Monitoring and characterization of toxic cyanobacterial blooms

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Abstract

Molecular tools were used to monitor the dynamics of *Microcystis* populations and the potential microcystin producers in Missisquoi Bay (the Canadian part of Lake Champlain). This study showed the GF/C filters, which are typically used to prepare samples for microcystin analysis, were not ideal for the characterization of total bacterial communities, but were sufficient for the chemical analyses of cyanobacterial microcystins. The *mcyD* gene copy number determined by Q-PCR correlated well with the microcystin concentration determined by ELISA and HPLC in 2006; therefore *mcyD* Q-PCR could be used as a rapid and sensitive method and as an effective monitoring system for toxic cyanobacterial blooms in Lake Champlain. *Escherichia coli* was also monitored as an indicator of manure contamination of lake water. Manure application and rain events positively related to the high nitrogen concentration in the lake in 2006, suggested that good manure management is necessary for the reduction of agricultural nutrient loads into Missisquoi Bay, believed to be a major cause of cyanobacterial blooms.

Résumé

Un suivi de la dynamique des populations de *Microcystis* qui ont la capacité de produire des toxines a été effectué à la baie Missisquoi (partie canadienne du lac Champlain) à l'aide d'outils moléculaires. Deux types de membranes ont été utilisés : un filtre standard (GF/C) pour quantifier les microcystines, et un filtre pour capturer les bactéries. Cette étude a démontré que le filtre standard (GF/C) est approprié pour déterminer les concentrations de microcystines, mais qu'il n'est pas adéquat pour caractériser avec des techniques moléculaires, les populations de bactéries et de cyanobactéries. Une bonne corrélation a été obtenue en 2006, entre les concentrations de microcystines déterminées par les méthodes ELISA et HPLC et le nombre de copies du gène *mcyD* (impliqué dans la biosynthèse des microcystines). Les résultats ont donc démontré le potentiel de cette méthode de PCR en temps réel, ciblant le gène *mcyD,* pour la détection et le suivi des proliférations de cyanobactéries qui produisent des toxines. La présence du pathogène fécal *Escherichia coli* a été évaluée afin de déterminer la relation entre l'application de lisier et de fumier et l'augmentation dans l'eau du lac, des nutriments qui causent les proliférations. Une bonne corrélation a été obtenue entre l'application du lisier et du fumier et les précipitations ainsi qu'entre les concentrations élevées d'azote dans le lac, suggérant qu'une meilleure gestion des matières fertilisantes est nécessaire pour réduire les apports de nutriments dans la baie Missisquoi.

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Abbreviations

Adda : 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid
BSA: Bovine serum albumin
DGGE: Denaturing Gradient Gel Electrophoresis
D-MeAsp: D-*erythro*-β-methylaspartic acid
dNTPs: deoxynucleoside triphosphates
ELISA: Enzyme Linked Immunosorbant Assay
GC-MS: Gas Chromatography-Mass Spectrometry
GF/C: Glass microfibre filters with a pore size of 1.2 µm
HPLC: High Performance Liquid Chromatography
IBDG: Indoxyl-β-D-glucuronide
MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization Time of Flight
MC-LA: microcystin-LA
MC-LF: microcystin-LF
MC-LR: microcystin-LR
MC-RR: microcystin-RR
mcy : microcystin synthetase
MC-YR: microcystin-YR
Mdha : N-methyl-dehydroalanine
MUGal: 4-Methylumbelliferyl-β-D-galactopyranoside
N: nitrogen
ndaF: nodularin synthetase gene subunit F
NH_4: ammonium
NOx: NO, NO_2 and NO_3
NRPS: nonribosomal peptide synthetase
NT: total nitrogen
NTD: total dissolved nitrogen
ORFs: open reading frames
P: phosphorus
PKS: polyketide synthase
PPIA: Protein Phosphatase Inhibition Assay
PT: total phosphorus
PTD: total dissolved phosphorus
PVPP: polyvinylpolypyrrolidone
Q-PCR: Quantitative-PCR
RFLP: Restriction Fragment Length Polymorphism
SDS: Sodium dodecyl sulfate
TC: total coliforms
UPGMA: unweighted pair group method with arithmetic mean algorithm
USEPA : United States Environmental Protection Agency
VBNC: viable but non-culturable

Introduction

Cyanobacterial blooms have become a worldwide concern in recent years, because of the risk of cyanotoxins produced by cyanobacteria to public safety. In the province of Quebec, waterbodies affected by cyanobacterial blooms increased from 34 in 2004 to almost 200 in 2007 (Blais 2008). Toxic cyanobacterial blooms have occurred in Missisquoi Bay from 2001 to 2008, and the beaches have been closed each year for safety reasons. These blooms pose a hazard to public health and the ecosystem, and have had a negative impact on the local economy.

The best way to protect public health is to avoid any direct contact with water containing cyanotoxins. The rapid and accurate detection of cyanotoxins is necessary for successful monitoring and warning systems. Toxin-producing and non-toxin producing species of cyanobacteria coexist in the same water body, and can not be differentiated by microscopy (Neilan 1996; Carrillo et al. 2003; Janse et al. 2003). The traditional bioassay method provides initial toxicity data of an unknown toxin: poor sensitivity and ethical issue have limited its use. The Protein Phosphatase Inhibition Assay (PPIA) is a sensitive but also expensive method to detect toxin. Specialized equipment and regulations are necessary for this assay. The accuracy of the Enzyme Linked Immunosorbant Assay (ELISA) depends on the cross reactivity of the tested samples to the standard, and this variable cross-reaction may interfere with concentration estimation, resulting in over- or under-estimation of toxin concentration (Carmichael and An 1999; van

Apeldoorn et al. 2007). Preparing samples is time consuming for High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS). Moreover, it is difficult to obtain the pure toxin standards, especially in the case of microcystins when so many isoforms are possible (Msagati et al. 2006; Pearson and Neilan 2008). Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometry is a rapid and sensitive method, and microcystin isoforms can also be identified. However, for most laboratories it is not feasible since this equipment is expensive (de Figueiredo et al. 2004). In conclusion, these methods have their limitations in providing a rapid and sensitive monitoring technique for cyanotoxins.

Molecular techniques based on the detection of genes involved in cyanotoxin biosynthesis are promising and widely used methods, especially in Australia and several European countries. Many genes involved in cyanotoxin production have been sequenced: for example, the microcystin synthetase (*mcy*) genes (Nishizawa et al. 1999; Nishizawa et al. 2000; Tillett et al. 2000; Christiansen et al. 2003; Rouhiainen et al. 2004), cylindrospermopsin (Mihali et al. 2008) and nodularin genes (Moffitt and Neilan 2004). This information has provided the basis for the development of molecular detection methods for potential toxin-producing cyanobacteria.

Of the total eight or ten genes involved in the microcystin synthetase (*mcy*) gene, *mcyA* (Tillett et al. 2001; Baker et al. 2002; Hisbergues et al. 2003; Yoshida et al. 2003; Kurmayer et al. 2004; Via-Ordorika et al. 2004; Wilson et al. 2005; Yoshida et al. 2005) and *mcyB* (Kurmayer et al. 2002; Nonneman and Zimba 2002;

Mikalsen et al. 2003; Via-Ordorika et al. 2004) were most often used as molecular probes for the detection of potential microcystin producers. The *mcyD* and *mcyE* genes are essential for the synthesis of the Adda and D-glutamate moieties, and the Adda is critical for the toxicity of microcystin. The *mcyD* or *mcyE* genes were also chosen as the target region for *mcy* detection in other studies (Rinta-Kanto et al. 2005; Rantala et al. 2006; Hotto et al. 2007). In these studies, a good correlation was established between the *mcy* gene detection and microcystin production.

Cyanobacterial toxins were first documented in Lake Champlain in 1999: microcystin was the main toxin detected in this area from 2001 to 2005, and *Microcystis* and *Anabaena* are potential microcystin producers found in the Lake (Watzin et al. 2002; 2003; Watzin et al. 2004; Watzin et al. 2005; Watzin et al. 2006).

Objectives

A genome based technique was developed to monitor the toxic blooms in Missisquoi Bay. The standard filter (GF/C: Glass microfiber filters with a pore size of 1.2 µm) method for capturing and quantifying microcystins, as well as a filter with a pore size of 0.22 µm for capturing bacteria were used to process water samples. The efficiency of the filters for the molecular detection of toxic cyanobacterial producers was compared. The *mcyD* gene, indicative of microcystin synthesis, was amplified from the Millipore filtrand (pre-filtered on the GF/C) as well as from the GF/C filtrand in the preliminary test. Was the GF/C filter effective for microcystin analysis? To answer this question, molecular techniques were employed to compare the GF/C filtrand and the Millipore filtrand, to determine the *Microcystis* 16S rRNA and *mcyD* gene diversity between the two filters.

Nutrient runoff from agricultural lands is a direct cause of eutrophication resulting in cyanobacterial blooms in Missisquoi Bay. Manure application on agricultural land is a common practice in Quebec. In 2006, there were heavy rain events in May and June during manure application in the spring. Our hypothesis was that the nutrients in the manure were carried into the lake with the rain, resulting in increased nutrient concentrations in the lake. To test our hypothesis, *Escherichia coli* was monitored as an indicator of manure contamination in the lake water and the relationship of the nutrients from the manure, *E. coli* densities and

cyanobacterial bloom events were examined to determine if any relationship could be established.

The overall objectives of the research were:

1) To develop a genome based technique to monitor toxic cyanobacterial blooms in Missisquoi Bay.
2) To evaluate the efficiency of the GF/C filter method for the molecular detection of toxin producing cyanobacteria.
3) To monitor *Escherichia coli* as an indicator of manure contamination in the lake water, to assess if there is a link between farmland manure application in association with rain events and the occurrence of cyanobacterial blooms.

Chapter 1 Literature review

1.1 Cyanobacteria

Cyanobacteria, commonly known as blue-green algae, are a group of prokaryotes producing oxygen as a by-product of the photosynthetic process. They are a unique group of Gram-negative bacteria, and also one of the largest groups of bacteria. They usually contain chlorophyll *a* for photosynthesis, which is the same as green algae and plants. They also contain phycobiliproteins as photosynthetic accessory pigments. Phycobiliproteins provide cyanobacteria with a wider light spectrum for photosynthesis than plants. They are classified into two large groups based on their color, the phycoerythrins (red) and the phycocyanins (blue). Phycocyanins provide the blue-green characteristic color for many cyanobacteria (Whitton and Potts 2000).

Cyanobacteria are among the very earliest life forms on earth, and they play an important role in the history of the planet. The oldest microfossil record of cyanobacteria in the archaean rocks from northwestern Western Australia may trace back to around 3.5 billion years ago (Schopf et al. 2002). Moreover, it is widely accepted that cyanobacteria are the ancestors of the chloroplasts found in higher plants and algae (Tomitani et al. 2006).

Cyanobacteria have a considerable variety of morphologies and include 150 genera and 2000 species (Herrero and Flores 2008). They have a diversity of habitats and colonize nearly all illuminated environments on Earth. As an

important primary producer, cyanobacteria play a significant role in the nitrogen, oxygen and carbon cycles in the biosphere (Whitton and Potts 2000). Cyanobacteria contribute around 30% of the total annual oxygen production on the planet (Herrero and Flores 2008).

Cyanobacteria are an essential part of the ecosystem, and are natural inhabitants of aquatic systems. However, under favorable conditions, especially in warm, shallow and slow moving water, they can quickly multiply and become the dominant population, resulting in the formation of scums or foams. The water looks like thick pea soup, often blue-green in color. Blooms of this nature may occur at any time, but most often occur in the late summer or early fall. In recent years, cyanobacterial blooms have been reported worldwide, for instance, Lake Taihu in China (Guo 2007), Lake Wannsee in Germany (Kurmayer et al. 2003), Lake Erie in North America (Rinta-Kanto et al. 2005) and the Baltic Sea in Europe (Koskenniemi et al. 2007).

Cyanobacteria have several competitive advantages over algae or other aquatic organisms, which contribute to their proliferation in the aquatic environments. First, they generally favor higher temperatures (>25°C), temperatures at which diatoms and green algae do not grow well. Rising temperatures due to global warming may be a factor contributing to the frequent occurrence of cyanobacteria blooms (Paeri and Huisman 2008).

Second, some cyanobacteria are capable of fixing atmospheric nitrogen. Some cyanobacteria have specialized cells called heterocysts to fix nitrogen: *Anabaena* and *Aphanizomenon* are examples in this group (Falconer 2005). A single

heterocyst is separated by several vegetative cells to form a multicellular organism. Heterocysts are cells with thick membranes, having a unique structure and physiology for performing nitrogen fixation under aerobic conditions. Nitrogen fixation is performed by nitrogenase, a highly conserved multimeric enzyme complex. The activity of nitrogenase is irreversibly inhibited by oxygen, so the heterocysts form a micro-anaerobic environment (Whitton and Potts 2000; Berman-Frank et al. 2003; Golden and Yoon 2003).

The surface bloom forming cyanobacteria usually have a buoyancy-regulating mechanism, which provides another distinct ecological advantage in obtaining light and nutrients. Gas vacuoles are composed of an array of gas vesicles: gas vesicles are hollow, gas-filled hollow cylindrical structures formed by protein. The possession of gas vacuoles allows cyanobacteria to regulate their depth in the water column to reach the optimal conditions for their growth (Walsby 1994).

Cyanobacterial blooms are a serious public health concern. On one hand, they greatly reduce the value and quality of both recreational and drinking water due to odor and taste factors, and contribute to fish mortality by depleting the water of oxygen (Nonneman and Zimba 2002). Another more important reason is that cyanobacteria produce a variety of bioactive secondary metabolites, most of which are potent toxins, posing a significant health hazard to human and other animals (Huisman et al. 2005). On average, 59% of the freshwater cyanobacterial blooms contain toxins (Chorus and Bartram 1999), and these toxic blooms have adverse impacts both on the ecosystems and the local economies (Bird 2005).

1.2 Cyanotoxins

Cyanotoxins are potent toxins released by cyanobacteria when the cells lyse and decompose. Cyanotoxins are classified as secondary metabolites, so they do not play a role in primary metabolism, but benefit the producing organisms in some way (Neilan et al. 1999). The biological function of cyanotoxins has not been definitively determined despite considerable research in this area.

At least 46 cyanobacterial species produce different kinds of toxin. The most common toxic cyanobacteria in freshwater are *Microcystis* sp., *Anabaena* sp., *Aphanizomenon* sp., *Cylindrospermopsis raciborskii*, *Planktothrix* sp., *Synechococcus* sp., *Gloeotrichia* sp., *Schizothrix* sp. and *Synechocystis* sp. (Chorus and Bartram 1999).

Cyanotoxins are very diverse in structure and toxicity. Cyanotoxins can be divided into three major groups based on their toxic effect on animals. Hepatotoxins, including microcystins, nodularins and cylindrospermopsins, affect the liver. Neurotoxins are toxins that affect the nervous system, and include anatoxin-a, anatoxin-a(s) and saxitoxins. Dermatotoxins are toxins that cause allergic reactions to the skin, such as lipopolysaccharides (de Figueiredo et al. 2004; Huisman et al. 2005). These toxins fall into three categories based on their chemical structure: cyclic peptides (microcystins and nodularins), alkaloids (anatoxin-a, anatoxin-a(s), cylindrospermopsins and saxitoxins) and lipopolysaccharides. In recent years, many other bioactive peptides commonly found in cyanobacterial blooms have been identified (Tonk et al. 2008).

Over the past century, deaths among domestic and wild animals resulting from drinking toxic cyanobacterial blooms have been reported worldwide (Falconer 2005; Huisman et al. 2005). Human exposure to cyanotoxin is mainly through ingestion and/or direct contact with water. Symptoms caused by cyanotoxins include skin irritation, nausea or vomiting, weakness, and symptoms of common gastrointestinal illnesses (Falconer 2005). In November 1979, Palm Island, in tropical northern Queensland, Australia, more than 100 children of Aboriginal families suffered from various symptoms of gastroenteritis. A later epidemiological study linked the "mystery disease" with the water supply contaminated with toxic *Cylindrospermopsis raciborskii* (Griffiths and Saker 2003).

Microcystin is one of the most widespread hepatotoxins in freshwater blooms and is often the cyanotoxin involved in animal and human poisonings.

1.2.1 Microcystin

"The fast death factor" was first partially identified from a NRC-1 strain of *Microcystis aeruginosa* and microcystin was named after this species (Bishop et al. 1959).

Microcystins are a family of cyclic heptapeptides, the basic cyclic structure composed of five non-protein amino acids and two protein amino acids (-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) (Fig 1.1). The five unusual amino acids are, namely, D-alanine, D-methylaspartic acid, Adda, D-glutamic acid and Mdha. D-MeAsp is D-*erythro*-β-methylaspartic acid; Adda is 3-amino-9-methoxy-2,6,8-

trimethyl-10-phenyl-4,6-decadienoic acid; and Mdha is N-methyl-dehydroalanine. X and Z indicate two variable L-amino acids (Nishizawa et al. 1999; Tillett et al. 2000; Falconer 2005).

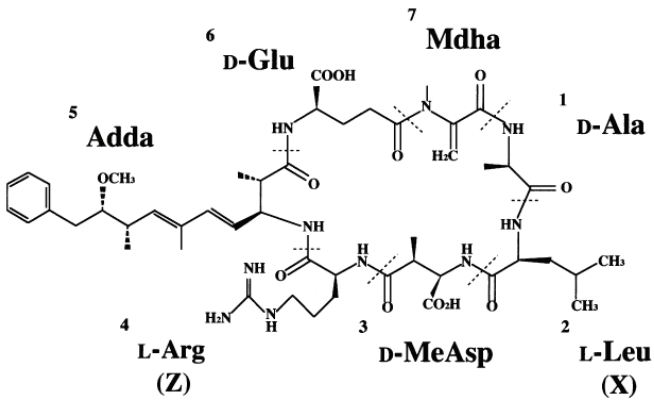

Fig.1.1 General chemical structure of microcystin-LR. Adapted from Nishizawa et al. (2001).

Microcystin isoforms have variable chemical structure: variations occur in all amino acids but mainly in the two protein amino acids. At least 89 microcystin isoforms have been identified (Welker and Von Döhren 2006). The isoforms have different degrees of toxicity, ranging from 50 to >1200 μg kg-1 body weight (based on 50% lethal dose in mice) (Hotto et al. 2007). Some microcystin isoforms are distinguished and named based on these two protein amino acids. For example, the most common and the most toxic microcystin, microcystin-LR (MC-LR) contains Leucine (L) and Arginine (R). Several isoforms frequently determined in blooms are: MC-RR containing two Arginine (R); MC-YR containing Tyrosine (Y) and Arginine (R); MC-LA containing Leucine (L) and Alanine (A); MC-LF containing Leucine (L) and Phenylalanine (F) (Jungblut and Neilan 2006).

All the microcystin isoforms had a strong peak at 238 nm of UV absorption spectrum due to the conjugated diene of the Adda residue. The detection of microcystin by HPLC is based on this point. The Adda and D-Glu moieties are most conserved in microcystin isoforms and are essential for the inhibition activity and the toxicity. In general, any structural modifications to the Adda-glutamate region of the toxin molecule changed the microcystins to be non-toxic (Rouhiainen et al. 2004).

The toxicity of microcystins is caused by the inhibition of eukaryotic serine/threonine protein phosphatase 1 and 2A by a thiotemplate system (Honkanen et al. 1990; MacKintosh et al. 1990; Dawson 1998). Microcystins are actively transported into liver cells by the bile acid transport system (Rantala et al. 2006). Microcystins cause acute poisoning and are potential cancer promoters (de Figueiredo et al. 2004; Dittmann and Wiegand 2006). Microcystins are responsible for deaths in wild and domesticated animal populations and have various acute and chronic pathogenic effects on humans. Microcystins can also bioaccumulate in aquatic food webs (mussels, crayfish and fish), posing a hazard to human consumers (Kotak et al. 1996; Prepas et al. 1997; de Figueiredo et al. 2004).

In February 1996, in Caruaru, Brazil, 126 patients at a hemodialysis unit suffered from a toxic illness with varying severity of acute neurotoxicity and subacute hepatotoxicity, leading to the death of 52 patients. It was related to the water used for dialysis, which was contaminated with mainly microcystin produced by

different species of cyanobacteria (Pouria et al. 1998). This was one of the first human death events directly related to microcystin toxicity.

The total MC-LR concentration of 1.0 µg/L was set as a guideline by the World Health Organization for drinking water (Chorus and Bartram 1999), and most countries have adopted this value as a guideline. In Canada a total MC-LR concentration of 1.5 µg/L is used instead (Health Canada 2003) and 1.3 µg/L is used in Australia (Westrick 2003). For recreational water, the WHO set a guideline of three risk levels related to cyanobacterial abundance: more than 4,000/mL cyanobacterial cells trigger the vigilance level, and public health officials are notified (Watzin et al. 2006).

Microcystins are mainly produced in freshwater habitats by the distantly related genera *Microcystis, Anabaena* and *Planktothrix* (Rouhiainen et al. 2004). *Microcystis* is a genus commonly found in freshwater blooms from cold temperate to tropical climates. *Microcystis,* Group I (Chroococcales), is a group of unicellular, colonial cyanobacteria containing vacuoles. In general, *Microcystis. aeruginosa*, *M. ichthyoblabe, M. novacekii, M. viridis, and M. wesenbergii* are the five dominant *Microcystis* species. They are defined based on cell size, colony formation and other morphological characteristics (Kaneko et al. 2007).

Anabaena is another bloom forming genus, belonging to Group IV (Nostocales). It belongs to a group of filamentous, heterocyst-forming cyanobacteria, capable of nitrogen fixation. *Planktothrix* is another filamentous cyanobacteria producing microcystin. *Planktothrix rubescens* with phycoerythrin-rich (red-pigmented) genotypes are most commonly found in deep, stratified and oligo- to mesotrophic

waters, while *Planktothrix agardhii*, phycocyanin-rich genotypes (green pigmented), inhabit shallower, polymictic and mesotrophic to hypertrophic waterbodies (Kurmayer et al. 2005).

1.2.2 Characterization of microcystin biosynthesis genes

Earlier biochemical and genetic studies have suggested that a mixed polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) origin of microcystin synthetase enzyme complex are involved in microcystin biosynthesis (Arment and Carmichael 1996; de Figueiredo et al. 2004; Finking and Marahiel 2004). Although the chemical structure of polyketides and nonribosomal peptides are different, PKS are assembled in a similar way as NRPS (Dittmann et al. 2001; Finking and Marahiel 2004).

NRPS are mechanisms commonly used by bacteria and fungi to produce natural products - small polypeptides of about 50 amino acids or less. Nonribosomal synthesis pathway is similar to the ribosomal system in the activity of the enzymes, but with different enzyme structures, and it is a process that requires energy (Finking and Marahiel 2004).

NRPS are a family of multifunctional enzymes, consisting of a variety of molecules; each module is responsible for incorporating one building block into the growing polypeptide chain. Each module can be divided into several domains harboring the catalytic activities, namely, substrate recognition and activation (A-domain), covalent loading (CP-domain), and peptide bond formation (C-domain).

Modules can be divided into initiation and elongation modules depending on whether they harbor a C-domain or not (C-domain lacks the initiation module) (Finking and Marahiel 2004).

In ribosomal peptide synthesis, only 20 amino acids can be used as substrates, and several proofreading mechanisms are involved in the process. In contrast, several hundred substrates can be incorporated into the polypeptide building blocks without proofreading in NRPS (Finking and Marahiel 2004; Grünewald and Marahiel 2006). The numerous microcystin isoforms can be partly explained by the characteristics of the NRPS machinery.

The microcystin synthetase (*mcy*) gene cluster sequences have been investigated in detail in *M. aeruginosa* strain K-139 (Nishizawa et al. 1999; Nishizawa et al. 2000) and *M. aeruginosa* PCC7806 (producing MC-LR) (Tillett et al. 2000), *Anabaena* strain 90 (producer of MC-LR, MC-RR, and D-Asp-MC-LR) (Rouhiainen et al. 2004) and *Planktothrix agardhii* CYA 126 (producer of mainly [D-Asp]-MC-RR) (Christiansen et al. 2003).

In *M. aeruginosa* PCC7806, the *mcy* gene cluster spans about 55 kb in the chromosome, and contains a total of 10 genes (Fig.1.2). The 10 genes encode nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS) and tailoring enzymes. Six large open reading frames (ORFs) (*mcyABCDE* and *mcyG*) contain 45 catalytic domains of the total 48 catalytic reactions involved in microcystin synthesis: other genes are involved as modifying enzymes (*mcyF, I* and *J*) and a putative ABC transporter (*mcyH*) (Tillett et al. 2000; Huisman et al. 2005). The larger operon (*mcyDEF* and *G*) encodes the PKS and NRPS modules

involved in the formation of the Adda and its linkage to D-glutamate. The *mcyD* encodes polyketide synthase, containing two of the type I modules. The *mcyE* and *mcyG* are hybrid enzymes of mixed NRPS and PKS. The smaller operon *mcyABC* encodes three NRPS containing five modules, which activate and incorporate the rest of the five amino acids (Mdha, DAla, X, D-MeAsp and Z) of microcystins into the peptide structure (Tillett et al. 2000; Huisman et al. 2005).

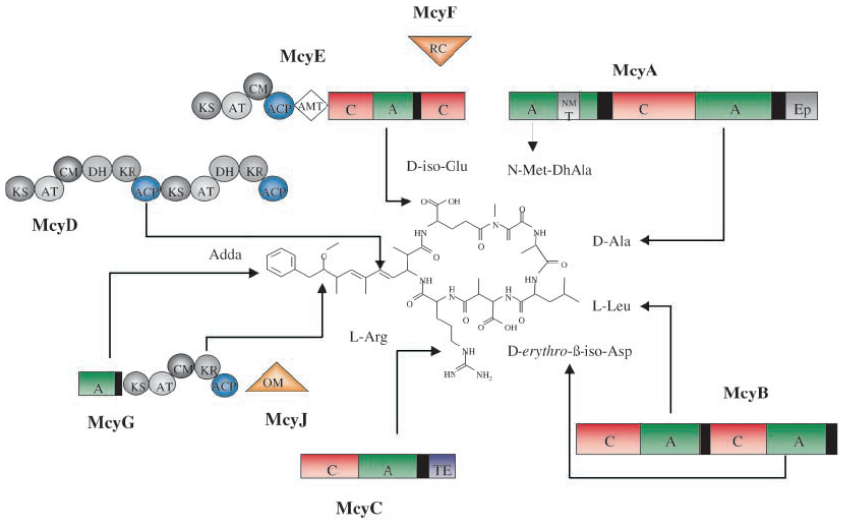

Fig.1.2 Model for predicted domain structures of microcystin synthetase and the formation of microcystin-LR. Adapted from Dittmann et al. (2001).

The entire genome of toxic *M. aeruginosa* NIES-843 and PCC 7806 have been sequenced, with genome sizes of 5,842,795 and 5,172,804 base pairs, respectively, in a single, circular chromosome. There is only one microcystin synthase gene cluster per genome (Kaneko et al. 2007; Frangeul et al. 2008).

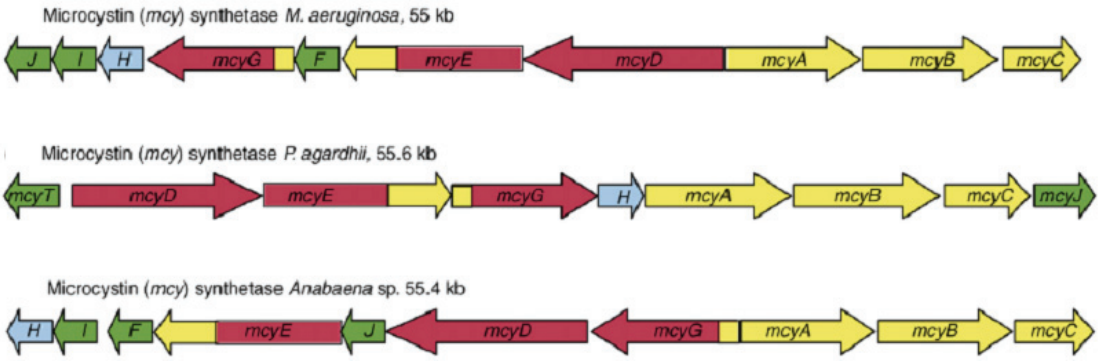

Fig.1.3 Model for microcystin synthetase gene clusters in *M. aeruginosa*, *P. agardhii* and *Anabaena* sp. Adapted from Pearson and Neilan (2008).

The *mcy* gene structure cluster in *M. aeruginosa* strain K-139 is highly similar to that in *M. aeruginosa* strain PCC7806 (Nishizawa et al. 1999; Nishizawa et al. 2000; Tillett et al. 2000). The *mcy* genes in *M. aeruginosa*, *Anabaena* and *P. agardhii* are distinct. The *mcyABCDEGJ* genes are present in all strains (Fig.1.3): the *mcyABC* have the same order, but the other genes have a different order. The *mcyF* and *mcyI* are lacking in *Planktothrix*, and *mcyT* is missing in *Microcystis* and *Anabaena*. The gene identities at the nucleotide and the amino acid levels are low among the three genera (Rouhiainen et al. 2004; Pearson and Neilan 2008).

This suggests that there is a common ancestor for *Microcystis*, *Anabaena* and *Planktothrix* in terms of *mcy* gene evolution. The *mcy* genes are of ancient origin and have been lost in many cyanobacterial genera (Huisman et al. 2005). Recombination (intragenic and intergenic), horizontal gene transfer, point mutations, and insertions/deletions are events that contributed to the natural variation of the *mcy* gene cluster (Tanabe et al. 2004; Tooming-Klunderud et al. 2008).

1.3 Cyanotoxin detection by molecular methods

Molecular methods are relatively simple, rapid and cost effective compared to most chemical methods for cyanotoxin detection, and have been widely used in many different areas. The toxicity of a cyanobacterial strain depends on whether or not it contains the genes for cyanotoxin production. Several genes involved in the biosynthesis of cyanotoxins have been identified, including the genes for microcystin (Tillett et al. 2000), cylindrospermopsin (Mihali et al. 2008) and nodularin (Moffitt and Neilan 2004). These genes have provided the basis for the molecular detection of toxic cyanobacteria.

1.3.1 Microcystin-producing cyanobacterial detection using PCR

An individual cyanobacterial strain can produce more than one microcystin isoform. One *mcy* gene cluster is involved in the synthesis of all the microcystin isoforms in a given strain (Huisman et al. 2005). For instance, *Microcystis* S-70 containing one set of the *mcy* genes, produces MC-LR, MC -RR, and MC -YR (Nishizawa et al. 2000).

The relationship between microcystin detection and the presence of *mcy* genes has been well established. The *mcy* genes have also been identified, however, in several *Microcystis* strains and *Planktothrix* strains that were lacking detectable levels of microcystins (Meiβner et al. 1996; Nishizawa et al. 1999; Kaebernick et al. 2001; Tillett et al. 2001; Mikalsen et al. 2003; Kurmayer et al. 2004). In

general, the false positive *mcy* gene is rare in *Microcystis*, and molecular detection of microcystin-producer is accurate.

The involvement of the *mcyA*, *B*, *D*, *E*, and *F* genes in microcystin production has been demonstrated by gene disruption studies (Dittmann et al. 1997; Tillett et al. 2000; Christiansen et al. 2003). Single *mcyA*, *mcyB, mcyD* or *mcyE* gene or combination of several *mcy* genes (*mcy ABCDE* and *mcyG*) have been successfully used as the targets for the detection of microcystin-producing strains from pure cultures and environmental samples (Baker et al. 2001; Tillett et al. 2001; Baker et al. 2002; Vaitomaa et al. 2003; Via-Ordorika et al. 2004; Ouahid et al. 2005; Rinta-Kanto et al. 2005; Jungblut and Neilan 2006; Hotto et al. 2007).

PCR amplification of the *mcy* gene cluster has also been used to differentiate microcystin-producing genera. Amplification of the *mcyA* gene followed by Restriction Fragment Length Polymorphism (RFLP) analysis allowed the differentiation between microcystin producing strains from the genera *Microcystis*, *Anabaena* and *Planktothrix*. The analyses were performed with cyanobacterial strains and water samples from Lake Wannsee (Berlin, Germany) (Hisbergues et al. 2003). Genus-specific *mcyE* primers were also used to identify potential microcystin-producing *Microcystis*, *Planktothrix* and *Anabaena* in 70 Finnish lakes (Rantala et al. 2006).

1.3.2 Detection of cyanotoxin producers by Q- PCR

PCR is a qualitative tool, able to detect the presence of the target gene or not, but it can not provide quantitative data on the abundance of the target gene. Quantitative-PCR (Q-PCR) is a more sensitive and quantitative method that has been applied to detect various cyanotoxins including microcystin, nodularin and cylindrospermopsin. Q-PCR provides more information related to the dynamics of potential cyanotoxin-producers and bloom-forming cyanobacterial genera, which will help gain a better understanding of the interaction of potential cyanotoxins producers with various environmental factors, leading to the possibility of better control of cyanobacterial blooms.

The *mcyE* was used for quantification of the microcystin producing *Microcystis* and *Anabaena* in Lake Tuusulanjärvi in the summer of 1999 and Lake Hiidenvesi on 15 August 2001 in Finland (Vaitomaa et al. 2003). The abundance of total cyanobacteria, *Microcystis*, and toxic *Microcystis* in western Lake Erie in August of 2003 and August of 2004 were quantified by cyanobacterial specific 16S rRNA genes, *Microcystis* specific 16S rRNA gene and the microcystin synthetase gene *mcyD*, respectively. The study provided an evaluation of the composition of cyanobacterial blooms within this lake (Rinta-Kanto et al. 2005). The relationship between microcystin production and the detection of the *mcy* gene was also confirmed by other researchers (Kurmayer and Kutzenberger 2003; Furukawa et al. 2006).

The Q-PCR method targeting the nodularin synthetase gene subunit F (*ndaF*) was developed to quantify the toxin-producing and bloom-forming *Nodularia*

spumigena in the Baltic Sea in July 2004. The *ndaF* copy numbers correlated with the nodularin concentrations (Koskenniemi et al. 2007). A duplex Q-PCR was developed for determining a cylindrospermopsin-specific and *Cylindrospermopsis raciborskii*-specific DNA sequence from culture and water samples in Australia (Rasmussen et al. 2008).

1.4 Eutrophication

"Eutrophication is the word used by scientists to describe the over-fertilization of lakes with nutrients and the changes that occur as a result" (Schindler and Vallentyne 2008). Eutrophication is characterized by excessive algal growth, frequent occurrence of toxic cyanobacterial blooms and a decrease in the abundance of species (Scholten 2005). Natural eutrophication is a process where waterbodies gradually age and become more productive, and it takes thousands of years to reach under normal conditions. However, human activities have increased the nutrient loading in lakes, greatly accelerating this process in thousands of lakes all over the world (Sharpley 2006). This is called cultural eutrophication, which can cause oligotrophic or mesotrophic lakes to become eutrophic, and eutrophic lakes to become hypereutrophic (Schindler and Vallentyne 2008).

In lakes, diatoms, green algae, cyanobacteria and other types of phytoplankton are the primary producers at the bottom of the food chain: their abundance is related to nutrient concentrations, especially nitrogen and phosphorus.

Eutrophication problems are generally the consequence of enhanced phosphorus and nitrogen loadings. Phosphorus is the key nutrient for the control of eutrophication, because many cyanobacteria have the ability to fix atmospheric nitrogen (Carpenter et al. 1998; Schindler and Vallentyne 2008).

1.4.1 Phosphorus

Fertilizers rich in phosphorus (P), nitrogen (N) and other nutrients are applied to agricultural land for crop production. However, the continuous long-term application of fertilizers will increase the soil nutrient levels especially P concentrations. When soil P levels are in excess of crop needs, the excess P in different forms will leach from the land into water, resulting in many adverse environmental problems (Hu 2008).

The phosphorus in waterbodies mainly comes from point sources or non-point sources. Point sources include factories or sewage treatment plants, detergent and other sources. Point sources are easier to control compared with the non-point sources. Non-point sources are from suburban lawns and agricultural lands, which are difficult to identify, measure and control (Sharpley 2006).

In Quebec, phosphorus concentrations in many rivers draining agricultural fields are higher than water quality guideline of 0.03 mg/L, the limit set by the Ministère de l'Environnement du Quebec to prevent eutrophication of fresh waterbodies. The construction of new wastewater plants and bans of phosphorus in detergents over the last 20 years have contributed to reducing the number of point sources

in aquatic systems. Non-point sources of phosphorus from agriculture are now regarded as the major contributors to high phosphorus levels in the rivers. Phosphorus is transported from agricultural land though surface runoff and drainage systems (Enright 2004; Simard et al. 2004).

Missisquoi Bay lies in the State of Vermont in the United States and the Province of Quebec in Canada. The water quality in the bay has decreased due to excess P input over the last two decades. The governments of Vermont and Quebec have taken action to address the P problem. In August 2002, a Missisquoi Bay Phosphorus Reduction Agreement was signed between the governments of Vermont and Quebec, to reduce phosphorus loading into Missisquoi Bay from point and non-point sources in its watershed. But it will take a long time to see the effectiveness of these actions and the recovery of the water quality in Missisquoi Bay due to the high phosphorus levels already present. Phosphorus concentrations in Missisquoi Bay have been at an average of 0.045 mg/L from 1999 to 2003. The water quality criteria for phosphorus set by Vermont, Quebec and New York are 0.025 mg/L (annual mean) for Missisquoi Bay (Potamis et al. 2004). The reduction of phosphorus loads in waterbodies might play a role in preventing the health hazards that toxic cyanobacterial blooms pose, by decreasing the cyanobacterial abundance, as well as by decreasing the cyanotoxin content (Watanabe and Oishi 1985; Sivonen 1990; Rapala et al. 1997; Orr and Jones 1998; Oh et al. 2000).

The Missisquoi Bay watershed was the largest contributor of phosphorus to the lake, compared to all other parts of Lake Champlain. It is estimated that over

90% of the phosphorus load to Missisquoi Bay comes from non-point sources. About 25% of the watershed is used for agriculture (62% forested and 5% urban), 79% of the non-point source phosphorus comes from agricultural areas. Agricultural fields are obviously an important non-point source of phosphorus into the Missisquoi Bay region (Potamis et al. 2004).

1.4.2 Manure application

Manure has traditionally been spread on land and used as a fertilizer. Manure is a valuable resource, containing nitrogen, phosphorus and other effective nutrients essential for crop growth, and maintaining soil structure and improving soil physical properties by the addition of organic matter. A good manure practice includes safe storage, proper handling, application and distribution. Otherwise, poor manure management has many environmental risks. For instance, surface application of manure creates a high risk of being carried into waterbodies (Brown et al. 2005; Cantin 2006). Losses of phosphorus and nitrogen from agricultural fields have been regarded as a major source of these elements in waterbodies.

In Quebec, more than 20 million tons of solid manure and 15 million tons of liquid manure are applied annually on the land (Cantin 2006). Farmers usually apply manure twice a year, in the spring and in the fall, both as a fertilizer and as a way to dispose of the manure. Recent research has demonstrated that soils fertilized

with manure would have a potential to release more phosphorus into waterbodies than chemically fertilized soil (Jiao 2005).

In the spring, manure is applied before planting; some nutrients may be lost by leaching or runoff. The maximum use of nutrients and availability of the fertilizer to the crop is closely related to the weather conditions between the time of the spreading and plant uptake. Rain events are a key point determining the effectiveness of the manure as a fertilizer. Rapid incorporation of manure into the soil after spreading is a good practice to reduce odors and reduce the transport of different forms of nitrogen and phosphorus, and pathogenic microorganisms to surface waters (Cantin 2006).

Manure application in the fall after harvesting, as a way of manure disposal is a common practice for many agricultural producers in Quebec. Now this practice is viewed as highly risky, because there is no crop to absorb the nutrients on the surface. The nutrients have a high risk of entering surface waters during snow-melt and rain (Brown et al. 2005). In southeastern Quebec, at least 25 percent of the annual precipitation is in the form of snow. As much as 30 percent of the annual runoff may occur between March 1 and April 15 due to snow melting on frozen soil (Gangbazo et al. 1997). In Quebec, manure spreading is not allowed between October 1 and March 31 (Srinivasan et al. 2006).

Manure also contains a rich variety of bacteria, viruses and parasites. Although manure is a beneficial soil amendment, disease-causing organisms and other pathogens from manure can affect animals and humans. In agricultural runoff,

according to a project in Vermont, the mean *Escherichia coli* levels are 10^6 *E. coli* /100mL in runoff from fresh manure application (Braun and Meals 2005).

The Missisquoi Bay watershed is an area with high density of livestock, and corn is the most common crop (Simard 2005). Manure application produced by livestock is an issue related to nutrient levels in the lake in this area. Manure is a major source of microorganisms in the surface and ground waters. The presence of *E. coli* in the lake water is a strong indication of recent sewage or animal waste contamination. The presence of *E. coli* in the lake water indicates potential fecal contamination and the possible presence of other enteric pathogens.

1.5 Indicator organisms

Indicator organisms, commonly being found in the digestive tract of animals and humans, are widely used to assess the microbiological safety of drinking water and recreational water. In the past, total coliforms have been used as indicators of water quality, but there are many other genera in this group, such as *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Aeromonas*, that are commonly present in unpolluted water. In recent years, *Escherichia coli*, enterococci, and fecal coliforms are considered as better fecal indicators. The U. S. Environmental Protection Agency recommends the use of *E. coli* as an indicator organism for recreational freshwaters (Anderson et al. 2005).

For the microbiological guideline in drinking water, *E. coli*, fecal coliforms and total coliforms should not be detectable. In 1992, Health Canada recommended

microbiological guideline values for recreational water quality “In fresh recreational water, the standard is 200 *E. coli*/100 mL or 200 fecal coliforms/100 mL when experience demonstrates that over 90% of the fecal coliforms are *E coli*.”

A number of studies have demonstrated that some strains of *E. coli* can persist and multiply in water reservoirs, and free-living *E. coli* surviving in external environments has been reported (Korhonen and Martikainen 1991; Power et al. 2005). This limits the effectiveness using *E. coli* as an indicator of fecal contamination. But the increase of *E. coli* numbers in waterbodies over season was an important indicator of recent facal contamination.

Plate counts based on specialized culture media is a traditional method for *E. coli* detection. In 1976, Kilian and Bulow first reported the association of the enzyme β-glucuronidase with the genus *Escherichia* (97% positive) and a β-glucuronidase assay was suggested as a useful identifier of *Escherichia* (Kilian and Bulow 1976a; b). Since then, different media that incorporate β-glucuronidase substrates have been developed for *E. coli* detection in food, clinical samples, water and other materials (Rice et al. 1990).

Plate counting methods only identify viable cells: cells with the ability to divide and grow. The viable but nonculturable state is a survival mechanism of bacteria under stress conditions, and this state has been investigated in many Gram-negative bacteria, including *E. coli* and *E. coli* O157:H7. Viable but nonculturable bacteria are unable to grow and form colonies on conventional culture media, but

maintain metabolic activity and pathogenicity. They can return to a normal state under optimal conditions in some cases (Lleo et al. 2005).

PCR based methods for *E. coli* detection allows the detection of viable but nonculturable cells. The *uidA* gene, coding for β-glucuronidase, is often chosen as the target region (Bej et al. 1991a; Bej et al. 1991c; Juck et al. 1996). β-glucuronidase is the first enzyme in the metabolic pathway of the degradation of hexuronides and hexuronates in *E. coli* (Novel and Novel 1976). Feng and colleagues (1991) demonstrated that the *uidA* gene was present in about 94% of *E. coli* and 44% of *Shigella* sp. and 29% of *Salmonella sp*. The *uidA* sequences were detectable in MUG-negative *E. coli* isolates. Similarly, an average of 34% of *E. coli* isolated from 35 human fecal samples were β-glucuronidase negative as tested by MUG media (Chang et al. 1989). So for *E. coli* detection, the sensitivity of *uidA* PCR is higher than the culturing method. The *uidA* sequences in *E. coli* and some *Shigella* strains have 98% similarity based on GeneBank, but as *Shigella* species are naturally found in the intestinal tracts of humans and other primates, the chance of their appearance in Missisquoi Bay lake water was small. Therefore, the *uidA* was used as a target for *E. coli* detection in the lake water.

Chapter 2 Materials and Methods

2.1 Description of sampling sites and water sampling

The study was conducted in Missisquoi Bay, Quebec, the Canadian part of Lake Champlain. Lake Champlain, the sixth largest freshwater lake in the USA, has a surface area of 1,124 km^2, which lies in the states of Vermont and New York and the Province of Quebec, Canada (Meals 2001). Missisquoi Bay is a shallow bay with a surface area of approximately 77.5 km^2 (19,150 acres); and 58% of the watershed area lies in Vermont and the remaining 42% is in Quebec. The maximum depth of the bay is 5 m and the average depth is 2.8 m. Approximately 23,000 people reside in the Quebec portion of the Missisquoi Bay basin (Gray et al. 2005). Pike River, Rock River and Missisquoi River are the three major tributaries flowing into the bay (Fig. 2.1).

FIG. 2.1. Sampling locations in Missisquoi Bay from 2006 to 2008 and the two sampling sites of Pike River in 2008. The three major tributaries of Missisquoi Bay are indicated. (Modified from http://www.ijc.org/rel/boards/missisquoi_bay/map_champlain_e.pdf)

Sampling was conducted in Missisquoi Bay from May to November in 2006, from May to December in 2007 and from May to July in 2008, at two week or one week intervals, depending on the biomass of phytoplankton. One to two liters of water were collected at the surface of the water using pre-sterilized bottles. Samples were collected from the two sites indicated in Fig. 2.1. One was offshore (the pelagic station) and the other was in the shore area where dense blooms usually accumulated (the littoral station). When there were strong winds on the sampling date, only the littoral station was sampled (Bird 2005). The mouth of the Pike River and a site far from the mouth were sampled as illustrated in Fig. 2.1 for *E. coli* detection in 2008.

Filtration was performed under vacuum pressure (10 psi) using an autoclaved filtration unit. For cyanobacterial analysis, after receiving the water samples (usually within 48h), around 250 mL water (400mL for July 18, 2006) was aseptically passed through a 1.2-μm pore size GF/C glass fiber filter (Whatman, Florham, NJ, USA). The GF/C filters were wrapped in aluminum foil and heated at 500 °C for 3 hours before being used for filtration. The filtrate of the GF/C was collected aseptically and then filtered though a Millipore filter (polyvinylidene fluoride) (Millipore, Bedford, MA) with a 0.22-μm pore size. Samples were processed in duplicate or triplicate. One hundred milliliter of water (on July 12 and July 18, 2006) was filtered though the Millipore filters. Each filter was put in a 50 mL Falcon tube and stored frozen at -80 °C until the DNA was extracted.

Filtration for *E. coli* detection by PCR was performed close to a flame. After receiving the lake samples (usually within 48h, sometimes in 72h), a 100 mL

water sample was aseptically filtered through 0.2- μm pore size hydrophilic polyethersulfone filters (PAL Corporation, Ann Arbor, MI, USA). Each sample was prepared in duplicate or triplicate and each filter was placed in a 50 mL Falcon tube and stored frozen at -80 °C until the DNA was extracted.

2.2 *Escherichia coli* detection of lake samples using MI agar plates

E. coli detection by culturing was conducted according to USEPA Method 1604 (United States Environmental Protection Agency 2002). BBL™ MI agar was purchased from Becton, Dickinson and company (Mississauga, ON). Two enzymes substrates, fluorogen 4-Methylumbelliferyl-β-D-galactopyranoside (MUGal) and chromogen Indoxyl-β-D-glucuronide (IBDG), are included in the MI agar media. β-D-glucuronidase produced by *E. coli* cleaves the chromogen IBDG to form a blue or indigo colored compound; β-galactosidase produced by total coliforms (TC) cleaves the fluorogen MUGal to produce a fluorescent compound when exposed to 366 nm UV light.

Briefly, 10mL, 25mL or 50mL water samples were aseptically filtered through 47mm, 0.45-μm pore size cellulose ester membrane filters (Whatman) under vacuum (10 psi); the filter with grid-side up was placed on MI agar, then the plate was incubated at 35°C for up to 24 hours. Colonies growing on plates were examined for the presence of blue color (*E. coli*) and fluorescence under long-wave ultraviolet light (366 nm) (TC).

E. coli O157:H7, the most common Shiga toxin producing *E. coli*, as it is MUG negative, MI agar is unable to differentiate it. BBL™ CHROMagar™ O157 purchased from Becton, Dickinson and company, is a selective medium for the isolation and differentiation of pathogenic *E. coli* O157:H7. Fifty mL or 100 mL water samples were aseptically filtered as for the MI agar, then the filters were placed on CHROMagar™ O157 plates, incubated at 35°C for up to 24 hours, and checked for light mauve to mauve colonies under ambient light. The mauve colored colonies were put on MacConkey II Agar (Becton, Dickinson and company) to confirm the results; MacConkey II is a selective and differential medium for the detection of coliforms and enteric pathogens.

2.3 Molecular characterization of water samples

2.3.1 Total genomic DNA extraction from filters

2.3.1.1 Total genomic DNA extraction from the GF/C and the Millipore filters

The DNA captured on the GF/C filter (GF/C filtrand) and the Millipore filter (Millipore filtrand, pre-filtered on the GF/C) was recovered using a modified Hisbergues protocol (2003).

First, 5 mL TES buffer (50mM Tris-HCl, pH8.0, 100mM EDTA, pH8.0, 0.25% sucrose) was added to the filter in a 50 mL Falcon tube; lysozyme was added to a final concentration of 0.5 mg/mL, and incubated at 37°C for 1 h while mixing with the Roto-torque rotator (Cole Parmer, Anjou, QC) at high speed 4; 1 h later,

proteinase K was added to a final concentration of 150 µg/mL. The sample was mixed with the Roto-torque rotator at high speed 4 for another 1 h. Sodium dodecyl sulfate (SDS) was added to a final concentration of 2%, and incubated at 50°C for 2 h. The supernatant was transferred to a new Falcon tube and the membrane washed with an additional 2.5 mL TES buffer. The solution was extracted with one volume of chloroform/isoamyl alcohol (24:1) by centrifuging 5 min at room temperature at 3823 X g. Ribonuclease (RNase) (10mg/mL) was added to a final concentration of 100 ng/µL and incubated at 37°C for 1 h. The solution was extracted with one volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by chloroform/isoamyl alcohol (24:1). The DNA was precipitated overnight at -20°C by the addition of an equal volume of cold 2-propanol. The DNA mixture was centrifuged 30 min at 4°C at 12,100 X g. The pellet was washed with 70% ethanol, dried, and resuspended in TE pH 8.0. The DNA was aliquoted and stored at -20°C.

2.3.1.2 Total genomic DNA extraction from hydrophilic polyethersulfone filters

DNA captured on the 0.2-µm pore size hydrophilic polyethersulfone filters was extracted according to a modified hot phenol method (Fortin et al. 2009).

First, 5 mL lysis buffer (50 mM Tris-HCl, pH8.0, 50 mM EDTA, pH8.0, 0.5% SDS, 50 mM sucrose, 100 mM NaCl) was added to the Falcon tube containing the filter; lysozyme was added to a final concentration of 5 mg/mL; 125 µL 20% SDS and 92.5 µL proteinase K (20mg/mL) was added to each tube. The mixture was

incubated at 37°C for 2 h, while mixing with the Roto-torque rotator at high speed 4. One volume of phenol/chloroform/isoamyl alcohol (25:24:1) preheated to 56 °C was added to the tube and incubated at 56°C for 10 min, making sure that filers were completely melted. The solution was extracted with one volume of chloroform/isoamyl alcohol (24:1). RNase A (10 mg/mL) was added to a final concentration of 100 ng/uL and incubated at 37°C for 1h. The solution was then extracted with one volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by a chloroform/isoamyl alcohol (24:1) extraction. DNA was precipitated by the addition of an equal volume of cold 2-propanol overnight at -20°C, and finally centrifuged 30 min at 4°C at 12,100 X g. The pellet was washed with 70% ethanol, dried and resuspended in TE pH 8.0.

DNA extraction from the hydrophilic polyethersulfone filters of the 2006 and the 2007 samples (except samples in November and December in 2007) was performed by Nathalie Fortin (Biotechnology Research Institute, Montreal, Quebec) and Hongmei Jing (the Université du Québec à Montréal, UQAM).

The recovered DNA from the filters was quantified by agarose gel electrophoresis using the ChemiImager ^{TM} system (Alpha Innotech Corporation, San Leandro, CA, USA), or by the PicoGreen method using a Fluorometer Tecan Safire (Tecan, Männedorf, Switzerland) according to the manufacturer's instructions.

2.3.2 PCR amplification of target genes

2.3.2.1 PCR amplification using CYA-MIC and *mcyD* primers

The *Microcystis* and *mcyD* diversity between the GF/C filtrand and the Millipore filtrand was evaluated using oligonucleotide primers (Primers designed by Nathalie Fortin) (Table 2.1) for the *Microcystis* 16S rRNA gene (CYA-MICF1GC and CYA-MICR1) and part of the polyketide synthase module (587bp) of the *mcyD* gene (*mcyD*F1GC and *mcyD*R2) (Nishizawa et al. 1999; Nishizawa et al. 2000; Tillett et al. 2000).

TABLE 2.1. Oligonucleotide sequences of primers used in this study

Target gene	Direction	Primer sequence(5' to 3')	Annealing (°C)	Fragments (bp)
mcyD	*mcyD*F1GC	[a] TGG GGA TGG ACT CTC TCA CTT C	55	587
	*mcyD*R2	AAC TCC CGT CTG ACT GTT GCG	55	
	*mcyD*F1	TGG GGA TGG ACT CTC TCA CTT C	58	127
	*mcyD*R1	GGC TTC AAC ATT CGG AAA ACG	58	
Microcystis 16S rRNA	CYA-MICF1GC	[a] GCT TGC GTC TGA TTA GCT AGT TG	55	327
	CYA-MICR1	CGG ACG CTT TAC GCC CAA TAA T	55	
	CYA-MICF1	GCT TGC GTC TGA TTA GCT AGT TG	65	
uidA	*uidA* 858	ATCACCGTGGTGACGCATGTCGC	60	486
	*uidA*1343	CACCACGATGCCATGTTCATCTGCC	60	
	*uidA*1047	TATGAACTGTGCGTCACAGCC	60	186
	*uidA*1232	CATCAGCACGTTATCGAATCC	60	

[a] (5'- GCG GGC GGG GCG GGG GCA CGG GGG GCG CGG CGG GCG GGG CGG GGG-3') was the sequences of the GC-clamp added for DGGE analyses.

PCR was performed in a total volume of 50 µL, containing 1-10 µL template DNA, 0.125 mg/mL bovine serum albumin (BSA), 25 pmol of forward (with a GC clamp) or reverse oligonucleotide primers (Table 2.1), 200 µM deoxynucleoside triphosphates (dNTPs), 1 X PCR buffer, 1mM $MgCl_2$, and 2.5 units of Taq

polymerase (GE Healthcare). Oligonucleotides were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). PCR was performed in a Biometra T 3000 Thermocycler (Montreal Biotech Inc. Montreal, QC) or Icycler Thermal Cycler (Biorad, Mississauga, ON).

A touch down PCR was performed to increase the specificity of the PCR amplification. The annealing temperature was set at 65°C and decreased by 1°C at each cycle for 10 cycles, followed by 20 additional cycles with the annealing temperature at 55°C. The PCR starts with 5 min at 96°C for denaturation of the template DNA. The PCR was conducted with total 30 cycles 1 min denaturation at 94°C, 1 min of annealing time, and 1 min extension at 72°C.

2.3.2.2 *E. coli* detection by PCR using the *uidA gene*

PCR was performed to detect *E. coli* in the lake samples. All the samples for *E. coli* analysis for 2006 and 2007 were aliquots of the samples used for *mcyD* Q-PCR analysis by Nathalie Fortin.

Most of the 2006 and 2007 samples used for *uidA* detection amplified well for the *mcyD* Q-PCR by Nathalie Fortin, so the interferences due to PCR inhibitors were negligible. For the very turbid samples taken after heavy rain events and used only for *E. coli* PCR, a spiking test was performed to test the presence of inhibitors. Aliquot of DNA from pure *E. coli* K12 and the DNA template from the sample was added into each PCR tube. The PCR products from the tested samples were compared with the positive controls. The products having negative

or weaker band than the positive controls were samples with inhibitors. Samples with inhibitors were purified with polyvinylpolypyrrolidone (PVPP) and Sephacryl S-400 (Amersham Biosciences) according to the protocol described in Jugnia et al. (2009).

The *uidA* nested PCR primers were from Juck et al. (1996) (Table 2.1). The first PCR conditions were as follows: 96°C for 5 min, 30 cycles of 94°C for 1 min, 1 min at 60°C, and 1 min at 72°C. The second PCR conditions were 96°C for 5 min, followed by 25 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. An *E. coli* K12 strain was used as the positive control.

PCR fragments were separated on gels according to the laboratory manual (Sambrook and Russell 2003) and photographed using the ChemiImager ™ system (Alpha Innotech Corporation).

2.3.3 Denaturing Gradient Gel Electrophoresis (DGGE) analysis using CYA-MIC and *mcyD* primers

DGGE using CYA-MIC and *mcyD* primers was performed using a Bio-Rad DCode system (Bio-Rad, Mississauga, ON) according to the protocol (Fortin et al. 2004). Four to eight PCR tubes from the same sample were combined, precipitated and quantified by gel. For DGGE using CYA-MIC primers, 600 ng PCR products were loaded onto an 8% polyacrylamide gel containing a 40-60% denaturant gradient (100% denaturant consisted of 7 M urea and 40%

formamide); 100 ng PCR products from pure strain of *M. aeruginosa* UTCC 300 and UTCC 632 were used as markers of pure strains.

For the *mcyD* DGGE, 200 ng PCR products were loaded onto an 8% polyacrylamide gel containing a 35-60% denaturant gradient; 100 ng PCR products from pure strains of *M. aeruginosa* UTCC 299 and UTCC 300 were used as markers of pure strains. Electrophoresis was performed at 80 volts for 16h in 1× TAE buffer; the gel was stained with Vistra Green (GE Healthcare) and photographed on a FluorImager System model 595 (Molecular Dynamics, Sunnyvale, CA, USA).

Pure strains of *Microcystis aeruginosa* UTCC 299, UTCC 300 and UTCC 632 were kindly provided by Dr. Frances Pick, from the University of Ottawa. *M. aeruginosa* UTCC 299 and UTCC 300 are microcystin producers, while *M. aeruginosa* UTCC 632 does not produce microcystin.

2.3.4 DGGE fragment isolation and sequencing

Several bands were cut from the polyacrylamide gel, eluted in 30 ul sterile deionized water. DNA was reamplified using the same primers without the GC clamp; PCR was performed in an Eppendorf Mastercycler Gradient (Brinkmann, Montreal, QC) with the following conditions: 96°C for 5 min, 25 cycles of 1 min at 94°C, 30 sec at 65°C, and 1 min at 72°C. PCR products were purified with the GFX^{TM} DNA purification kit (GE Healthcare) according to the manufacturer's

instruction, and prepared for sequencing at the University of Laval in Quebec, Canada.

2.3.5 Cluster and phylogenetic analyses of DGGE gels

The forward and reverse sequences were aligned using BioEdit program with manual correction to create consensus sequences. Consensus sequences of 16S rRNA gene were analyzed for chimeras using the chimera program on the Ribosomal Database Project II 8.1 online database (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) (Cole et al. 2003), and none were detected. The BLAST algorithm was used to compare the DGGE band sequences with the GeneBank database (Altschul et al. 1990). The *mcyD* sequences were checked by verifying the open reading frame using the MacVector program version 7.2 (Accelrys, Madison, WI, USA). Sequences were submitted for comparison to the EMBL databases using the FASTA algorithm (Pearson and Lipman 1988; Pearson 1990).

The ClustalW program in MacVector or the Clustalw2 tool in the EMBL-EBI website (http://www.ebi.ac.uk/Tools/clustalw2/) was used for multiple alignment analyses of the 16S rRNA gene and the *mcyD* gene sequences using the default setting.

GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium) was used for the analyses of the DGGE banding profile. The Band based Dice method and the unweighted pair group method with arithmetic mean algorithm (UPGMA)

were used for analysis, and the band-based comparison using the similarity coefficient defined by Dice was used to create a similarity matrix. Phylogenetic trees for the 16S rRNA sequences were constructed using the MacVector program version 7.2 software, based on neighbor-joining algorithm and Jukes-Cantor distance to build the best tree. The statistical significance and reliability of inferred topologies were tested by 1,000 bootstrap resamplings of the neighborjoining data.

2.3.6 Quantitative real-time PCR (Q-PCR) using the *mcyD* gene

Oligonucleotide primers (*mcyD*F1 and *mcyD*R1) amplifying a 127bp of the polyketide synthase module of the *mcyD* gene (Nishizawa et al. 1999; Nishizawa et al. 2000; Tillett et al. 2000) were designed for Q-PCR analyses. The forward primer is the same as the one used for the *mcyD* DGGE analysis. Absolute quantification was used for quantitative analysis by creating a standard curve. The standard curve was generated with serial dilutions of plasmid DNA containing part of the *mcyD* gene. The *mcyD* gene copy numbers in the plasmid were calculated based on the plasmid DNA concentration. The *mcyD* standard curve ranging from 23.1 to 2,310,000 gene copies/µL was prepared by Nathalie Fortin, together with the optimization of primers, and $MgCl_2$ concentrations for the *mcyD* Q-PCR (Fortin et al. 2009).

Q-PCR was used to compare the abundance of cells carrying the *mcyD* genes in the GF/C filtrand and the Millipore filtrand. The total reaction volume was 20 µL,

containing 4mM $MgCl_2$, 0.8 uM of both primers and 10 µL reagent of 2x QuantiTect™ SYBR® Green PCR (Qiagen, Mississauga, ON), 2.2 µL RNase free water, 5 µL of a series of 10 fold dilution of lake sample DNA. One standard plasmid (2,310 *mcyD* copies/µL) and 5 mM Tris-Cl pH 8.0 as negative control were included in each run. Each sample dilution was performed in duplicate. The *mcyD* amplification and quantification was performed with Rotor-Gene 3000 software version 6.0 (Corbett Research, Mortlake, NSW, Australia). A hot start at 95°C for 15 min was followed by 45 cycles of 95°C for 10 sec, 58°C for 15 sec, and 72°C for 20 sec. The melting curve analysis was done by raising the temperature from 65 to 95°C, holding for 45 sec on the first step, and holding for 5 sec on the subsequent steps. The melting curve was used to determine the melting temperature of the amplification products including the target and artifact signals (non-specific products and primer dimers). The target products was allowed to be differentiated based on the melt peaks of different fragments.

Dynamic tube normalization was used as a normalization method. Threshold cycle (United States Environmental Protection Agency) values of the samples were calculated by importing the standard curve and adjusting to the Ct values of the plasmid DNA standard used. Samples with PCR efficiency above 0.83 were included in further data analysis (Fortin et al. 2009).

2.3.7 Statistics analysis

The Pearson correlation coefficient function in the Microsoft Excel was used for correlation analysis between the *mcyD* copy number and microcystin concentration, and the *mcyD* copy number and *Microcystis* cells numbers.

2.4 Identification and abundance of cyanobacterial biomass

The identification and abundance of cyanobacterial species were evaluated by Serge Paquet from the laboratory of Dr. David Bird at the Université du Québec à Montréal (UQAM), which was done under an inverted microscope using the Utermöhl's method (1958). The biomass data of 2006 and 2007 were therefore kindly provided by Dr. Bird (Principal Investigator) as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms."

2.5 Chemical analyses of microcystin concentration

Microcystin concentrations in Missisquoi Bay in 2006 and 2007 were determined by Dr. Rocio Aranda-Rodriguez at Health Canada, Ottawa, according to the procedure described in Aranda-Rodriguez et al (2005). In 2006, HPLC and ELISA were used for microcystin analysis; in 2007, only ELISA was used due to the low microcystin concentrations. Microcystin data were therefore kindly provided by Dr. Rocio Aranda-Rodriguez as part of a collaborative NSERC

Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms."

2.6 Nutrient concentrations in Missisquoi Bay

The concentration of total nitrogen (NT), total dissolved nitrogen (NTD), NOx (NO, NO_2 and NO_3), ammonium (NH_4), total phosphorus (PT) and total dissolved phosphorus (PTD) in Missisquoi Bay in 2006, 2007 and 2008 were determined at UQAM. Data was kindly provided by Dr. Bird (Principal Investigator) as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms."

2.7 Contributions of the author and other researchers

This research represents part of a multidisciplinary project involving several research organizations, and as such the thesis contains data that was produced by other researchers. These data were used to help interpret the results reported here, which have been identified in the appropriate figures and tables, and include: Microcystin concentration (HPLC and ELISA) data from Dr. Rocio Aranda-Rodriguez (Health Canada, Ottawa); nutrient data (nitrogen and phosphorus) from Dr. David Bird (UQAM); and cyanobacterial count data from S. Paquet (UQAM). In addition, some *E. coli* count data was produced by C. Maynard (BRI). And some of the DNA extractions for the *E. coli* work were

performed by N. Fortin (BRI) and H. Jing (UQAM). In all cases in the thesis, these data have been identified and credited to the appropriate individuals.

All other data presented here were produced by the author of the thesis.

Chapter 3 Results

3.1 Bloom forming cyanobacteria and microcystin concentration in Missisquoi Bay

Missisquoi Bay is one of the most eutrophic areas of Lake Champlain (Meals and Budd 1998; Watzin et al. 2006). The bay has been affected by cyanobacterial blooms from 2001-2008 and all beaches have been closed each year by the Quebec government due to water safety issues. *Microcystis* and *Anabaena* were potential toxin-producing cyanobacterial genera in Lake Champlain in 2000-2004 (Rosen et al. 2001; Watzin et al. 2002; 2003; Watzin et al. 2004; Watzin et al. 2005) and microcystin was the major cyanotoxin detected in the bay (Bird 2005).

Missisquoi Bay was affected by two blooms in 2006. One was on August 18, where *Microcystis aeruginosa* and *Anabaena spiroides* were the dominant species; the second bloom was on September 12, where *Microcystis aeruginosa and Aphanizomenon flos-aquae* were the dominant species. No obvious bloom occurred in 2007, but a one day bloom was observed on September 21. Extensive blooms formed in mid July in 2008. The biomass data for *Microcystis, Anabaena* and *Aphanizomenon* in 2006 and 2007 are listed in Table 1 and 2 in Appendix.

In 2006, the microcystin concentrations determined by ELISA and HPLC were similar. Microcystins were detectable in the littoral station on all sampling dates, varying from 0.2 to 3.1 μg/L by HPLC (Fig.1 in Appendix). The microcystin

concentration exceeded the 1 µg/L drinking water guideline set by the World Health Organization (Chorus and Bartram 1999) and the 1.5 µg/L Canadian guideline (Health Canada 2003) in most sampling dates from early August to late September. Microcystin concentrations in the pelagic station were detectable on certain sampling dates, with the highest value on September 12 of 2.3 µg/L by HPLC.

In 2007, microcystin concentrations were below the 1 µg/L drinking water guideline throughout the summer. The highest microcystin concentration detected in the littoral station was around 7 ng/L by ELISA, which was nearly 600-fold lower than the maximum value (4, 200 ng/ L) by ELISA in 2006.

3.2 DGGE analysis using the CYA-MIC primers

The *mcyD* gene, indicative of microcystin synthesis, was amplified from the Millipore filtrand as well as the GF/C filtrand in most selected dates in 2006. Fig. 3.1 shows the *mcyD* fragments generated by *mcyD* regular PCR from the littoral station on August 8, 2006. The *mcyD* amplicons were present in the three replicates of both the Millipore filtrand and the GF/C filtrand. The *mcyD* primers designed in this study were able to distinguish microcystin-producing *Microcystis* strains from non-microcystin producing *Microcystis* strains. Molecular tools were used to compare material on the two filters, to characterize the *Microcystis* 16S rRNA genes in both fractions.

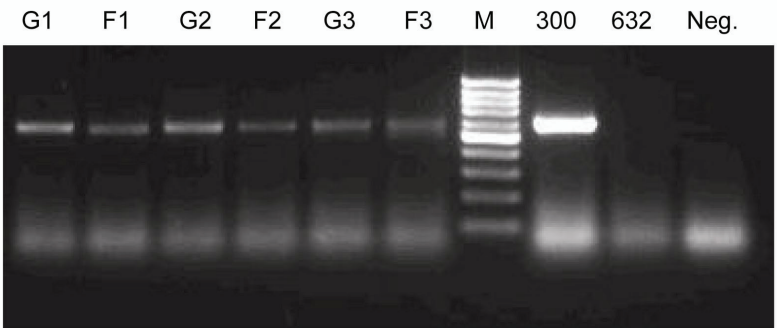

FIG. 3.1. PCR amplification of the *mcyD* gene from samples collected from the littoral station on August 8, 2006 from both the GF/C filtrand and the Millipore filtrand with the *mcyD1* F1GC & *mcyD1*R2 primers. G1, G2, G3: three replicates of the GF/C filtrand; F1, F2, F3: three replicates of the Millipore filtrand; M: 100bp molecular weight ladder; 300: *Microcystis. aeruginosa* UTCC 300 (positive control); 632: *M. aeruginosa* UTCC 632 (negative control); Neg: sterile water.

3.2.1 DGGE analysis using CYA-MIC primers (filters with different pore sizes)

To determine whether there were differences in the DGGE patterns by using filters with different pore sizes, the GF/C (1.2 μm) and the Millipore (0.22 μm) filters, the DNA recovered from the GF/C filtrand and the Millipore filtrand (not pre-filtered on the GF/C) was analyzed. As shown in the Fig. 3.2, on July 12 and July 18, the banding pattern of the Millipore filtrand (MI) was the same as the GF/C filtrand (G) using CYA-MIC primers. Although only two samples were tested, the banding pattern of the GF/C filtrand represented the major banding pattern of the whole sample.

3.2.2 DGGE analysis using CYA-MIC primers (the GF/C filtrand and the Millipore filtrand)

High molecular weight nucleic acids were isolated from both the GF/C filtrand and the Millipore filtrand (pre-filtered on GF/C) from all selected samples in 2006, 2007 and 2008. 16S rRNA gene amplicons using CYA-MIC primers were present on both filters of selected samples in 2006 and 2007 using regular PCR analyses. DGGE using CYA-MIC primers of the GF/C filtrand and the Millipore filtrand were performed in 2006 and 2007 to compare the two fractions and the seasonal dynamics of *Microcystis*. The numbered bands were excised from the gel (Fig. 3.2) and sequenced. The bands in the same position in a gel were identified by the same number. The closest BLAST matches of the sequenced DGGE bands are listed in Table 3.1. Their sequences are listed in the Appendix.

TABLE 3.1 Characterization of the 16S rRNA gene sequences from the GF/C filtrand and the Millipore filtrand in the DGGE analyses using CYA-MIC primers of selected samples in 2006 and 2007

	Band No.	Accession number	Database Closest Match	Similarity (%)
2006	06-1	DQ065390	Uncultured freshwater bacterium	99
		DQ522211	*Leptospira interrogans*	96
	06-2	AJ565421	*Beta proteobacterium* MWH-UniP1	100
	06-3	FJ204841	Uncultured phototrophic eukaryote	100
		FJ002181	*Aulacoseira granulata var. angustissima*	100
	06-4	U40340	*Microcystis aeruginosa* PCC 7941	97
	06-5	AM259220	*Synechococcus* sp. 0tu30s01	99
	06-6	EF583861	*Anabaena* sp.	99
		DQ264167	*Aphanizomenon gracile*	99
	06-7	EU636199	*Anabaena circinalis*	100
		EU157999	*Aphanizomenon flos-aquae*	100
	06-8	EU803737	Uncultured bacterium (Lake Gatun)	100
		EU907928	*Limnobacter* sp.	97
	06-9	EF152370	*Synechococcus* sp.	100
		AM710380	*Cyanobium* sp.	100
	06-10	U40340	*Microcystis aeruginosa* PCC 7941	100
2007	07-1	DQ065390	Uncultured freshwater bacterium	99
		DQ522211	*Leptospira interrogans*	96
	07-2	AJ565421	*Beta proteobacterium* MWH-UniP1	100
	07-3	AM710355	*Cyanobium* sp. JJ21RS4	100
		AM259221	*Synechococcus* sp. 0tu28s07	100
	07-4	EF152370	*Synechococcus* sp. KUAC 3039	99
		AM710378	*Cyanobium* sp. JJ9-A3	100
	07-5	U40340	*Microcystis aeruginosa* PCC 7941	100

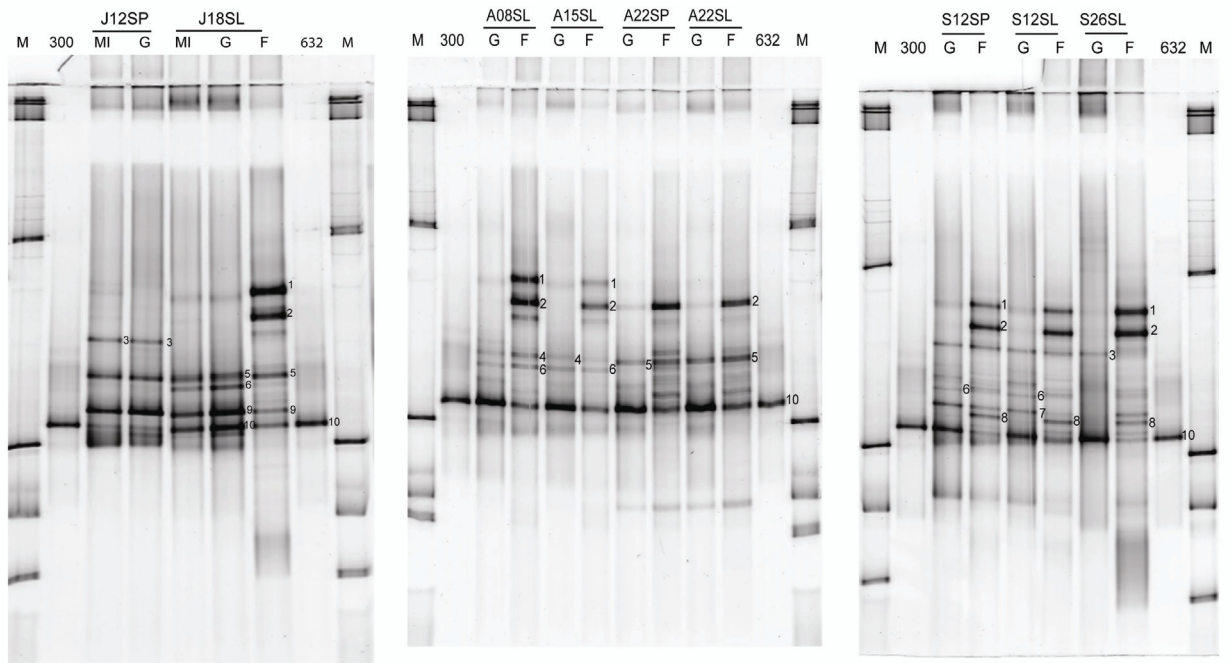

FIG. 3.2. DGGE pattern using CYA-MIC primers of the GF/C filtrand and the Millipore filtrand from selected samples collected in Missisquoi Bay of Lake Champlain in 2006. PCR products (600 ng) of lake samples were loaded onto an 8% polyacrylamide gel containing a 40-60% denaturant gradient. PCR products (100 ng) of *M. aeruginosa* UTCC 300 (300) and UTCC 632 (632) were used as positive controls. The samples were labeled by sampling month-day-station, (ie. J12SP: the sample was taken on July 12 from the pelagic station). M: 100bp molecular weight ladder; G: the GF/C filtrand; F: the Millipore filtrand, pre-filtered on the GF/C; MI: the Millipore filtrand, not pre-filtered on the GF/C; SL: the littoral station; SP: the pelagic station; J: July; A: August; S: September. The number on the gel indicates the band to the left of the number. The bands with numbers were excised and sequenced.

3.2.2.1 DGGE analyses using CYA-MIC primers of selected samples in 2006

DGGE using CYA-MIC primers was performed with the samples collected in July, August and September of 2006 (Fig. 3.2). *M. aeruginosa* UTCC 300 and UTCC 632 were used as markers of pure strains, and bands no. 4 and 10 were highly similar to *Microcystis,* as confirmed by sequencing. *Microcystis* was identified in all the GF/C filtrand in July, August and September, with the lowest band density

on July 12. The other sequences identified were highly similar to *Anabaena*, *Aphanizomenon*, *Synechcoccus* and algae. The sequences identified in sampling date were different. It clearly indicated the banding profile changed over time, before and after the blooms (around August 15 and September 12).

Microcystis was identified in the Millipore filtrand of selected samples. Other sequences identified in the GF/C filtrand were also identified in the Millipore filtrand. However, there were unique bands (no. 1, 2 and 8) only identified in the Millipore filtrand, with high similarity to the uncultured freshwater bacterium *Leptospira*, a *Betaproteobacterium* and an uncultured lake bacterium, *Limnobacter* sp., respectively.

The bacteria identified from the DGGE were compared with the biomass data of 2006 (Table 1 in Appendix). The abundance of *Microcystis* was high (> 9,000 cells /mL) on most of the dates selected for DGGE with minimum abundance on July12 (< 2600 cells/mL), and this agreed with the *Microcystis* detection by DGGE. *Anabaena spiroides* and *Aphanizomenon flos-aquae* were bloom forming species in 2006, and sequences similar to *Anabaena* and *Aphanizomenon* were also identified by DGGE.

3.2.2.2 DGGE analyses using CYA-MIC primers of selected samples in 2007

Fig. 3.3 shows the results of DGGE using CYA-MIC primers for the August and September 2007 samples. *Microcystis* was identified in the GF/C filtrand on August 30 in both stations, on September 5 in the pelagic station and on

September 12 in the littoral station. The band intensity of *Microcystis* was much weaker in 2007 than in 2006. The gels were comparable because about 600 ng PCR products from lake samples were put in each lane. Other sequences identified were mainly *Synechcoccus* sp. and *Cyanobium* sp., belonging to the picocyanobacteria. *Anabaena* and *Aphanizomenon* sequences were not identified in 2007. The GF/C filtrand banding pattern of 2007 was different from that of 2006.

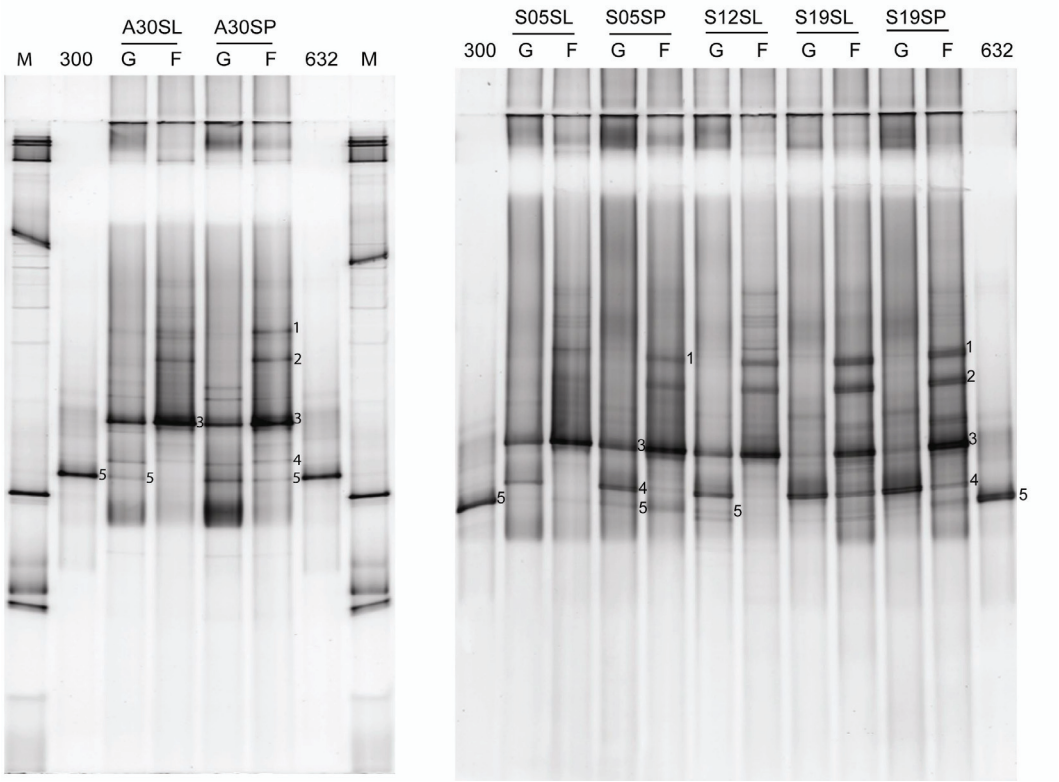

FIG. 3.3. DGGE pattern using CYA-MIC primers of the GF/C filtrand and the Millipore filtrand from selected samples collected in Missisquoi Bay of Lake Champlain in 2007. PCR products (600 ng) of lake samples were loaded onto an 8% polyacrylamide gel containing a 40-60% denaturant gradients. PCR products (100 ng) of *M. aeruginosa*. UTCC 300 (300) and UTCC 632 (632) were used as positive controls. The samples were labeled by sampling month-day-station, (ie. A30SL: the sample was taken on August 30 from the littoral station). M: 100bp molecular weight ladder; G: the GF/C filtrand; F: the Millipore filtrand; SL: littoral station; SP: pelagic station; A: August; S: September. The number on the gel indicated the band to the left of the number. The bands with numbers were excised and sequenced.

For the Millipore filtrand in 2007, a *Microcystis* sequence was only confirmed on August 30 in the pelagic station. *Synechcoccus* sp. and *Cyanobium* sp. sequences were also detected. The same unique bands (1 and 2) of 2006 were also identified.

The biomass of *Microcystis* was much lower in 2007 than in 2006. The biomass on August 30 in the littoral station, on September 5 in the littoral and the pelagic stations were 256, 427, 211 cells/mL (Table 2 in Appendix), respectively, with no detectable values for other samples. Green algae and *Synechcoccus* sp. were identified by microscopy from the 2007 lake samples (data not shown).

3.2.2.3 Cluster and phylogenetic analysis of DGGE using CYA-MIC primers

Using GelCompar II to analyze the DGGE banding pattern of 2006 in the littoral station (the pelagic station was not considered as only two samples were available), it clearly showed that the Millipore filtrand grouped into a cluster and separated from the GF/C filtrand (Fig. 3.4. I). For 2006, in the littoral station of the GF/C filtrand group, August 8 and August 15 (close to the first bloom) had highly similar banding profiles, September 26 (after the second bloom) had a different pattern and was separated from the rest of the dates. The results clearly demonstrated that the GF/C filtrand banding profiles changed over time.

The 2007 GelCompar II cluster results also showed that the GF/C filtrand and the Millipore filtrand separated into two distinct clusters. In both stations, the GF/C filtrand pattern of August 30 was different from that of the September samples

(Fig. 3.4. II and III). Phylogenetic trees were constructed to analyze the relationship of the DGGE sequences using CYA-MIC primers in 2006 and 2007 (Fig. 3.5 and 3.6). *Aquifex pyrophilus* was chosen as the outgroup.

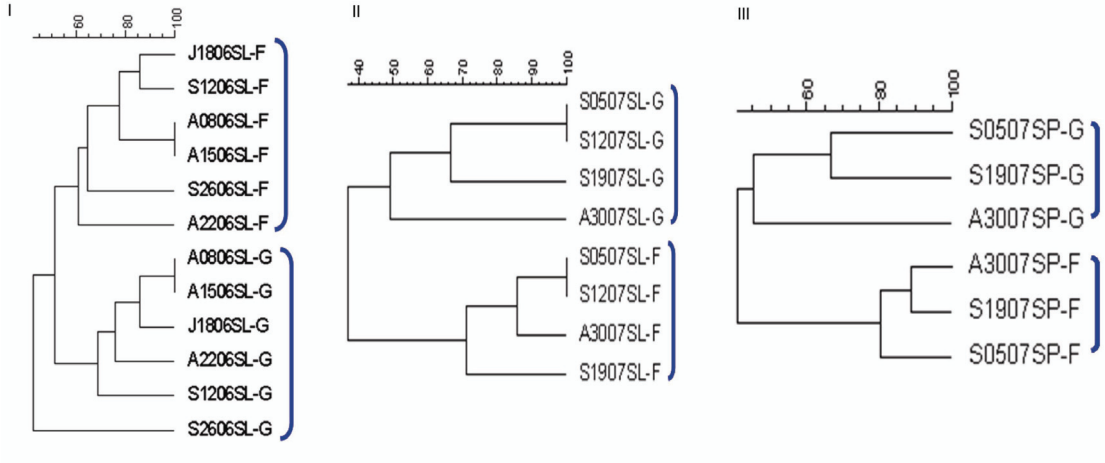

FIG. 3.4. Cluster analysis of DGGE banding profile using CYA-MIC primers, using GelCompar II (band based Dice method and UPGMA). I: the littoral station in 2006; II: the littoral station in 2007; III: the pelagic station in 2007. The samples were labeled by sampling month-day-station-G or -F (ie. J18SL-F: the Millipore filtrand sample was taken on July 18 from the littoral station). G: the GF/C filtrand; F: the Millipore filtrand; J: July; A: August; S: September; SL: the littoral station; SP: the pelagic station. The scale bar on the top of each panel indicates the similarity coefficient defined by the Dice method.

As shown in Fig. 3.5, all 10 bands identified in 2006 were divided into 4 branches, the first and the fourth branches constituted bacteria identified only in the Millipore filtrand. The second branch contained *Microcystis* and *Synechcoccus* species. The third branch contained *Anabaena*, *Aphanizomenon* and eukaryotes. Most sequences detected in 2006 in the GF/C filtrand fraction were related to bloom forming cyanobacteria and picocyanobacteria.

In 2007, all five bands fell into 4 clusters; the first and the fourth clusters were bacteria in the Millipore filtrand; the second was *Microcystis*; the third was

Synechcoccus and *Cyanobium* (Fig. 3.6). The main sequences detected in 2007 in the GF/C filtrand fraction were picocyanobacteria.

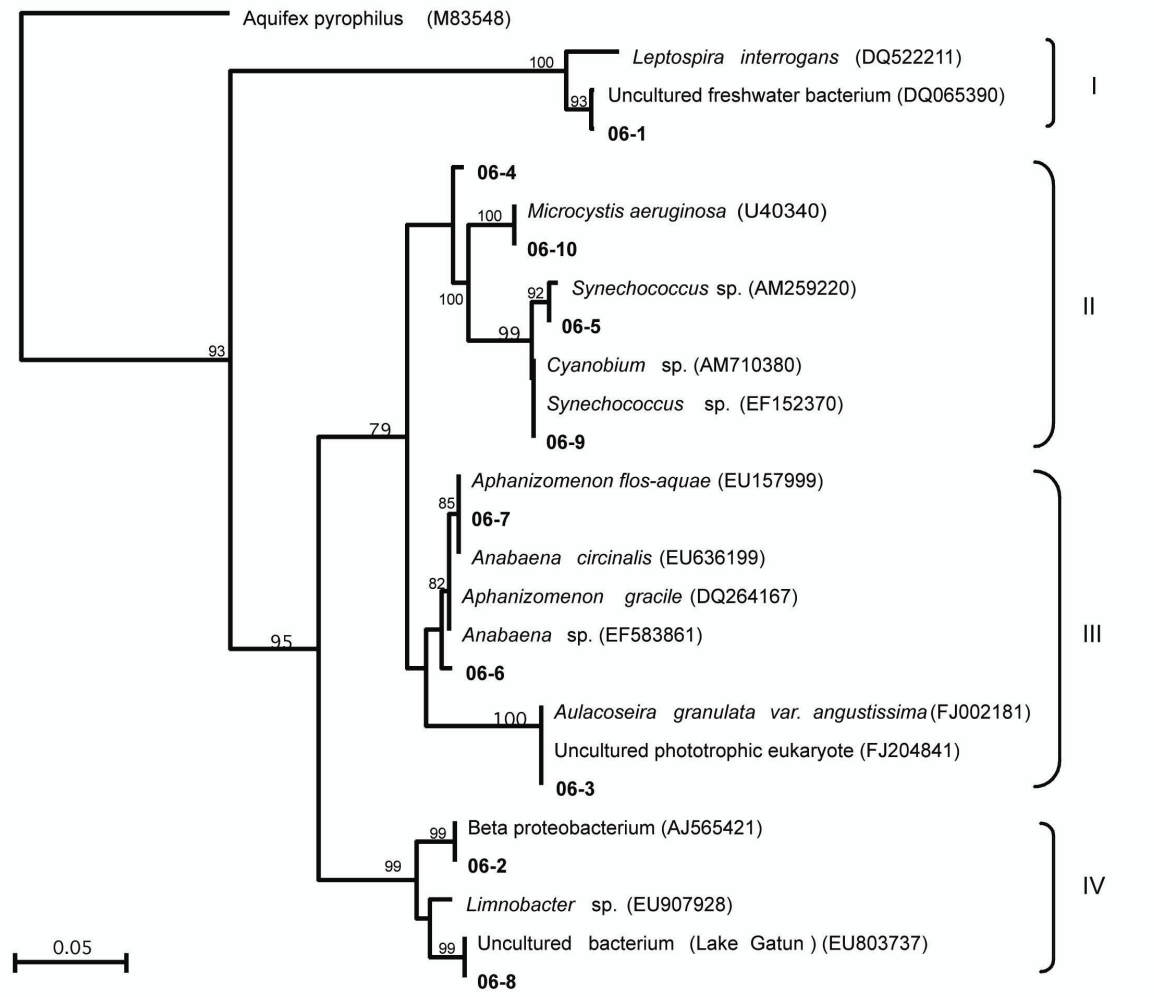

FIG. 3.5 Phylogenetic tree of DGGE gene sequences using CYA-MIC primers identified during the summer of 2006 (Fig. 3.2). Neighbor-joining algorithm and Jukes-Cantor corrections were carried out using the Clustal W in MacVector. DGGE bands were indicated as in Fig. 3.2 with the addition of 06, and the reference sequences were those with accession numbers in parenthesis. *Aquifex pyrophilus* was chosen as the outgroup. I, IV: bacteria in the Millipore filtrand; II: *Microcystis*, *Synechcoccus* and *Cyanobium;* III: *Anabaena, Aphanizomenon and eukaryotes.* Numbers on the nodes are the percentage of bootstrap values based on 1,000 replicates. Bootstrap values lower than 50 are not indicated on the nodes. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

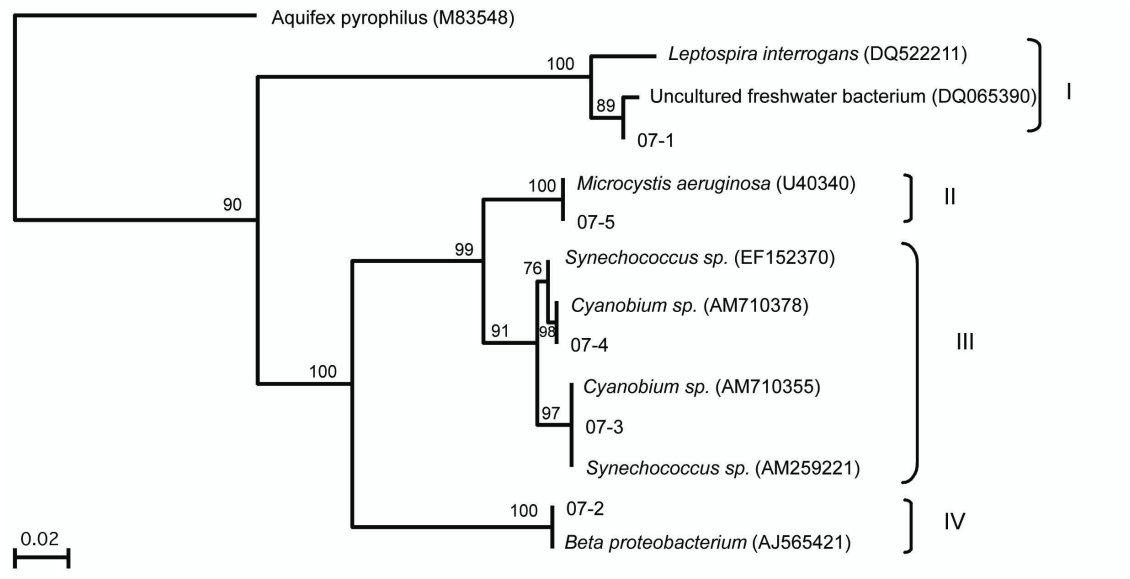

FIG. 3.6. Phylogenetic tree of DGGE gene sequences using CYA-MIC primers identified during the summer of 2007 (Fig. 3.3). Neighbor-joining algorithm and Jukes-Cantor corrections were carried out using the Clustal W in MacVector. DGGE bands were indicated as in Fig. 3.3 with the addition of 07, and the reference sequences were those with accession number in parenthesis. *Aquifex pyrophilus* was chosen as the outgroup. I, IV: bacteria in the Millipore filtrand; II: *Microcystis*; III: *Synechcoccus* and *Cyanobium*. Numbers on the nodes are the percentage of bootstrap values based on 1,000 replicates. Bootstrap values lower than 50 are not indicated on the nodes. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

In general, the GF/C filtrand banding pattern using CYA-MIC primers was different from the Millipore filtrand in 2006 and 2007. DGGE and sequencing analyses revealed seasonal changes in the dynamics of cyanobacterial populations. The unique bands high similar to freshwater bacteria were only identified in the Millipore filtrand and also *Microcystis*, *Anabaena* and *Aphanizomenon* cells were present in the Millipore filtrand. The GF/C filter is not ideal for characterization of all bacterial species in the sample, because some bacteria, including picocyanobacteria, can pass through the larger pore size of

the GF/C filters. Using filters with a smaller pore size such as 0.22 μm for the characterization of cyanobacterial blooms is a better choice to reduce the possible error.

3.3 Filter comparison of the microcystin synthetase gene (the GF/C filtrand and the Millipore filtrand)

Sequences highly similar to *Microcystis*, *Anabaena* and *Aphanizomenon* were identified in the Millipore filtrand. Because *Microcystis* and *Anabaena* are potential microcystin producers, it was necessary to know the genotype of the microcystin polyketide synthase gene and its abundance in the Millipore filtrand. The *mcyD* DGGE and Q-PCR were performed to compare *mcyD* genotypes and gene abundance between the GF/C filtrand and the Millipore filtrand.

3.3.1 The *mcyD* DGGE analyses of selected samples

Oligonucleotide primers (*mcyD1*F1GC & *mcyD1*R2) were designed to compare the *mcyD* genotypes between the GF/C filtrand and the Millipore filtrand by DGGE in 2006. As shown in Fig. 3.7, *M. aeruginosa* UTCC 299 and UTCC 300 were used as markers of pure strains, and a 15 bp deletion was identified between the two *mcyD* fragments on the gel (Fig.3.8). The *mcyD* fragments were detected on both fractions in 2006. The *mcyD* sequences identified in the Millipore filtrand were identical to the sequences identified in the GF/C filtrand.

The *mcyD* sequences (listed in Appendix) identified in the lake samples could be divided into two genotypes, and their sequence similarity to the polyketide synthase in different strains is presented in Table 3.2. One genotype was the same as *M. aeruginosa* UTCC 300 and was identical to *M. aeruginosa* PCC7806 (AM778952). The other type contained a 15 bp deletion and was highly similar to *M. aeruginosa* UTCC 299 and *M. aeruginosa* NIES-843 (AP009552), with several nucleic acid differences as indicated in Fig. 3.8 A, but highly similar based on amino acid sequences (Fig. 3.8. B).

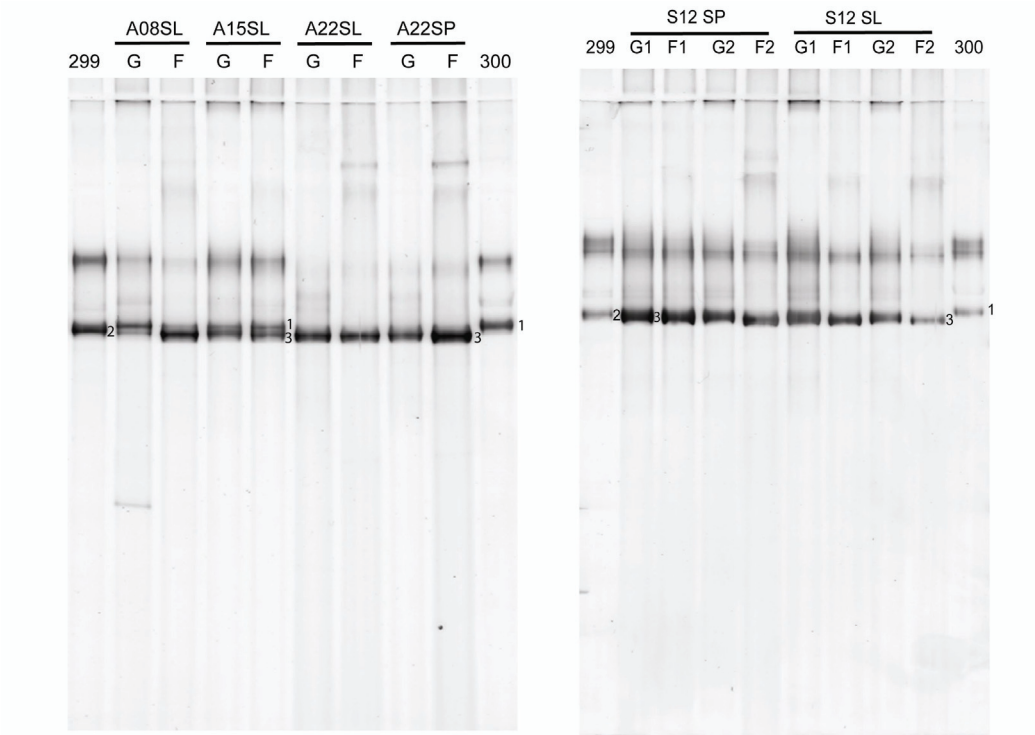

FIG. 3.7. DGGE analyses of the *mcyD* gene of the GF/C filtrand and the Millipore filtrand in August and September of 2006. PCR products (200 ng) were loaded onto an 8% polyacrylamide gel containing 35-60% denaturant gradients; PCR products (100 ng) from pure strains of *M. aeruginosa* UTCC 299 (299) and 300 (300) were used as positive controls. The samples were labeled by sampling month-day-station (ie. A08SL: the sample was taken on August 8 from the littoral station). G: the GF/C filtrand; F: the Millipore filtrand; SL: the littoral station; SP: the pelagic station; A: August; S: September. The numbers on the gel indicate the bands to the left of the number. On September 12, two replicates were collected at both stations.

A

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3-3         GTTCTCTCCCTTCGACGATTACTTTTCGTTTTCCGAATGTTGAAGCCTTAGCTAATTATT 109
3-2         GTTCTCTCCCTTCGACGATTACTTTTCGTTTTCCGAATGTTGAAGCCTTAGCTAATTATT 120
3-1         GTTCTCTCCCTTCGACAATTACTTTTCGTTTTCCGAATGTTGAAGCCTTAGCTAATTATT 110
AP009552    GTTCTCTCCCTTCGACAATTACTTTTCGTTTTCCGAATGTTGAAGTCTTAGCTAATTATT 110
2           GTTCTCTCCCTTCGACAATTACTTTTCGTTTTCCGAATGTTGAAGTATTAGCTAATTATT 110
1           GTTCTCTCCCTTCGACAATTACTTTTCGTTTTCCGAATGTTGAAGCCTTAGCTAATTATT 110
AM778952    GTTCTCTCCCTTCGACAATTACTTTTCGTTTTCCGAATGTTGAAGCCTTAGCTAATTATT 107
            *************** ***************************  *************

3-3         TACAGCGAGAAGTTCTAGATAATTCTCAACCGGTATTTACCCCTCAAATAAAAGCAGAAA 169
3-2         TACAGCGAGAAGTTCTAGATAATTCTCAACCGGTATTTACCCCTCAAATAAAAGCAGAAA 180
3-1         TACAGCGAGAAGTTCTAGATAATTGTCAACCGGTATTTACCCCTCAAATAAAAGCAGAAA 170
AP009552    TACAGCGAGAAGTTCTAGATAATTGTCAACCGGTATTTACCCCTCAAATAAAAGCAGAAA 170
2           TACAGCGAGAAGTTCTAGATAATTGTCAACCGGTATTTACCCCTCAAATAAAAGCAGAAA 170
1           TACAGCAAGAAGTTCTAGATAATTGTCAACCAGTATTGACCCCTCAAATAAAAGCAGAAA 170
AM778952    TACAGCAAGAAGTTCTAGATAATTGTCAACCAGTATTGACCCCTCAAATAAAAGCAGAAA 167
            ****** ***************** ****** ***** **********************

3-3         TTTCTCAAAAACAGTCAGAAAAATC----------------ACAACTCGATGATGATCCTA 214
3-2         TTTCTCAAAAACAGCCAGAAAAATC----------------ACAACTCGATGATGATCCTA 225
3-1         TTTCTCAAAAACAGCCAGAAAAATC----------------ACAACTCGATGATGATCCTA 215
AP009552    TTTCTCAAAAACAGCCAGAAAAATC----------------ACAACTCGATGATGATCCTA 215
2           TTTCTCAAAAACAGCCAGAAAAATC----------------ACAACTCGATGATGATCCTA 215
1           TTTCTCAAAAACAGCCAGAAAAATCCTCTCTGTAGAAAAATCACAACTCGATGATGATCCTA 230
AM778952    TTTCTCAAAAACAGCCAGAAAAATCCTCTCTGTAGAAAAATCACAACTCGATGATGATCCTA 227
            ************** **********               ********************
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B

```
2           SLTSTELRNLLQTDFNCSLPSTITFRFPNVEVLANYLQREVLDNCQPVFTPQIKAEISQK 60
BAG03682    SLTSTELRNLLQTDFNCSLPSTITFRFPNVEVLANYLQREVLDNCQPVFTPQIKAEISQK 60
3-2         SLTSTELRNLLQTDFNCSLPSTITFRFPNVEALANYLQREVLDNSQPVFTPQIKAEISQK 60
3-3         SLTSTELRNLLQTDFNCSLPSTITFRFPNVEALANYLQREVLDNSQPVFTPQIKAEISQK 60
3-1         SLTSTELRNLLQTDFNCSLPSTITFRFPNVEALANYLQREVLDNCQPVFTPQIKAEISQK 60
1           SLTSTELRNLLQTDFNCSLPSTITFRFPNVEALANYLQQEVLDNCQPVLTPQIKAEISQK 60
CAO90228    SLTSTELRNLLQTDFNCSLPSTITFRFPNVEALANYLQQEVLDNCQPVLTPQIKAEISQK 60
            ********************************.******:******.***:************

2           QPEKS-----QLDDDPIVIVGMACRFPGGAKNLQSFWELLEQGKDAITEIPQERWDQETW 115
BAG03682    QPEKS-----QLDDDPIVIVGMACRFPGGAKNLQSFWELLEQGKDAITEIPQDRWDQETW 115
3-2         QPEKS-----QLDDDPIVIVGMACRFPGGAKNLQSFWELLEQGKDAITEIPQDRWDQETW 115
3-3         QSEKS-----QLDDDPIVIVGMACRFPGGAKNLQSFWELLEQGKDAITEIPQDRWDQETW 115
3-1         QPEKS-----QLDDDPIVIVGMACRFPGGAKNLQSFWELLEQGKDAITEIPQDRWDQETW 115
1           QPEKSSVEKSQLDDDPIVIVGMACRFPGGAKNLQSFWELLEQGKDAITEIPQDRWDQETW 120
CAO90228    QPEKSSVEKSQLDDDPIVIVGMACRFPGGAKNLQSFWELLEQGKDAITEIPQDRWDQETW 120
            *.***     ****************************************:*******
```

FIG. 3.8. Multiple alignment of the *mcyD* sequences from the 2006 DGGE analyses and two sequences from GeneBank (AP009552 and AM778952 for DNA, BAG03682 and CAO90228 for protein). A represents DNA sequences and B represents protein sequences. Alignments showed the deletion in DNA and protein sequences and the nucleic acid and the amino acid differences between sequences.

TABLE 3.2. Characterization of the *mcyD* gene sequences from the GF/C filtrand and the Millipore filtrand: comparison of *mcyD* DGGE analyses of the selected samples in 2006

	Band1	Band 2	Band 3
Polyketide synthase in strain *Microcystis aeruginosa* NIES-843 (AP009552)	95.2%	98.9%	98.9%, 99.3%,99.5%
Polyketide synthase in strain *Microcystis aeruginosa* PCC 7806 (AM778952)	100.0%	95.6%	95.2%, 95.3%,95.4%

The *mcyD* genotypes determined on the different sampling dates changed over time in 2006. Before August 15, two sequence genotypes were identified; after August 15, only sequence genotype with the 15 bp deletion was identified. The main microcystin isoforms determined by HPLC changed as indicated in Fig. 1 in Appendix. In the littoral station, MC-LR and MC-LA were the two main microcystin isoforms before August 15, while after August 15, MC-LA was the main microcystin isoform with a higher concentration than the other MC isoforms. In the pelagic station, MC-LA was the major isoform on September 12.

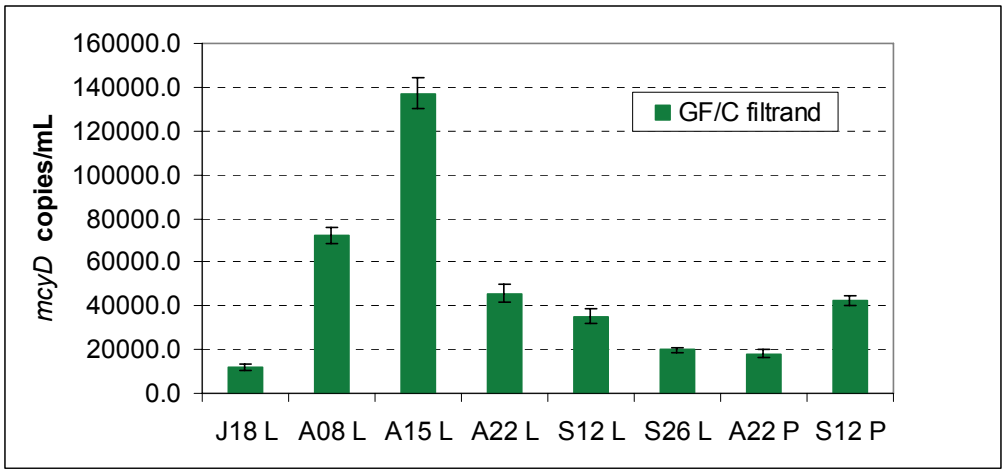
M. aeruginosa UTCC 299 mainly produced MC-YR, and *M. aeruginosa* UTCC 300 produced mainly MC-LR (Susan Leblanc, University of Ottawa, personal communication). The hypothesis was that the *mcyD* sequences that were the same as *M. aeruginosa* UTCC 300 might also produce the MC-LR, and that the sequences similar to *M. aeruginosa* UTCC 299 might be responsible for the production of the MC-LA. There is currently not enough evidence to support the hypothesis that the two *mcyD* sequence types might be related to the observed differences in microcystin isoforms.

In 2007, the *mcyD* gene was not identified on the GF/C filtrand or the Millipore filtrand in selected samples by regular PCR (data not shown).

3.3.2 Q-PCR comparative analysis of *mcyD* gene abundance

The *mcyD* gene was detected in most of the Millipore filtrand fractions during *mcyD* DGGE analyses, so Q-PCR was performed to assess the *mcyD* gene abundance in the GF/C filtrand and the Millipore filtrand.

The *mcyD* Q-PCR results of both filters in July, August and September in 2006 are shown in Fig. 3.9. In 2006 in the GF/C filtrand fraction, the *mcyD* copy numbers were above 20,000 copies/mL in most selected samples. The copy numbers of the *mcyD* gene reached a maximum on August 15 in the littoral station. The *mcyD* copy number in the Millipore filtrand were much lower than in the GF/C filtrand: 5 of 8 samples were below 100 copies/mL, while the maximum copy number of almost 300 copies/mL was determined on August 15. The *mcyD* copy number ratio of the GF/C filtrand versus the Millipore filtrand was high, as shown in Table 3.3, which meant that most of the *mcyD* genes were detected in the GF/C filtrand. Although the *mcyD* gene was identified in the Millipore filtrand, its abundance was much lower, and would not result in an underestimation of the total *mcyD* copy numbers determined by GF/C filtrand. It was concluded that the GF/C filter was effective for microcystin analysis.



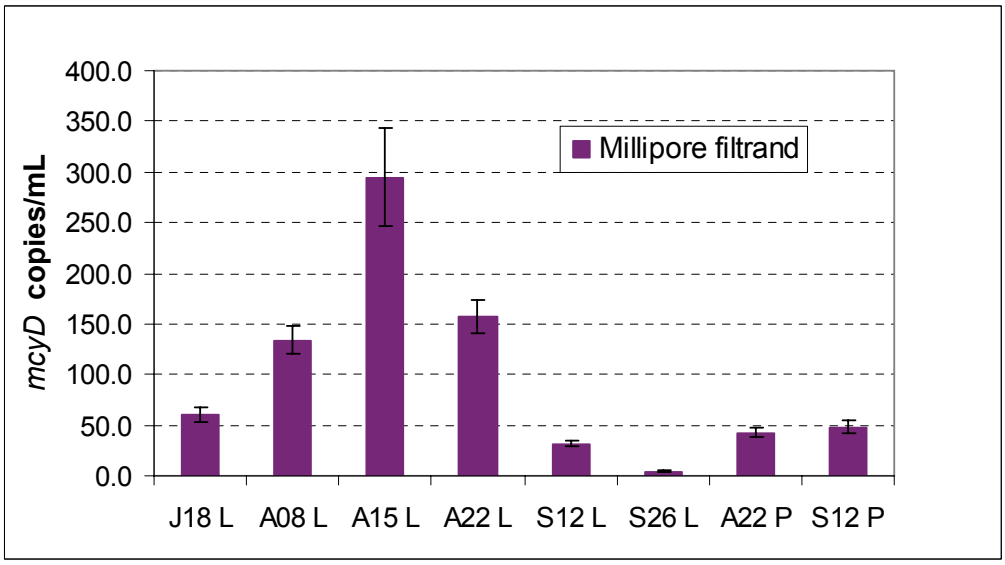


FIG. 3.9. Q-PCR analyses of the *mcyD* gene detected in the GF/C filtrand and the Millipore filtrand during the summer of 2006. The samples were labeled by sampling month-day-station (ie. J18L: the sample was taken on July 18 from the littoral station). J: July; A: August; S: September; L: littoral station; P: pelagic station.

TABLE 3.3. Ratio of *mcyD* copy number determined by Q-PCR in the GF/C filtrand (G) versus the Millipore filtrand (F) of selected samples in 2006 and 2008

Station	Sampling date	G/F (copies/mL) ratio
Littoral	18/07/2006	198
	08/08/2006	539
	15/08/2006	466
	22/08/2006	291
	12/09/2006	1103
	26/09/2006	4570
Pelagic	22/08/2006	425
	12/09/2006	883
Littoral	15/07/2008	2392
	28/07/2008	333
Pelagic	15/07/2008	448

The *mcyD* Q-PCR was performed on August 30 and September 19 in 2007 in both stations to confirm the results of regular PCR. The *mcyD* copy number in the GF/C filtrand did not have good reaction efficiency, and was below the reliable detection limit of the *mcyD* Q-PCR (33 copies/25μL PCR reaction). The *mcyD* gene copy number in the Millipore filtrand was below the lowest detection limit of Q-PCR (data not shown).

The *mcyD* copy number ratio of the GF/C versus the filtrate on July 18, 2006 was lower than the other samples in 2006. Based on observation during the filtration process, our hypothesis was that before the blooms, the colonies were relatively smaller than during the bloom, and that these small potentially toxic cells would very likely pass through the GF/C filters. During the 2008 bloom, *mcyD* Q-PCR was performed in several selected samples (Fig. 3.10). The *mcyD* abundance ratio of the GF/C filtrand versus the Millipore filtrand was high, similar to 2006, which confirmed the low *mcyD* copy numbers in the Millipore filtrand.

Unfortunately, the data in 2008 could not be used to test our hypothesis because we did not perform the double filtration strategy prior to the bloom.

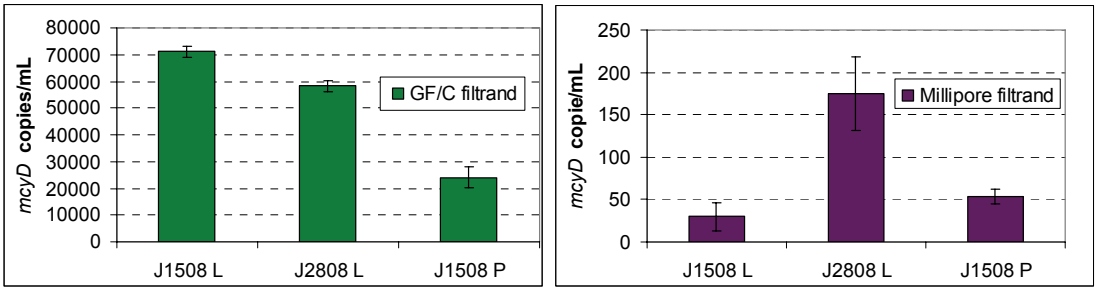
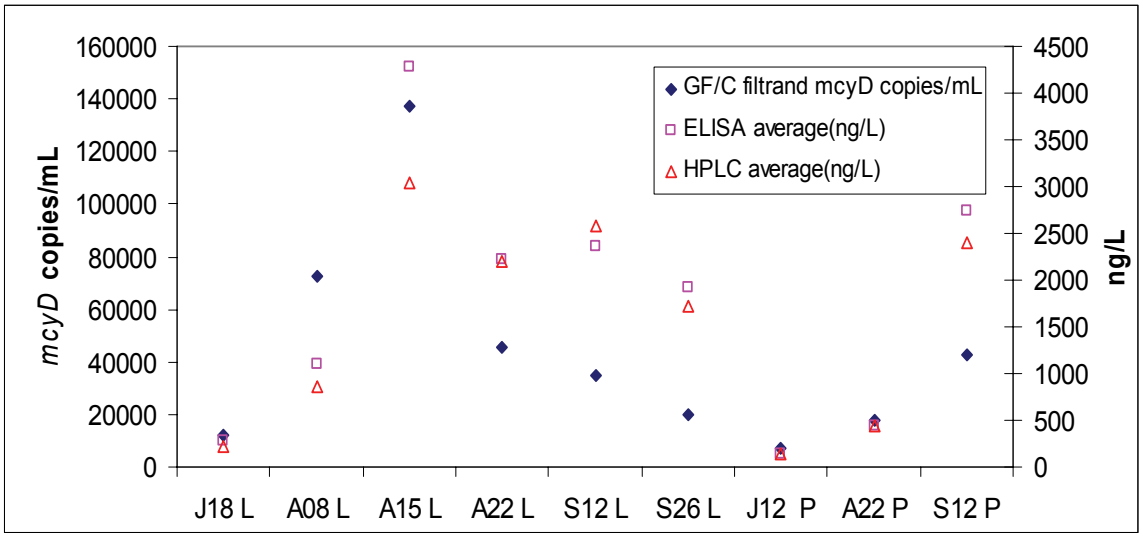


FIG. 3.10. Q-PCR analyses of the *mcyD* gene detected in the GF/C filtrand and the Millipore filtrand in July 2008. The samples were labeled by sampling month-day-year-station (ie. J1508L: the sample was taken on July 15, 2008 from the littoral station). J: July; L: littoral station; P: pelagic station.

3.3.3 Relationship between *mcyD* Q-PCR copy number and microcystin concentration

The relationship between the *mcyD* copy number in the GF/C filtrand fraction (the Millipore filtrand fraction was not considered due to the low copy number) determined by Q-PCR and microcystin concentration by ELISA and HPLC in 2006 is presented in Fig. 3.11. The Pearson coefficients of the *mcyD* copy number and ELISA, and of the *mcyD* copy number and HPLC were 0.79 and 0.65, respectively. In 2007, the microcystin concentration detected by ELISA was low, and on August 30 and September 19 was 1.1 ng/L and 0.6 ng/L, respectively. The *mcyD* copy number in these selected samples was very low or below the detection limit on both filters.



Note: The microcystin concentration data determined by ELISA and HPLC was kindly provided by Dr. Rocio Aranda-Rodriguez (Health Canada) as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms."

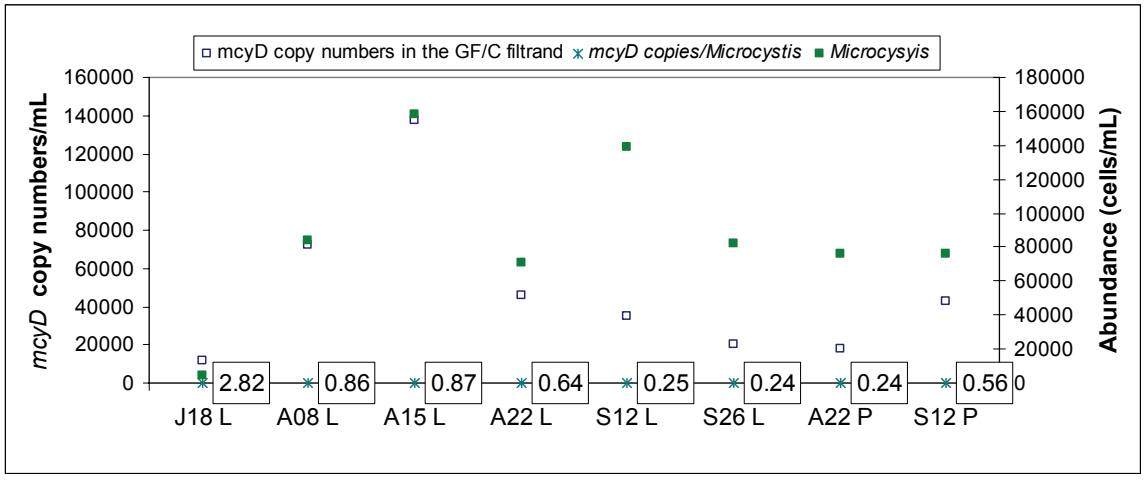
FIG. 3.11. The relationship between the *mcyD* copy number/mL and microcystin concentration determined by ELISA and HPLC in Missisquoi Bay during the summer of 2006. The samples were labeled by sampling month-day-station (ie. J18L: the sample was taken on July 18 from the littoral station). J: July; A: August; S: September; L: littoral station; P: pelagic station.

The *mcyD* Q-PCR results had a good correlation with the microcystin concentration determined by ELISA and HPLC in 2006. The *mcyD* Q-PCR can be used as a rapid and sensitive method for microcystin detection in Lake Champlain, compared to chemical analysis.

3.3.4 Relationship between *mcyD* copy number and *Microcystis* abundance in 2006

To determine the relationship between the *mcyD* copy number and *Microcystis* abundance, the *mcyD* copy number in the GF/C filtrand (the *mcyD* copy number in the Millipore filtrand was negligible) was plotted against the abundance of

Microcystis as determined microscopically. The *mcyD* copy number correlated with the biomass of *Microcystis* with a Pearson coefficient of 0.69. The percentage of total *Microcystis* carrying the *mcyD* gene was calculated based on the assumption that each *Microcystis* cell contained only one copy of the *mcyD* gene. As presented in Fig. 3.12, three of the eight samples had a percentage around 0.2, and 5 out of 8 samples were above 0.5, with the highest value of about 2.8 on July 18. Microcystin and non-microcystin producing *Microcystis* were coexisting in the lake water. In the littoral station, the portion of microcystin producing *Microcystis* cells changed over time. This may be a factor in determining microcystin production.



Note: The *Microcystis* biomass data was kindly provided by Dr. David Bird (Principal Investigator) as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms."

FIG. 3.12. Relationship of the *mcyD* copies/mL by Q-PCR and the abundance of *Microcystis* during the summer of 2006. The value in the square at the bottom was the *mcyD* copy number versus total biomass of *Microcystis.* The samples were labeled by sampling month-day-station (ie. J18L: the sample was taken on July18 from the littoral station). J: July; A: August; S: September; L: littoral station; P: pelagic station.

Anabaena is a potential microcystin producer, but we do not know if the *mcyD* primers designed in this study were able to identify microcystin-producing *Anabaena* because we did not have a microcystin-producing *Anabaena* pure strain to confirm this.

In conclusion, the *mcyD* gene copy number detected in the Millipore filtrand was much lower than in the GF/C filtrand. The GF/C filter captured most of the *mcyD* gene, which meant it could capture most microcystin. The GF/C filter was a good choice for identification of the *mcyD* genes, and for microcystin analysis especially considering the chemical characteristics of glass fiber filters, the fast flow rate and the low cost. The *mcyD* Q-PCR closely correlated to the microcystin concentration in the bloom events, and could be used as a rapid and sensitive way for microcystin detection, monitoring and to serve as a warning system for potential algal bloom.

3.4 *Escherichia coli* detection

3.4.1 *E. coli* detection in 2006 by nested PCR

Nested PCR was only performed for the 2006 samples, and the results are shown in Table 3.4. Each sample had two replicates, and three kinds of PCR results (both negative, or one negative and one positive or both positive) are shown for the different samples.

Based on the culture and PCR results of 2007, we believed that when both replicates were positive, there was a high possibility that there were more *E. coli*

colonies than from other samples. In 2006, positive replicates were detected on May 30 (in the littoral station), June 13 (in both stations), September 26 (in the littoral station), and November 6 (in both stations).

TABLE 3.4. PCR analyses of Missisquoi Bay samples in 2006 using nested primers for β-glucuronidase (*uidA*) to detect *E. coli*

Station	Sampling date	Nested PCR results
Littoral	23/05/2006	+/+
	30/05/2006	-/-
	13/06/2006	+/+
	12/07/2006	-/-
	18/07/2006	+/-
	02/08/2006	+/-
	15/08/2006	+/-
	22/08/2006	+/-
	12/09/2006	-/-
	26/09/2006	+/+
	16/10/2006	+/+
	06/11/2006	+/+
Pelagic	30/05/2006	+/-
	13/06/2006	+/+
	12/07/2006	+/-
	02/08/2006	-/-
	22/08/2006	+/-
	12/09/2006	+/-
	16/10/2006	-/-
	06/11/2006	+/+

Note: +/+: positive for two replicates; -/-: negative for two replicates; +/-: two replicates, one positive and one negative.

3.4.2 *E. coli* detection by plates and nested PCR in 2007 and 2008

E. coli O157:H7 was not detected by CHROMagar in all tested samples, and some putative colonies checked by MacConkey II plate proved to be negative.

Both culture and PCR methods were used for *E. coli* detection in the 2007 and 2008 samples. Total coliforms were detected in all selected samples with

different values. The colonies of total coliforms were underestimated due to the very small colonies and difficulty to distinguish the fluorescent colonies from the non-fluorescent ones in some samples. The total coliforms results together with the *E. coli* results are presented in Tables 3.5 and 3.6.

In 2007, *E. coli* detection results by the culture method showed that at least one *E. coli* colony per 100 mL lake water was detected with the exception of two samples (July 6 in the littoral station and August 2 in the pelagic station).

TABLE 3.5. Colony numbers of *E. coli* and total coliforms on MI agar plates and PCR analyses using *uidA* nested primers for Missisquoi Bay samples in 2007

Station	Date	*E. coli* /100mL	Nested PCR	TC/100mL
Littoral	23/05/2007	ND	+/-	ND
	06/06/2007	ND	+/+	ND
	20/06/2007	120	+/+	ND
	06/07/2007	0	-/-	290
	19/07/2007	3	-/-	55
	02/08/2007	2	-/-	42
	22/08/2007	2	-/-	ND
	05/09/2007	2	-/-	TNTC
	21/10/2007	33	+/+	503
	11/11/2007	10	+/+	170
	18/11/2007	32	+/+	112
	21/11/2007	68	+/+	116
	03/12/2007	70	+/+	340
Pelagic	23/05/2007	3 *	+/-	ND
	06/07/2007	1	-/-	144
	19/07/2007	1	-/-	81
	02/08/2007	0	+/-	196
	22/08/2007	5	-/-	ND
	05/09/2007	8	+/-	468

Note:*: results from DC plates; +/+: positive for two replicates; -/-: negative for two replicates; +/-: two replicates, one positive and one negative; ND: data not available; TNTC: Too Numerous To Count. Some results were kindly provided by Christine Maynard.

More *E. coli* colonies (>10) were detected on June 20 and samples from October 21 to December 3 than on the other sampling dates in 2007. The results of

nested PCR based on 30 ng DNA template (each had two replicates) listed in Table 3.2 showed that 8 of the total of 17 samples were negative for two replicates, and positive replicates were the same samples having more *E. coli* colonies on MI agar plate.

In 2008, Pike River was sampled from May to July, and more than 230 *E. coli* colonies per 100mL were cultured from the samples. The value was higher than the standard for recreational water (200 *E.coli*/100mL) in Canada. At least one *E. coli* colony was cultured on MI agar plates in Missisquoi Bay, except on June 25 in the pelagic station. More *E. coli* colonies were detected in Pike River than in Missisquoi Bay. All *uidA* nested PCR results for the Pike River were positive. In Missisquoi Bay, on May 19 and June 1 the littoral station had more *E. coli* colonies by culture and PCR methods (Table 3.6).

TABLE 3.6. Colony numbers of *E. coli* and total coliforms on MI agar plates and PCR analyses using *uidA* nested primers for Missisquoi Bay (MB) and Pike River (PR) samples in 2008

Site	Sampling date	*E. coli* /100mL	Nested PCR	TC/100mL
PR	22/05/2008	234	+/+	TNTC
PR	01/06/2008	2050	+/+	2450
PR mouth	02/06/2008	454	+/+	520
PR mouth	25/06/2008	480	+/+	TNTC
PR	02/07/2008	246	+/+	349
MB littoral	19/05/2008	28	+/+	87
	22/05/2008	2	+/-	310
	01/06/2008	48	+/+	108
	25/06/2008	1	+/-	58
	02/07/2008	1	+/-	36
	15/07/2008	1	+/+	181
MB pelagic	02/06/2008	2	+/-	39
	25/06/2008	0	+/-	TNTC
	15/07/2008	1	+/-	TNTC

Note: +/+: positive for two replicates; -/-: negative for two replicates; +/-: two replicates, one positive and one negative; TNTC: Too Numerous To Count.

At first, we tried to find a relationship between rain events and the presence of *E. coli.* Our hypothesis was that *E. coli* was be more detectable after rain events, as *E. coli* from sewage, land and other sources are being carried into the lake together with the rain. Based on the precipitation results in the Philipsburg, Quebec site from Environment Canada (http://climat.meteo.ec.gc.ca), it was hard to find a link between these two events. *E. coli* was not detected after all the rain events, but most spring and fall samples (in May, June, September, October and November) contained a substantial amount of *E. coli*, and could positively be related to the rain events and manure application.

Samples from May to July in 2007 were used for both culturing and *uidA* (858-1343) PCR after DNA extraction. All samples were negative after 30 cycles of PCR including samples having *E. coli* colonies on plates. Nested PCR of *uidA* was then performed and the positive PCR results agreed with the culture results. Thirty ng of DNA template for the first round of PCR were used for all samples. After all samples were analyzed, it seemed that the nested PCR results based on 30 ng of DNA template had a lower detection limit than the culture method; PCR results were negative for samples that contained *E. coli* colonies by the culture method.

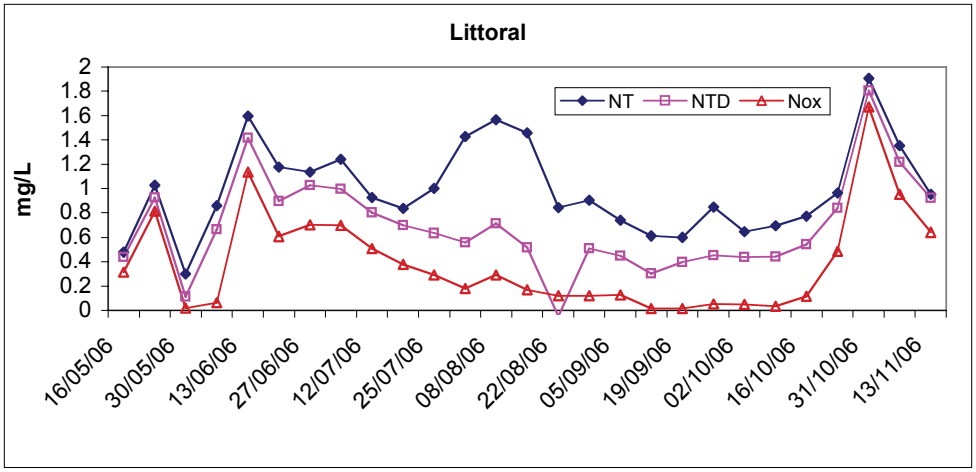
We later realized that this was because we did not use all the sample DNA as a template in the PCR. In most cases, only 1/25 (10µL /250 µL), 1/50 (5µL /250 µL) or less of the total DNA was used as template. The PCR results did not therefore represent the whole sample; in contrast, the culture results represented the total sample as the filters captured all the *E. coli* cells from the water sample. In 2008,

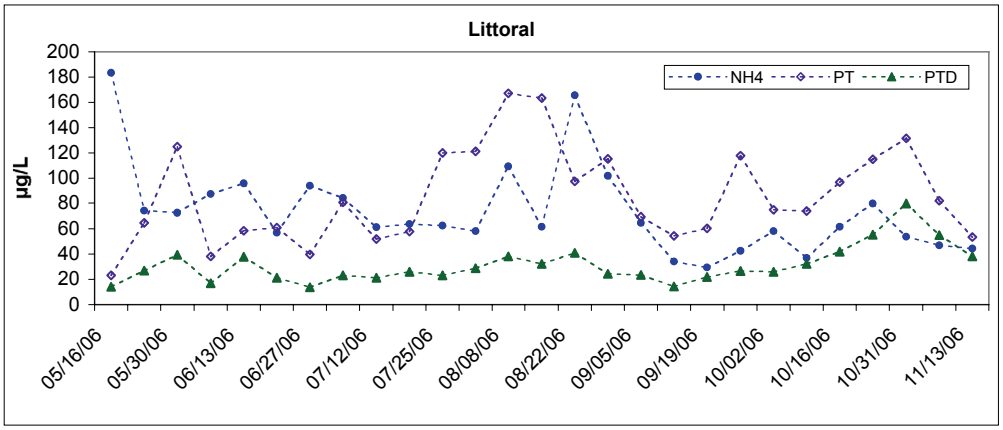
another set of nested PCR were performed by reducing the volume of TE dissolving the template DNA from 250 µL to 50 µL. By increasing the DNA template concentration from 30 ng to 100 ng, the PCR results were all positive for all the 2008 samples (data not shown). The PCR method allowed the detection of viable but non-culturable cells, as there were samples with no *E. coli* colonies on MI agar plates with positive *uidA* results.

3.5 Nitrogen and phosphorus concentrations in Missisquoi Bay

Nitrogen and phosphorus concentrations in Missisquoi Bay in the littoral station in 2006, 2007 and 2008 are plotted in Figs. 3.13-3.15.

In 2006 (Fig. 3.13), the concentration of NT, NTD and NO_X in the littoral station had three peaks in May, June and at the end of October. The total precipitation in May, June and October was 212.4 mm, 161.6 mm and 143.2 mm (higher than the other months in 2006), respectively (http://climat.meteo.ec.gc.ca). The high nitrogen concentration could be positively related to the rain events and the dates with more *E. coli* colonies detected by *uidA* nested PCR. For the PT, PTN and NH_4, there was not an obvious linkage to the rain events and *E. coli* detection as for the NT, NTD and NO_X.



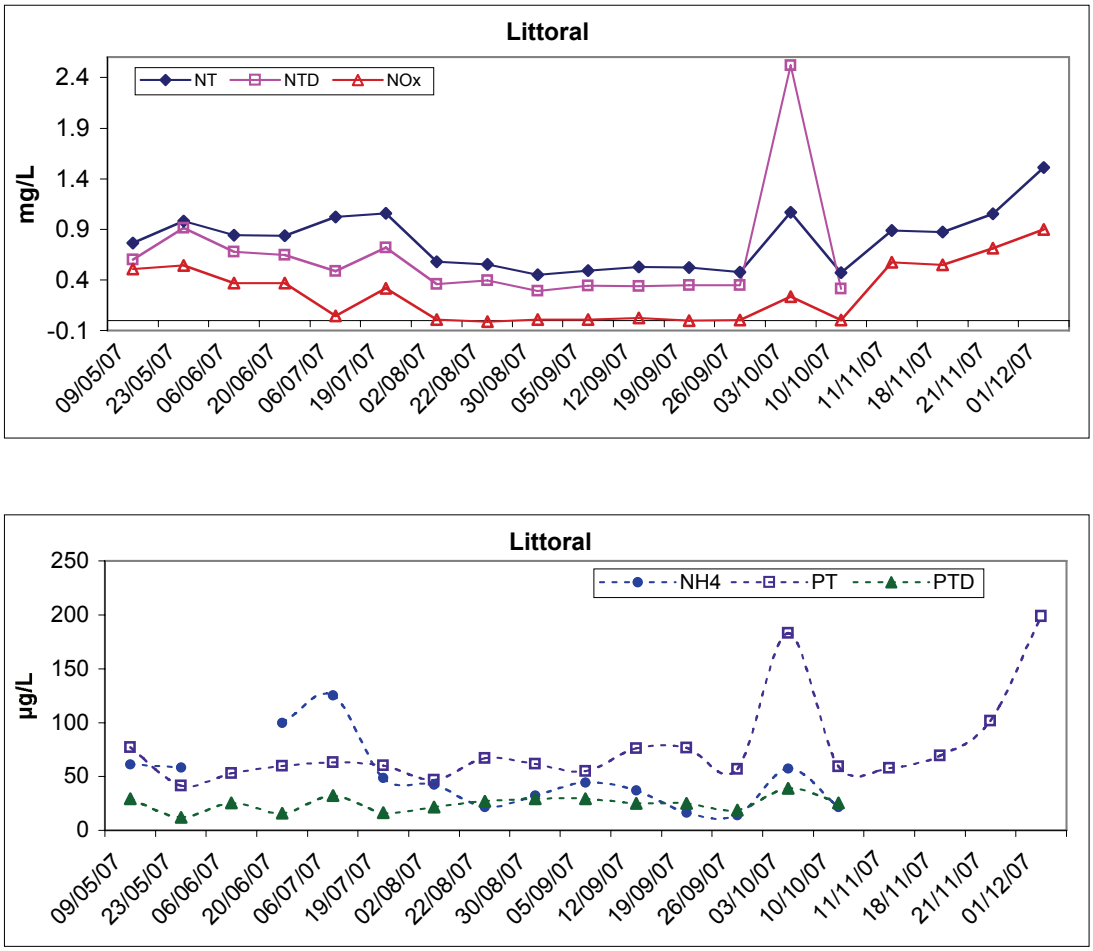


Note: data kindly provided by Dr. David Bird (Principal Investigator) as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms."

FIG. 3.13. Nitrogen and phosphorus concentrations in Missisquoi Bay in the littoral station in 2006. The total nitrogen (NT), total dissolved nitrogen (NTD), NOx (NO, NO_2 and NO_3) were plotted in one figure and expressed in mg/L, ammonium (NH_4), total phosphorus (PT) and total dissolved phosphorus (PTD) plotted in another figure and expressed in µg/L.

In 2007 (Fig.3.14), the concentration of NT, NTD and NO_X did not have obvious peaks as were seen in 2006. The concentration of NT, NTD and NO_X at the end of May were relatively high, however, the concentration of NT, NO_X and PT increased in November and reached the maximum at the end of November 2007,

and also more *E. coli* were detected in those dates. This period followed the application of manure on agricultural lands.

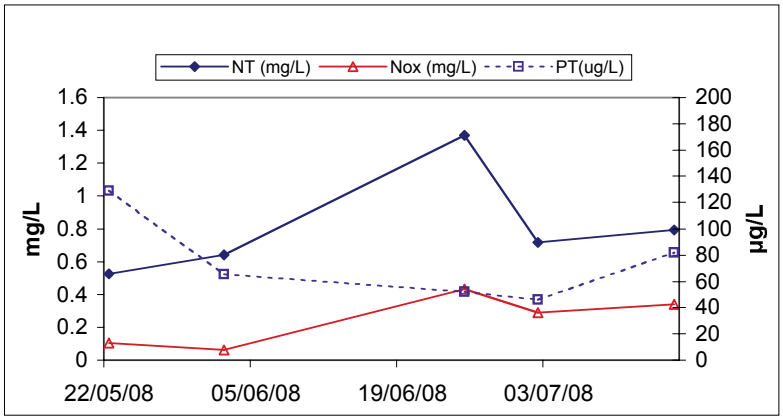


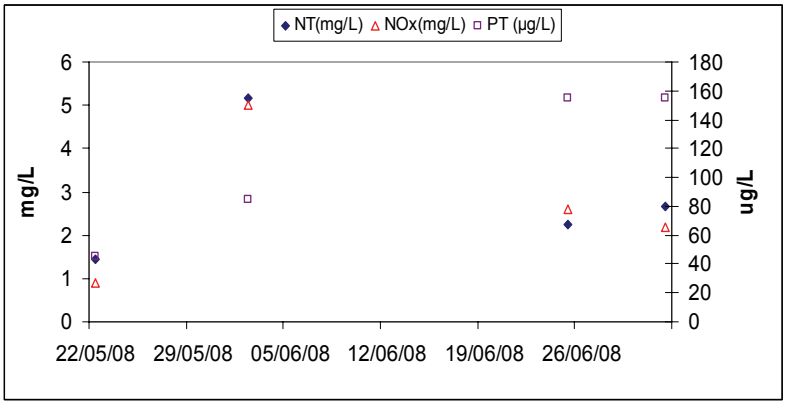
Note: same as in Fig. 3.1.3

Fig. 3.14. Nitrogen and phosphorus concentrations in Missisquoi Bay in the littoral station in 2007. The total nitrogen (NT), total dissolved nitrogen (NTD), NOx (NO, NO_2 and NO_3) were plotted in one figure and expressed in mg/L, ammonium (NH4), total phosphorus (PT) and total dissolved phosphorus (PTD) plotted in one figure and expressed in µg/L.

In 2008, even though limited nutrient data for the Pike River and Missisquoi Bay were available, the NT, NO_X and PT in the Pike River was higher than in Missisquoi Bay, which meant the nutrients were carried by the Pike River into the bay. The dates where more *E. coli* colonies were identified could be related to

the rain events and manure application in the spring. The phosphorus concentration in the bay in the three years were much higher, and had an annual average of 50 µg/L, higher than the 30 µg/L guideline set by the Quebec government.





Note: same as in Fig.3.13

Fig. 3.15. Nitrogen and phosphorus concentrations in 2008 in Missisquoi Bay and Pike River. The total nitrogen (NT), NOx (NO, NO_2 and NO_3) were expressed in mg/L, total phosphorus (PT) was expressed in µg/L.The figure at the left was in Missisquoi Bay, and the figure at the right was in Pike River. In Pike River, the May 22 sample was collected from Pike River site; other samples were collected from the Pike River mouth site.

In conclusion, *Escherichia coli* was chosen as an indicator organism for fecal contamination in the lake water: For three years, *E. coli* detection could be positively related to the rain events and manure application in the spring and the fall. In 2006 and the winter of 2007, the greater number of *E. coli* colonies also corresponded to high NT, NTD and NO_X concentrations. It is very likely that nutrients from agricultural lands were carried into Missisquoi Bay. These data suggested that there is a high possibility that the rain events during manure application contributed to the nutrient concentrations in the lake. At this time, we do not have sufficient data to clearly establish the extent of the correlation between *E. coli* detection, major rain events and manure application. A better manure management practice, for example, avoiding manure spread on lands just prior to heavy rain events, may be necessary for reducing nutrient inputs into Missisquoi Bay from agricultural land, although more data is needed to confirm this point.

Chapter 4 Discussion and Conclusions

4.1 Discussion

In this study, molecular tools were used to characterize the dynamic of CYA-*Microcystis* populations, and evaluate the efficiency of the 1.2 μm GF/C filter at capturing all of the microcystin-producing cyanobacteria in samples destined for microcystin analysis. The relationship between the cyanobacteria blooms and manure application was also evaluated.

Microbial population in the GF/C filtrand and the Millipore filtrand

Using the same primer set, the DGGE banding patterns of the GF/C filtrand differed from the Millipore filtrand. Most sequences related to the cyanobacteria were identified in the GF/C filtrand, but sequences related to freshwater bacteria were only identified in the Millipore filtrand. In environmental microbiology, the PCR products of a specific primer set depend on the components of the microbial population. The GF/C filter captured most cells larger than or equal to 1.2 μm, and cyanobacteria were the major component of the cells in this fraction. The Millipore filter captured most cells of approximately 1.2-0.2 μm in the filtrate of the GF/C filter. Bacteria and picocyanobacteria were the abundant cells in this fraction.

Becker and colleagues (2000) demonstrated that in many PCR reactions, the abundance of the template is a factor affecting amplification: the more abundant templates are more readily amplified, while minor templates are not as efficiently amplified. This seemed to explain the different sequences identified from the GF/C filtrand and the Millipore filtrand. In the Millipore filtrand, bacteria were the dominant population, while in the GF/C filtrand, the abundance of heterotrophic bacteria was much smaller.

Picocyanobacteria

Sequences highly similar to *Synechococcus* and *Cyanobium* were present in both the GF/C filtrand and the Millipore filtrand. *Synechococcus* and *Cyanobium* are single-cell picocyanobacteria, and together with eukaryotic phototrophs are members of the autotrophic picoplankton (size 0.2 – 2 μm). *Synechococcus* cells were identified by microscopy in 2007, but there was not much information available related to the presence of picocyanobacteria in Lake Champlain. Although the lack of specificity of the primers targeting *Microcystis* 16S rRNA was one reason for the identification of *Synechococcus* and *Cyanobium* like sequences, it also demonstrated the abundance of picocyanobacteria in Missisquoi Bay. The dominance of picocyanobacteria in several lakes has been reported. Picocyanobacteria were the major component of picophytoplankton found in the halocline of Lake Suigetsu (Japan) (Okada et al. 2007) and in Lake Ontario (Caron et al. 1985). In Lake Erie, one of the Laurentian Great Lakes (North America), the dominance of *Synechococcus* and *Cyanobium* was

confirmed by sequences in a cyanobacteria 16S rRNA library, and also confirmed by phytoplankton community analysis (Ouellette et al. 2006).

Microcystin analysis

The GF/C filter captured the majority (close to 100%) of the *mcyD* gene; the portion of *mcyD* captured in the Millipore filtrand was negligible. The relationship between colony size and microcystin production has been carried out in several studies. Microcystin concentrations were greatest in the >100 μm colony size class, and decreased with size class (Graham and Jones 2007). In Lake Wannsee in 2000, colonies larger than 500 μm contained 42 to 73% of the microcystin genotype, but the colonies smaller than 500 μm had only 10 to 15% of the microcystin genotype (Kurmayer et al. 2002). The larger size classes of *Microcystis* colonies (>100 μm) showed the highest proportion of microcystin-producing genotypes, microcystin cells, and 83% ± 34% of the total microcystin concentration (Kurmayer et al. 2003). From these studies, it was concluded that the GF/C filter could capture the large size cells together with almost all the microcystin. For microcystin analysis by HPLC, a large volume of water is usually required. Filtration using filters with small pore sizes, such as the 0.2 μm filter, can take hours, especially during a bloom event. Considering the physico-chemical stability, the high flow rate and the cost (Lee et al. 1995), the GF/C filter was a good choice for microcystin analysis.

The *mcyD* copy numbers and microcystin concentration

The *mcyD* copy numbers detected in the GF/C filtrand had a good correlation with the microcystin concentration determined by ELISA and HPLC in 2006. The abundance of *Microcystis* sp. cells containing *mcy* related genes, positively correlated to the microcystin concentration in many studies (Foulds et al. 2002; Kurmayer and Kutzenberger 2003; Vaitomaa et al. 2003; Yoshida et al. 2003; Rinta-Kanto et al. 2005).

Q-PCR is now a more affordable technique in most molecular labs. The amount of water required to obtain the necessary DNA for Q-PCR was much lower than that needed for HPLC analysis. The *mcyD* Q-PCR can be used as a rapid, sensitive and effective method for microcystin monitoring in Lake Champlain. The reliability of *mcyD* Q-PCR in other waterbodies needs to be validated.

Total *Microcystis* and *mcyD* copy number

The proportion of *mcyD* carrying cells in the total *Microcystis* cells ranged from 0.22 to 2.8 in 2006 based on the assumption that each cell contains one *mcyD* copy per genome. It demonstated that toxic and non-toxic *Microcystis* strains coexist in the bay. This result is in agreement with many studies conducted on field samples (Kurmayer et al. 2002; Kurmayer et al. 2003; Kurmayer et al. 2004;

Via-Ordorika et al. 2004; Rinta-Kanto et al. 2005; Kurmayer and Gumpenberger 2006).

The *mcyD* copy numbers were almost three times the total *Microcystis* cells in those studies. A likely possibility is that cyanobacterial species other than *Microcystis* also carry the target gene. *Anabaena* was a bloom-forming genus observed in Missisquoi Bay in 2006, and they also produce microcystins. The *mcyD* primers may also be able to detect the *mcyD* gene in *Anabaena*, but this could not be confirmed experimentally here because a microcystin-producing pure strain of *Anabaena* was not available.

The relationship of *mcy* related gene copy number and the cyanobacterial cell number was different in several previous studies. The ratio of *mcyA* genotypes to colony-forming *Microcystis* cells was 0.01, 0.37 and 2.37 in Lake Mikata in Japan for three samples (Yoshida et al. 2005). The *mcyE* copy numbers determined in *Microcystis* and *Anabaena* were 2 to 200 times more than the cell numbers in Lake Tuusulanjärvi and Lake Hiidenvesi in Finland (Vaitomaa et al. 2003). In all of these studies, the *mcy* gene was quantified by Q-PCR or competitive PCR and the total *Microcystis* abundance was determined by microscopy.

The percentage of microcystin-producing cells from different studies is not comparable. The different portion of microcystin-producing and non-producing cells may be an important factor controlling the total microcystin production profile.

Phosphorus levels

The phosphorus levels from 2006 to early 2008 from both the littoral and the pelagic stations had an annual average higher than 0.05 mg/L, which was much higher than the water quality guideline of 0.03 mg/L set by the Ministère de l'environnement du Québec (MENV) (Simard 2005). Phosphorus concentrations in Missisquoi Bay had an average level of 0.045 mg/L from 1999 to 2003 (Potamis et al. 2004). The phosphorus concentration in Missisquoi Bay has been consistently high for decades, which may be attributed to the high nutrient inputs from agricultural land in this area. The recovery rate of phosphorus has been very slow over the last 10 years, so reducing phosphorus concentrations in Missisquoi Bay could take a long time.

The presence of *E. coli* in 2006 was positively related to nitrogen levels, but not closely related to phosphorus. This may be attributed to the different chemical characteristics of nitrogen and phosphorus and their different cycles. Brown and colleagues (Brown et al. 2005) demonstrated that the concentration of nitrogen was almost twice the concentration of phosphorus in liquid manure (dairy, swine and poultry). Nitrogen was much easier to leach into waterbodies than phosphorus, and that is one reason why the concentration of all the nitrogen forms (except NH_4) is much higher than that of phosphorus in the waterbodies.

Nutrients and cyanobacterial biomass

The dynamics of cyanobacteria and toxin production is controlled by the interaction of many environmental factors. The concentration of nutrients is an important factor controlling the biomass of cyanobacteria. The average nutrient concentrations in Missisquoi Bay in 2006 and 2007 were similar, but extensive toxic blooms only occurred in 2006. The relationship among the nutrients, the cyanobacterial biomass and microcystin production is not well understood. Other factors may play a role in the cyanobacterial blooms. The relationship of nutrients and cyanobacterial biomass has been studied in several lakes. In a study conducted in 22 lakes in southern Quebec, Canada, total phosphorus (PT) was strongly related to the total phytoplankton biomass, the total cyanobacterial biomass, and the total toxigenic cyanobacterial biomass. The microcystin equivalents were better predicted by total nitrogen (TN) concentration than total phosphorus concentration (Giani et al. 2005). Cyanobacterial dominance is more strongly correlated with PT and TN than the ratio of N:P, based on the analysis of data from 99 lakes around the world (Downing et al. 2001). PT was a better indicator of total cyanobacterial biomass than TN and the TN to PT ratio, based on the data collected from16 Alberta lakes (Trimbee and Prepas 1987). PT was strongly correlated with both *M. aeruginosa* biomass and cellular microcystin-LR (MC-LR) in a survey of 12 lakes in central Alberta, Canada, from 1990 to 1994 (Kotak et al. 2000). Different results were reported about the effects of nutrients on cyanobacterial biomass and toxin production, which means that the controlling factors of cyanobacteria were different from one location to another. These

studies suggest that it may not be possible to find a formula that is applicable to all the waterbodies.

Rain events and nitrogen concentrations

A clear trend was found between heavy rain events and the nitrogen concentration in the lake in 2006, but not in 2007. More than 50% of the annual rainfall occurred in the spring and the autumn in Quebec (Cantin 2006), which corresponded to the manure application period. The major portion of annual losses in runoff occurred during one or two intense storms (Cantin 2006). In another study, nitrogen loss was closely related to the rain events and the volume of the runoff (Converse et al. 1976). These studies demonstrated that the manure should not be spread right before heavy rains. This change in agricultural practice would therefore greatly reduce the amount of nutrients carried into the rivers and lakes together with the rain.

PCR method used in *E. coli* detection

The PCR method is more sensitive than culturing assays for the detection of *E. coli*, as confirmed from earlier studies (Bej et al. 1990; Bej et al. 1991a; Bej et al. 1991b). The results of 30 cycles of PCR were negative in this study, due to the low amounts of total DNA in the samples used as template. Nested PCR results were positive based on the modified protocol of 2008. PCR allowed the detection of viable but nonculturable (VBNC) *E. coli* cells. Lleo and colleagues (2005)

applied competitive PCR to quantify the VBNC *E. coli* cells in groundwater in Italy, and the number of VBNC *E. coli* ranged from 24/mL to 70/mL. In this study the presence of VBNC *E. coli* cells was detected by PCR, but the results were not quantified. Q-PCR results will provide more quantitative data for monitoring *E. coli* dynamics in these systems. The *E. coli* number was relatively low at most sampling times. The Q-PCR results using DNA directly from the water samples may be close to the detection limit. An enrichment process may be necessary as was done in two other studies. Credit River water samples were enriched by plating 100 µL of water, followed by growth on LB plates for 5 days (Foulds et al. 2002). Overnight enrichment was used for the detection of *Enterococcus* sp. and *E. coli* in drinking water samples in Mainz, Germany (Frahm and Obst 2003).

PCR also has its limitation, since inhibitors were present in most 2008 samples. Similarly, PCR inhibitors were present in 80% of the tested natural waterbodies in the Credit River in Mississauga, Ontario (Foulds et al. 2002). Inhibitors, which are common in environmental samples, will affect the effectiveness of PCR, and should be considered in all PCR applications. The purification of DNA by PVPP and Sephacryl S-400 used in this study was an effective treatment to remove the inhibitors.

Microbial source tracking

Although the detection of *E. coli* indicates that there is fecal contamination of the lake water, the method used in this study was unable to directly link the *E. coli* to

the origin of the manure. Microbial source tracking can identify the source of the pathogen. Chemical and molecular methods have been used in this research area (Meays et al. 2004; Seurinck et al. 2005). A ribotyping protocol was applied to analyze *E. coli* isolates from one location in Idaho and three locations in Georgia in the USA. The origin of the *E. coli* isolates (cattle, horse, swine and chicken) was separated among host animal species at each location (Hartel et al. 2002). The human and animal origins of *E. coli* have been differentiated in several studies by different methods, which have provided useful information for pollution management strategies (Bernhard and Field 2000a; b; Carson et al. 2001; Scott et al. 2003). If the *E. coli* detected in this study can be linked to livestock origin, it would be possible to establish a more direct link between the manure and the nutrient levels in the lake. This would also provide an incentive to improve manure application strategies to reduce nutrient inputs into Missisquoi Bay.

4.2 Conclusions

In this study, molecular tools were used to characterize the dynamics of *Microcystis* populations and the potential microcystin producers in Missisquoi Bay. *E. coli* was monitored as an indicator of manure contamination of the water, and hence as an indicator source of nutrient input into the lake.

- The efficiency of the 1.2 μm GF/C filter at capturing all the microcystin-producing cyanobacteria was evaluated. The GF/C filter is not a good choice for characterization of all bacterial species in the sample, because some bacteria, including picocyanobacteria, can pass through the larger pore size. Filters with small pore sizes such as 0.22 μm would be a better choice to reduce possible errors, especially when picocyanobacteria are an important component of the research objectives.

- The *mcyD* fragments were identified in the Millipore filtrand in selected samples in 2006 and 2008. The *mcyD* sequences identified in the Millipore filtrand were identical to the sequences identified on the GF/C filtrand. The GF/C filter captured the majority (close to 100%) of the *mcyD* gene: the portion of *mcyD* captured in the Millipore filtrand was negligible. It was concluded that the GF/C filter was appropriate for microcystin analysis and the identification of the *mcyD* gene .

- The *mcyD* copy numbers detected in the GF/C filtrand had a good correlation with the microcystin concentration determined by ELISA and

HPLC in 2006. The *mcyD* Q-PCR can be used as a rapid, sensitive and effective method for the detection and monitoring of microcystin in Lake Champlain.

- *E. coli* detection was positively related to major rain events and manure application in the spring and the fall over a three year period. There was a trend between heavy rains and the nitrogen concentration in the lake in 2006. At this time, we do not have sufficient data to clearly establish the extent of the correlation between *E. coli* detection, major rain events and manure application. Improving manure application strategies (especially avoiding manure spreading before forecast heavy rain events) would be an important factor for reducing the nutrient inputs into Missisquoi Bay, although more data is needed to confirm this point.

Appendix

TABLE 1 *Microcystis, Anabaena and Aphanizomenon* biomass in 2006

Station	Date	Species	Biomass µg C per L	Total biomass ppb	Percent of biomass	Abundance cell per mL
littoral	2006/07/25	*Anabaena helicoidea*	2.01	5652.08	0.04	56.99
	2006/06/27	*Anabaena solitaria planctonica*	70.79	5002.10	1.42	185.46
	2006/07/12	*Anabaena solitaria solitaria*	10.36	3472.16	0.30	19.79
	2006/07/18		79.77	11515.50	0.69	152.35
	2006/06/20	*Anabaena spiroides*	45.58	10753.32	0.42	170.01
	2006/06/27		12.29	5002.10	0.25	68.44
	2006/07/07		45.74	3113.01	1.47	170.60
	2006/07/18		1414.22	11515.50	12.28	12504.48
	2006/07/25		2590.31	5652.08	45.83	22903.37
	2006/08/02		6835.14	13953.59	48.98	60435.90
	2006/08/08		8395.15	17882.11	46.95	46745.06
	2006/08/15		10080.31	22504.04	44.79	56128.19
	2006/08/22		2972.81	9416.56	31.57	11089.16
	2006/08/29		209.27	11885.82	1.76	1165.22
	2006/09/05		75.55	14062.73	0.54	281.81
	2006/09/12		1286.85	23192.33	5.55	7165.30
	2006/09/19		260.43	9456.74	2.75	1450.12
	2006/09/26		162.80	12958.33	1.26	426.51
	2006/10/02		93.06	7965.69	1.17	243.81
	2006/07/12	*Anabaena spiroides crassa*	129.64	3472.16	3.73	186.02
	2006/07/18		184.11	11515.50	1.60	264.18
	2006/07/25		56.58	5652.08	1.00	62.54
	2006/08/02		1852.29	13953.59	13.27	2047.23
	2006/08/08		433.61	17882.11	2.42	622.19
	2006/08/15		83.85	22504.04	0.37	120.32
	2006/09/12		175.43	23192.33	0.76	251.73
	2006/09/19		133.50	9456.74	1.41	191.57
	2006/09/26		20.05	12958.33	0.15	22.16
	2006/10/02		70.19	7965.69	0.88	77.58
	2006/10/10		48.55	9438.90	0.51	69.66
	2006/06/27	*Aphanizomenon flos-aquae*	16.65	5002.10	0.33	220.79
	2006/07/07		15.74	3113.01	0.51	178.90
	2006/07/12		6.02	3472.16	0.17	34.04
	2006/07/18		175.50	11515.50	1.52	1276.87
	2006/07/25		28.94	5652.08	0.51	383.86
	2006/08/02		1167.33	13953.59	8.37	15482.17
	2006/08/08		727.84	17882.11	4.07	8274.22
	2006/08/15		836.10	22504.04	3.72	11089.16
	2006/08/22		1635.76	9416.56	17.37	18595.66
	2006/08/29		117.96	11885.82	0.99	1340.96
	2006/09/05		140.69	14062.73	1.00	1023.61
	2006/09/12		307.64	23192.33	1.33	3497.35
	2006/09/19		1695.79	9456.74	17.93	19278.07
	2006/09/26		2836.32	12958.33	21.89	32243.86
	2006/10/02		2836.32	7965.69	35.61	32243.86
	2006/10/10		2641.23	9438.90	27.98	30026.02
	2006/10/16		2232.29	4534.13	49.23	25377.11
	2006/10/24		943.80	4272.60	22.09	6866.75

Station	Date	Species	Biomass µg C per L	Total biomass ppb	Percent of biomass	Abundance cell per mL
	2006/10/31		330.15	1575.17	20.96	3753.25
	2006/11/06		840.39	3359.20	25.02	9553.73
	2006/11/13		10.31	1519.86	0.68	117.16
	2006/05/23	*Microcystis aeruginosa*	23.05	2364.03	0.98	352.24
	2006/07/07		54.79	3113.01	1.76	484.45
	2006/07/12		22.59	3472.16	0.65	345.13
	2006/07/18		276.65	11515.50	2.40	4226.87
	2006/07/25		1558.05	5652.08	27.57	13776.14
	2006/08/02		2105.26	13953.59	15.09	62824.34
	2006/08/08		4773.42	17882.11	26.69	72932.53
	2006/08/15		10294.96	22504.04	45.75	157295.42
	2006/08/22		2352.52	9416.56	24.98	70202.89
	2006/08/29		7564.90	11885.82	63.65	115583.13
	2006/09/05		2004.28	14062.73	14.25	30623.13
	2006/09/12		15445.39	23192.33	66.60	136567.23
	2006/09/19		3656.83	9456.74	38.67	55872.29
	2006/09/26		5398.71	12958.33	41.66	82486.27
	2006/10/02		1378.99	7965.69	17.31	21069.40
	2006/10/10		1384.57	9438.90	14.67	21154.70
	2006/10/16		61.46	4534.13	1.36	1833.98
	2006/10/24		33.77	4272.60	0.79	298.55
	2006/10/31		1.70	1575.17	0.11	50.66
	2006/11/06		5.58	3359.20	0.17	85.30
	2006/06/06	*Microcystis flos-aquae*	5.23	2955.13	0.18	79.97
	2006/06/27		37.93	5002.10	0.76	579.57
	2006/08/02	*Microcystis weisenbergei*	636.72	13953.59	4.56	5629.88
	2006/08/08		2006.87	17882.11	11.22	11174.46
	2006/08/15		65.18	22504.04	0.29	576.28
	2006/08/29		48.49	11885.82	0.41	740.93
	2006/09/05		19.33	14062.73	0.14	107.66
	2006/09/12		256.05	23192.33	1.10	2263.95
	2006/09/19		47.27	9456.74	0.50	417.96
	2006/10/02		7.88	7965.69	0.10	69.66
pelagic	2006/07/12	*Anabaena flos-aquae intermedia*	45.45	2579.27	1.76	401.84
	2006/07/07	*Anabaena solitaria solitaria*	5.92	9792.40	0.06	22.08
	2006/07/12		4.21	2579.27	0.16	11.04
	2006/06/27	*Anabaena spiroides*	5.18	2642.79	0.20	19.32
	2006/08/02		9781.58	16163.42	60.52	54464.82
	2006/08/22		1240.89	10110.00	12.27	6909.40
	2006/09/12		2149.57	16535.93	13.00	8018.31
	2006/07/07	*Anabaena spiroides crassa*	40.01	9792.40	0.41	57.41
	2006/08/02		1159.22	16163.42	7.17	1663.37
	2006/08/22		247.15	10110.00	2.44	354.63
	2006/09/12		60.51	16535.93	0.37	115.57
	2006/07/12	*Aphanizomenon flos-aquae*	140.05	2579.27	5.43	792.54
	2006/08/02		800.73	16163.42	4.95	10620.00
	2006/08/22		667.81	10110.00	6.61	7591.81
	2006/09/05		24.65	4321.47	0.57	280.22
	2006/09/12		1530.71	16535.93	9.26	17401.45
	2006/10/16		2149.75	3922.12	54.81	24438.80
	2006/11/06		187.59	2720.68	6.89	2132.53
	2006/11/13		7.59	2182.86	0.35	86.28
	2006/05/30	*Microcystis aeruginosa*	40.34	2527.05	1.60	616.42
	2006/07/07		155.61	9792.40	1.59	2377.61

Station	Date	Species	Biomass µg C per L	Total biomass ppb	Percent of biomass	Abundance cell per mL
	2006/08/02		2188.52	16163.42	13.54	33438.07
	2006/08/22		5002.32	10110.00	49.48	76429.88
	2006/09/05		1261.75	4321.47	29.20	19278.07
	2006/09/12		4918.58	16535.93	29.74	75150.36
	2006/10/16		137.21	3922.12	3.50	4094.46
	2006/11/06		0.64	2720.68	0.02	19.00
	2006/11/13		5.58	2182.86	0.26	85.30
	2006/06/27	*Microcystis flos-aquae*	19.92	2642.79	0.75	176.12
	2006/08/02	*Microcystis weisenbergei*	1374.75	16163.42	8.51	12155.42
	2006/09/12		115.67	16535.93	0.70	1022.74

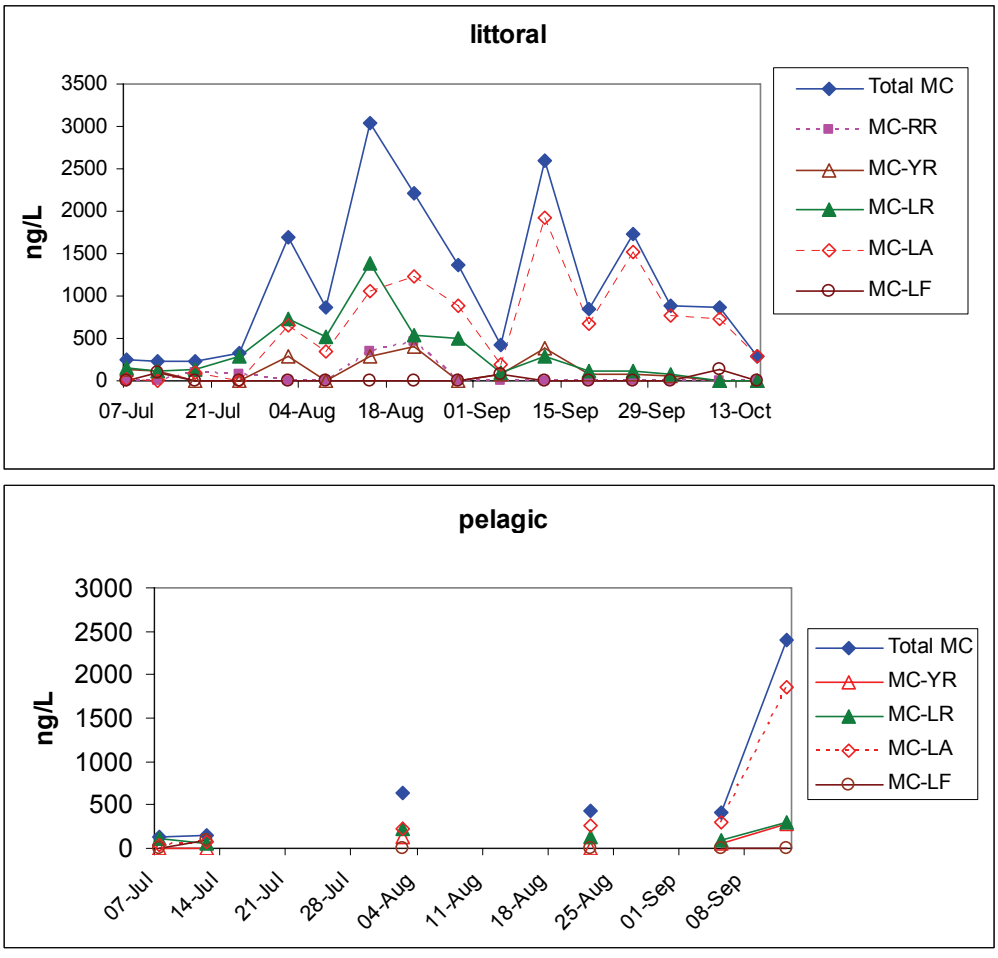
Note: Data kindly provided by Dr. David Bird (Principal Investigator) as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms.

TABLE 2 *Microcystis, Anabaena and Aphanizomenon* biomass in 2007

Station	Species	Date	Abundance (cells/mL)	biovolume (um3)
littoral	*Anabaena flos-aquae*	02/08/2007	114	197.9
		26/09/2007	2859	230.9
		03/10/2007	597	197.9
	Anabaena flos-aquae intermedia	30/08/2007	796	113.1
		05/09/2007	4692	113.1
		11/09/2007	90	113.1
	Anabaena spiroides	10/10/2007	21	268.1
	Anabaena spiroides crassa	30/08/2007	38	696.9
		05/09/2007	218	696.9
		11/09/2007	40	696.9
		19/09/2007	104	696.9
		26/09/2007	92	696.9
		10/10/2007	341	904.8
	Aphanizomenon flexuosum	09/05/2007	14	42.4
		06/07/2007	682	42.4
	Aphanizomenon flos-aquae	23/05/2007	40	75.4
	Aphanizomenon gracile	20/06/2007	51	42.4
	Microcystis aeruginosa	06/06/2007	76	33.5
		20/06/2007	184	65.4
		22/08/2007	3839	65.4
		05/09/2007	427	65.4
pelagic	*Anabaena circinalis*	26/09/2007	35	381.7
	Anabaena flos-aquae	22/08/2007	621	226.2
		30/08/2007	1122	197.9
		05/09/2007	507	226.2
		26/09/2007	1111	197.9
	Anabaena flos-aquae intermedia	19/09/2007	4606	113.1
	Anabaena spiroides	05/09/2007	3071	268.1

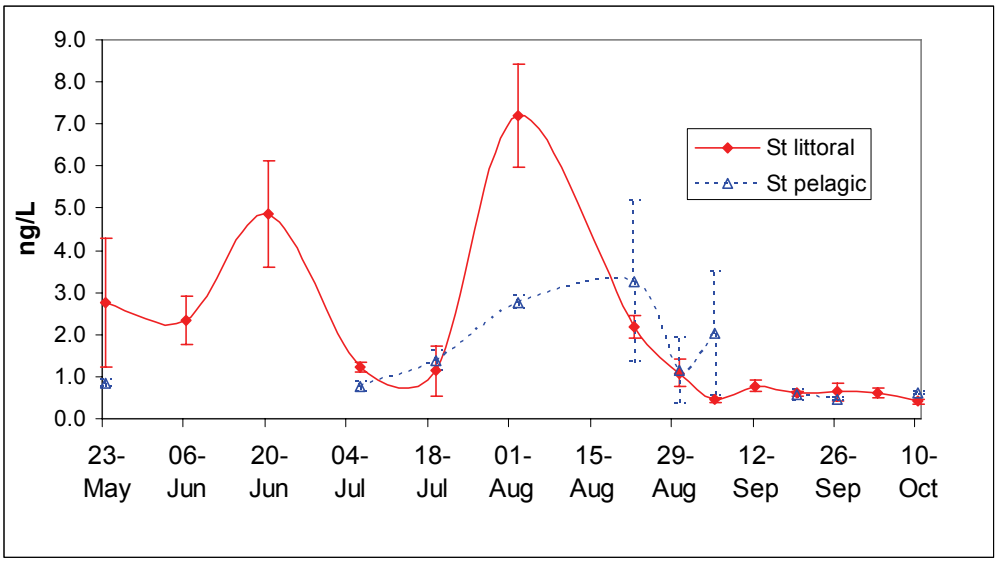
Station	Species	Date	Abundance (cells/mL)	biovolume (um3)
	Anabaena spiroides crassa	30/08/2007	60	523.6
		05/09/2007	114	904.8
		19/09/2007	1706	523.6
		26/09/2007	111	904.8
	Aphanizomenon gracile	02/08/2007	71	35.3
	Microcystis aeruginosa	19/07/2007	74	65.4
		02/08/2007	255	33.5
		22/08/2007	1529	65.4
		30/08/2007	256	65.4
		05/09/2007	211	33.5

Note: Data kindly provided by Dr. David Bird (Principal Investigator) as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms.



Note: Data kindly provided by Dr. Rocio Aranda-Rodriguez as part of a collaborative NSERC Strategic Research Grant entitled "Chemical and genome-based quantitative tracing of toxic cyanobacterial blooms."

FIG.1. Microcystins total and variants concentration determined by HPLC in 2006. littoral: littoral station; pelagic: pelagic station.



Note: same as in Fig.1

FIG.2 Microcystin concentration of 2007 in both station determined by ELISA. St:Station.

DGGE sequences in 2006 and 2007

>06-1

CCAAGGCGACGATCAGTAGCCGGCCTGAGAGGGTGAACGGCCACATTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTTAAGAATCTTGCTCAATGGGGGAAACCCTGAAG
CAGCGACGCCGCGTGAACGACGAAGGTCTTCGGATTGTAAAGTTCAATAAGGAGGGAAAAA
TAAGCAGTAATGTGATGATTGTACCTCCCTAAAGCACCGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTATGGTGCAAG

>06-2

CCAAGGCTTCGATCAGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATC
CAGCAATGCCGCGTGTGCGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCAGGGAAGAA
ATCTTTTAGGCTAATACCTTGGAAGGATGACGGTACCTGAAGAATAAGCACCGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGGTGCAA

>06-3

CCAAGGCGACGATCAGTATCTGGTTTGAGAGGATGATCAGACACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACG
GAGCAATACCGCGTGAGGGAAGAAAGCCTACTGGGTTGTAAACCTCTTTTTTCAGGGAGGA
AAAAAATGACGTGTACCTGAAGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
GACGGAGGATGC

>06-4

GCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGC
AACGCCGCGTGAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAAGATC
TGACGGTACTTGAGGAATCAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGG
GGAGGC

>06-5

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAACGCCGCGTGAGGGATGAAGGCCTCTGGGCTGTAAACCTCTTTTATCAAGGAAGA
AGATCTGACGGTACTTGATGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATA
CGGGAGTGGCA

>06-6

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAATACCGCGTGAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGA
AGATATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGATGC

>06-7

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAATACCGCGTGAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGA
ACAGAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGA

>06-8

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGCAACCCTGAT
CCAGCAATGCCGCGTGTGCGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCAGGGAAGA
AATCTTTTGGGCGAATACCCCGGAAGGATGACGGTACCTGAAGAATAAGCACCGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGG

>06-9

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAACGCCGCGTGAGGGATGAAGGCCTCTGGGCTGTAAACCTCTTTTCTCAAGGAAGA
AGATCTGACGGTACTTGAGGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAA

TACGGGAGTGGCAAGCG

>06-10

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGAGCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAACGCCGCGTGAGGGAGGAAGGTCTTTGGATTGTAAACCTCTTTTCTCAAGGAAGA
AGTTCTGACGGTACTTGAGGAATCAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGGGGAGGCA

>07-1

CCAAGGCGACGATCAGTAGCCGGCCTGAGAGGGTGAACGGCCACATTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTTAAGAATCTTGCTCAATGGGGGAAACCCTGAAG
CAGCGACGCCGCGTGAACGACGAAGGTCTTCGGATTGTAAAGTTCAATAAGGAGGGAAAAA
TAAGCAGTAATGTGATGATTGTACCTCCCTAAAGCACCGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTA

>07-2

CCAAGGCTTCGATCAGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATC
CAGCAATGCCGCGTGTGCGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCAGGGAAGAA
ATCTTTTAGGCTAATACCTTGGAAGGATGACGGTACCTGAAGAATAAGCACCGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGGTGCAA

>07-3

CCAAGGCTTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACG
GAGCAACGCCGCGTGAGGGATGAAGGCCTCTGGGCTGTAAACCTCTTTTATCAAGGAAGAA
GATCTGACGGTACTTGATGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATAC
GGGAGTGGCAAG

>07-4

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAACGCCGCGTGAGGGACGAAGGCCTCTGGGCTGTAAACCTCTTTTCTCAAGGAAGA
AGATCTGACGGTACTTGAGGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATA
CGGGAGTGGC

>07-5

CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGAGCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAACGCCGCGTGAGGGAGGAAGGTCTTTGGATTGTAAACCTCTTTTCTCAAGGAAGA
AGTTCTGACGGTACTTGAGGAATCAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGGGGAGGC

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