngbya martensiana* *Oscillatoria limnetica* *Trichodesmium lacustris*	*Lyngbya limnetica* *Oscillatoria limnetica* *Oscillatoria ornata* *Oscillatoria splendida* *Oscillatoria tenuis* *Oscillatoria terrebriformis* *Oscillatoria utermoehlii* *Phormidium mucicola* *Trichodesmium lacustre*

Table 19. Summary of studies examining the effects of both abiotic and biotic variables on microcystin concentrations and on the presence of toxigenic strains of cyanobacteria capable of producing microcystins. Those variables listed were significant in either correlation or regression analyses.

Study	*Environmental Variables*	*Biotic Variables*
Vaitomaa *et al.* (2003)	N/A	*Microcystis* (*mcy*E)
Kotak *et al.* (1993)	Temperature, total phosphorus, Secchi depth	*Microcystis aeruginosa* biomass
Kotak *et al.* (1995)	Total phosphorus, pH	*Microcystis aeruginosa* biomass
Jacoby *et al.* (2000)	Soluble Reactive Phosphorus (SRP)	N/A
Albay *et al.* (2005)	Dissolved oxygen, chl a, salinity, PAR, temperature, N:P, pH	*Microcystis* biomass
Rolland *et al.* (2005)	Chl a, TN, alkalinity	*Anabaena* and *Microcystis* biomass
Graham *et al.* (2006)	Latitude, nutrients, N:P, chl a, Secchi	N/A
Yepremian *et al.* (2007)	N/A	*Planktothrix agardhii* biomass
Koskenniemi *et al.* (2007)	N/A	Nda gene
Yoshida *et al.* (2007)	Nitrate (*mcy*A)	N/A
Kardinaal *et al.* (2007)	N/A	*Microcystis* biovolumes
Lehman *et al.* (2008)	High water temperature, low stream flow	*Microcystis* biomass
Gobler *et al.* (2008)	Nitrogen-loading	*Microcystis* biomass
Fahnenstiel *et al.* (2008)	N/A	*Microcystis aeruginosa* biomass
Rogalus & Watzin (2008)	N/A	Potentially toxic cyanobacteria cell density
Arnaud *et al.* (2008)	N/A	*Planktothrix agardhii* biomass
Briand *et al.* (2008)	N/A	*Planktothrix agardhii* biomass

Table 20. Pearson correlations for environmental variables measured in Constance Lake 2006.

	TEMPERATURE	DO	pH	ORP	SPC	LEC	TP	RP	TKN	NH3NH4	SECCHI	N:P
TEMPERATURE	1.000											
DO	-0.695	1.000										
pH	0.800	-0.745	1.000									
ORP	-0.375	0.725	-0.816	1.000								
SPC	-0.149	-0.456	0.274	-0.718	1.000							
LEC	0.882	-0.557	0.529	-0.117	-0.338	1.000						
TP	-0.166	-0.217	0.318	-0.631	0.737	-0.411	1.000					
RP	-0.764	0.850	-0.720	0.593	-0.132	-0.748	0.176	1.000				
TKN	0.231	0.240	0.132	0.149	-0.417	0.006	-0.229	0.107	1.000			
NH3NH4	-0.214	0.062	-0.086	-0.127	0.329	-0.313	-0.213	-0.088	0.179	1.000		
SECCHI	0.541	-0.363	0.069	0.231	-0.468	0.789	-0.787	-0.635	-0.074	-0.047	1.000	
N:P	0.208	0.257	-0.259	0.614	-0.776	0.378	-0.972	-0.136	0.452	0.239	0.703	1.000

Table 21. Pearson correlations for environmental variables measured in Constance Lake 2007.

	TEMPERATURE	DO	pH	ORP	SPC	LEC	TP	RP	TKN	NH3NH4	SECCHI	N:P
TEMPERATURE	1.000											
DO	0.326	1.000										
pH	-0.160	-0.618	1.000									
ORP	-0.162	0.051	-0.074	1.000								
SPC	-0.457	-0.362	0.548	-0.160	1.000							
LEC	0.417	0.161	-0.494	0.257	-0.364	1.000						
TP	0.223	0.003	-0.008	0.136	-0.501	0.458	1.000					
RP	-0.069	-0.014	0.135	0.364	-0.289	-0.311	0.310	1.000				
TKN	0.201	0.302	-0.455	0.332	-0.827	0.339	0.629	0.509	1.000			
NH3NH4	0.223	-0.111	-0.289	0.062	-0.387	0.214	0.063	0.488	0.478	1.000		
SECCHI	-0.085	0.320	0.179	-0.371	0.210	-0.677	-0.344	0.159	-0.101	-0.200	1.000	
N:P	0.147	0.365	-0.550	0.345	-0.786	0.212	0.328	0.483	0.941	0.553	0.027	1.000

Table 22. Pearson correlations for environmental variables measured in Constance Lake 2006-2007.

	TEMPERATURE	DO	pH	ORP	SPC	LEC	TP	RP	TKN	NH3NH4	SECCHI	N:P
TEMPERATURE	1.000											
DO	0.235	1.000										
pH	0.527	-0.184	1.000									
ORP	-0.435	-0.315	-0.494	1.000								
SPC	-0.328	-0.774	0.296	-0.061	1.000							
LEC	0.783	-0.008	0.224	-0.060	-0.243	1.000						
TP	-0.269	-0.482	0.271	-0.229	0.653	-0.308	1.000					
RP	-0.603	-0.347	-0.150	0.560	0.310	-0.600	0.310	1.000				
TKN	0.010	-0.024	-0.182	0.203	-0.265	0.057	0.123	0.259	1.000			
NH3NH4	0.020	0.370	-0.105	-0.215	-0.059	-0.108	-0.365	-0.039	0.044	1.000		
SECCHI	0.498	0.237	0.038	-0.034	-0.456	0.480	-0.648	-0.256	0.123	0.113	1.000	
N:P	0.127	0.109	-0.341	0.431	-0.462	0.315	-0.587	0.032	0.578	0.240	0.497	1.000

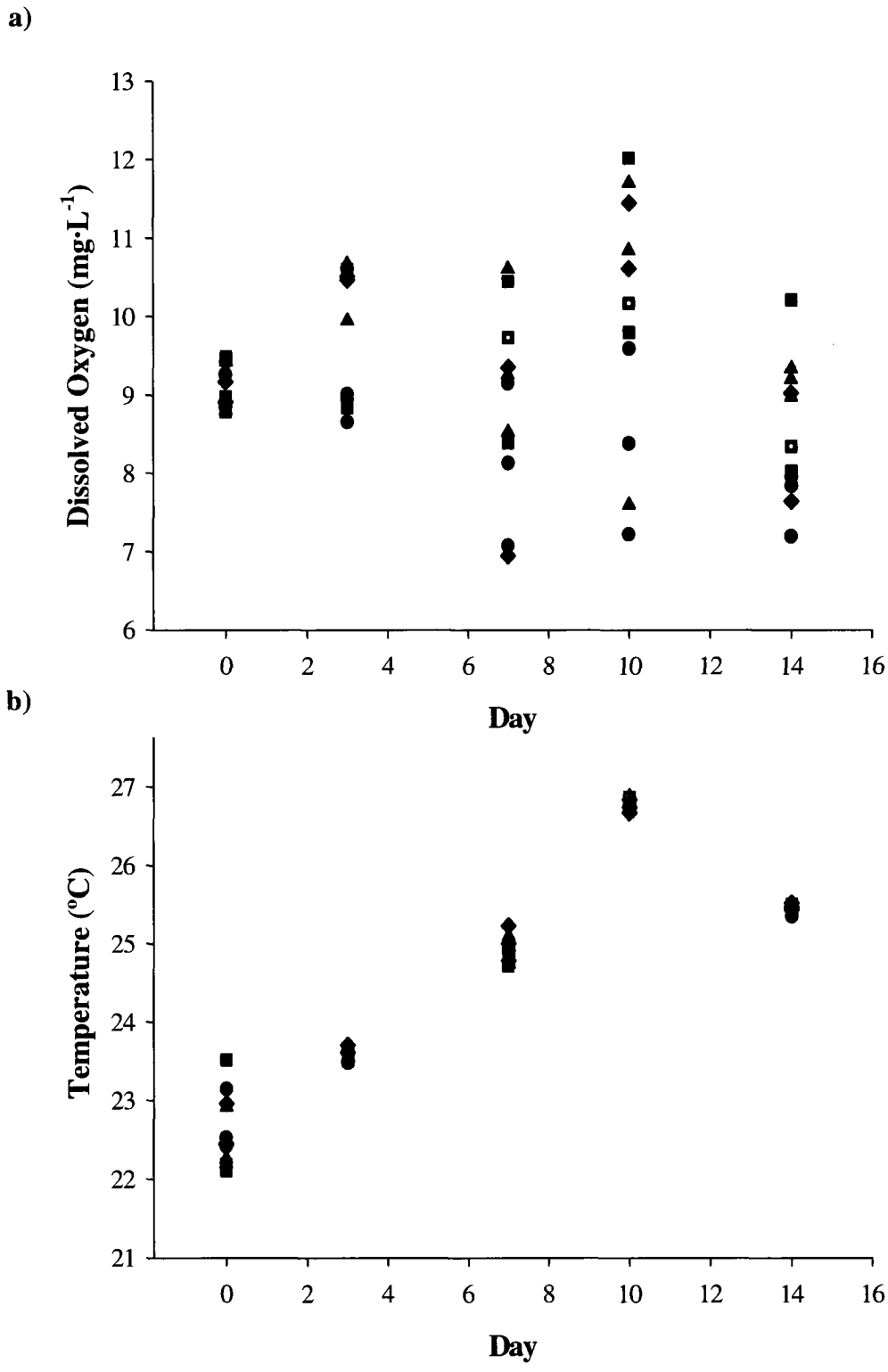


Figure 66. a) Dissolved Oxygen, and **b)** Temperature for all 12 enclosures on days 0, 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Each symbol represents a different treatment. Circles represent control enclosures (un-shaded, no nutrients added); squares represent enclosures that are shaded with no nutrients added; triangles represent enclosures that are shaded with nutrients; and diamonds represent enclosures that are un-shaded with nutrients added.

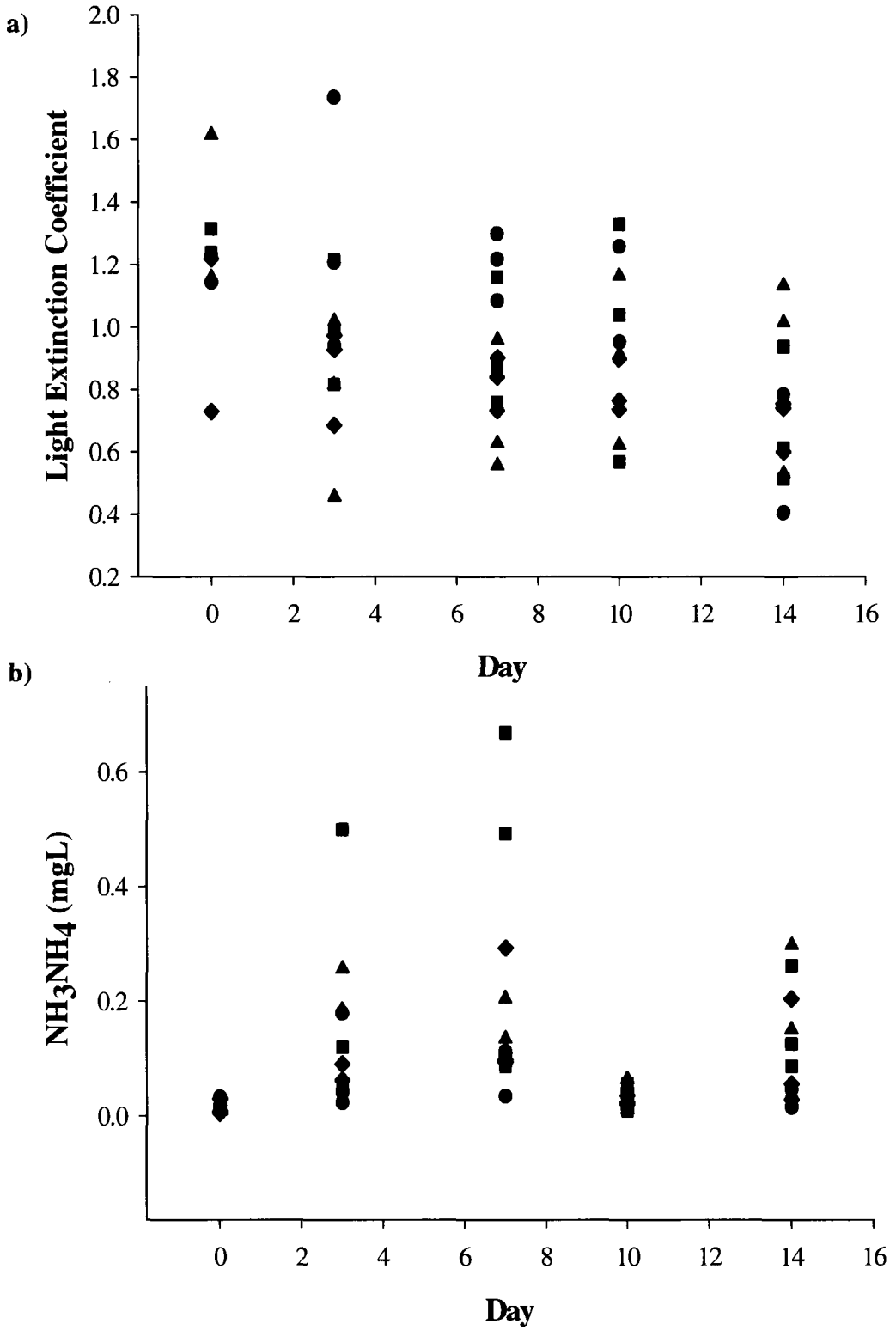
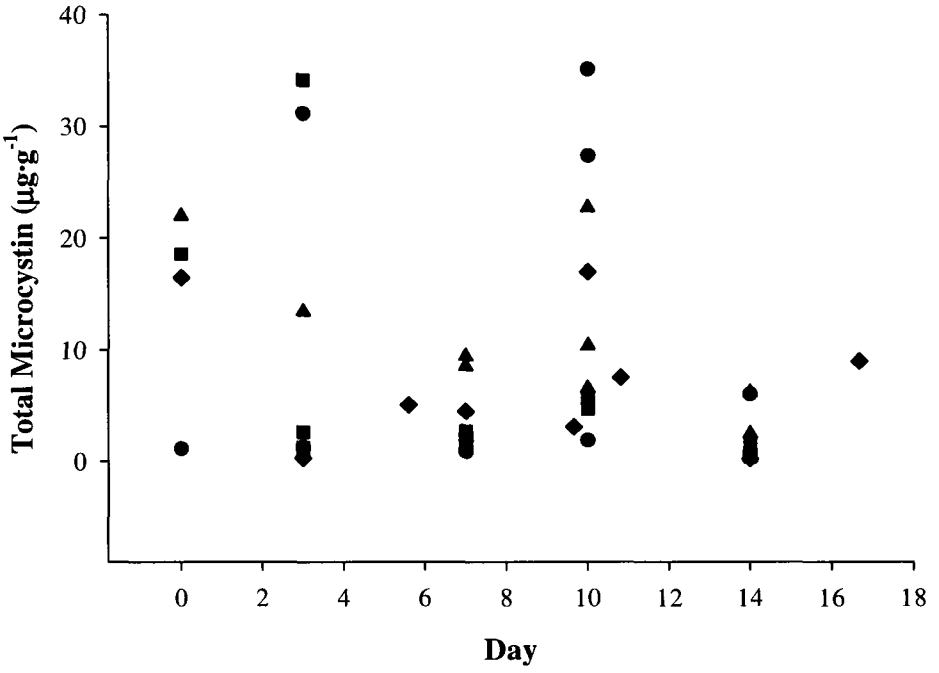


Figure 67. a) Light Extinction, and **b)** NH_3+NH_4 for all 12 enclosures on days 0, 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Each symbol represents a different treatment. Circles represent control enclosures (un-shaded, no nutrients added); squares represent enclosures that are shaded with no nutrients added; triangles represent enclosures that are shaded with nutrients; and diamonds represent enclosures that are un-shaded with nutrients added.

a)



b)

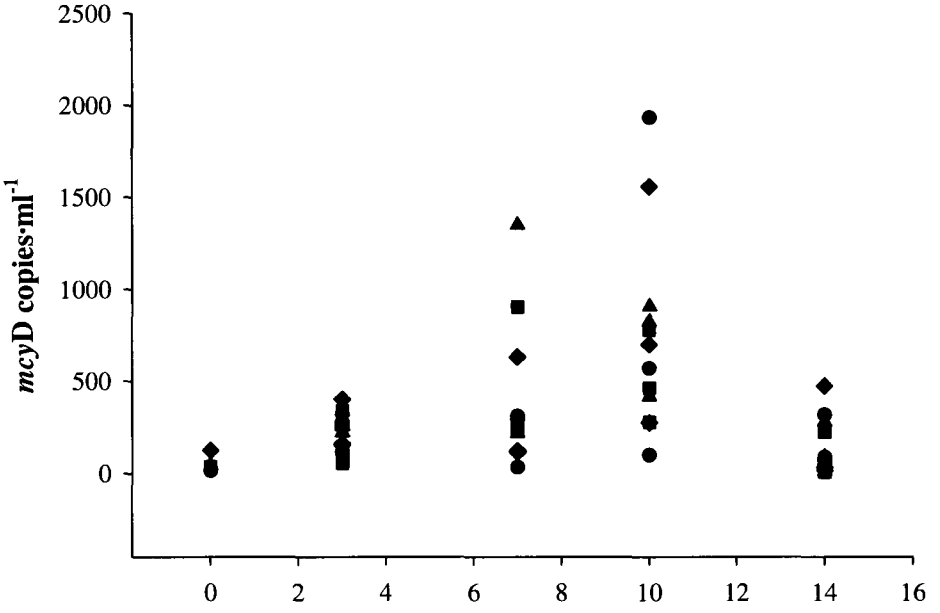


Figure 68. a) Total Microcystin µg·g^{-1}, and **b)** *mcy*D copies·ml^{-1} for all 12 enclosures on days 0, 3, 7, 10, and 14 of the Constance Lake 2007 enclosure experiment. Each symbol represents a different treatment. Circles represent control enclosures (un-shaded, no nutrients added); squares represent enclosures that are shaded with no nutrients added; triangles represent enclosures that are shaded with nutrients; and diamonds represent enclosures that are un-shaded with nutrients added.

Table 23. Pearson correlations for environmental variables measured in Constance Lake Enclosures 2007.

	TEMPERATURE	DO	pH	ORP	SPC	LEC	TP	RP	TKN	NH3NH4	N:P
TEMPERATURE	1.000										
DO	0.131	1.000									
pH	0.306	0.621	1.000								
ORP	-0.565	-0.288	-0.545	1.000							
SPC	-0.005	-0.503	-0.743	0.335	1.000						
LEC	-0.326	-0.118	-0.427	0.494	0.150	1.000					
TP	0.155	0.190	0.407	-0.462	-0.257	-0.451	1.000				
RP	0.108	0.068	0.034	-0.325	-0.103	-0.236	0.733	1.000			
TKN	0.245	0.107	0.406	-0.508	-0.254	-0.446	0.924	0.642	1.000		
NH3NH4	0.001	-0.072	0.072	-0.405	-0.044	-0.300	0.697	0.716	0.745	1.000	
N:P	-0.026	-0.248	-0.335	0.327	0.214	0.375	-0.904	-0.703	-0.674	-0.518	1.000