Development and field evaluation of molecular techniques for monitoring toxigenic cyanobacteria in water

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Dedication

This thesis is dedicated to my dearest wife Pamela Ngum and son Ridley Ngwa, for their devotion, boundless love, and support all along this journey.
Abstract

Increased incidences of toxigenic cyanobacterial blooms in freshwater bodies pose significant threats to human and ecosystem health worldwide. Microcystins (MCs), produced mostly by *Microcystis, Anabaena,* and *Planktothrix* spp., are amongst the most prevalent freshwater cyanotoxins. Characterization of toxigenic blooms by conventional microscopy is often challenging because of co-occurrence of morphologically indistinguishable toxic and non-toxic strains of cyanobacteria. This research project therefore sought to develop and evaluate polymerase chain reaction (PCR) approaches for improved monitoring of toxigenic cyanobacteria in Canadian freshwater lakes.

Preliminary studies evaluating the utility of a suite of microcystin synthetase (*mcy*) genes for quantitative PCR-based detection of microcystin-producing *Microcystis* revealed that assays targeting portions of the *mcyA, mcyE,* and *mcyG* genes successfully estimated potential microcystin-producing *Microcystis* genotypes in water samples collected from Baie Missisquoi (Missisquoi Bay), Quebec. The qPCR-based *Microcystis mcyA, mcyE,* or *mcyG* toxigenic cell equivalents showed significant associations (p<0.05; $R^2>0.90$) with total *Microcystis* counts determined by microscopy. Furthermore, all three assays successfully quantified potentially toxic *Microcystis* cells in samples with undetectable microcystin concentrations, suggesting their potential usefulness in early warning systems for toxigenic cyanobacteria.

To further ascertain the utility of developed molecular assays in estimating toxigenic *Microcystis* concentrations, laboratory studies were conducted to investigate how *mcy* gene concentrations and biomass of mixed assemblages of a toxic *Microcystis* sp. and non-toxic *Anabaena* sp. varied under different nitrogen, phosphorus, temperature, and light regimes. Results demonstrated dependence of growth rates, microcystin cellular and *mcyE* gene quotas not only on nutrients and temperature conditions, but also the presence of competing cyanobacteria. The fact that changes in *mcyE* copies often mirrored changes in *M. aeruginosa* CPCC 299 cellular growth rates implied possible coupling of *mcyE* production to cellular
growth; thus validating use of \textit{mcyE} gene concentrations as indicators of toxigenic \textit{Microcystis}.

The third phase of this study involved development and utilization of a multiplex qPCR approach for simultaneous quantification of microcystin-producing \textit{Anabaena}, \textit{Microcystis}, and \textit{Planktothrix} genotypes in Missisquoi Bay. Laboratory evaluation showed the multiplex assay to be highly sensitive and specific for \textit{mcyE}-containing \textit{Anabaena}, \textit{Microcystis}, and \textit{Planktothrix} genotypes, with assay standards achieving R$^2$ values above 0.99 and reaction efficiencies greater than 90%. Analyses of water samples from Missisquoi Bay showed \textit{Microcystis} spp. as the main putative microcystin producer, with patchy occurrence of toxigenic \textit{Anabaena} and \textit{Planktothrix} genotypes during the 2010 and 2011 sampling periods.

Finally, the developed qPCR assays were utilized to study \textit{Microcystis} and \textit{Planktothrix} \textit{mcyE} gene expression, concomitantly with changes in \textit{mcyE} copies, cyanobacterial biomass and MC concentrations, in order to derive the most reliable indicator of microcystin risk. McyE transcripts were generally lower in mixed cultures relative to monocultures, in agreement with depressed growth recorded in the mixed cultures. Whereas concentrations of \textit{mcyE} gene copies, cell counts, and chl-a correlated significantly (p<0.01) with microcystins in laboratory cultures, McyE gene transcripts levels associated very poorly with MC. Furthermore, \textit{mcyE} copies showed the strongest positive association with MCs in field samples, suggesting that \textit{mcyE} copies are better indicators of MC risks rather than McyE transcripts or traditional biomass proxies.
Résumé

L’occurrence plus fréquente d’efflorescences en eau douce constitue, à l’échelle mondiale, une importante menace à la santé humaine et environnementale. Les microcystines, produites principalement par *Microcystis*, *Anabaena*, et *Planktothrix* spp., sont les cyanotoxines les plus communes en eau douce. La caractérisation microscopique d’efflorescences toxigènes pose souvent un défi étant donné la co-occurrence de souches de cyanobactéries toxigènes et non-toxigènes qu’on ne peut distinguer morphologiquement. Ce projet de recherche visa donc à développer et évaluer des approches axées sur l’amplification en chaîne par polymérase (ACP) pour améliorer le suivi des cyanobactéries toxigènes dans les lacs d'eau douce du Canada.

Des études préliminaires évaluant l’utilité, dans la détection quantitative par ACP (qACP) de souches de *Microcystis* actives ou inactives en production de microcystine, d’une série de gènes codant pour les sous-unités de la microcystine synthétase (*mcy*), démontra que les tests visant l’identification d’une portion des gènes *mcyA*, *mcyE*, ou *mcyG* permet l’estimation de la quantité de souches de *Microcystis* toxigènes dans des échantillons d’eau provenant de la Baie Missisquoi, Québec. Les équivalents en cellules de *Microcystis* toxigènes pour les gènes *mcyA*, *mcyE*, ou *mcyG* furent fortement associés (p<0.05; R²>0.90) au compte total de *Microcystis* obtenu par microscopie. De plus, les trois tests réussirent à quantifier les cellules de *Microcystis* ayant le potentiel d’être toxigènes dans des échantillons ayant une teneur en microcystine indéTECTABLE, laissant présager leur utilité potentielle dans un système d'alerte précoce pour les cyanobactéries toxigènes.

Afin d’évaluer davantage si les tests moléculaires élaborés pour l’estimation de la teneur en *Microcystis* toxigènes sont utiles, des études en laboratoire furent entreprises afin d’évaluer comment la teneur en gènes *mcy* et la biomasse de différents assemblages mixtes d’une espèce toxigène de *Microcystis* et d’une espèce non-toxigène d’*Anabaena* pourraient varier sous divers régimes d’azote, de phosphore, de température et de lumière. Les résultats démontrent une
dépendance des taux de croissance, quotas en microcystine cellulaire et gène \textit{mcyE}, non seulement sur les nutriments et conditions de température, mais aussi sur la présence de cyanobactéries compétitrices. Le fait que les changements dans le nombre de copies de \textit{mcyE} furent souvent le reflet du taux de croissance de \textit{M. aeruginosa} CPCC 299 implique un jumelage entre la production de \textit{mcyE} et la croissance cellulaire, ce qui valide l’utilisation de la concentration du gène \textit{mcyE} comme indicateur de \textit{Microcystis} toxigène.

Le troisième volet de cette étude impliqua le développement et l’utilisation d’une approche qACP multiplexe pour la quantification simultanée des génotypes d’\textit{Anabaena}, \textit{Microcystis}, et \textit{Planktothrix} produisant de la microcystine dans la Baie Missisquoi. En laboratoire le test multiplex s’avéra très sensible et spécifique aux souches d’\textit{Anabaena}, \textit{Microcystis}, et \textit{Planktothrix} portant le gène \textit{mcyE}. Les valeurs de R\textsuperscript{2} pour la courbe d’étalonnage excéderent 0,99, et les efficacités de réaction excéderent 90\%. L’analyse d’échantillons d’eau provenants de la Baie Missisquoi souligna que \textit{Microcystis} spp. était le principal présumé producteur de microcystine, tandis que les génotypes toxigènes d’\textit{Anabaena} and \textit{Planktothrix} présentèrent une distribution irrégulière durant les périodes d’échantillonnage de 2010 et 2011.

Enfin, les tests qACP développés servirent à l’étude de l’expression génique de \textit{mcyE} dans \textit{Microcystis} et \textit{Planktothrix}, en parallèle au changement du nombre de copies de \textit{mcyE}, la biomasse cyanobactérienne et les concentrations en microcystine, afin d’en dériver un indicateur fiable du risque associé à la microcystine. Les produits de transcription de \textit{mcyE} furent généralement moins élevés dans les cultures mixtes (vs. monocultures), ce qui s’accorde avec la diminution du taux de croissance en cultures mixtes. Lorsque, en cultures maintenues en laboratoire, les concentrations en copies du gène \textit{mcyE}, le nombre de cellules, et la teneur en chlorophylle a étaient significativement corrélés (p<0,01) avec la teneur en microcystine, le niveau de transcriptions du gène \textit{mcyE} s’avéra faiblement corrélé au niveau de microcystine. De plus, dans les échantillons prélevés dans la Baie Missisquoi, le nombre de copies de \textit{mcyE}}
montra une forte association à la teneur en microcystine, suggérant que le nombre de copies de mcyE pourrait être un meilleur indicateur du risque de contamination en microcystine, en comparaison aux substituts traditionnels que sont les produits de transcription de McyE ou simplement la biomasse.


**Contribution of Authors**

All manuscripts presented in this dissertation were authored by Felexce Ngwa (first author), Dr. Chandra Madramootoo, and Dr. Suha Jabaji. Felexce Ngwa designed all experiments, conducted the field work, extracted nucleic acids (DNA and RNA) from laboratory and field samples, designed primers and probes, performed all PCR/qPCR, cloning, and sequence analyses, extracted toxins and performed ELISA analyses. He also performed statistical analyses and wrote all the manuscripts. Dr. Chandra Madramootoo, the second author, supervised and funded the research project, provided guidance on various aspects of experimental design and reviewed all the manuscripts. The third author, Dr. Suha Jabaji, provided bench space and equipment for all molecular techniques, guidance on primer and probe design and also reviewed all the manuscripts.
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Listing everyone who contributed in the realisation of an endeavour like this is near impossible. To all those who in one way or another helped make this dream come true, I pray you accept this general appreciation.

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**Acronyms**

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<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
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<tr>
<td>Adda</td>
<td>3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid; a constituent C$_{20}$β-amino acid of microcystins and nodularins</td>
</tr>
<tr>
<td>AMT</td>
<td>Amino transferase</td>
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<tr>
<td>AT</td>
<td>Acyl transferase</td>
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<td>BGA-PC</td>
<td>Phycocyanin Blue-Green Algae</td>
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<td>BHQ</td>
<td>Black hole quencher</td>
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<td>BLAST</td>
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<td>C-methyl transferase</td>
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<td>CPCC</td>
<td>Canadian Phycological Culture Centre</td>
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<td>C$_T$</td>
<td>Cycle threshold in qPCR</td>
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<td>CyanoHABs</td>
<td>Cyanobacterial harmful algal blooms</td>
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<td>Cy5</td>
<td>Cyanine fluorophore dye</td>
</tr>
<tr>
<td>CYN</td>
<td>Cylindrospermopsin</td>
</tr>
<tr>
<td>CyrA-J</td>
<td>Cylindrospermopsin biosynthetic genes</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton, also known as the unified atomic mass unit</td>
</tr>
<tr>
<td>DH</td>
<td>Dehydratase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Ep</td>
<td>Epimerase</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein dye</td>
</tr>
<tr>
<td>HARNESS</td>
<td>Harmful Algal Research and Response National Environmental Science Strategy</td>
</tr>
<tr>
<td>HEX</td>
<td>Hexachlorofluorescein dye</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance (or pressure) liquid chromatography</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribed spacer</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases ≡ 1000 base pairs (bp)</td>
</tr>
<tr>
<td>KR</td>
<td>Keto acyl reductase</td>
</tr>
<tr>
<td>KS</td>
<td>Keto acyl synthase</td>
</tr>
<tr>
<td>LacZ</td>
<td>Gene that encodes β-galactosidase enzyme involved in lactose metabolism in <em>E. coli</em> and other enteric bacteria</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth/agar</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LD$_{50}$</td>
<td>Dose causing 50% mortality of a tested population</td>
</tr>
<tr>
<td>LDR</td>
<td>Ligase detection reaction</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>MC</td>
<td>Microcystin, a potent cyanobacterial heptapeptide hepatotoxin</td>
</tr>
<tr>
<td>mcyA-J</td>
<td>Genes encoding the microcystin synthetase (<em>mcy</em>) enzyme subunits A to J</td>
</tr>
<tr>
<td>McyA-J</td>
<td>Proteins encoded by the equivalent <em>mcyA-J</em> genes</td>
</tr>
<tr>
<td>mcyD$_{DH}$</td>
<td>First dehydratase domain of the <em>mcyD</em> gene</td>
</tr>
<tr>
<td>mcyD$_{KS}$</td>
<td>β-keto synthase domain of the <em>mcyD</em> gene</td>
</tr>
<tr>
<td>MDDEP</td>
<td>Ministère du Développement Durable de l'Environnement et des Parcs</td>
</tr>
<tr>
<td>Mdha</td>
<td>N-methyl dehydroalanine, an amino acid found in microcystins</td>
</tr>
<tr>
<td>Mdhb</td>
<td>N-methyl dehydrobutyrine, an amino acid found in nodularins</td>
</tr>
<tr>
<td>MEGA</td>
<td>Molecular evolutionary genetics analysis</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ndaA-I</td>
<td>Genes encoding the nodularin synthetase subunits A to I</td>
</tr>
<tr>
<td>nifH</td>
<td>Genes encoding a component of the nitrogenase enzyme</td>
</tr>
<tr>
<td>NIVA</td>
<td>Norsk Institute for Vannforsning</td>
</tr>
<tr>
<td>NMT</td>
<td>N-methyl transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NOD</td>
<td>Nodularin, a cyanobacterial pentapeptide hepatotoxin</td>
</tr>
<tr>
<td>NRPS</td>
<td>Non-ribosomal peptide synthetase</td>
</tr>
<tr>
<td>OM</td>
<td>O-methyl transferase</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation</td>
</tr>
<tr>
<td>PC-IGS</td>
<td>Phycocyanin operon intergenic spacer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode array detector</td>
</tr>
<tr>
<td>PKS</td>
<td>Polyketide synthase</td>
</tr>
<tr>
<td>PPIA</td>
<td>Protein phosphatase inhibition assay</td>
</tr>
<tr>
<td>REML</td>
<td>Restricted maximum likelihood: a variance estimation method</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rpoC1</td>
<td>Gene encoding RNA polymerase subunit C1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative PCR</td>
</tr>
<tr>
<td>sp. (spp.)</td>
<td>Species (Plural)</td>
</tr>
<tr>
<td>sxt</td>
<td>Saxitoxin biosynthetic gene</td>
</tr>
<tr>
<td>Taq</td>
<td>Heat-resistant Taq DNA polymerase produced by <em>Thermus aquaticus</em> bacteria</td>
</tr>
<tr>
<td>TE</td>
<td>Thioesterase</td>
</tr>
<tr>
<td>TN</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>TNA</td>
<td>Taq nuclease assay</td>
</tr>
<tr>
<td>TP</td>
<td>Total phosphorus</td>
</tr>
<tr>
<td>TSI</td>
<td>Trophic state index</td>
</tr>
<tr>
<td>V\text{\textsubscript{max}}</td>
<td>Maximum nutrient uptake rate</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3 indolyl-β-D galactoside</td>
</tr>
<tr>
<td>YSI</td>
<td>Yellow Springs Instrument Co</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Chapter 1 General introduction

1.1 Background: cyanobacterial blooms

Cyanobacteria, otherwise referred to as blue-greens, blue-green algae, Myxophyceae, Cyanophyceae, or Cyanophyta (Carmichael, 2008), are prokaryotic organisms known to have colonized aquatic and terrestrial habitats over three billion years ago (Schopf, 2000). These organisms, however, have the potential to proliferate massively under favourable conditions to form so-called freshwater or marine cyanobacterial (algal) blooms. Over the last three decades, there have been global increases in incidence and intensity of cyanobacterial harmful algal blooms (CyanoHABs) as evidenced by the exponential increase in reports of CyanoHAB events in the period spanning 1960-2004 (Figure 1.1). Such events have indeed been reported on all continents of the world. Similarly, all Canadian provinces have reported potentially toxic blooms of cyanobacteria, with prairie lakes and ponds being bloom hotspots (Environment Canada, 2011). According to a 2012 report on the state of Quebec’s freshwater bodies, over 250 freshwater bodies were contaminated by cyanobacterial blooms from 2004 to 2008, with yearly bloom recurrence in 61% of these water bodies (MDDEP, 2012). Increasing incidence and recurrence of CyanoHABs therefore pose significant threats to recreational and drinking water resources not only in Canada, but globally.

1.2 Causes and impacts of cyanobacterial blooms

Environmental conditions such as sunlight, warm temperatures, low water turbulence, and high nutrients are amongst the most common promoters of cyanobacterial growth (Carmichael, 2008). Although the formation of a cyanobacterial bloom is a complex event which is often simultaneously driven by multiple factors (Heisler et al., 2008), cultural eutrophication of water bodies (Anderson et al., 2002; Carmichael, 2008; Glibert et al., 2005; Hallegraeff, 1993; Heisler et al., 2008) and rising temperatures (Fernald et al., 2007; Paerl &
Huisman, 2009; Robarts & Zohary, 1987) are believed to have significantly contributed to the increased global incidence of potentially toxic cyanobacterial blooms over the last three decades.

Blooms of cyanobacteria cause several water quality problems including but not limited to; toxin production, release of taste and odor compounds, creation of anoxic conditions during bloom die-off as well as related aesthetic issues. Annual estimates of economic losses in water usage as result of cyanobacterial bloom contamination and eutrophication of water bodies in the United States alone exceed US $ 2 billion (Dodds et al., 2009), suggesting an even more dire global picture.

The presence of cyanobacterial toxins in water bodies is particularly worrisome to water resource managers and public health authorities because of documented human, domestic animal, and bird fatalities resulting from acute exposure (Briand et al., 2003; Jochimsen et al., 1998; Wood et al., 2010a) as well as the carcinogenic potential of several cyanobacterial toxins (Falconer, 1991; Ito et al., 1997; Ueno et al., 1996; Zhou et al., 2002). The most prevalent cyanotoxins include the hepatotoxins (microcystins, nodularins, cylindrospermopsins) and neurotoxins (mainly anatoxin-a, anatoxin-a(S), saxitoxins, and recently beta-methylamino-l-alanine; BMAA) (Cox et al., 2003; Sivonen & Börner, 2008; Sivonen & Jones, 1999). The most widespread of the cyanobacterial toxins, microcystins, are produced mostly by Microcystis, Anabaena, and Planktothrix (formerly Oscillatoria) species (Sivonen & Jones, 1999).

1.3 Problem definition

Direct and indirect evidence from existing literature suggest that current eutrophication trends coupled with rising temperatures could potentially lead to future global increases not only in the occurrence but also the duration and intensity of freshwater cyanobacterial blooms (Carmichael, 2008; O’Neil et al., 2012), thereby exacerbating the potential risks of human exposure to cyanotoxins. For this reason, early detection and quantification of potentially toxic cyanobacteria would be crucial to effective risk management. Management of cyanobacteria and cyanotoxin risks in Canadian water bodies still relies on
conventional microscopy coupled with various toxin detection techniques. However, the morphological plasticity of cyanobacteria with environmental conditions, coupled with morphological changes induced by selective culturing techniques (Casamatta & Vis, 2004; Rasmussen et al., 2007; Soares et al., 2013) render microscopy incapable of discriminating toxic from non-toxic cyanobacterial strains that often co-exist within the same bloom (Vezeie et al., 1998).

In order to better assess the health risks associated with the increasing cyanobacterial blooms, there is need for improved detection of toxigenic cyanobacteria and cyanotoxins, both in Canada and globally. Although much effort has been devoted towards elucidating the chemistry, biosynthesis, toxicology, and detection of most cyanotoxins globally, there is general consensus on the need to develop more robust cyanobacterial monitoring techniques. The US Harmful Algal Research and Response National Environmental Science Strategy report (HARRNESS, 2005) indeed identified priority areas for scientific effort to include: improved ability to detect HAB species and analyze cyanoHAB toxins, improved capacity for monitoring and forecasting cyanoHABs in a cost effective and timely manner, improved protection of human health, improved protection of endangered species and improved ecological health, amongst others. This project therefore builds on these identified research needs to develop an approach for improved monitoring of cyanoHABs.

1.4 Research objectives

The overarching goal of this research project was to develop and evaluate molecular techniques for improved monitoring of toxigenic cyanobacteria in freshwater bodies, with the Missisquoi Bay, Quebec, being a case study. This study should provide water resource managers with robust tools that could be incorporated into early warning systems for more efficient management of impending toxigenic cyanobacterial blooms. This goal was attained through the following specific objectives:
I. To determine toxin biosynthetic genes suitable for monitoring toxigenic cyanobacteria by evaluating PCR assays targeting a suite of microcystin synthetase gene loci;

II. To investigate changes in microcystin synthetase gene copies and biomass of mixed assemblages of toxic and non-toxic cyanobacteria under various nutrient, temperature, and light regimes;

III. To develop and optimize robust molecular techniques for characterizing multiple microcystin-producing cyanobacteria in a freshwater body, with the Missisquoi Bay, Quebec, as case study;

IV. To evaluate the utility of developed molecular techniques relative to conventional monitoring approaches for indicating microcystin risks in laboratory cultures and field samples.

1.5 Thesis organisation

This dissertation comprises eight chapters and appendices arranged as follows;

Chapter 1 General introduction: provides a general background on cyanobacterial blooms and their impacts, defines the research problem and objectives of this project, and outlines the organization of the thesis.

Chapter 2 Literature review: provides a synopsis of literature on cyanobacteria and bloom occurrence in Canada and globally, cyanobacterial toxins and existing detection approaches, regulation of cyanotoxin biosynthesis, and cyanobacterial detection approaches.

Chapter 3 Monitoring toxigenic Microcystis strains in the Missisquoi Bay, Quebec, by PCR targeting multiple toxic gene loci: This chapter describes the selection of suitable microcystin synthetase (encodes microcystin synthesis in cyanobacteria) genetic loci and design, testing and validation of PCR assays for specific detection of microcystin-producing Microcystis (the most common toxic cyanobacteria) spp. Objective one of this research project was addressed in this chapter.
Chapter 4  Growth dynamics and toxin production in mixed assemblages of *Anabaena* and *Microcystis* spp. under varying nutrient, temperature, and light conditions: In order to use toxin gene copies as proxies for toxigenic cyanobacteria concentration, it is necessary to understand how they respond to changes in biotic and abiotic factors. This chapter therefore addressed the second objective of the project.

Chapter 5  Development and application of a multiplex qPCR technique to study the dynamics of multiple microcystin-producing cyanobacterial genera in a freshwater lake: *Microcystis, Anabaena,* and *Planktothrix* spp. are three potential microcystin producing cyanobacteria that have been detected in Canadian lakes. This chapter which addressed the third objective of this research project describes the design, testing, and validation of a novel quantitative PCR assay for simultaneous and specific estimation of microcystin-producing *Microcystis, Anabaena,* and *Planktothrix* concentrations. Furthermore, the qPCR assay is used to study the response of toxigenic cyanobacteria to various environmental and/or physicochemical factors.

Chapter 6  Evolution of cyanobacterial microcystin synthetase E transcription with changes in gene copies and biomass under laboratory and field conditions: addressed the fourth and last objective of this research project.

Chapter 7  General summary and conclusions: The general summary and conclusions emanating from this research are presented in this chapter. Also included are the contributions made by this research endeavour towards the improvement of scientific knowledge as well as suggestions for future research.

Chapter 8  Bibliography: This chapter presents all literature cited in this dissertation.

Appendix A  Presents selected photomicrographs of cyanobacterial spp. identified in Missisquoi Bay, Quebec.
Appendix B  Presents the melting curves of PCR amplicons generated in this study.

Appendix C  Presents plots of qPCR C$_T$ values against $mcyE$ gene copy number for the *Anabaena*, *Microcystis*, and *Planktothrix* singleplex and multiplex standard curves.

Figure 1.1: Published CyanoHABs events between 1960 and 2004 (Source: Carmichael, 2008)
Chapter 2 Literature review

2.1 Nature and occurrence of cyanobacteria

Cyanobacteria are photoautotrophic bacteria that are believed to have inhabited the earth ca.3500 million years ago (Hedges et al., 2001; Schopf, 2000). These organisms were originally misclassified as algae because they possess both photosystems I and II and like most phytoplankton, undergo mostly plant-like oxygenic photosynthesis using water as the principal electron donor for photosynthetic carbon reduction (Tooming-Klunderrud, 2007). However, the absence of membrane-bound organelles, presence of cell wall components and protein synthesis apparatus akin to those of prokaryotes necessitated their reclassification as bacteria (Castenholz, 2001). The ability of several cyanobacteria species to perform facultative anoxygenic bacteria-like CO\textsubscript{2} photoassimilation in the presence of sulfides as electron donors via photosystem I driven reaction (Padan, 1979) further attests to this reclassification. The capability of these organisms to undergo both photosystem I driven facultative anoxygenic photosynthesis and photosystem II based aerobic CO\textsubscript{2} photoassimilation supports the thesis that cyanobacteria might have predated the oxidized biosphere and probably contributed to the emergence of an oxygen-rich atmosphere via oxygen-releasing photosynthetic processes. It is widely believed that today’s green algae and plants owe their oxygenic photosynthetic ability to cyanobacteria. Indeed, the endosymbiosis theory suggests that the photosynthetic organelle of green algae and plants (the plastid) probably resulted from engulfment and enslavement of a cyanobacterium by a previously non-photosynthetic protist (Bhattacharya et al., 2004).

Cyanobacteria exist in diverse forms ranging from unicellular to colonial or filamentous forms. Some filamentous colonies possess the ability to differentiate into specialized cell types such as the thick-walled akinetes and heterocysts which serve survival/vegetative and nitrogen fixation roles respectively. Morphological characterization places these organisms into five main orders viz Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales.
These organisms are ubiquitous, occupying diverse niches with temperature ranging from near-zero temperatures, for Arctic dwelling species (Quesada et al., 1999), to temperatures as high as 73°C for the hot spring dwelling cyanobacterium *Synechococcus* (Miller et al., 1998). They can either occur as free floating plankton in marine, brackish and freshwater bodies or may be attached to rocks, on plant surfaces or in the bottom sediments. Terrestrial species capable of inhabiting hot and polar deserts, sand dunes, and rocks have been reported, while others form symbiotic associations with plants, fungi or even animals (Whitton & Potts, 2000). Survival and success of cyanobacteria in diverse and most often unfavourable environments has been attributed not only to their modest demands for water, light, carbon dioxide, and organic nutrients but also their capability to: fix atmospheric N₂, regulate buoyancy, tolerate desiccation, high ultra violet radiation levels, and high salt concentration, as well as wide temperature variations (Castenholz, 2001; Whitton & Potts, 2000).

2.2 Reproduction and genomics of cyanobacteria

Cyanobacteria undergo mostly asexual reproduction, with binary fission being most common. The filamentous Oscillatoriales and Nostocales, on one hand, undergo binary division mainly in one plain whereas division in Stigonematales commonly occurs in more than one plain (Whitton and Potts, 2000). Binary cell division in the non-filamentous Chroococcales, on the other hand, occurs either in one, two, or three plains whereas Pleurocapsales undergo multiple internal fissions or mixtures of binary and multiple fissions (Whitton and Potts, 2000). Prior to fission, the parent genetic material replicates such that each daughter cell inherits an exact copy of parental genes.

The nucleoid of most cyanobacterial cells usually contains a single, large chromosome consisting of a circular double stranded DNA, although some cyanobacteria are also known to host a range of extrachromosomal DNA elements or plastids (Bolch et al., 1997; van den Hondel et al., 1979). Complete genomes of many cyanobacterial species have been sequenced and annotated. The Cyanobase database (Nakao et al., 2010) lists genome projects for close to 40 cyanobacterial
species as of 2013. Comparison of the genomes of several cyanobacteria species shows high genetic plasticity and fluidity, exemplified by multiplicity of multilocus repeats, insertion sequences, as well as genes that encode transposases and DNA modification enzymes (Meeks et al., 2001). It is believed that diversity in these organisms might be the result of horizontal gene transfer or gene mutations.

Reported cyanobacterial genome sizes range from as low as 1.7Mb (with 1756 genes) in Prochlorococcus marinus MED4 to as high as 9.1Mb (with 6794 genes) in Nostoc punctiforme ATCC 29133(http://genome.microbedb.jp/cyanobase).

2.3 Cyanobacterial blooms

Cyanobacteria are essential components of terrestrial and aquatic biota, but become problematic when they proliferate massively to bloom proportions. Cyanobacteria are believed to have attained bloom levels in water bodies when there is visible discoloration of water into green, blue-green, or reddish-brown tinges as a result of the presence of suspended cyanobacterial cells, filaments, and/or colonies. Proliferation and persistence of cyanobacterial blooms is influenced by physicochemical and hydrologic factors such as temperature, light availability, nutrient loads (particularly phosphorus [P] and nitrogen [N]), pH, turbidity, and flow regimes of a particular water body. Extensive reviews of the effects of these factors on bloom dynamics are available (e.g. Huisman & Hulot, 2005; Paerl, 2008; Paerl et al., 2011; Saker & Griffiths, 2001; Soranno, 1997). The inherent ability of cyanobacteria to resist UV radiation damage via production of UV-absorbing compounds (Castenholz & Garcia-Pichel, 2000; He & Häder, 2002; Xue et al., 2005) has contributed to the success of these organisms over other phytoplankton. Furthermore, production of toxic compounds by some cyanobacteria is thought to serve as deterrence against zooplankton grazing (Capper et al., 2006; Gustafsson et al., 2005; Rohrlack et al., 2005), hence perpetuating the blooms.
2.3.1 Global occurrence

Cyanobacterial blooms are a common occurrence in freshwater bodies globally. Blooms of cyanobacteria are more prevalent in eutrophic or hypereutrophic waters than oligotrophic water bodies. In temperate lakes, blooms often occur during the late summer to early fall period whereas sub-tropical and tropical water bodies might experience this phenomenon at any time of the year (Falconer, 2005). Cyanobacterial blooms have been reported in almost all countries around the world (Figure 2.1). Between 10-92% (mean 59%) of blooms surveyed worldwide are toxic, with hepatotoxic blooms being more common than neurotoxic ones (Sivonen & Jones, 1999). Most blooms are usually made up of more than one cyanobacterial species, each often containing morphologically indistinguishable toxic and non-toxic strains (Sivonen & Jones, 1999; Vezie et al., 1998).

Cyanobacterial blooms occur in many Canadian freshwater bodies and although the extent of the problem on a national scale is unknown, many prairie lakes and ponds seem to be bloom hotspots (Environment Canada, 2011). A recent analyses of data from studies of 246 Canadian water bodies showed prevalence of cyanobacterial toxins (microcystins) in water bodies from all Canadian provinces (Orihel et al., 2012). This implies the presence of microcystin-producing cyanobacteria in all Canadian provinces. The most common bloom-forming toxigenic cyanobacteria genera in Canadian freshwater bodies include *Anabaena*, *Aphanizomenon*, *Microcystis*, and *Oscillatoria* (Fortin et al., 2010; Ngwa et al., 2012; Winter et al., 2011).

2.3.2 Cyanobacterial blooms in Quebec

Cyanobacterial blooms in Quebec’s freshwater bodies are a recent but increasing phenomenon. According to the Quebec Ministry of Environment, over 250 water bodies in the province were affected by cyanobacterial blooms from 2004-2008, with a yearly recurrence in 61% of these water bodies (MDDEP, 2012). The most affected water bodies were found in the Laurentians, Eastern Townships, and Outaouais administrative regions. One of the most eutrophic areas of Lake Champlain—the Missisquoi Bay—located between Quebec, Canada and
Vermont, USA has experienced recurrent summer blooms of cyanobacteria over the last decade (Blais, 2002; Watzin et al., 2006). Phosphorus is believed to be one of the major causes of eutrophication (Adhikari et al., 2010; Eastman et al., 2010) and probably of cyanobacterial blooms in the Missisquoi Bay. The Pike River watershed has been identified as the major contributor of P-loads into the Missisquoi Bay, with annual P-discharges from snowmelt and rainfall-generated runoff events accounting for over 80% of the watershed’s annual P discharges into the bay (Adhikari et al., 2010). In addition to P, physical factors such as temperature, light intensity, and water column stability are believed to be potential drivers of cyanobacterial blooms in this region. Though there have been efforts by the governments of Quebec and Vermont to reduce phosphorus loads (MDDEP, 2002) there has been no noticeable improvement in summer cyanobacterial bloom incidences.

### 2.3.3 Impacts of cyanobacterial blooms

Blooms of cyanobacteria are emerging threats to water resources worldwide due to the plethora of problems they cause. Senescence of massive blooms of toxic cyanobacteria, for example, can release large amounts of toxins (Glibert & Burkholder, 2006) into water bodies, thereby adversely affecting the health of humans and animals that drink such water (Briand et al., 2003; Jochimsen et al., 1998; Wood et al., 2010a). In addition to potential toxicity problems, massive growth and decline of cyanobacteria might lead to localized oxygen stress (anoxia/hypoxia), decline in benthic plant communities (through light shading), mineralization and re-suspension of sediment entrapped nutrients, and consequently perpetuation of the bloom (Sinclair et al., 2008).

Socio-economic impacts of cyanobacterial blooms usually emanate from loss of revenue from recreational activities, tourism, and decreased lakefront property value. Furthermore, the increased cost associated with additional water treatment to remove cyanobacterial toxins and taste-and-odour compounds in portable water supplies could further exacerbate the economic fallout from cyanobacterial contamination of water resources. Annual losses in restriction of use of water resources for recreational, drinking, and agricultural purposes due to
eutrophication and harmful cyanobacterial bloom contamination have been estimated at US$ 2 billion for the USA alone (Dodds et al., 2009). This figure suggests even greater global economic losses from cyanobacterial infestation of water resources.

2.4 Cyanobacterial toxins (cyanotoxins)

Many cyanobacterial species produce a wide range of bioactive secondary metabolites, including nonribosomal peptides, alkaloids, polyketides, lipopolysaccharides, and depsipeptides, some of which might be hepatotoxic, neurotoxic, dermatotoxic, or generally cytotoxic in nature (Neilan et al., 1999; Sivonen & Börner, 2008; Sivonen & Jones, 1999). Although the primary role of cyanotoxins in cyanobacterial primary metabolism, growth or reproduction remains largely unknown (Neilan et al., 1999), fatalities resulting from their toxic effects have been documented in birds, animals, and even humans (Cadel-Six et al., 2009; Jochimsen et al., 1998; Sivonen & Börner, 2008; Wood et al., 2010a). Epidemiological evidence linking human death to cyanotoxins has however been established only in a few countries such as Brazil (Jochimsen et al., 1998) and China (Yu, 1995) but not Canada or the US. Evidence of cyanotoxin-related animal and bird mortalities nevertheless exists in many countries worldwide (Codd et al., 2005b).

Globally, over 40 cyanobacterial genera have been reported to produce cyanotoxins, out of which, Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nodularia, Nostoc, and Planktothrix (formerly Oscillatoria) are believed to be the main producers (Carmichael, 2001). Cyanotoxins are most often associated with planktonic cyanobacteria that inhabit fresh and brackish waters than their marine occurring counterparts and even less frequently with benthic and terrestrial cyanobacteria (Sivonen and Börner, 2008). Cyanotoxins are commonly classified according to their mode of toxicosis either as hepatotoxins, neurotoxins, dermatotoxins, cytotoxins or endotoxins.
2.4.1 Hepatotoxins

This group includes the cyclic peptides microcystin (MC) and nodularin (NOD) as well as the tricyclic alkaloid cylindrospermopsin (CYN).

2.4.1.1 Microcystins and nodularins

Microcystins have seven amino acids in a ring structure whose general structure is: cyclo-(D-Alanine\textsuperscript{1}-X\textsuperscript{2}-D-erythro-β-methylisoAspartate\textsuperscript{3}-Z\textsuperscript{4}-Adda\textsuperscript{5}-D-isoGlutamate\textsuperscript{6}-N-methyldehydroAlanine\textsuperscript{7}), with X and Z being variable L-amino acids (Figure 2.2A) whereas nodularins have five amino acids in a ring structure with general formula: cyclo-(D-erythro-β-methylisoAspartate\textsuperscript{1}-Z\textsuperscript{2}-Adda\textsuperscript{3}-D-isoGlutamate\textsuperscript{4}-2-(methylamino)-2-dehydrobutyricacid\textsuperscript{5} (Figure 2.2B). Microcystin and nodularin exhibit great similarity in chemical structure especially the Adda-glutamate portion which is known to interact with the protein phosphatase molecule (Barford & Keller, 1994; Goldberg et al., 1995) thereby eliciting toxicity. Indeed structural modification of the Adda-glutamate moieties or linearization of microcystin or nodularin ring structures has been shown to render the resulting molecules either non-toxic or less toxic (Rinehart et al., 1994).

A majority of microcystin and nodularin structural variants are highly toxic within a relatively narrow range with intra-peritoneal (i.p.) mouse toxicities ranging between 50-300µg kg\textsuperscript{-1} body weight (bw) (Sivonen & Jones, 1999). Microcystins and nodularins elicit liver injury via inhibition of eukaryotic serine/threonine protein phosphatase types 1 and 2A (Honkanen et al., 1990; MacKintosh et al., 1990; Sivonen & Jones, 1999; Welker & von Döhren, 2006) - two enzymes which are vital for important cellular processes including cell growth and tumour suppression. These toxins are therefore believed to be initiators and/or promoters of tumour progression. Data from animal model studies points to a probable greater tumour promoting potential of nodularins over microcystins (Health Canada, 2012).

Symptoms of acute hepatotoxicosis in laboratory animals may include anorexia, diarrhoea, pallor of mucous membranes, weakness and death (within 1-2 hours) mainly due to intrahepatic haemorrhage and hypovolaemic shock (Carmichael,
Microcystins are the most commonly reported cyanobacterial toxins in Canadian waters (Fortin et al., 2010; Health Canada, 2012; Orihel et al., 2012).

2.4.1.2 Cylindrospermopsins

Cylindrospermopsins (CYN), unlike microcystins and nodularins, are three membered cyclic alkaloids consisting of tricyclic guanidine moiety combined with hydroxymethyluracil (Figure 2.2C). Cylindrospermopsin elicits toxicity mainly through inhibition of protein synthesis (Froscio et al., 2003), although covalent modification of DNA or RNA molecules has also been reported (Shaw et al., 2000). The uracil moiety of cylindrospermopsins is believed to be crucial for CYN toxicity. Laboratory studies suggest that such toxicity could stem from the inhibition of in vitro activity of uridine monophosphate (UMP) synthase complex that is involved in synthesis of the pyrimidine nucleotides (Reisner et al., 2004).

Cylindrospermopsin production was first reported in Cylindrospermopsis raciborskii (Hawkins et al., 1985) though other cyanobacteria genera have also been implicated in the production of this toxin (see Table 2.1). Cylindrospermopsins are generally less toxic than microcystins or nodularins, with toxicities ranging between 0.2-50 mg kg\(^{-1}\) bw (Falconer & Humpage, 2006; Harada et al., 1994). Although cylindrospermopsin occurrence in Canadian freshwaters is rather infrequent, their increasing incidence in US and other temperate lakes makes them an emerging threat to Canadian recreational water bodies (Health Canada, 2012).

2.4.2 Neurotoxins

Neurotoxins encompass structurally diverse, very rapidly acting alkaloid toxins. Death by respiratory arrest within minutes (Sivonen & Börner, 2008; Sivonen & Jones, 1999) or a few hours (Carmichael, 1992) has been reported in mice. The main types of alkaloid neurotoxins include; anatoxin-a, anatoxin-a(S), and saxitoxins.
2.4.2.1 Anatoxin-a

Anatoxin-a is a secondary amine with chemical structure 2-acetyl-9-azobicyclo-[4-2-1] non-2-ene. This compound is a structural analogue of the neurotransmitter acetylcholine, but cannot be degraded by the enzyme acetylcholinesterase; hence poisoning by anatoxin-a usually results in muscle fasciculation, convulsions and rapid death by respiratory failure due to blockage of postsynaptic cholinergic transmission (Dow & Swoboda, 2000). The intra-peritoneal (i.p.) LD$_{50}$ of anatoxin for mice is 200µg kg$^{-1}$ bw, with death usually occurring within 30 minutes (Carmichael & Biggs, 1978).

2.4.2.2 Anatoxin-a(S)

Anatoxin-a(S) is a naturally occurring methyl phosphate ester of N-hydroxyguanidine that is known to inhibit acetylcholinesterase. Poisoning by anatoxin-a(S) produces symptoms similar to those of anatoxin-a in addition to ataxia, diarrhoea, hypersalivation, and tremors. However, anatoxin-a(S) exhibits lethality ten times that of anatoxin-a, with an LD$_{50}$ (i.p., mouse) of 20 µg kg$^{-1}$ bw (Dow & Swoboda, 2000).

2.4.2.3 Saxitoxins

Saxitoxins are commonly referred as paralytic shellfish poisons (PSPs) because of their original isolation from shellfish. They occur either as non-sulphated (saxitoxin—STX), singly (gonyautoxin—GTX) or doubly (C-toxin) sulphated carbamate alkaloids that are known to interfere with neurotransmission through blockage of nerve cell sodium channels. Saxitoxins, which have LD$_{50}$ (i.p., mouse) values of 8-10 µg/kg bw and oral LD$_{50}$ of 260 µg/kg bw (Falconer, 2008; Funari & Testai, 2008), elicit symptoms that may include irregular breathing, muscle cell paralysis, twitching and death by respiratory failure (Carmichael, 1992).

2.4.3 Dermatoxins/cytotoxins

Aplysiatoxins and lyngbyatoxins are cyclic alkaloid toxins commonly known to elicit severe contact dermatitis (“Swimmer’s Itch”) as well as other cytotoxic effects in humans. The mode of toxicity of both toxins is very similar and it has
been speculated that these compounds might be of similar biogenetic origin (Ito et al., 2002).

2.4.3.1 Aplysiatoxins

Aplysiatoxin and debromoaplysiatoxin are the brominated and debrominated structural analogues of a class of polyacetate alkaloid algal compounds with characteristic dermal toxicity and tumor promotion properties (Nagai et al., 1996; Ueno, 1986). These compounds promote tumor development by acting as powerful activators of protein kinase (PK) C (Nakagawa et al., 2009; Yanagita et al., 2010). In addition, both analogues inhibit phorbol-12,13-dibutyrate-receptor binding, with aplysiatoxin being about 10-fold stronger than debromoaplysiatoxin in inhibiting epidermal growth factor and phorbol-12,13-dibutyrate-receptor binding (Horowitz et al., 1983). Symptoms of aplysiatoxin poisoning include, amongst others, vomiting, diarrhoea, and a burning sensation in the mouth and throat (Nagai et al., 1996).

2.4.3.2 Lyngbyatoxins

Three structural analogues of this toxin have been identified: lyngbyatoxin-A, -B, and –C, the first being the most common (Osborne et al., 2001). Just like aplysiatoxins, lyngbyatoxin-A (LA) is a recognised potent irritant and vesicant, as well as tumor promoter. It elicits its effects by activating the calcium-activated, phospholipid-dependent protein kinase C (Dow & Swoboda, 2000). LA has an LD_{100} (i.p., mouse) of 300µg kg^{-1} bw whereas lyngbyatoxin-B and lyngbyatoxin-C are only 1/200th and 1/20th times as potent, respectively, as LA (Osborne et al., 2001). Symptoms of lyngbyatoxin poisoning include: oral and esophageal inflammation, diarrhea, vomiting, ulceration of the skin, facial rash, amongst others.

2.4.4 Endotoxins

Lipopolysaccharides (LPS) which are found on the outer membrane of most Gram negative bacterial and cyanobacterial cell walls have been implicated in eliciting endotoxic as well as contact irritant responses in human and animals (Dow & Swoboda, 2000). Potency of LPS seems to be genera-dependent, with LPS from
pathogenic bacteria such as *Salmonella* spp. being more potent than cyanobacterial LPS. Cyanobacterial LPS, therefore, do not pose the health risks as enterbacteriaceae LPS, with the common symptoms of cyanobacterial LPS poisoning being skin and eye irritation, respiratory problems, headaches, dizziness, and blistering of mucous membranes are amongst (Stewart et al., 2006).

### 2.5 Detection of cyanotoxins

Detection of cyanobacterial toxins in water can be performed using either biological assays or physicochemical analytical techniques. A majority of the methods developed so far focused on detection of hepatotoxic microcystins, given the global prevalence and importance of this toxin.

#### 2.5.1 Whole organism bioassays

Toxicity of cyanobacterial material was determined in the past by use of mouse (Ito et al., 1997; Vasconcelos & Pereira, 2001) or invertebrate (Delaney & Wilkins, 1995; Kim et al., 2003; Kiviranta et al., 1991) bioassays. Cyanotoxin toxicity in this case was generally assessed based on the concentration/dose of the toxicant that caused fatalities in 50% of test organisms. Although the toxic response of test organisms generally permitted inference of the putative toxin class that elicited a particular response, whole organism bioassays only provided qualitative information on whether a bloom was toxic or not. Furthermore, ethical issues regarding *in vivo* mammal bioassays coupled with lack of sensitivity and specificity have limited use of such assays in monitoring specific cyanotoxins in environmental samples.

#### 2.5.2 Protein phosphatase inhibition assays

Protein phosphatase inhibition assays (PPIAs) which estimate microcystin and nodularin concentration in samples by measuring the inhibitory effects of these toxins on protein phosphatases (PP1 and PP2A) have been used in recent years to screen microcystins and nodularins due to the sensitivity and rapidity of the assays, as well as availability and commercialization of enzymes (McElhiney & Lawton, 2005). PPIAs employ either radioisotopic (Lambert et al., 1994; Xu et al., 2000) or colorimetric (An & Carmichael, 1994; Heresztyń & Nicholson, 2001;
Robillot et al., 2000; Sassolas et al., 2011; Ward et al., 1997; Wirsing et al., 1999; Wong et al., 1999) techniques to quantify toxin concentrations in laboratory cultures and environmental samples.

Although radioisotopic PPIAs tend to be more sensitive than colorimetric approaches, the latter are preferred cyanotoxin screening tools in most laboratories because they are more convenient to handle and less expensive than radioisotopic assays. Though toxin concentrations estimated by PPIAs have been shown to correlate strongly with high performance liquid chromatography (HPLC) data (Rapala et al., 2002; Ward et al., 1997), the inability of PPIAs to detect different toxin variants to the same extent coupled with assay sensitivity to environmental protein phosphatases unrelated to microcystins and nodularin (Rapala et al., 2002) makes PPIA quantification of specific toxin concentrations in environmental samples problematic.

2.5.3 Immunological assays

Immunological assays such as enzyme linked immunosorbent assay (ELISA) are amongst the most commonly used cyanotoxin detection tools because of their specificity, simplicity, and minimum requirement for sample processing (McElhiney & Lawton, 2005). Most ELISA formats employ antibodies raised against particular cyanotoxin variants (e.g. MC-LR) to recognise and consequently quantify the amount of target toxin in test samples. ELISA platforms currently used in cyanotoxin monitoring were developed using either monoclonal (Mikhailov et al., 2001; Nagata et al., 1997; Rivasseau et al., 1999; Ueno et al., 1996; Zeck et al., 2001) or polyclonal (An & Carmichael, 1994; Brooks & Codd, 1988; Chu et al., 1990; Metcalf et al., 2000; Moreno et al., 2011; Mountfort et al., 2005) antibodies. A common drawback of commonly used ELISAs lies in the fact that antibodies for assay development are usually raised against a particular toxin variant (e.g. MC-LR); hence non-target toxin variants that often co-occur in environmental samples might be poorly detected or not detected at all. Development of cross-reactive ELISAs using polyclonal antibodies raised against an MC-LR conjugate (Metcalf et al., 2000) or the Adda moiety of microcystins (Fischer et al., 2001) has resulted in sensitive detection of
up to seven microcystin variants as well as nodularins in laboratory cultures and natural samples.

Other immunologically-based formats such as immunoaffinity chromatography (Kondo et al., 2002), direct time resolved fluoroimmunometric assay (Mehto et al., 2001), impedimetric immunoelectrode (Sun et al., 2013), and enzyme linked immunoparticle assay (ELIPA) (Reverté et al., 2013) have also been successfully used to detect cyanotoxins in laboratory cultures and field samples, though there are currently no available commercial formats.

2.5.4 Analytical chromatography

The only analytical techniques capable of separating microcystins for specific identification and quantification of individual toxin variants are chromatography and capillary electrophoresis (Meriluoto, 1997).

Various variants of chromatography have been successfully employed in cyanotoxin detection, with high performance liquid chromatography (HPLC) being the analytical technique most commonly used in microcystin and nodularin detection. A crucial step in HPLC characterization of cyanotoxins is efficient separation of extracted toxins from co-extracted compounds that might confound (Ikawa et al., 1999; Moollan et al., 1996) downstream toxin detection and quantification.

Resolution of microcystins and/or nodularins from unwanted co-extracted compounds has been achieved in HPLC using a wide range of analytical columns comprising amongst others; reversed phase C18 cartridges (Allis et al., 2007; Baker et al., 2002; Lawton et al., 1994; Wirsing et al., 1999), amide C16 columns (Méjean et al., 2009; Spoof et al., 2003), ion exchange columns (Cadel-Six et al., 2009; Martin et al., 1990), or internal surface reversed columns (Aboal & Puig, 2005; Gjølme & Utkilen, 1996; Meriluoto et al., 1990) and an aqueous mobile phase consisting of acetonitrile, trifluoroacetic acid, methanol, or mixtures where necessary (Nicholson & Burch, 2001). Detection of toxins following chromatographic separation is performed either by: (1) direct measurement of ultra-violet (UV) absorbance at the toxin’s absorption maxima (238 nm for most
MCs and NODs or 222nm for MC-LW) (Lawton et al., 1994), (2) use of a photo-diode array (PDA) detector (Lawton et al., 1995; Lawton et al., 1994; Spoof et al., 2009), or (3) mass spectrometric detection (LC/MS) (Geis-Asteggiante et al., 2012; Hummert et al., 2000; Kondo et al., 1992; Lawton et al., 1995). The use of more sophisticated post-liquid chromatography toxin detection approaches such as matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) (Robillot et al., 2000) and tandem mass spectrometry (MS/MS)(Allis et al., 2007; Bateman et al., 1995; Geis-Asteggiante et al., 2012; Yuan et al., 1999) though more expensive has resulted in increased sensitivity of the chromatographic analytical techniques.

Recent development of ultra-performance liquid chromatography (UPLC) (Beltrán et al., 2012; Li et al., 2011; Spoof et al., 2009) has further improved toxin resolution, identification and quantification. Although other analytical techniques such as capillary electrophoresis (Sirén et al., 1999), thin layer chromatography (Luukkaninen et al., 1994; Pelander et al., 2000; Sivonen et al., 1992), gas chromatography (Harada et al., 1996; Sivonen et al., 1992) as well as pre-chromatography fluorescence derivatization (Hayama et al., 2012; Kam-Wing Lo et al., 2003) have also been used to detect cyanotoxins in laboratory cultures and natural samples, HPLC-PDA remains the most validated analytic method.

In spite of the added sensitivity and specificity afforded by HPLC and related analytical techniques, the high set-up and operation costs, expertise requirement, and lack of reference standards for most toxin variants still hampers the use of these analytical tools in routine environmental monitoring programs that require measurement of total toxin concentrations.

2.6 Genes regulating microcystin and nodularin biosynthesis

Biosynthesis of microcystins and nodularins takes place non-ribosomally via a thiotemplate mechanism that is regulated by respectively 55- and 48-kb multi-enzyme complexes consisting of both non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules (Figure 2.3).
Genome sequencing and characterization of the microcystin synthetase (mcy) gene clusters in *Anabaena* (Rouhiainen et al., 2004), *Microcystis* (Nishizawa et al., 2000; Tillett et al., 2001) and *Planktothrix* (Christiansen et al., 2003) as well as the nodularin synthetase (nda) gene cluster in *Nodularia spumigena* (Moffitt & Neilan, 2004) has revealed some similarities in the multi-enzyme components encoded by the different gene clusters. This notwithstanding, there exist salient differences not only in the organization of the various clusters but also the way various genes are arranged within a cluster (Figure 2.3). The microcystin synthetase gene cluster, for example, is made up of ten open reading frames (ORFs; *mcyA* to *mcyJ*) whereas nodularin synthetase comprises only nine ORFs (*ndaA* to *ndaI*). Transcription of *Microcystis*, *Anabaena* and *Nodularia* toxin biosynthetic genes, for example, seems to occur bidirectionally from a central promoter region whereas transcription of all but one *Planktothrix mcy* genes, *mcyT*, occurs unidirectionally from a promoter located upstream of the *mcyD* gene (Figure 2.3). Each *mcy* gene encodes unique Mcy proteins that are putatively involved in synthesis of different portions of the microcystin molecule as depicted in Figure 2.4. The similarities and differences in the various toxin biosynthetic genes can be exploited to design assays to target particular gene loci for specific detection and differentiation of different toxin producing cyanobacterial genera.

### 2.7 Detection of cyanobacteria in water

#### 2.7.1 Microscopic identification and enumeration

Detection of cyanobacteria in environmental water samples has traditionally been achieved via classical microscopic techniques following strain isolation and cultivation (Sivonen & Börner, 2008). Characterization of these organisms into different groups by microscopy is based on differences in phenotypic features including morphology, chemotaxonomic and/or physiological traits (Anagnostidis & Komárek, 1990).

Microscopic identification followed by isolation and subsequent testing for toxin producing capability over the years has, indeed, led to the creation of invaluable culture collections of toxic and non-toxic cyanobacteria. That notwithstanding,
microscopic classification of cyanobacteria sometimes fails to reflect true evolutionary relationships (Lyra et al., 2001; Wilmotte, 1994). The morphological plasticity of cyanobacteria with environmental conditions coupled with morphological changes induced by selective culturing techniques (Casamatta & Vis, 2004; Rasmussen et al., 2007; Soares et al., 2013) render microscopy incapable of discriminating toxic from non-toxic cyanobacteria strains that often co-exist in the same bloom (Sivonen & Jones, 1999; Vezie et al., 1998). The fact that microscopic detection of cyanobacteria in environmental samples is labor intensive, requires expert knowledge, and might be prone to operator subjectivity, necessitates development of more robust detection tools. Advances in genetic characterization of cyanobacteria have opened new avenues for specific detection of toxigenic cyanobacteria.

2.7.2 Polymerase chain reaction detection of cyanobacteria

The advent of polymerase chain reaction (PCR) in the early 1980s has revolutionized DNA amplification as well as detection of microorganisms in complex environmental samples. PCR in principal exploits the DNA amplifying ability of thermostable DNA polymerase enzymes, to initiate amplification of target gene sequences generating millions or even billions of DNA copies following repeated heating and cooling cycles. The level of sensitivity afforded by PCR amplification has resulted in development and utilization of various PCR-based techniques in the characterization of microbial life forms including cyanobacteria.

2.7.2.1 Conventional PCR

Conventional PCR is an end-point based approach wherein amplified PCR products (amplicons) are analyzed only at the end of the reaction using agarose or denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), sequencing, amongst others. As such the technique is useful mainly as a qualitative diagnostic tool for detecting presence or absence of particular genes in organisms. The presence of various types of internal heterogeneity in the genomic DNA sequences of many microorganisms (Karlin et al., 1997) has permitted use of conventional PCR in phylogenetic and taxonomic
characterization of cyanobacteria. Neilan et al. (1997), for example, used *Microcystis*-specific PCR and post-PCR sequencing to classify *Microcystis* spp. wherein they observed monophyletic clustering of 16S rDNA sequences from ten *Microcystis* spp. with only two *Microcystis* sequences clustering with the *Synechococcus* genus. Nubel et al. (1997) used conventional PCR amplification of the 16S rRNA gene in combination with denaturing gradient gel electrophoresis for rapid and phylogenetically meaningful identification of cyanobacteria. Despite reported successes at specific identification of different cyanobacterial genera, the 16S rRNA gene has been shown to be a poor target for distinguishing toxic from non-toxic cyanobacteria strains (Gugger et al., 2002; Neilan et al., 1997).

Attempts at differentiating toxic from non-toxic cyanobacteria by use of the more variable 16S-23S internal transcribed spacer (ITS) gene locus have generated mixed results. Whereas nodularin-producing *Nodularia spumigena* strains were successfully differentiated from non-toxic counterparts using either 16S-23S ITS or phycocyanin operon intergenic spacer (PC-IGS) regions (Laamanen et al., 2001), Otsuka et al. (1999) were unable to separate toxic from non-toxic *Microcystis* using ITS genes. The discovery and sequencing of cyanotoxin biosynthetic genes provided suitable targets for design of more specific PCR-based assays for detection and differentiation of toxic from non-toxic cyanobacteria.

A majority of the conventional PCRs to date have targeted the microcystin synthetase genes in *Microcystis* genera due to the global prevalence of microcystin-producing *Microcystis* as well as availability of genome sequences for the *mcy* genes from these organisms. The non-ribosomal peptide synthetases—*mcyA* and *mcyB*—are frequently used targets for PCR-based detection of toxigenic cyanobacteria. Tillett et al. (2001), for example, designed a PCR assay targeting the *mcyA* N-methyl transferase domain and used this to screen for toxicity in 37 *Microcystis* spp. as well as environmental samples. Results from this study showed good correlation between *mcyA* gene detection and microcystin production. An *mcyB*-based assay designed to target the first adenylation domain of the *Microcystis mcyB* gene (Kurmayer et al., 2002) was
able to detect mcyB genes in colonies of *M. aeruginosa* (73%) and *M. ichthyoblabe* (16%) but not in *M. wessenbergii* colonies isolated from Lake Wansee, Germany.

Despite the popularity of the mcyA and mcyB gene targets in conventional PCR detection of hepatotoxic cyanobacteria in both laboratory and field samples (Baker et al., 2002; Foulds et al., 2002; Hisbergues et al., 2003; Hotto et al., 2005; Nonneman & Zimba, 2002; Pan et al., 2002; Saker et al., 2005; Via-Ordorika et al., 2004; Wilson et al., 2005; Yoshida et al., 2005) the mixed NRPS/PKS mcyE gene locus is increasingly becoming a popular target for PCR-based detection of toxigenic cyanobacteria. This gene is considered a more suitable target because of the important role it plays in the synthesis and incorporation of Adda and D-glutamate into the microcystin structure. The relatively invariant nature of Adda and D-glutamate coupled with their crucial roles in MC toxicity further reinforces the utility of mcyE genes in toxigenicity detection. Vaitomaa et al. (2003), for example designed and tested via conventional PCR, genus-specific primers for *Microcystis* and *Anabaena* mcyE using 36 toxic and non-toxic strains of *Microcystis*(13), *Anabaena*(14), *Planktothrix*(8), and *Nostoc*(1). The *Anabaena* and *Microcystis* specific assays were found to produce amplicons solely with DNA from the respective toxic *Anabaena* and *Microcystis* spp. A *Planktothrix* mcyE PCR assay was designed and utilized to amplify mcyE genes solely in the seven toxic *Planktothrix* species out of the 63 toxic and non-toxic cyanobacteria species tested (Rantala et al., 2006).

Conventional PCR detection of toxic cyanobacteria has not been limited to microcystin-producing genera but has been extended to profiling other toxin producers following discovery and characterization of the nodularin (Moffitt & Neilan, 2004), and recently cylindrospermopsin (Mazmouz et al., 2010; Mihali et al., 2008), saxitoxin (Kellmann et al., 2008; Mihali et al., 2009), and anatoxin (Méjean et al., 2009) biosynthetic genes. Moffitt and Neilan (2001), for example, designed an ndaA-specific PCR assay which they used to amplify putative peptide synthetase amplicons exclusively from nodularin-producing *Nodularia spumigena* isolates as well as blooms samples that tested positive for nodularins. A number
of laboratory and field studies (Koskenniemi et al., 2007; Lyra et al., 2005; Rantala et al., 2008) have also reported successful detection of Nodularin-producing Nodularia spp. using PCR assays targeting the ndaF gene.

PCR-based differentiation of cylindrospermopsin-producing C. raciborskii from cylindrospermopsin-producing Anabaena bergii and Aphanizomenon ovalisporum was initially accomplished via a multiplex conventional PCR assay incorporating primers specific for Cylindrospermopsis raciborskii rpoC1 gene, polyketide synthase toxicity determinant, and peptide synthetase determinant (Fergusson & Saint, 2003). More recently, PCR and subsequent sequence analyses have resulted in specific detection of cylindrospermopsin-producing Oscillatoria spp. (Mazmouz et al., 2010), Aphanizomenon ovalisporum (Yilmaz & Phlips, 2011), or multiple cylindrospermopsin producers (Barón-Sola et al., 2012).

PCR detection of the neurotoxin- (anatoxin- or saxitoxin-) producers, unlike the hepatotoxin-producers, is a more recent development, probably due to tardy characterization of the neurotoxin biosynthetic genes. Cadel-six et al. (2009) described the design and utilization of an assay, targeting a 400 bp fragment within the polyketide synthase (ks2) locus of the anatoxin biosynthetic gene, to reliably detect anatoxin-a and homoanatoxin-a-producing Oscillatoria in field samples. In a similar manner, a ks2-based PCR was used to study the spatial variability of anatoxin-a and homoanatoxin-a-producing cyanobacteria in benthic cyanobacterial mats (Wood et al., 2010b). Specific detection of toxic Anabaena and Oscillatoria genera was achieved by PCR targeting anatoxin biosynthetic gene C (anaC) (Rantala-Ylinen et al., 2011).

Detection of saxitoxin-producing cyanobacteria has mostly been accomplished by PCR assays that target the sxtA gene locus of the saxitoxin biosynthetic gene cluster (sxt). Ledreux et al. (2010), for example, utilized a sxtA-specific PCR assay to successfully amplify fragments of the sxtA gene from 14 French isolates of Aphanizomenon gracile that tested positive for saxitoxin production, but also reported detection of the target sxtA gene fragment in Aphanizomenon aphanizomenoides from which saxitoxins could not be detected. Whereas sxtA-based PCR coupled with ELISA and LC-MS/MS has also been successfully
applied to differentiate saxitoxin-producing from non-saxitoxin producing *Aphanizomenon gracile* isolates from German lakes (Ballot et al., 2010), a PCR assay capable of detecting *sxtA* fragments in *Anabaena circinalis, Lyngbya wollei, Aphanizomenon* sp., or *Cylindrospermopsis raciborskii* was successfully used to detect saxitoxin-producing *Anabaena circinalis* in Australian freshwater samples (Al-Tebrineh et al., 2010).

Although PCR-based detection of toxin biosynthetic genes has the potential to provide vital information on the toxigenicity of samples, usage of such assays as the sole basis for cyanotoxin risk assessment, especially in uncharacterized environmental samples might be problematic owing to the possibility of false positive results. False positives occur when cyanobacteria possess the target toxin biosynthetic genes but lack toxin producing capacity. Occurrence of cyanobacteria with inactive *mcy* genes has indeed been reported amongst natural populations of *Anabaena* (Fewer et al., 2011), *Microcystis* (Mikalsen et al., 2003; Via-Ordorika et al., 2004), and *Planktothrix* (Christiansen et al., 2006; Kurmayer et al., 2004). While reasons for prevalence of these inactive *mcy* genotypes remain largely unknown, insertional and/or deletional mutagenesis within the *mcy* gene cluster are thought to be implicated (Christiansen et al., 2008; Kaebernick et al., 2001; Ostermaier & Kurmayer, 2009). Though reports of inactive anatoxin, cylindrospermopsin, or saxitoxin genotypes are relatively uncommon, probably due to the limited number of studies involving these toxin biosynthetic clusters, the looming potential for false positives warrants application of stringent PCR design and testing criteria.

### 2.7.2.2 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) is a variation of PCR which allows for simultaneous amplification and quantification of target genes in a sample. Real-time PCR estimation of amount of target DNA molecules can be achieved either by absolute or relative quantification methods. Whereas absolute quantification methods estimate the number of target DNA copies in a sample by use of DNA standard curves (Dhanasekaran et al., 2010), relative quantification
relies on reference genes to produce relative amounts of the target (Pfaffl et al., 2002; Schmittgen & Livak, 2008).

Real-time measurement of DNA amplification after each PCR cycle is made possible by incorporating fluorescent dyes into the PCR reaction mixture. Fluorescent chemistries commonly used in qPCR detection of toxigenic cyanobacteria include DNA intercalating dyes (e.g., SYBR® Green dye) that bind non-specifically with any double stranded (ds) DNA (Glazer & Rye, 1992; Zipper et al., 2004) or hydrolysis probes (e.g., TaqMan®) consisting of target-specific dual labelled oligonucleotide sequences that fluoresce only after hybridization with complementary target DNA (Holland et al., 1991). The intensity of fluorescence emitted using either detection chemistry is usually proportional to the initial concentration of target DNA molecules in the test sample. Measurement of the fluorescence intensity after each PCR cycle allows for determination of the PCR cycle at which fluorescence signal significantly exceeds background [commonly known as threshold cycle (C_T) and recently quantification cycle (C_q; Benes et al., 2009)]. Knowledge of these C_T values permits quantification of target gene concentration using either absolute or relative quantification methods.

Although SYBR® Green assays are more prone to generating false positive results than TaqMan® assays, they are nevertheless easier to set up and as such have been widely used in detecting toxigenic cyanobacteria in both laboratory and field situations. A majority of such real-time PCR studies have utilized uniplex PCR assays that target the mcy and/or nda gene clusters to characterize microcystin- and/or nodularin-producing cyanobacteria populations. Vaitomaa et al. (2003), for example, used a SYBR® Green qPCR assay targeting the mcyE gene to estimate microcystin-producing Anabaena and Microcystis concentrations in two Finnish lakes wherein Microcystis was identified as the main microcystin producer. The researchers further reported significant positive correlation between Microcystis mcyE copies and the microcystin concentration in lake water. Similar real-time PCR assays that target different portions of the Microcystis mcyE gene have also been used to study toxic Microcystis in Chinese (Otten et al., 2012) and Singaporean (Te & Gin, 2011) freshwater bodies.
Furukawa et al. (2006), in contrast, quantified microcystin-producing *Microcystis* concentrations in two Japanese lakes using a SYBR® Green assay that targeted the N-methyl transferase domain of the *mcyA* gene and had a limit of detection of 8.8 toxigenic *Microcystis* cells per reaction. The *mcyA* gene has also been targeted for specific detection of toxigenic *Microcystis* in Lake Mikata, Japan (Yoshida et al., 2007) as well as prominent microcystin-producing cyanobacteria in an Indian pond (Srivastava et al., 2012).

Detection of microcystin-producing cyanobacteria by qPCR targeting the *mcyD* gene has also been attempted. Fortin et al. (2010), for example, used SYBR® Green-based qPCR assays targeting the first dehydratase and β-keto synthase domains of the *mcyD* gene to characterize microcystin-producing *Microcystis* in water samples from the Missisquoi Bay, Lake Champlain. Results from this study showed strong association between *Microcystis mcyD* copies and microcystin concentration.

Koskenniemi et al. (2007) designed and utilized a real-time PCR assay targeting the *ndaF* gene to quantify nodularin-producing *Nodularia* spp. in laboratory cultures and Baltic Sea water samples, wherein between 30-45000 *ndaF* copies mL⁻¹ of Baltic Sea water were detected. Recently, SYBR® Green-based qPCR assays incorporating general primers that target both *mcyE* and *ndaF* gene clusters have been used to characterize all known microcystin- and nodularin-producing cyanobacteria in environmental water samples (Al-Tebrineh et al., 2011; Rantala et al., 2008).

TaqMan®-based qPCR assays which, unlike SYBR® Green assays, require the use of target specific probes whose design and optimization can be very tedious, are increasingly being used in estimating toxigenic cyanobacterial concentrations in environmental samples not only due to their specificity but also the capacity for detecting multiple gene targets within a single reaction tube.

Although much effort has been devoted towards detection of microcystin-producing *Microcystis*, the increasing incidence of mixed blooms of potentially toxic cyanobacteria spanning multiple genera warrants a shift towards multiple target detection. Foulds et al. (2002), for example, utilized uniplex TaqMan®
assays targeting the lacZ and mcyA genes to detect as low as three copies of E. coli lacZ and Microcystis mcyA genes in sterile water samples initially inoculated with E. coli and toxic Microcystis cells. Kurmayer and Kutzenberger (2003) employed Taq nuclease assays (TNAs) targeting the mcyB and PC genes to estimate the proportions of toxic to total Microcystis (range 1-38%) in Lake Wannsee (Germany) samples. Okello et al. (2010) also utilized the Kurmayer and Kutzenberger TNAs to study the spatial distribution of toxic Microcystis in five east African lakes, while studies by Rinta-Kanto and colleagues employed uniplex TNAs targeting mcyD and 16S rDNA gene fragments to: (1) quantify toxic Microcystis concentrations as well as total Microcystis proportions in Lake Erie (USA) bloom samples (Rinta-Kanto et al., 2005), and (2) elucidate how toxigenic and total Microcystis concentrations in Lake Erie associate with microcystins and other environmental parameters (Rinta-Kanto et al., 2009a). TNAs that target different portions of Microcystis 16S rDNA and mcyD gene sequences have also been successfully used to estimate toxic Microcystis abundance in the San Francisco Estuary (USA), where Microcystis carrying the mcyD gene were found to constitute 0.4-20% of the total Microcystis population, which in itself made up 28-96% of the total cyanobacterial population (Baxa et al., 2010).

The only TNA to specifically detect microcystin-producing Planktothrix so far is a duplex assay designed to simultaneously target Planktothrix 16S rRNA and mcyA genes in preserved sediment samples from three lakes in the French Alps (Savichtcheva et al., 2011). This assay was able to reliably estimate Planktothrix mcyA and 16S rRNA copy numbers in samples from all three lakes.

Recently, a quadruplex TNA targeting the saxitoxins (sxtA), cylindrospermopsin (cyrA), microcystin/nodularin (mcyE/ndaF) biosynthetic genes as well as a general cyanobacterial 16S rRNA gene was designed, optimized, and validated for detection of all saxitoxin-, cylindrospermopsin-, and microcystin/nodularin-producing cyanobacteria irrespective of their genera (Al-Tebrineh et al., 2012b). This assay was subsequently utilized to estimate sxtA, cyrA, mcyE/ndaF, and 16S rRNA gene copies from Murray River water samples (Al-Tebrineh et al., 2012a). Results from this study showed predominance of cyrA-containing cells early in
the bloom while *sxtA*-containing cells became more common towards the bloom’s decline. Cyanobacterial cells containing *mcyE/ndaF* genes, on the contrary, were present at low levels throughout the sampling period.

There are presently no PCR assays that can specifically detect and estimate concentrations of multiple toxin-producing cyanobacterial genera within a single reaction tube. It is believed that use of such assays in toxic cyanobacteria monitoring could result in: (1) significant reductions in sample analysis cost, and (2) generation of vital information on specific dominant toxin-producing cyanobacteria genera that could be very useful in the design of strategies to prevent massive development of targeted noxious algal species.

### 2.7.2.3 Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is another variation of PCR used to detect and quantify gene expression levels. This technique also employs various fluorescent chemistries to provide relative or absolute quantitative measures of gene expression levels (Pfaffl, 2004; Pfaffl et al., 2002) after creation of complementary DNA (cDNA) transcripts from RNA using reverse transcriptase enzymes. Thus the use of RT-qPCR in monitoring toxic cyanobacteria, unlike qPCR which detects toxigenic cyanobacteria genotypes (i.e. genotypes with active and/or inactive toxin biosynthetic genes), results in detection and estimation of concentrations of genotypes able to synthesize the toxin. It is assumed that measurement of concentrations of actual toxin producers in a given bloom could permit more reliable assessment of the risk of exposure to target cyanotoxins.

Although RT-qPCR can potentially provide vital information on bloom toxicity that cannot be otherwise obtained from current qPCR approaches, use of this technique in monitoring toxigenic cyanobacteria is not widespread, probably due to the greater lability of RNA relative to DNA as well as inherent difficulties in extracting and preserving high quality RNA from complex environmental samples (Gadkar & Filion, 2012; Sharkey et al., 2004). The few attempts at implementing RT-qPCR in cyanobacterial monitoring have nevertheless provided valuable insight into expression of cyanotoxin biosynthetic genes under field and
laboratory conditions. Gobler et al. (2007) using a qualitative RT-PCR assay to study *Anabaena* and *Microcystis mcyE* gene expression in Lake Agawan (USA) water samples, showed frequent expression of *Microcystis mcyE* in water samples collected between May and August but rarely in those sampled in September and October. The fact that the overall *Microcystis mcyE* gene expression patterns were mostly consistent with microcystin concentration coupled with non-detection of *Anabaena* McyE transcripts lead these researchers to suggest that *Microcystis* was the putative microcystin producer in this lake system. Laboratory studies of *Microcystis* McyE transcript levels using an armored RNA standard showed *mcyE* expression levels to be growth-phase-dependent, with gene expression highest at the mid-log phase and lowest during the stationary phase (Rueckert & Cary, 2009). Recently, RT-qPCR assays were successfully utilized to quantify *Microcystis* McyE transcripts in lake water (Wood et al., 2011) and buried sediment samples (Misson et al., 2012). Transcriptional regulation of *Microcystis mcyA* and *mcyH* genes under iron starvation and limitation conditions has also been investigated (Alexova et al., 2011). Apart from *Microcystis* or *Anabaena* microcystin biosynthetic genes, quantitative estimation of nodularin biosynthetic gene F (*ndaF*) expression in Baltic Sea *Nodularia* spp. has been reported (Koskenniemi et al., 2007).

### 2.7.3 Microarray based detection methods

DNA hybridization-based techniques such as DNA microarrays (DNA chips) have also been used in the detection and differentiation of toxic and non-toxic cyanobacteria. Although generally more laborious, time-consuming, and costly than PCR amplification, the higher throughput potential of microarrays (Rantala et al., 2008) nevertheless makes them appealing alternatives. Most microarrays developed so far have focused on 16S rRNA gene polymorphisms to detect, differentiate, and quantify different cyanobacteria genera irrespective of their toxigenicities. Rudi and colleagues (Rudi et al., 2000), for example, designed a cyanobacterial-specific 16S rDNA microarray that relied on competitive PCR coupled with conventional DNA-DNA hybridization techniques to specifically
detect and discriminate between several cyanobacteria genera including *Microcystis, Nostoc, Planktothrix*, amongst others.

The microarray developed by Castiglioni and colleagues (Castiglioni et al., 2004), although also consisting of probes targeting the cyanobacterial 16S rRNA gene rather utilized the ligase detection reaction (LDR) with ‘zip code’ hybridization (Gerry et al., 1999) for specific and sensitive (down to 1 fmol DNA) detection of 19 cyanobacterial groups. A MAG microarray that incorporates magnetic capture hybridization techniques and DNA microarray (Matsunaga et al., 2001) for detecting cyanobacterial 16S rRNA genes has also been reported. This assay was able to specifically detect *Anabaena, Microcystis, Nostoc, Oscillatoria*, and *Synechococcus* spp. irrespective of their toxigenicity.

Although effective for detecting even single nucleotide differences, 16S rRNA-based microarrays are generally incapable of discriminating toxic from non-toxic cyanobacteria due to high similarity in 16S rRNA genes from toxic and non-toxic strains of various cyanobacterial genera (Lyra et al., 2001). The only DNA chip assay to specifically target toxin biosynthetic genes was developed by Rantala et al. (2008). This assay which utilized probes targeting the *mcyE/ndaF* genes in conjunction with the LDR ‘zip code’ hybridization techniques was successfully used to detect as low as 1-5 fmol of PCR product from fresh and brackish water samples containing hepatotoxin-producing *Anabaena, Microcystis, Planktothrix, Nostoc*, and *Nodularia* spp.

### 2.7.4 Other detection approaches

Cyanobacterial cells contain the photosynthetic pigments chlorophyll a and phycocyanin, with absorption maxima around 670nm and 620nm respectively. Chl-a concentrations determined by extractive approaches have traditionally been used as quick indicators of algal biomass and trophic status of water bodies (Lawton et al., 1999). However, chl-a measurements are incapable of differentiating cyanobacterial biomass from biomass arising from other green algae and diatoms. As a result, measurement of phycocyanin (mostly present in cyanobacteria) concentration has been proposed as a better indicator of cyanobacterial biomass (Ahn et al., 2007; Ramos et al., 2010).
Recent developments in sensor technology have resulted in increased estimation of cyanobacterial biomass via \textit{in vivo} chl-a and/or phycocyanin measurements using various fluorescent sensors (Chang et al., 2012; Izydorczyk et al., 2005; Song et al., 2013). Spectral properties of these pigments have also been exploited in optical teledetection of phytoplankton and cyanobacteria either via shipboard, aircraft, or satellite observations (e.g., Alikas et al., 2010; Gons et al., 2005; Tebbs et al., 2013; Wheeler et al., 2012). Despite the novelty of some of these approaches, none is capable of differentiating between toxic and non-toxic blooms of cyanobacteria.

2.8 Conclusion of literature review

The extraordinary ability of cyanobacteria to thrive in adverse environments has enabled these organisms to colonize a wide array of niches ranging from hot springs to cold arctic waters (Miller et al., 1998; Quesada et al., 1999). These organisms normally constitute natural flora in these unperturbed aquatic and terrestrial ecosystems. Under favourable conditions, however, most cyanobacteria tend to proliferate massively to out-compete other phytoplankton producing cyanobacterial blooms. Eutrophication of water bodies over the last couple of decades has led to recurring incidents of cyanobacterial blooms and degradation of water resources in most countries globally. The economic losses from this resource degradation exceed US$ 2 billion per annum in the United States alone (Dodds et al., 2009). In addition to the economic impacts, a majority (approximately 60 \%) of blooms produce toxins (Sivonen & Jones, 1999) that have been implicated in the morbidity or mortality of animals, birds and humans (e.g., Jochimsen et al., 1998; Matsunaga et al., 1999; Wood et al., 2010a). There is therefore an urgent need for effective monitoring and control of these noxious organisms. The following section summarized approaches that have traditionally been used for cyanotoxin detection and bloom characterization.

Detection of cyanotoxins is often accomplished by use of either biological or physicochemical analytic methods. Biological techniques that have been utilized for cyanotoxin detection include mouse or invertebrate bioassays, protein phosphatase inhibition assays, or enzyme linked immunosorbent assays, amongst
others. Physicochemical analytical techniques mostly involve chromatographic separation and detection using either photo-diode array detectors or mass spectrometry (Nicholson & Burch, 2001). Either approach (Biological or physicochemical analytical detection) has its advantages and disadvantages (McElhiney & Lawton, 2005; Nicholson & Burch, 2001), hence the choice of cyanotoxin detection technique depends on expected outcome and budgetary constraints. Enzyme linked immunosorbent assays, for example, are preferred tools in routine monitoring programs that require total estimates of MCs and NODs because of their specificity, simplicity, and minimum requirement for sample processing (McElhiney & Lawton, 2005) whereas the more sophisticated and costly HPLC-PDAs might be preferable for identification and quantification of individual toxin variants (e.g., Meriluoto, 1997).

Characterization of environmental bloom samples has traditionally been performed by microscopy identification coupled with biological or physicochemical analytical toxin detection methods (Sivonen & Börner, 2008). Although valuable information on bloom characteristics can be obtained through this approach, the high dependence on expert judgement, its labour intensive nature, and the inability of microscopy to distinguish between morphologically similar toxic and non-toxic cyanobacterial strains necessitates development of more specific and reliable tools. The final section of this review summarised the characterization of common toxin biosynthetic gene clusters as well as the approaches that have been utilized for toxigenic cyanobacteria detection.

The discovery, sequencing, and characterization of genomes of toxic and non-toxic cyanobacteria has led to exploitation of molecular approaches for detection and differentiation of toxic from non-toxic cyanobacteria. Microcystin, nodularin, cylindrospermopsin, anatoxin, and saxitoxin biosynthetic genes in common bloom forming cyanobacteria have been sequenced and characterized. This review focused on genetic regulation of microcystin biosynthesis because of the global importance of microcystins and its relevance to this research project. Microcystin biosynthesis in bloom forming Anabaena, Microcystis, and Planktothrix genera has been shown to be regulated by a 55 kb microcystin synthetase (mcy) gene.
cluster comprising ten open reading frames (mcyABCDEFGHIJ) transcribed bidirectionally from a central promoter in *Anabaena* (Rouhiainen et al., 2004) and *Microcystis* (Tillett et al., 2000) but of only nine ORFs (mcyABCDEGHJT) in *Planktothrix* (Christiansen et al., 2003) which, except for mcyT, are mostly unidirectionally transcribed. The similarities and differences amongst the gene clusters have been exploited in designing molecular tools for specific detection and differentiation of toxic from non-toxic cyanobacteria.

Cyanobacteria genetic loci such as the 16S rRNA, 16S-23S internal transcribed spacer, and phycocyanin operon intergenic spacer were initial targets for conventional PCR-based differentiation of cyanobacteria genera (Laamanen et al., 2001; Neilan et al., 1997; Nubel et al., 1997; Otsuka et al., 1999). Despite reported successes at differentiating cyanobacteria into phylogenetically meaningful classes, PCR assays targeting these gene loci were unable to distinguish toxic from non-toxic cyanobacteria (Lyra et al., 2001). Although subsequent conventional PCRs targeting various toxin biosynthetic gene fragments were more successful at discriminating between toxic and non-toxic cyanobacterial strains (e.g., Kurmayer et al., 2002; Tillett et al., 2001), these assays nevertheless only generated qualitative information on presence or absence of particular toxin producers. Recent quantitative PCR assays that target toxin biosynthetic genes have successfully utilized either DNA intercalating dyes (mostly SYBR green) (e.g., Fortin et al., 2010; Vaitomaa et al., 2003) or hydrolysis probes (mostly TaqMan®) (e.g., Kurmayer & Kutzenberger, 2003; Rinta-Kanto et al., 2009a) to specifically detect, differentiate, and quantify different toxigenic cyanobacteria genera. These assays, however, mostly utilized uniplex reactions incapable of quantifying more than one toxin-producing genus per reaction and only provided quantities of potential and not actual toxin producers. Although use of reverse transcription quantitative PCR to measure toxin gene expression levels (e.g., Misson et al., 2012; Wood et al., 2011) provides vital information on active toxin producers, there are, nevertheless, few gene expression studies done this way, probably due to difficulties associated with extraction and preservation of high quality RNA. DNA microarray detection of toxic cyanobacteria has equally been attempted (Rantala et al., 2008) but this
technique is not commonly used in cyanobacteria monitoring as it is more laborious, time consuming, and costly than PCR.

To conclude, this review identified incidence of cyanobacterial bloom as an emergent and important water quality issue which might be exacerbated in a warmer future climate where increasing population growth would spur demand for increased food production through intensive agricultural practices that release excess nutrients into water bodies. Increasing cyanobacterial blooms coupled with the realization of the inadequacies of conventional approaches for monitoring potentially toxic cyanobacteria will necessitate a shift towards more robust molecular detection techniques (such as PCR) which target the toxin biosynthetic genes. Although such PCR-based tools have been and are currently being explored in some countries around the world, there is no consensus regarding the most suitable toxin biosynthetic gene (in a repertoire of nine or more putative genes in most toxigenic cyanobacteria) target. In addition, most of the reported assays are only capable of detecting one toxin-producing cyanobacterial genus per assay, thereby warranting the use of multiple assays to detect multiple toxin-producing genera that often co-occur within the same bloom. Furthermore, there is a severe paucity in literature regarding use of these futuristic molecular tools in profiling toxigenic cyanobacteria in Canadian freshwater bodies. The present study was thus aimed at developing novel PCR-based techniques to address the local knowledge gap while also contributing towards current global lack of single PCR assays for simultaneous detection of multiple toxigenic cyanobacterial genera.
<table>
<thead>
<tr>
<th>Toxin group</th>
<th>Number of structural variants</th>
<th>Primary mammalian target organ and elicited effects</th>
<th>Toxigenic genera</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hepatotoxins</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Microcystins</td>
<td>90</td>
<td>Liver; hepatotoxic, protein phosphatase inhibition, disruption of membrane integrity and conductance, tumour promoters and conductance, tumour promoters and/or initiators</td>
<td>Anabaena, Anabaenopsis, Hapalopsiphon, Microcystis, Nostoc, Phormidium, Planktothrix (Oscillatoria)</td>
</tr>
<tr>
<td>Nodularins</td>
<td>9</td>
<td>Liver; hepatotoxic, protein phosphatase inhibition, disruption of membrane integrity and conductance, tumour promoters and/or initiators</td>
<td>Nodularia, Cyanobacterial symbiont of Theonella</td>
</tr>
<tr>
<td>Cylindrospermopsins</td>
<td>3</td>
<td>Necrotic injury (liver, kidneys, spleen, lungs, intestines), protein synthesis inhibition, genotoxic                                                                ordial agents, protein kinase C activators</td>
<td>Aphanizomenon, Anabaena, Cylindrospermopsis, Rhaphidiopsis, Umezakia</td>
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<td><strong>Neurotoxins</strong></td>
<td></td>
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<tr>
<td>Anatoxin-a</td>
<td>5</td>
<td>Nerve synapse; postsynaptic, depolarising neuromuscular blockers</td>
<td>Aphanizomenon, Anabaena, Arthrosira, Cylindrospermum, Phormidium, Oscillatoria, Rhaphidiopsis</td>
</tr>
<tr>
<td>Anatoxin-a(S)</td>
<td>1</td>
<td>Nerve synapse; acetylcholinesterase inhibitor</td>
<td>Anabaena</td>
</tr>
<tr>
<td>Saxitoxins</td>
<td>20</td>
<td>Nerve axons; sodium channel blockers</td>
<td>Aphanizomenon, Anabaena, Cylindrospermopsis, Lyngbya, Planktothrix, Scytonema</td>
</tr>
<tr>
<td><strong>Dermatotoxins and cytotoxins</strong></td>
<td></td>
<td></td>
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<tr>
<td>Lyngbyatoxin-a</td>
<td>1</td>
<td>Skin and gastrointestinal tract; inflammatory agents, protein kinase C activators</td>
<td>Lyngbya, Schizothrix, Oscillatoria</td>
</tr>
<tr>
<td>Aplysiatoxins</td>
<td>2</td>
<td>Skin; inflammatory agents, protein kinase C activators</td>
<td>Lyngbya, Schizothrix, Oscillatoria</td>
</tr>
<tr>
<td><strong>Endotoxins</strong></td>
<td></td>
<td></td>
<td>Most cyanobacteria</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>Various</td>
<td>Affects any exposed tissue; inflammatory agents, gastrointestinal tract irritants</td>
<td></td>
</tr>
</tbody>
</table>

*a*; Adapted from Codd et al. (2005b), *b*; Toxin not necessarily produced by all species or strains within the genus, Updates from; *c*; Welker and von Doren (2006), *d*; Smith et al. (2012), *e*; Ballot et al. (2005), *f*; Teneva et al. (2005)
Figure 2.1: Countries that have reported potentially toxic cyanobacterial bloom incidents. Adapted from Carmichael (2008) with updates (black triangles) from other literature sources (Akin-Oriola et al., 2006; Codd et al., 2005a; Nyenje et al., 2010)
Figure 2.2: General structure of the hepatotoxins microcystins (A), nodularins (B), and cylindrospermopsin (C). R₁ and R₂ represent either H or CH₃ while X and Z are variable L-amino acids in the different toxin conformers. In MCYST-LR, for example, X= L-Leucine(L) and Z= L-Arginine(R). Z represents L-Arginine in nodularins but L-Valine in motuporin (Redrawn from Sivonen and Jones 1999).
Figure 2.3: Gene clusters encoding synthesis of nodularin in *Nodularia* (Moffitt & Neilan, 2004) and microcystins in *Microcystis* (Nishizawa et al., 2000; Tillett et al., 2000), *Anabaena* (Rouhiainen et al., 2004), and *Planktothrix* (Christiansen et al., 2003). (Redrawn from Börner & Dittman, 2005)
Figure 2.4: Putative roles of various microcystin synthetase (Mcy) proteins in the synthesis of the microcystin molecule. PKS domains: AT: acyltransferase; ACP: acyl carrier protein; KS: β-ketoacyl synthase; KR: ketoacyl reductase; DH: dehydratase; CM: C-methyltransferase; AMT: aminotransferase; NRPS domains: A: aminoacyl adenylation; C: condensation; NMT: N-methyl transferase; Ep: epimerase; TE: thioesterase; McyF: racemase; OM (McyJ): O-methyl transferase. Black bars represent the thiolation motif of NRPS modules while arrows indicate steps of microcystin synthesis controlled by individual proteins. (Adapted from Borner and Dittman, 2005)
Connecting text to chapter 3

Chapter two of this dissertation reviewed the available literature on cyanobacterial bloom, cyanobacterial toxins and their biosynthesis, as well as approaches for detecting cyanobacteria and their toxins in water. The need for supplementation of current microscopy-based cyanobacteria detection approaches with more sensitive and robust molecular tools was identified. Whereas much progress has been made in many countries to evaluate the suitability of PCR-based toxigenic cyanobacteria detection techniques, little effort has been devoted to this futuristic approach in Canada. Chapter three of this thesis addressed this local knowledge gap by exploring the suitability of three microcystin synthetase genetic loci (mcyA, E, and G) for quantitative PCR estimation of microcystin-producing Microcystis spp. prevalent in many Canadian freshwater bodies. The manuscript from this study, which addressed objective one of this dissertation, was accepted for publication in Environmental Toxicology Journal on February 1, 2012. This manuscript which was co-authored by Drs. Madramootoo, C.A., and Jabaji, S., forms the basis of the following chapter.

Chapter 3 Monitoring toxigenic *Microcystis* strains in the Missisquoi Bay, Quebec, by PCR targeting multiple toxic gene loci

Felexce Ngwa, Chandra Madramootoo, and Suha Jabaji

Abstract

The increasing incidence of mixed assemblages of toxic and non-toxic cyanobacterial blooms in Quebec’s freshwater bodies over the last decade, coupled with inherent inadequacies of current monitoring approaches, warrant development of sensitive and reliable tools for assessing the toxigenic potential of these water blooms.

In this study, we applied three independent PCR assays that simultaneously target the microcystin synthetase (*mcy*) genes A, E and G to rapidly and reliably detect and quantify potentially toxic *Microcystis* genotypes in the Missisquoi Bay, Quebec, Canada. Linear regressions of quantitative PCR threshold cycles (*C*_\text{\textsubscript{T}}) against the logarithm of their respective *Microcystis* cell number equivalents resulted in highly significant linear curves with coefficients of determination (*R*_\text{\textsuperscript{2}}) greater than 0.99 (\textit{p}< 0.0001, \textit{n} = 6) and reaction efficiencies of 91.0, 95.8, and 92.7 percent respectively for the *mcyA*, *mcyE* and *mcyG*-based qPCR assays. The three assays successfully estimated potential microcystin-producing *Microcystis* genotypes from all field samples.

The proportions of *Microcystis* *mcyA*, *mcyE* and *mcyG* genotypes to total *Microcystis* cell counts showed substantial spatial variability ranging between 1.7-21.6%, 1.9-11.2% and 2.2-22.6% respectively. Correlation of microscopically determined total *Microcystis* counts to qPCR-based *Microcystis* *mcyA*, *mcyE* or *mcyG* cell number equivalents resulted in highly significant associations with *R*_\text{\textsuperscript{2}} > 0.90. Thus, PCR-based assays targeting the *mcyA*, *mcyG* and/or *mcyE* genes can serve as powerful screening tools for rapid and sensitive estimation of microcystin-producing *Microcystis* genotypes in freshwater water bodies.
Key words: Microcystin synthetase; Microcystis genotypes; Missisquoi Bay; Quantitative real-time PCR; Toxigenic cyanobacteria; Water quality monitoring

3.1 Introduction

Freshwater bodies in Quebec, Canada, have witnessed an increased incidence of summer blooms of potentially toxic cyanobacteria (blue-green algae) genera over the last decade (Blais, 2002), with over 150 lakes contaminated by different strains of the Microcystis, Anabaena, Aphanizomenon, or Planktothrix genera or mixtures of these. A majority of these genera have been associated with production of varying concentrations of the cosmopolitan cyanotoxin, microcystin (MC). The Missisquoi Bay located on the north eastern arm of Lake Champlain has been one of the hardest hit freshwater bodies in Quebec with reported microcystin concentrations often exceeding the Canadian drinking water guideline of 1.5µg L⁻¹ during the summer months. This has resulted in almost yearly issuance of public health advisories and beach closures since 2001, which have had a tremendous toll on the economy of the surrounding regions that rely heavily on tourism.

Microcystins are cyclic heptapeptides with about 90 known congeners (Welker & von Döhren, 2006) exhibiting different hepatotoxicities. Their toxicities are believed to emanate from inhibition of eukaryotic protein serine/threonine phosphatases 1 and 2A (Honkanen et al., 1991; Welker & von Döhren, 2006). Acute effects of microcystin poisoning including liver injury and/or death have been reported in humans and animals (Jochimsen et al., 1998; Wood et al., 2010a). In addition, chronic exposure to low levels of microcystins in drinking water has been implicated in incidence of liver carcinomas (Falconer et al., 1983; Fujiki & Suganuma, 1999). These potential problems from toxigenic cyanobacteria blooms have made monitoring of these organisms and their toxins in freshwater bodies a priority for most governments.

Monitoring cyanobacteria in Quebec’s freshwater bodies relies predominantly on use of microscopy in conjunction with analytical chemical methods for cyanotoxin detection, which most often are cumbersome and incapable of pinpointing the toxin-producing species in the commonly occurring mixed
populations of toxic and non-toxic strains. Thus, there is a need for development of reliable and sensitive tools for estimation of toxigenic cyanobacteria genera. Gene-based approaches such as the polymerase chain reaction (PCR) that target the microcystin biosynthesis genes offer a more sensitive and specific approach for discriminating toxic from non-toxic genotypes. In addition to shorter turnaround times for sample analyses, PCRs are in general not only less expensive but also more amenable to high throughput than conventional cyanobacterial monitoring approaches.

Microcystin biosynthesis is encoded by the microcystin synthetase (mcy) gene cluster—a multienzyme complex consisting of both non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules (Tillett et al., 2000). Characterization of this gene cluster in *Microcystis* spp. has revealed the presence of ten genes (mcyA-J) arranged in two bi-directionally transcribed operons (mcyA-C and mcyD-J) that encode the synthesis and incorporation of various amino acid residues into the microcystin structure. Microcystin biosynthetic processes in *Anabaena* and *Planktothrix* spp. are remarkably similar to the *Microcystis* process despite noticeable variations in arrangement of *mcy* genes in the *Anabaena* (Rouhiainen et al., 2004) and *Planktothrix* (Christiansen et al., 2003) *mcy* gene clusters. Insertional (Christiansen et al., 2006) or deletional (Tillett et al., 2000) mutagenesis of the *mcy* genes has been shown to result in lack of microcystin production although the phenomenon is less common for *Microcystis* than *Planktothrix* spp. (Christiansen et al., 2006).

Quantitative real-time PCR (qPCR) assays so far developed target mostly single genes in either of the *mcy* operons with a majority of these assays either targeting the *mcy*A (Furukawa et al., 2006; Ha et al., 2009; Rinta-Kanto et al., 2009b; Ye et al., 2009; Yoshida et al., 2007), *mcy*B (Kurmayer & Kutzenberger, 2003; Okello et al., 2010), *mcy*D (Baxa et al., 2010; Fortin et al., 2010; Hotto et al., 2008; Rinta-Kanto et al., 2005) or *mcy*E (Rantala et al., 2006; Vaitomaa et al., 2003) genes. Whereas much effort has been made towards quantification of potentially toxic cyanobacteria by use of qPCR assays targeting single *mcy* genes, very few attempts (Valério et al., 2010) have been made to evaluate performance of assays
targeting multiple genes. Although Fortin and coworkers (Fortin et al., 2010) employed qPCR to estimate toxic *Microcystis* concentrations in Quebec, Canada, their analyses focused on two different regions of the same mcyD gene. Considering that the detection of one mcy gene in a sample does not necessarily imply the presence of other equally vital microcystin biosynthetic genes, single gene-based PCR quantification might be more prone to yielding false positive results, especially in the absence of toxin analysis data. Indeed the discovery of mutations in the mcy gene cluster that result in inactive mcy genotypes mainly in *Planktothrix* (Kurmayer et al., 2004) but also *Microcystis* (Mikalsen et al., 2003; Tillett et al., 2001) and *Anabaena* (Fewer et al., 2011) genera emphasizes the need for evaluating qPCR approaches that target multiple mcy genes as the basis for estimating potentially toxic cyanobacteria in environmental samples. The mcyA, mcyE and mcyG genes were considered suitable targets for qPCR-based estimation of toxigenic *Microcystis* in the present study because of their roles in the synthesis and incorporation of vital amino acids into the microcystin structure (Tillett et al., 2000; Vaitomaa et al., 2003).

In this study, we applied three independent quantitative PCR assays to simultaneously target the mcyA, mcyE, and mcyG genes in a bid to rapidly and reliably detect potentially toxic *Microcystis* in a freshwater body, thereby decreasing the likelihood of obtaining false positive results. *Microcystis* sp. was considered a suitable target for detection given that it is often one of the most dominant potentially toxic cyanobacteria genera in the Missisquoi Bay during summer and early fall months (Fortin et al., 2010). Using real-time qPCR assays, we were able to reliably estimate microcystin-producing *Microcystis* concentrations from field samples as well as laboratory cultures. To the best of our knowledge, this is the first study to target the mcyG gene for qPCR-based estimation of toxigenic cyanobacteria while also providing a comparative evaluation of the suitability of three mcy gene targets for quantification of toxigenic *Microcystis* spp.
3.2 Materials and methods

3.2.1 Laboratory culture conditions

Microalgal strains (Table 3.1), except for *Microcystis* sp. VQ-C009, used in the present study were purchased from the Canadian Phycological Culture Centre (CPCC, Waterloo, ON, Canada). Strain VQ-C009 was isolated from bloom samples collected from the Missisquoi Bay, Quebec, Canada and identified according to morphological and phylogenetic characteristics (Kurmayer et al. 2004). All strains were cultured in 250mL Erlenmeyer flasks containing 100mL nutrient rich mineral medium BG-11 (Stanier et al., 1971) and maintained in a growth chamber under conditions of 25±1 °C temperature, 12:12 LD (light and dark) cycles at a photon flux density of 30 ±2 µmol photon m⁻² s⁻¹ provided by cool white fluorescent bulbs. Cultures harvested after three weeks of growth were used to extract DNA that was utilized in primer specificity testing and quantitative real-time PCR standard curve development.

3.2.2 Field sampling and analyses

Seven surface water samples were collected from the Missisquoi Bay, Quebec on August 12 and 25, 2009. Three near-shore (littoral) stations (VQ-A, VQ-B, VQ-C) and a fourth (PK-D) located at the mouth of the Pike River were sampled on August 12 while the three open water (pelagic) stations (BM-A, BM-B, and VQ-D8) were sampled on August 25 (Figure 3.1). Between 50-250mL water samples for DNA, chlorophyll a (chl-a), and microcystin analyses were immediately filtered onto GF/C glass microfiber filters (47mm diameter, Whatman, England), wrapped in aluminum foil paper and transported on ice to the lab. Chlorophyll a analysis was performed within 24 hours whereas samples for DNA and microcystin analyses were stored at -20°C for subsequent analyses. A 100mL aliquot of water sample from each station was preserved in acid Lugol for subsequent phytoplankton enumeration.

Water samples for total phosphorus (TP) and nitrogen (TN) estimation were collected in acid-washed 500mL high density polyethylene (HDPE) bottles (VWR International, PA, USA) and transported on ice to the lab. Total phosphorus (TP)
and nitrogen (TN) concentrations were determined by the peroxodisulfate oxidation method (Ebina et al., 1983). In situ chlorophyll and cyanobacterial concentrations as well as physicochemical parameters were measured at the time of sample collection using a pre-calibrated multi-parameter water quality sonde YSI 6600V2-4 (YSI Inc., Yellow Springs, OH, USA).

### 3.2.3 Cyanobacterial biomass estimation

Lugol-preserved field samples were analyzed using a Leitz Diavert phase contract inverted microscope (Ernst Leitz Inc, Rockleigh, NJ, USA) at magnifications between 125× and 930× following the modified Utermohl method (Nauwerck, 1963) described by Findlay and Kling (1998). Laboratory cultures were enumerated using a hemocytometer mounted on a Hund Wetzlar microscope (Helmut Hund GmbH, Wetzlar, Germany). Filters for laboratory analyses of chl-a were boiled in 90% ethanol for 5 min and allowed to stand overnight in a refrigerator prior to spectrophotometric analysis (Sartory & Grobbelaar, 1984).

### 3.2.4 Cell harvesting and DNA extraction

Cyanobacterial cells were harvested from lab cultures by centrifuging 2mL aliquots at 20,000 × g for 5 min using a Denville™ 260D bench top centrifuge (Denville Scientific Inc, Metuchen, NJ, USA). The supernatant was removed by sterile pipetting and DNA was extracted from the resulting pellets using the Qiagen DNeasy® Plant Mini kit (Qiagen Inc., Mississauga, ON, Canada). DNA from environmental water samples was extracted from cells collected on GF/C filters. Briefly, the frozen filters were cut into pieces with a sterile scalpel and placed in 2 mL screw-cap microcentrifuge tubes (Fisher Scientific, Whitby, ON, Canada) containing 400 µL lysis buffer (AP1) and 4 µL RNase A stock solution. Approximately 0.3g glass beads (0.5mm dia, BioSpec Products Inc, Bartlesville, OK, USA) were added to each tube prior to cell lysis and homogenization in a Precellys 24 homogenizer (Bertin Technologies, France). The resulting homogenate was subjected to DNA extraction and purification following the Qiagen DNeasy® Plant Mini kit protocol. Purified DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE,
USA), and its quality was confirmed by running 100ng of DNA on a 1.5% agarose (Applied Biological Materials Inc., Richmond, B.C., Canada) gel electrophoresis. All extracted DNA was stored in 1.5mL Eppendorf microcentrifuge tubes at -20°C for later PCR analyses.

### 3.2.5 Primer design and specificity testing

Three primer pairs targeting respectively, a portion of the 16S rRNA gene specific for cyanobacteria (Nubel et al., 1997), N-methyl transferase motif of the mcyA gene (Furukawa et al., 2006) and the glutamate-activating adenylation domain of the mcyE gene (Vaitomaa et al., 2003) of microcystin-producing *Microcystis* genera were selected from literature and synthesized by Alpha DNA (Montreal, Canada). Prior to design of the novel *Microcystis mcyG* primer pairs, a clustal W multiple sequence alignment (Thompson et al., 1994) of the twenty cyanobacterial mcyG gene sequences present in GenBank as of January 2009 was performed. The mcyG 67F and mcyG 310R primers used in this study were designed from the most conserved region of the *Microcystis mcyG* gene using Primer3 software (Rosen & Skaletsky, 2000). A nucleotide basic local alignment search tool (BLASTN) analysis of GenBank sequences using these primer sequences produced significant hits solely for *Microcystis mcyG* genes, thereby asserting primer specificity.

All primers (Table 3.2) were tested in conventional PCR assays to confirm that they amplify only the target genes. Conventional PCR was performed on 25 µL reaction volumes using the Gene Amp® PCR System 9700 (Applied Biosystems, Carlsbad, CA, U.S.A), with each reaction containing 2µL of template DNA (10ng µL⁻¹), 1 × PCR buffer, 1.5mM MgCl₂, 250 µM dNTPs, 1U of AmpliTaq Gold (Applied Biosystems) and 500nM of each primer, all brought to a final volume of 25µL with autoclaved distilled water. PCR thermal cycling conditions included an initial denaturation at 95 °C for 5min, followed by 40 cycles of PCR, each consisting of 94 °C for 30s, 56°C for 30s, and 72 °C for 60s. Final extension was at 72 °C for 10 min. All samples were run with a positive as well as a negative control containing no template DNA. The presence of the various gene amplification products was verified by gel electrophoresis.
3.2.6 PCR amplicon analysis

PCR amplification products from mcyA, E and G primer pairs originating from station VQ-C (which recorded the highest Microcystis concentration) as well as the M. aeruginosa CPCC 299 standard strain were selected for sequence analysis. A total of six PCR amplification products (two from each primer pair) were ligated into the pDrive cloning vector (Qiagen Inc.) and transformed with Qiagen EZ Competent Cells following kit manufacturer’s recommendations. Transformants were selected on Luria-Bertani (LB) agar plates containing 30μg mL\(^{-1}\) kanamycin, 64 μg mL\(^{-1}\) 5-bromo-4-chloro-3 indolyl-β-D galactoside (X-Gal) and 0.50μM isopropyl-β-D-thiogalactoside (IPTG). Recombinant Plasmid DNA was purified using the Purelink® Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s recommendations, and sent for sequencing at Génomique Québec Innovation Centre, Montreal, Qc. Four of the six sequences generated were deposited in GenBank under accession numbers JN936964-JN936967. In order to visualize the phylogenetic relationship between the two mcyG sequences generated from this study and published GenBank mcyG sequences, a multiple sequence alignment was performed using Clustal W (Version 1.81) software package (Thompson et al., 1994) while the phylogenetic reconstruction of the aligned sequences was conducted in the Molecular Evolutionary Genetics Analysis (MEGA) tool (version 5.05; Tamura et al., 2011) using the Neighbour-Joining method of Saitou and Nei (1987).

3.2.7 Quantitative real-time PCR

Quantitative real-time PCR was performed using a Stratagene Mx3000P qPCR System (Stratagene Inc., La Jolla, CA, USA). The PCR mixture was made up of 2μL of template containing various amounts (0.003-30ng) of genomic DNA, 200 nM of forward and reverse primer pairs for mcyA, mcyE or mcyG genes, and 12.5 μL of hot start Quantifast™ SYBR® Green I PCR master mix (Qiagen Inc.), all brought to a final volume of 25 μL using autoclaved distilled water. The thermal cycling program consisted of an initial activation step of 5 min at 95°C, followed by 40 cycles of 10s at 95°C and 30s at 60°C. Real-time monitoring of PCR products was performed by measuring the fluorescence emitted by SYBR® Green
I dye following its intercalation into the double-stranded DNA generated after each PCR cycle. All samples were amplified in quadruplicates and the C<sub>T</sub> determined by the base-line corrected normalized fluorescence (dRn) method of the Mx3000P software. Cell number equivalents of toxigenic Microcystis spp. in unknown samples were determined as previously described (Furukawa et al., 2006). Melting curve analyses were performed after PCR from the fluorescence intensity data collected by gradually increasing the temperature from 55°C to 95°C at a rate of 0.5°C per second using the Mx3000P software.

3.2.7.1 Generation of standard curves

Tenfold serially diluted template DNA of M. aeruginosa CPCC299 cultures was used to develop standard curves for the mcyA, mcyE and mcyG qPCR assays. Template DNA dilutions ranging from 1:10 to 1:10<sup>6</sup> and corresponding to 1.58 × 10<sup>7</sup> to 1.58 × 10<sup>2</sup> M. aeruginosa CPCC 299 cell equivalents as estimated by microscopic enumeration (Rinta-Kanto et al., 2005) were used in all three qPCR assays. The limit of detection (LOD) for the three assays was determined by use of qPCR data generated from two-fold serially diluted DNA templates with cell equivalents ranging from 1.024 × 10<sup>3</sup> to 1.0 cells per reaction. Six replicates from each of these dilutions were analyzed using the limit of detection tool of GenEx Ent software (version 5.3.2; MultiD Analyses AB, Göteborg, Sweden). All three assays were found to reliably detect (95% confidence level, cutoff cycle 40) as low as 2 M. aeruginosa cell equivalents µL<sup>-1</sup> of PCR reaction mixture.

3.2.8 Microcystin extraction and analysis

Frozen filters for microcystin analyses were cut into pieces with a sterile scalpel, placed into 2 mL safe-lock Eppendorf tubes containing 500µL double de-ionized water and incubated in a boiling water bath for 5min (Metcalf et al., 2000). The resulting samples were subjected to shaking (30 min, 250rpm) on an Excella™ E-5 classic platform shaker (New Brunswick Scientific Co., Edison, NJ, USA) followed by centrifugation (6 min , 14000 rpm) on a Denville™ 260D bench top centrifuge (Denville Scientific). The supernatant was analyzed using an Envirogard Microcystin Plate kit (Strategic Diagnostics, Newark, DE, USA),
following manufacturer’s instructions. Absorbance was measured at 450nm using a BioTek Elx 800 absorbance microplate reader (BioTek Inc., Winooski, VT, USA).

The concentration of MC-LR equivalent was calculated from the regression curve $y = 27.70 - 27.08 \log_{10}(x)$, ($R^2 = 0.96$), with $y$ being the ratio of the absorbance (at 450nm) of the standard or sample relative to that of a negative control, while $x$ was the concentration of MC-LR equivalent. The assay detection limit was 0.1µg L$^{-1}$.

3.2.9 Statistical analysis

To test the accuracy and statistical significance of qPCR data, the REG procedure of the SAS statistical package version 9.2 (SAS Institute Inc, Cary, NC, USA) was used to fit the qPCR $C_T$ values to toxic Microcystis counts determined by microscopy. The following linear model was used:

$$Y_i = \beta_o + \beta_1 X_i + \varepsilon_i \quad [3.1]$$

where

$Y_i$ is the $C_T$ value,

$X_i$ is the logarithm of toxic Microcystis cell equivalents,

$\beta_o$ is the $y$-intercept,

$\beta_1$ is the slope, and

$\varepsilon_i$ is the error term.

Linear curves were fitted using the least-square approximation and associated statistical tests. Before regression analyses, data was log-transformed and tested for normality using the Kolmogorov-Smirnoff test at default P-values < 0.05.

3.3 Results

3.3.1 Field sampling

Blooms of cyanobacteria were reported in the Missisquoi Bay, Quebec during the summer of 2009 with periodic surface accumulations (scum) at various near-shore locations. Chl-a concentrations during the August 12 sampling varied between 3 to 207µgL$^{-1}$ with stations VQ-B and VQ-C, where visible surface scums were found, also recording the highest chl-a levels (Table 3.3). On the second sampling
date (August 25, 2009), chl-a levels were between 5 to 9 µgL⁻¹ with no reported sightings of surface scum of cyanobacteria. Carlson trophic state index (TSI) calculations based on chl-a (Carlson, 1997; Ha et al., 2009) data gave values that were below 60 in over 70% of the sampling stations. A majority of the sampling stations were therefore eutrophic, with samples from near-shore stations (VQ-A, VQ-B and VQ-C) generally showing higher TSI values relative to pelagic stations.

3.3.2 PCR specificity, cloning and sequence analyses

Genomic DNA extracted from cultured strains (Table 3.1) and from field samples yielded high quality DNA that was successfully amplified using the general cyanobacterial primer pair CYA359F/781R. The MSF/MSR-2R, mcyE-F2/mcyE-R8 and the mcyG-67F/mcyG-310R primer pairs, produced putative PCR amplicons within the expected sizes of 190, 247 and 244bp respectively (Figure 3.2A-F), exclusively for microcystin-producing Microcystis strains (Table 3.1). Comparison of generated mcyA, mcyE, and mcyG nucleotide sequences with existing GenBank sequences as of March 2011 resulted in highly significant homologies with sequence identities ranging from 94-100 % for mcyA, 91-100 % for mcyE and 95-96 % for mcyG. Figure 3.3 shows the Neighbour-Joining tree deduced from Clustal W alignment of twenty-three GenBank Anabaena, Microcystis, Nostoc, Planktothrix mcyG sequences and the mcyG sequences produced in this study. The generated mcyG sequences were found to cluster exclusively with Microcystis mcyG genes but not with orthologous genes from other cyanobacteria genera. Within each mcy gene clade, respective mcy sequences obtained from this study formed distinctive sub-clades from the rest of the GenBank mcy genes (Data not shown). Indeed the mcyA, mcyE and mcyG sequences from M. aeruginosa CPCC 299 were found to be highly identical to their corresponding homologs from field station VQ-C, with identities of respectively 98, 99, and 96% for the mcyA, mcyE, and mcyG sequences.

3.3.3 Sensitivity of qPCR assays

All three mcy primer sets yielded highly significant linear curves (R² ≥ 0.99, P < 0.0001, n = 6) on plotting the respective qPCR C_T values against starting DNA
concentration (in log *Microcystis* cell number equivalents). The three qPCR assays were highly sensitive as evidenced by the relatively high reaction efficiencies of 91.0, 95.8 and 92.7 % for the mcyA, mcyE and mcyG standards, respectively. Melting peak analysis showed that PCR product peaks were found at temperatures of 82.8, 81.8, and 80.8°C for the mcyA, mcyE, and mcyG specific primers, respectively, in agreement with results of previous studies (Furukawa et al., 2006; Vaitomaa et al., 2003). Samples containing no DNA template yielded negative results (Data not shown).

### 3.3.4 Estimation of microcystin-producing *Microcystis* genotypes in field samples

Total *Microcystis* cell concentrations ranged from $2.1 \times 10^3$ to $2.8 \times 10^6$ cells mL$^{-1}$, with station VQ-C recording the highest and PK-D the lowest cell counts (Figure 3.4). Indeed stations VQ-B and VQ-C had a massive visible scum of cyanobacteria during the field surveys while no scum was observed at any of the remaining sampling sites except for VQ-A which had blooms with minimal surface accumulations.

Although the qPCR assays targeting the three mcy genes predicted different potentially toxic *Microcystis* cell number equivalents in all field samples, the trends were nevertheless remarkably similar. Pearson correlation analyses of cell concentrations obtained using the three different gene targets showed highly significant associations ($p< 0.0001$, $n = 7$) with coefficient of determination ($R^2$) values greater than 0.99 (Data not shown). *McyA*-containing *Microcystis* cell number equivalents ranged between $1.60 \times 10^2$ – $5.1 \times 10^5$ cells mL$^{-1}$ while cell concentrations based on the mcyE gene ranged between $1.74 \times 10^2$ – $3.08 \times 10^5$ cells mL$^{-1}$. The estimated cell number equivalents for mcyG gene ranged from $1.51 \times 10^2$ – $6.22 \times 10^5$ cells mL$^{-1}$. As with total *Microcystis*, sites VQ-C and PK-D also had respectively the highest and lowest potential microcystin-producing *Microcystis* cell concentrations (Figure 3.4). However, statistical analysis of the data generated showed no significant differences ($\alpha = 0.05$) between *Microcystis* cell concentrations determined by the three assays at stations with no visible bloom (PK-D, BM-A, BM-B, VQ-D8), whereas the mcyA- and mcyG-based cell
concentrations were often significantly greater than \( mcyE \)-based concentrations at stations with visible blooms (VQ-A, VQ-B and VQ-C). There was generally no statistically significant difference between cell concentrations from the \( mcyA \) and \( mcyG \) assays at any station.

Total \( Microcystis \) cell numbers and \( mcyA, mcyE \) and \( mcyG \) cell number equivalents were highly correlated, with coefficients of determination (\( R^2 \)) of respectively 0.999, 0.985, and 0.994 (\( p<0.0001, n=7 \)). This notwithstanding, total \( Microcystis \) concentrations were found to be at least an order of magnitude higher than microcystin-producing \( Microcystis \) cell numbers for all sampling stations (Figure 3.4). The proportions of microcystin-producing \( Microcystis \) to total \( Microcystis \) in all sampling stations ranged between 1.7-21.6 % (mean= 10.1, \( n=7 \)) for \( mcyA \), 1.9-11.2% (mean = 6.9, \( n=7 \)) for \( mcyE \) and 2.2-22.6% (mean = 10.6, \( n=7 \)) for \( mcyG \) genes (Figure 3.5). Station VQ-C had the highest proportion of potentially toxic \( Microcystis \) relative to total \( Microcystis \) based on \( mcyE \) and \( mcyG \) analyses whereas \( mcyA \)-based analyses predicted the highest proportion for station VQ-B.

### 3.3.5 Detection of microcystins in field samples

Microcystins were detected at three out of the seven stations, with toxin concentrations ranging between 0.13-28.90 \( \mu g \) L\(^{-1} \) (Table 3.3). Stations VQ-B and VQ-C, which witnessed massive blooms, had microcystin concentrations almost 20 times the Canadian drinking water guideline value of 1.5 \( \mu g \) L\(^{-1} \). Microcystins were below the limit of detection of the ELISA analytic method at stations with no visible blooms (PK-D, BM-A, BM-B VQ-D8).

### 3.3.6 Cyanobacterial population composition

Microscopic analyses of lake water samples resulted in identification of over 12 cyanobacterial genera, with \( Microcystis, Anabaena, Aphanizomenon, Aphanocapsa, \) and \( Pseudanabaena \) spp. widespread in many stations (Fig. 3.6). \( Microcystis \) spp. were found to be the dominant cyanobacterial genera mostly at bloom stations VQ-A, VQ-B, and VQ-C, wherein they accounted for respectively 42, 89, and 94 % of the total cyanobacterial population. The potential microcystin
producers *M. aeruginosa, Anabaena flos aquae*, and *A. spiroides kleb* were present at most sampling stations (data not shown). Other important but less prevalent microcystin producers also identified included *M. wesenbergi* and *M. ichthyoblabe*. Interestingly, *Aphanizomenon flos aquae* and *Cylindrospermopsis raciborskii*, which have been implicated in the production of the hepatotoxic tricyclic alkaloid cylindrospermopsin (Schembri et al., 2001) mostly in tropical and sub-tropical waters, were identified at a few stations in the Missisquoi Bay.

The cyanobacterial population in the non-bloom stations (PK-D, BM-A, BM-B, and VQ-D8) on the contrary was dominated by picocyanobacteria, which accounted for over 90% of the total cyanobacterial population. The proportions of total *Microcystis* relative to the total cyanobacterial populations at these stations were very low with values of 1.6, 4.6, 3.2 and 2.1 for PK-D, BM-A, BM-B and VQ-D8 respectively.

### 3.4 Discussion

Real-time qPCR targeting three *mcy* genes was successfully applied to estimate potential microcystin-producing *Microcystis* concentrations in environmental samples. Statistical analyses of qPCR data revealed that of the three assays, the *mcyA* and *mcyG*-based assays consistently estimated significantly higher toxigenic *Microcystis* cell number equivalents at stations with high cyanobacterial biomass than the *mcyE* assay, although all three yielded comparable cell concentrations at stations with low biomass. While it is presently unclear why the *mcyE* assay results differed significantly from those of the other two assays under bloom conditions, the fact that the *mcyE* assay predicted cell concentrations which correlated less with microcystin concentrations (Data not shown) could mean that exclusive use of the *mcyE*-based assay for estimation of potentially toxic *Microcystis* concentrations in water under bloom conditions might be problematic. Vaitomaa et al. (2003) utilized the same *mcyE* primers to quantify putative microcystin-producing *Microcystis* in two Finnish lakes wherein they observed significant positive correlations between *Microcystis mcyE* copy numbers and microcystin concentration and concluded that the *mcyE* gene copy number could be a useful surrogate for hepatotoxic *Microcystis*. Although these
authors did not perform a comparative study with different \textit{mcy} genes, it is not uncommon for assays targeting different \textit{mcy} loci to predict different toxigenic potential for the same sample. Fortin and coworkers (Fortin et al., 2010), for example, were able to generate significantly higher numbers of \textit{Microcystis mcyD} copies in 2006 and 2007 samples from the Missisquoi Bay, Quebec, with primers for the \textit{mcyD}_{KS} gene than with those designed for \textit{mcyD}_{DH} gene and attributed this difference to nucleotide mismatches in the reverse primer of \textit{mcyD}_{DH}. The differences in detection observed in this study could not, however, be linked to primer sequence nucleotide mismatch given that all primer sequences were 100\% homologous to sequences from the reference strain \textit{M. aeruginosa} CPCC 299 as well as the \textit{Microcystis} strain identified in the study lake. However, given the limited number of samples sequenced and the likelihood of presence of unidentified strains with sequence mismatches to primer sequences, we cannot entirely discount the probable role of nucleotide mismatches in the observed discrepancies.

The distribution of both total and potentially toxic \textit{Microcystis} spp. showed substantial spatial variability, with the near-shore stations in general recording higher cell concentrations, compared to pelagic stations. Indeed Fortin et al. (2010), using an \textit{mcyD} qPCR-based assay reported variability between pelagic and littoral toxigenic cell quotas in the Missisquoi Bay in the summers of 2006 and 2007. The clear trend showing a general increase in potentially toxic \textit{Microcystis} genotypes with increase in total \textit{Microcystis} concentration lends credence to the thesis that the increasing incidence of cyanobacteria in Quebec’s fresh water bodies could lead to increased toxigenic potential and thereby exacerbate the risk to public health.

The proportions of toxigenic \textit{Microcystis} genotypes relative to the total \textit{Microcystis} population (approximately 2-23\%) reported in the present study are in agreement with values reported elsewhere. Whereas Baxa et al. (2010) reported \textit{Microcystis mcyD} to total \textit{Microcystis} population ratios of 0.37-20.2 \% in the San Francisco Estuary (USA), Rinta-Kanto et al. (2009b) reported ratios as low as 0.4-11.5\% in Lake Erie (USA) samples. Studies by Kurmayer and Kutzenberger (2003) in Lake Wannsee, Germany, however, showed toxic to non-toxic
Microcystis ratios ranging between 1-38% while Yoshida et al. (2007) reported proportions between 0.5-35% in Lake Mikata, Japan. Thus, the Microcystis population reported in this study lake, just like in other eutrophic lakes, was dominated by non-toxic strains. When compared to the total cyanobacterial biomass, toxigenic Microcystis seem to constitute a very tiny fraction of the cyanobacterial population in this lake.

The substantial spatial variation in toxigenic Microcystis proportions, especially the tendency for lake shore accumulations highlighted in this and other lake systems is probably the result of a complex interaction of factors controlling not only the growth but also their movement and distribution within the lake systems. The availability of nutrients coupled with shallower and warmer lakeshore waters (Fortin et al., 2010) further favors the growth and accumulation of both toxic and non-toxic cyanobacteria in the embayment areas such as the Missisquoi Bay, thereby increasing the risks to public health. It should, however, be emphasized that since a limited number of samples and sampling locations were used in these analyses, it might not be appropriate to interpret the spatial distribution reported here as representative of the situation in the whole lake. This notwithstanding, the study proves the versatility of the developed qPCR assays as tools for profiling toxigenic cyanobacteria proportions in freshwater bodies.

The striking similarity between nucleotide sequences from the field samples and the respective GenBank Microcystis mcyA, mcyE, and mcyG suggests that toxigenic Microcystis spp. were indeed present in the Missisquoi Bay during summer 2009. Confirmatory ELISA tests in fact confirmed the presence of microcystins, at least in stations with blooms. Although not detected at non-bloom stations (PK-D, BM-A, BM-B, and VQ-D8), it could be hypothesized that microcystins were probably present, but at very low concentrations that could not be detected owing to the very small volumes of water (50-250mL) that were filtered and analyzed.

The relatively high homology between the respective mcyA, mcyE or mcyG sequences from field samples and corresponding M. aeruginosa CPCC 299 mcy genes could probably be a result of the fact that being the only sequences in our
analyses originating from Canadian waters; they might have evolved in such a way as to adapt to specific local environmental conditions (Slatkin, 1987). However, considering that the field sequences used in the phylogenetic analysis were obtained from single clones from one station, it is unlikely that these results are representative of all possible Microcystis mcyA, mcyE and mcyG genotypes in this lake.

To the best of our knowledge, this is the first study to target the mcyG gene for qPCR-based estimation of toxigenic cyanobacteria while also providing a comparative evaluation of the suitability of three mcy genes as targets for quantification of toxigenic Microcystis spp. in freshwater bodies. Application of our qPCR assays led to estimation of potentially toxic Microcystis cell concentrations even at stations where microcystin concentrations were still below the ELISA assay detection limit. Potentially toxic Microcystis concentrations estimated by both mcyA and mcyG assays at all sampling stations did not show any statistically significant difference whereas cell concentrations based on the mcyE assay were significantly lower than those determined by both mcyA and G assays only at the bloom stations, implying that use of the mcyE assay for estimation of toxigenic Microcystis under bloom conditions might be problematic.

Nonetheless, the very low detection limits attained in this study as well as the strong agreement amongst all three assays under non-bloom conditions imply incorporation of the mcyA, mcyG and/or mcyE assays into early warning systems for toxigenic cyanobacteria monitoring could enable resource managers take swift and appropriate measures to minimize risks to public health from impending harmful algal blooms. Use of more than one of these assays has the potential to substantially increase the confidence level of managerial decisions.

3.5 Acknowledgements

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### Table 3.1: Microalgal strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Origin</th>
<th>Microcystin&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Conventional PCR Amplification&lt;sup&gt;a&lt;/sup&gt;</th>
<th>mcyA</th>
<th>mcyE</th>
<th>mcyG</th>
<th>CYA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena sp</em></td>
<td>CPCC 426</td>
<td>Lake Biwa, Japan</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>A. flos-aquae</em></td>
<td>CPCC 64</td>
<td>Lake Ontario, Canada</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>A. flos-aquae</em></td>
<td>CPCC 67</td>
<td>Mississippi, USA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Aphananta flocculosa</em></td>
<td>CPCC 597</td>
<td>Manitoba, Canada</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Aphanocapsa rivularis</em></td>
<td>CPCC 596</td>
<td>Rotoura, New Zealand</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Microcystis sp.</em></td>
<td>VQ-B009</td>
<td>Missisquoi Bay, Canada</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. aeruginosa</em></td>
<td>CPCC 299</td>
<td>Alberta, Canada</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. aeruginosa</em></td>
<td>CPCC 300</td>
<td>Alberta, Canada</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. aeruginosa</em></td>
<td>CPCC 468</td>
<td>Ontario, Canada</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. aeruginosa</em></td>
<td>CPCC 632</td>
<td>Madison, USA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. aeruginosa</em></td>
<td>CPCC 633</td>
<td>Ontario, Canada</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. flos-aquae</em></td>
<td>CPCC 461</td>
<td>Fox river, USA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Scenedesmus acutus</em></td>
<td>CPCC 10</td>
<td>Falconbridge, Canada</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Synechococcus leopoliensis</em></td>
<td>CPCC 102</td>
<td>Austin, USA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, PCR amplicon; -, no PCR amplicon yielded with primer pairs MSF/MSR-2R (*mcyA*), mcyE-F2/mcyE-R8 (*mcyE*), mcyG-67F/mcyG-310R (*mcyG*) or CYA359F/CYA781R (16S rRNA)

<sup>b</sup> +, detected; -, Not detected by ELISA
Table 3.2: List of primers used in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer pair</th>
<th>Sequence (5'-3')</th>
<th>Product size (bp)</th>
<th>$T_a$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mcyA</td>
<td>MSF</td>
<td>ATCCAGCAGTTGAGCAAGC</td>
<td>190</td>
<td>56</td>
<td>Tillett et al., 2001</td>
</tr>
<tr>
<td></td>
<td>MSR-2R</td>
<td>GCCGATGGTGGGATCTGAAT</td>
<td>190</td>
<td>56</td>
<td>Furukawa et al., 2006</td>
</tr>
<tr>
<td></td>
<td>mcyE-F2</td>
<td>GAAATTTGTGAGGAAGGTGC</td>
<td>247</td>
<td>56</td>
<td>Vaitomaa et al., 2003</td>
</tr>
<tr>
<td>mcyE</td>
<td>mcyE-R8</td>
<td>CAATGGGAGCTGAAAG</td>
<td>247</td>
<td>56</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>mcyG-67F</td>
<td>CAACCAACAGGTATCTTAAAGC</td>
<td>244</td>
<td>56</td>
<td>This study</td>
</tr>
<tr>
<td>mcyG</td>
<td>mcyG-310R</td>
<td>TGAGGCAAGGTTTCTCTTG</td>
<td>244</td>
<td>56</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>CYA 359F</td>
<td>GGGGATTTCCGCAATGGG</td>
<td>244</td>
<td>56</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>CYA 781R(a)</td>
<td>GACTACTGGGGTTTCAATCCCATT</td>
<td>450</td>
<td>60</td>
<td>Nubel et al., 1997</td>
</tr>
<tr>
<td></td>
<td>CYA 781R(b)</td>
<td>GACTACTGGGGTTTCAATCCCATT</td>
<td>450</td>
<td>60</td>
<td>Nubel et al., 1997</td>
</tr>
</tbody>
</table>

$^a$ Annealing temperature; $^b$ Y, C/T nucleotide degeneracy

Table 3.3: Variation in selected water quality parameters at different stations in the Missisquoi Bay

<table>
<thead>
<tr>
<th>Station</th>
<th>GPS coordinate</th>
<th>Water temp ($^\circ$C)</th>
<th>pH</th>
<th>TN (mg L$^{-1}$)</th>
<th>TP (µg L$^{-1}$)</th>
<th>Chl a (µg L$^{-1}$)</th>
<th>TSI (Chl a)</th>
<th>Microcystin (µg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VQ-A</td>
<td>N45°03.669' W73°09.436'</td>
<td>24.25</td>
<td>9.28</td>
<td>3.37</td>
<td>27.07</td>
<td>14.81</td>
<td>57</td>
<td>0.13</td>
</tr>
<tr>
<td>VQ-B</td>
<td>N45°04.956' W73°08.925'</td>
<td>26</td>
<td>9.48</td>
<td>0.74</td>
<td>28.62</td>
<td>207.34</td>
<td>82.9</td>
<td>28.9</td>
</tr>
<tr>
<td>VQ-C</td>
<td>N45°05.095' W73°08.525'</td>
<td>NA</td>
<td>NA</td>
<td>5.1</td>
<td>19.13</td>
<td>59.24</td>
<td>70.6</td>
<td>27.49</td>
</tr>
<tr>
<td>PK-D</td>
<td>N45°04.352' W73°05.775'</td>
<td>25.9</td>
<td>8.7</td>
<td>45.9</td>
<td>21.5</td>
<td>2.96</td>
<td>41.2</td>
<td>ND</td>
</tr>
<tr>
<td>BM-A</td>
<td>N45°02.240' W73°08.580'</td>
<td>25.21</td>
<td>8.9</td>
<td>1.35</td>
<td>26.17</td>
<td>4.74</td>
<td>45.8</td>
<td>ND</td>
</tr>
<tr>
<td>BM-B</td>
<td>N45°03.432' W73°04.930'</td>
<td>26.04</td>
<td>9.3</td>
<td>0.52</td>
<td>23.72</td>
<td>9.48</td>
<td>52.6</td>
<td>ND</td>
</tr>
<tr>
<td>VQ-D8</td>
<td>N45°04.887' W73°08.472'</td>
<td>25.29</td>
<td>8.71</td>
<td>1.67</td>
<td>30.27</td>
<td>5.92</td>
<td>48</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ 0-2.6: oligotrophic; 2.6-7.3: mesotrophic; 7.3-56: eutrophic; 56-155+: hypereutrophic. NA: data not available; ND: not detected
Figure 3.1: Map of the Missisquoi Bay, Lake Champlain (Quebec portion) showing the seven sampling stations. Black squares: sampling of August 12, 2009. Brown triangles: sampling of August 25, 2009. GPS coordinates of sampling sites are shown in Table 3.3
Figure 3.2: PCR products amplified from laboratory cultures and field samples. **TOP GEL:** PCR products amplified from laboratory cultures of cyanobacteria with primer sets specific to *Microcystis mcyA* gene (A), *Microcystis mcyE* gene (B), and *Microcystis mcyG* (C): Lanes (numberings and descriptions are identical for gels A, B, and C): (M) DNA size maker; (1) *Microcystis aeruginosa* CPCC 299 (toxic); (2) *M. aeruginosa* CPCC 300 (toxic); (3) *M. aeruginosa* CPCC 632 (nontoxic); (4) *Microcystis* sp. VQ-B009 (toxic); (5) *M. aeruginosa* CPCC 124 (nontoxic); (6) *Anabaena flos aquae* CPCC 64 (toxic); (7) No template control. **BOTTOM GEL:** PCR products amplified from field samples with primers for *Microcystis mcyA* gene (D), *Microcystis mcyE* gene (E), and *Microcystis mcyG* gene (F): Lanes (numberings and descriptions are identical for gels D, E, and F): (M) DNA size marker; (1) VQ-A sample; (2) VQ-B sample; (3) VQ-C sample; (4) PK-D sample; (5) BM-A sample; (6) BM-B sample; (7) VQ-D8 sample; (8) No template control.
Figure 3.3: Neighbor-joining tree illustrating the phylogenetic relationship between mcyG sequences generated in this study and 22 GenBank/EMBL mcyG sequences. The distance tree was built using the neighbor-joining algorithm with bootstrap resampling. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the respective branches. Evolutionary distances were computed using the p-distance method as implemented in MEGA 5 (Tamura et al., 2011). GenBank/EMBL accession numbers of the sequences used in the analyses are shown at the beginning of sequence name.
Figure 3.4: *Microcystis* cell concentrations determined by direct microscopy counts and real-time qPCR for seven sampling stations in the Missisquoi Bay, Quebec. Total *Microcystis* counts obtained by direct microscopy are expressed in cell counts mL\(^{-1}\) while toxigenic *Microcystis* concentrations obtained by qPCR are expressed in *M. aeruginosa* CPCC 299 cell equivalents mL\(^{-1}\). All plots are on a log\(_{10}\) scale.

Figure 3.5: Proportion of toxigenic *Microcystis* to total *Microcystis* cell concentrations at various stations in the Missisquoi Bay during the summer 2009 sampling period.
Figure 3.6: Distribution of cyanobacterial population in the Missisquoi Bay during summer 2009.
In chapter three, three mcy gene targets were successfully utilized in quantitative PCR estimation of microcystin-producing *Microcystis* spp. during the 2009 field exploratory study. However, in order to establish the reliability of such assays for more routine monitoring of toxigenic cyanobacteria, it is critical to understand how changing physicochemical/environmental factors affect both cellular growth and mcy gene concentration as well as toxin production in mixed cultures of toxic and non-toxic cyanobacterial spp. commonly encountered in freshwater bodies in Canada and globally. To this end, laboratory culture experiments were designed to investigate the effects of nutrient levels, light intensity, and temperature on growth rates, microcystin concentration, and changes in mcyE gene copies for a microcystin-producing *Microcystis* sp. grown under monoculture and mixed culture conditions. Findings from these experiments were used to inform objective two of this research project. The following manuscript prepared from this study and co-authored by Drs. Madramootoo C.A. and Jabaji S. has been submitted to the Journal of Environmental Microbiology for publication in 2013.

**Ngwa, F., Madramootoo, C.A., Jabaji, S. 2013. Growth dynamics and toxin production in mixed assemblages of *Anabaena* and *Microcystis* spp. under varying nutrient, temperature, and light conditions. (Submitted to Environmental Microbiology Journal)**
Chapter 4 Growth dynamics and toxin production in mixed assemblages of *Anabaena* and *Microcystis* spp. under varying nutrient, temperature, and light conditions

Felixce Ngwa, Chandra Madramootoo, and Suha Jabaji

Abstract

Mixed blooms of toxic and non-toxic cyanobacteria spanning multiple genera are a common occurrence in water bodies worldwide, with one to two genera often dominating the cyanobacterial population at a given time. The proportion of toxic to non-toxic cyanobacterial genotypes within a given bloom is most often determined by the dominant cyanobacterial genera. Although the factors affecting proliferation and toxicity of cyanobacteria have been intensely researched for individual strains, the response of mixed assemblages of multiple cyanobacterial genera to changes in physicochemical/environmental factors is poorly understood.

In this study, we investigated the response of mono- and mixed cultures of *Microcystis aeruginosa* CPCC 299 and *Anabaena flos aquae* CPCC 67 to changes in nitrogen (N) and phosphorus (P), light, and temperature via qPCR estimation of the microcystin synthetase (*mcy*) E gene copies, coupled with cellular biomass and microcystin analyses. The overarching goal was to elucidate how growth, *mcy*E gene copies, and microcystin concentration were impacted in the mono- and mixed cultures under varying nutrient, light, and temperature conditions.

Results from this study showed a strong dependence of growth rates, *mcy*E copies, microcystin cellular and gene quotas not only on nutrients, and temperature conditions but also the presence or absence of competing cyanobacterial strains. The highest cellular counts, *mcy*E copies, and growth rates were recorded in the respective monocultures receiving the most nutrients (P and N), the highest temperature and strongest light treatments, whereas the highest cell- and gene-based microcystin quotas and concentrations were recorded in the corresponding mixed cultures.
These results show that inasmuch as interaction between cyanobacterial species can lead to mutual growth inhibition within mixed cultures, the rather higher cellular toxin quotas resulting from this interaction might indeed render mixed blooms of such toxigenic cyanobacteria more toxic. Furthermore, the fact that changes in \( mcyE \) copies often mirrored changes in \( M. \ aeruginosa \) CPCC 299 cellular growth rates implied possible coupling of \( mcyE \) production to cellular growth.

**Keywords:** Monocultures; Quantitative PCR; \( mcyE \) gene; Microcystin quotas, Mixed blooms

**4.1 Introduction**

The predominance of cyanobacteria in eutrophic water bodies worldwide is a menace to human and ecosystem health because of their ability to produce bioactive secondary metabolites that might be toxic to humans and animals (Fujiki & Suganuma, 1999; Hawkins et al., 1985; Jochimsen et al., 1998; Wood et al., 2010a) or cause noxious taste and odour problems (Graham et al., 2010; Smith et al., 2008) in drinking and recreational water supplies. Furthermore, bloom die-off results in creation of anoxic conditions detrimental to most aquatic biota. The success of cyanobacteria over other algal taxa has often been attributed to their ability to successfully exploit prevailing physicochemical/environmental conditions such as nutrients, light, temperature, water column stability, amongst others. Nutrient (N and P) concentrations in the surrounding medium are known to strongly influence proliferation of cyanobacteria, though optimal growth concentrations tend to differ among species or even strains.

Previous laboratory studies reported significant differences in the growth of \( N_2 \)-fixing and non-fixing cyanobacteria under changing phosphate and nitrate concentrations. Growth rates of non-heterocystous cyanobacteria like \( Microcystis \) spp. (Sabour et al., 2009a; Vézie et al., 2002) and \( Oscillatoria \) sp. (Sivonen, 1990), for example, have been shown to increase with increase in surrounding phosphate and nitrate concentrations, whereas growth of diazotrophic species such as \( Anabaena \) (Rapala et al., 1997; Sabour et al., 2009a), \( Nodularia \), and \( Aphanizomenon \) (Lehtimaki et al., 1997) were found to correlate positively with
increasing phosphate but not nitrate. Field studies have so far generated rather contradictory results, with some studies (Carey et al., 2009; Xu et al., 2010b) reporting P as the primary growth-limiting nutrient, a few pinpointing the importance of both N and P (Wilhelm et al., 2011; Xu et al., 2010a) and a good number identifying N as the key driver of cyanobacterial growth dynamics (Liu et al., 2011; Moisander et al., 2009).

The optimal temperature for growth of cyanobacteria, like nitrogen and phosphorus requirements, is known to be species-specific although subtle differences in growth response of toxic and non-toxic strains have been reported (Davis et al., 2009). Though most temperate planktonic cyanobacteria are capable of tolerating temperatures in the range of 15-35°C (Fogg et al., 1973; Tang et al., 1997) a good number of studies have shown increases in growth rates with increase in temperature (Castro et al., 2004; Rapala et al., 1997; Sivonen, 1990; Watanabe & Oishi, 1985; Zeng & Wang, 2011).

Apart from nutrients and temperature, cyanobacteria, like most phytoplankton, require light for photosynthesis and growth. Although growth rates of most cyanobacteria tend to increase with increase in light intensity, the survival and dominance of a given species in mixed assemblages is believed to be a function of their “critical light intensity” requirements (Huisman & Weissing, 1994). Kardinaal et al. (2007), for example, found non-toxic Microcystis strains to be better competitors for light than the toxic strains and attributed this to the possible higher energy requirements for toxin production in the toxic strains, whereas LeBlanc Renaud et al. (2011), on the contrary, reported dominance of toxic over non-toxic Microcystis strains at low light intensities. Though the original biological role of most cyanotoxins in cyanobacterial metabolism remains unclear, a good number of studies have postulated a putative photoadaptative role for microcystins (Hesse et al., 2001; Kaebernick et al., 2000; Phelan & Downing, 2011), suggesting that toxic cyanobacteria should have a competitive edge over non-toxic counterparts under high illumination.

Although the importance of the aforementioned factors for photosynthesis and growth of cyanobacteria has been demonstrated, a majority of these studies were
carried out using either monocultures or mixed cultures of toxic and non-toxic strains from the same genus, with little attention to interspecific differences. Furthermore, there is scant literature on variations in concentrations of toxin encoding gene with changes in the aforementioned variables. A study of the response of mixed assemblages of cyanobacteria from different genera to changes in nutrient, light and temperature under controlled laboratory conditions is crucial not only to understanding freshwater toxigenic bloom dynamics but also holistic management of these noxious organisms.

The present study employed microscopy coupled with quantitative PCR assays and enzyme linked immunosorbent assay (ELISA) to monitor the changes in cellular counts, mcyE, and microcystin concentration in mono- and mixed culture *M. aeruginosa* CPCC 299 and *A. flos aquae* CPCC 67 exposed to various nutrient, light, and temperature conditions. *Microcystis* and *Anabaena* spp. were chosen in our study because the two genera often co-occur in Canadian freshwater bodies; hence laboratory evaluation of their growth dynamics using qPCR approaches could provide valuable insight for water resource managers regarding use of quantitative PCR in routine monitoring of these noxious organisms.

### 4.2 Materials and methods

#### 4.2.1 Experimental design and culture conditions

The *Microcystis aeruginosa* CPCC 299 (hereafter *M. aeruginosa*) and *Anabaena flos aquae* CPCC 67 (hereafter *A. flos aquae*) used in this study were purchased from the Canadian Phycological Culture Centre (CPCC; formerly the University of Toronto Culture Centre [UTCC]) and maintained in a sterile manner in 250 mL Erlenmeyer flasks containing 100mL BG-11 medium (Stanier et al., 1971). The *M. aeruginosa* strain used in this study is known to produce microcystins whereas the *A. flos aquae* strain is non-toxic based on information from the CPCC. All cultures were grown in a Conviron growth chamber under conditions of 25±1 °C temperature, 12:12 light:dark cycles at a photon flux density of 30 ± 2 µmol photons m⁻²s⁻¹ provided by cool white fluorescent bulbs. Cultures grown in standard BG-11 medium for three weeks were harvested by centrifuging at 3220 ×
g for 10 min at 15°C (Schreiter et al., 2001) and rinsed once in phosphate- and nitrate-free BG-11 medium, prior to the start of the experiments.

4.2.2 Nutrient experiments

*M. aeruginosa* and/or *A. flos aquae* were transferred into 250 mL Erlenmeyer flasks each containing 100mL modified BG-11 at nitrogen (N) and phosphorus (P) concentrations specified in Table 4.1, to obtain final cell concentrations of $10^6$ cells mL$^{-1}$. Each nutrient combination was replicated four times for both mono- and mixed culture experiments. The resulting 48 batch cultures were randomly distributed into a Conviron growth chamber and incubated for two weeks under the conditions stipulated in section 4.2.1.

4.2.3 Temperature and light experiments

Cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of unmodified BG-11. In the temperature experiment, mono- and mixed culture Erlenmeyer flasks containing $10^6$ *M. aeruginosa* and/or *A. flos aquae* cells per millilitre were randomly placed in replicate Conviron growth chambers maintained at 20 or 25°C and a constant light intensity of 30 (± 2) µmol photons m$^{-2}$s$^{-1}$. For the light experiment, temperature in all four growth chambers was kept constant at 25(±1) °C while light intensities were maintained at 30 ± 2 µmol photons m$^{-2}$s$^{-1}$ in two chambers and 60(± 2) µmol photons m$^{-2}$s$^{-1}$ in two others.

4.2.4 Sample collection

All experiments were run for two weeks after which the cyanobacterial biomass was harvested for various analyses. Briefly, three 25mL subsamples from each flask were filtered onto GF/C filter papers (Whatman, England), wrapped in aluminum foil paper and either immediately analyzed for chl-a as described previously (Ngwa et al., 2012) or preserved at -20°C for subsequent DNA and microcystin extraction and analysis. Subsamples for cyanobacteria cell counts were aseptically transferred into 15mL falcon tubes containing acid Lugol solution and preserved in the dark prior to enumeration using a light microscope (Helmut Hund GmbH, Wetzlar, Germany).
4.2.5 Microcystin extraction and analysis

Intracellular microcystins were extracted from frozen filters using 75 % (v/v) aqueous methanol and ultra-sonication in a bath sonicator as described in Spoof et al. (2003). The microcystin extract clarified by centrifuging (10000 g, 10 min) was subsequently diluted with double distilled water to obtain a methanol concentration below 0.5% (Rivasseau et al., 1999) prior to enzyme linked immunosorbent assay (ELISA) analysis using an EnviroGard® Microcystin Plate kit (Strategic Diagnostics, Newark, DE, USA) and a Synergy H4 Hybrid Multi-Mode microplate reader (BioTek, Winooski, VT, USA). The ELISA detection limit was 0.1µg L⁻¹.

4.2.6 DNA extraction, PCR and sequencing

Frozen filters for DNA analysis were treated with 400 µL lysis buffer AP1, bead beaten, and homogenized as previously described (Ngwa et al., 2012) prior to DNA extraction and purification using the Qiagen DNeasy® Plant Mini kit (Qiagen Inc., Mississauga, ON, Canada) following the manufacturer’s recommendations. Extracted DNA concentration and purity were verified using NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) prior to preservation at -20°C for downstream applications. Only DNA with A₂₆₀/A₂₈₀ ratios between 1.7 and 2.0 was used in subsequent PCR processes.

In order to prepare standards for subsequent qPCR estimation of mcyE gene copies, conventional PCR was performed on DNA extracted from *M. aeruginosa* CPCC 299 using primer pairs specific for the *Microcystis mcyE* gene (MicmcyE415F; 5'-CCTGCACTCCCTGAGGAAAC-3’ and MicmcyE-581R; 5’-AATGACCGCCAATTTCAAGA-3’). The PCR reaction mixture contained 20ng of purified gDNA, 1× GeneAmp® PCR Buffer I (Applied Biosystems, Carlsbad, CA, and U.S.A), 1.5mM MgCl₂, 250µM dNTPs, 1U Hot Start AmpliTaq Gold® DNA polymerase (Applied Biosystems), and 500nM of each primer in a 25µL reaction volume. All reactions were carried out on a GeneAmp® PCR System 9700 (Applied Biosystems) using the thermal programme described in section 3.2.5. Amplified PCR products were purified using the QIAquick PCR
purification kit (Qiagen Inc.), ligated with the pDrive cloning vector (Qiagen Inc.) and transformed using One Shot® TOP10 chemically competent E. coli kit (Invitrogen, Carlsbad, CA, USA) following the kit manufacturer’s procedure. Purified plasmid DNA was sent to the Génome Québec Innovation Centre (Montreal, QC) for sequencing using ABI’s 3730xl DNA Analyzer technology (Applied Biosystems). Generated nucleotide sequences were compared with GenBank database nucleotide sequences using BLASTN algorithms. Portions of purified plasmid DNA were preserved at -80°C and subsequently used to establish standard curves for quantitative PCR.

4.2.7 Quantitative PCR

*Microcystis mcyE* gene copies in samples from nutrient, light and temperature experiments were estimated via quantitative PCR amplification of extracted gDNA. Standard curves were generated using purified recombinant plasmid DNA containing *Microcystis mcyE* gene inserts. Briefly, tenfold serial dilutions of plasmid DNA samples were performed to obtain solutions with the respective gene copy numbers ranging from $10^8$ to $10^2$ per microliter. Subsequently, qPCR was performed on duplicate dilutions using Mx3005P qPCR System (Agilent Technologies Inc., Santa Clara, CA, USA). Each 25 µL qPCR reaction mixture contained 2µL DNA template, 1x hot start QuantiFast® SYBR® Green I PCR master mix (Qiagen Inc.), *mcyE* forward (300nM) and reverse (200nM) primers. Thermal cycling conditions included initial activation at 95°C for 5min, followed by 40 cycles consisting of denaturation at 95°C for 10s and combined annealing/extension at 60°C for 30s. All fluorescence data were collected at the combined annealing/extension step, with the threshold cycle ($C_T$) automatically determined using the base-line corrected normalized fluorescence (dRn) algorithms of the MxPro qPCR software v4.10 (Agilent Technologies Inc.). Generated threshold cycle values were plotted against the $\log_{10}$ of the corresponding copy numbers to generate standard curves for *mcyE* copy number estimation. In order to determine the *mcyE* copy numbers in the experimental samples, gDNA from each of the culture experiments was diluted 100fold and analysed by qPCR as described previously to generate $C_T$ values. Linear
interpolation of $C_T$ values on the standard curve, generated $mcyE$ gene copies per PCR reaction, which were then related to the amount of DNA extracted to obtain the number of $mcyE$ gene copies per millilitre of culture.

4.2.8 Data analysis

The effects of nutrients, light, and temperature on mono- and mixed culture growth of *M. aeruginosa* and *A. flos aquae* were evaluated using the restricted maximum likelihood (REML) procedure for mixed models. Model residuals were checked for normality using the Shapiro-Wilk test at the 5% significance level. All statistical tests were performed with SAS v9.3 (SAS Institute Inc., Cary, NC, USA).

4.3 Results

4.3.1 Growth rate

The cellular growth rate of *M. aeruginosa* was strongly impacted not only by nutrient levels but also by the presence of *A. flos aquae* in the growth media. Mono- and mixed culture growth of *M. aeruginosa* was poorest in media with lowest N (1.0 mg L$^{-1}$) irrespective of the level of P (Figure 4.1A). *M. aeruginosa* growth was highest in medium D (P: 20 mg L$^{-1}$, N: 1000 mg L$^{-1}$), reaching 0.17 ± 0.01 day$^{-1}$ and 0.13 ± 0.01 day$^{-1}$ in monocultures and mixed cultures respectively. The difference between mono- and mixed culture growth of *M. aeruginosa* was however significant (p<0.0001) only in media C (P: 0.2 mg L$^{-1}$, N: 1000 mg L$^{-1}$) and D. Growth of *A. flos aquae*, like *M. aeruginosa*, was generally slower in mixed cultures than the corresponding monocultures, with the difference between mono- and mixed culture growth being statistically (p<0.001) significant in media A (P: 0.2 mg L$^{-1}$, N: 1.0 mg L$^{-1}$), B (P: 20 mg L$^{-1}$, N: 1.0 mg L$^{-1}$) and D. The highest growth rate (0.22 ± 0.01 day$^{-1}$) was recorded in monoculture medium D whereas the lowest growth rate (0.09 ± 0.02 day$^{-1}$) occurred in mixed culture medium A.

The effects of light intensity on mono- and mixed culture growth of *M. aeruginosa* and *A. flos aquae* are presented in Figure 4.1B, where growth rates in monocultures were again consistently higher than those in mixed cultures at both
high (60 µmol m⁻² s⁻¹) and low (30 µmol m⁻² s⁻¹) light intensities. The highest growth rate for *M. aeruginosa* (0.16 ± 0.01 day⁻¹) and *A. flos aquae* (0.19 ± 0.01 day⁻¹) occurred in monocultures under high light while the lowest growth rate for *M. aeruginosa* (0.12 ± 0.01 day⁻¹) and *A. flos aquae* (0.07 ± 0.02 day⁻¹) occurred in mixed cultures at high light intensities. Although there was no significant difference between *M. aeruginosa* or *A. flos aquae* growth rates at low and high light intensities, monoculture growth of each strain was nevertheless always significantly (p < 0.01) higher than mixed culture growth.

Figure 4.1C shows the growth response of *M. aeruginosa* and *A. flos aquae* to changes in temperature. Growth rate of monoculture *M. aeruginosa* was significantly (p = 0.013) higher at 25°C than at 20°C, with mixed culture growth being significantly (p < 0.05) lower than monoculture culture growth under all temperature conditions. Similarly, growth rate for *A. flos aquae* was significantly higher (p < 0.01) in monoculture than corresponding mixed cultures at both temperatures. The difference between high and low temperature growth of *A. flos aquae* was nevertheless statistically insignificant.

4.3.2 Quantitative PCR analyses of mcyE gene copies

Linear regression of the resulting Cₜ values against the logarithm of mcyE gene copies produced highly significant linear (R² > 0.99, Efficiency >96%) curves (Table 4.2). The resulting linear models (Table 4.2) were used to estimate mcyE copies in unknown samples by linear interpolation of qPCR generated Cₜ values. The rate of change of mcyE gene copies in nutrient manipulation experiments mirrored the growth rate of *M. aeruginosa* CPCC 299 described in section 4.3.1 (Figure 4.2A). The maximum rate of change of mcyE copy number (0.22 ± 0.01 day⁻¹) was observed in *M. aeruginosa* monocultures grown in medium D (P: 20 mg L⁻¹, N: 1000 mg L⁻¹). The net change in mcyE copies was generally higher in monocultures than mixed cultures, though statistically significant (p<0.01) only in media A (P: 0.2 mg L⁻¹, N: 1.0 mg L⁻¹) and D. The net change in mcyE copies tended to increase with increasing P and/or N concentrations.

The rate of change of *Microcystis* mcyE copies in monocultures was significantly higher than in mixed cultures only at low light (Figure 4.2B). This
notwithstanding, net changes in mcyE genes at low light were not significantly different from those at the high light intensity, irrespective of culture condition.

Temperature had a more pronounced effect on the rate of change of mcyE genes, with the rate of change at 20°C being significantly (p = 0.003) lower than at 25°C. Furthermore, significantly (p < 0.01) higher mcyE rates were recorded in monocultures relative to mixed cultures. The highest rate of change of Microcystis mcyE copies was witnessed in monocultures grown at 25°C whereas the lowest change was in mixed cultures at 20°C (Figure 4.2C).

4.3.3 Variation in microcystin quotas

The cellular microcystin (MC) quotas for the microcystin producing M. aeruginosa CPCC 299 ranged between 6.38 (± 1.73) to 23.7 (± 7.90) fg cell⁻¹ for all phosphorus and nitrogen concentrations tested (Figure 4.3A). There was evidently strong dependence of cellular MC quotas on both nutrient levels and presence or absence of A. flos aquae in culture media. Cellular toxin quotas were, in general, lower in M. aeruginosa monocultures than the corresponding mixed cultures, although these differences were significant (p < 0.01) in media C (P: 0.2 mg L⁻¹, N:1000 mg L⁻¹) and D (P: 20 mg L⁻¹, N: 1000 mg L⁻¹) only.

The proportion of intracellular microcystins relative to the mcyE gene copies ranged from 0.14(±0.02) to 1.25(±0.85) fg mcyE copy⁻¹ (Figure 4.3B). Although mcyE toxin quotas in mixed cultures were equally higher than in monocultures, the difference between mono- and mixed culture mcyE microcystin quotas was nevertheless significant (p < 0.01) only in media A (P: 0.2 mg L⁻¹, N: 1.0 mg L⁻¹) and D.

Mean microcystin quotas under various light and temperature treatments are presented in Tables 4.3 and 4.4, respectively. Though there was no significant difference in cellular and mcyE microcystin quotas at low and high light intensities for monoculture growth, mix cultured toxin quotas at low light intensity were significantly (p < 0.0001) higher than those at high light intensity. Furthermore, monoculture quotas were significantly (p < 0.0001) lower than mixed culture quotas only at the low light intensity. Microcystin quotas at high temperatures were not significantly different from low temperature quotas. These
toxin quotas were rather significantly impacted by co-occurring cyanobacteria in culture media; with mixed cultured *M. aeruginosa* having significantly (p < 0.0001) higher cellular and *mcyE* toxin quotas than the monocultures.

### 4.4 Discussion

In the present study, we showed the growth of *M. aeruginosa* CPCC 299 to be significantly impacted not only by nutrient levels but also, temperature, as well as co-occurring *A. flos aquae*. The N, P levels, and co-occurring cyanobacteria (*A. flos aquae* or *M. aeruginosa*) had significant (p < 0.05) interactive effects on growth of *M. aeruginosa* and *A. flos aquae*. Comparison of the growth patterns of *M. aeruginosa* and *A. flos aquae*, however, revealed that whereas nitrogen concentrations ≤ 1.0 mg L\(^{-1}\) severely depressed mono- and mixed culture growth of *M. aeruginosa*, irrespective of phosphorus levels, growth of *A. flos aquae* on the contrary was not significantly impacted by low N. Although *M. aeruginosa* is a non-fixer of nitrogen and should be more prone to nitrogen limitation than the diazotrophic *A. flos aquae*, it was nevertheless surprising that its growth was severely limited at such unnaturally high levels of N. Results from previous studies of the growth dynamics of other toxigenic cyanobacteria provide useful insights on the present observations. Sivonen (1990), for example, reported extremely poor growth, inadequate to support high toxin production, in the non-diazotrophic *Oscillatoria* spp. at nitrogen concentrations below 0.1 mg L\(^{-1}\). Vézie et al. (2002) showed that toxic *M. aeruginosa* strains grew better than non-toxic strains at high nutrient concentration whereas the reverse was true at low nutrient concentrations. Furthermore, these researchers observed severe inhibition of the toxic *Microcystis* strains at nitrogen concentrations below 2.9 mg L\(^{-1}\); hence the suggestion that toxic strains probably needed more nutrients for sustained growth at low nutrient concentrations than their non-toxic counterparts. A plausible explanation for this phenomenon was that the toxic strains, unlike non-toxic strains, need more energy not only for cellular division but also for the energy intensive process of microcystin biosynthesis (Briand et al., 2008). Nitrogen plays a vital role in microcystin biosynthesis and cellular growth given that the MC molecule is N-rich (10 N atoms per molecule). Furthermore, the requirement for
N in toxic cyanobacteria might also be aggravated by the presence of additional light harvesting pigments (Hesse & Kohl, 2001) probably absent in non-toxic strains. Thus under nutrient (particularly N) limiting conditions, the cost of microcystin production might outweigh any potential benefits of producing this toxin; hence the success of non-toxic Microcystis strains at low N and P concentrations. Batch culture studies of the growth dynamics of Microcystis ichthyoblabe Kütz. 1843 and Anabaena aphanizomenoides Forti 1912 under varying N and P concentrations confirmed that growth of the toxic Microcystis sp. was indeed strongly limited by low N & P concentrations whereas the Anabaena sp. was limited by low P alone (Sabour et al., 2009a). Furthermore, there was a marked increase in heterocysts in A. aphanizomenoides grown in media with low nitrogen, suggesting plausible onset of N2-fixation at low N. A study by Briand et al. (2008), on the contrary, showed superior growth of non-toxic Planktothrix agardhii strains over their MC-producing counterparts under non-growth-limiting conditions while the reverse was true under growth-limiting conditions. These findings led these researchers to suggest that the benefits of producing MC probably outweighed the cost associated with MC biosynthesis, at least for P. agardhii, under cell-growth limiting conditions.

The findings from our study, however, align more with those from several previous studies (Sabour et al., 2009a; Sivonen, 1990; Vézie et al., 2002; Yang et al., 2012), thereby suggesting that the depressed growth of M. aeruginosa CPCC 299 at nitrogen concentrations below 1.0 mg L\(^{-1}\) was probably due to insufficient energy at such low N levels for maintenance of cellular processes and microcystin biosynthesis. The negative cellular growth rates at low N concentrations imply that M. aeruginosa biomass at the end of the 14 days growth period was less than the starting level. This was probably because M. aeruginosa cells were nitrogen-starved to the extent that they were incapable of sustaining cellular metabolic processes at such low N concentrations leading to higher die-off rates towards the end of the 14 days experimental period. This assertion is substantiated by evidence from Vézie et al. (2002), who reported initial increases in biomass of nutrient-starved toxic Microcystis spp. during the first week of growth, after which growth came to a halt for strains grown in low nutrient (N & P) media.
The resilience of *A. flos aquae* at the low N concentrations reported in our study led us to consider the idea that the low nutrient concentrations were either seldom below the critical nutrient requirement (Huisman & Hulot, 2005) for this organism, or that critically low N-levels might have been augmented by N₂-fixation (Davis et al., 1966) in the diazotrophic *A. flos aquae*.

The present study also showed that temperature, unlike nutrients, had varied effects on cellular growth of *M. aeruginosa* and *A. flos aquae*. Whilst *M. aeruginosa* recorded significantly higher growth rate at high temperatures than low temperatures, *A. flos aquae* growth was relatively unaffected by the temperature treatments investigated thereby suggesting that *A. flos aquae* was less sensitive to temperature changes than *M. aeruginosa*. These observations are in agreement with previous a study that reported severe limitation of *Microcystis* growth and respiration at temperatures below 20°C but only slight variation in *Anabaena* growth between 20 and 30°C (Robarts & Zohary, 1987). Zeng and Wang (2011) also reported higher specific growth rates for *M. aeruginosa* at higher (30°C) than at lower (18°C) temperatures. Sabour et al. (2009b), however, reported an increase in the growth rates of both *M. ichthyoblabe* and *Anabaena aphanizomenoides* with increase in temperature from 10-35°C. Although the response of *A. flos aquae* to changes in temperature was at variance with the results reported for *A. aphanizomenoides* previously (Sabour et al., 2009b), our results nevertheless emphasize the plausibility of intra- and/or interspecific difference in response to changes in temperature. Furthermore, the fact that *M. aeruginosa* and *A. flos aquae* monocultures consistently witnessed significantly higher growth rates than the mixed cultures at the temperatures investigated further highlights the need for consideration of competitive interaction when assessing impacts of environmental variables on bloom dynamics.

Results from our light experiment studies showed that although the high and low light treatments did not significantly affect the cellular growth in either *M. aeruginosa* CPCC299 or *A. flos aquae* CPCC67, monocultures of both strains nevertheless witnessed significantly higher growth rates than their mixed culture counterparts. These results are consistent with those from Renaud LeBlanc et al.
who also observed no significant differences in the growth rate of toxigenic *M. aeruginosa* CPCC (UTCC) 300 at 20 and 80 µ mol photons m\(^{-2}\) s\(^{-1}\). Imai et al. (2009) also reported no significant differences in *M. aeruginosa* and *M. wesenbergii* growth at 30 and 60 µ mol photons m\(^{-2}\) s\(^{-1}\). These notwithstanding, effect of light on the growth rate of cyanobacteria in laboratory and field studies remains contentious, with some studies (Yang et al., 2012) reporting significant two fold interaction effect of temperature with light whereby there was increased growth of *M. aeruginosa* with increased temperature at high (80 µ mol photons m\(^{-2}\) s\(^{-1}\)) light intensity but a decrease with increased temperature at low (35 µ mol photons m\(^{-2}\) s\(^{-1}\)) light intensity. Tomioka et al. (2011), on the other hand, reported significant positive correlations between the specific growth rate of the *M. aeruginosa* population in Lake Kasumigaura, Japan and ambient photosynthetically active radiation (PAR). The observance of no significant difference between low and high light growth reported in our study suggests that the investigated light intensities were probably above the minimum light requirement for growth for both species, but never exceeded maximum values beyond which growth could be photoinhibited.

The similarity in cellular growth rates of *M. aeruginosa* and net changes in *Microcystis mcyE* genes under most of the nutrient, temperature and light treatments investigated suggests possible coupling of cellular growth and *mcyE* copy production rates. Furthermore the fact that the growth rates of both *M. aeruginosa* and *A. flos aquae* as well as net change in *Microcystis mcyE* genes were generally higher in monocultures under most of the nutrient, temperature, and light treatments whereas significantly higher cellular and *mcyE* gene-based microcystin quotas were mostly observed in *M. aeruginosa* mixed cultures suggest that interaction between *M. aeruginosa* and *A. flos aquae* might have resulted in the depressed growth but elevated toxin quotas in the mixed cultures. Although we could not directly determine the nature of this interaction using the present experimental design, results from previous studies lead us to consider the possible release of infochemicals into the mixed cultures due to interaction between *M. aeruginosa* CPCC 299 and *A. flos aquae* CPCC 67. Indeed several *Anabaena* and *Microcystis* spp. are known to produce bioactive allelochemicals
and microcystins that have been implicated in growth inhibition of aquatic macrophytes and algae. Although the function of microcystins in cyanobacterial metabolism has been a subject of intense research, its role in growth inhibition of other aquatic organisms remains debatable. Whereas some studies have attributed growth inhibition of macrophytes (Jang et al., 2007; Pflugmacher, 2002) or other cyanobacterial genera (Singh et al., 2001) to microcystins, others have rather ascribed the growth inhibiting property of microcystin-producing *Microcystis* spp. to release of allelopathic compounds other than microcystins (Schatz et al., 2005; Sukenik et al., 2002). In a similar manner, both toxic and non-toxic *Anabaena* spp. (including *Anabaena flos aquae*) have been reported to elicit varied inhibitory effects on aquatic bacteria (Østensvik et al., 1998) and microalgae (Kearns & Hunter, 2000; Suikkanen et al., 2004), making it difficult to discriminate allelochemical and cyanotoxin-based effects.

A recent study showed that exposure of *M. aeruginosa* to exudates from mixed cultures of *M. aeruginosa* and *C. raciborskii* resulted in significant growth inhibition whereas exudates from monocultures of either species produced insignificant results (Mendes e Mello et al., 2012), suggesting that the production of growth inhibitors was probably contingent on competitive stress between interacting species. Some studies have suggested that the presence of infochemicals (i.e., allelopathic or toxigenic) in culture media might serve as cues triggering increased microcystin production in *Microcystis* spp. (Engelke et al., 2003; Schatz et al., 2007).

Results from the present study indeed showed elevated cellular toxin quotas in mixed culture *M. aeruginosa* compared to the corresponding monocultures, thereby suggesting that elevated toxin levels might have emanated from the response of mixed culture *M. aeruginosa* cells to infochemicals resulting from its interaction with *A. flos aquae*. Furthermore, the decrease in growth and *mcyE* copies observed in mixed cultures implies that mixed cultures contained growth inhibiting compounds absent in the respective monocultures. We can however not completely rule out the possibility that growth inhibition in mixed cultures could have resulted from competition for resources, although this seems less likely.
considering that even culture medium D (with the highest nutrient concentrations and least limited by N and P limitation) also recorded low mixed culture growth and \textit{mcyE} copies.

### 4.5 Conclusions

This study demonstrated reduced growth of \textit{M. aeruginosa} CPCC 299 and \textit{A. flos aquae} CPCC 67 in the mixed cultures under most of the nutrient, temperature, and light conditions investigated. Cellular microcystin quotas, on the contrary, were higher in the mixed cultures than the corresponding monocultures. These experiments therefore showed that although interaction between cyanobacterial species led to decreased bloom density, the resulting blooms were in fact more toxic.

Whereas \textit{A. flos aquae} CPCC 67 was unaffected by nitrogen concentrations in the growth media, \textit{M. aeruginosa} CPCC 299 growth was found to be severely inhibited at nitrogen concentrations below 1.0 mg L\textsuperscript{-1} but co-limited by nitrogen and phosphorus at higher N levels, hence simultaneous reductions of N and P levels in water bodies can potentially reduce incidence of toxigenic blooms of \textit{Microcystis}.

The fact that changes in \textit{mcyE} copies often mirrored changes in cellular growth rate of \textit{M. aeruginosa} CPCC 299 under most of the investigated treatments suggest a possible coupling of \textit{mcyE} production to cellular growth. Consequently, changes in \textit{mcyE} copies can be reliable indicators of changes in biomass of the microcystin-producing \textit{M. aeruginosa} strains.

### 4.6 Acknowledgements

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grants to C. Madramootoo and S. Jabaji and the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) grant to C. Madramootoo.
Table 4.1: Experimental design for nutrient experiments involving *M. aeruginosa* CPCC 299 and *A. flos aquae* CPCC 67

<table>
<thead>
<tr>
<th>Media</th>
<th>Nutrient concentrations</th>
<th>Monoculture growth</th>
<th>Mixed culture growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>PO$_4^-$</strong> (mg L$^{-1}$)</td>
<td><strong>NO$_3^-$</strong> (mg L$^{-1}$)</td>
<td>CPCC67</td>
</tr>
<tr>
<td>A</td>
<td>0.2</td>
<td>1.0</td>
<td>4*</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>0.2</td>
<td>1000</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>1000</td>
<td>4</td>
</tr>
</tbody>
</table>

*; Number of replicates for each nutrient treatment

Table 4.2: Models used to estimate *mcyE* gene copies in *M. aeruginosa* CPCC 299

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gene</th>
<th>Model*</th>
<th>Efficiency</th>
<th><strong>R$^2$</strong> value</th>
<th><strong>p-</strong> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient</td>
<td><em>mcyE</em></td>
<td>$Y = -3.339\log X + 40.61$</td>
<td>99.28</td>
<td>0.999</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Temperature/light</td>
<td><em>mcyE</em></td>
<td>$Y = -3.413\log X + 40.45$</td>
<td>96.33</td>
<td>0.999</td>
<td>p&lt;0.0001</td>
</tr>
</tbody>
</table>

* Y; C$_T$ value, X; *mcyE* gene copies

Table 4.3: Mean microcystin quotas under low (30 $\mu$ mol photons m$^{-2}$ s$^{-1}$) and high (60 $\mu$ mol photons m$^{-2}$ s$^{-1}$) light intensities

<table>
<thead>
<tr>
<th>CPCC strain</th>
<th>MC quota, fg cell$^{-1}$ (Mean± SD)</th>
<th>MC quota, fg <em>mcyE</em> copy$^{-1}$ (Mean± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>299</td>
<td>9.30 ± 2.75</td>
<td>0.52 ± 0.21</td>
</tr>
<tr>
<td>299+67</td>
<td>161.03 ± 57.97</td>
<td>11.19 ± 5.04</td>
</tr>
</tbody>
</table>

Table 4.4: Mean microcystin quotas under low (20°C) and high (25°C) temperatures

<table>
<thead>
<tr>
<th>CPCC strain</th>
<th>MC quota, fg cell$^{-1}$ (Mean± SD)</th>
<th>MC quota, (fg) <em>mcyE</em> copy$^{-1}$ (Mean± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>299</td>
<td>51.29 ± 5.35</td>
<td>0.42 ± 0.15</td>
</tr>
<tr>
<td>299+67</td>
<td>512.77 ± 162.36</td>
<td>13.80 ± 2.74</td>
</tr>
</tbody>
</table>

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Figure 4.1: Cellular growth rates of *M. aeruginosa* CPCC 299 and *A. flos aquae* CPCC 67 under various nutrient (A), light (B) and temperature (C) treatments. Nutrient medium A (P: 0.2 mg L\(^{-1}\), N: 1.0 mg L\(^{-1}\)), B (P: 20 mg L\(^{-1}\), N: 1.0 mg L\(^{-1}\)), C (P: 0.2 mg L\(^{-1}\), N: 1000 mg L\(^{-1}\)), D (P: 20 mg L\(^{-1}\), N: 1000 mg L\(^{-1}\))
Figure 4.2: Variation of mcyE genes in *M. aeruginosa* CPCC 299 under various nutrient (A), light (B), and temperature (C) treatments. Nutrient medium A (P: 0.2 mg L\(^{-1}\), N: 1.0 mg L\(^{-1}\)), B (P: 20 mg L\(^{-1}\), N: 1.0 mg L\(^{-1}\)), C (P: 0.2 mg L\(^{-1}\), N: 1000 mg L\(^{-1}\)), D (P: 20 mg L\(^{-1}\), N: 1000 mg L\(^{-1}\)).
Figure 4.3: Changes in microcystin cellular (A) and mcyE (B) quotas in *M. aeruginosa* CPCC 299 under various nutrient concentrations. Nutrient medium A (P: 0.2 mg L\(^{-1}\), N: 1.0 mg L\(^{-1}\)), B (P: 20 mg L\(^{-1}\), N: 1.0 mg L\(^{-1}\)), C (P: 0.2 mg L\(^{-1}\), N: 1000 mg L\(^{-1}\)), D (P: 20 mg L\(^{-1}\), N: 1000 mg L\(^{-1}\))
Connecting text to chapter 5

Chapters three and four respectively, explored the suitability of three mcy gene targets for qPCR estimation of microcystin-producing Microcystis spp., and the variation of mcy gene concentration with changing physicochemical and/or environmental conditions. Results from these studies affirmed the utility of the mcyE gene in qPCR-based estimation of microcystin-producing Microcystis spp. The periodic dominance of Canadian freshwater blooms by other potential microcystin-producing cyanobacteria necessitated the development of more robust tools for simultaneous detection of these noxious organisms.

Chapter five of this dissertation, which addressed the third objective of the research project, involved the design, testing, and validation of a novel multiplex qPCR technique for simultaneous detection and quantification of microcystin-producing Anabaena, Microcystis, and Planktothrix species. Furthermore, this tool was used to study the response of these toxigenic cyanobacterial genera to changes in physicochemical/environmental conditions in the Missisquoi Bay during the summers of 2010 and 2011.

The following manuscript, co-authored by Drs. Madramootoo, C.A. and Jabaji, S., has been submitted to Harmful Algae Journal for publication.

Chapter 5 Development and application of a multiplex qPCR technique to study the dynamics of multiple microcystin-producing cyanobacterial genera in a freshwater lake

Felexce Ngwa, Chandra Madramootoo, and Suha Jabaji

Abstract

The emergence and persistence of complex blooms comprising multiple toxigenic cyanobacteria genera pose significant challenges for water quality management in Canada and globally. The co-occurrence of morphologically indistinguishable toxic and non-toxic strains makes monitoring and control of these noxious organisms particularly challenging. Conventional monitoring approaches are not only incapable of discriminating toxic from non-toxic strains, but have proven to be less sensitive and specific.

In this study, a multiplex real-time quantitative polymerase chain reaction (qPCR) approach was developed and tested for its sensitivity and specificity at detecting, differentiating, and estimating potentially toxic Anabaena, Microcystis and Planktothrix genotype compositions in complex environmental samples. The oligonucleotide primers and probes utilized were designed to target portions of the microcystin synthetase gene E (mcyE) that encode the synthesis of the unique Adda moiety of microcystins in the three target genera.

Laboratory evaluation showed the developed assay to be highly sensitive and specific at detecting and quantifying targeted species. Indeed, the assay standards for the Anabaena, Microcystis and Planktothrix reactions attained efficiencies above 90%, with coefficients of determination consistently above 0.99. Analysis of environmental water samples resulted in detection and estimation of target toxigenic cyanobacterial genera in water samples even when cell numbers were below the detection limit for most conventional detection methods. Furthermore, the concentration of toxigenic Microcystis (the main putative microcystin-
producing cyanobacteria) in the study lake correlated significantly (p < 0.01) with total phosphorus concentration, but not total nitrogen or other measured physicochemical parameter. Use of the robust qPCR technique with the capacity for simultaneous detection, differentiation, and quantification of multiple toxigenic cyanobacteria genera enabled us to generate vital information that otherwise cannot be provided by currently used monitoring approaches.

**Key words:** Multiplex qPCR; Microcystin synthetase; Microcystin; Water quality; Cyanobacterial blooms

### 5.1 Introduction

Cyanobacterial blooms are an emerging threat to freshwater bodies globally, with the Province of Quebec, Canada, reporting significant increases in contamination of its freshwater lakes since 2004 (MDDEP, 2012). This increased incidence, which has resulted in periodic beach closures and substantial loss in revenue from lake side communities (Fortin et al., 2010), has been attributed not only to an increase in the number of new outbreaks, but also increased public awareness about cyanobacterial pollution. As a result, there is mounting pressure on government agencies to provide timely and reliable information on toxigenic cyanobacterial concentrations as well as the potential risks to public health.

Blooms of cyanobacteria are known for their proclivity at perturbing the ecological and aesthetic quality of water as well as production of bioactive secondary metabolites that elicit a myriad of hepatotoxic-, neurotoxic-, and/or cytotoxic effects in humans and domestic animals (Briand et al., 2003; Fujiki & Suganuma, 1999; Jochimsen et al., 1998; Wood et al., 2010a). Hepatotoxic microcystins (MCs) and nodularins (NODs) are the most prevalent cyanotoxins (Sivonen & Jones, 1999), with over 90 known MC congeners globally (Welker & von Döhren, 2006). The cyclic heptapeptide microcystins are predominantly produced by cyanobacteria belonging to the *Anabaena*, *Microcystis* and *Planktothrix* genera, although *Anabaenopsis*, *Hapalosiphon*, and *Nostoc* have also been implicated (Sivonen & Börner, 2008; Sivonen & Jones, 1999).
Most approaches for monitoring cyanobacteria in water bodies rely heavily on conventional microscopic techniques (Ouellette et al., 2006) coupled with analytical chemical detection of toxins (Kaebernick et al., 2002; Rueckert, 2007). Unfortunately, the morphological plasticity of toxic and non-toxic cyanobacteria strains co-occurring in complex environmental samples (Vezie et al., 1998) coupled with the limited number of toxin standards renders these conventional approaches incapable of differentiating toxic from non-toxic strains (Lyra et al., 2001).

Molecular biomarker techniques based on detection of toxin biosynthetic genes are rapidly gaining ground in water quality monitoring owing not only to their greater sensitivity and specificity but also shorter turnaround times, high throughput capabilities and lower costs (Fortin et al., 2010; Ouellette & Wilhelm, 2003). In addition, molecular techniques are capable of detecting potentially toxic cyanobacteria even when toxin concentrations are well below detection limits for most conventional toxin analyses methods, making them highly amenable for incorporation into early warning systems.

Microcystin biosynthesis follows a thio-template mechanism (Dittmann et al., 1997) that is encoded by the microcystin synthetase (mcy) gene cluster—a multi-enzyme complex consisting of both non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules (Tillett et al., 2000). Characterization of this gene cluster in *Microcystis* (Nishizawa et al., 1999; Tillett et al., 2000) *Anabaena* (Rouhiainen et al., 2004), and *Planktothrix* (Christiansen et al., 2003) species has revealed the presence of 9-10 open reading frames (ORFs) arranged either in two (*Microcystis* and *Planktothrix* spp.) or three (*Anabaena* spp.) bi-directionally transcribed operons encoding the synthesis and incorporation of various amino acid residues into the microcystin structure. The *mcyE* gene, in conjunction with *mcyD* and *mcyG* genes, directs synthesis and incorporation of the two vital amino acid moieties, Adda and D-Glu, into the MC structure. The importance of these two amino acids to MC toxicity as well as their relatively invariant nature makes the *mcyE* regions reliable biomarkers for detection of microcystin-producing cyanobacteria. Although *mcy* genes have been shown to be
requisite for toxin production (Tillett et al., 2000), some studies have nevertheless identified cyanobacterial strains that contain the mcy genes but are incapable of producing MCs (Kurmayer et al., 2004; Mikalsen et al., 2003). This phenomenon is, however, more common for Planktothrix sp. (Christiansen et al., 2006; Kurmayer et al., 2004) than other cyanobacterial genera. This notwithstanding, molecular approaches including quantitative real-time polymerase chain reaction (qPCR) remain the best approach for estimating potentially toxic cyanobacterial genotype relative proportions in water.

Most qPCR assays so far developed are, however, uniplex reactions that target mcy genes either in Microcystis (e.g., Fortin et al., 2010; Ngwa et al., 2012; Okello et al., 2010; Rinta-Kanto et al., 2009b; Srivastava et al., 2012; Te & Gin, 2011), Anabaena (e.g., Rueckert, 2007; Sipari et al., 2010), Planktothrix (e.g., Halstvedt et al., 2007; Kurmayer et al., 2011; Savichtcheva et al., 2011), or all three genera (e.g., Rantala et al., 2006; Vaitomaa et al., 2003).

Current multiplex PCR methods are either qualitative in nature (Ouahid & del Campo, 2009; Valério et al., 2010) or are quantitative but use primers and probes that target all major microcystin, nodularin, cylindrospermopsin, and/or saxitoxin-producing cyanobacteria (Al-Tebrineh et al., 2012a; Al-Tebrineh et al., 2012b).

Such assays, unlike the multiplex qPCR developed in this study, are incapable of discriminating and estimating relative proportions of specific toxin-producing genera within a given bloom. This information is vital for understanding factors affecting proliferation of the different noxious cyanobacteria genera within the context of designing effective algal bloom control strategies.

We report in this study the design and validation of a multiplex qPCR technique for simultaneous detection, differentiation, and quantification of microcystin-producing cyanobacteria from the Anabaena, Microcystis, and Planktothrix genera in the Missisquoi Bay water samples collected during Spring to Fall seasons of 2010 and 2011. These assays, which employ the TaqMan® (hydrolysis) probe chemistry to simultaneously target the mcyE genes in these three commonly occurring microcystin-producing cyanobacterial genera, are capable of generating vital information that otherwise cannot be obtained from currently used
monitoring approaches. We believe incorporation of these assays into water quality monitoring programs would go a long way towards improving the characterization of toxic algal blooms.

5.2 Materials and methods

5.2.1 Study site, sample collection and analyses

Two littoral stations – VQ-B2 (45° 04’ 57"N; 73° 08’ 52"W) and PH-A (45° 02’ 22"N; 73° 04’ 46"W) - located on the Quebec portion of the Missisquoi Bay, Lake Champlain (Figure 5.1) were sampled (0.5m depth) on a weekly or bi-weekly basis during the months of June-November of 2010 and 2011. Whole water samples for DNA (500mL), chlorophyll a (500mL) and toxin (500mL) analyses were collected in acid washed 2000mL Kimble Kimax glass bottles (Fisher Scientific, Ottawa, ON, Canada), transported on ice to the laboratory where they were filtered onto GF/C filter papers (47mm diameter, Whatman, England), wrapped in aluminum foil paper and either analyzed within 24 hours (for chl-a) or stored at -20°C for subsequent analyses. Samples for total phosphorus (TP) and total nitrogen (TN) were placed into acid-washed 125mL high density polyethylene (HDPE) bottles (VWR International, PA, USA) and transported on ice to the laboratory where TP and TN concentrations were determined by the peroxodisulfate oxidation method (Ebina et al., 1983).

Phytoplankton samples were collected in amber 60mL HDPE bottles (Wheaton Corporation, Millville, NJ, USA) containing acid Lugol and preserved in the dark for later analysis. A YSI 6600V2-4 Sonde (YSI Inc., Yellow Springs, OH, USA) was used to measure pH, dissolved oxygen (DO), water temperature, conductivity, and turbidity while surface and underwater light intensity at the sampling stations were measured using a LI-COR LI-250A light meter and LI-193 sensor (LI-COR Inc., Lincoln, NB, USA).

5.2.2 Phytoplankton enumeration and chlorophyll a analysis

Lugol preserved field samples were analyzed using a Leitz Diavert phase contrast inverted microscope (Ernst Leitz Inc, Rockleigh, NJ, USA) at magnifications between 125× and 930× following the modified Utermohl method as previously
described (Findlay & Kling, 1998). Counting of laboratory cultures and chl-a analyses were performed as described in a previous study (Ngwa et al., 2012).

5.2.3 Cyanobacterial strains and culture conditions

A total of 37 toxic and non-toxic cyanobacterial strains (Table 5.1) were used to test the specificity of the developed PCR assays. *Anabaena lemmermannii var. minor* NIVA-CYA 83/1, *Planktothrix prolifica* NIVA-CYA 128, and *Planktothrix agardhii* NIVA-CYA 127 were purchased from Norsk Institute for Vannforsning (NIVA, Oslo, Norway) and maintained in BG-11 medium as previously described (Ngwa et al., 2012). Unialgal, non-axenic cyanobacterial strains bearing the CPCC designation were purchased from the Canadian Phycological Culture Centre (CPCC, Waterloo, Canada) and maintained in BG-11 medium. Lyophilized cells of *Anabaena* spp., *Aphanizomenon* spp., *Cylindrospermopsis* sp. and *Microcystis* spp. with strain designations LME-CYA were kindly donated by the Estella Sousa e Silva Algal Culture Collection (ESSACC, Lisbon, Portugal) (Paulino et al., 2009) while *Anabaena* spp. (strains 14, 66A, 86, 90, 202A1, and 318) and *Planktothrix* spp. (strains 45, 49, 97 and 213) (Vaitomaa et al., 2003) were graciously donated by Prof. Kaarina Sivonen of the University of Helsinki (Helsinki, Finland).

5.2.4 DNA extraction

Cyanobacterial genomic DNA was extracted either from cell pellets or frozen GF/C filters (Whatman) containing cyanobacterial cells collected from field samples as previously described (Ngwa et al., 2012). The concentration and purity of extracted DNA was verified on a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) using the ND-1000 software (v3.2.1) and only gDNA with $A_{260}/A_{280}$ ratios between 1.7 and 2.0 was used in PCRs.

5.2.5 Probe and primer design

The novel *Anabaena, Microcystis, and Planktothrix mcyE* primers and probes (Table 5.2) used in this study were designed using Primer3 (Rosen & Skaletsky, 2000) and synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). In silico specificity tests were performed by running nucleotide basic
alignment search tool (BLASTN) searches of primer and probe sequences against the non-redundant (nr) nucleotide database of GenBank wherein significant hits were registered solely for designated targets. All primers were tested by conventional PCR on gDNA from reference cyanobacterial strains and expected PCR amplicons were produced only for strains containing the target mcyE gene (Table 5.1).

5.2.6 Conventional PCR, cloning and sequencing

To ascertain that only target *Anabaena*, *Microcystis*, and *Planktothrix* mcyE genes were being amplified, conventional PCR was performed on the reference strains (Table 5.1) using GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA, U.S.A). Each PCR reaction contained 1U AmpliTaq Gold (Applied Biosystems), 1 × PCR buffer, 1.5 mM MgCl₂, 250 µM dNTPs, 500nM of each primer, 400ng µL⁻¹ bovine serum albumen (BSA) (Life Technologies Inc., Burlington, ON, Canada) and autoclaved distilled water to a final volume of 25µL. Thermal cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 40 cycles of PCR, each consisting of denaturation at 94 °C for 30s, annealing at 56°C for 30s, strand extension at 72 °C for 60s and a final extension step at 72 °C for 10 min. PCR inhibition was checked via conventional PCR using the cyanobacterial 16S rRNA primer pair CYA359F(GGG GAA TYT TCC GCA ATG GG) and CYA781R (GAC TAC TG GGT ATC TAA TCC CAT T) as previously described (Nubel et al., 1997). Presence of target PCR amplicons was verified by 1.5 % agarose gel electrophoresis followed by ethidium bromide staining, visualization and documentation using the Kodac Gel Logic 200 Imaging System (Carestream Health Inc., Rochester, NY, USA). Purified plasmid DNA containing the *Anabaena*, *Microcystis*, and *Planktothrix* mcyE inserts produced as described in section 4.2.6 was sent to the Génome Québec Innovation Centre (Montreal, QC) for sequencing using ABI’s 3730xl DNA Analyzer technology (Applied Biosystems). Generated nucleotide sequences were compared with GenBank database nucleotide sequences using BLASTN algorithms.
Portions of plasmid DNA from *Anabaena* sp. 202A1, *M. aeruginosa* CPCC 299, and *Planktothrix* sp. 97 were preserved at -80°C for subsequent use as external standards for multiplex qPCR.

### 5.2.7 Quantitative real-time PCR

The multiplex qPCR used in estimating potential microcystin-producing *Anabaena*, *Microcystis*, and *Planktothrix* genera in both field and laboratory cultures utilized three different TagMan® fluorescent chemistries to detect the respective genera within a single reaction tube. The *Anabaena mcyE* probe was a novel ZEN double-quenched probe containing 6-carboxyfluorescein (FAM) fluorescent reporter dye covalently attached to its 5′ end, an internal ZEN moiety, and Iowa Black® FQ (IABkFQ) quencher at the 3′ end. In contrast, the *Microcystis* and *Planktothrix mcyE* probes had respectively Hexachlorofluorescein (HEX) and the cyanine fluorophore (Cy5) reporter dyes at their 5′ ends with IABkFQ and black hole quencher 2 (BHQ-2), respectively, at the 3′ ends (Table 5.2).

PCR amplifications were performed using the Mx 3005P QPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The optimized multiplex qPCR reaction mixture (25µL) contained 12.5 µL of 2× Brilliant Multiplex QPCR Master Mix (Agilent Technologies, Inc.), 30nM ROX reference dye, 400 ng µL⁻¹ BSA, optimized concentrations of *Anabaena*, *Microcystis*, and *Planktothrix* specific primers and probes (Table 5.2), and DNA template concentrations ranging between 0.5-14.0 ng per reaction. The thermal cycling program included an initial activation step at 95°C for 10 min, followed by 40 cycles of two-step PCR, consisting of a denaturation step at 95°C for 15s and annealing/elongation steps at 60°C for 60s. Fluorescence data was collected at the elongation step and analyzed using the MxPro QPCR software version 3.0 (Agilent Technologies, Inc.).

### 5.2.8 Multiplex qPCR assay optimization and performance evaluation

Concentrations of primers and probes were initially optimized in uniplex qPCR reactions for the target cyanobacterial genera, after which uniplex and multiplex
reactions were run concomitantly to ensure that threshold cycle ($C_T$) values and efficiencies of amplification for equivalent uniplex and multiplex reactions did not vary by more than one $C_T$ point and 5% respectively.

In order to assess the sensitivity of the optimized multiplex assays to non-target background gDNA, different amounts of template DNA from four non-target organisms were added to known amounts of target DNA. Briefly, gDNA from *Anabaena spiroides* LMECYA 16, *Aphanizomenon flos aquae* LMECYA 88, *M. aeruginosa* LMECYA 144, and/or *Planktothrix* sp. 45 were added to gDNA from *Anabaena* sp. 202A1 (13.0 ng per reaction), *M. aeruginosa* CPCC 299 (14.0 ng per reaction), and *Planktothrix* sp. 97 (14.0 ng per reaction) at dilutions of 1:10 and 1:100. The resulting mixtures were analyzed by multiplex qPCR assay to generate $C_T$ values for spiked and non-spiked DNA samples. In order to test the effects of addition of non-target (background) DNA on the multiplex qPCR results, multiple comparisons of $C_T$ values from spiked and non-spiked samples were performed using the restricted maximum likelihood (REML) method of SAS.

5.2.9 Standard curves and reaction efficiencies

Plasmid DNA containing target *mcyE* gene regions in *Anabaena* sp. 202A1, *M. aeruginosa* CPCC 299, or *Planktothrix* sp. 97 were used as external standards for estimating the respective *mcyE* copies. Tenfold serial dilutions ($10^0 - 10^8$) of the plasmid DNA template, corresponding to $10^8$-$10^1$ copies of *Anabaena, Microcystis*, and *Planktothrix* *mcyE* genes were performed and the resulting dilution series qPCR amplified in triplicates to obtain the corresponding $C_T$ values. The numbers of copies of the respective *mcyE* genes in a given qPCR reaction was calculated using the formula:

$$\text{Gene copies} \, \mu\text{L}^{-1} = \frac{\text{[Plasmid DNA conc}( \text{g} \mu\text{L}^{-1}) \times 6.022 \times 10^{23}\text{(gene copies mol}^{-1})]}{[(\text{Plasmid + insert size})(\text{bp}) \times \text{MW of DNA base pair}(\text{g mol}^{-1}\text{bp}^{-1})]} \quad [5.1]$$

In all copy number calculations, the molecular weight (MW) of one DNA base pair (bp) was assumed to be equal to 660 g mol$^{-1}$ (Vaitomaa et al., 2003). Standards curves for the respective *mcyE* gene copy number calculation were
generated by linear regression of the \( C_T \) values of various dilutions against the \( \log_{10} \) of corresponding \( mcyE \) copy numbers. Goodness of fit of the curves was assessed using MxPro QPCR software algorithms. The amplification efficiencies (E) of both the uniplex and multiplex qPCR reactions were automatically calculated from the slopes (S) of the respective linear regression equations using the formula: \( E(\%) = \left[ 10^{-\left(\frac{S}{2}\right)} - 1 \right] \times 100 \). Reactions were considered acceptable when amplification efficiencies were between 90% and 110% and \( \Delta C_T \leq 1 \) for replicate amplification reactions.

In order to determine the assay limit of detection (LOD), multiplex qPCR was performed on two-fold serially diluted template DNA containing between \( 1.020 \times 10^3 \) to \( 1.0 \) \( mcyE \) gene copies per reaction. The qPCR data generated served as input to GenEx Ent software (version 5.3.2; MultiD Analyses AB, Göteborg, Sweden) and the LOD determined as described previously (Ngwa et al., 2012).

### 5.2.10 Microcystin analyses

Cell-bound microcystin isoforms were extracted from GF/C glass filters, containing entrapped cells from known volumes of field samples or laboratory cultures, using 75% (v/v) aqueous methanol as described previously by Spoof et al. (2003), with slight modifications. Briefly, GF/C filters were placed in 2mL microcentrifuge tubes containing 1mL 75% (v/v) aqueous methanol, ultrasonicated in a bath sonicator for 15 min and subsequently centrifuged at 10000 g for 10 min. The clarified supernatant was transferred into new microcentrifuge tubes and diluted with double distilled water to obtain aqueous methanol concentration below 0.5% (v/v) (Rivasseau et al., 1999) prior to ELISA analyses. Microcystin concentration in duplicate samples was spectrophotometrically determined using a 96 well EnviroGard® microcystin plate kit (Strategic Diagnostics, Newark, DE, USA) and a Synergy H4 Hybrid Multi-Mode microplate reader (BioTek, Winooski, VT, USA). The EnviroGard® ELISA microtiter plates utilized polyclonal antibodies that allowed for detection of microcystin-LR, -RR, and -YR. All toxin concentrations are reported as MC-LR equivalents, with the assay having a limit of detection of 0.1µg L\(^{-1}\).
5.2.11 Statistical analysis

Statistical analysis was performed using SAS statistical software package version 9.3 (SAS Institute Inc., Cary, NC, USA). All data was tested for normality (Shapiro-Wilk, p<0.05) and any nonconforming data were log transformed to meet these conditions prior to further analyses. The multiple regression analyses utilized PROC REG options of SAS, with forward selection of significant variables at the 5% probability level.

5.3 Results

5.3.1 Microscopic analysis of field samples

Periodic blooms of cyanobacteria occurred in the Missisquoi Bay, Quebec during the 2010 and 2011 sampling seasons, with over 12 different cyanobacterial genera identified microscopically at both stations VQ-B2 and PH-A (data not shown). Cyanobacterial biomass was, however, either dominated by Microcystis, Anabaena, or Aphanizomenon species, especially during the summer months (June-September). Whereas station VQ-B2 had over 60% of its mean seasonal cyanobacterial biomass represented by Microcystis (27.8±31.5%), Aphanizomenon (18.2 ± 21.4%), and Anabaena (17.7 ± 25.3%) species for the 2011 sampling season, station PH-A on the contrary had over 70% of its mean seasonal cyanobacterial biomass made up of Microcystis (47.7±34.3%), Anabaena (13.7± 19.4%), and Aphanizomenon (11.5 ± 15.5%) species. These three cyanobacterial genera were found to coexist at both sampling stations most (>60%) of the time. Total cyanobacterial concentration for 2010 (Figure 5.2) was relatively lower than for 2011(Figure 5.3) at both sampling locations, with Anabaena, Aphanizomenon, and Microcystis species again being widespread. Potential microcystin producers Anabaena flos aquae, M. wesenbergii and M. aeruginosa were spotted on several occasions whereas Planktothrix spp. appeared only sporadically (data not shown).

5.3.2 Variation in physicochemical parameters in Missisquoi Bay

Seasonal averages of the physicochemical parameters measured at the two sampling stations during the 2010 and 2011 seasons are shown in Table 5.3.
There was a marked spatiotemporal variability in all parameter values over the sampling periods as exemplified by the relatively high standard deviations from mean values (Table 5.3) Mean TN: TP ratios for the four sample-season-years were consistently below the benchmark value of 29, which is indicative of the potential for dominance of cyanobacteria over other phytoplankton species (Smith, 1983).

5.3.3 Conventional PCR, cloning and assay specificity

All genomic DNA from reference strains was successfully amplified using the 16S rRNA cyanobacterial primer pair CYA359F/781R (Table 5.1), signifying absence of PCR inhibitors. The *Anabaena*, *Microcystis* and *Planktothrix mcyE* primer pairs produced putative PCR amplicons within the expected sizes of 160, 167 and 184 bp, respectively solely for respective microcystin-producing strains (Table 5.1). Blasting of generated *Anabaena*, *Microcystis*, or *Planktothrix mcyE* nucleotide sequences against existing GenBank sequences as of February 2012 produced significant hits mainly with the *mcyE* sequences from respective genera.

5.3.4 Standard curves, sensitivity and efficiency of qPCR assays

Figure 5.4 shows the standards curves generated from a simultaneous qPCR run for optimized uniplex and multiplex reactions. $C_T$ values for both uniplex and multiplex reactions targeting the *mcyE* gene in the same genera varied by less than one $C_T$ while the amplification efficiencies for both uniplex and multiplex reactions were respectively 95.03% and 99.46% for *Anabaena mcyE*, 95.99% and 94.51% for *Microcystis mcyE* and 92.71% and 94.73% for *Planktothrix mcyE* genes. The multiplex qPCR assay had a linear dynamic range of $10^1$-$10^7$ copies per reaction for each of the target *Anabaena*, *Microcystis* and *Planktothrix mcyE* genes, with lower limits of detection being respectively 33, 63, and 3 *mcyE* copies per PCR reaction at the 95% confidence level.

Comparison of $C_T$ values of spiked and non-spiked samples showed that the presence of as much as 5.8 ng of background DNA from non-target *Planktothrix*, *Anabaena*, *Microcystis*, and/or *Aphanizomenon* spp. in the qPCR reaction mixture had no significant effect on the resulting PCR cycle thresholds (Table 5.4).
5.3.5 Microcystin analyses

Microcystin concentrations ranged from below the detection limit to 24.0 µg L$^{-1}$, with 26% of the samples recording MC concentrations exceeding the World Health Organization (WHO) drinking water guideline value of 1.0 µgL$^{-1}$. The WHO recreational waters guideline value (20.0 µgL$^{-1}$) was only exceeded once (August 29, 2011) while the Quebec recreational guideline value (16.0 µgL$^{-1}$) was exceeded twice in 2011, specifically, on August 29 (Station PH-A) and September 26 (Station VQ-B2). Microcystin concentrations, just like cyanobacterial concentrations, showed substantial spatiotemporal variability as depicted in Figure 5.5. Mean MC concentrations for 2010 and 2011 at station PH-A were respectively 0.67±0.81 µgL$^{-1}$ and 3.63±5.94 µgL$^{-1}$ while VQ-B2 recorded 0.11 ± 0.16 µgL$^{-1}$ and 1.40 ± 4.68 µgL$^{-1}$ for 2010 and 2011 respectively.

5.3.6 Abundance of microcystin-producing cyanobacteria and association with physicochemical parameters

Three potential microcystin-producers belonging to the *Anabaena*, *Microcystis* and *Planktothrix* genera were detected in water samples collected from the two sampling stations during the 2010 and 2011 seasons. However, the distribution of *Anabaena* and *Planktothrix mcyE* copies was rather sporadic when compared to *Microcystis mcyE*, which was more widespread. Indeed *Anabaena* and *Planktothrix mcyE* copies were only detected in 6% and 14% of the samples (n = 50), respectively, with gene copies only ranging from below the detection limit to slightly over 10$^3$ copies L$^{-1}$. On the contrary, *Microcystis mcyE* copies were detected in over 90% of the samples with copy numbers ranging from below the limit of detection to 6.44 × 10$^6$ copies L$^{-1}$. Seasonal mean toxigenic *Microcystis* genes for station PH-A were (5.7±15.3) × 10$^5$ copies L$^{-1}$ and (1.5±2.0) × 10$^6$ copies L$^{-1}$ for the 2010 and 2011 sampling season while station VQ-B2 had respectively (4.8±3.7) × 10$^3$ and (2.9±9.0) × 10$^5$ copies L$^{-1}$. Gene copies at both stations thus exhibited high spatiotemporal variability with station PH-A, on average, recording significantly (p<0.05) higher *Microcystis mcyE* copies relative to station VQ-B2. Assuming that each toxigenic *Microcystis* cell contains only one *mcyE* gene per genome, based on BLASTN searches of NCBI
whole genome sequences using target *Microcystis mcyE* sequence and results from previous studies (Al-Tebrineh et al., 2011; Vaitomaa et al., 2003), trends for toxigenic *Microcystis* cells should mirror those for *Microcystis mcyE* copies. Proportions of toxigenic *Microcystis* to the total *Microcystis* population for the 2011 sampling seasons thus ranged between 0.1-14.3% (mean = 4.4%; n = 13) for station PH-A and 0.1-13.5% (mean = 3.2%; n = 13) for station VQ-B2, with peaks in the month of October. Cell proportions for 2010 could not be reliably estimated given that microscopic analyses resulted in identification of *Microcystis* genera in just over 30% of the samples whereas qPCR resulted in detection of toxigenic *Microcystis* in over 90% of the samples. Linear regressions displaying the strengths of associations between microcystin and the biomass variables chl-a, total *Microcystis* and *Microcystis mcyE* for 2011 samples are presented in equations 5.2-5.4.

\[
\log(MC) = -2.139 + 1.781 \log(chl \, a), \quad Adj. \, R^2 = 0.59, \, p < 0.0001 \quad [5.2]
\]

\[
\log(MC) = -5.3078 + 0.7193 \log(Mcrsp), \quad Adj. \, R^2 = 0.66, \, p < 0.0001 \quad [5.3]
\]

\[
\log(MC) = -4.7339 + 0.8395 \log(MicmcyE), \quad Adj. \, R^2 = 0.80, \, p < 0.0001 \quad [5.4]
\]

where

- *MC* is the total microcystin concentration (µg L^{-1}),
- *chl a* is the total chlorophyll a concentration (µg L^{-1}),
- *Mcrsp* is the total number of *Microcystis* cells per liter, and
- *MicmcyE* is the concentration of *Microcystis mcyE* genes (copies L^{-1}).

*Microcystis mcyE* gene copies thus appeared to be the best indicator of microcystins concentration, explaining about 80 percent of the variability in the MC concentration [eq.5.4], whereas total *Microcystis* and chl-a could only explain respectively 66 and 59 percent of the variability in MC in the study lake. For the 2010 samples, only *Microcystis mcyE* showed significant but weaker correlation with MC (data not shown).

There was very weak association between total cyanobacteria concentration and MC in both years (Adj. \, R^2 = 0.1, \, p =0.06), suggesting that MC-producing cyanobacteria constituted a very small proportion of the total cyanobacterial population.
Correlation analyses showed significant positive associations (p<0.01, r > 0.45) amongst chl-a concentration, *Microcystis* spp. counts, and *Microcystis mcyE* gene copies (Table 5.5). Furthermore, whereas total phosphorus concentrations correlated significantly (p<0.01, r>0.43) with the three biomass proxies, total nitrogen on the contrary, showed no significant association with any of three variables (Table 5.5).

### 5.3.7 Multiple linear regressions of cyanobacterial biomass proxies and physicochemical parameters

Parameter estimates for the multiple linear regression models relating chlorophyll a concentration, *Microcystis* spp. counts, and *Microcystis mcyE* copies to various environmental variables are shown in Table 5.6. The chl-a, *Microcystis* spp. counts, and *Microcystis mcyE* copies models all had total phosphorus (TP) as one of the predictor variables, accounting for respectively 65, 28, and 20% of the variability (Table 5.6). Approximately 70% of the variability in chl-a concentration was explained by TP concentration and pH of the water while only 44% of the variability in *Microcystis mcyE* copies was explained by TP, turbidity and underwater light intensity.

### 5.4 Discussion

#### 5.4.1 Characterization of Missisquoi Bay cyanobacterial population

Blooms of mixed assemblages of toxic cyanobacteria spanning multiple genera are becoming increasingly prevalent in freshwater bodies globally (Al-Tebrineh et al., 2012a; Rantala et al., 2006; Vaitomaa et al., 2003). The Missisquoi Bay of Lake Champlain has witnessed a yearly incidence of toxic cyanobacterial blooms over the last decade, with potentially toxic *Microcystis* and *Anabaena* genera being widespread (Fortin et al., 2010; Watzin et al., 2006). Most bloom monitoring approaches implemented in this lake so far have either utilized conventional methods (Watzin et al., 2006) that are incapable of distinguishing toxic from non-toxic cyanobacterial genotypes or molecular techniques that target single toxin- producing genera (Fortin et al., 2010). The present study utilized a validated multiplex qPCR technique in conjunction with conventional microscopy
and toxin analyses to better characterize, over two summer seasons, the cyanobacterial bloom composition in one of Quebec’s most impacted freshwater bodies. Unlike previous studies, the multiplex qPCR technique reported here is the first to estimate toxigenic Microcystis, Anabaena, and Planktothrix concentrations in a single reaction tube.

Microscopic examination of field samples during the present study also revealed widespread occurrence of species from the Microcystis, Anabaena, and/or Aphanizomenon genera, each dominating the cyanobacteria population at different points in time, in agreement with previous studies in temperate lakes (Fortin et al., 2010; Sipari et al., 2010; Vaitomaa et al., 2003; Watzin et al., 2006). Total cyanobacterial densities in most of the samples (>90%) were above 2000 cells mL\(^{-1}\) (Figure 5.2), thus exceeding the WHO Alert Level 1 (Bartram et al., 1999) which triggers toxin analyses. Alert Level 2 (>100,000 cells mL\(^{-1}\)) at which WHO recommends provision of alternative drinking water supplies was exceeded in 49% of the samples, most of which were collected between August and September 2011. Despite these total cyanobacterial alert level exceedances, MC concentrations during the study period were mostly below WHO and Canadian drinking and recreational water guideline values (Health Canada, 2012), with average toxin concentrations generally within the 0.5-4.6 µg/L range reported in other temperate lakes (Gobler et al., 2007; Hotto et al., 2008; Rantala et al., 2006).

Although microscopy showed periodic dominance of the Missisquoi Bay cyanobacterial population by potential microcystin-producing cyanobacteria of the Anabaena or Microcystis genera, qPCR analyses nevertheless showed a majority (over 90%) of field samples to contain Microcystis mcyE genes with rather sporadic occurrence of Anabaena (6% of samples) and Planktothrix (14% of samples) mcyE genotypes. The highest Microcystis mcyE copies recorded in our study were, indeed, about three orders of magnitude higher than the corresponding Anabaena or Planktothrix mcyE copy numbers. The dominance of potential microcystin-producing Microcystis genotypes over Anabaena and Planktothrix genotypes observed in this study was not surprising given that Microcystis spp. comprise the most important microcystin producers worldwide.
and have previously been reported to dominate the cyanobacterial population on the Vermont (USA) portion of Missisquoi Bay (Watzin et al., 2006). This notwithstanding, it is possible we might have underestimated toxigenic *Anabaena* and *Planktothrix mcyE* concentrations in this study owing to the fact that analyses were performed on water samples collected just beneath the water surface (maximum 0.5m depth), whereas these two genera are known to form both epilimnic and metalimnic blooms (Ernst et al., 2001; Halstvedt et al., 2007; Mitrovic et al., 2001).

An intriguing observation of the present study was the detection of *Microcystis mcyE* genes in samples where *Microcystis* spp. could not be positively identified by microscopy. The phenomenon was particularly common for samples with low levels of *Microcystis mcyE* copies. Indeed, discrepancies between results from molecular and microscopy detection of cyanobacteria in complex environmental samples are not entirely uncommon (Sipari et al., 2010; Tomioka et al., 2008). Zamor et al. (2012), attributed such discordance between microscopic and qPCR detection of low levels of toxigenic algae to the higher detection limit and measurement error of microscopy compared to molecular methods. A plausible explanation for the discrepancies recorded in the present study could be that our sampling protocol as well as the relatively lower sensitivity of microscopic techniques did not allow for detection of such low levels of *Microcystis* cells, whereas the qPCR technique was able to detect these low concentrations due to its higher sensitivity.

One caveat of the present study is that use of gDNA in our multiplex qPCR assay only allowed for detection of potential and not actual microcystin-producing genotypes from the three target genera. Considering that genotypes containing the *mcy* genes but lacking toxin-producing ability have been reported in *Planktothrix* strains (Christiansen et al., 2006; Kurmayer et al., 2004) and to a lesser extent *Microcystis* (Mikalsen et al., 2003; Via-Ordorika et al., 2004) and Baltic Sea *Anabaena* (Fewer et al., 2011) strains, it is plausible that results reported here could be masked by such occurrences. Although this phenomenon has not been reported for the study lake to date, it would be interesting in future studies to
evaluate various McyE transcript levels in this lake through quantitative reverse transcriptase (RT)-PCR using the assays described in the present study.

### 5.4.2 Physicochemical conditions and cyanobacterial blooms dynamics in the Missisquoi Bay

In the multiple regression models, TP but not TN partially explained the variability in chl-a, total *Microcystis* spp., and toxigenic *Microcystis* concentration. In addition to TP, water turbidity and light intensity also partially accounted for the variability in *Microcystis mcyE* while variability in chl-a concentration was further explained by water pH. That notwithstanding, the fact that TP was the sole variable that associated significantly with all the three cyanobacterial biomass proxies suggests that TP could be an important driver of the cyanobacterial blooms dynamics in the Missisquoi Bay. The ratios of TN/TP in samples collected during the August-September sampling periods ranged from 2.7-20, suggesting potential dominance of N$_2$-fixing cyanobacteria (Smith, 1983) over non-fixers. Heterocystous *Anabaena* and *Aphanizomenon* filaments were indeed detected in the samples collected during this period implying some form of N-limitation and reliance of at least some of the *Anabaena* and *Aphanizomenon* population on fixed nitrogen. Curiously, total *Microcystis* spp. counts as well as *Microcystis mcyE* copies also peaked during August-September period when TN/TP ratios were at the lowest. A plausible explanation for this anomaly could be that luxury uptake and storage of nutrients by *Microcystis* spp. (Kromkamp et al., 1989; Stal, 2012) under nutrient replete conditions might have led to its success at such low nutrient levels.

### 5.4.3 Association between cyanobacterial and microcystin concentrations

The concentration of microcystins in Missisquoi Bay water samples was significantly positively correlated with chl-a, total *Microcystis* and *Microcystis mcyE* copies, with genes copies showing the strongest association with MC ($R^2 = 0.80$, $p<0.0001$). The lack of association between total *Anabaena* (potential MC producers) counts coupled with the patchy occurrence of *Anabaena* and
Plankthothrix mcyE genes shows that Microcystis mcyE copies were the best indicator of MC concentration in the Missisquoi Bay during summer 2011. Fortin et al. (2010) also reported highly significant positive correlation between Microcystis mcyD copies and MC concentration in Missisquoi Bay water samples collected in the summer of 2006. These authors further reported a two months uninterrupted constant rate of increase in toxin (+15% per day) and mcyD copies (+7.5%) and used this information to accurately predict the date on which a public health advisory was to be posted by responsible Quebec government agency.

We did not, however, witness any such uninterrupted increase in neither microcystin nor Microcystis mcyE gene copies during our 2010 and 2011 surveys probably due to year-to-year variability in toxin and cyanobacterial dynamics. Nevertheless, we observed that toxin concentrations in lake water samples generally exceeded the WHO and Canadian drinking water guideline values (1.0 and 1.5 µg L\(^{-1}\) respectively) mostly when concentrations of Microcystis mcyE genes were above 10\(^5\) copies L\(^{-1}\). Based on the strength of empirical relationships between MCs and gene copies reported here and other studies (Fortin et al., 2010; Otten et al., 2012), we suggest development of new guidelines based on toxin gene concentrations instead of cell counts. The relatively poor association between microcystin concentration and total cyanobacterial counts (Adj. R\(^2\) = 0.1, p = 0.06) further suggests that use of total cyanobacteria counts as an indicator of cyanotoxins hazards might lead to implementation of unnecessarily stringent control on water use in this lake.

A cautionary note regarding use of toxin gene copy numbers as indicators of toxigenicity is that although most qPCR standards for estimating toxigenic cell proportions are based on the assumption of one copy of toxigenic gene per Anabaena, Microcystis, or Nodularia cell (Koskenniemi et al., 2007; Rantala et al., 2006; Vaitomaa et al., 2003), the presence of more than one copy of these genes per genome of uncharacterized toxigenic cyanobacterial cells or rapidly growing cyanobacterial cells with multiple replication origins (Nadkarni et al., 2002) might confound the predictive power of these toxigenic gene copy based tools.
5.5 Conclusions

A qPCR assay for simultaneous detection, differentiation and quantification of three common microcystin-producing cyanobacteria genera was developed and validated. This assay was able to detect and estimate toxigenic gene copies in lake water samples even when cyanobacterial cells and toxin concentrations were well below the limits of detection of conventional microscopy and ELISA respectively, thereby reinforcing their potential applicability in early warning systems for monitoring noxious algal blooms.

Analyses of field samples using the developed assays resulted in identification of *Microcystis* spp. as the main putative microcystin-producers in the Missisquoi Bay, with only patchy appearance of toxigenic *Anabaena* and/or *Planktothrix* genotypes.

Of all the measured physicochemical parameters, only total phosphorus showed significant positive associations with chl-a concentration, *Microcystis* counts, and *Microcystis mcyE* copies, suggesting TP was an important driver of cyanobacteria bloom dynamics in the Missisquoi Bay, hence management action aimed at reducing P-loads in this water body might lead to abatement of toxigenic *Microcystis* blooms. However, identification of *mcyE*-containing *Anabaena* and *Planktothrix* genotypes in this lake implies that management action should not only focus on approaches aimed at eliminating *Microcystis* spp., but must also avoid creating conditions that could lead to shifts to community dominance by either toxigenic *Anabaena* or *Planktothrix* spp.

Empirical regression models relating MC to *Microcystis mcyE* copies, chl-a and total *Microcystis* concentrations revealed that although all three biomass proxies were suitable predictors of MC concentration in most instances, *Microcystis mcyE* copies were more robust at predicting MC concentration in the Missisquoi Bay. Thus qPCR-based toxigenic cyanobacterial concentrations might be more reliable indicators of potential cyanotoxin hazards compared to commonly used biomass proxies, especially where toxin concentration data is lacking.
To the best of our knowledge, this is the first study to apply hydrolysis (TaqMan®) probe chemistry for differential estimation of concentrations of the three common microcystin-producing genera within the same reaction tube. We believe that application of this assay in water quality monitoring could lead not only to differential estimation of concentrations of multiple microcystin-producing cyanobacterial genera but also evaluation of environmental variables driving toxigenic cyanobacterial population dynamics at relatively lower costs and turnaround times.

5.6 Acknowledgements

The authors thank T. Coplay and R. Chamoun for helpful discussions on assay design and optimization.
<table>
<thead>
<tr>
<th>#</th>
<th>Cyanobacterial strain</th>
<th>Produces microcystin</th>
<th>Specificity(^b) of primers for;</th>
<th>Reference(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Anabaena</em> sp. 66A</td>
<td>+</td>
<td>+, -</td>
<td>NT Vaitomaa et al., 2003</td>
</tr>
<tr>
<td>2</td>
<td><em>Anabaena</em> sp. 90</td>
<td>+</td>
<td>+, -</td>
<td>NT Vaitomaa et al., 2003</td>
</tr>
<tr>
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<td><em>Anabaena</em> sp. 202A1</td>
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<td>-</td>
<td>NT Vaitomaa et al., 2003</td>
</tr>
<tr>
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<td>-</td>
<td>NT Vaitomaa et al., 2003</td>
</tr>
<tr>
<td>5</td>
<td><em>A. Lemmermannii</em> NIVA CYA 83/1</td>
<td>+</td>
<td>-</td>
<td>+ Vaitomaa et al., 2003</td>
</tr>
<tr>
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<td>-</td>
<td>+ Vaitomaa et al., 2003</td>
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<tr>
<td>7</td>
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<td>-</td>
<td>+ Vaitomaa et al., 2003</td>
</tr>
<tr>
<td>8</td>
<td><em>Anabaena</em> sp CPCC 426</td>
<td>-</td>
<td>-</td>
<td>+ Ngwa et al., 2012</td>
</tr>
<tr>
<td>9</td>
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<td>-</td>
<td>+ Valerio et al., 2010</td>
</tr>
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<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>+ Valerio et al., 2010</td>
</tr>
<tr>
<td>12</td>
<td><em>A. flos aquae</em> CPCC 67</td>
<td>-</td>
<td>-</td>
<td>+ Ngwa et al., 2012</td>
</tr>
<tr>
<td>13</td>
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<td>+ Ngwa et al., 2012</td>
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<td>14</td>
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<td>-</td>
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</tr>
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<td>15</td>
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<td>-</td>
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<td><em>Aph. flos aquae</em> LMECYA 88</td>
<td>NT</td>
<td>-</td>
<td>+</td>
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<td>17</td>
<td><em>Aph. Issatschenkoi</em> LMECYA 166</td>
<td>NT</td>
<td>-</td>
<td>+</td>
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<tr>
<td>18</td>
<td><em>C. raciborskii</em> LMECYA 135</td>
<td>NT</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#</td>
<td>Cyanobacterial strain</td>
<td>Produces microcystin</td>
<td>Specificity(^b) of primers for;</td>
<td>Reference(^a)</td>
</tr>
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<td>-----------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anabaena (mcyE)</td>
<td>Microcystis (mcyE)</td>
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<td>20</td>
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<td><em>P. agardhii</em> NIVA CYA 127</td>
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<td>24</td>
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<td>25</td>
<td><em>M. aeruginosa</em> LMECYA 59</td>
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<td>26</td>
<td><em>M. aeruginosa</em> LMECYA 87</td>
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<td>27</td>
<td><em>M. aeruginosa</em> CPCC 299</td>
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<tr>
<td>28</td>
<td><em>M. aeruginosa</em> CPCC 300</td>
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<td>29</td>
<td><em>M. aeruginosa</em> LMECYA 144</td>
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<td>30</td>
<td><em>M. aeruginosa</em> LMECYA 183</td>
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<td>31</td>
<td><em>M. aeruginosa</em> CPCC124</td>
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<td>32</td>
<td><em>M. aeruginosa</em> CPCC468</td>
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<td>33</td>
<td><em>M. aeruginosa</em> CPCC632</td>
<td>-</td>
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<td>36</td>
<td><em>M. aeruginosa</em> CPCC459</td>
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<td>37</td>
<td><em>S. leopoliensis</em> CPCC102</td>
<td>-</td>
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</tbody>
</table>

\(^a\) Studies from which microcystin production(+) or lack of production (-) were inferred; \(^b\) Production(+) or non-production(-) of microcystin synthetase \((mcyE)\) or cyanobacterial 16S rRNA amplicons by the respective \(mcyE\) and 16S rRNA primers.

NT; not tested
Table 5.2: Primers and probes used in this study

<table>
<thead>
<tr>
<th>DNA target</th>
<th>Primer/probe name</th>
<th>Sequence (5′-3′)</th>
<th>Amplicon size (bp)</th>
<th>Tm °C</th>
<th>Concentration nM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anabaena</strong></td>
<td></td>
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<tr>
<td>mcyE Gene</td>
<td>AnamcyE-424F</td>
<td>GCTCATCGCTCTCAAGCC</td>
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<td>60</td>
<td>200</td>
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<td>AnamcyE-583R</td>
<td>CAGAGAGTGTCCGCCGAT</td>
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<td>60</td>
<td>150</td>
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<td></td>
<td>AnamcyE-FAM</td>
<td><strong>FAM/CAC AAGTCT/ZEN/CTTATACTGCACC/CGA/IABkFQ/</strong></td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Microcystis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcyE Gene</td>
<td>MicmcyE-415F</td>
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<td>167</td>
<td>60</td>
<td>300</td>
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<td>MicmcyE-581R</td>
<td>AATGACCGCCAA TTTCAA AG</td>
<td></td>
<td>60</td>
<td>200</td>
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<tr>
<td></td>
<td>MicmcyE-Hex</td>
<td><strong>HEX/TTCTAATTACGTGGCACCC/CGA/IABkFQ/</strong></td>
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<td>67</td>
<td>200</td>
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<tr>
<td><strong>Planktothrix</strong></td>
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</tr>
<tr>
<td>mcyE Gene</td>
<td>PlamcyE-427F</td>
<td>GATTGCACCTCA TGAAACCG</td>
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<td>59</td>
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<td>PlamcyE-610R</td>
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<td>60</td>
<td>200</td>
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<tr>
<td></td>
<td>PlamcyE-Cy5</td>
<td><strong>Cy5/TTGGCGGACATTCTGATGCTTT/BHQ_2/</strong></td>
<td></td>
<td>68</td>
<td>200</td>
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</table>
Table 5.3: Water quality parameters (averages ± SD) at VQ-B2 and PH-A for 2010 and 2011 Spring-Fall sampling seasons

<table>
<thead>
<tr>
<th>Variables</th>
<th>Station VQ-B2</th>
<th></th>
<th>Station PH-A</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>2010</td>
<td>2011</td>
<td>2010</td>
<td>2011</td>
</tr>
<tr>
<td>TP (mgL(^{-1}))</td>
<td>0.03±0.02(9)*</td>
<td>0.04±0.03(16)</td>
<td>0.03±0.01(9)</td>
<td>0.05±0.04(16)</td>
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<tr>
<td>TN (mgL(^{-1}))</td>
<td>0.42±0.14(9)</td>
<td>0.60±0.34(16)</td>
<td>0.49±0.23(9)</td>
<td>0.53±0.20(16)</td>
</tr>
<tr>
<td>TN/TP</td>
<td>28.49±16.18(9)</td>
<td>26.52±24(16)</td>
<td>14.28±5.49(9)</td>
<td>20.76±21.12(16)</td>
</tr>
<tr>
<td>pH</td>
<td>8.52±0.58(9)</td>
<td>8.44±0.48(16)</td>
<td>8.51±0.59(9)</td>
<td>8.26±0.53(16)</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>20.76±7.13(9)</td>
<td>9.44±8.81(16)</td>
<td>18.40±4.99(9)</td>
<td>14.68±20.54(16)</td>
</tr>
<tr>
<td>DO (mgL(^{-1}))</td>
<td>9.48±1.17(9)</td>
<td>9.73±1.63(16)</td>
<td>9.43±0.96(9)</td>
<td>9.30±1.30(16)</td>
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<td>TDS (gL(^{-1}))</td>
<td>0.09±0.0(9)</td>
<td>0.09±0.01(16)</td>
<td>0.09±0.01(9)</td>
<td>0.09±0.01(16)</td>
</tr>
<tr>
<td>Conductivity (µScm(^{-1}))</td>
<td>136.58±18.37(9)</td>
<td>118.15±11.88(16)</td>
<td>136.47±21.81(9)</td>
<td>123.64±13.22(16)</td>
</tr>
<tr>
<td>Chl-a (µgL(^{-1}))</td>
<td>10.14±9.53(9)</td>
<td>14.29±18.21(16)</td>
<td>9.57±6.21(9)</td>
<td>10.62±7.58(16)</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>21.72±5.73(9)</td>
<td>19.91±5.62(16)</td>
<td>22.13±6.01(9)</td>
<td>20.04±6.65(16)</td>
</tr>
<tr>
<td>Light (µmolm(^{-2})s(^{-1}))</td>
<td>533.2±300.4(8)</td>
<td>897.4±458.4(16)</td>
<td>609.4±256.6(8)</td>
<td>728.9±458.7(16)</td>
</tr>
</tbody>
</table>

TP; total phosphorus, TN; total nitrogen, DO dissolved oxygen, TDS; total dissolved solids, Chl-a; chlorophyll a

* Numbers in brackets represent total number of samples analysed.
Table 5.4: Evaluation of multiplex qPCR specificity by comparing C_{T}s obtained from qPCR analyses of mixtures of gDNA from target and non-target species

<table>
<thead>
<tr>
<th>Organism(s)</th>
<th>C_{T} value (Mean±SD)</th>
<th>Anabaena mcyE</th>
<th>Microcystis mcyE</th>
<th>Planktothrix mcyE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dilution of Background DNA added to standard mixture</td>
<td>1:10</td>
<td>1:100</td>
<td>1:10</td>
</tr>
<tr>
<td>Non-spiked standard</td>
<td>14.87 ± 0.07$^{†}$</td>
<td>14.03 ± 0.51$^{†}$</td>
<td>13.70 ± 0.21$^{†}$</td>
<td></td>
</tr>
<tr>
<td>Standard/Planktothrix sp 45</td>
<td>14.88 ± 0.12</td>
<td>14.75 ± 0.06</td>
<td>14.53 ± 0.16</td>
<td>14.37 ± 0.75</td>
</tr>
<tr>
<td>Standard/LMECYA 161</td>
<td>14.74 ± 0.11</td>
<td>14.83 ± 0.09</td>
<td>14.19 ± 0.29</td>
<td>14.08 ± 0.95</td>
</tr>
<tr>
<td>Standard/LMECYA 144</td>
<td>14.73 ± 0.07</td>
<td>14.80 ± 0.21</td>
<td>14.35 ± 0.21</td>
<td>14.31 ± 0.52</td>
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<tr>
<td>Standard/LMECYA 88</td>
<td>14.77 ± 0.03</td>
<td>14.73 ± 0.07</td>
<td>13.78 ± 0.89</td>
<td>13.86 ± 0.54</td>
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<tr>
<td>Standard/ (Planktothrix sp 45+LMECYA 161)</td>
<td>14.82 ± 0.06</td>
<td>14.74 ± 0.05</td>
<td>14.49 ± 0.74</td>
<td>14.13 ± 0.43</td>
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<td>Standard/ (Planktothrix sp 45+LMECYA 161+LMECYA 144)</td>
<td>14.71 ± 0.10</td>
<td>14.74 ± 0.07</td>
<td>14.32 ± 0.30</td>
<td>14.24 ± 0.23</td>
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<tr>
<td>Standard/ (Planktothrix sp 45+LMECYA 161+LMECYA 144+ LMECYA 88)</td>
<td>14.77 ± 0.18</td>
<td>14.85 ± 0.18</td>
<td>14.47 ± 0.73</td>
<td>13.74 ± 0.63</td>
</tr>
</tbody>
</table>

$^{a}$ Mixes of reference standards (Anabaena sp 202A1, M. aeruginosa CPCC 299, and Planktothrix sp 97) and background DNA from non-target organisms [Planktothrix sp 45(18.0 ng/µL), Anabaena spiroides LMECYA 161 (12.0 ng/µL), M. aeruginosa LMECYA 144 (20.1 ng/µL), and/or Aphanizomenon flos aquae LMECYA 88(58.0 ng/µL)] at mixing ratio of 1:1.

$^{b}$ Mixture containing genomic DNA from target reference strains; Anabaena sp 202A1, M. aeruginosa CPCC 299, and Planktothrix sp 97, all at respectively 13, 14, and 14 ng per reaction.

$^{†}$ No statistical significance between non-spiked and spiked C_{T} values at both 1:10 and 1:100 dilutions of background DNA
Table 5.5: Pearson correlation coefficients amongst environmental variables, chl-a, *Microcystis* counts, and *Microcystis mcyE* copies

<table>
<thead>
<tr>
<th></th>
<th>Chl-a</th>
<th>Micrsp</th>
<th>MicmcyE</th>
<th>TP</th>
<th>TN</th>
<th>pH</th>
<th>Turbidity</th>
<th>Temp</th>
<th>Light</th>
<th>DO</th>
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<tbody>
<tr>
<td>chl-a</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrsp</td>
<td>0.47**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MicmcyE</td>
<td>0.45**</td>
<td>0.84**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>0.75**</td>
<td>0.47**</td>
<td>0.43**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>0.24</td>
<td>0.1</td>
<td>0.13</td>
<td>0.27</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pH</td>
<td>0.48**</td>
<td>0.13</td>
<td>0.07</td>
<td>0.14</td>
<td>-0.17</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turbidity</td>
<td>0.46**</td>
<td>0.21</td>
<td>0.03</td>
<td>0.64**</td>
<td>0.19</td>
<td>0.08</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp</td>
<td>0.23</td>
<td>0.17</td>
<td>0.25</td>
<td>-0.08</td>
<td>-0.26</td>
<td>0.45**</td>
<td>-0.05</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>-0.17</td>
<td>-0.02</td>
<td>0.21</td>
<td>-0.34*</td>
<td>-0.14</td>
<td>0.11</td>
<td>-0.29*</td>
<td>-0.17</td>
<td>1.00</td>
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<tr>
<td>DO</td>
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<td>0.06</td>
<td>-0.11</td>
<td>0.14</td>
<td>0.25</td>
<td>0.22</td>
<td>0.15</td>
<td>-0.68**</td>
<td>-0.12</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Chl-a; Chlorophyll a, Micrsp; Total *Microcystis* spp., MicmcyE; *Microcystis mcyE* copies, Light; Underwater light intensity.
*; p <0.05, **; p <0.01
Table 5.6: Parameter estimates for predictor variables of multiple linear regression models explaining *Microcystis* spp., *Microcystis mcyE*, and chlorophyll-a in the Missisquoi Bay

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>Parameter estimates</th>
<th>Parameter estimates</th>
<th>Parameter estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Microcystis</em> spp.</td>
<td><em>Microcystis mcyE</em></td>
<td>Chlorophyll a copies</td>
</tr>
<tr>
<td>Turbidity</td>
<td>-</td>
<td>-1.14**</td>
<td>-</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>1.84**</td>
<td>3.19*</td>
<td>0.97**</td>
</tr>
<tr>
<td>Light intensity</td>
<td>-</td>
<td>1.23*</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>-</td>
<td>6.01**</td>
</tr>
<tr>
<td>Constant</td>
<td>9.378**</td>
<td>7.156**</td>
<td>-3.219*</td>
</tr>
<tr>
<td>R²</td>
<td>0.28</td>
<td>0.44</td>
<td>0.71</td>
</tr>
<tr>
<td>Model significance</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*; Parameter estimate significant at p< 0.05
**; Parameter estimate is significant at p< 0.01
-; Variable does not significantly affect model
Figure 5.1: Location of sampling stations VQ-B2 and PH-A in the Missisquoi Bay, Quebec, Canada
Figure 5.2: Distribution of dominant cyanobacteria genera at stations VQ-B2 (Top) and PH-A (Bottom) during the 2010 sampling season.
Figure 5.3: Distribution of dominant cyanobacteria genera at stations VQ-B2 (Top) and PH-A (Bottom) during the 2011 sampling season.
Figure 5.4: Uniplex (open circles) and multiplex (filled squares) qPCR standard curves for *Anabaena* (A), *Microcystis* (B), and *Planktothrix* (C) *mcyE* genes. Broken lines represent multiplex curves while uniplex reactions are represented by smooth lines.
Figure 5.5: Seasonal variation in total Microcystis (shaded bars), toxigenic Microcystis (open bars) and microcystin concentration (triangles) at stations VQ-B2 (Top) and PH-A (Bottom) during the 2010 and 2011 sampling seasons. Notice the logarithm scale for the Microcystis and microcystins axes.
Connecting text to chapter 6

The manuscript presented in chapter six of this dissertation addressed the fourth and last objective of the research project. This manuscript which is co-authored by Drs. Madramootoo, C.A. and Jabaji, S. has been submitted to Microbiology Open Journal for publication.

The real-time quantitative PCR approach which was used in the preceding chapters to estimate microcystin-producing cyanobacteria in different environmental matrices is often critiqued for its inability to indicate if detected mcy genes are indeed being actively transcribed into the toxin. It is commonly assumed that reverse transcription quantitative PCR measurement of Mcy transcript levels and consequently the proportion of active toxin biosynthetic genes might be better indicators of toxin levels than mcy gene copies. The fourth objective was therefore aimed at utilizing the assays described in chapter five to monitor the progression of McyE gene transcript levels, mcyE gene copies, microcystin concentration, and various biomass proxies in laboratory cultures and environmental water samples, in order to further explore the utility of mcyE expression and/or copies as indicators of microcystin concentration in freshwater blooms. This is the topic of the article that follows.

Chapter 6 Evolution of cyanobacterial microcystin synthetase E transcription with changes in gene copies and biomass under laboratory and field conditions

Felexce Ngwa, Chandra Madramootoo, and Suha Jabaji

Abstract

Increased incidences of freshwater blooms comprising mixed assemblages of microcystin-producing cyanobacterial genera constitute a serious threat to human and ecosystem health. Microcystin biosynthesis is regulated by a multi-enzyme complex comprising non-ribosomal peptide synthetase and polyketide synthase modules encoded by the microcystin synthetase (mcy) gene cluster. Although much effort has been devoted towards elucidating the microcystin biosynthetic mechanisms in many cyanobacteria genera, little is known about the impacts of co-occurring cyanobacteria on cellular growth, mcy gene expression and mcy gene copy distribution.

The present study utilized a reverse transcription qPCR assay and ELISA, coupled with measurements of a range of cyanobacterial biomass variables and qPCR to study how competition between microcystin-producing Microcystis aeruginosa CPCC299 and Planktothrix agardhii NIVA-CYA 126 impacts mcyE gene expression, mcyE gene copies, microcystin concentration, and various biomass variables under controlled laboratory conditions. Furthermore, analyses of environmental water samples from the Missisquoi Bay, Quebec, enabled us to determine how mcyE gene expression, mcyE gene copies, and various biomass proxies correlated with cellular microcystin concentrations in a freshwater lake.

Results from our laboratory study indicated significant down-regulation of mcyE gene expression in mixed cultures of M. aeruginosa plus P. agardhii on most sampling days in agreement with depressed growth recorded in the mixed cultures, suggesting that interaction between the two species probably resulted in release of compounds that suppressed both growth and mcyE gene expression in
the mixed cultures. Furthermore, although mcyE gene copies and McyE transcripts were detected in all samples with measureable microcystin levels, only mcyE gene copies showed significant positive correlations with microcystin concentrations while McyE transcript levels did not. These results suggest that mcyE gene copies are indeed better indicators of potential risks from microcystins than McyE transcript levels.

Key words: microcystin; reverse transcription qPCR; cyanobacterial bloom; gene expression; mcyE

6.1 Introduction

Microcystins (MCs) are amongst the most widespread cyanotoxins in freshwater bodies, with over 90 structural variants identified globally (Welker & von Döhren, 2006). Human, animal, and bird fatalities resulting from acute exposure to lethal doses of various microcystin structural variants have been reported (Briand et al., 2003; Jochimsen et al., 1998; Wood et al., 2010a). Chronic exposure to sub-lethal doses of microcystins has been shown to initiate tumor development in laboratory animals (Falconer, 1991; Ito et al., 1997; Nishiwaki-Matsushima et al., 1992). Although there is no direct experimental evidence associating microcystins to tumorigenesis in humans, epidemiological studies have nevertheless found correlations between incidence of microcystins in drinking water and/or food, to development of certain types of cancers in China (Ueno et al., 1996; Yu, 1995; Zhou et al., 2002). These studies coupled with their demonstrable tumor promoting effects in laboratory rodents (Falconer, 1991; Ito et al., 1997; Nishiwaki-Matsushima et al., 1992) has led to the classification of microcystins as ‘possible human carcinogens (Class 2B)’ by the International Agency for Research on Cancer (IARC; Grosse et al., 2006).

Microcystins are mostly produced by members of the Microcystis genera but also strains belonging to Anabaena, Planktothrix, and Nostoc genera, amongst others (Chorus & Bartram, 1999; Sivonen & Börner, 2008). Blooms of cyanobacteria can contain mixed assemblages of microcystin-producing cyanobacteria from different genera (Al-Tebrineh et al., 2012a; Rantala et al., 2006; Vaitomaa et al.,
or morphologically indistinguishable strains of microcystin-producing and non-producing cyanobacteria of the same genus (Vezie et al., 1998).

The global relevance of microcystins and *Microcystis* has led to dedicated efforts towards elucidating the role of this toxin in cyanobacteria metabolism and the factors regulating its biosynthesis. A good number of studies have shown that nutrients (Orr & Jones, 1998; Rapala et al., 1997; Vézie et al., 2002; Xu et al., 2010a), temperature (Amé & Wunderlin, 2005; Davis et al., 2009; Watanabe & Oishi, 1985), light (Kaebernick et al., 2000; Kardinaal et al., 2007; LeBlanc Renaud et al., 2011), and pH (Krüger et al., 2012; van der Westhuizen & Eloff, 1983) affect cyanobacterial growth and microcystin production in various ways. Results from many of these studies are rather contradictory; hence the precise biological function of microcystins and the factors regulating its production are still contentious.

Microcystin-producing cyanobacteria contain the microcystin synthetase (*mcy*) genes that are generally absent in their non-toxic counterparts. Thus detection of these toxin biosynthetic genes in samples usually indicates presence of potentially toxic cyanobacteria. Genome sequencing and characterization of the microcystin synthetase gene clusters in *Anabaena* (Rouhiainen et al., 2004), *Microcystis* (Nishizawa et al., 2000; Tillett et al., 2000), and *Planktothrix* (Christiansen et al., 2003) species has elucidated the roles of various *mcy* genes in the microcystin biosynthesis pathway.

Elucidation of the molecular basis for microcystin biosynthesis, for example, has resulted in increased use of molecular techniques to discriminate toxic from non-toxic cyanobacterial blooms. A majority of molecular methods employ PCR amplification of cyanobacterial genomic DNA to either detect presence/absence of various *mcy* genes in samples as in conventional PCR (e.g., Kurmayer et al., 2002; Tillett et al., 2001; Via-Ordorika et al., 2004; Yoshida et al., 2005) or quantify concentrations of various *mcy* gene copies as in quantitative real-time PCR (e.g., Al-Tebrineh et al., 2012a; Fortin et al., 2010; Kurmayer & Kutzenberger, 2003; Vaitomaa et al., 2003). Although DNA-based PCR detection, differentiation, and quantification of *mcy* genes provide vital information on
proportions of potential microcystin-producing cyanobacteria, it nevertheless offers little insight on active mcy gene transcription. Considering that deletional or insertional mutagenesis of mcy genes has been shown to produce non-toxic mutants incapable of expressing the mcy genes (Christiansen et al., 2008; Kaebernick et al., 2001; Ostermaier & Kurmayer, 2009), genomic DNA-based qPCR methods might be subject to toxigenicity overestimation in environmental samples with high proportions of such mutants.

Synthesis of microcystins follows a series of steps starting with transcription of mcy genes into mcy mRNAs, translation of mRNAs into polyketide synthases, non-ribosomal peptide synthetases, mixed peptide synthetases, and tailoring enzymes that assemble various constituent amino acids into the microcystin structure (Misson et al., 2012). Reverse transcription quantitative real-time PCR (RT-qPCR) analysis of total cyanobacterial RNA allows for measurement of transient mcy mRNA transcripts, and putatively the concentrations of potential microcystin-producing cyanobacteria which are actively transcribing the mcy genes at the time of sampling. It is thus assumed that knowledge of concentrations of actual microcystin producers in a given bloom could potentially result in a more reliable assessment of the risk of exposure to this toxin.

Although RT-qPCR can potentially provide vital information on bloom toxicity that cannot be otherwise obtained from current DNA-based qPCR approaches, use of this technique in monitoring toxigenic cyanobacteria is not widespread probably due to the greater lability of RNA relative to DNA as well as inherent difficulties in extracting and preserving high quality RNA from complex environmental samples (Gadkar & Filion, 2012; Sharkey et al., 2004). The few studies on reverse transcription PCR analysis of toxigenic cyanobacteria have mostly involved qualitative measurement of presence or absence of Mcy transcripts in laboratory cultures (e.g., Kaebernick et al., 2002; Kaebernick et al., 2000; Mikalsen et al., 2003) or field samples (Gobler et al., 2007). Quantitative estimation of various Microcystis Mcy gene transcript levels under laboratory (e.g., Rueckert & Cary, 2009; Schatz et al., 2007; Sevilla et al., 2008) and field (e.g., Misson et al., 2012; Wood et al., 2011) conditions have also been
successfully realized. Nevertheless, in order to reliably interpret \textit{mcy} gene expression data from environmental samples, it is important to understand how co-occurring cyanobacteria might impact gene expression patterns and MC concentrations. Furthermore, understanding the relationship between Mcy transcript levels and actual cellular microcystin concentrations could provide insight on the utility of Mcy gene transcript levels in assessing the potential risks from microcysts in water blooms of cyanobacteria.

The aim of the present study was therefore to investigate the variation of McyE gene transcript levels, \textit{mcyE} copies, biomass, and microcystin concentration in monocultures and mixed cultures of microcystin-producing \textit{Microcystis aeruginosa} CPCC299 and \textit{Planktothrix agardhii} NIVA-CYA 126 strains. Analyses of water samples collected from a freshwater lake during summer 2011 enabled us to further evaluate how variation of \textit{mcyE} gene expression, \textit{mcyE} gene copies, or cyanobacterial biomass with microcystin production, could impact the utility of these putative proxies as indicators of microcystin risks in a freshwater body.

6.2 Materials and methods

6.2.1 Organisms and culture conditions

Unialgal cultures of toxic \textit{Microcystis aeruginosa} CPCC 299 (hereafter \textit{M. aeruginosa}) and \textit{Planktothrix agardhii} NIVA-CYA 126 (hereafter \textit{P. agardhii}) were purchased from the Canadian Phycological Culture Collection (CPCC; formerly the University of Toronto Culture Collection [UTCC]) and the Norsk Institutt for Vannforskning (NIVA), respectively. The organisms were grown in 125 mL sterile BG-11 media in 250 mL Erlenmeyer flasks placed in a Conviron growth chamber maintained at a temperature of 25°C and illuminated with 12h of white fluorescent light supplied at a photon flux density of 30 \(\mu\) mol m\(^{-2}\) s\(^{-1}\). Cultures were allowed to acclimatize for one week in fresh BG-11 under stipulated culture conditions prior to start of competition experiments.
6.2.2 Competition experiments

Acclimatized cultures of *M. aeruginosa* and *P. agardhii* were each aseptically transferred into separate triplicate 2L Erlenmeyer flasks containing 1L of BG-11 for the monoculture assays. For the mixed culture experiments, *M. aeruginosa* and *P. agardhii* were added into 2L Erlenmeyer flasks (in triplicates) containing 1L BG-11 such that each flask contained approximately the same starting concentration (10^5 cells mL^{-1}) of each strain. All nine flasks were randomly placed in a Conviron growth chamber maintained at 25°C temperature and 12 h:12 h light:dark cycle at 30 µmol photons m^{-2} s^{-1} provided by cool white fluorescent bulbs. Each flask was aseptically sampled on November 5th, 9th, 13th, 17th, and 21st 2012 (hereafter days 5, 9, 13, 17, and 21 of the experiment), with 20mL aliquots immediately filtered onto GF/C filter paper (Whatman, England), wrapped in aluminum foil and preserved at -80°C (for nucleic acid and microcystin analyses) or analyzed immediately for chlorophyll a. Samples for microscopy were preserved in acid Lugol and kept in the dark for subsequent cell counting.

6.2.3 Field samples

A total of 32 surface water samples were collected from two littoral stations VQB2 (45° 04' 57"N; 73° 08' 52"W) and PH-A (45° 02' 22"N; 73° 04' 46"W), located in the Missisquoi Bay, Quebec, between May and November 2011. Five hundred millilitre aliquots were filtered onto on GF/C filters (Whatman, England), wrapped in aluminum foil and preserved at -20°C for nucleic acid extraction and toxin analyses.

6.2.4 Biomass measurement

The growth of *M. aeruginosa* and *P. agardhii* was assessed by measuring the chlorophyll a and cell concentrations on days 5, 9, 13, 17, and 21 of culture experiments. Chlorophyll-a was determined using both extractive and non-extractive approaches. In the extractive approach, the pigment was extracted from GF/C filters using hot 90% ethanol followed by spectrophotometric analysis as described previously (Sartory & Grobbelaar, 1984). Enumeration of cyanobacterial cells was carried out using a light microscope as previously
described (Ngwa et al., 2012). In order to estimate *Microcystis aeruginosa* and *Planktothrix agardhii* biovolumes, *Microcystis* cells were assumed to be spherical in shape and *Planktothrix* cylindrical. Dimensions of at least 50 *Microcystis* cells (diameters) and *Planktothrix* filaments (length and diameter) were measured using a SteREO Discovery.V20 microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada). *P. agardhii* cell numbers were estimated from total filament counts and mean filament length by assuming a mean cell length of 5 µm (Jacquet et al., 2005; Suda et al., 2002). Mean volumes of the respective spherical and cylindrical shapes were calculated as described previously (Lawton et al., 1999).

*In situ* chlorophyll (non-extractive) and blue green algal phycocyanin (BGA-PC) concentrations were measured at the time of sampling using a pre-calibrated multi-parameter water quality sonde YSI 6600V2-4 (YSI Inc., Yellow Springs, OH, USA).

### 6.2.5 Microcystin analysis

Preserved frozen filters were placed in 2 mL microcentrifuge tubes, allowed to thaw at room temperature for 5 min and rapidly subjected to two freeze-thaw cycles (liquid N₂ @ 1 min, 37°C water bath @ 5 min). Samples were subsequently extracted in 75% (v/v) aqueous methanol following the procedure described previously (Section 5.2.10). In order to prevent methanol interference with downstream ELISA quantification of microcystin (Metcalf et al., 2000), all extracts were diluted with double distilled water to bring methanol concentration below 0.5%(v/v) as suggested by Rivasseau et al. (1999). Microcystin concentration of each sample was measured in duplicate using an Envirogard® microcystin plate kit (Modern Water Inc., New Castle, DE, USA) as described in section 5.2.10.

### 6.2.6 Nucleic acid isolation and reverse transcription

Preserved GF/C filters for DNA and RNA isolation were separately ground into powder in liquid N₂ cooled mortars and pestles. To avoid nucleic acid degradation, samples were completely submerged in liquid N₂ throughout the grinding process.
Total RNA was extracted from entire ground filters using RNeasy® Plant Mini kit (Qiagen Inc., Mississauga, ON, Canada) following the manufacturer’s recommendations. RNA concentration and integrity were assessed spectrophotometrically with NanoDrop® ND1000 (NanoDrop, Wilmington, DE, USA) followed by 1.2% formaldehyde-agarose gel electrophoresis. Prior to reverse transcription (RT), residual gDNA co-extracted with RNA was destroyed with DNase wipeout buffer. A total of 100ng of purified RNA was reverse transcribed using the QuantiTech® reverse transcription kit (Qiagen Inc.) following the kit manufacturer’s protocol. Transcribed cDNA concentration and integrity were checked with ND1000 spectrophotometer. Genomic DNA contamination of reverse transcribed cDNA was verified via conventional PCR of no reverse transcriptase (NoRT) samples with 16S rRNA, rpoC1, MicmcyE and PlamcyE primer pairs (Table 6.1), followed by 1.5% agarose gel electrophoresis. Transcribed cDNA was preserved at -20°C for subsequent PCR applications.

Genomic DNA was extracted from ground filters with the DNeasy® Mini Plant kit (Qiagen Inc.) as per the kit manufacturer’s instructions. DNA quality and quantity were assessed with ND1000 spectrophotometer (NanoDrop), followed by electrophoresis on 1.5 % agarose gel. Purified DNA was preserved at -20°C until further analysis.

6.2.7 Primer design

Primer pairs targeting the microcystin synthetase E (mcyE) gene in Microcystis and Planktothrix genera designed with Primer 3 (Rosen & Skaletsky, 2000) were tested as previously described (Sections 5.2.4 & 5.2.5). Primer pairs for the16S rRNA (Imamura et al., 2006) and rpoC1 (Alexova et al., 2011) reference genes were selected from literature. Primers were tested via conventional PCR of cDNA to ensure that they amplified the target genetic locus.

6.2.8 Quantitative PCR determination of mcyE gene copies

The concentrations of Microcystis and Planktothrix mcyE copies were determined by quantitative PCR of gDNA, using standard curves generated from Microcystis and Planktothrix mcyE plasmid DNA as described previously (Section 5.2.9). All samples were amplified in duplicate using the Mx3005P qPCR System (Agilent
Technologies, Inc., Santa Clara, CA, USA) and QuantiFast® SYBR® Green master mix (Qiagen Inc.) as per the manufacturers’ recommendations. Amplifications were performed in 25 µL reaction mixtures containing respectively 300 and 200 nM of forward and reverse primers for Microcystis or Planktothrix mcyE gene, 1× QuantiFast® SYBR® Green I master mix (with ROX dye), and 2 µL gDNA template (10²-10⁴ fold dilutions). The PCR thermal cycle consisted of a hot start cycle at 95°C for 5 min, followed by 40 cycles comprising denaturation at 95°C for 10 sec, and a combined annealing/extension step at 60°C for 30s. All fluorescence data was collected at 60°C and C_T values determined using MxPro qPCR software v4.10 (Agilent Technologies, Inc.).

6.2.9 Analysis of mcyE gene expression by RT-qPCR

Microcystin synthetase E (mcyE) gene transcript levels under laboratory and field conditions were estimated by the relative quantification approach described by Zhao and Fernald (2005). The PCR assays were performed on two target genes (Microcystis and Planktothrix mcyE) and two reference genes (16S rRNA and rpoC1) using the primer pairs shown in Table 6.1. The Microcystis and Planktothrix mcyE assays were performed as described in section 6.2.8 using 2 µL (500ng) of cDNA template instead of gDNA in each 25 µL PCR reaction mixture. Amplification of reference genes on the other hand was performed in 25 µL reactions each containing either 300 nM of each 16S rRNA primer or 500 nM rpoC1 forward and reverse primers, 1× QuantiFast® SYBR® Green I master mix, and 500ng cDNA template. All RT-qPCR amplifications were performed using the thermal program described in section 6.2.8. Melt curve analyses of data from all qPCR and RT-qPCR assays were performed as previously described (Ngwa et al., 2012).

6.2.9.1 Quantification of gene expression

In order to quantify gene expression, raw fluorescent data were imported from the Stratagene Mx3005P qPCR machine into Miner software v4.0 (Zhao & Fernald, 2005) and various software algorithms utilized to calculate C_T values and efficiencies based on kinetics of individual PCR reactions. The resulting C_T values and efficiencies were subsequently inputted into equation [6.1] to calculate
relative expression of target and references genes as described by Zhao and Fernald (2005).

\[
R_0 = \frac{1}{(1 + E)^{C_T}} \quad [6.1]
\]

where,

\( R_0 \) is initial template concentration,
\( E \) is the efficiency of the PCR reaction,
\( C_T \) represents the PCR cycle at which fluorescence significantly exceeds the background fluorescence.

In order to correct target gene expression data for differences in RNA quality and reverse transcription efficiency between samples, the relative expression of *Microcystis* or *Planktothrix* mcyE genes in various samples was normalized against expression of 16S rRNA and rpoC1 genes in the respective samples.

### 6.2.10 Statistical analysis

All statistical analyses were performed with SAS software package (v9.3, SAS Institute Inc., Cary, NC, USA). Normality of data was checked using the Shapiro-Wilk normality test at the 5% significance level. Non-conforming data was log transformed to meet the normality condition and a mixed model procedure using the restricted maximum likelihood (REML) variance estimation method was implemented to detect any significant differences in cyanobacterial growth, mcyE copies, mcyE gene expression and microcystin concentration under mono- and mixed culture treatments.

### 6.3 Results

#### 6.3.1 Quantification of mcyE gene expression and copy number

All primers used in this study successfully amplified a single product within the expected size range with gDNA or cDNA from the respective target organisms, but gave no amplicons with non-target organisms, negative controls, or NoRT samples. Furthermore, melt curve analyses of qPCR and RT-qPCR fluorescence data showed that each primer pair generated only one product with a specific melting temperature that was uniform across all samples containing target nucleic acids. Negative controls containing no template did not produce any peaks and all
fluorescence data generated from such samples was always below the detection threshold.

Standard curves used to estimate *Microcystis* and *Planktothrix mcyE* copies were highly linear (R² of 0.999 for *Microcystis* and 0.998 for *Planktothrix mcyE* standards) over seven orders of magnitude.

**6.3.1.1 QPCR quantification of *mcyE* copies**

*Microcystis* and *Planktothrix mcyE* copies in the mono- and mixed culture treatments were determined on gDNA extracted from samples collected on days 5, 9, 13, 17, and 21 of the experiments. *Microcystis mcyE* copies witnessed a steady increase from day 5 to 21 in both treatments, with values ranging from \(9.41(\pm 3.25) \times 10^6\) copies mL\(^{-1}\) to \(4.75(\pm 0.10) \times 10^9\) copies mL\(^{-1}\) in *M. aeruginosa* monocultures and \(4.32(\pm 0.33) \times 10^6\) copies mL\(^{-1}\) to \(2.85(\pm 0.10) \times 10^9\) copies mL\(^{-1}\) in mixed *M. aeruginosa* plus *P. agardhii* cultures (hereafter mixed culture *M. aeruginosa*; Figure 6.1). *Microcystis mcyE* copies showed significant treatment*day interaction (p<0.0001). The number of *Microcystis mcyE* copies was higher in monocultures than mixed cultures on most sampling days, but for day 9 when mixed cultures registered higher *mcyE* copies than monocultures. The differences in *mcyE* copies between monocultures and mixed cultures were statistically significant on all but the last sampling date (Figure 6.1). *Planktothrix mcyE* copies, like *Microcystis mcyE* copies, showed significant treatment*day interaction (p < 0.05). Whereas *Planktothrix mcyE* copies in monoculture *P. agardhii* showed a similar trend to *Microcystis mcyE* copies, rising from \(3.11(\pm 1.06) \times 10^6\) copies mL\(^{-1}\) on day 5 to \(9.15(\pm 0.48) \times 10^7\) copies mL\(^{-1}\) on day 21, *mcyE* copies in mixed cultures of *P. agardhii* plus *M. aeruginosa* (hereafter mixed culture *P. agardhii*), on the contrary, increased from \(1.64(\pm 0.12) \times 10^7\) copies mL\(^{-1}\) on day 5 to a maximum \(4.81(\pm 0.72) \times 10^8\) copies mL\(^{-1}\) on day 17 before dropping to \(2.10(\pm 0.94) \times 10^8\) copies mL\(^{-1}\) by day 21. Although *Planktothrix mcyE* copies were relatively higher in mixed culture *P. agardhii* than the monocultures on each sampling date, the difference was significant only on days 9, 13, and 17 (Figure 6.2).
6.3.1.2 Quantification of mcyE gene expression

Expression of mcyE in competition experiments

Expression of mcyE genes in M. aeruginosa and P. agardhii showed significant treatment*day interaction effects (p < 0.0001). Microcystis mcyE expression was 19.7 folds higher in monoculture M. aeruginosa than the mixed cultures on day 5, decreasing slightly to 19.2 folds on day 9 before falling steeply to equivalent values on days 17 and 21 (Figure 6.3). Whereas Microcystis mcyE in mixed cultures of M. aeruginosa witnessed a steady increase from day 5 to 21, mcyE expression in monoculture M. aeruginosa increased from day 5 to 13, then decreased to day 17 before increasing again on day 21. Expression of Planktothrix mcyE genes in monoculture P. agardhii cultures, on the contrary, witnessed a steady increase from day 5 to 21, whereas in mix cultured P. agardhii there was a gradual increase from day 5 to 13 and then a drop through days 17 to 21 (Figure 6.4). Expression of Planktothrix mcyE in monocultures was significantly higher than the corresponding mixed cultures on all sampling days, but for day 5 where there was no significant difference between the two treatments. The highest fold change in mcyE expression between the two treatments was on day 21 when mcyE expression was 161 folds higher in monoculture P. agardhii than the mixed culture whereas the lowest fold change was on day 5.

Expression of mcyE gene in environmental samples

Twenty-three out of the 32 environmental water samples which produced RNA of quality suitable for RT-PCR were analysed for Microcystis and Planktothrix mcyE gene expression. Microcystis mcyE gene was expressed in all samples analyzed but no sample produced detectable Planktothrix McyE gene transcript levels. Expression of Microcystis mcyE genes was generally higher in samples collected from station PH-A than station VQ-B2 samples collected on the same day, except for samples of August 15 and September 26, which showed higher mcyE expression in station VQ-B2 than station PH-A samples (Figure 6.5). Microcystis mcyE expression did not show any association with Microcystis mcyE copies or MC concentration determined in a previous study (Section 5.3.5).
6.3.2 Microcystin analysis

Intracellular microcystin (MC) concentration in monoculture *M. aeruginosa* witnessed a steady increase from 0.16 ±0.03 mg L\(^{-1}\) on day 5, to 9.72±0.39 mg L\(^{-1}\) on day 21 of the culture experiments (Figure 6.6). In a similar manner, monoculture *P. agardhii* had intracellular microcystin concentrations ranging from 0.06 ±0.03 mg L\(^{-1}\) to 6.41±1.11 mg L\(^{-1}\) on days 5 to 21 respectively (Figure 6.7). For the mixed cultures, total intracellular microcystin concentration was 0.42±0.1 mg L\(^{-1}\) on day 5, increasing by more than 26 folds to 10.98±0.78 mg/L on day 21 (Figures 6.6 and 6.7). Whereas MC concentrations in monoculture *P. agardhii* were significantly lower than concentrations in mixed cultures on all but one sample days (day 21) (Figure 6.7), MC concentrations in monoculture *M. aeruginosa* and mix cultures were not significantly different from each other on three out of five sampling days (Figure 6.6).

6.3.3 Variation of cyanobacterial biomass

The cell counts for *M. aeruginosa* and *P. agardhii* under mono- and mixed culture conditions are shown in Figures 6.8 and 6.9 respectively. *M. aeruginosa* cell counts in monocultures varied from 1.91(±0.34) \times 10^5 cells mL\(^{-1}\) on day 5 to 1.50(±0.17) \times 10^7 cells mL\(^{-1}\) on day 21 and from 1.63(±0.50) \times 10^5 cells mL\(^{-1}\) to 1.48(±0.03) \times 10^7 cells mL\(^{-1}\) on days 5 to 21, respectively, in mixed cultures of *M. aeruginosa*. *M. aeruginosa* cell counts were consistently higher in monoculture than mixed cultures, although the differences were significant only on days 9, 13, and 17 (Figure 6.8). *P. agardhii* cell counts in monoculture and mixed culture treatments ranged between 1.70(±0.23) \times 10^6 to 2.04(±0.0.09) \times 10^7 cells mL\(^{-1}\) and 1.20(±0.15) \times 10^6 to 6.64(±0.68) \times 10^6 cells mL\(^{-1}\) respectively on the first and last sampling days. Except for sampling day 5, monoculture *P. agardhii* counts were mostly higher than mixed cultured *P. agardhii* (Figure 6.9). The difference in counts was, however, statistically significant only on day 21. Cyanobacterial biomass measured in terms of extractive chlorophyll a concentration as well as YSI Probe-based chlorophyll and BGA-PC concentrations showed similar trends to cell counts determined by microscopy (data not shown).
6.3.4 Correlation analyses

In order to determine the strength of associations between \textit{mcyE} gene expression and copies, microcystin, and various biomass proxies, Pearson correlation coefficients were calculated using log$_{10}$ transformed data. The results of these analyses for \textit{M. aeruginosa} and \textit{P. agardhii} are presented in Tables 6.2 and 6.3 respectively.

Microcystin concentration correlated significantly ($r > 0.7, p < 0.01$) with \textit{mcyE} copies, chlorophyll a, BGA-PC, and cell counts but not with \textit{mcyE} gene expression for both \textit{M. aeruginosa} and \textit{P. agardhii} (Tables 6.2&6.3). Expression of \textit{mcyE} in \textit{M. aeruginosa} showed significant but weak positive association with \textit{mcyE} copies, chlorophyll a, cell counts (microscopy and YSI Probe-based) whereas \textit{Planktothrix} \textit{mcyE} gene expression on the contrary correlated weakly with cell counts only. The association of \textit{Microcystis} \textit{mcyE} copies with various biomass proxies had Pearson correlation ($r$) values between 0.82-0.96 (Table 6.2), whereas that between \textit{Planktothrix} \textit{mcyE} copies and biomass variables ranged from 0.55-0.94 (Table 6.3).

6.4 Discussion

Cyanobacterial blooms often contain mixed assemblages of toxic and non-toxic strains from the same genus (Vezie et al., 1998) or multiple cyanobacterial genera (Al-Tebrineh et al., 2012a; Rantala et al., 2006; Vaitomaa et al., 2003). \textit{Anabaena}, \textit{Microcystis}, and \textit{Planktothrix} are amongst the most common potential microcystin-producing cyanobacteria genera in many freshwater lakes. Although some advances have been made towards elucidating the roles of various environmental factors on cyanobacterial growth and toxin production (e.g., Amé & Wunderlin, 2005; Kardinaal et al., 2007; Orr & Jones, 1998; van der Westhuizen & Eloff, 1983; van der Westhuizen & Eloff, 1985), little has been done to comprehend the impacts of co-occurring cyanobacteria on bloom dynamics. In order to address this knowledge gap, we monitored the changes in biomass, \textit{mcyE} gene copies, McyE gene transcripts, and microcystin concentration in mono- and mixed cultures of \textit{M. aeruginosa} CPCC 299 and \textit{P. agardhii} NIVA-CYA 126 during 21 days of growth. Subsequently, expression of
mcyE genes in environmental samples was investigated in order to elucidate how gene expression data correlates with toxin production in freshwater blooms.

Results from the laboratory experiments showed the concentrations of the cyanobacterial cells, McyE transcripts, mcyE copies, and MC in culture media to be strongly dependent not only on the presence or absence of competing cyanobacteria, but also the sampling day. That is, there were significant treatment*day interaction effects. *Microcystis mcyE* copies in mono- and mixed culture *M. aeruginosa* showed uninterrupted increase from day 5 to 21, with the concentration of mcyE gene copies being at least 2-3 folds higher in monocultures on most sampling days, in agreement with the higher *M. aeruginosa* cell concentrations recorded in monocultures. *Planktothrix mcyE* copies, on the contrary, were between 2-31 folds higher in mixed cultures than in monoculture *P. agardhii*, in stark contrast to the relatively lower *P. agardhii* cell concentrations in the mixed cultures. The lower cell concentrations in mixed culture *M. aeruginosa* and *P. agardhii* suggest possible interaction between *M. aeruginosa* and *P. agardhii* resulting in growth inhibition. Although not directly comparable to the present study, competition experiments between toxic and non-toxic *Microcystis* have indeed shown severe inhibition of growth of non-toxic strains by the toxic strain (Schatz et al., 2005). Further analyses, however, led these researchers to suggest that other secondary metabolites (e.g., micropeptin, microviridin or microgenin) but not microcystins were probably responsible for the growth inhibition (Schatz et al., 2005). Other studies have, however, associated the antiagal properties of toxic *Microcystis* spp. to the release of microcystins (Singh et al., 2001) or other unknown allelopathic compounds (Sukenik et al., 2002) into the surrounding media. A recent study (Mendes e Mello et al., 2012) showed that allelochemicals from mixed cultures of *Cylindrospermopsis raciborskii* and *M. aeruginosa* caused growth inhibition in different strains of *M. aeruginosa*. Considering that the *P. agardhii* strain used in this study, like *M. aeruginosa*, is also known to produce a wide array of secondary metabolites including aeruginosides, anabaenopeptins, cyanopeptolins, and microviridins (Philmus et al., 2008) which have exhibited toxicity in *Daphnia* sp. (Rohrlack et al., 2004), it is conceivable to link growth depression witnessed in the mixed cultures to
release of growth inhibiting compounds due to interaction between *M. aeruginosa* and *P. agardhii*.

Generally, the presence of cyanotoxin or other allelopathic compounds in the cyanobacterial growth medium is believed to signal cyanotoxin biosynthesis in intact toxic cyanobacterial cells. Schatz et al. (2007), for example, observed marked increases in the concentrations of microcystins and McyB transcripts in cells of *Microcystis* sp. previously exposed to cell-free extracts from lysed *Microcystis* sp. compared to an unexposed control. Engelke et al. (2003), on the other hand, reported elevated microcystin and nodularin production in, respectively, *Microcystis aeruginosa* PCC 7820 and *Nodularia* sp. PCC7802 grown in spent medium of non-toxic *Planktothrix agardhii*. Although we noted elevated MC in mixed cultures in many instances, the fact that MC concentrations of mixed cultures originated from both *M. aeruginosa* and *P. agardhii* and could not be explicitly allocated to any of the two competing strains made it difficult to determine if such elevated toxin levels were driven by external cues or were simply the result of the coupling of microcystin production to cell division as previously postulated (Lyck, 2004; Orr & Jones, 1998).

The gene expression data showed significant down-regulation of both *Microcystis* and *Planktothrix mcyE* gene expression in the mixed cultures relative to the respective monocultures, with up to 161-folds change for *Planktothrix mcyE* and 20-folds for *Microcystis mcyE* gene expression. Whereas *mcyE* genes in monoculture *P. agardhii* were continuously up-regulated from days 5 through 21, *mcyE* genes in monoculture *M. aeruginosa* were up-regulated from day 5 to day 9 and then down-regulated to levels lower than that of day 5, by days 17 and 21. Although *Microcystis mcyE* expression levels in this study were relatively lower than those reported elsewhere (Rueckert & Cary, 2009), the up-/ down-regulation patterns were rather similar. According to Rueckert and Cary (2009), *Microcystis mcyE* was significantly up-regulated to a peak by the mid-log phase (day 8 to 12) before being down-regulated to almost insignificant levels by day 38 of the experiment. Unfortunately, these researchers did not provide microcystin concentration data to corroborate the observed gene expression patterns. The
microcystin concentrations recorded in the monoculture *M. aeruginosa* did not seem to corroborate our *mcyE* expression pattern as MC concentration increased continuously from day 5 to day 21 whilst *mcyE* expression witnessed periodic peaks and dips during this period. Whereas the *mcyE* expression trend in monoculture *P. agardhii* were in agreement with the increased cellular MC content from day 5 to 21, *mcyE* expression patterns in mixed cultured *P. agardhii* were in discord with the observed continuous increase in MC.

Examination of the environmental data also showed substantial up- and down-regulation of *mcyE* gene expression over the sampling period, with PH-A samples generally recording higher expression than the corresponding VQ-B2 samples. As with laboratory samples, *mcyE* expression patterns did not always match toxin concentration data (not shown). The June 27th sample at station PH-A, for example, recorded the highest McyE transcript levels but relatively low *mcyE* copies (1.11×10⁵ copies L⁻¹) and MC concentration (0.1µg L⁻¹) whereas the September 26th sample at VQ-B2, with the highest MC concentration (18.9 µg L⁻¹) also had relatively high *mcyE* copies (3.64×10⁶ copies L⁻¹) but extremely low McyE transcript levels.

The poor correlation between *mcy* gene expression and microcystin concentration reported in the present laboratory and field studies contradicted several previous studies. Schatz et al. (2007), for example, reported simultaneous increases in MC and McyB transcripts levels when laboratory cultures of *M. aeruginosa* KLL MG were exposed to cell-free extracts from *M. aeruginosa* cultures. Similarly, Sevilla et al. (2008) reported correlative increases in *mcyD* transcription and microcystin-LR levels in *M. aeruginosa* PCC7806 grown under iron-depleted conditions. Another laboratory evaluation of the response of *Planktothrix* sp. to changing light intensities found positive correlations between *mcyA* transcription rates and the total microcystin production rate in *P. agardhii* (Tonk et al., 2005). Kaebernick et al. (2000), on the contrary, showed transcription of *Microcystis mcyB* and *mcyD* genes to be light dependent but found no correlation between increasing McyB or McyD transcript levels and cellular toxin concentration,
which they attributed to possible transport of microcystin out of the cell at high light intensities (68 μmol of photons m$^{-2}$ s$^{-1}$).

At the field level, Gobler et al. (2007) reported somewhat consistent patterns in mcyE expression and microcystin levels in samples from Lake Agawam, USA, but also noted peculiar instances when microcystins were very high despite non-detection of McyE gene transcripts. Wood et al. (2011) reported significant up- and down-regulation of mcyE gene expression over short periods of time (2-5h) but only detected McyE transcripts in 29 % of the samples from Lake Rotorua, New Zealand, that had detectable levels of mcyE copies and microcystins. Reports of continuous MC production by declining cultures of M. aeruginosa held in the dark (Lyck, 2004), nevertheless, suggest that microcystin production can proceed even under conditions known to down-regulate mcy gene expression. Current literature on the associations between mcy transcription and MC concentrations in laboratory cultures and field samples is therefore somewhat contradictory.

The inconsistencies in mcyE copies, McyE transcript levels and microcystin concentration reported in our study are conceivable when we consider the complexity of the cascade of biosynthetic steps involved in microcystin biosynthesis. In order to produce a microcystin molecule, genetic information must be dedicatedly transcribed from ten (in Microcystis spp.) or nine (in Planktothrix spp.) mcy genes into various mcy mRNA transcripts (measured in RT-qPCR), before translation into the respective Mcy enzymes that catalyze the biosynthesis of the toxin molecule. The final concentration of microcystins in a given cell might, therefore be independent of ribosome or mRNA concentrations (Rueckert & Cary, 2009), but could rather be influenced by posttranscriptional regulation of any of the ten or nine Mcy proteins catalyzing the various steps in toxin biosynthesis as well as posttranslational modifications of MC precursors. A previous study suggested that posttranslational modification of NRPSs and PKSs via dedicated phosphopantetheinyl transferase mediated linkages might indeed be critical to the catalytic activity of NRPSs and PKSs (Walsh et al., 1997). Results from this study therefore lead us to posit that posttranscriptional and/or posttranslational regulation of Mcy proteins or MC precursors might have resulted
in the mismatch between measured cellular microcystin concentrations and McyE transcript levels. Additionally, these discrepancies might as well be attributed to conceivable temporal decoupling between mcyE transcription and presence of microcystins in the cells as suggested by Gobler et al. (2007). The fact that most prokaryotic mRNA are transient molecules that are continuously degraded during translation (Rueckert & Cary, 2009; Voet & Voet, 2004), led us to theorize that the signals responsible for particularly high or low levels of microcystins could have occurred well in advance of our sampling date.

Exploration of the correlations between microcystins and mcyE copies and McyE transcripts, or various biomass variables revealed that although microcystins were detected in all samples where mcyE genes were actively transcribed, McyE transcript levels were the poorest indicators of microcystin concentrations in both laboratory and environmental samples. Whereas BGA-PC and extractive chl-a concentrations were the best predictors of microcystin concentration in laboratory cultures, followed closely by mcyE copies, microscopic cell counts and YSI chl-a equivalent, mcyE copies were on the contrary the best indicators of microcystin concentrations in environmental water samples. The better association of MC concentration with BGA-PC and extractive chl-a over mcyE copies recorded in laboratory samples was expected considering that correlations of Microcystis or Planktothrix mcyE copies against total microcystins in mixed cultures did not account for the microcystin resulting from either P. agardhii or M. aeruginosa respectively whereas correlations of BGA-PC or chl-a against microcystins accounted for this differences, as both related total cyanobacterial biomass to total microcystin concentration. The reverse was true in environmental samples where mcyE copies (a measure of potentially toxic Microcystis) had better correlations with microcystin concentrations, whereas microscopic cell counts and chl-a, which measured total Microcystis (toxic and non-toxic) and phytoplankton biomass performed rather poorly.

We therefore submit that although YSI-based BGA-PC counts and extractive chl-a, like mcyE gene copies, can be equally good indicators of microcystins levels in samples with high proportion of microcystin-producing cyanobacteria (as was the
case with our laboratory cultures), mcyE gene copies are preferable proxies in environmental samples dominated by non-toxic cyanobacteria and/or green algae.

6.5 Conclusions

Results from this laboratory study showed the biomass of microcystin-producing M. aeruginosa CPCC 299 and P. agardhii NIVA-CYA 126 to be significantly lower in the mixed cultures compared to the monocultures on most sampling days, suggesting release of growth-inhibiting compounds into the mixed culture media by interacting M. aeruginosa and P. agardhii.

Furthermore, mcyE gene copies and McyE transcripts in the cyanobacterial strains were affected not only by presence of competing cyanobacteria in growth media, but also by the sampling day. That is, there was significant treatment*day interaction effects. Microcystis or Planktothrix mcyE gene expression was generally down-regulated in mixed cultures relative to monocultures, in agreement with the lower biomass recorded in the mixed cultures. Although both McyE transcripts and mcyE copies were detected in all samples containing measurable microcystins, only mcyE copies showed significant positive associations with cellular microcystin concentrations.

Examination of the associations of conventional cyanobacterial biomass proxies (chl-a, and cell counts) with microcystins under laboratory and field conditions further affirmed the utility of genomic DNA-based qPCR measurement of mcyE gene concentrations as indicators of potential risks from microcystins, especially in freshwater blooms comprising mixed assemblages of cyanobacteria.

The general discordance between mcyE expression and intracellular microcystin concentrations in laboratory cultures and field samples led us to postulate greater role of posttranscriptional or posttranslational regulation processes, rather than Mcy transcript levels, in determining cellular MC concentrations.

6.6 Acknowledgements

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la Recherche sur la Nature et les Technologies (FQRNT) and Max Bell Foundation grants to C. Madramootoo.
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Table 6.2: Correlation of *Microcystis mcyE* gene expression and copies, microcystin concentration and various biomass variables

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<th>BGA-PC</th>
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<th>Relative mcyE Expression</th>
<th>Microcystis cell counts</th>
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**; p < 0.01, *; p < 0.05, nss; not statistically significant
Table 6.3: Correlation of Planktothrix *mcyE* gene expression and copies, microcystin concentration, and various biomass variables

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<td>0.04&lt;sub&gt;ns&lt;/sub&gt;</td>
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<td><em>Planktothrix</em> cell counts</td>
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<td>0.84**</td>
<td>0.91**</td>
<td>0.42&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>YSI Chlorophyll</td>
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<td>0.92**</td>
<td>0.92**</td>
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**; p < 0.01, *; p < 0.05, ns; not statistically significant

Blue-green algae phycocyanin (BGA-PC) and cell counts are expressed per mL, *mcyE* copies (mL<sup>-1</sup>), while chlorophyll and microcystin are expressed as µgL<sup>-1</sup>.

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Figure 6.1: Variation in Microcystis mcyE copies in *M. aeruginosa* CPCC 299 monocultures and mixed cultures containing *P. agardhii* NIVA-CYA 126

**p < 0.01, *; p<0.05, nss; not statistically significant**

Figure 6.2: Variation in Planktothrix mcyE copies in *P. agardhii* NIVA-CYA 126 and mixed cultures containing *M. aeruginosa* CPCC 299

**p < 0.01, *; p<0.05, nss; not statistically significant**
Figure 6.3: Relative expression (histograms) and fold change (triangles) of the *Microcystis mcyE* gene in mono- and mixed cultures of *M. aeruginosa* CPCC 299

Figure 6.4: Relative expression (histograms) and fold change (triangles) of the *Planktothrix mcyE* gene in mono- and mixed cultures of *P. agardhii* NIVA-CYA 126. Notice the different in scales of the y-axis for the *Planktothrix* and *Microcystis* graphs

**; p < 0.01, *; p<0.05, nss; not statistically significant
Figure 6.5: Relative expression of *Microcystis mcyE* genes in Missisquoi Bay samples of summer 2011
**Figure 6.6:** Evolution of microcystin concentration in *M. aeruginosa* CPCC 299 monocultures and mixed cultures

**Figure 6.7:** Evolution of microcystin concentration in *P. agardhii* NIVA-CYA 126 monocultures and mixed cultures

**p < 0.01, *; p<0.05, nss; not statistically significant**
Figure 6.8: Changes in *M. aeruginosa* CPCC 299 cell counts in mono- and mixed cultures

Figure 6.9: *Changes in P. agardhii* NIVA-CYA 126 cell counts in mono- and mixed cultures

**; p < 0.01, *; p<0.05, nss; not statistically significant
Chapter 7 General summary and conclusions

7.1 General summary

The increasing incidences of toxigenic cyanobacterial blooms in freshwater bodies pose significant risks to human and ecosystem health worldwide. In Canada, the most prevalent freshwater cyanobacterial toxins are the hepatotoxic microcystins, which are produced mostly by cyanobacteria from the *Microcystis*, *Anabaena*, and *Planktothrix* genera. The co-occurrence of morphologically indistinguishable toxic and non-toxic strains of cyanobacteria within the same bloom has made risk assessment of cyanobacterial blooms by commonly used conventional microscopy very challenging.

The overarching goal of this study was to develop and evaluate molecular approaches for improved monitoring of microcystin-producing cyanobacteria species given their prevalence in freshwater bodies in Canada and globally. To this end, a three year project (2009-2011) entailing laboratory studies of toxic and non-toxic cyanobacterial dynamics as well as surveys of field stations located in the Missisquoi Bay, Quebec, was designed to generate knowledge to inform this objective. Chapter 3 of the dissertation evaluated the reliability of three microcystin biosynthetic genes (*mcyA, E* and *G*) for qPCR estimation of microcystin-producing *Microcystis* spp. (most common microcystin-producing cyanobacteria) in a Canadian freshwater lake while chapter 4 studied how changes in various physicochemical factors impacted changes in *mcyE* gene concentrations and biomass of a microcystin-producing *Microcystis* sp. of Canadian origin. Building from chapters 3 and 4, we developed in chapter 5 a multiplex qPCR assay that simultaneously detected microcystin-producing *Anabaena, Microcystis*, and *Planktothrix* spp. and utilized this to study the response of these toxic cyanobacteria to changes in environmental conditions in a freshwater lake in southern Quebec during the summers of 2010 and 2011. Finally, in chapter 6, the qPCR assays developed in the previous chapter were utilized concomitantly with traditional cyanobacterial monitoring approaches to
study how McyE gene transcript levels, mcyE gene copies, and various biomass proxies associated with microcystin concentrations in order to further confirm the reliability of mcyE gene targets as indicators of toxigenic cyanobacterial concentration and consequently toxicity of blooms. The following conclusions are drawn from this research.

7.2 Conclusions

Objective I: To determine toxin biosynthetic genes suitable for monitoring toxigenic cyanobacteria by evaluating PCR assays targeting a suite of microcystin synthetase gene loci.

Three independent PCR assays targeting portions of the mcyA, mcyE mcyG genes were successfully applied to detect and estimate potential microcystin-producing *Microcystis* cell concentrations in the Quebec portion of the Missisquoi Bay, Lake Champlain. Cloning and sequencing of PCR amplicons from field samples generated nucleotide sequences that clustered with the respective GenBank *Microcystis* mcyA, mcyE and mcyG nucleotide database entries, thereby confirming the specificity of the PCR assays. Toxigenic *Microcystis* cell concentrations determined using the mcyA, mcyE, and mcyG assays were not significantly different at stations with no visible bloom. Toxic cell concentrations determined with the mcyE assay were however significantly lower than concentrations determined using the mcyA and mcyG assays at the bloom stations. In spite of this, all three assays showed similar trends in toxigenic cell concentrations for the different sampling stations.

The high correlation coefficients ($R^2 > 0.90$) between the potentially toxic *Microcystis* concentrations estimated as mcyA, mcyE, and mcyG equivalents and total *Microcystis* counts from microscopy suggests that real-time qPCR-based measurement of these mcy genes can be a robust tool for monitoring toxigenic cyanobacteria in freshwater bodies. Furthermore, the very low detection limits attained in this study implies that incorporation of the mcyA, mcyG, and/or mcyE assays into early warning systems for toxigenic cyanobacteria monitoring can enable resource managers to take swift and appropriate measures to minimize risks to public health from impending harmful cyanobacterial blooms.
Objective II: To investigate changes in microcystin synthetase gene copies and biomass of mixed assemblages of toxic and non-toxic cyanobacteria under various nutrient, temperature, and light regimes

This study demonstrated depressed growth of *M. aeruginosa* CPCC 299 and *A. flos aquae* CPCC 67 in the mixed cultures under most of the nutrient, temperature, and light conditions investigated. Cellular and *mcyE* gene-based microcystin quotas, on the contrary, were higher in the mixed cultures than the corresponding monocultures. These experiments therefore showed that although interaction between cyanobacterial species led to a decrease in bloom density, the resulting blooms were in fact more toxic.

Whereas *A. flos aquae* CPCC 67 was unaffected by nitrogen concentrations in the growth media, *M. aeruginosa* CPCC 299 growth was found to be severely inhibited at nitrogen concentrations below 1.0 mg L$^{-1}$ but co-limited by nitrogen and phosphorus at higher N levels, hence simultaneous reductions of N and P levels in water bodies can potentially reduce incidence of toxigenic blooms of *Microcystis*.

The fact that changes in *mcyE* copies often mirrored changes in cellular growth rate of *M. aeruginosa* CPCC 299 under most of the investigated treatments suggest a possible coupling of *mcyE* production to cellular growth. Consequently, changes in *mcyE* copies can be reliable indicators of changes in biomass of the microcystin-producing *M. aeruginosa* strains.

**Objective III: To develop and optimize robust molecular techniques for characterizing multiple microcystin-producing cyanobacteria in a freshwater body.**

A novel qPCR assay for simultaneous detection, differentiation and quantification of three common microcystin-producing cyanobacteria genera was developed and validated. This assay was able to detect and estimate *mcyE* gene copies in lake water samples even when cyanobacterial cells and toxin concentrations were well below the limits of detection for conventional microscopy and ELISA.
respectively, reinforcing the applicability of developed assays in early warning systems for monitoring noxious algal blooms. Analyses of field samples using the developed assays resulted in identification of *Microcystis* spp. as the main putative microcystin-producers in the study lake, with only patchy appearances of toxigenic *Anabaena* and *Planktothrix* genotypes. Toxic *Microcystis* concentration correlated significantly with TP but not TN, suggesting that TP was an important determinant of toxic *Microcystis* blooms in the study lake.

Empirical regression models relating MC to *Microcystis mcyE* copies, chl-a and total *Microcystis* concentrations revealed that although all three biomass proxies were reliable indicators of MC concentration in water, *Microcystis mcyE* copies were more robust indicators of MC concentration in the Missisquoi Bay.

**Objective IV:** To evaluate the utility of developed molecular techniques relative to conventional monitoring approaches for indicating microcystin risks in laboratory cultures and field samples.

Results from laboratory experiments showed expression of the *mcyE* genes in a given cyanobacterial strain to be affected not only by presence of competing cyanobacteria in growth media, but also by the sampling day. That is, there was significant treatment*day interaction effects. However, *Microcystis* or *Planktothrix mcyE* gene expression was generally down-regulated in mixed cultures relative to monocultures, in agreement with the lower cellular counts recorded in the mixed cultures.

Although McyE gene transcripts and *mcyE* copies were detected in all samples containing measurable microcystins, only *mcyE* copies showed significant positive association with cellular microcystin concentrations under laboratory and field conditions. Furthermore, extractive chl-a, microscopic cell counts, and YSI chlorophyll concentrations showed stronger, significant positive associations (p < 0.01, r > 0.7) with microcystin concentration in laboratory cultures than in the field samples.
The strong positive association between mcyE copies and microcystins under laboratory and field conditions further affirmed the utility of genomic DNA-based qPCR measurement of mcyE gene concentrations as indicators of potential risks from microcystins, especially in freshwater blooms comprising mixed assemblages of cyanobacteria.

7.3 Contributions to knowledge

The work presented in this thesis generates important information on application of molecular techniques in monitoring toxigenic cyanobacteria in freshwater bodies and presents further insight on reliability of various biomass proxies for predicting toxigenicity of cyanobacterial blooms. This research therefore lays claim to the following contributions to scientific understanding regarding use of molecular tools in monitoring toxic cyanobacteria:

1. This research project led to the development of a novel multiplex real-time quantitative PCR approach capable of simultaneously detecting and quantifying up to three common microcystin-producing cyanobacteria genera in a single reaction tube. The level of sensitivity and specificity of this tool coupled with the ability to simultaneously and specifically quantify up to three toxigenic cyanobacterial genera can result in a reduction in sample processing time and cost. This research therefore provides water resource managers a reliable and cost-effective tool for use in early warning systems for harmful algae.

2. This research for the first time performed a comparative analysis of the utility of traditional cyanobacterial biomass proxies (chlorophyll a and cell counts), microcystin synthetase (mcy) E gene copies, and mcyE transcript levels for assessment of the toxigenicity of cyanobacterial blooms. It was demonstrated that whereas cell counts, chl-a, and mcyE gene concentrations could be equally reliable indicators of microcystin risk in water bodies dominated by microcystin-producing cyanobacteria, mcyE gene concentrations were, nevertheless, the most robust indicators in water bodies dominated by non-toxic cyanobacteria.
3. We were able to detect, for the first time, co-occurrence of \textit{mcyE}-containing \textit{Anabaena}, \textit{Microcystis}, and \textit{Planktothrix} genotypes in a Canadian freshwater body. This information can assist water resource managers to design targeted strategies to prevent occurrence of these noxious organisms in this water body.

4. This research also demonstrated that the effects of the various physicochemical parameters on growth, \textit{mcyE} genes, and toxin production were contingent on interactions between co-occurring cyanobacterial strains. Such information serves as a starting point towards understanding data from environmental samples that often comprise mixed populations of different cyanobacteria.

5. For the first time, a comparative analysis of toxigenic cyanobacteria concentrations determined by qPCR assays targeting up to three different toxin biosynthetic genes was performed wherein it was discovered that toxigenic cyanobacterial cell concentrations determined using different PCR assays can vary considerably depending on the target gene utilized. Thus absolute toxigenic cell concentrations determined by qPCR assays that target different gene loci may not be directly comparable but might serve as useful indicators of general trends in toxigenicity of blooms.

7.4 \textbf{Recommendations for future research}

1. \textbf{Development of multiplex qPCR assays targeting other toxigenic cyanobacteria.}

Microscopic examination of field samples during the present study revealed widespread occurrence of \textit{Anabaena} and \textit{Aphanizomenon} spp. which are also potential producers of anatoxins, saxitoxins, and/or cylindrospermopsins. Studies from the US and other temperate lakes have shown a gradual invasion by the cylindrospermopsin-producing \textit{Cylindrospermopsis} spp., suggesting that occurrence of multiple toxin-producing cyanobacteria within the same lake might become more rampant in the future. Health Canada recognizes cylindrospermopsin-producing cyanobacteria as emerging threats to Canadian
freshwater ecosystems. A natural progression to the present work is to explore possibilities of incorporating into the present multiplex platform, assays that target anatoxin, saxitoxin, and cylindrospermopsin-producing cyanobacteria to produce a one-shot molecular technique for specific monitoring of major toxin-producing cyanobacteria genera.

2. Harmonization of standards for qPCR monitoring of toxigenic cyanobacteria.

Application of quantitative PCR in routine monitoring of CHABs is currently constrained by lack of universal standards for absolute quantification as well as absence of information on the most appropriate toxigenic loci for qPCR-based quantification. The absence of such standards makes comparison of qPCR results from different researchers problematic. The logical next step towards implementing qPCR in routine monitoring programs should involve an exhaustive comparative evaluation of the different toxin biosynthetic genes in order to determine the genetic locus that accurately reflects changes in toxin concentrations in various toxigenic cyanobacteria under different environmental conditions. Knowledge generated from such studies can be used for developing and harmonizing standards and procedures for estimating toxigenic cyanobacteria.

3. Development of portable real-time PCR for toxigenic cyanobacteria

Portable real-time PCR systems capable of rapid (<25mins) identification of viral loads are gradually gaining ground in biomedical research. A plausible future area of research can involve evaluation and adaptation of portable real-time PCR technology for field-based detection of toxigenic cyanobacteria in unprocessed (or minimally processed) water samples. Utilization of such miniaturized PCR systems could provide actual real-time data on concentrations of potentially toxic cyanobacteria, hence speeding up the response to impending toxigenic cyanobacterial blooms.
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Appendix A- Selected photomicrographs of cyanobacterial spp. identified in Missisquoi Bay, Quebec.

A-1 Photomicrographs of cyanobacteria species identified in Missisquoi Bay, Quebec during summer 2011. *Dolichispermum (Anabaena) crassa* with heterocysts (A), *Aphanizomenon flos-aquae* complex with heterocysts (B) *Microcystis botrys* (small colonies; C), *Microcystis botrys* (large colonies) showing wide marginal mucilage (D). Photo credit: H.J. Kling
A-2 Photomicrographs of selected *Microcystis* species identified in Missisquoi Bay, Lake Champlain, Quebec during summer 2011. *Microcystis flos-aquae* (E), *Microcystis cf novacekii* with *Pseudanabaena mucicoli* in margin (F), *Microcystis viridis* (G), and *Microcystis wesenbergii* (H). Photo credit: H.J. Kling
Appendix B- Melting curve analyses of PCR amplicons generated during this research project

B-1: Melting curve analysis of *Microcystis mcyA* (A), *mcyE* (B), and *mcyG*(C) on 2009 field samples using SYBR green detection (Chapter 3)
B-2: Melting curve analysis of *Microcystis* and *Planktothrix* mcyE amplicons from laboratory samples. (D) *Microcystis* mcyE from nutrient experiments (Chapter 4). (E) *Microcystis* mcyE from temperature and light experiments (Chapter 4). (F) *Microcystis* mcyE from gDNA of mono- and mixed culture experiment (Chapter 6) (G) *Planktothrix* mcyE from gDNA of mono- and mixed culture experiment (Chapter 6)
B-3: Melting curve analysis of *mcyE*, rpoC1, and 16S rRNA amplicons from laboratory and field samples. (H) Melting curve for *Microcystis mcyE*, rpoC1, and 16S rRNA from cDNA of mono- and mixed culture experiment (Chapter 6). (I) Melting curve for *Microcystis mcyE*, rpoC1, and 16S rRNA from cDNA of summer 2011 field samples (Chapter 6). (J) Melting curve for *Planktothrix mcyE* and 16S rRNA from cDNA of mono- and mixed culture experiment (Chapter 6).
Appendix C: Plot of qPCR $C_T$ values against $mcyE$ gene copy number for the *Anabaena*, *Microcystis*, and *Planktothrix* standard curves described in Chapter 5

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