MOLECULAR CHARACTERIZATION OF TOXIC CYANOBACTERIA IN NORTH AMERICAN AND EAST AFRICAN LAKES

by

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ABSTRACT

Toxic cyanobacterial blooms constitute a threat to the safety and ecological quality of aquatic environments worldwide. Cyclic hepatotoxin, especially microcystin, is the most widely occurring of the cyanotoxins. The aim of this study was to identify the cyanobacterial genotypes present including how many toxic genotypes were present in two North American lakes and one African Lake. All three lakes are prone to cyanobacterial blooms and were sampled in 2005 and 2006: Lake Ontario (Bay of Quinte, Canada), Lake Erie (Maumee Bay, Canada) and Lake Victoria (Nyanza Gulf, Kenya). The cyanobacterial genotypic community was assessed using DNA based analyses of the hypervariable V3 region of the 16S rRNA gene. In addition, the aminotransferase (AMT) domain in modules *mcyE* and *ndaF* of the microcystin and nodularin gene cluster respectively was used to detect the presence of hepatotoxic genotypes. Denaturing gradient gel electrophoresis (DGGE) results from this study suggested that hepatotoxin producers were present in all study sites sampled and were most likely members of the genus *Microcystis*. This study was the first to report the potential for microcystin production in the in-shore and off-shore open lake of Nyanza Gulf in Kenya. A seasonal study of the Bay of Quinte and Maumee Bay showed differences in the cyanobacterial genotypic community from early to late summer. In addition, the cyanobacterial genotypic community from the Bay of Quinte differed from 2005 to 2006 and quantification of the North American samples revealed an increase in cyanobacterial cells from early to late summer. The Bay of Quinte saw relatively no change in hepatotoxic cells from early to late summer but in Maumee Bay hepatotoxic cells increased from undetectable in early summer to dominating the cyanobacterial community by late

summer. This study demonstrated the use of DGGE and qPCR of the 16S rRNA-V3 and AMT gene region in monitoring the cyanobacterial community of waterbodies susceptible to toxic cyanobacterial blooms.

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CHAPTER 1.0: GENERAL INTRODUCTION

1.1 TOXIC CYANOBACTERIAL BLOOMS

The phylum Cyanobacteria is a morphologically diverse group of Gram-negative, photosynthetic prokaryotes (Codd 1995). In eutrophic freshwater bodies, cyanobacteria are frequently the dominant phytoplankton group (Reynolds, 2007; Negri *et al*., 1995) and under favorable conditions, extensive growth of cyanobacteria can result in intense blooms on the water surface. Several environmental factors are involved in the promotion of these cyanobacterial blooms such as high phosphorous concentrations, low nitrogen to phosphorus ratio (N:P), increased water temperature, and a stable water column (Ferber *et al*., 2004; Jacoby *et al*., 2000; Lean and Pick 1981). Cyanobacterial blooms often result in a variety of water quality issues such as dissolved oxygen (OD) depletion resulting in subsequent fish kills, aesthetic nuisances, and unsafe drinking water (Jacoby *et al*., 2000). In addition, cyanobacteria are capable of producing a broad range of secondary molecules which include biologically toxic metabolites such as microcystin. These toxins are produced during cellular growth or released during cell lysis and exposure to these cyanotoxins presents waterborne hazards in the aquatic system and organisms using the water body as a source of drinking water (Codd *et al*., 2005; Dietrich and Hoeger 2005). The first documented report in the scientific literature of the toxic effects of cyanobacteria appeared in 1878 by George Francis who attributed the deaths of sheep, horses, dogs, and pigs to the ingestion of toxic *Nodularia spumigena* Mertens from Lake Alexandrina (Francis 1878).

1.2 CYANOTOXIN CLASSIFICATION

Given their 3.5 billion years of evolution, cyanobacteria have developed a broad spectrum of secondary metabolites. Several of these secondary metabolites have been shown to have anticancer, antibacterial, antiviral, and protease inhibitory effects (Moore 1996; Namikoshi and Rinehart 1996; Panda *et al*., 1998; Patterson, Larsen, More 1994; Smith *et al*., 1994). The toxins produced by cyanobacteria can be classified according to their mode of action (hepatotoxic, neurotoxic, cytotoxic, dermatotoxic, or irritant toxic) or by their chemical structure (cyclic peptides, alkaloids, and lipopolysaccharides) (Sivonen and Jones 1999; Wiegand and Pflugmacher 2005) (Table 1.1).

Lipopolysaccharide (LPS) endotoxins are common to all cyanobacteria since they are a part of the Gram-negative cell wall where they form complexes with proteins and phospholipids (McElhiney and Lawton 2005). The effects of LPS from enteric bacteria, such as *Escherichia coli*, *Salmonella* sp., and *Pseudomonas aeruginosa* are well known. Unlike enteric bacterial LPS, cyanobacterial LPS contains a great variety of long chain unsaturated fatty acids and hydroxyl fatty acids and lacks phosphate groups (Martin *et al*., 1989). Generally, the fatty acid component of the LPS molecule is responsible for allergenic reactions in humans and mammals to this endotoxin (Sivonen and Jones 1999). In contrast to LPS, neurotoxins and hepatotoxins are intracellular secondary metabolites and only certain strains of cyanobacteria are capable of synthesizing these compounds (McElhiney and Lawton 2005). Neurotoxins consist of two groups of low molecular weight alkaloids: anatoxins (anatoxin-a, homoanatoxin-a and anatoxin-a(S)) and saxitoxins (McElhiney and Lawton 2005). Both anatoxin-a and homoanatoxin-a mimic the effect of acetylcholine and bind to neuronal nicotinic acetylcholine receptors

TABLE 1.1 **General summary of cyanobacterial toxins including their respective cyanobacterial producing genera and mode of action.** Table was adapted from (Sivonen and Jones 1999)

Toxin group	**Cyanobacteria genera**	**Mode of action**
Lipopolysaccharide (LPS)	All	Potential irritant; affects any exposed tissue
Anatoxin-a	*Anabaena*, *Aphanizomenon*	Irreversibly binds to nicotinic acetylcholine receptors
Anatoxin-a(S)	*Anabaena*	Inhibitor of acetylcholinesterase
Saxitoxins	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*	Blocks nerve cell sodium channels
Nodularin	*Nodularia*	Inhibition of protein phosphatases (PP1 and PP2A)
Microcystin	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*	Inhibition of protein phosphatases (PP1 and PP2A)
Cylindrospermopsin	*Cylindrospermopsis, Aphanizomenon, Umezakia*	Inhibitor of protein synthesis

causing muscle cells to become overstimulated (McElhiney and Lawton 2005). These two toxins have been detected within species and strains of *Anabaena*, *Aphanizomenon*, and *Planktothrix* (*Oscillatoria*) (Edwards *et al*., 1992; Sivonen and Jones 1999). Anatoxin-a (S) is a potent inhibitor of acetylcholinesterase causing interference with normal muscle contraction (McElhiney and Lawton 2005). Anatoxin-a(S) can be produced by strains of *Anabaena flos-aquae* (Lyng.) Brèb. and *Anabaena lemmermannii* P. Richter (Carmichael and Gorham 1979; Henriksen *et al*., 1997). The other group of neurotoxic alkaloid is saxitoxin which blocks nerve cell sodium channels halting impulse propagation between neurons (McElhiney and Lawton 2005). Saxitoxins are produced by some strains of *Aphanizomenon* sp.*, Anabaena* sp.*, Lyngbya* sp. and *Cylindrospermopsis* sp. (Humpage *et al*., 1993; Sivonen and Jones 1999).

Since hepato– (liver) toxic cyanobacterial blooms are more prevalent than neurotoxic blooms; poisoning by cyclic hepatopeptides remains the most common cause of toxicosis involving cyanobacteria (Carmichael *et al*., 1997; Sivonen and Jones 1999). Cyclic hepatotoxins include the cylindrospermopsins, the nodularins, and the microcystins. The cylindrospermopsin is a cyclic sulfated-guanidinium alkaloid produced by *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju (Ohtani *et al*., 1992), *Umezakia natans* M. Watanabe (Harada *et al*., 1994), and *Aphanizomenon ovalisporum* Forti (Banker *et al*., 1997; Shaw *et al*., 2000). Cylindrospermopsin mediates its toxicity by targeting the liver and inhibits protein synthesis (Terao *et al*., 1994). Nodularins are a hepatotoxic penta-peptide that is only known to be synthesized by one species of cyanobacteria, *Nodularia spumigena* Mertens (Sivonen and Jones 1999). The structure and mode of toxicity of nodularins are similar to microcystins (discussed in the

following sections) (figure 1.1) (McElhiney and Lawton 2005). Of the numerous toxins produced by cyanobacteria, microcystins are the most prevalent and studied of the cyanotoxins (Hisbergues *et al*., 2003).

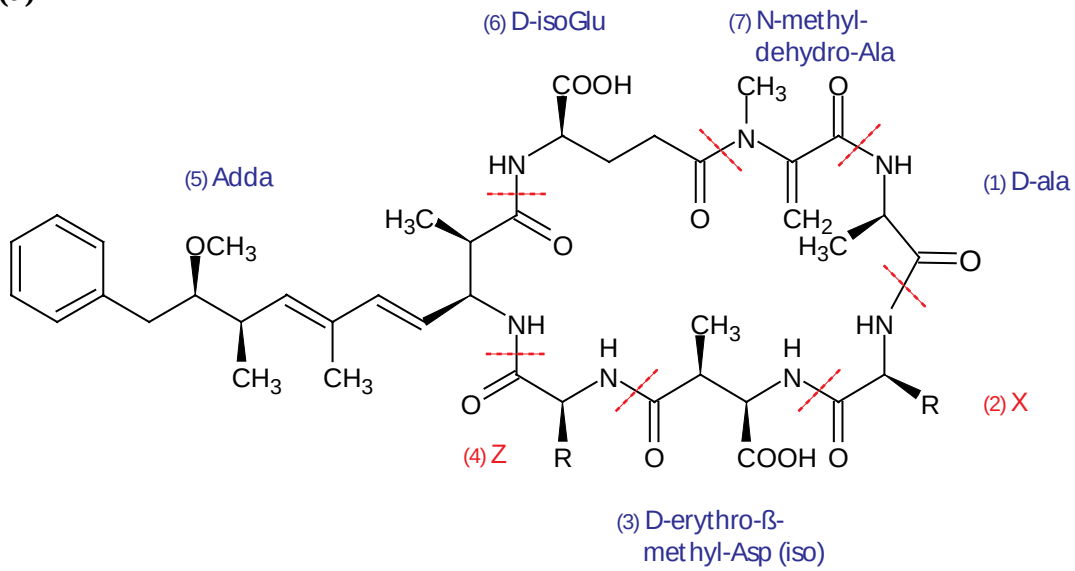
1.3 HEPATOTOXIN – MICROCYSTIN

Both microcystin and nodularin are monocyclic hepatotoxins and consist of either five (nodularins) or seven (microcystins) amino acids (Sivonen and Jones 1999). The cyclic peptides are relatively large natural products (molecular weight, MW ≈ 800–1100) but relatively small compared with other cell oligopeptides and polypeptides (MW > 10 000) (Sivonen and Jones 1999). Microcystin's chemical nature makes it stable in water and tolerant of extreme changes in water chemistry such as pH (Harada *et al*., 1996; Harada and Tsuji 1998). Microcystin-producing strains are from a group of cyanobacterial genera that include unicellular, multicellular filamentous, heterocystous, and nonheterocystous forms (Christiansen *et al*., 2003). Each toxic strain is capable of producing more than one variant of microcystin as well as other peptides simultaneously (Fastner, Erhard, von Dohren 2001; Sivonen *et al*., 1992). Bloom forming species of *Microcystis*, *Anabaena*, and *Planktothrix* (*Oscillatoria*) are the most common microcystin producers and more rarely are species of *Anabaenopsis*, *Nostoc*, and *Hapalohsiphon* (observed in soils) (Carmichael 1992; Skulberg *et al*., 1992). Generally, five *Microcystis* species (*M. aeruginosa*, *M. ichthyoblabe*, *M. novacekii*, *M. viridis*, and *M. wesenbergii*) are recognized as the dominant microcystin-producing species (Tanabe *et al*., 2004).

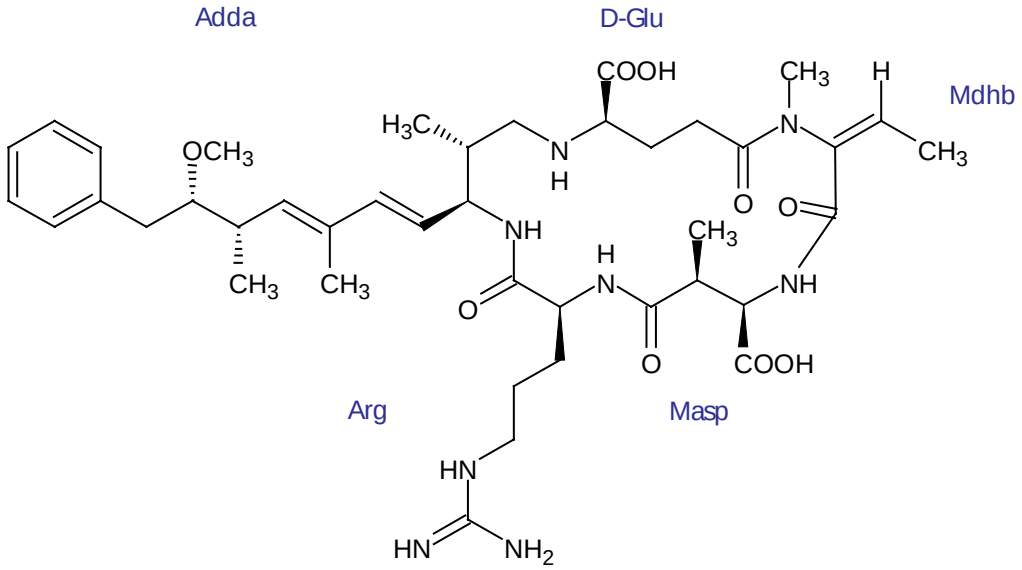
There are over 70 isoforms of microcystin that exhibit varying levels of toxicity; with microcystin-LR (L, L-leucine; R, L-argentine), -RR, and -YR being the most

FIGURE 1.1 Chemical structures of the cyanobacteria hepatotoxins, microcystin, and nodularin. (a) General structure of microcystin (D-Ala–L-X–D-MeAsp–L-Z–Adda–D-Glu–Mdha), in which X and Y are variable L-amino acids. (b) Structure of nodularin showing similarities to microcystin.

(a)


(6) D-isoGlu
(7) N-methyl-dehydro-Ala
(5) Adda
(1) D-ala
(2) X
(4) Z
(3) D-erythro-ß-methyl-Asp (iso)

(b)


Adda
D-Glu
Mdhb
Arg
Masp

common (McElhiney and Lawton, 2005; Sivonen and Jones 1999). All the isoforms share the common cyclo (D-Ala–L-X–D-MeAsp–L-Z–Adda–D-Glu–Mdha) structure, with X and Z denoting variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid, DMeAsp is 3-methyl-aspartic acid, and Mdha is *N*-methyldehydroalanine (Honkanen *et al*., 1990; Rinehart *et al*., 1994) (Figure 1.1). The unusual amino acid Adda gives microcystin its toxicity and is key to the biological activity of the microcystin molecule (Goldberg *et al*., 1995).

1.3.1 MICROCYSTIN MODE OF TOXICITY

With the exception of a few hydrophobic microcystins variants, microcystin is water soluble (hydrophilic) thus it cannot directly permeate the lipid membranes (hydrophobic) of animals and plants cells (Sivonen and Jones 1999). For the toxin to elicit an effect it requires active uptake mechanisms to enter the cells via membrane proteins. Primary route of hepatotoxin exposure to humans and animals is through the ingestion of toxin contaminated waters during recreational activities (Chorus *et al*., 2000; Pilotto *et al*., 1997; Sivonen and Jones 1999) or contaminated foods such as shellfish (Eriksson *et al*., 1989) and fish (Ernst *et al*., 2001; Ernst *et al*., 2007). Once ingested, it is postulated that microcystin reabsorbed from the gastrointestinal tract is taken up from the blood by membrane transporters of hepatocytes, via multispecific bile acid transport mechanisms (Eriksson *et al*., 1990a; Runnegar *et al*., 1991). In vertebrates, toxicity is linked to the active transport of microcystin into hepatocytes by the bile acid organic anion transport system, which then inhibits serine/threonine phosphatase 1 (PP 1) and 2A (Goldberg *et al*., 1995; MacKintosh *et al*., 1990). Hydrophobic interactions between the long hydrophobic Adda side-chain of microcystin and the hydrophobic grove of PP 1 and

2A renders the protein phosphatase inactive (Goldberg *et al*., 1995). However, microcystin is not inhibitory to protein phosphatase activity in *Microcystis* itself (Shirai *et al*., 1991).

Exposure to high doses of microcystin will cause liver damage as a result of cytoskeletal disorganization that eventually leads to death by hemorrhagic shock (Ding *et al*., 1998; Eriksson *et al*., 1990b; Hermansky *et al*., 1990; Honkanen *et al*., 1990). This led the World Health Organization (WHO) to set a safe concentration threshold of 1.0 $\mu g \cdot L^{-1}$ of microcystin in drinking water. However, several studies showing long-term chronic exposure to low concentrations of microcystin has been linked to primary liver cancer in humans and animals (Nishiwaki-Matsushima *et al*., 1992; Ueno *et al*., 1996; Yu 1988). Based on these findings, Ueno *et* al. (1996) propose that the concentration threshold be set to 0.01-$\mu g \cdot L^{-1}$.

1.3.2 ECOLOGICAL ROLE

Numerous studies have investigated the biological function(s) of microcystin but its potential role(s) and function(s) remain highly debated. Several hypotheses regarding the biological function of microcystin have been proposed such as the evolution of these molecules as a defense mechanism in response to grazing pressure by zooplankton (DeMott, *et al*., 1991). The zooplankton defense theory is contradicted by recent studies which postulate that microcystin-producing cyanobacteria predate the metazoan lineage (Rantala *et al*., 2004). This means that microcystin-producing strains are "phylogenetically older" than the zooplankton that graze on them (Rantala *et al*., 2004). Hence, Rantala *et al*. (2004) suggest that microcystin and nodularins have adapted to serve other functions such as siderophoric scavenging of trace metals (e.g. chelating of

iron ions) (Utkilen and Gjolme 1995) or signaling gene regulation (Dittmann *et al*., 2001). The role of microcystin in photosynthesis or other light related reactions within the cell have also been speculated. Hess et al. (2001) observed that cellular concentrations of photosynthetic pigments are approximately 20% lower in non-microcystin producing mutants compared to wild-type microcystin-producing strains. Also, subcellular localization of microcystins in thylakoid membranes (Shi *et al*., 1995; Young *et al*., 2005) and light regulated transcription of the microcystin synthetase gene cluster (Kaebernick *et al*., 2000) suggest a role in light-dependent processes. Involvement as growth regulators (quorum sensing) has also been speculated. Sedmak and Kosi (1998) observed that *Microcystis aeruginosa* Kützing is permeable to microcystin-RR and cells exposed to the toxin proliferate faster than controls. They suggest that microcystin production is a self-enhancing mechanism for cell division that leads to denser blooms and gives microcystin producers a competitive advantage over non-toxic cyanobacterial strains. Although microcystin is classified as a secondary metabolite, the linear correlation between microcystin production and cell division suggests constitutive production of microcystins and a possible role in cellular metabolism (Orr and Jones 1998).

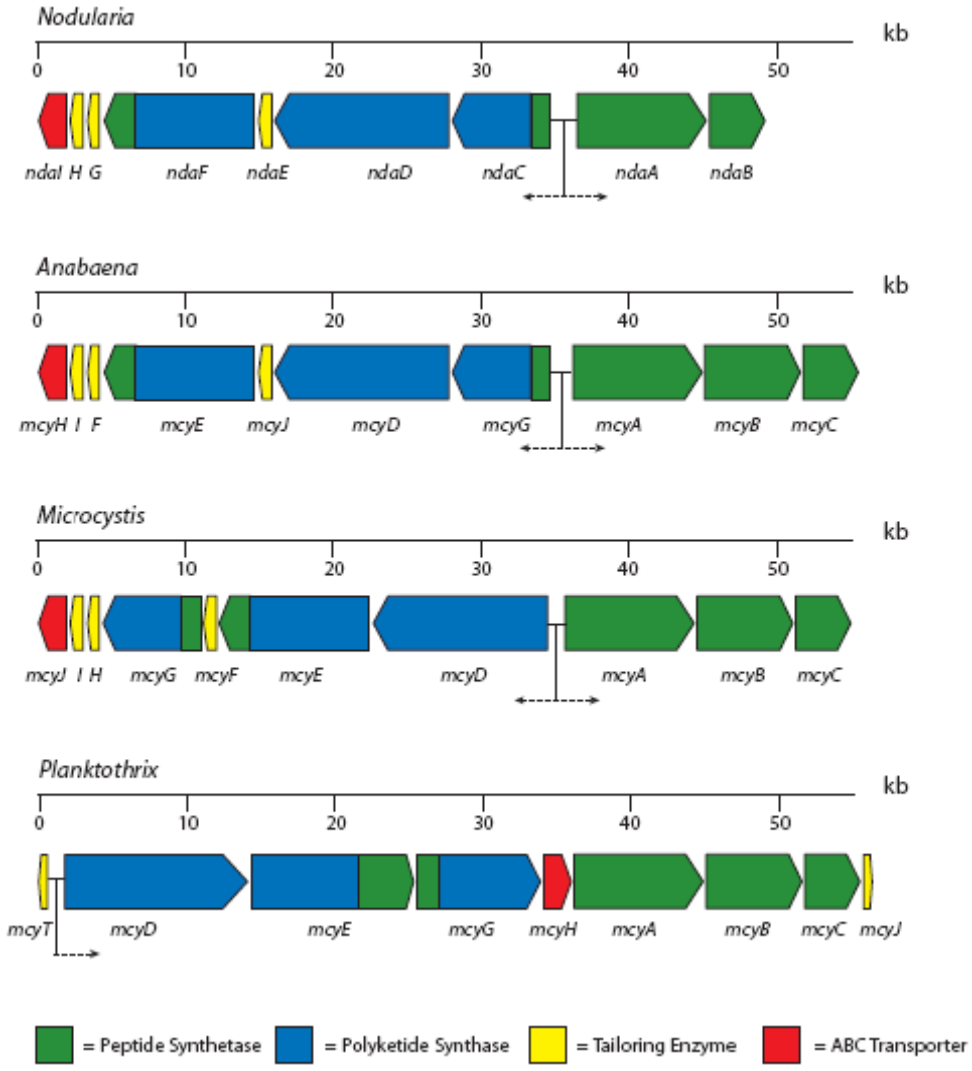
Since the role of microcystin is not well understood, the influences of environmental factors on microcystin production are complex and difficult to identify (Rolland *et al*., 2005). Conflicting results are reported in the literature concerning the effects of various ecological conditions, such as nutrients, light and temperature, on microcystin production (Graham *et al*., 2004; Jacoby *et al*., 2000; Kaebernick *et al*., 2000; Kotak *et al*., 2000; Wicks and Thiel 1990).

1.3.3 TOXIC AND NON-TOXIC MICROCYSTIN-PRODUCING STRAINS

Morphologically toxic and nontoxic microcystin-producing strains are identical. Genetically, only toxic strains carry a functional microcystin synthetase gene cluster (Figure 1.2) transcribing the enzymes involved in microcystin biosynthesis (Dittmann *et al*., 1997; Meissner *et al*., 1996; Neilan *et al*., 1999; Nishizawa *et al*., 1999). All genera with microcystin-producing strains also contain related strains that are unable to produce the toxin (Rantala *et al*., 2004). The literature remains unclear as to why closely related strains differ in their ability to produce microcystin and nodularins. LeFlaive and Ten-Hage (2007) suggested that non-toxic strains may take advantage of the production of microcystin produced by co-occurring strains without the energy cost of synthesis. Rantala *et al*. (2004) hypothesized that strains not carrying the genes for microcystins or nodularins biosynthesis in their genome contain other genes for the synthesis of other nonribosomal peptides that serve similar functions as the hepatotoxins. Similar peptide synthetase complexes of unknown function found in several cyanobacterial genera support this hypothesis (Neilan *et al*., 1999).

The sporadic distribution of microcystin synthetase (*mcy*) genes in several cyanobacterial lineages has raised a few theories about the origins of the *mcy* genes. Congruent evolution between specific *mcy* gene regions (fragments of the *mcyA*, *mcyD*, and *mcyE* genes), 16S rRNA and *rpo*C1 genes in hepatotoxic cyanobacteria suggests that the microcystin synthetase genes were present in the last common ancestor of a large number of cyanobacteria (Rantala *et al*., 2004). Thus, the sporadic distribution of the ability to synthesize microcystin in current cyanobacteria is attributed to repeated gene loss (Rantala *et al*., 2004). Moreover, the distribution of gene cluster and variation of the

FIGURE 1.2 **Organization of the gene cluster for nodularin biosynthesis in *Nodularia*** (Moffitt and Neilan 2004) **and microcystin biosynthesis in *Anabaena*** (Rouhiainen *et al*., 2004)**, *Microcystis*** (Tillett *et al*., 2000)**, and *Planktothrix*** (Christiansen *et al*., 2003). Figure shows the order of genes and direction of gene transcription. The arrows indicate the transcriptional start sites from the putative promoter regions. Figure adapted from (Dittmann and Börner 2005).


Nodularia
kb
0
10
20
30
40
50
ndaI H G
ndaF
ndaE
ndaD
ndaC
ndaA
ndaB
Anabaena
kb
0
10
20
30
40
50
mcyH I F
mcyE
mcyJ
mcyD
mcyG
mcyA
mcyB
mcyC
Microcystis
kb
0
10
20
30
40
50
mcyJ I H
mcyG
mcyF
mcyE
mcyD
mcyA
mcyB
mcyC
Planktothrix
kb
0
10
20
30
40
50
mcyT
mcyD
mcyE
mcyG
mcyH
mcyA
mcyB
mcyC
mcyJ
= Peptide Synthetase
= Polyketide Synthase
= Tailoring Enzyme
= ABC Transporter

70 or more microcystin isoforms may also be explained by horizontal gene transfer, lateral recombination, and gene loss (Mikalsen *et al*., 2003; Moffitt and Neilan 2004; Rantala *et al*., 2004).

1.3.4 MICROCYSTIN SYNTHETASE GENE (*MCY*) AND MICROCYSTIN BIOSYNTHESIS

The microcystin synthetase gene (*mcy*) cluster encodes for non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), and tailoring enzymes, which modify the peptide chain (Dittmann *et al*., 1997; Meissner *et al*., 1996; Nishizawa *et al*., 1999; Nishizawa *et al*., 2000; Tillett *et al*., 2000). Microcystin's cyclic structure, unusual peptide bonds, and occurrence of modified amino acid moieties suggests that microcystin and nodularin are nonribosomally synthesized (Arment and Carmichael 1995; Dittmann *et al*., 1997). Peptide synthetase genes identified in *Microcystis sp.* and *Anabaena sp.* contain the characteristic modular structure of the peptide synthetase genes and specific conserved sequence motifs observed in other bacteria and fungi (Dittmann *et al*., 1996). Microcystin is synthesized by the large enzyme complex, microcystin synthetase, utilizing the nonribosomal thio-template mechanism (Arment and Carmichael 1995). According to this mechanism, peptide synthesis is performed by NRPSs and generally, the order of the modules (genes) is co-linear to the amino acid sequence of the peptide (Mootz *et al*., 2002). Each amino acid is activated as an amino acid adenylate precursor and transferred to the enzyme complex bound (Laland and Zimmer 1973; Stein and Vater 1996).

The gene clusters involved in microcystin biosynthesis were identified and sequenced in *Microcystis aeruginosa* strain K139 (Nishizawa *et al*., 1999; Nishizawa *et al*., 2000) and PCC7806 (Tillett *et al*., 2000), *Planktothrix agardhii* strain CYA 126

(Christiansen *et al*., 2003), and *Anabaena* sp. strain 90 MCS (Rouhiainen *et al*., 2004) (Figure 1.2). The size of the gene cluster among these three genera is approximately 55-kb. The multienzyme complexes in the three genera are highly similar but the arrangements of the *mcy* genes differ between the genera (Dittmann and Borner 2005; Rouhiainen *et al*., 2004). Only the NRPS genes, *mcyA*, *mcyB*, and *mcyC*, from the *mcy* gene cluster are in the same order for all three genera. The microcystin gene cluster is comprised of 10 microcystin genes (*mcyA-J*) in *M. aeruginosa* K139 and *Anabaena* strain 90 and 9 genes in *P. agardhii* CYA 126. The *mcyF* and *mcyI* genes are absent in the *P. agardhii* CYA 126 *mcy* gene cluster but an additional gene, *mcyT*, is present which is not observed in *Microcystis* and *Anabaena*. The *mcy* genes are organized into two clusters in *M. aeruginosa* K139 and *Anabaena* strain 90 and transcribed from a bidirectional promoter. In contrast, the *mcy* genes in *P. agardhii* CYA 126 are in one cluster and transcribed in the same direction except for *mcyT* as depicted in Figure 1.2.

For *Microcystis aeruginosa* PCC7806, Tillett and colleagues (2000) observed that the 10 open reading frames (ORFs) are flanked on both sides by genes not involved in microcystin biosynthesis but show high similarity to genes localized on the chromosome of *Synechocystis* sp. PCC 6803. This strongly suggests that the *mcy* gene cluster is located on the chromosome rather than on a large plasmid as previously proposed by (Bolch *et al*., 1997). Previously mentioned, the ten *mcy* genes reside in two putative operons (*mcyA-C* and *mcyD-J*) (Nishizawa *et al*., 1999; Nishizawa *et al*., 2000; Tillett *et al*., 2000). One operon consists of the NRPS genes (*mcyA*, *mcyB*, *mcyC*), and the larger operon contain one PKS gene (*mcyD*) and two hybrid corresponding to the PKS-NRPS modules (*mcyE* and *mcyG*) (Tillett *et al*., 2000). The PKS modules of McyG and E along

with the two-modular polyketide synthase McyD are responsible for the synthesis of the unique Adda-D-glutamic acid precursor (Tillett *et al*., 2000). Tailoring enzymes encoded by the *mcy* cluster include *mcyF* that contains the racemase gene involved in the synthesis of D-glutamate residues in the microcystin molecule (Nishizawa *et al*., 2000). The *mcyH* gene encodes for the putative ATP-binding cassette (ABC) transporter protein (a membrane transport system) that is potentially involved in microcystin transport (Tillett *et al*., 2000). The presence of a putative ABC transporter protein sequence in the microcystin biosynthesis gene cluster suggests that extracellular microcystins may also be the result of active transport rather than passive release via cell lysis (Pearson *et al*., 2004).

The nodularin gene cluster spans 49-kb and consists of 9 ORFs (*ndaA* to *ndaI*) (Moffitt and Neilan 2001). Similarities in the biosynthesis, chemical structure, and biological action of microcystins and nodularins implicates that the two groups of hepatotoxins are closely related (Sivonen and Jones 1999). The study by Rantala *et al*. (2004) indicates that the nodularin synthetase genes were formed from the ancestral microcystin synthetase gene set through a deletion and mutation in the *mcyA* gene of the microcystin cluster. The authors suggested that nodularins can be regarded as the most extreme structural variant of the numerous known microcystin variants. As in *Microcystis* and *Anabaena*, the Nodularin genes are transcribed from a central bidirectional promoter region (Dittmann and Börner 2005).

1.4 MOLECULAR ANALYSES OF POTENTIAL TOXIGENIC CYANOBACTERIA

To reduce the risk of poisoning in humans and animals there is a need for sensitive and robust methods for detecting and monitoring of toxin producing cyanobacteria in drinking and recreational waters. Since microcystin producing and non-producing genotypes within a particular species are closely related, identification based on cellular morphological characteristics is extremely difficult (Hisbergues *et al*., 2003). Furthermore, morphological plasticity has also been observed in culture which adds to the difficulty of morphological identification (Otsuka *et al*., 2000). To overcome these issues, molecular analyses have been developed to help with the identification of cyanobacteria to the strain level. Molecular DNA tools provide a reliable method of detecting toxic from non-toxic cyanobacterial strains (Janse *et al*., 2004).

Genomic analysis and sequence analysis of various genes and variable non-coding regions have been used to detect microcystin producing cyanobacteria. Past studies included random amplified polymorphic DNA (RAPD) (Nishihara *et al*., 1997), restriction fragment length polymorphism (RFLP) (Neilan *et al*., 1995) repetitive DNA elements (Asayama *et al*., 1996; Rouhiainen *et al*., 1995), and 16S rRNA genes (Neilan *et al*., 1997; Otsuka *et al*., 1998; Tillett *et al*., 2001) but none of these analyses have been shown to be reliable in identifying toxic microcystin producers from non-producers. On the other hand, studies analyzing the *mcy* gene cluster have shown a direct relationship between gene detection and toxin production.

1.4.1 PCR OF *MCY* GENES FOR IDENTIFICATION OF POTENTIAL TOXIGENIC *MICROCYSTIS* SPECIES

Despite the genotypic similarities between toxic and non-toxic strains, only potential microcystin producing species contain the microcystin gene cluster (Baker *et al*., 2002; Tillett *et al*., 2000). Elucidation of the microcystin genes and its biosynthesis has led to a number of studies utilizing the *mcy* region to detect toxin-producing strains. A study by Janse *et al*. (2004) of the *mcyA* gene, the *mcyB* gene, and the adenylation domain within the *mcy* gene cluster revealed a good correlation with microcystin production but showed some irregularities. Some of their samples showing no microcystin production tested positive for the presence of *mcy* genes. This may be due to the similarity of the *mcyB* region with other loci, or possibly the presence of inactive *mcy* gene cluster in non-toxic *Microcystis* strains (Janse *et al*., 2004).

As previously stated above, the genes *mcyD, mcyE* and *mcyG* are responsible for the assembly of the unique Adda moiety. PCR primers flanking sequences of the *mcyD* and *mcyE* gene have been successful at identifying potentially harmful hepatotoxic *Microcystis* and *Anabaena* species (Vaitomaa *et al*., 2003). To increase sensitivity, some researchers opted to use several primer sets. Ouahid *et al*. (2005) used two to three sets of different primers targeting the *mcy* genes in their whole cell PCR reaction with success. Regardless of the primer combination, PCR products were only obtained from microcystin producing strains (Ouahid *et al*., 2005). For most of these studies, the primers used were only specific towards one microcystin producing genera (Bertasi *et al*., 2003; Christiansen *et al*., 2003; Kurmayer and Kutzenberger 2003; Kurmayer *et al*., 2005; Neilan 1996; Tillett *et al*., 2001; Vaitomaa *et al*., 2003). Thus, using these genus specific primers could result in an under estimation of total potential toxin-producers in water

samples. Primers targeting the *mcy* genes in more than one genus allows for the monitoring of multiple microcystin-producing species in freshwater. A study by Hisbergues *et al*. (2003) using PCR primers targeting the condensation domain of McyA have been shown to amplify the corresponding *mcyA* fragment in *Anabaena*, *Planktothrix*, and *Microcystis*, the main genera responsible for most of the microcystin-producing species. Recently, PCR primers designed by Jungblut and Neilan (2006) were able to detect all known microcystin and nondularin-producing strains by targeting the aminotransferase (AMT) domain of the *mcyE* and *ndaF* genes. Amplifying regions of the *mcy* gene cluster is a rapid and easy way of identifying the presence of potentially toxic microcystin-producing blooms in advance. However, focusing solely on the *mcy* gene regions ignores the non-microcystin producing cyanobacteria species that may also be present in the sample. Thus, information about the diversity of cyanobacteria in the water body will be missed.

1.4.2 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) OF CYANOBACTERIAL 16S RRNA GENES & THE INTERNAL TRANSCRIBED SPACERS (ITS)

The combination of PCR and denaturing gradient gel electrophoresis (DGGE) is widely used in analyzing microbial diversity based on target nucleic acid sequences (Bernard *et al*., 2000; Gill *et al*., summitted; Muyzer, de Waal, Uitterlinden 1993; Muyzer and Smalla 1998; Schafer *et al*., 2000; van Hannen *et al*., 1999). PCR-DGGE is capable of surveying entire microbial communities without cultivation (Muyzer *et al*., 1993). DGGE is a sequence-dependent separation of PCR products that can be used to evaluate the genotypic diversity in natural samples and to assess the purity of isolated cultures or colonies (Muyzer *et al*., 1993). In DGGE, separation of PCR products is based on the electrophoretic mobility of partially melted DNA molecules in a polyacrylamide

gel of increasing denaturing conditions (urea and formamide) (Muyzer *et al*., 1993). The degree to which a DNA strand dissociates depends on the nucleotide composition and melting properties of the molecule (Nollau and Wagener 1997). As nucleotide domains in the DNA molecule separate, there is a change in the double stranded DNA structure from helical to partially dissociated DNA molecule which halts the migration of the DNA molecule (Muyzer *et al*., 1993). This results in unique fingerprints when DGGE is used to separate PCR products from environmental samples. To guarantee sequence-specific strand separation and to prevent a complete denaturation of the double-stranded DNA molecule, GC-rich sequences (GC-clamp) is incorporated onto the 5' end of the forward primer (Myers *et al*., 1985; Sheffield *et al*., 1989).

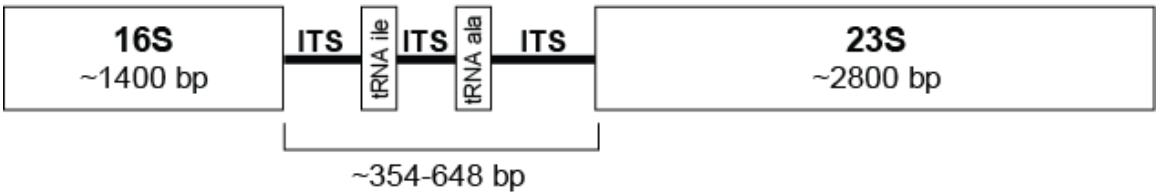
The numbers, positions, and intensities of the DGGE bands can be used to determine the diversity of environmental samples (Garcia-Pichel *et al*., 2003; Lyautey *et al*., 2005; Muyzer 1999; Nakatsu *et al*., 2000). However, it should be noted that DGGE diversity profiles are not completely reflective of the true diversity of the field (Janse *et al*., 2003). There are inherent problems associated with both the DNA extraction step and the PCR-DGGE step. Misinterpretation of DGGE profiles can be due to amplification errors, preferential amplification of DNA templates (Reysenbach *et al*., 1992), varying numbers of rRNA operons between genera (Speksnijder *et al*., 2001), heteroduplex formation (Jensen & Straus, 1993), and co-migration of different DNA fragments (Sekiguchi *et al*., 2001). To reduce possible inter-sample PCR variation, all PCR reactions can be run in triplicates and pooled together before loading on a DGGE gel (Li *et al*., 2006).

To visualize the genotypic diversity of bacteria including cyanobacteria in natural samples, DGGE analysis of the small-subunit ribosomal RNA (16S rRNA) gene sequence (Figure 1.3) has been widely used (Kolmonen *et al*., 2004; Li *et al*., 2006; Nakatsu *et al*., 2000; Nübel *et al*., 1997; Zwart *et al*., 1998). Sequence analysis of the 16S rRNA gene is useful in the phylogenetic classification of taxonomic levels above species (Wilmotte 1994). Due to its high degree of conservation, 16S rRNA genes often fail to discriminate between closely related species (Janse *et al*., 2003). For higher taxonomic resolution of closely related species, there is an increased focus on the faster evolving rRNA 16S–23S internal transcribed spacer (rRNA-ITS) within the ribosome cistron (Hillis and Dixon 1991) (Figure 1.3). Janse *et al*., (2003, 2004) showed that DGGE analyses of natural and cultured samples were very reliable in differentiating between closely related cyanobacterial species. The authors determined that rRNA-ITS DGGE is a valuable method for the analyses of the *Microcystis* genus due to the high resolution and single bands generated. These studies showed that DGGE can be a powerful tool for examining and comparing cyanobacterial diversity.

1.4.3 QUANTIFICATION OF CYANOBACTERIA USING QUANTITATIVE REAL-TIME PCR

Adding fluorescent dye in a PCR reaction permits monitoring of amplicon accumulation in a closed tube using a laser or a camera in real time (Walker 2001). Quantitative real-time PCR (qPCR) combines the amplification step and detection step into a single process (Wong and Medrano 2005). During qPCR reactions, data is collected throughout the process by monitoring fluorescence intensity which correlates with PCR product concentrations (Walker 2001; Wong and Medrano 2005). PCR reaction can be divided into four phases: the linear ground phase, early exponential phase,

FIGURE 1.3 **Map of prokaryotic rRNA cistron.** Functional constraints of rRNA makes the gene highly conserved and is therefore extensively used in diversity studies.


16S
~1400 bp
ITS
tRNA ile
ITS
tRNA ala
ITS
23S
~2800 bp
~354-648 bp

log-linear phase (exponential phase), and plateau phase (Wong and Medrano 2005). In regards to qPCR, the early exponential phase is where the threshold cycle is reached – the fluorescent signal is higher than the background (Ct) (Wong and Medrano 2005). The Ct value is inversely reflective of the target starting copy number in the original sample (Wong and Medrano 2005). There are a variety of applications for qPCR such as single-step detection and quantification of target nucleic acids from food, vectors in gene therapy protocols, genetically modified organisms, viral load, and areas of microbiology and oncology (Mackay *et al*., 1993). With respect to cyanobacteria, qPCR has improved the speed and resolution at which toxic and nontoxic *Microcystis* populations can be examined (Rinta-Kanto *et al*., 2005). Rinta-Kanto *et al*., (2005) surveyed the cyanobacterial community in western Lake Erie by quantifying the gene copy numbers of cyanobacteria-specific 16S rRNA genes, *Microcystis*-specific 16S rRNA genes, and *mcy*D genes. The authors noted that prokaryotes have varying numbers of 16S rRNA genes (ranging from one to four or more per genome) within the genome that may be a source of error for quantification based on the 16S rDNA. The enumeration of toxigenic *Microcystis* populations based on the *mcyD* gene is more sensitive since it appears that the cells carry only one *mcyD* gene copy per genome (Rinta-Kanto *et al*., 2005). The quantification of *mcyA* genotypes in comparison with direct microscopic counts of *Microcystis viridis* (A. Braun) Lemmermann showed relatively good correlation between the two (Furukawa *et al*., 2006). This suggests that qPCR can accurately quantify the number of cyanobacteria. Earlier studies by (Kurmayer and Kutzenberger 2003) and (Vaitomaa *et al*., 2003) utilized other *mcy* genes to quantify the toxigenic *Microcystis* population. Kurmayer and Kutzenberger (2003) monitored the microcystin content of

Lake Wannsee by comparing the copy number of the *mcyB* gene region with the intergenic spacer region within phycocyanin operon. By comparing the two gene regions, the amount of toxic genotypes in relation to the total *Microcystis* concentration can be quantified. Vaitomaa *et al*. (2003) used the *mcyE* gene region to quantify and discriminate toxigenic *Microcystis* and *Anabaena* in lake samples. These studies show that qPCR is a sensitive and rapid tool for monitoring potential toxic blooms.

1.5 THESIS OBJECTIVES

1. **Use PCR-DGGE analysis of the 16S-V3 spacer to profile the cyanobacterial genetic community and aminotransferase (AMT) domain of *mcyE* and *ndaF* genes to detect the presence of potential hepatotoxic cyanobacterial genotypes.**

PCR-DGGE analyses of the 16S-V3 spacer will be used to evaluate the cyanobacterial phytoplankton community from the three study sites (Lake Ontario, Lake Erie, and Lake Victoria). The 16S-V3 hypervariable region spans the 357-518 nucleotide region of the 16S ribosomal DNA of cyanobacteria (Yu and Morrison 2004). Cyanobacteria specific V3 primers (Cya-b-F371 and Cya-R783) designed by (Zwart *et al*., 2005) were used to characterize the planktonic cyanobacterial genotypes present from each sample. Primers (HEPF and HEPR) designed by Junglunt and Neilan (2006) that amplify the AMT domain of the *mcyE* and *ndaF* genes in all known microcystin and nodularin producing strains will be used to determine the presence of potential hepatotoxin-producers from the collected filter samples. PCR-DGGE of the AMT domain will determine if multiple hepatotoxic cyanobacterial genotypes were present from each

sampling station for each of the three lakes. For each DGGE profile, the presence of bands and band intensity will be used to statistically calculate genotypic diversity and similarity between each site. Predominate and unique bands from certain profiles will be excised from the gel and sequenced in an attempt to identify the corresponding cyanobacteria species.

2. Quantify the number of cyanobacteria and potential hepatotoxic cyanobacteria present at each sampling site using quantitative real-time PCR (qPCR) of the 16S-V3 and AMT fragment respectively.

The same cyanobacteria specific 16S-V3 primers (Cya-b-F371 and Cya-R783; Zwart *et al*., 2005) used in DGGE will be used to estimate the number of cyanobacteria present at each sampling site. The same primers for the AMT gene region (HEPF and HEPR; Junglunt and Neilan, 2006) as mentioned above will used to determine the number of toxic cyanobacteria present in the filter sample. For each sample, data obtained from qPCR of AMT fragment will be compared with the 16S qPCR data to estimate the percentage of potentially toxic cyanobacteria.

CHAPTER 2: METHODS

2.1 STUDY SITES

2.1.1 BAY OF QUINTE, LAKE ONTARIO

The Bay of Quinte (257.4 km^2) at the northeastern end of Lake Ontario is a Z-shaped bay that is divided into three regions: upper (Trenton to Green Point, Ontario), middle (Green Point to Glenora), and lower bays (Glenora to Lake Ontario) (Figure 2.1). The upper bay spans 36.4 km^2 in area, with a mean depth of 3.5 m, and is the shallowest of the three regions; the middle bay, 49.2 km^2, has a mean depth of 5.2 m; and the deep lower bay, 71.8 km^2, has a mean depth of 24.4 m (Chu *et al*., 2004; Minns 1995).

In this study, the Bay of Quinte was sampled during the summer of 2005 and 2006. In 2005, three stations: Napanee (NA), Hay Bay (HB) and Big Bay (BB) were sampled on three separate dates (June 28, August 3, and August 30) on the fourth collecting trip only Napanee was sampled due to poor weather conditions. During the summer of 2006, the number of sampling stations was increased to six: NA, GPT, NR, MBO, F1, and DS. Two collecting trips were made in 2006: one in early summer (July 4) and one in late summer (September 22).

2.1.2 MAUMEE BAY, LAKE ERIE

Lake Erie (12,893 km^2) is the most southern and shallowest of the five Laurentian (North American) Great Lakes (Figure 2.2). The lake is divided into three distinct physiographic basins: western, central, and eastern with a mean depth of 10, 25, and 50 m respectively. Six stations were sampled in early summer (June 20, 2006) and late summer (August 22, 2006) from Maumee Bay, western Lake Erie (Toledo, Ohio). The six stations

Figure 2.1 **Map of the Bay of Quinte station locations in 2005 and 2006.** Locations of stations are indicated by station code and asterisk. (A) Lake Ontario with location of the Bay of Quinte circled in red, (B) station locations sampled in 2005, (C) station locations within the Bay of Quinte in 2006.

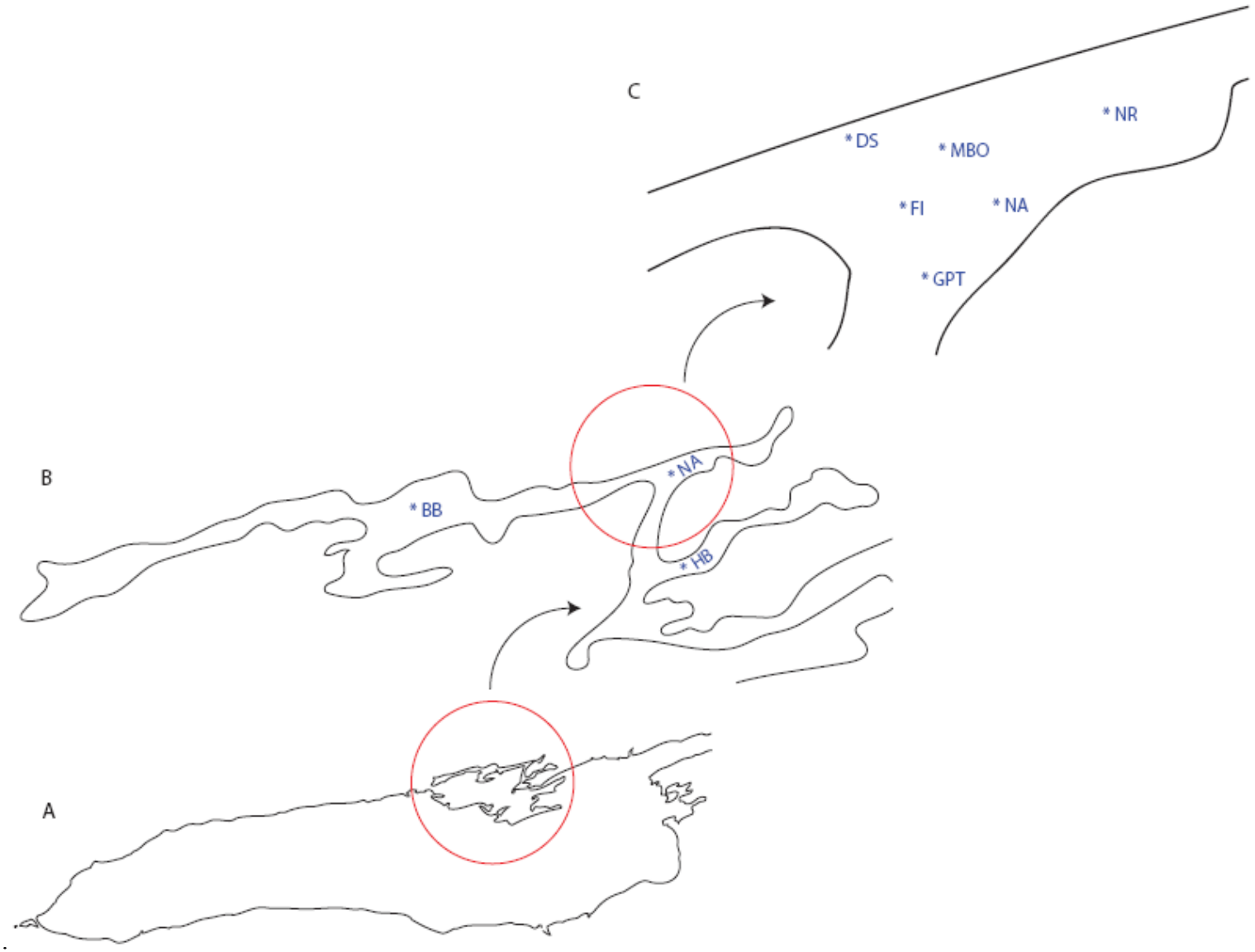

C
* NR
* DS
* MBO
* FI
* NA
* GPT
B
* NA
* BB
* HB
A

Figure 2.2 **Map of Maumee Bay station locations in 2006.** Locations of stations are indicated by station code and asterisk. (A) Lake Erie with location of Maumee Bay circled in red, (B) station locations sampled in 2006.

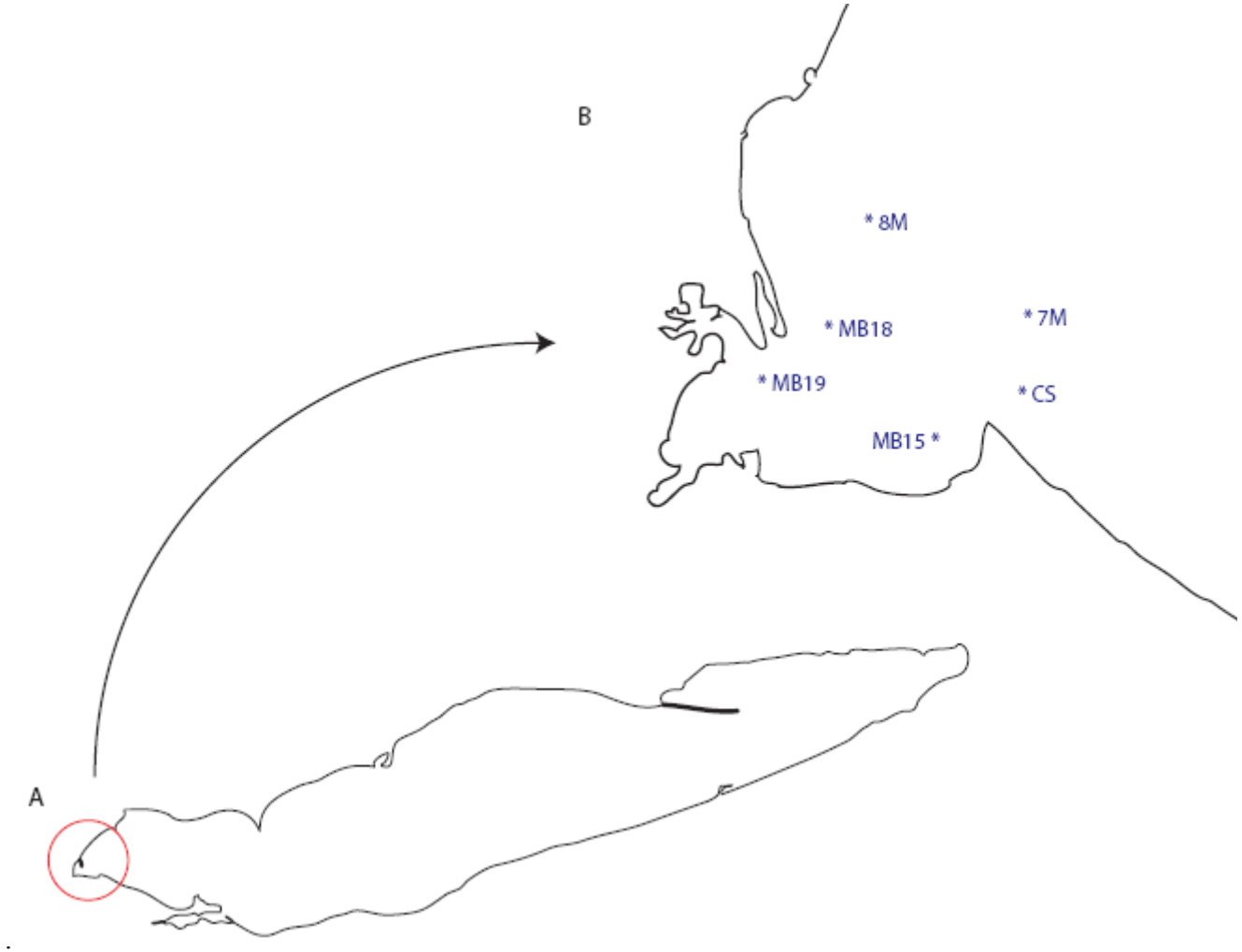
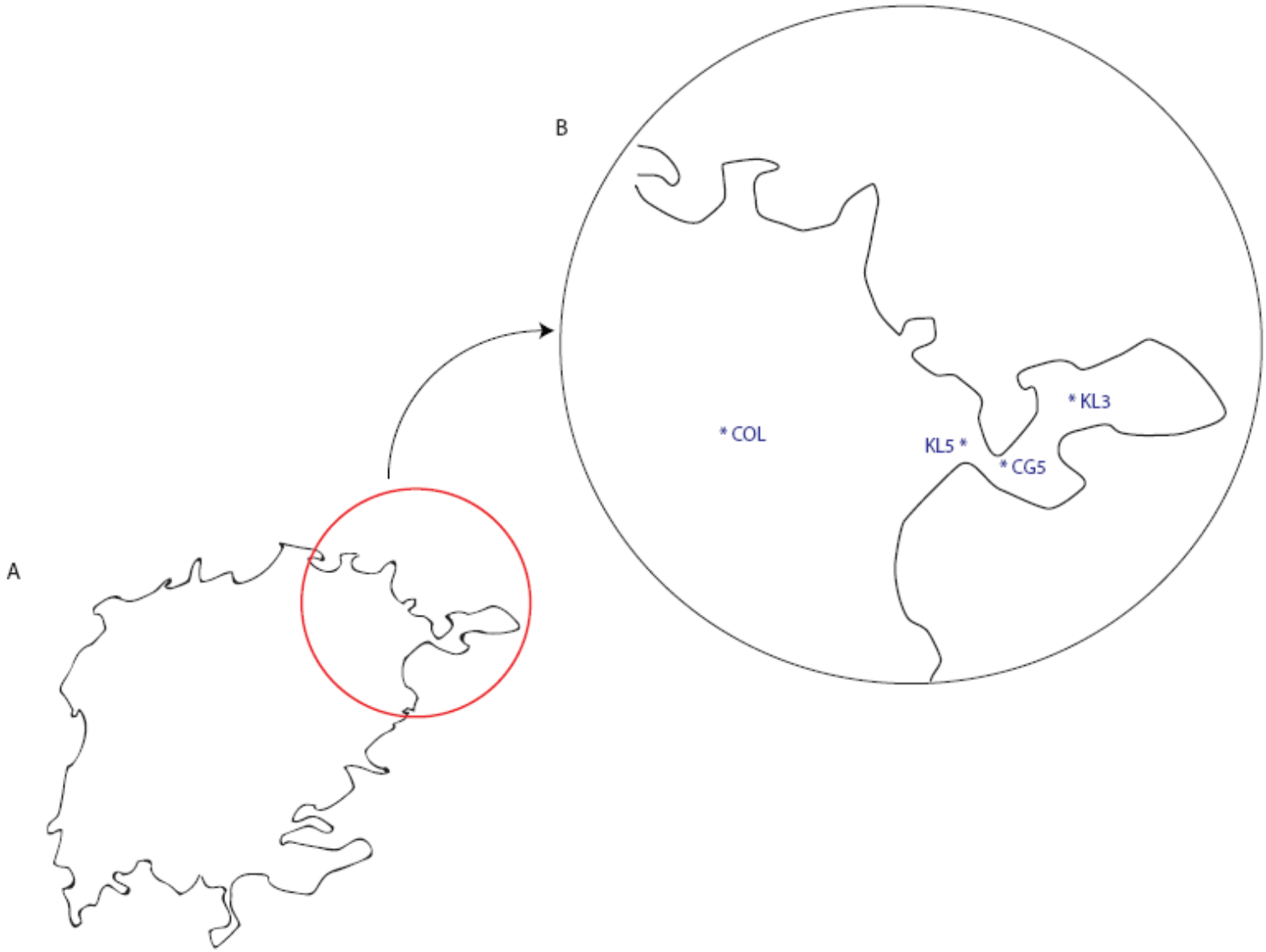

B
* 8M
* MB18
* 7M
* MB19
* CS
MB15 *
A

Figure 2.3 **Map of Nyanza Gulf station locations in 2005.** Locations of stations are indicated by station code and asterisk. (A) Lake Victoria with location of Nyanza Gulf circled in red, (B) station locations sampled in 2005.


A
B
* COL
KL5 *
* CG5
* KL3

include: MB19 (N 41.72714, W 83.43214), MB18 (N 41. 74238, W 83.40142), 8M (N41.78887, W 83.35550), MB15 (N 41.70132, W 83.36464), Clear Site (N 41.70004, W 83. 32247), and 7M (N 41.73333, W 83.29720).

2.1.3 NYANZA GULF, LAKE VICTORIA

Relative to the Laurentian Great Lakes (<13 000 BP), Lake Victoria (East Africa) is an ancient lake (>23 000 BP) (Figure 2.3). Its water is shared among three countries: Tanzania, Kenya, and Uganda in east Africa. In terms of surface area, Lake Victoria is the largest tropical lake and second largest freshwater lake in the world. It spands 68 800 km^2 with a mean depth of 40 m and volume of 2 760 km^3 (Guildford *et al*., 2003). In this study, four stations were sampled between November 14 and 15, 2005: 3 from Nyanza Gulf, (also called Winam Gulf or Kavirondo Gulf) and one from the open lake. The gulf is a shallow embayment that connects to the open lake via the Rusinga channel. The locations of the sites are as follows: KL3 (S 00^o15.371', E 34^o 31.173'), KL5 (channel; S 00^o 21.177', E 34^o 14.302), and CG5 (channel; S 00^o 25.193', E 034^o 18.149); and one from the open lake COL (channel open lake; S 00^o 18.597', E 34^o 08.798'). As well, a phytoplankton bloom sample was taken from Nyanza Gulf on November 16, 2005.

2.2 SAMPLING

From each station sampled, known volumes of water sample were taken from depths that ranged from surface water to 2.0 m below the water surface. Water sample volumes ranging from 0.5 L to 1.0 L were vacuum-filtered through a glass fibre filter (Whatman GF/F; 47 mm diameter; N.J., U.S.A.). Each filter was stored in individual polystyrene petri dishes (Falcon no. 1006; 50 x 9 mm; Becton-Dickinson Labware; NJ, U.S.A.) and kept in a dewer containing dry ice during transportation for long-term

storage in a –80^{o}C freezer until use. At each station sampled, 6 filter replicates were taken and about 25-50 ml of phytoplankton sample was preserved in Lugol's iodine for future microscopic identification.

2.3 STRAINS AND STRAIN CULTIVATION

Five non-toxic cyanobacterial isolates and three microcystin-producing isolates (Table 2.1) were obtained from Dr. Susan Watson (Environment Canada, University of Calgary), Dr Freiderich Jüttner (University of Zurich), and four culture collections: Culture Collection of Algae (NIVA), Norway; Pasteur Culture Collection (PCC), France; University of Toronto Culture Collection (UTCC), Canada; and the University of Texas Culture Collection (UTEX), USA were amongst the studied cyanobacteria. These cultures were grown at 23ºC in liquid mediums of Bold's basal medium (BBM) (Stein, 1973), B3N (Bold-Basal Medium with 3-fold nitrogen and vitamins; modified), or cyano media (Jüttner *et al*., 1983) in a 150 ml Erlenmeyer flask containing approximately 50 ml of media at an irradiance of approximately 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for a cycle of light-to-dark ratio of 16:8 h. To maintain healthy cell growth, subcultures (1-5 ml) of each isolate were transferred to a new sterile 150 ml Erlenmeyer flask with fresh media monthly and grown under the same conditions as the initial culture. The isolates were monitored microscopically using an Olympus BX51 light microscope (Olympus Optical Co., LTD; Tokyo, Japan) to eliminate cross-contamination and dominance of bacteria. All steps were performed in a laminar flow hood using aseptic technique and sterile equipment.

2.4 DNA EXTRACTION FROM CELL CULTURES

DNA from cell cultures of cyanobacterial isolates were extracted by transferring 250 µl of each culture into a sterile 1.5 ml microfuge tube and centrifuged at 8000 rpm for 2 min to pellet the cells. For cell lysis, the supernatant was removed and the pelleted cells were freeze-thawed three times by immersing each tube in liquid nitrogen for 20 s immediately followed by immersion in a 65°C water bath for 30 s. DNA was isolated using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc.; Mississauga, Canada) and DNA was eluted in 50-150 µl of either 50 mM Tris-HCl (pH 8.0) or DNA/RNA free water.

2.5 DNA EXTRACTION FROM FROZEN FILTERS

Bead-beating technique was used for the extraction of bulk community DNA from phytoplankton samples collected on GF/F filters. A combination of protocols by Kim *et al*. (2006), Giovannoni *et al*. (1990) and Zwart *et al*. (1998) were applied for the extraction method. In general, $1/16^{th}$ of the GF/F filter was cut using a sterile razor blade and transferred into a 2.0 ml screw-cap tube containing zirconia/silica beads (0.5 g of 0.5 mm in diameter and 0.2 g of 0.1 mm in diameter; Biospec, Bartlesville, OK, USA) and 700 µL of lysis buffer (40 mM EDTA, 400 mM NaCl, 50 mM Tris-hydrochloride, pH 9.0). The sample was bead-beaten for 90 seconds at 3500 rpm followed by cooling on ice for 1.0 minute. The sample was then centrifuged for 2.0 minutes at 10 000 x g and supernatant was transferred to a new 1.5 ml microfuge tube. RNase was added to a final concentration of 1.0 µg μL^{-1} and incubated for 10 minutes at 65^{o}C. Following incubation, proteinase K was added to a final concentration of 50 µL ml^{-1} and sodium dodecyl sulfate (SDS) to a final concentration of 0.5%. The cell suspension was then incubated at 60^{o}C

for 1 hour. DNA was isolated with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 [vol/vol/vol]) and centrifuged for 5 minutes at 13 000 g. The aqueous layer was transferred to a new 1.5 ml microfuge tube and the previous step was repeated again. The aqueous phase was then purified with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]) to the aqueous phase followed by centrifuging for 5 minutes at 13 000 g. DNA was then precipitated with 3 M sodium acetate (pH 5) (0.1x volume of aqueous phase) and iso-propanol alcohol and incubated in a –80^{o}C freezer for 15 minutes followed by centrifuging in a 4^{o}C centrifuge for 20 minutes at 14 500 g. The supernatant was discarded followed by a wash with 500 µL of 70% ethanol and centrifuged in a 4^{o}C centrifuge at 14 500 x g for 10 minutes. The supernatant was discarded and the DNA pellet was dried in a SpeedVac for 2-5 minutes. The DNA pellet was rehydrated in 50 µL of DNA/RNA free water and stored in a –20^{o}C freezer until further use.

2.6 PCR AMPLIFICATION OF 16S rRNA-V3 REGION

Using DNA extracts from filter samples or cyanobacterial isolates, a 161 base pair (bp) region of the 16S rRNA gene was amplified in two successive PCRs, conducted as a nested PCR first using cyanobacterial specific primers to reduce bacterial DNA interference (Zwart et *al.*, 2005). The first cyanobacteria-selective round of amplification was performed with 2 µl DNA extract, 2.5 µl 10x taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 (at 25°C), 1.0% Triton® X-100, and 15 mM $MgCl_2$) (Promega; W.I., U.S.A.), 0.625 U Taq DNA polymerase (Promega; W.I., U.S.A.), 200 µM each dNTP (Promega, Madison, W.I., U.S.A.), 0.5 µM of each cyanobacterial specific primer Cya-b-F371 (5'-CCTACGGGAGGCAGCAGTGGGGAATTTTCCG-3') and Cya-R783

(5'-GACTACWGGGGTATCTAATCCW-3') (Zwart *et al*., 2005) in a total reaction volume of 25 µl. A 20-cycle touchdown procedure was followed using the Eppendorf Mastercycler® Gradient 5331 (Eppendorf, C.A., U.S.A.). After an initial denaturation step at 95°C for 1 min 30 s, a 20 cycle touchdown procedure was performed at 94°C for 1 min, 65°C for 1min, and 72°C for 1 min, in which the annealing temperature decreased by 0.5°C each cycle to end at 55°C in the final cycle. A final extension step at 72°C for 10 min concluded the end of the pre-amplification. The touchdown procedure reduces the formation of spurious by-products during amplification (Muyzer *et al*., 1993).

The second round of amplification was performed with similar reagent concentrations as the first round but with the following changes: 2 µl template DNA, 0.5mM of each general bacterial primer (GC)-F357 (5'-CCTACGGGAGGCAGCAG-3') and R518 (5'-CCAGCAGCCGCGGTAAT-3') (Muyzer *et al*., 1993) in a total reaction volume of 25 µl. A 40 bp GC-clamp 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC-3' was added to the 5' end of primer F357GC to increase the separation of DNA bands in DGGE gel (Muyzer *et al*., 1993). Following the initial denaturation step at 95ºC for 1 min 30 s, a touchdown procedure consisting of 20 cycles at 94°C for 1 min, 65°C for 1min, and 72°C for 1 min, in which the annealing temperature decreased by 0.5°C each cycle was performed. Subsequently five additional cycles were performed at 94°C for 1 min, 55°C for 1min, and 72°C for 1 min. A final extension step at 72°C for 10 min concluded the nested round of the procedure. Each Amplification product was electrophoresed through a 1.0% agarose gel in 1x TBE buffer at 100 V for 45 min. Products were visualized using the Syngene Bioimaging System (Synoptics Ltd., United Kingdom). PCR product size and concentrations were estimated

using a DNA marker (ΦX174 DNA digested with *Hae* III restriction enzyme) that was electrophoresed along side the PCR products.

2.7 PCR AMPLFICATION OF AMINOTRANSFERASE REGION (AMT)

PCR amplification of a 472 bp fragment of the aminotranferase domain of PKS and NRPS in *mcy*E and *nda*F respectively was similar as above but with 0.5 μM of each primer: (GC)-HEPF (TTTGGGGTTAACTTTTTTGGGCATAGTC) and HEPR (AATTCTTGAGGCTGTAAATCGGGTTT) (Jungblut and Neilan 2006). A 40 bp GC-clamp was added to the 5' end of primer (GC)-HEPF. Thermal cycle program consisted of initial denaturation step at 92^{o}C for 2 min and 35 cycles of 92 ^{o}C for 20 s, 52 ^{o}C for 30 s, and 72 ^{o}C 1 min, with a final extension step at 72^{o}C for 5 min. PCR amplicons were visualized as above.

2.8 DGGE STANDARD

The standards were a modification of those used by (Gill *et al*., submitted). DNA concentration was estimated from agarose gel electrophoresis. Approximately 100 ng·μl^{-1} of amplified 16S rDNA fragments from 9 well resolved single band cyanobacterial isolates (Table 2.1) were mixed to create a 16S rRNA-V3 DGGE standard. When electrophoresed in a DGGE gel, each band in the mixture was representative of one DNA sequence at a single position on the gel and associated with one or more known cyanobacterial isolates used in this study. An AMT (*mcyE/ndaF*) DGGE standard consisted of 3 known microcystin producing strains: UTCC 299 (*Microcystis aeruginosa*), UTCC 507 (*Planktothrix rubescens*), and NIVA-CYA 83/1 (*Anabaena lemmermannii* var.

TABLE 2.1 **Cyanobacteria isolates of the Chroococcales, Nostocales, and Oscillatoriales used in the development of the16S rRNA-V3 region DGGE standard marker.** The asterisk denotes the three known microcystin-producing strains used in the development for the AMT region (of the *mcyE and ndaF* gene) DGGE standard marker.

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Taxon		Strain	Origin	Source Collection
Chroococcales	*Microcystis aeruginosa* Kutz.em. Elenkin*	UTCC 299	Pretzlaff Pond, Alberta, Canada	University of Toronto Culture Collection
Nostocales	*An. flos-aquae*	UTCC 64	Western Lake Ontario, Canada	University of Toronto Culture Collection
	An. lemmermannii var minor*	NIVA-CYA 83/1	Unknown	Culture Collection of Algae (NIVA), Norwegian Institute for Water Research
	Anabaena viguieri Denis et Frémy		Unknown	Dr Freiderich Juttner (University of Zurich)
	*Aphanizomenon flos-aquae (*Linneaus) Ralfs	HHAFA	Hamilton, Harbour, Lake Ontario, Canada	Dr. Susan Watson (Environment Canada, University of Calgary)
	Calothrix parietina (Thuret) Bornet et Flahault	PCC 6303	Wisconsin, USA	Pasteur Culture Collection c/o Dr Freiderich Juttner (University of Zurich)
	Gloeotrichia ghosi Singh	LB 1920	Unknown	The Culture Collection of Algae, University of Texas
Oscillatoriales	*Oscillatoria limosa*		Unknown	Dr Freiderich Juttner (University of Zurich)
	*Planktothrix rubescens**	UTCC 507	Lake Wilcox, Ontario, Canada	University of Toronto Culture Collection

minor). The DGGE standards allowed for the comparison of banding profiles from natural samples.

2.9 DGGE PROFILING OF 16S rRNA-V3 AND AMT AMPLICONS

A vertical DGGE was performed using the D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Inc.; C.A., U.S.A.). PCR amplicons (25 µl) of from the 16S rRNA–V3 region from natural samples were applied to 8% (wt/vol) polyacrylamide gel (1.0 mm, 16 by 16 cm; acrylamide-bisacrylamide, 37.5:1) with a linear denaturing gradient of 35-55%, and 40-55% (100% denaturant is 7 M urea and 40% deionized formamide [vol/vol]) for 16S rRNA-V3 and AMT amplicons respectively. Samples were electrophoresised in a 0.5x TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA, [pH 7.6]) at a constant voltage and temperature of 60 V and 60°C respectively for 16 h. After electrophoresis, the gel was stained in 0.5 µg ethidium bromide ml^{-1} for 1 h and subsequently visualized using the Syngene Bioimaging System (Synoptics Ltd.; Cambridge, United Kingdom). Band matching to the DGGE marker and determination of band intensities were performed using the GeneTools software program for Windows (Synoptics Ltd.; Cambridge, United Kingdom).

2.10 SEQUENCING OF DNA FROM DGGE PCR PRODUCTS AND BANDS

Bands were spot check by cutting out unique and common bands in each profile using a pipette tip and incubated in 50 µl DNA/RNA free water overnight at 4^{o}C. An aliquot of 1.0 µl of the eluant was re-amplified using the same programs as above (second round of amplification protocol for 16S rRNA-V3 only) and PCR products were purified using UltraClean™ PCR Clean-up™ Kit (Mo Bio Laboratories, Ltd.; C.A., U.S.A.). All

purified PCR products were sequenced using the Applied Biosystems 3130XL Genetic Analyzer. Sequence reaction products were visualized using Bioedit sequence alignment editor and analysis program (Hall 1999) and similarity to sequences deposited in the GenBank databases were verified by using the program BLAST (Altschul *et al*., 1990) (www.ncbi.nlm.nih.gov/blast/).

2.11 STATISTICAL ANALYSIS OF DGGE PROFILES

Banding patterns on gels were statistically analyzed to infer diversity. The presence or absence of bands and/or band intensities in each sample was taken into account. Shannon-Weaver diversity index (*H'*) ($H' = -\sum p_i \times \ln p_i$, where p_i is the proportion of members that a particular species contributes to the total in the sample) was used to calculate a relative estimate of the degree of genetic variation within a population (Girvan *et al*., 2003; Kowalchuka *et al*., 2006; Shannon and Weaver 1949). *H'* takes into account both the "richness" and "eveness" component of diversity (Lynch *et al*., 2004). Similarity indices between sample banding patterns were calculated using Jaccard similarity index ($J = 100(c/[a + b - c])$, where a is the number of bands of sample A, b the number of bands in sample B and c the number of bands common to A and B) (Jaccard 1908; Lyautey *et al*., 2005).

2.12 QUANTITATIVE REAL-TIME PCR (QPCR)

To quantitatively describe the number of cyanobacteria and potential toxic Cyanobacteria present in each sample, the gene copy numbers of cyanobacteria-specific 16S rRNA genes, and AMT (*mcyE/ndaF*) were determine by quantitative real-time PCR (qPCR) in two separate assays. PCR amplification and quantification were performed

using a 48-well plate MiniOpticon Real-Time PCR Detection System and MJ Opticon Monitor™ Analysis Software, version 3.1.32 (Bio-Rad Laboratories, Inc.; C.A., U.S.A). The same cyanobacteria-specific 16S rRNA primers (Cya-b-F371 and Cya-R783) and AMT (*mcyE/ndaF*) primers (HEPF and HEPR) as above but without the 40-nucleotide GC clamp were used in the qPCR reaction. For both cyanobacterial 16S and AMT (*mcyE/ndaF*) assays, qPCR was performed with a total volume of 20 µl containing: 10 µl of the hot start reaction mix (2x iQ SYBR Green Supermix; Bio-Rad Laboratories, Inc.; C.A., U.S.A), 0.5 µM concentrations of each primer (Sigma-Genosys, Ltd., T.X., U.S.A), and 2 µl of DNA extract. All the samples were amplified in triplicates with a no template control (NTC) included in each run. The thermal cycle program for cyanobacterial 16S rRNA-V3 consisted of initial preheating step of 95^{o}C for 3 min, followed by 40 cycles of 95^{o}C for 20 s, 52 ^{o}C for 20s, and 72 ^{o}C for 20 s. The thermal cycle program for AMT (*mcyE/ndaF*) consisted of 95^{o}C for 3 min, followed by 40 cycles of 95^{o}C for 20 s, 56^{o}C for 30s, and 72^{o}C for 60 s. The generation of amplicons was monitored after each annealing and extension step by measuring the fluorescence intensity from double-stranded DNA binding SYBR Green I dye using MiniOpticon (Bio-Rad Laboratories, Ltd.; C.A., U.S.A.). To assess the homogeneity of a sample, fluorescence melting (dissociation) curve analysis was performed on the amplified products in each reaction tube. Following the qPCR reaction, the temperature was gradually increased from 56^{o}C to 95^{o}C with 1^{o}C increment and a hold time of 2 s at each increment before the fluorescence is read. To check that the expected amplicons were amplified, PCR products were run on a 1% agarose gel along side a ΦX174 DNA-*Hae* III standard. The number of total cyanobacterial and potential toxic cyanobacterial cells in each sample were determined

by converting the obtained Ct values into cell numbers according to the to the regression equations of the external standards (cell number vs Ct). Data from qPCR analysis was subjected to an ANOVA (analysis of variance) using Systat® version 9 for Windows to determine if there was a significant differences between lakes, stations, and dates.

2.13 CONSTRUCTION OF QPCR STANDARD CURVES

External standards of the 16S rRNA gene and AMT (*mcyE/ndaF*) were prepared from genomic DNA of *Microcystis aeruginosa* UTCC 299. *M. aeruginosa* UTCC 299 was grown in 1.0 L Erlenmeyer flask containing approximately 350 ml of Bold's basal medium (BBM) (Stein 1973). An aliquot of the culture was directly counted in a Spencer Brite-line® hemocytometer (American Optical, Scientific Instruments; N.Y., U.S.A.). The cells were enumerated under 100x magnification with an Olympus BX51 System compound light microscope. Five cell-count replicates were performed and an average cells·L^{-1} was calculated. Immediately following cell counts, a 25 ml volume was filtered through a GF/F filter and frozen prior to extraction. The number of cells present on the GF/F filter was calculated by multiplying the cells·L^{-1} (determined hemocytometer) by the volume filtered (25 ml). Genomic DNA extraction process was the same as above and total DNA yield was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc.; Wilmington, D.E., U.S.A). To determine how much DNA is yielded from one cell, DNA yield (ng of DNA) was divided by total number of cells on the 1/16 filter piece. Six dilutions of template DNA ranging from 5 x 10^5 cells to 5 cells equivalent were prepared from the DNA extract to serve as external standards. Cycle threshold line (C_t) was position to obtain the highest possible correlation coefficient (R^2) and amplification efficiency (e, $e = 10^{-1/S} - 1$, where S is the

slope of the linear regression; e = 1.0, means a doubling of product in each cycle) with greater weight placed on the latter. An R^2 value greater than 0.990 indicates proper positioning of the threshold line and a slope of -3.32 indicates perfect doubling.

2.14 PROTEIN PHOSPHATASE INHIBITION ASSAY AND DIRECT CELL COUNT

The protein phosphatase inhibition assay (PPIA) used to determine microcystin concentration was performed by Sarah Yakobowski (University of Waterloo, Ontario, Canada) on all samples collected from the Bay of Quinte and Maumee Bay. One Bay of Quinte (NA September 22, 2006) sample was sent to Hedy Kling (Freshwater Institute, Winnipeg, Manitoba, Canada) for direct cell count.

CHAPTER 3.0: RESULTS

3.1 DGGE RESULTS

3.1.1 CYANOBACTERIA 16S RRNA-V3 GENETIC PROFILES FROM THE BAY OF QUINTE, MAUMEE BAY, AND NYANZA GULF USING DGGE

Profiles of the cyanobacterial genetic communities using the 16S rRNA-V3 gene region are depicted in Figures 3.1.1 to 3.1.4. For consistency, all samples on each DGGE polyacrylamide gel were extracted, PCR amplified, and DGGE analyzed together. Each cyanobacterial population was differentiated on the basis of the position of their 16S rRNA-V3 amplicons on DGGE gels. In this study, each band was assumed to be a unique genotype. The two blurred bands at the bottom of the 16S rRNA-V3 DGGE gels are presumed to be primer-dimers formed during the amplification process. The intensities of each band provided information about the abundance of a particular genetic population relative to the other bands within the same sample.

3.1.1.1 BAY OF QUINTE, 2005

The 16S rRNA-V3 DGGE profile for the Bay of Quinte in the summer of 2005 (Figure 3.1.1) indicated the presence of a predominant unidentified genotype A from all three sampling stations (NA, HB, BB) in June 28, 2005. Re-amplification and sequencing of genotype A (Figure 3.1.1) provided no further information other than the sequence is of cyanobacterial origin. Microscopic examination of the June 28, 2005 planktonic samples indicated that a large portion of the planktonic community was the genus *Gloeotrichia*. It is speculated that genotype A was from a strain of *Gloeotrichia* other than *G. ghosi* UTEX LB 1920 since genotype A did not match with the standard. A band

corresponding to the *M. aeruginosa* UTCC 299 standard was faintly present in the HB June 28, 2005 filter sample but absent from the other two filters collected on the same date from stations NA and HB. In contrast, bands corresponding to the *A. viguieri*/*A. planktonica* and *M. aeruginosa* UTCC 299 standards were predominant in all three sampling stations from the August and October filters.

3.1.1.2 BAY OF QUINTE, LAKE ONTARIO 2006

The 16S rRNA-V3 profile for the Bay of Quinte samples (Figure 3.1.2) showed differences in the cyanobacteria genetic population between early and late summer. Based on band intensities, *Microcystis* genotypes were predominant in early summer (July 4, 2006) from all six sampled stations. Furthermore, present at each station in relatively high abundance was an unidentified band, genotype B (Figure 3.1.2) between the *A. flos-aquae* UTCC64 and *A. lemmermannii* NIVA-CYA 83/1 standards. Genotype B in the July 4, 2006 samples was at the same position as genotype A in the June 28, 2005 samples (Figure 3.1.1). Sequencing of these two bands, A (Figure 3.1.1), and B (Figure 3.1.2), showed that they were identical. Another unknown band, genotype A (Figure 3.1.2), above the genotype B band (Figure 3.1.2) was present in all the samples but in lower abundance relative to the previous two bands mentioned. In five of the six early summer samples (NA, GPT, NR, MBO, DS) a faint band corresponding to the *A. viguieri*/*A. planktonica* standard was present.

By late summer (September 22, 2006), the band corresponding to the *Microcystis* standard was only faintly present in all the six samples collected from each station. In contrast, there were two discrete genetic populations in relatively high abundance in all six samples (Figure 3.1.2). One was a band matching the *A. viguieri*/*A. planktonica*

standard and the other was genotype C. Genotype C (Figure 3.2) was the most intense band (most abundant) in the late summer samples.

3.1.1.3 MAUMEE BAY, LAKE ERIE 2006

Similar to the Bay of Quinte 2006 samples, profiles of the 16S rRNA-V3 amplicons from the Maumee Bay samples showed differences in the cyanobacteria genetic populations between early and late summer (Figure 3.1.3A and 3.1.3B). Due to slanted banding patterns in the June 20, 2006 samples (Figure 3.1.3A), they were re-analyzed on a separate DGGE gel (Figure 3.1.3B). There were slight differences between the two DGGE gels (Figure 3.1.3A and 3.1.3B) for the June 20, 2006 samples which are discussed below.

Profiles of the early summer (June 20, 2006) samples showed a very faint band corresponding to the *Microcystis* standard only from station MB18 (Figure 3.1.3A). In a second gel run the *Microcystis* band was present in two samples: MB19 and 8M (Figure 3.1.3B). Sample MB18 in early summer showed the only band (faint) matching to the *O. limosa* standard for both gels. Five distinct bands (A, B, D, E, F) that did not migrate to the same position as any of the standards were present in all the early summer samples for both gels (Figure 3.1.3A and 3.1.3B). Unknown genotype A and B bands (Figure 3.1.3A and B) migrated to gel positions above the first *C. parietina* standard. Unknown genotype D, E, and F bands were in close proximity to one another and migrated between the *A. flos-aquae* and *A. lemmermannii* standards. The two gels (Figure 3.1.3A and B) differed by one distinguishable band, genotype C, above the *A. flos-aquae* standard and present in all the samples in the gel depicted in Figure 3.1.3A but absent in the second gel (Figure 3.1.3B).

By late summer (August 22, 2006) *Microcystis* genotypes were present in high abundance from all stations sampled. Based on band intensities, all the other genotypes were in lower abundance relative to the bright bands corresponding to the *Microcystis* standard (Figure 3.1.3A). Two other bands were predominant in the late summer samples: unknown genotype G and H bands (Figure 3.1.3A). Band G appeared to have a similar melting temperature as the *C. parietina* standard since it migrated closely to the same position on the DGGE gel.

3.1.1.4 NYANZA GULF (WINAM GULF), LAKE VICTORIA 2005

Profile of the 16S-rRNA-V3 amplicons showed two bands matching the *Microcystis* and *A. viguieri* standards in all the samples with varying intensities (Figure 3.1.4). The bands corresponding to the *Microcystis* genotype was in higher abundance in samples KL3, KL5, and COL. Sample KL5 had a third band matching the *O. limosa* standard. Unknown genotype A band (Figure 3.1.4) was present in all the station samples collected from the 4 stations but the band was barely visible in the phytoplankton bloom sample. Unknown genotype B band was present in all five samples but lower in abundance relative to the other bands.

3.1.2 PROFILES OF THE TOXIC CYANOBACTERIA COMMUNITY USING AMT REGION OF *MCYE* AND *NDAF* USING DGGE

Profiles of the AMT amplicons for each study site are represented in Figures 3.1.5 to 3.1.9. The profile of the aminotransferase (AMT) domain of the microcystin gene *E* (*mcyE*) and nodularin gene *F* (*ndaF*) showed far less genotype richness than the 16S rRNA-V3 DGGE profiles since there were less bands observed in the AMT DGGE gels.

Figure 3.1.1 DGGE profile of the 16S rRNA-V3 amplicons amplified from the Bay of Quinte 2005 filter samples. Unknown genotype A bands visible in all the samples is highlighted with a red box. Bands that migrated to the same gel position as a standard band are indicated with an asterisk. Standard mix consisted of: (1) *Calothrix parietina* PCC 6303, (2) *Anabaena flos-aquae* UTCC 64, (3) *Anabaena lemmermannii* NIVA-CYA 83/1, (4) *Anabaena viguieri*/*Anabaena planktonica**, (5) *Gloeotrichia ghosi* UTEX LB 1920, (6) *Microcystis aeruginosa* UTCC 299/*Microcystis wesenbergii**, (7) *Aphanizomenon flos-aquae* HHAFA, (8) *Oscillatoria limosa*, (9) *Planktothrix rubescens* UTCC 507

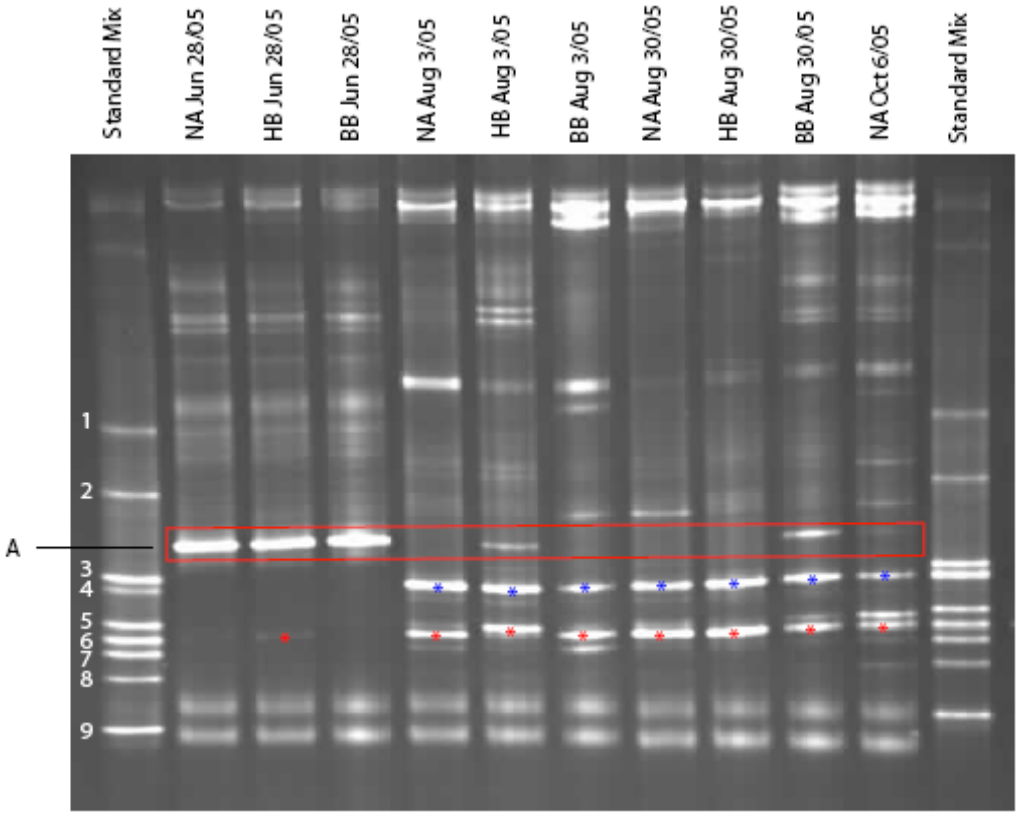

Standard Mix
NA Jun 28/05
HB Jun 28/05
BB Jun 28/05
NA Aug 3/05
HB Aug 3/05
BB Aug 3/05
NA Aug 30/05
HB Aug 30/05
BB Aug 30/05
NA Oct 6/05
Standard Mix
A
1
2
3
4
5
6
7
8
9

Figure 3.1.2 DGGE profile of the 16S rRNA-V3 amplicons amplified from the Bay of Quinte 2006 filter samples. Unknown genotypes A, B, and C bands visible in all the samples are highlighted with a red box. Bands that migrated to the same gel position as a standard band are indicated with an asterisk. Standard mix consisted of: (1) *Calothrix parietina* PCC 6303, (2) *Anabaena flos-aquae* UTCC 64, (3) *Anabaena lemmermannii* NIVA-CYA 83/1, (4) *Anabaena viguieri*/*Anabaena planktonica**, (5) *Gloeotrichia ghosi* UTEX LB 1920, (6) *Microcystis aeruginosa* UTCC 299/*Microcystis wesenbergii**, (7) *Aphanizomenon flos-aquae* HHAFA, (8) *Oscillatoria limosa*, (9) *Planktothrix rubescens* UTCC 507

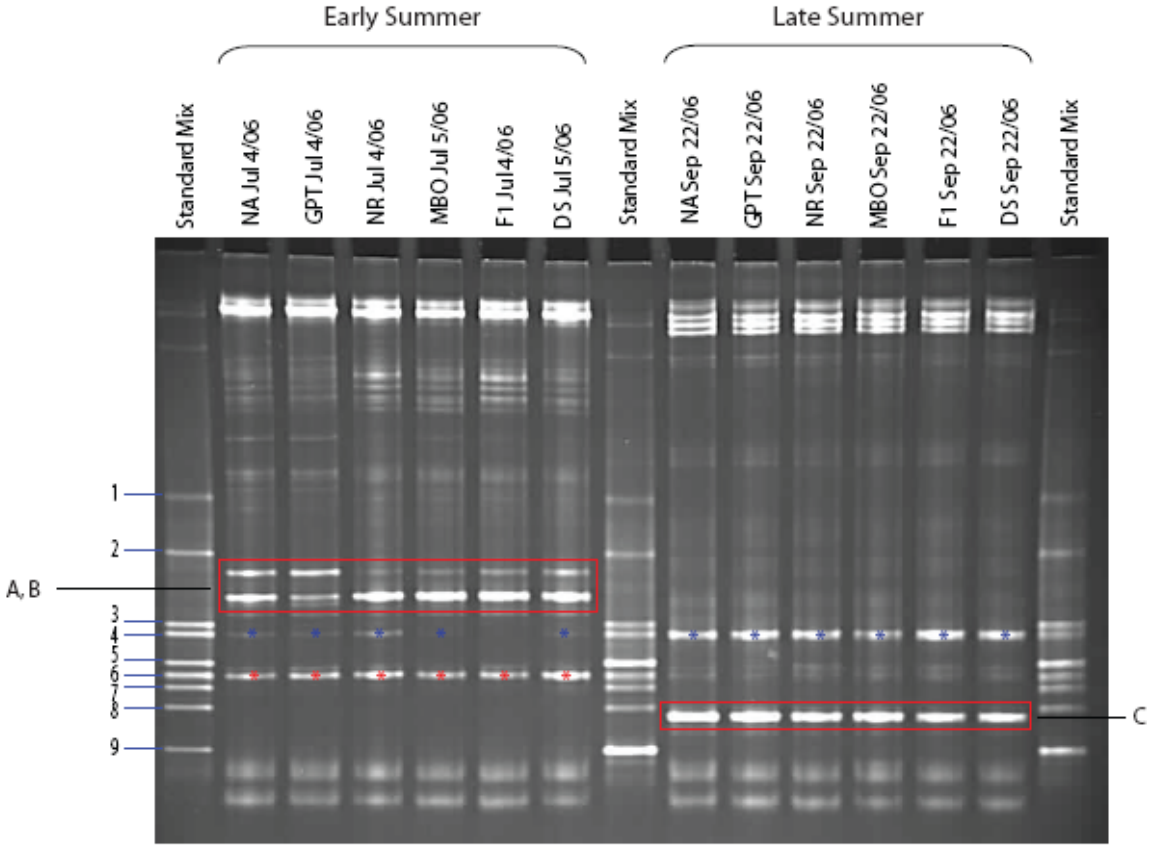

Early Summer
Late Summer
Standard Mix
NA Jul 4/06
GPT Jul 4/06
NR Jul 4/06
MBO Jul 5/06
F1 Jul4/06
DS Jul 5/06
Standard Mix
NA Sep 22/06
GPT Sep 22/06
NR Sep 22/06
MBO Sep 22/06
F1 Sep 22/06
DS Sep 22/06
Standard Mix
1
2
A, B
3
9
C

Figure 3.1.3A DGGE profile of the 16S rRNA-V3 amplicons amplified from the Maumee Bay 2006 filter samples. Unknown genotypes A, B, C, D, E, F, G, and H bands visible in all the samples are highlighted with a red box. Bands that migrated to the same gel position as a standard band are indicated with an asterisk. Standard mix consisted of: (1) *Calothrix parietina* PCC 6303, (2) *Anabaena flos-aquae* UTCC 64, (3) *Anabaena lemmermannii* NIVA-CYA 83/1, (4) *Anabaena viguieri*/*Anabaena planktonica**, (5) *Gloeotrichia ghosi* UTEX LB 1920, (6) *Microcystis aeruginosa* UTCC 299/*Microcystis wesenbergii**, (7) *Aphanizomenon flos-aquae* HHAFA, (8) *Oscillatoria limosa**, (9) *Planktothrix rubescens* UTCC 507

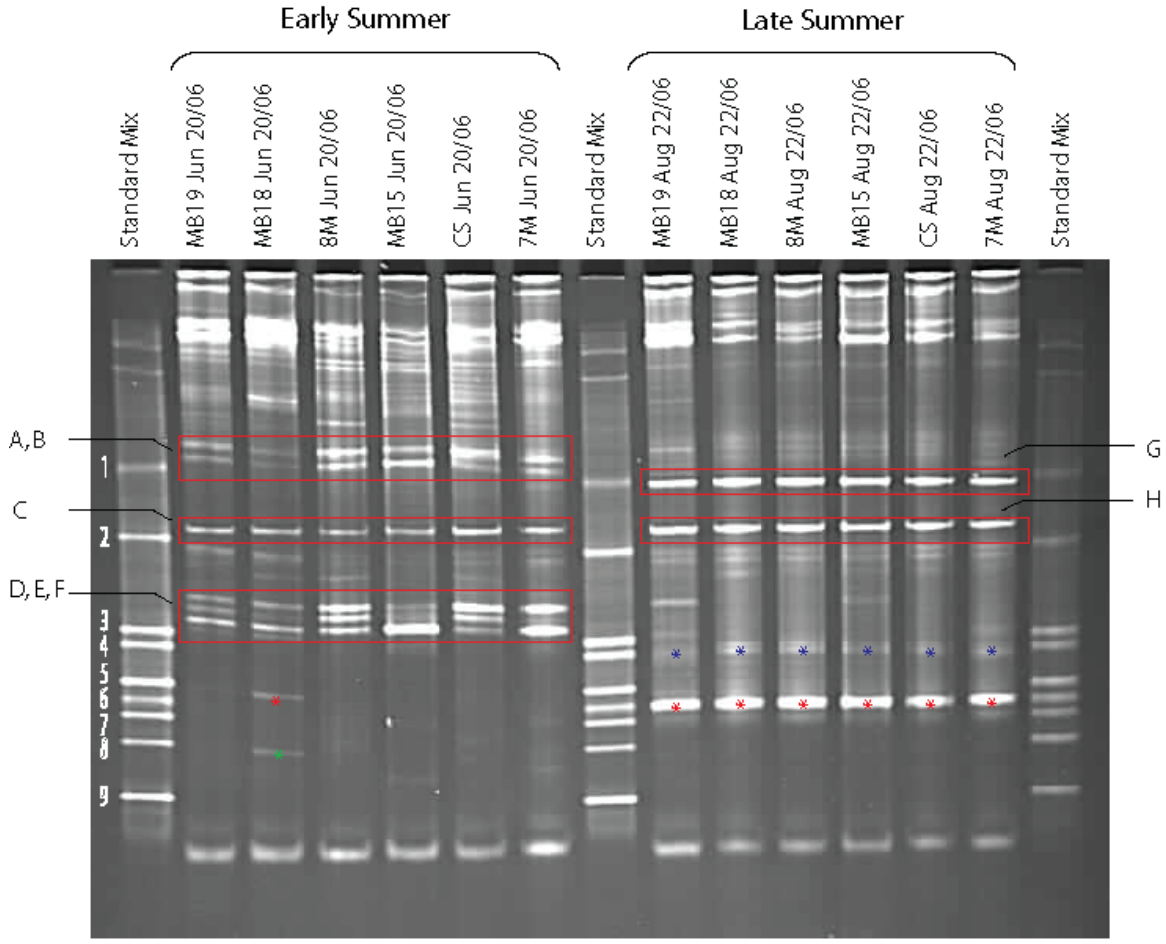

Early Summer
Late Summer
Standard Mix
MB19 Jun 20/06
MB18 Jun 20/06
8M Jun 20/06
MB15 Jun 20/06
CS Jun 20/06
7M Jun 20/06
Standard Mix
MB19 Aug 22/06
MB18 Aug 22/06
8M Aug 22/06
MB15 Aug 22/06
CS Aug 22/06
7M Aug 22/06
Standard Mix
A, B
C
D, E, F
G
H
1
2
3
4
5
6
7
8
9

Figure 3.1.3B DGGE profile of the 16S rRNA-V3 amplicons amplified from the Maumee Bay June 20, 2006 filter samples. Unknown genotype A, B, C, D, E, and F bands visible in all the samples are highlighted with a red box. Bands that migrated to the same gel position as a standard band are indicated with an asterisk. Standard mix consisted of: (1) *Calothrix parietina* PCC 6303, (2) *Anabaena flos-aquae* UTCC 64, (3) *Anabaena lemmermannii* NIVA-CYA 83/1, (4) *Anabaena viguieri*/*Anabaena planktonica*, (5) *Gloeotrichia ghosi* UTEX LB 1920, (6) *Microcystis aeruginosa* UTCC 299/*Microcystis wesenbergii**, (7) *Aphanizomenon flos-aquae* HHAFA, (8) *Oscillatoria limosa**, (9) *Planktothrix rubescens* UTCC 507.

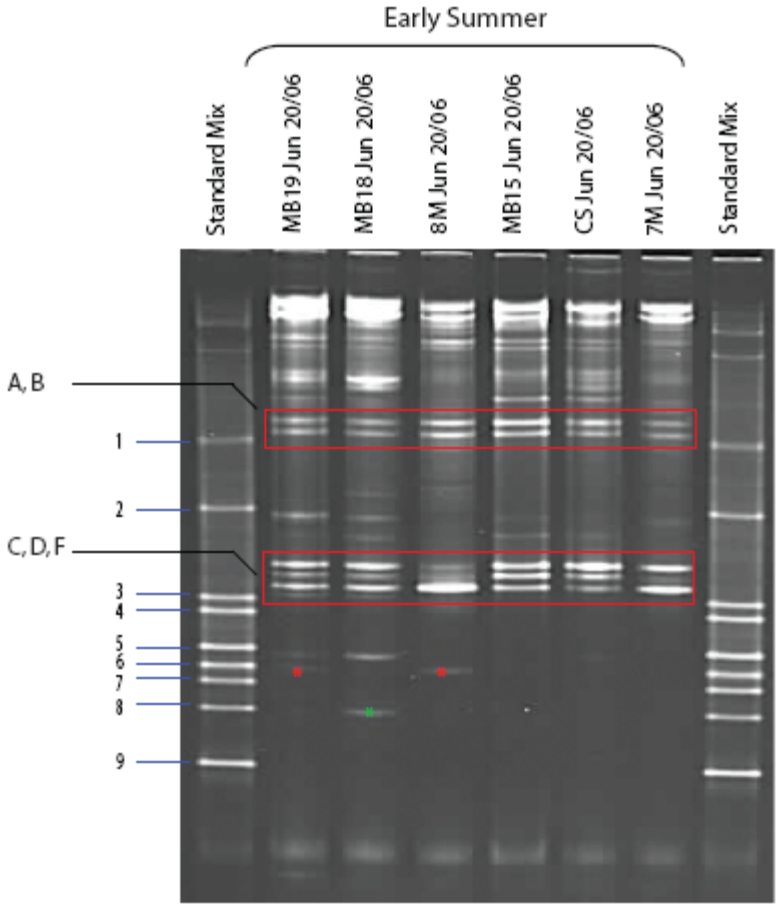

Early Summer
Standard Mix
MB19 Jun 20/06
MB18 Jun 20/06
8M Jun 20/06
MB15 Jun 20/06
CS Jun 20/06
7M Jun 20/06
Standard Mix
A, B
1
2
C, D, F
3
4
5
6
7
8
9

Figure 3.1.4 **DGGE analysis of the 16S rRNA-V3 amplicons amplified from the Nyanza Gulf 2005 filter samples.** Unknown genotypes A and B bands visible in all the samples are highlighted with a red box. Bands that migrated to the same gel position as a standard band are indicated with an asterisk. Standard mix consisted of: (1) *Calothrix parietina* PCC 6303, (2) *Anabaena flos-aquae* UTCC 64, (3) *Anabaena lemmermannii* NIVA-CYA 83/1, (4) *Anabaena viguieri*/*Anabaena planktonica**, (5) *Gloeotrichia ghosi* UTEX LB 1920, (6) *Microcystis aeruginosa* UTCC 299/*Microcystis wesenbergii**, (7) *Aphanizomenon flos-aquae* HHAFA, (8) *Oscillatoria limosa**, (9) *Planktothrix rubescens* UTCC 507

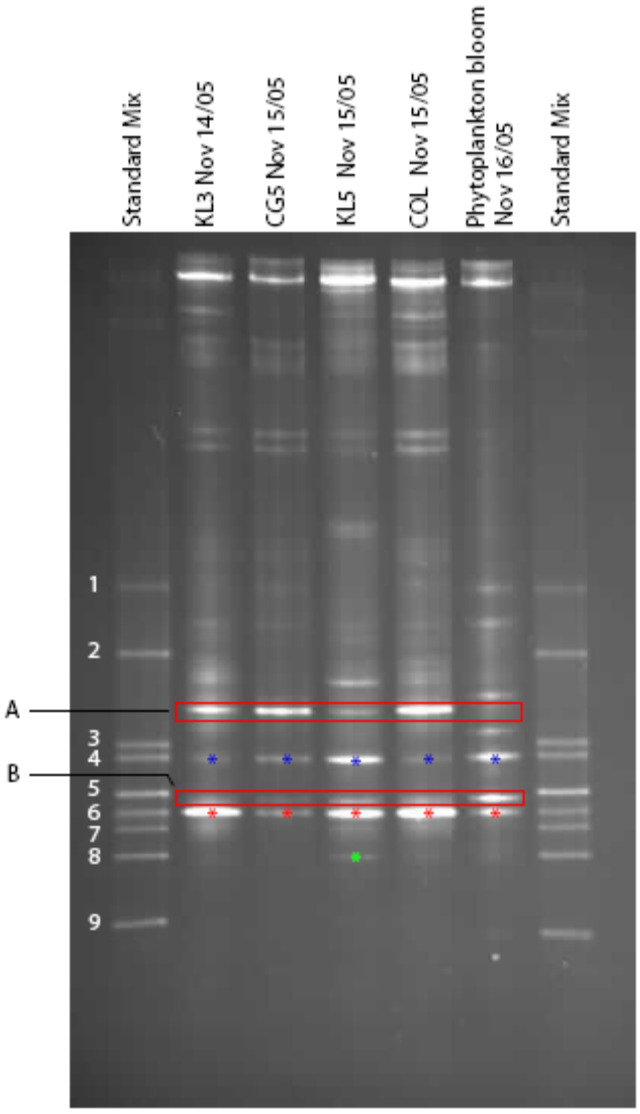

Standard Mix
KL3 Nov 14/05
CG5 Nov 15/05
KL5 Nov 15/05
COL Nov 15/05
Phytoplankton bloom Nov 16/05
Standard Mix
1
2
A
3
4
B
5
6
7
8
9

Figure 3.1.5 DGGE gel of the AMT amplicons amplified from the Bay of Quinte 2005 filter samples. Negative image of ethidium bromide stained gel. Standard mix consisted of (from top to bottom): (1) *Planktothrix rubescens* UTCC 507, (2) *Anabaena lemmermannii* NIVA-CYA 83/1, and (3) *Microcystis aeruginosa* UTCC 299. Samples "BB Jun 28/05" and "HB Aug 3/05" contained faint bands at the same gel position as the *M. aeruginosa* UTCC 299 standard but were too faint to be visible in the gel picture.

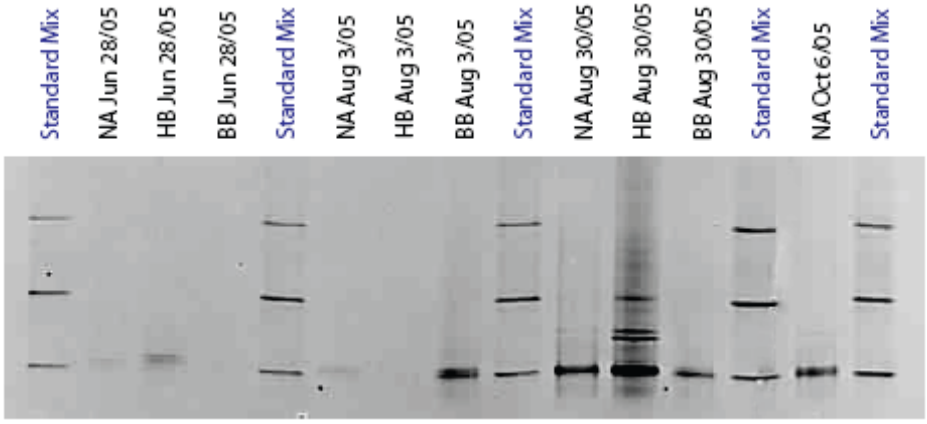

Standard Mix
NA Jun 28/05
HB Jun 28/05
BB Jun 28/05
Standard Mix
NA Aug 3/05
HB Aug 3/05
BB Aug 3/05
Standard Mix
NA Aug 30/05
HB Aug 30/05
BB Aug 30/05
Standard Mix
NA Oct 6/05
Standard Mix

Figure 3.1.6 DGGE gel of the AMT amplicons amplified from the Bay of Quinte 2006 filter samples. Negative image of ethidium bromide stained gel. Standard mix consisted of (from top to bottom): (1) *Planktothrix rubescens* UTCC 507, (2) *Anabaena lemmermannii* NIVA-CYA 83/1, and (3) *Microcystis aeruginosa* UTCC 299

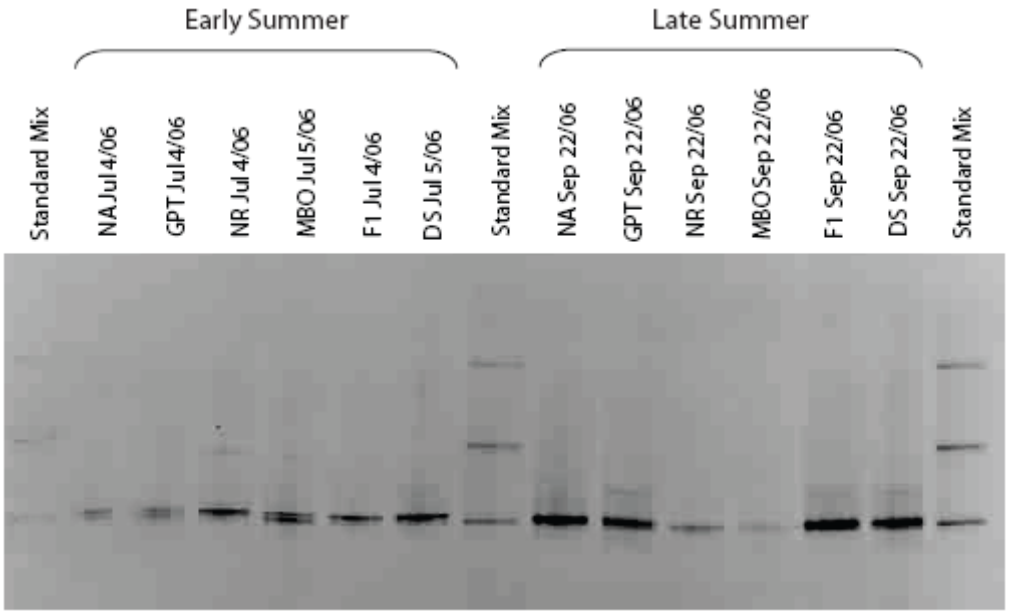

Early Summer
Late Summer
Standard Mix
NA Jul 4/06
GPT Jul 4/06
NR Jul 4/06
MBO Jul 5/06
F1 Jul 4/06
DS Jul 5/06
Standard Mix
NA Sep 22/06
GPT Sep 22/06
NR Sep 22/06
MBO Sep 22/06
F1 Sep 22/06
DS Sep 22/06
Standard Mix

Figure 3.1.7 DGGE gel of the AMT amplicons amplified from Maumee Bay 2006 filter samples. Three distinct bands in the gel are labeled A, B, and C. Negative image of ethidium bromide stained gel. Standard mix consisted of (from top to bottom): (1) *Planktothrix rubescens* UTCC 507, (2) *Anabaena lemmermannii* NIVA-CYA 83/1, and (3) *Microcystis aeruginosa* UTCC 299.

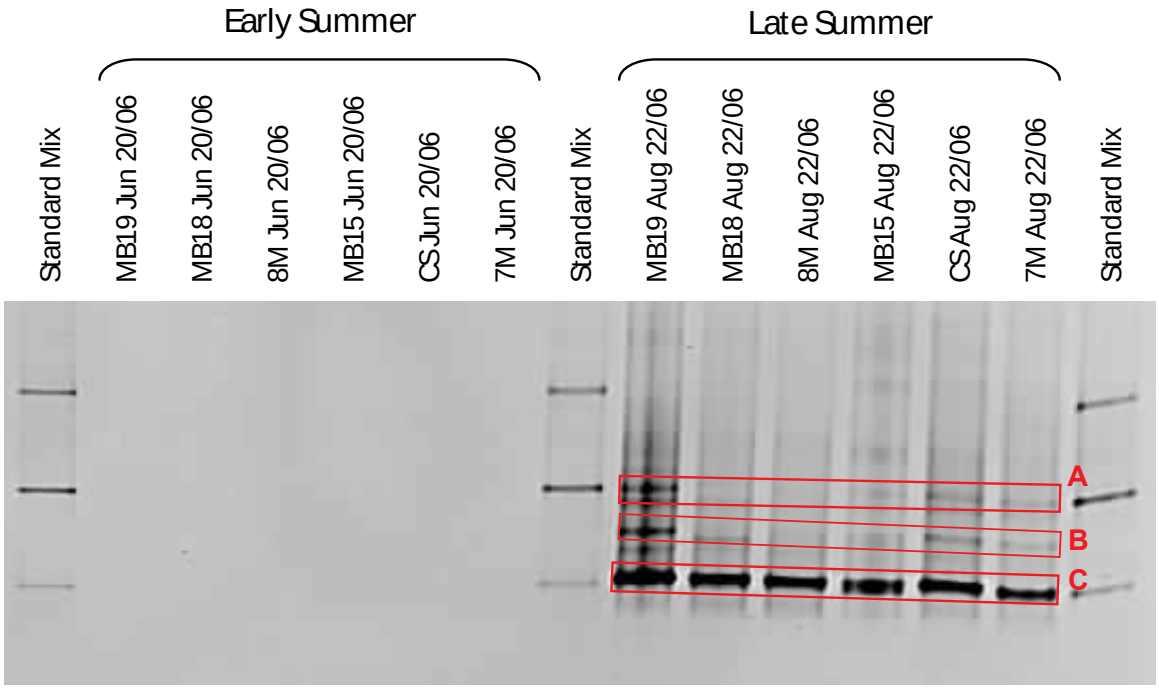
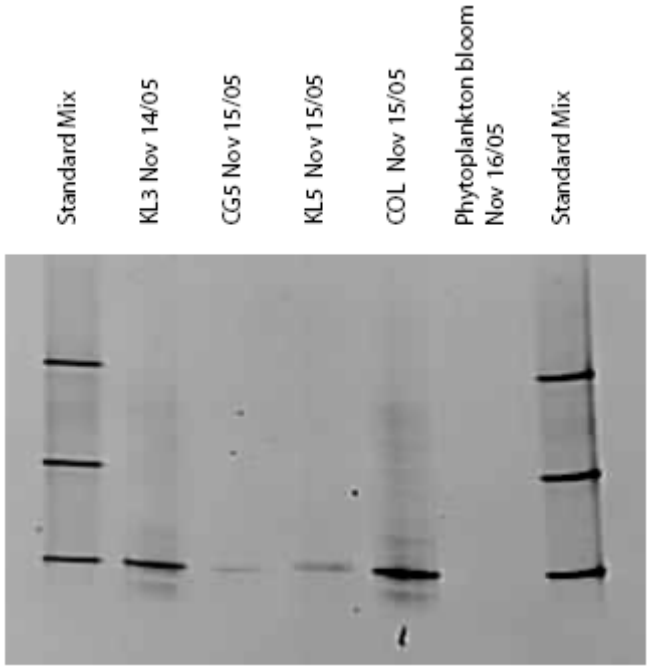

Early Summer
Late Summer
Standard Mix
MB19 Jun 20/06
MB18 Jun 20/06
8M Jun 20/06
MB15 Jun 20/06
CS Jun 20/06
7M Jun 20/06
Standard Mix
MB19 Aug 22/06
MB18 Aug 22/06
8M Aug 22/06
MB15 Aug 22/06
CS Aug 22/06
7M Aug 22/06
Standard Mix
A
B
C

Figure 3.1.8 DGGE gel of the AMT amplicons amplified from Nyanza Gulf 2005 filter samples. Negative image of ethidium bromide stained gel. Standard mix consisted of (from top to bottom): (1) *Planktothrix rubescens* UTCC 507, (2) *Anabaena lemmermannii* NIVA-CYA 83/1, and (3) *Microcystis aeruginosa* UTCC 299


Standard Mix
KL3 Nov 14/05
CG5 Nov 15/05
KL5 Nov 15/05
COL Nov 15/05
Phytoplankton bloom Nov 16/05
Standard Mix

3.1.2.1 BAY OF QUINTE, LAKE ONTARIO 2005 AND 2006

Except for the HB August 30, 2005 sample, there appears to be only one or two AMT genotype populations present in all the samples (Figure 3.1.5). Each sample on the gel contained a band at the same position as the *M. aeruginosa* UTCC 299 standard. The multiple banding patterns in the HB August 30, 2005 samples showed that four AMT genotypes were present. One of the four AMT bands in sample HB August 30, 2005 migrated to the same position as the *A. lemmermannii* NIVA-CYA 83/1 standard. Based on band intensities, very little AMT amplicons were detected in the three June 28, 2005 samples (NA, HB, BB) and two of the August 3, 2005 samples (NA, HB). By late August, the band intensity was greater suggesting an increase in abundance of toxic genotypes.

DGGE analysis of AMT amplicons from the 2006 samples (Figure 3.1.6) showed one or two distinct toxic genetic populations present at each site in early and late summer. Both the AMT bands (separated by on 1 mm) migrated nearly close to the same position as the *M. aeruginosa* UTCC 299 standard.

3.1.2.2 MAUMEE BAY, LAKE ERIE 2006

Profiles of the Maumee Bay samples (Figure 3.1.7) showed no detectable toxic genotypes in early summer (June 20, 2006). Filter extraction and PCR-DGGE analysis of all Maumee Bay samples were performed several times to ensure that the result was not due to errors in the extraction and PCR process. The same extract used in AMT DGGE analysis was used for the 16S rRNA-V3 analysis to ensure that the extracts were amplifiable. By late summer (August 22, 2006), multiple AMT genotypes were visible in each sample on the gel. There were more than 5 different bands present in each of the

sample. Most notable was the band corresponding to the *M. aeruginosa* UTCC 299 standard in each of the sample in relatively high abundance over the other bands present. There was also a band in the same gel position as the *A. lemmermannii* NIVA-CYA 83/1 standard present in all 6 late summer samples.

3.1.2.3 NYANZA GULF (WINAM GULF), LAKE VICTORIA 2005

PCR and DGGE analysis of the AMT domain (*mcyE*/*ndaF*) showed a band in the same gel position as the *M. aeruginosa* UTCC 299 standard in the four samples collected from each of the station in Nayza Gulf (Figure 3.1.8). No AMT band was detected in the phytoplankton bloom sample while COL contained more than one visible AMT band.

3.1.3 SEQUENCING OF EXCISED DGGE BANDS

Predominant and unique bands were excised and sequenced to provide further identification of the cyanobacterial species represented in the particular band. On average, only short sequences approximately 100 base pair long were obtained from each of the excised 16S rRNA-V3 DGGE bands. This small region did not provide enough information to identify the species and sometimes the genus of the excised bands. The 16S rRNA-V3 sequences were compared to sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990) (www.ncbi.nlm.nih.gov/blast/). The 16S rRNA-V3 sequences showed high degrees of sequence similarities (95-100%) to multiple cultured and uncultured cyanobacteria species.

Sequences obtained from selected AMT bands were about 400 nucleotides long. BLAST nucleotide (BLASTn) (Altschul *et al*., 1997) results of sequences obtained from bands at gel positions close to the *M. aeruginosa* UTCC 299 standard (Figure 3.1.7, band

C) returned matches to several *Microcystis* species (*aeruginosa*, *wesenbergii*, and *viridis*) with 98% nucleotide matches.

Comparison of the 16S rRNA-V3 sequences obtained from excised bands showed that genotypes that migrated to the same gel position have identical sequences. As shown with the Bay of Quinte, sequences of all genotype A bands in the 2005 samples (Figure 3.1.1) and band B of the 2006 early summer sample (Figure 3.1.2) were the same. Bands with the same gel position as the *A. viguieri*/ *A. planktonica* standard in the 2005 and 2006 Bay of Quinte gels had identical sequences to the standard. All bands that migrated to the same gel position as the *M. aeruginosa* UTCC 299 standard in both Maumee Bay and Bay of Quinte samples are a 99%-100% match with the standard.

3.1.4 GENERAL STATISTICAL ANALYSIS

3.1.4.1 DIVERSITY

Shannon's diversity indices were calculated based on the number and relative intensity of bands present in the 16S rRNA-V3 profiles. Diversity index of the three study sites showed some differences between the lakes, but sampling stations within a study site did not differ significantly from each other ($P>0.05$). The Nyanza Gulf samples had the highest diversity index (*H'*) of 3.09 followed by Maumee Bay, and Bay of Quinte. Samples from Maumee Bay showed a slight decrease in diversity between early (*H'* = 2.72) to late summer (*H'* = 2.61) although not significant ($P = 1.00$). The diversity index for the Bay of Quinte 2006 samples decreased between early (*H'* = 2.42) and late summer (*H'* = 2.18) ($P = 0.02$). For the Bay of Quinte 2005 samples, both the June 28 and August 30 samples had the same calculated diversity (*H'* = 2.02); and the August 3 and October

6 samples had nearly similar diversities (*H'* = 2.38 and 2.39 respectively). Evenness values for all the samples ranged from 92% to 98%.

3.1.4.2 REPRODUCIBILITY DGGE PROFILES

Two separate 16S rRNA-V3 DGGE gels for each study site (Bay of Quinte 2005 samples, Maumee Bay early summer samples, and Nyanza 2005 samples) were compared for similarities in banding patterns. Sample sets from each gel were extracted, PCR amplified, and DGGE analyzed at different times. Genomic DNA was extracted from filter pieces taken from different GF/F filters. Based on the presence and absent of bands in each sampling station, the Jaccard similarity coefficient was determined for each duplicate set of 16S rRNA-V3 DGGE gels. The Bay of Quinte 2005 sample profiles showed the highest similarity (84%) between the two gels followed by the Nyanza Gulf 2005 samples (75%) and Maumee Bay 2006 early summer samples (71%).

3.2 QPCR RESULTS

3.2.1 STANDARD CURVE

3.2.1.1 16S RRNA-V3 STANDARD CURVE

A standard curve was developed using six dilutions of template DNA extracted from *M. aeruginosa* UTCC 299 that ranged from 5 cells per reaction to 5.0 x 10^5 cells per reaction (Figure 3.2.1A). The Cya-b-F371 and Cya-R783 primer set by (Zwart *et al*., 2005) were used to develop the 16S rRNA-V3 standard curve. The regression equation was y = 36.00 – 3.719x (R^2 = 0.996, 86% efficiency) where *y* is the threshold cycle (Ct: the PCR cycle at which an increase in fluorescence signal is detected above a set baseline signal) at the set fluorescence threshold level (0.038) and *x* is the amount of starting DNA (represented as $\log_{10}$ cell number equivalents). A melt curve made from the qPCR

products (Figure 3.2.1B) showed that all products had similar melting curves with only one peak at 85^{o}C, no primer peaks were observed in samples with DNA template. There were small peaks in the NTC (no temple control) tubes (blue peaks in Figure 3.2.1B) but it was insignificant to the larger peaks of the DNA template samples.

3.2.1.2 AMT (MCYE/NDAF) STANDARD CURVE

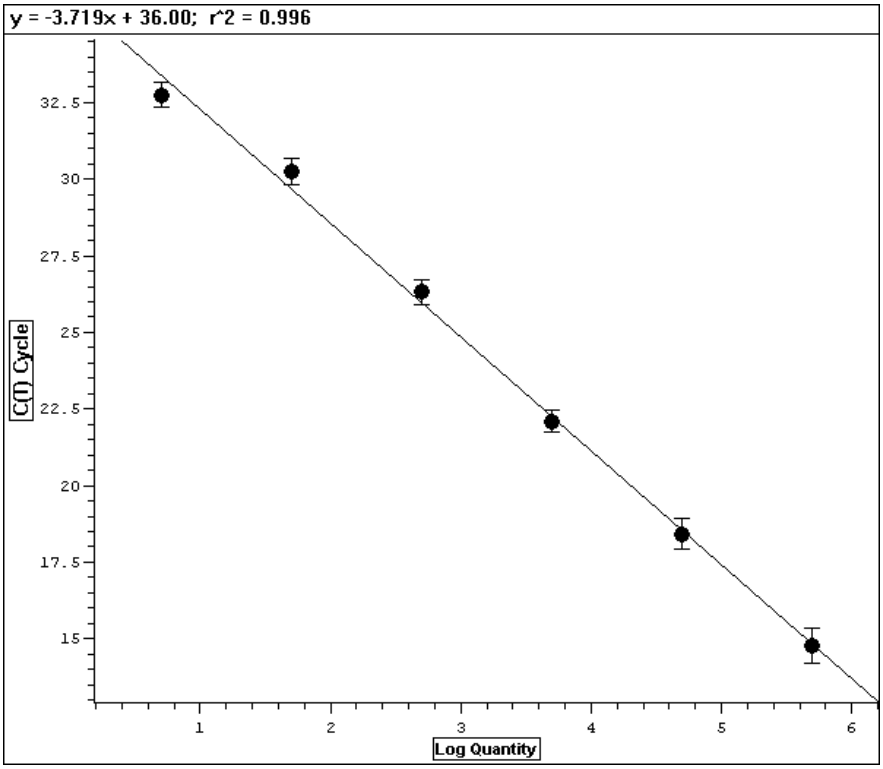
A highly significant linear curve (Figure 3.2.2A) between the amount of starting DNA and Ct was obtained for AMT standard curve using the primer set HEPF and HEPR (Jungblut and Neilan 2006). The regression equation was y = 31.88 – 3.665x (R^2 = 0.999, 87% efficiency), where *y* is the Ct at the set fluorescence threshold level (0.014) and *x* is the amount of starting DNA (represented as $\log_{10}$ cell number equivalents). AMT sequence detection ranged from 5 x 10^1 to 5 x 10^5 cells per 20-µl reaction. Quantification of 5 cells per 20-µl reaction resulted in a melting curve with two distinct peaks indicating presence of primer-dimers. For this reason, the 5 cells per reaction standard was excluded from the AMT standard curve, thus the standard curve ranged from 5 x 10^5 cells per reaction to 5 x 10^1 cells per reaction. A melt curve for all the qPCR products showed only one peak at 85^{o}C and no primer peak from the DNA template samples. The NTC samples produced a peak at ~76^{o}C (blue peaks in Figure 3.2.2B).

3.2.2 QUANTIFICATION OF TOTAL CYANOBACTERIA AND TOTAL POTENTIAL HEPATOTOXIC CYANOBACTERIAL CELL COUNTS IN LAKE WATER SAMPLES

Quantification of total cyanobacteria was based on the *M. aeruginosa* UTCC 299 16S rRNA-V3 standard curve. On the other hand, the quantification of potential hepatotoxic cyanobacteria was calculated from the *M. aeruginosa* UTCC 299 AMT standard curve. Numbers indicated in Tables 3.2.1 and 3.2.2 were the mean values of three or more

Figure 3.2.1 Standard curve and dissociation/melting curve of the 16S rRNA-V3 amplicons. (A) Standard curve of 16S rRNA-V3 amplicons was based on predetermined *M. aeruginosa* UTCC 299 cell concentrations by relating known DNA concentration (in cell equivalent) to Ct values of diluted samples using primers Cya-b-F371 and Cya-R783 (Muyzer *et al*., 1993). All plots represent the mean of triplicate determinations and error bar provide the standard deviation. (B) Dissociation/melting curve of the 16S rRNA-V3 amplicons. The melting curve of the three no template control (NTC) samples are indicated by the red circle.

A


y = -3.719x + 36.00; r^2 = 0.996
C(T) Cycle
32.5
30
27.5
25
22.5
20
17.5
15
1
2
3
4
5
6
Log Quantity

B

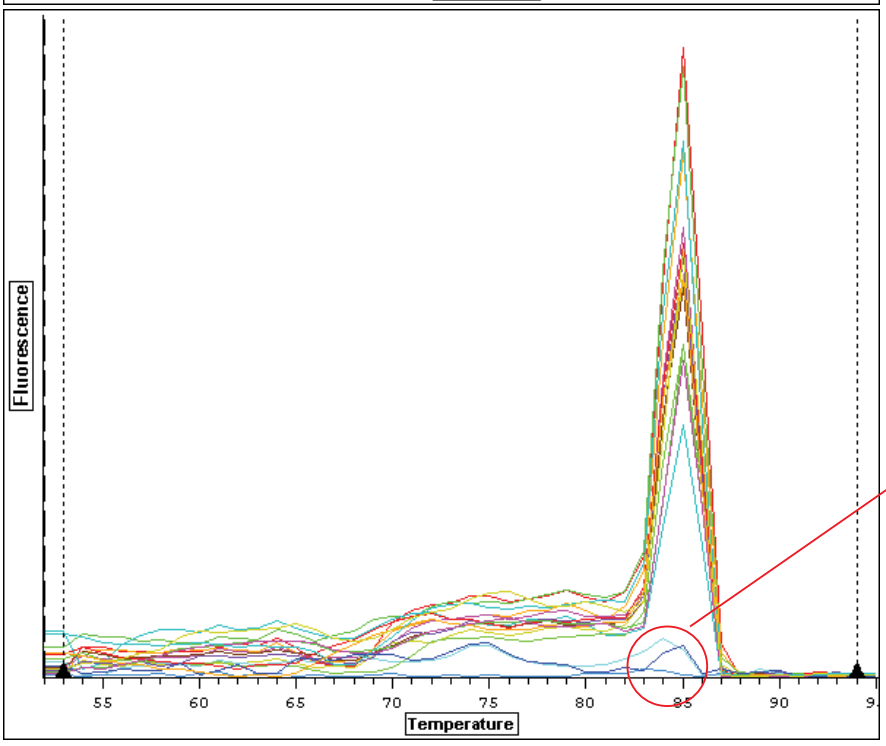
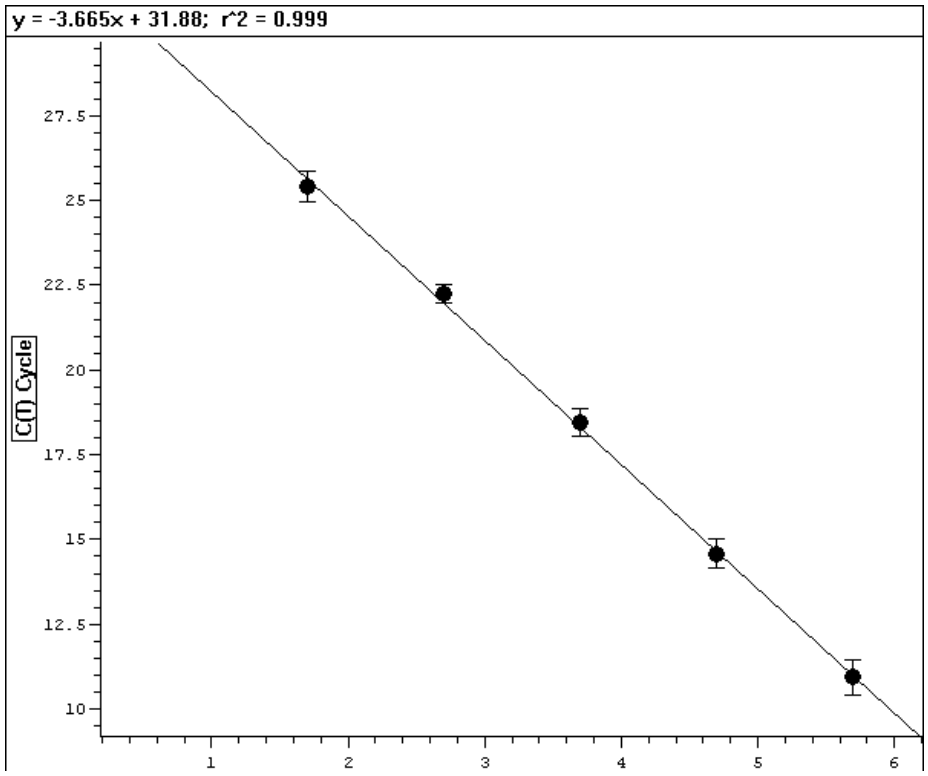

Fluorescence
55
60
65
70
75
80
85
90
Temperature
NTC

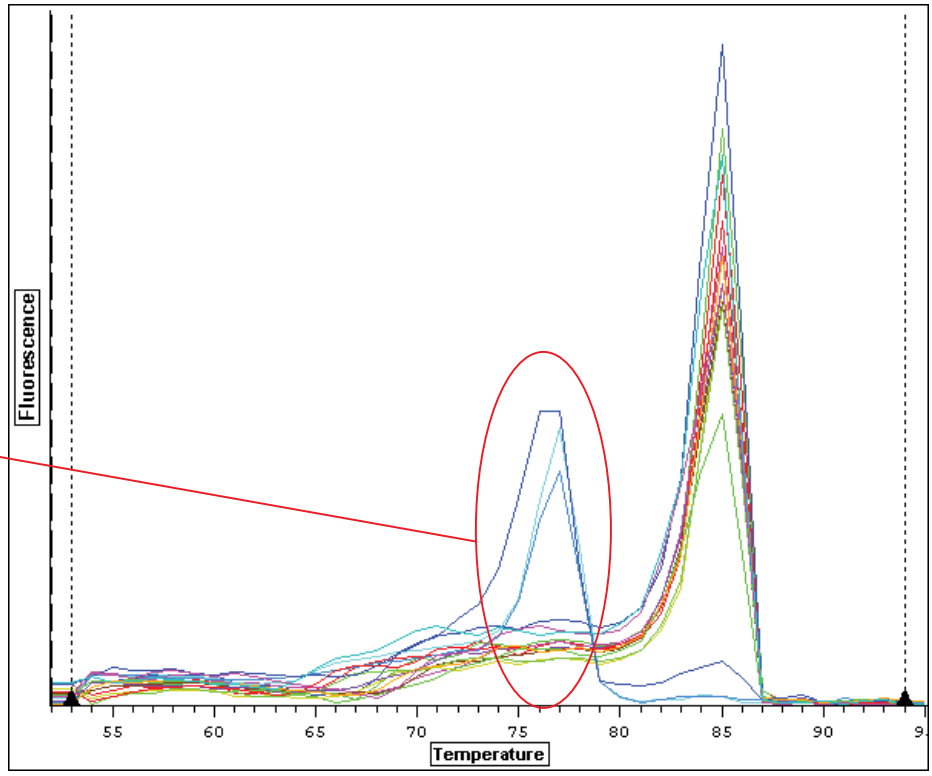
Figure 3.2.2 Standard curve and dissociation/melting curve of the AMT amplicons. (A) Standard curve of AMT amplicons was based on predetermined *M. aeruginosa* UTCC 299 cell concentrations by relating known DNA concentration (in cell equivalent) to Ct values of diluted samples using primers HEPF and HEPR (Jungblut and Neilan 2006). All plots represent the mean of triplicate determinations and error bar provide the standard deviation. (B) Dissociation/melting curve of the AMT amplicons. The melting curves from the three NTC samples are highlighted by the red oval in the figure.

A


y = -3.665x + 31.88; r^2 = 0.999
C(T) Cycle
27.5
25
22.5
20
17.5
15
12.5
10
1
2
3
4
5
6

B

NTC


Fluorescence
55
60
65
70
75
80
85
90
Temperature

determinations ± standard deviation. For consistency and comparison purposes, the genomic DNA extract used for qPCR quantification of the 16S rRNA-V3 gene fragment was also used for qPCR of the AMT gene region.

3.2.2.1 BAY OF QUINTE (LAKE ONTARIO)

Water samples were collected and filtered through GF/F filters in early (July 4, 2006) and late summer (August 22, 2006) from six sampling stations in the Bay of Quinte. Majority of the sampling stations showed no significant differences ($P > 0.05$) in total cyanobacteria (cells·L^{-1}) and potential hepatotoxic cell (cells·L^{-1}) concentrations for both early and late summer. Hence, the six stations were treated as one sampling replicate and averaged. Quantification of the six sampling stations by qPCR assay determined that the average cyanobacterial cell concentration was significantly higher ($P = 0.000$) from late summer ($1.76 \pm 0.70 \times 10^9$ cells·L^{-1}) to early summer ($1.22\ 76 \pm 1.00 \times 10^8$ cells·L^{-1}) by an order of one magnitude (Table 3.2.1). For early summer, the cell concentration ranged from 4.01 (± 1.70) x 10^7 cells·L^{-1} (GPT) to 3.18 (± 0.73) x 10^8 cells·L^{-1} (DS). For late summer, the cell concentration ranged from 9.85 (± 1.07) x 10^8 cells·L^{-1} (F1) to 3.00 (± 0.95) x 10^9 cells·L^{-1} (NR). Quantification of cyanobacterial cell concentration from station NA September sample ($1.82 \pm 1.31 \times 10^9$ cells·L^{-1}) was almost one magnitude higher than direct cell count numbers (3.69×10^8 cells·L^{-1}) as determined by Hedy Kling (Freshwater Institute, Winnipeg, Manitoba, Canada).

Hepatotoxic genotypes were detected from all six stations by qPCR. On average, the hepatotoxic cell concentration was 2.99 (± 3.60) x 10^6 cells·L^{-1} in early summer and decreased almost 50% to 1.46 (± 0.20) x 10^6 cells·L^{-1} by late summer; however, this was not a significant decrease ($P = 0.372$). Potential hepatotoxic cell concentrations ranged

from 4.17 (± 1.47) x 10^5 cells·L^{-1} (NA) to 9.28 (± 3.63) x 10^6 cells·L^{-1} (DS) in early summer and 1.20 (± 0.35) x 10^6 cells·L^{-1} (F1) to 1.75 (± 1.07) x 10^8 cells·L^{-1} (NA) in late summer. In both the early and late summer samples, the hepatotoxic population was less than 1.0% of the total cyanobacteria population. PPIA (protein phosphatase inhibition assay) reported detectable levels of microcystin in early and late summer samples (Sarah Yakobowski, University of Waterloo, Ontario, Canada). The average total microcystin concentration ranged from 2.18 (± 0.57) µg·L^{-1} in early summer to 0.58 (± 0.58) µg·L^{-1} by late summer (Sarah Yakobowski, University of Waterloo, Ontario, Canada).

3.2.2.2 MAUMEE BAY (LAKE ERIE)

Quantitative real-time PCR of the filter samples collected from Maumee Bay in early (June 20, 2006) and late summer (August 22, 2006) showed no significant differences ($P > 0.05$) in cyanobacterial cell concentrations (cells·L^{-1}) among the six sampling stations in both early and late summer (Table 3.2.2). As well, in late summer, there were no significant differences ($P > 0.05$) in potential hepatotoxic cell concentration (cells·L^{-1}) among the six sampling stations. Thus, cell concentrations from the six sampling stations were averaged. As observed in the Bay of Quinte, there was a significant increase ($P = 0.0$) of more than one order of magnitude in cyanobacterial cell abundance from early to late summer (Table 3.2.2). Cell concentration in Maumee Bay in early summer ranged from 1.12 (± 0.98) x 10^7 cells·L^{-1} (CS) to 3.62 (± 0.52) x 10^7 cells·L^{-1} (7M). Cell concentration from station MB18 samples in early summer was undetermined because the samples failed to amplify. An average of the five sites (station MB18 excluded) sampled in early summer showed that the cell abundance was 2.35 (±

TABLE 3.2.1 qPCR data of total cyanobacteria and total potential hepatotoxic cyanobacteria concentration (cells·L^{-1}) from the Bay of Quinte filter samples. Cyanobacterial cell abundance was based on the *M. aeruginosa* UTCC 299 16S rRNA-V3 standard curve. Number of potential hepatotoxic cyanobacteria based on the *M. aeruginosa* UTCC 299 AMT standard curve. The values represent the mean of triplicate determinations ± standard deviation.

	No. of Cyanobacteria (cells·L^{-1}) (*M. aeruginosa* UTCC 299 equivalent)		No. of Potential Hepatotoxic Cyanobacteria (cells·L^{-1}) (*M. aeruginosa* UTCC 299 equivalent)	
sampling station	**early summer (4-Jul-2006)**	**late summer (22-Sept-2006)**	**early summer (4-Jul-2006)**	**late summer (22-Sept-2006)**
NA	6.26×10^{7} ($\pm 2.95 \times 10^{7}$)	1.82×10^{9} ($\pm 1.31 \times 10^{9}$)	4.17×10^{5} ($\pm 1.47 \times 10^{5}$)	1.75×10^{6} ($\pm 7.21 \times 10^{5}$)
GPT	4.01×10^{7} ($\pm 1.70 \times 10^{7}$)	1.25×10^{9} ($\pm 8.47 \times 10^{7}$)	5.34×10^{6} ($\pm 9.07 \times 10^{6}$)	1.47×10^{6} ($\pm 1.42 \times 10^{5}$)
NR	9.40×10^{7} ($\pm 5.12 \times 10^{7}$)	3.00×10^{9} ($\pm 9.50 \times 10^{8}$)	6.49×10^{5} ($\pm 1.47 \times 10^{5}$)	1.60×10^{6} ($\pm 2.88 \times 10^{5}$)
MBO	1.23×10^{8} ($\pm 8.76 \times 10^{6}$)	1.65×10^{9} ($\pm 1.76 \times 10^{8}$)	5.51×10^{5} ($\pm 2.92 \times 10^{4}$)	1.33×10^{6} ($\pm 3.15 \times 10^{5}$)
F1	9.67×10^{7} ($\pm 1.64 \times 10^{7}$)	9.85×10^{8} ($\pm 1.07 \times 10^{8}$)	1.71×10^{6} ($\pm 3.08 \times 10^{5}$)	1.20×10^{6} ($\pm 3.53 \times 10^{5}$)
DS	3.18×10^{8} ($\pm 7.33 \times 10^{7}$)	1.87×10^{9} ($\pm 3.20 \times 10^{1}$)	9.28×10^{6} ($\pm 3.63 \times 10^{6}$)	1.41×10^{6} ($\pm 5.00 \times 10^{6}$)
Avg. of 6 stations	1.22×10^{8} ($\pm 9.99 \times 10^{7}$)	1.76×10^{9} ($\pm 6.96 \times 10^{8}$)	2.99×10^{6} ($\pm 3.60 \times 10^{6}$)	1.46×10^{6} ($\pm 1.96 \times 10^{5}$)

Table 3.2.2 qPCR data of total cyanobacteria and total potential hepatotoxic cyanobacterial cell concentration (cells·L^{-1}) from Maumee Bay filter samples. Cyanobacterial cell abundance was based on the *M. aeruginosa* UTCC 299 16S standard curve. Number of potential hepatotoxic cyanobacteria based on the *M. aeruginosa* UTCC 299 AMT standard curve. The values represent the mean of triplicate determinations ± standard deviation (*M. aeruginosa* UTCC 299 equivalent per liter). Sample from station MB18 failed to amplify. BDL (below detection limit) means that amplification occurred but was below the set threshold

	No. of Cyanobacteria (cells·L^{-1}) (*M. aeruginosa* UTCC 299 equivalent)		No. of Potential Hepatotoxic Cyanobacteria (cells·L^{-1}) (*M. aeruginosa* UTCC 299 equivalent)	
sampling station	**early summer (20-Jun-2006)**	**late summer (22-Aug-2007)**	**early summer (20-Jun-2006)**	**late summer (22-Aug-2007)**
MB18	–	1.08×10^8 ($\pm 1.78 \times 10^7$)	BDL	1.40×10^8 ($\pm 4.55 \times 10^7$)
MB19	1.79×10^7 ($\pm 4.84 \times 10^6$)	5.33×10^7 ($\pm 1.32 \times 10^7$)	BDL	5.23×10^7 ($\pm 8.78 \times 10^6$)
8M	2.19×10^7 ($\pm 1.18 \times 10^7$)	1.39×10^8 ($\pm 1.12 \times 10^8$)	BDL	3.57×10^8 ($\pm 4.26 \times 10^8$)
MB15	3.03×10^7 ($\pm 3.31 \times 10^6$)	2.02×10^7 ($\pm 5.22 \times 10^6$)	BDL	2.78×10^7 ($\pm 6.11 \times 10^5$)
7M	3.62×10^7 ($\pm 5.17 \times 10^6$)	7.35×10^7 ($\pm 1.52 \times 10^7$)	BDL	6.04×10^7 ($\pm 4.17 \times 10^6$)
Clear Site	1.12×10^7 ($\pm 9.80 \times 10^6$)	2.31×10^8 (± 0.0)	BDL	7.17×10^7 ($\pm 1.22 \times 10^7$)
Avg. of 6 stations	2.35×10^7 ($\pm 9.92 \times 10^6$)	1.04×10^8 ($\pm 7.46 \times 10^7$)	BDL	1.18×10^8 ($\pm 1.23 \times 10^8$)

Table 3.2.3 Comparison of qPCR and PPIA data in this present study with Rinta-Kanto *et al*. (2005). Each numeric value for the Bay of Quinte and Maumee Bay in 2006 is an average of the 6 stations ± standard deviation. Protein phosphatase inhibition assay (PPIA) values were obtained from Sarah Yakobowski (University of Waterloo, Ontario, Canada). The qPCR and PPIA values for the Maumee Bay area are comparable to the values published by Rinta-Kinto *et al*. (2005).

	Site	Date	No. of cyano. (cells·L^{-1})	No. of potential hepatotoxic cyano. (cells·L^{-1})	PPIA (µg·L^{-1})
This present study	Bay of Quinte	Jul 2006	1.22 (± 0.99) x 10^8	2.99 (± 3.60) x 10^6	2.18 (± 0.57)
	Bay of Quinte	Sep 2006	1.76 (± 0.70) x 10^9	1.46 (± 0.20) x 10^6	0.58 (± 0.58)
	Maumee Bay	Jun 2006	2.35 (± 0.99) x 10^7	BDL	BDL
	Maumee Bay	Aug 2006	1.04 (± 0.75) x 10^8	1.18 (± 1.23) x 10^8	4.45 (± 2.19)
Rinta-Kanto *et al*. (2005)	Maumee Bay (station 1)	Aug 2003	9.9 (± 1.1) x 10^8	1.1 (± 0.3) x 10^6	15.4
	Maumee Bay (station 882)	Aug 2004	7.5 (± 1.3) x 10^5	3.4 (± 0.7) x 10^4	0.04

0.99) x 10^7 cells·L^{-1}. This number was one order of magnitude lower than the Bay of Quinte samples collected in early summer. In late summer in Maumee Bay cell abundance ranged from 2.02 (± 0.52) x 10^7 cells·L^{-1} (MB15) to 2.31 (± 0.0) x 10^8 cells·L^{-1} (CS) with an average of 1.04 (± 0.75) x 10^8 cells·L^{-1}. Again, the average cyanobacterial cell concentration of Maumee Bay was one magnitude lower than the Bay of Quinte samples in late summer.

Quantitative real-time PCR data from the quantification of the AMT gene region (*mcyE* and *ndaF*) for the Maumee Bay early summer samples were unreliable since PCR products in all the samples were detected only after PCR amplification cycles 28-36. A melt curve of all the PCR products showed two broad peaks indicating primer-dimers in all the sample reaction tubes. Thus, potential hepatotoxic cyanobacterial cells were out of the quantifiable range of this qPCR assay. However, potential hepatotoxic cyanobacterial cells were in high abundance by late summer. Cell concentration of potential hepatotoxic cyanobacteria in late summer ranged from 2.78 (± 0.06) x 10^7 cells·L^{-1} to 3.57 (± 4.26) x 10^8 cells·L^{-1} with an average of 1.18 (± 1.23) x 10^8 cells·L^{-1}. Consequently, this suggests that there were more toxic hepatotoxic cyanobacteria than there were total cyanobacteria in late summer which is not possible. A second qPCR reaction on another set of late summer DNA extracts yielded similar results (data not shown) as the first run. PPIA showed that MC levels were below detectable limits in early summer but increased to 4.46 (± 2.19) µg·L^{-1} by late summer (Sarah Yakobowski, University of Waterloo, Ontario, Canada).

CHAPTER 4.0: DISCUSSION

4.1 DGGE DISCUSSION

4.1.1 DGGE ANALYSIS OF 16S RRNA-V3 AND AMT (*MCYE*/*NDAF*) AMPLICONS FROM THE STUDY SITES

In this current study, DGGE analysis was used to identify and enumerate the number of different cyanobacterial genotypes present and to observe for any seasonal changes that may occur from early summer to late summer. The decision to use the 16S rRNA was based on its universal distribution in all prokaryotes, ease of amplification, and the relative abundance of sequence information in the public database (GenBank). The primers for AMT region of *mcyE* and *ndaF* was chosen for its ability to amplify in both microcystin and nodularin producing cyanobacteria from several genera (Jungblut and Neilan 2006). This permits detection of potentially hepatotoxic organisms to be surveyed for each station.

16S rRNA-V3 fingerprinting of the three lakes showed distinct banding patterns among them as well as distinct seasonal patterns from early to late summer. The different banding patterns among the three study sites (Bay of Quinte, Maumee Bay, and Nyanza Gulf) illustrates that the cyanobacterial genotypic community in the three sites are different from each other which is not unexpected. Likewise, the North American sites showed a change in cyanobacterial genotypic community from early to late summer. Multiple 16S rRNA-V3 bandings in all the stations sampled suggest a diverse cyanobacterial genotypic community with Nyanza Gulf having the highest diversity. Samples taken from the Bay of Quinte and Maumee Bay were similar in that there was a slight decrease in diversity from early to late summer. These two sites, however, differed

in the timing when *Microcystis* was most predominant. For the Bay of Quinte, *Microcystis* genotypes were detected in greater abundance (based on band intensity) in early summer than in late summer however, the reverse was observed for Maumee Bay. *Microcystis* genotypes were detected in all the Nyanza Gulf samples as well. Bay of Quinte samples had relatively similar diversity indices between 2005 and 2006 but showed different seasonal banding patterns between the two years.

AMT profiles indicated that hepatotoxic genotypes were present in all three lakes. Sequences from excised AMT bands suggested that they were all from *mcyE* gene of *Microcystis* species. No AMT sequences belonging to the nodularin *ndaF* gene were detected. This was expected since nodularin-producing *Nodularia* is commonly observed in brackish waters around the world (Koskenniemi *et al*., 2007).

4.1.1.1 Bay of Quinte 16S rRNA-V3 and AMT DGGE analysis

The Bay of Quinte stretches 100 km in length and is a major near-shore water body for Ontario. Similar to many other freshwater bodies, the Bay of Quinte has experienced a long history of severe eutrophication, especially in the upper bay (Nicholls 1999). As a result of high phosphorus loading and nuisance algae, the Bay of Quinte was named as an Area of Concerns by the International Joint Commission (Millard and Sager 1994). Following remedial actions after the signing of the Great Lakes Water Quality Agreement in 1972, there has been an approximately 90% reduction of point source phosphorous loading (Chu *et al*., 2004) and a 51% decrease in phytoplankton (Nicholls *et al*., 2002).

More recently, the colonization of dreissenid mussels are causing other water-quality issues, most importantly, the increase in *Microcystis* cells (Nicholls *et al*., 2002).

This is consistent with previous experiences in western Lake Erie and in Saginaw Bay, Lake Huron (Vanderploeg *et al*. 2001; Budd *et* al. 2002). Following the establishment of *Dreissena* in Lake Ontario, there has been a 13-fold increase in *Microcystis* biovolume at the Bay of Quinte (Nicholls *et al*., 2002). Preferential feeding and alteration of lake water nutrient cycling by *Driessena* is hypothesized to contribute to the increase in cyanobacterial blooms (Baker *et al*., 2000; Bykova *et al*., 2006; Nicholls *et al*., 2002). Previous studies by Vanderploeg *et al*. (2001) suggested that *Dreissena* are capable of selective feeding by expelling toxic *Microcystis* as pseudofeces while ingesting "desirable" algae. Dionisio Pires *et al*. (2005), however, observed no difference in the excretion of toxic and non-toxic *Microcystis* strains by *Dreissena* which is contradictory to what was observed by Vanderploeg *et al*. (2001). Dionisio Pires *et al*. (2005) suggested that the difference in results to Vanderploege *et al*. (2001) may be due to the type of *Microcystis* strains used in the two studies. Dionisio Pires *et al*. (2002) performed grazing experiments with *Dreissena polymorpha* using *Microcystis* strains that were colonial but lacked a mucilage matrix; whereas, Vanderploeg *et al*. (2001) used *Microcystis* strains that maintained their mucilage making it less palatable (Dionisio Pires *et al*., 2005). Nevertheless, the mussels' high density in combination with their high filtration rate, and excretion of feces/pseudofeces and soluble nutrients can alter the lake nutrient chemistry, which then affects the phytoplankton community structure (Arnott and Vanni 1996; Bykova *et al*., 2006; Makarewicz *et al*., 2000). Arnott and Vanni (1996) shown that mussels excreted more phosphorous (P) relative to nitrogen (N) thereby decreasing the N:P ratio which favours cyanobacteria dominance. The combinations of

decreased point source phosphorous loadings and dense driessenid population has altered the phytoplankton community structure in the Bay of Quinte (Nicholls *et al*., 2002).

In 2005 the samples were taken from the upper bay (BB) and middle bay (NA, top region of middle bay; HB, lower region of middle bay) (Figure 2.1). Despite the distance between the three stations, their cyanobacterial genotypic communities were similar. Several cyanobacterial genotypes including potential hepatotoxin-producing genotypes were detected in nearly all the Bay of Quinte samples using DGGE analysis of the 16S rRNA-V3 and AMT gene fragments. Based on DGGE separation of the 16S rRNA-V3, *Gloeotrichia* dominated the cyanobacterial community in early summer, but *Microcystis* and *Anabaena* dominated the cyanobacterial community by late summer. DGGE analysis AMT amplicons from the 2005 samples reported the presence of hepatotoxic *Microcystis* genotypes throughout the summer.

The high abundance of *Gloeotrichia* sp. (observed microscopically) during the June 28, 2005 and July 4, 2006 (early summer) collecting trips suggest that the dominant genotype A band, in early summer of 2005 (Figure 3.1.1) and distinct genotype B in early summer of 2006 (Figure 3.1.2), were possibly from a *Gloeotrichia* sp. Sequences of genotypes A (Figure 3.1.1) and B (Figure 3.1.2) were identical in sequence which suggest that they were the same *Gloeotrichia* genotype in 2005 and 2006. Other than knowing that genotypes A (Figure 3.1.1) and B (Figure 3.1.2) were not the same genotype as the *Gloeotrichia ghosi* UTEX LB 1920, the species of this particular presumed *Gloeotrichia* band remains unidentified. BLASTn of the sequences for genotype A and B provided no further information except that the sequence was derived from a cyanobacterium. The lack of a band or the presence of a faint band corresponding to the *Microcystis* sp.

standard indicates that species of *Microcystis* were in very low abundance during June 28, 2005. However, the presence of potential hepatotoxic genotypes were detected from all three sampled stations (NA, HB, BB) on June 28, 2005 in spite of the low detection of *Microcystis* 16S rRNA-V3 genotypes. All seven samples collected on August and October of 2005 showed similar banding patterns with *Microcystis* and *Anabaena* species detected from each site. This implied that throughout late summer to early fall of 2005 there was little change in the cyanobacterial community. Detection of hepatotoxic bands from all the 2005 samples indicated that toxic cyanobacteria were present throughout the summer months. Based on band intensities, potential hepatotoxic genotypes increased in abundance from early to late summer. AMT amplicons(s) present in all the 2005 samples migrated closely to the same gel position as the *M. aeruginosa* UTCC 299 AMT standard. This suggests that *Microcystis* was the primary potential hepatotoxin-producer in the Bay of Quinte. This is consistent with Nicholls *et al*. (2002) observations that *Microcystis* increases after the establishment of *Dreissena* and may only involve toxic strains because non-toxic strains are actively consumed by *Dreissena* according to Vanderploeg *et al*. (2001).

All samples from 2006 were only collected from the top region of the Bay of Quinte middle bay and are equivalent to station NA of 2005. A band corresponding to the *Microcystis* standard was present in all station samples collected in early summer (July 4-5, 2006). In contrast to the 2005 samples, band intensity for *Microcystis* was very faint in late summer of 2006 suggesting little abundance of *Microcystis*. Similar to the 2005 late summer samples, a band corresponding to the *Anabaena viguieri*/*Anabaena planktonica* standard was present in all sites for the 2006 late summer (September 22) samples. There

were some discrepancies from 2005 to 2006 (two distinct genotypes, A and C, [Figure 3.2.1] present in the 2006 samples but absent in the 2005 samples). These differences could be attributed to differences in environmental conditions between the summer of 2005 and 2006 that favoured certain cyanobacterial species over others. For example, studies showed that increases in nitrogen reduced the biomass of heterocystous (nitrogen fixing) *Aphanizomenon* and *Anabaena* species but favoured non-heterocystous *Microcystis* and *Synechococcus* (Barica *et al*., 1980; Ferber *et al*., 2004; Stockner and Shortreed 1988).

DGGE analysis of PCR amplified AMT amplicons indicated the presence of two hepatotoxic genotypes present throughout the summer of 2006 (Figure 3.1.6). Bands migrating closely together may be subspecies of a genus or different copies of the gene from the same organism (Iteman *et al*., 2002; Kolmonen *et al*., 2004). The close proximity of the two genotypes to each other and to the *Microcystis* standard suggested that the bands were derived from two *Microcystis* strains. Since it is believed that only a single copy of the *mcy* gene cluster is present within a single cyanobacterial genome (Dittmann *et al*., 1997; Nishizawa *et al*., 1999) it is unlikely that the two AMT bands present in the DGGE gel are from the same cyanobacterial cell. Results from AMT DGGE analyses of both 2005 and 2006 samples is supported by Hotto *et al*. (2007) who also observed that *Microcystis* was the dominant potential microcystin producer in southern and eastern shores of Lake Ontario (during 2001 and 2003) based on *mcyA* sequences.

4.1.1.2 MAUMEE BAY 16S RRNA-V3 AND AMT DGGE ANALYSIS

Historically, Lake Erie had experienced ongoing cultural eutrophication and dense algal blooms during the 1950s and 1960s (Beeton, 1969; Davis, 1968). Following the 1972 Great Lakes Water Quality Agreement to reduce phosphorous discharge to the Great Lakes, there was a significant reduction in cyanobacteria biomass and algal blooms by the mid 1980s (Makarewicz and Bertram, 1991). Surprisingly, in September of 1995, a dense toxic *Microcystis* bloom occurred that covered the entire surface of the western basin (Brittain *et al*., 2000). A second and larger toxic *Microcystis* bloom occurred several years later in August of 2003 and persisted for over a month (EPA 2007; Ouellette *et al*., 2006). Since then, toxic *Microcystis* blooms of varying magnitude have become common in late summers (EPA 2007). Similar to the Bay of Quinte, the spread of *Dreissena polymorpha* in the late 1980's to Lake Erie (Leach 1993) coincided with an increase in *Microcystis* blooms (Ouellette *et al*., 2006). Prior to the colonization of *Dreissena polymorpha* in the 1980s cyanobacteria was only a minor component of the phytoplankton community (Nicholls and Hopkins 1993). The mussel's establishment in Lake Erie is presumed to play a role in shifting the phytoplankton community structure from predominately nitrogen-fixing cyanobacteria (*Anabaena spiroides* and *Aphanizomenon flos-aquae*) to non-nitrogen-fixing *Microcystis* species in the western basin (Brittain *et al*., 2000). *Dreissenids* are believed to be responsible for making ammonium-nitrogen more available to non-nitrogen fixers thereby allowing them to take advantage of the increased soluble phosphorous levels produced by the mussels (Brittain *et al*., 2000). In this current study, non-nitrogen-fixing *Microcystis* were observed to dominate Maumee Bay's cyanobacterial community in late summer.

Figure 4.1 Cyanobacterial bloom in Maumee Bay on August 22, 2006. (A) View of the water surface of Maumee Bay. (B) Close-up view of the water surface. Photo by A. Chhun

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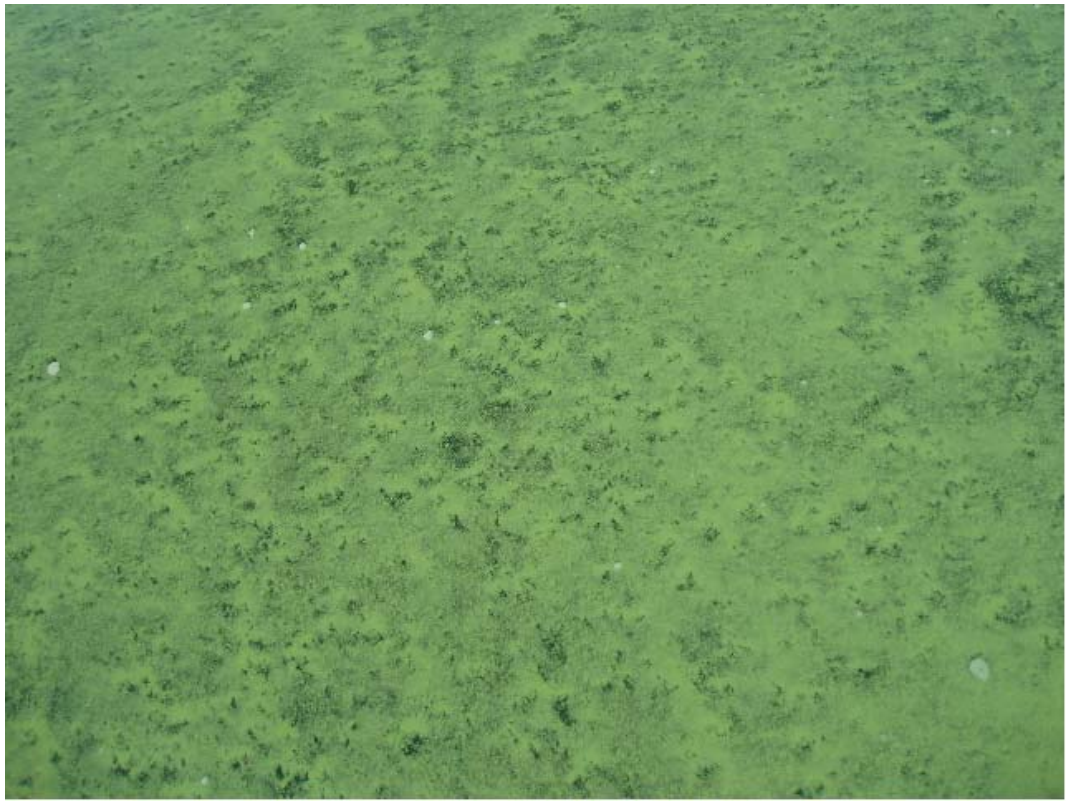
A

B



As with the Bay of Quinte samples, there were seasonal differences in the planktonic cyanobacterial community between early and late summer. Several cyanobacterial 16S rRNA-V3 genotypes, many of them unidentified, were present in both early and late summer. The 16S rRNA-V3 profiles reported little to no *Microcystis* genotypes in early summer but high abundance of *Microcystis* genotypes by late summer (Figure 3.1.3A and 3.1.3B). Profiles of the AMT amplicons showed no detectable hepatotoxic genotypes observed in early summer but multiple hepatotoxic genotypes were present in late summer when Maumee Bay was experiencing a cyanobacterial bloom (Figure 4.1).

Faint or absent of bands corresponding to the *Microcystis* standard in the early summer station samples were indications of low *Microcystis* biomass during early summer months. The absence of an AMT band (Figure 3.1.7) for the early summer samples implied that toxic strains were in such low abundance (<1% of total DNA) they were unable to be detected by PCR-DGGE. The distinct 16S rRNA-V3 bands (Figure 3.1.3A, A-F) that did not migrate to the same position as any of the standards in the early summer samples remain unknown. A comparison of these sequences to the nucleotide database (BLASTn) resulted in a 98% nucleotide match to *Microcystis* and numerous uncultured cyanobacteria. Due to the small sequence length and the highly conserved nature of the 16S rRNA gene, several cyanobacterial species or genera may share the same nucleotide sequences (Gill *et al*., summitted; Nubel *et al*., 1997). Thus, bands in figure 3.1.3A (A-F) cannot be assumed to belong to *Microcystis* due to sequence similarities. Microscopic observations suggest one or more of these bands may belong to a species of *Anabaena* since it was the cyanobacterial genus most commonly observed in

the samples. It is also possible that one or more of the 16S rRNA-V3 bands may belong to *Synechococcus* and *Cyanobium* species. Ouellette et al. (2006) observed that their 16S rRNA clone library of samples collected during July in 1999-2002 from near and off-shores of Lake Erie was dominated by *Synechococcus* and *Cyanobium*-like sequences. The authors also reported that the picoplankton population (0.2-2.0 μM) made-up 24% of the total chlorophyll *a* in the western basins of Lake Erie in July 2002. Dominance of *Synechococcus* and *Cyanobium* within the picoplankton population is common in oligotrophic freshwater systems (Callieri and Stockner, 2002; Postius and Ernst, 1999). Lake Erie is no different as its ecosystem is shifting from eutrophic to meso-oligotrophic the dominance of *Synechococcus* and *Cyanobium* in picoplankton size class is not surprising (Ouellette *et al*., 2006). Thus the picoplankton population makes up a large portion of the phytoplankton community and their presence were most likely represented by some of the unknown 16S rRNA-V3 bands.

August and September (late summer) are the months that *Microcystis* blooms typically occur in western Lake Erie (Brittain *et al*., 2000). Based on the visual observation of the lake, it was clear that in late summer of 2006 Maumee Bay was experiencing a cyanobacterial bloom (Figure 4.1). Microscopic examination of the phytoplankton samples collected in late summer revealed that it was predominantly colonies of *Microcystis*. This morphological observation was further supported by DGGE analysis of the 16S rRNA-V3 amplicons which indicated an intense band at the same position as the *Microcystis* standard in each sampling station from late summer. DGGE analysis of the AMT amplicons indicated that hepatotoxic strains, most likely a strain of *Microcystis*, were present in all the late summer samples. The high abundance of

Microcystis and presence of the hepatotoxin gene(s) are strong indications that the cyanobacterial bloom observed in late summer was a toxic *Microcystis* bloom. This was not surprising since toxic *Microcystis* blooms of varying degrees have been reported annually during the 2000s (EPA 2007; Rinta-Kanto *et al*., 2005).

Multiple AMT bands in some of the samples (Figure 3.1.7) suggest different hepatotoxic genotypes present at these stations. High sequence similarities of different AMT bands in the same sample to *Microcystis* standard indicated that there could be more than one *Microcystis* species present. One must keep in mind that sequence data for *mcy* genes are small relative to the 16S rRNA database for cyanobacteria. Thus sequence searches of the AMT sequence domain of *mcyE* may be biased towards *Microcystis* since the majority of data sequences are *Microcystis* sequences. Sequences from AMT bands that migrated to the same position as the *A. lemmermannii* NIVA-CYA 83/1 standard (Figure 3.1.7, band A) showed high sequence similarity to a partial sequence of the *mcyE* gene of *M. wesenbergii* (98%) in GenBank.

4.1.1.3 Nyanza Gulf 16S rRNA-V3 and AMT DGGE analysis

Over the last three decades, population growth, increasing demands for natural resources and land-use has lead to pollution loading and eutrophication of Lake Victoria in eastern Africa (Machiwa, 2003). Similar to the two Laurentian Great Lakes studied, there has been a shift of the phytoplankton community structure in Lake Victoria from diatoms to cyanobacteria dominance throughout the year (Kling *et al*., 2001). Cyanobacterial blooms have become common in the Nyanza Gulf and are most abundant from October to November when the water column is thermally stratified (Kling *et al*., 2001; Krienitz *et al*., 2002; Lung'ayia *et al*., 2001; Ochumba and Kibaara 1989). For this

reason, phytoplankton samples were collected in November for molecular analysis of the cyanobacterial community. Presently, nitrogen fixers make up most of the phytoplankton biomass (Kling *et al*., 2001). Kling, et al. (2001) observed that heterocystous nitrogen fixing cyanobacteria *Cylindrospermopsis* dominate the phytoplankton from December to June in 1990-1992. Nitrogen-fixers can overcome the nitrogen limited condition in Lake Victoria brought on by excessive phosphorous loading, from sewage effluents and agriculture (Kling *et al*., 2001; Lehman and Branstrator, 1994; Lung'ayia *et al*., 2001). In this study, nitrogen-fixing *Anabaena* species were commonly found in all samples examined microscopically.

Profiles of the Nyanza Gulf's 16S rRNA-V3 amplicons indicated *Microcystis* and *Anabaena* genotypes were present in all sampling stations. A band corresponding to the *Anabaena viguieri*/*Anabaena planktonica* standard was present in all the samples. *Microcystis* genotypes appeared to be the most dominant cyanobacterial genotype (based on band intensity) in 3 (KL3, KL5, COL) of the 4 station samples. Low Secchi transparency (0.5-1.2 m) at Nyanza Gulf is an advantageous condition for the taxa *Microcystis*. *Microcystis*, especially *M. aeruginosa*, are capable of developing many gas vacuoles within the cell to regulate its vertical buoyancy in the water column (Kromkamp *et al*., 1988; Zohary and Roberts, 1990). Examination of the preserved phytoplankton bloom sample revealed only *Anabaena* species, however, DGGE separation of the 16S rRNA-V3 amplicons revealed multiple cyanobacterial genotypes. This may be due to the degraded quality of the phytoplankton bloom sample. The genetic profile of the phytoplankton bloom sample indicated that it was composed of several 16S rRNA-V3 genotypes (>5) that included *Microcystis* and *Anabaena*-like genotypes. This is

comparable to a study by Krienitz *et al*. (2002) who observed that the toxic cyanobacterial bloom in Nyanza Gulf on November of 2001 was composed of *Microcystis* (*M. aeruginosa*) and *Anabaena* (*A. flos aquae* and *A. discoidea*).

PCR-DGGE of the AMT amplicons revealed that potentially hepatotoxic *Microcystis* strains were present at all four stations. The presence of AMT bands in station COL implied that potentially toxic *Microcystis* genotypes pose a threat to the open lake and not just the shallow depth of Nyanza Gulf. DGGE analysis of the phytoplankton sample produced no detectable AMT bands on the polyacrylamide gel. This indicated that the phytoplankton bloom was not toxic or that the potentially hepatotoxic population was too miniscule to be detected by PCR-DGGE. The detection of hepatotoxin producers in November 2001 (Krienitz *et al*. (2002) and potential hepatotoxin genotypes in November 2005 raises the question of whether cyanotoxic cells appear annually around November or if they are constantly present. More sampling trips to these stations are required to address seasonality of cyanobacteria in Lake Victoria.

4.1.2 GENERAL STATISTICAL ANALYSIS

4.1.2.1 DIVERSITY

The 16S rRNA-V3 sequence divergence between cyanobacteria species was used to infer diversity at each sampling station. The number of bands presents (richness) and relative band intensity (evenness) was used in the Shannon diversity index equation to mathematically measure the cyanobacterial community diversity. Each band present on the gel was considered as one operational taxonomic unit (OTU).

The Shannon diversity index of the Bay of Quinte and Maumee Bay samples showed temporal differences from early to late summer for both. Given the close

proximity of the 6 each stations to one another in the Bay of Quinte and Maumee Bay, the banding patterns showed no significant spatial differences. Hence, the diversity of the 6 sampling stations was averaged for early and late summer. Nyanza Gulf had a slightly higher 16S rRNA-V3 genotypic diversity relative to the two temperate lakes. All the sampling sites of the three lakes had a high evenness value (92-96%) indicating that the relative abundance of each OTU is equally spread. In terms of richness, Nyanza Gulf had a higher number of bands (OTU) which gave it a higher diversity index than the Bay of Quinte and Maumee Bay samples. There were no significant differences in cyanobacterial community diversity for Maumee Bay between early and late summer. However, there was a slight decrease in diversity for the Bay of Quinte 2006 samples from early and late summer, but the opposite was observed in the 2005 sample which saw a small increase in diversity. Nevertheless, diversity index for the Bay of Quinte in 2005 and 2006 were fairly similar. These small differences suggested that cyanobacterial blooms had little effect on the cyanobacterial genotypic diversity.

DGGE can be a powerful tool for surveying microbial community structures; however, caution should be used when interpreting DGGE profiles. Several factors in the DGGE analysis used in this study can result in an underestimation of genotypic diversity. The presence of bands that migrate to identical positions in a gel does not necessarily mean that they are the same sequence or originate from the same organism (Jackson *et al*., 2000; Muyzer *et al*., 1993). In addition, all PCR based techniques have biases in PCR amplification which affects the band intensity used to calculate diversity (Kowalchuka *et al*., 2006). Given that only a fraction (1/16) of the filter was analyzed and may not be representative of the actual population, the diversity estimation could have been biased

(Kirchman *et al*., 2001). In this present study, several DGGE analyses of samples from different filter pieces collected from the same station and date found that there were some discrepancies but overall, the bands were fairly reproducible. As well, underestimation of diversity can happen when species populations occurring in small numbers fail to be detected by PCR-DGGE. Generally, target DNA of a species whose abundance is greater than 1% of the total DNA extract mixture can be detected by PCR-DGGE (Muyzer *et al*., 1993). In contrast, there is the other possibility that heterogeneity of 16S rRNA genes within a single species may result in overestimation of diversity (de Souza *et al*., 2004; Nubel *et al*., 1997). Hence, diversity indices should only be used for relative comparison to other samples and not viewed as absolute numbers (Girvan *et al*., 2003).

4.1.2.2 REPRODUCIBILITY

Three different sample sets were run on two different DGGE gels to determine how reproducible banding patterns were for the same stations. The average Jaccard similarity coefficient of the three sample sets was 76.7%. This showed that when samples were extracted and analyzed on a DGGE gel and repeated again (extraction and analysis), the banding patterns were highly reproducible.

4.2 QPCR DISCUSSION

4.2.1 STANDARD CURVES

In this present study, quantitative real-time PCR (qPCR) was used to enumerate the abundance of cyanobacterial cells and hepatotoxic cells present at each station at the time the samples were collected. Only lake samples collected during the summer of 2006 were analyzed by qPCR. Filters collected in 2005 were too old and decayed (too much freeze thawing) to provide reliable results. Cyanobacterial cell concentration and

hepatotoxic cell concentration were calculated based on *M. aeruginosa* UTCC 299 cell equivalents. PCR amplification efficiency based on the slope of the standard curve was 86% for the 16S rRNA-V3 primers and 87% for the AMT primers. This is slightly below the ideal efficiency range of 90% to 110% with 100% meaning perfect doubling of PCR amplicons. PCR amplification efficiency is influenced by the amplicon size and GC content, and primers, fluorescent dye, and *Taq* polymerase used (Arezi *et al*., 2003; Labrenz *et al*., 2004). Nevertheless, quantification of target cell number was possible since both standard curves, 16S rRNA-V3 and AMT, had a high correlation value (R^2 = 0.996 and 0.999 respectively) between the amount of initial DNA template and Ct values. Dissociation curve of all PCR amplicons from both 16S rRNA-V3 and AMT amplification samples showed only one peak at 85^{o}C in tubes with DNA template added. This confirms that no primer-dimers and non-specific binding of the PCR primers occurred in the template samples. There was a smaller peak at around 76 ^{o}C to 77^{o}C illustrated in the dissociation curve of the AMT amplicons for the three NTC (no template control) reaction tubes. These peaks are most likely primer-dimers that occurred due to lack of DNA template in the NTC reaction tubes. PCR products that were run on an agarose gel showed only the band corresponding to the expected PCR size giving further support that only the target sequence was amplified.

Multiple copies of the 16S rRNA gene in the genome allowed for PCR amplification with only few cells present because there was more target DNA template available. Therefore, it was easy to establish a standard curve with as little as 5 cells per reaction. On the other hand, the AMT region in the *mcyE* or *ndaF* gene which may only exist as one copy per genome made constructing the AMT standard curve more difficult.

PCR amplification at 5 cells per reaction failed due to insufficient amount of DNA template. Hence, the detection limit of the AMT standard curve ranged from 50 to 5 x 10^5 cells per 20 µl reaction.

Numbers from qPCR analyses were only considered as estimates of the cell concentration for each sample. The log-linear relation of the standard curve makes qPCR very sensitive to minor changes to the slope of the curve resulting in systematic errors (Becker *et al*., 2002; Kurmayer and Kutzenberger, 2003). Moreover, there are inherent errors when using the 16S rRNA gene derived from only the *M. aeruginosa* UTCC 299 strain to create the standard. Redundancies of the 16S rRNA sequences in a single genome vary among different cyanobacteria. As well, a single cyanobacterial cell can have more than one copy of genomic DNA (polyploidy) depending on the environmental condition (Becker *et al*., 2002; Becker *et al*., 2002; Herdman *et al*., 1979; Labarre *et al*., 1987). Therefore, all cell abundances calculated from the two standard curves were interpreted as *M. aeruginosa* UTCC 299 cell equivalents.

4.2.2 QUANTIFICATION OF CYANOBACTERIAL COMMUNITY

Variations of cell concentration among the six sampling stations in the Bay of Quinte and Maumee Bay were not statistically significant, thus an average was taken of the six stations. In general, the Bay of Quinte contains a higher concentration of cyanobacterial cells than Maumee Bay by an order of one magnitude in both early and late summer months (Table 3.2.3). Both sampling sites showed a significant increase in cyanobacterial cell concentration from early to late summer but differed in their seasonal changes in potential hepatotoxic cell concentration.

4.2.2.1 BAY OF QUINTE

At the time of this study, there was no known literature reporting on the cyanobacterial cell concentration from the Bay of Quinte. To our knowledge, this is the first study to quantify the cyanobacterial cells from the Bay of Quinte uins qPCR. According to this current study there was a significant increase (P = 0.0) in cyanobacterial cell concentration from early to late summer in 2006; however, the concentration of hepatotoxic cells remained relatively the same (Table 3.2.1). The average total cyanobacterial cell concentration (cells·L^{-1}) was 1.22 x 10^8 (± 1.00) in early summer and increased more than one magnitude to 1.76 x 10^9 (± 0.70) by late summer. Direct cell counts of the NA sample collected in late summer was about one magnitude lower (3.69 x 10^8 cells·L^{-1}; Hedy Kling, Freshwater Institute, Canada) than the qPCR data (1.82 x 10^9 cells·L^{-1}). This difference could be due to errors in counting, not all the cyanobacterial cells were accounted for, or errors in the standard curve, or both. As stated earlier, the log-linear curve of the16S rRNA-V3 standard curve makes qPCR analysis very sensitive to variations in the slope.

Potential hepatotoxic cell population comprised only a small percentage (< 1%) of the total cyanobacterial community during both collecting trips. Hepatotoxic cell concentration decreased from 2.99 (± 3.60) x 10^6 to 1.46 (± 1.96) x 10^6 but the difference was not statically significant. Nevertheless, the average total microcystin concentration in early summer, 2.18 (± 0.57) μg·L^{-1} (Sarah Yakobowski, University of Waterloo, Canada) exceed the WHO guidelines of 1.0 μg·L^{-1} but by late summer the microcystin concentration, 0.58 (± 0.58) μg·L^{-1} (Sarah Yakobowski, University of Waterloo, Canada), was below the WHO threshold. Even though hepatotoxic cell concentrations were

statistically similar, microcystin concentration differed significantly. This may be due to differences in ecological conditions and different dominating hepatotoxic strains between early and late summer. Genetic regulation of microcystin are presumed to be influenced by certain environmental factors such as temperature (Billam *et al*., 2006), light (Graham *et al*., 2004; Kaebernick *et al*., 2000; Rolland *et al*., 2005; Utkilen and Gjolme 1992), total phosphorus and total nitrogen ratio (Jacoby *et al*., 2000; Kotak *et al*., 2000), and water column stability (Rolland et al., 2005). Graham et al. (2004) and Yang *et al*. (2006) observed that the relationship between microcystin concentration and environmental conditions are nonlinear and not related to a single environmental condition but rather a complex relationship between several ecological factors. Also, microcystin production between different hepatotoxic strains can differ by 3 orders of magnitude (Sivonen and Jones, 1999). Thus, it is possible that the toxic cyanobacterial strains in early summer produced more toxin than the toxic cyanobacterial strains observed in late summer. As observed from the Bay of Quinte 16S rRNA-V3 DGGE gel (Figure 3.1.2) the early summer profile contained bright distinguishable *Microcystis*-like genotypes versus late summer which contained only faint *Microcystis*. The decrease in microcystin concentration may be due to decrease in toxic *Microcystis* cells from early to late summer.

4.2.2.2 MAUMEE BAY

Maumee Bay also had an increase in cyanobacterial cell concentration from early to late summer. Unlike the Bay of Quinte, there was a considerable increase in hepatotoxic cell concentrations from early to late summer (Table 3.2.2). Cyanobacterial cell concentration demonstrated an average one-fold increase from 2.35 (± 0.99) x 10^7 cells·L^{-1} in early summer to 1.04 (± 0.75) x 10^8 cells·L^{-1} by late summer. These numbers

relate with an earlier study by Rinta-Kanto *et al*. (2005) who surveyed the western basin that included Maumee Bay (Table 3.2.3). Quantification of the 2003 *Microcystis* bloom (considered the most severe bloom to date; Bridgeman 2007) by qPCR, reported total cyanobacteria concentration for Maumee Bay as 9.9 (± 1.1) x 10^8 cells·L^{-1} in August (Rinta-Kanto *et al*., 2005). In 2004, cyanobacterial concentration of a "moderate" bloom was reported as 7.5 (± 1.3) x 10^5 cells·L^{-1}. Hence, based on these results, the 2006 bloom was not as severe as the 2003 bloom but greater than the 2004 bloom.

In early summer, the hepatotoxic cell concentration was below the detectable qPCR limits. The lack of detectable hepatotoxic genotypes from the qPCR assay is supported by the absence of hepatotoxic DNA bands observed in the AMT PCR-DGGE gel (Figure 3.1.7). This indicated that the hepatotoxin cell population was low and not a significant contributor to the cyanobacterial community structure in early summer. By late summer, the hepatotoxic cell concentration increased tremendously from undetectable to an average of 1.18 (± 1.23) x 10^8 cells·L^{-1}. The increased in hepatotoxic cells were observed as bright AMT bands on the DGGE gel (Figure 3.1.7). Based on the data, there were more hepatotoxic cells than total cyanobacterial cell which is not possible. Nonetheless, the data indicated that hepatotoxic cells made up the majority of the cyanobacterial community at the time the sample was collected in late summer. Rinta-Kanto *et al*. (2005) also quantified toxic *Microcystis* in August 2003 and August 2004 and reported that the concentrations were 1.1 (± 0.3) x 10^6 cells·L^{-1} and 3.4 (± 0.7) x 10^4 cells·L^{-1} respectively in Maumee Bay. The estimated potential hepatotoxic cells concentration [1.18 x (± 1.23) 10^8 cells·L^{-1}, August 2006] of the 2006 bloom from this present study was much higher than those observed in 2003 and 2004 by Rinta-Kanto *et*

al. (2005) (Table 3.2.3). A part of the reason for the differences in number is because the toxic cell assessment in this study included all possible hepatotoxin producing cells from different genera. Thus, toxin cell concentration would be greater than just surveying toxic *Microcystis* cells alone. In addition, extraction method, qPCR assay, and standard curve used in this study were different from Rinta-Kanto *et al*. (2005). This would account for the significant differences in cell concentration between the two studies.

Microcystin concentration also corresponded to the increase in hepatotoxic cell concentration (Table 3.2.3). Just as hepatotoxic cells were below the detectable limits by qPCR in early summer, microcystin levels were also below the detectable limits by PPIA (Sarah Yakobowski, University of Waterloo). In late summer, the average total microcystin concentration increased to 4.45 (± 2.19) $\mu g \cdot L^{-1}$ (Sarah Yakobowski, University of Waterloo), which is more than four times above the WHO threshold. The microcystin concentrations reported by Rinta-Kanto *et al*. (2005) for Maumee Bay using PPIA were 15.4 $\mu g \cdot L^{-1}$ in August of 2003 and 0.04 $\mu g \cdot L^{-1}$ in August of 2004. The microcystin concentration numbers further supports the earlier statement that the cyanobacterial blooms in late summer of 2006 were not as severe as the 2003 bloom but greater than the 2004 bloom.

CHAPTER 5: CONCLUSION

Cyanobacterial blooms are increasing worldwide as a consequence of accelerated eutrophication in freshwater bodies. Secondary products produced by bloom-forming cyanobacteria are implicated in a number of water quality issues, but the most concerning are toxin production. Human and animal illnesses and death due to exposure to hepatotoxins (microcystin and nodularin) makes it the most studied of the cyanotoxins (Hisbergues *et al*., 2003). In the current study, the cyanobacterial community structure was evaluated from three separate study sites: Bay of Quinte (Lake Ontario), Maumee Bay (Lake Erie), and Nyanza Gulf (Lake Victoria) using molecular techniques. The combination of DGGE and qPCR analysis of the 16S rRNA-V3 gene region and AMT region of *mcyE* and *ndaF* gene region was used to survey and quantify the cyanobacterial genotypic community in the studied sites. Primers Cya-b-F371 and Cya-R783 (Zwart *et al*., 2005) for the 16S rRNA-V3 region and primers HEPF and HEPR (Jungblunt & Neilan, 2006) for the AMT domain were effective in estimating the total cyanobacterial cell and total hepatotoxic cell concentrations respectively. DGGE separation of the 16S rRNA-V3 amplicons was shown to be a quick and reproducible method for visualizing the cyanobacterial genotypic community. DGGE analysis of the AMT amplicons showed that all three lakes contained potentially hepatotoxin-producing cells. The majority of toxic genotypes detected most likely came from species of the genus *Microcystis*. This was expected since *Microcystis* is one of the most dominate bloom-forming cyanobacterial genus in eutrophic waters with a majority of its strains capable of producing microcystins (Carmichael, 1992; Chorus and Bartram, 1999). Quantification of the 16S rRNA-V3 amplicons showed that the Bay of Quinte contained a higher

concentration of cyanobacteria than Maumee Bay in both early and late summer. Potential hepatotoxic cell concentration was higher in the Bay of Quinte than Maumee Bay in early summer but the by late summer Maumee Bay had far more toxin producers than the Bay of Quinte.

The Bay of Quinte cyanobacterial community structure differed from one year to another and from early summer to late summer. Based on DGGE analysis of the 2005 samples, *Microcystis* genotypes were not present in early summer but were present from August through October. The reverse was observed in 2006, *Microcystis* genotypes were detected in early summer but faint in late summer. However, potential hepatotoxin-producers were detected throughout the summer in both years. Quantitative real-time PCR analysis of the 2006 samples reported a significant increase (one order of magnitude) in cyanobacterial cell concentration from early to late summer but the potential hepatotoxic cell concentrations remained relatively similar. Although hepatotoxin population consisted of less than 4% of the cyanobacterial population in early summer, it was enough to produce harmful levels of microcystin. Analytical analysis of the same 2006 samples revealed a seasonal change in microcystin concentration from levels above the WHO 1.0 $\mu g \cdot L^{-1}$ set guideline (2.25 $\mu g \cdot L^{-1}$) in early summer to levels below the guideline (0.55 $\mu g \cdot L^{-1}$) (Sarah Yakobowski, University of Waterloo). It appears that certain environmental factors were involved in triggering the transcriptional proteins in the toxic cells to produce more microcystin in early summer than late summer.

In Lake Erie, *Microcystis* is the major microcystin-producer, thus the presence of *Microcystis* 16S rRNA-V3 genotypes were of particular interest. DNA bands of 16S rRNA-V3 amplicons that migrated to the same gel position as the *Microcystis* standard

were a good predictor of potential toxin producers in the lake. DGGE separation of the 16S rRNA-V3 amplicons revealed faint to undetectable *Microcystis*-like bands in the Maumee Bay samples suggesting that potential hepatotoxin producers were in low concentration. This is supported by the undetectable hepatotoxin genotypes by DGGE analysis in the early summer samples. Additionally, these results coincide with the lack of detectable heptatoxin-producers and microcystin by qPCR and PPIA (Sarah Yakobowski, University of Waterloo) respectively. Bright *Microcystis* 16S rRNA-V3 band intensity in all the late summer bloom samples supports the microscopic observation the bloom-forming cyanobacteria was dominated by species of *Microcystis*. The multiple AMT bands and high levels of potential hepatotoxin genotypes and microcystin confirmed that the bloom was toxic and consisted of possibly more than one hepatotoxic genotype.

DNA profile of the Nyanza Gulf suggested a richer cyanobacterial genotypic community than the North American sites sampled. Bands at the same gel position as the *Anabaena* and *Microcystis* 16S rRNA-V3 standard suggested the potential for hepatotoxin production. With the exception of the phytoplankton bloom sample, the presence of AMT band close to the same gel position as the *Microcystis* standard indicated that potential microcystin producing *Microcystis* species were present in Nyanza Gulf. The lack of an AMT band in the phytoplankton bloom sample on the DGGE gel suggested that the bloom consisted of multiple cyanobacterial genotypes but was not toxic.

Concerns of over- or underestimations of the genotypic diversity and cells concentrations may be overcome by using a single copy gene region such as RNA

polymerase beta subunit (*rpoB*). The *rpoB* gene appears to be a single copy gene found in all prokaryotes and consists of slow and fast evolving regions that can provide a lot of genetic information (Dahllöf *et al*., 2000; Mollet *et al*., 1997). Similar to the 16S rRNA gene, *rpoB* is a housekeeping gene which makes it less susceptible to horizontal gene transfer (Case *et al*., 2007). DGGE analysis of the *rpoB* gene by Peixoto *et al*. (2002) observed that *rpoB* profiles produced fewer bands than the 16S rRNA profile for the same soil sample, thus evaluation of diversity was easier to analyse than the 16S rRNA gene. As well, a microbial ecological study by Case *et al*. (2007) reported that *rpoB* had a higher taxonomic resolution, down to subspecies level, than the 16S rRNA gene. For these reasons *rpoB* is widely used as an alternative biomarker in ecological studies (Case *et al*., 2007; Dahllöf *et al*., 2000; Peixoto *et al*., 2002; Rantsiou *et al*., 2004).

The existence of potential hepatotoxic cells in the three surveyed sites confirmed the need for continual monitoring and detection of these lakes for harmful toxic blooms that may form. Although molecular techniques are powerful tools in ecological studies, it has its limitations. In this study, molecular data was only able to determine genotypes present and quantity present but could not explain the differences and changes in genotypic community and quantity that occurred. The molecular data in this study would need to be analyzed in combination with the physical and nutrient chemistry of the water to understand the seasonal changes in the cyanobacterial community and predict the causes of blooms and triggers of toxin production.

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APPENDIX A

Lake	Site	Date	Diversity (H')	OTU (S)	Evenness (E_H)
Erie	MB19	20-Jun-06	2.57	14	0.98
Erie	MB18	20-Jun-06	2.51	13	0.98
Erie	8M	20-Jun-06	2.44	12	0.98
Erie	MB15	20-Jun-06	2.54	14	0.96
Erie	CS	20-Jun-06	2.41	12	0.97
Erie	7M	20-Jun-06	2.50	13	0.97
Erie	MB19	22-Aug-06	2.57	14	0.98
Erie	MB18	22-Aug-06	2.51	13	0.98
Erie	8M	22-Aug-06	2.44	12	0.98
Erie	MB15	22-Aug-06	2.54	14	0.96
Erie	CS	22-Aug-06	2.41	12	0.97
Erie	7M	22-Aug-06	2.50	13	0.97
Quinte	NA	4-Jul-06	2.46	13	0.96
Quinte	GPT	4-Jul-06	2.31	11	0.96
Quinte	NR	4-Jul-06	2.32	11	0.97
Quinte	MBO	4-Jul-06	2.12	9	0.96
Quinte	F1	4-Jul-06	2.25	10	0.98
Quinte	DS	4-Jul-06	2.32	11	0.97
Quinte	NA	22-Sep-06	1.98	8	0.95
Quinte	GPT	22-Sep-06	2.20	10	0.96
Quinte	NR	22-Sep-06	2.19	10	0.95
Quinte	MBO	22-Sep-06	2.09	9	0.95
Quinte	F1	22-Sep-06	2.18	10	0.95
Quinte	DS	22-Sep-06	2.07	9	0.94
Victoria	KL3	14-Nov-05	2.97	25	0.92
Victoria	CG5	15-Nov-05	2.75	20	0.92
Victoria	KL5	15-Nov-05	2.76	19	0.94
Victoria	COL	15-Nov-05	2.96	23	0.94
Victoria	LV1	16-Nov-05	2.65	17	0.93
Quinte_05	NA	28-Jun-05	1.89	8	0.91
Quinte_05	HB	28-Jun-05	2.08	10	0.90
Quinte_05	BB	28-Jun-05	1.92	8	0.92
Quinte_05	NA	3-Aug-05	1.98	9	0.90
Quinte_05	HB	3-Aug-05	2.32	12	0.94
Quinte_05	BB	3-Aug-05	1.97	8	0.95
Quinte_05	NA	30-Aug-05	1.71	7	0.88
Quinte_05	HB	30-Aug-05	1.56	6	0.87
Quinte_05	BB	30-Aug-05	2.04	9	0.93
Quinte_05	NA	6-Oct-05	2.39	12	0.96

APPENDIX B

Lake	Station	Date	No. of Cyano. (cells/L)	No. of Potential Hepatotoxic Cyano.
Quinte	NA	4-Jul-06	9.67E+07	5.70E+05
Quinte	NA	4-Jul-06	4.40E+07	2.77E+05
Quinte	NA	4-Jul-06	4.71E+07	4.03E+05
Quinte	GPT	4-Jul-06	2.86E+07	1.58E+07
Quinte	GPT	4-Jul-06	3.21E+07	1.03E+05
Quinte	GPT	4-Jul-06	5.97E+07	1.01E+05
Quinte	NR	4-Jul-06	3.50E+07	4.81E+05
Quinte	NR	4-Jul-06	1.20E+08	7.10E+05
Quinte	NR	4-Jul-06	1.27E+08	7.56E+05
Quinte	MBO	4-Jul-06	1.20E+08	5.28E+05
Quinte	MBO	4-Jul-06	1.16E+08	5.42E+05
Quinte	MBO	4-Jul-06	1.33E+08	5.84E+05
Quinte	F1	4-Jul-06	1.05E+08	2.01E+06
Quinte	F1	4-Jul-06	7.78E+07	1.40E+06
Quinte	F1	4-Jul-06	1.07E+08	1.72E+06
Quinte	DS	4-Jul-06	2.97E+08	1.25E+07
Quinte	DS	4-Jul-06	3.99E+08	9.93E+06
Quinte	DS	4-Jul-06	2.57E+08	5.37E+06
Quinte	NA	22-Sep-06	3.33E+09	2.57E+06
Quinte	NA	22-Sep-06	1.04E+09	1.45E+06
Quinte	NA	22-Sep-06	1.09E+09	1.23E+06
Quinte	GPT	22-Sep-06	1.28E+09	1.32E+06
Quinte	GPT	22-Sep-06	1.15E+09	1.47E+06
Quinte	GPT	22-Sep-06	1.31E+09	1.61E+06
Quinte	NR	22-Sep-06	1.96E+09	1.35E+06
Quinte	NR	22-Sep-06	3.82E+09	1.92E+06
Quinte	NR	22-Sep-06	3.21E+09	1.55E+06
Quinte	MBO	22-Sep-06	1.49E+09	1.13E+06
Quinte	MBO	22-Sep-06	1.63E+09	1.69E+06
Quinte	MBO	22-Sep-06	1.84E+09	1.16E+06
Quinte	F1	22-Sep-06	9.26E+08	9.54E+05
Quinte	F1	22-Sep-06	9.20E+08	1.05E+06
Quinte	F1	22-Sep-06	1.11E+09	1.61E+06
Quinte	DS	22-Sep-06	1.87E+09	1.67E+06
Quinte	DS	22-Sep-06	1.87E+09	1.73E+06
Quinte	DS	22-Sep-06	1.87E+09	8.36E+05

APPENDIX C

Lake	Station	Date	No. of Cyano. (cells/L)	No. of Potential Hepatotoxic Cyano.
Erie	MB18	20-Jun-06	BDL	BDL
Erie	MB18	20-Jun-06	BDL	BDL
Erie	MB18	20-Jun-06	BDL	BDL
Erie	MB19	20-Jun-06	1.38E+07	BDL
Erie	MB19	20-Jun-06	1.67E+07	BDL
Erie	MB19	20-Jun-06	2.33E+07	BDL
Erie	8M	20-Jun-06	8.64E+06	BDL
Erie	8M	20-Jun-06	3.13E+07	BDL
Erie	8M	20-Jun-06	2.59E+07	BDL
Erie	MB15	20-Jun-06	2.67E+07	BDL
Erie	MB15	20-Jun-06	3.11E+07	BDL
Erie	MB15	20-Jun-06	3.31E+07	BDL
Erie	7M	20-Jun-06	3.33E+07	BDL
Erie	7M	20-Jun-06	3.31E+07	BDL
Erie	7M	20-Jun-06	4.22E+07	BDL
Erie	Clear Site	20-Jun-06	2.16E+07	BDL
Erie	Clear Site	20-Jun-06	9.72E+06	BDL
Erie	Clear Site	20-Jun-06	2.17E+06	BDL
Erie	MB18	22-Aug-06	1.23E+08	1.93E+08
Erie	MB18	22-Aug-06	1.14E+08	1.17E+08
Erie	MB18	22-Aug-06	8.83E+07	1.11E+08
Erie	MB19	22-Aug-06	4.00E+07	4.27E+07
Erie	MB19	22-Aug-06	5.35E+07	5.99E+07
Erie	MB19	22-Aug-06	6.64E+07	5.42E+07
Erie	8M	22-Aug-06	1.33E+08	8.90E+07
Erie	8M	22-Aug-06	1.52E+08	1.34E+08
Erie	8M	22-Aug-06	1.33E+08	8.49E+08
Erie	MB15	22-Aug-06	2.40E+07	2.96E+07
Erie	MB15	22-Aug-06	1.43E+07	2.10E+07
Erie	MB15	22-Aug-06	2.24E+07	3.28E+07
Erie	7M	22-Aug-06	7.14E+07	6.03E+07
Erie	7M	22-Aug-06	5.96E+07	6.46E+07
Erie	7M	22-Aug-06	8.97E+07	5.63E+07
Erie	Clear Site	22-Aug-06	2.31E+08	8.36E+07
Erie	Clear Site	22-Aug-06	2.31E+08	7.23E+07
Erie	Clear Site	22-Aug-06	2.31E+08	5.92E+07