Microbial Ecology

Toxic *Microcystis* is Widespread in Lake Erie: PCR Detection of Toxin Genes and Molecular Characterization of Associated Cyanobacterial Communities

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Abstract

During the past decade, algae blooms, which include the toxic cyanobacterium *Microcystis*, have reoccurred in the Laurentian Great Lakes, most commonly in the western basin of Lake Erie. Whereas the western basin is the most impacted by toxic *Microcystis* in Lake Erie, there has historically been little effort focused on identifying the spatial distribution of *Microcystis* throughout this lake. To address this lack of knowledge, we have employed a polymerase-chain-reaction-based detection of genes required for synthesis of the toxin microcystin (*mcy*D and *mcy*B), as well as 16S rDNA fragments specific to either all *Microcystis* or all cyanobacteria. Using a multiplex approach, we tested 21 samples from 13 field stations and found that toxigenic *Microcystis* were present in the western and eastern basins in the summers of 1999, 2000, and 2002 and the central basin in 1999 and 2002. This is the most extensive distribution of *Microcystis* reported in Lake Erie. Clone libraries (16S rDNA) of these cyanobacterial communities were generated from 7 of the 13 field stations (representing all three basins) to partially characterize this microbial community. These libraries were shown to be dominated by sequences assigned to the *Synechococcus* and *Cyanobium* phylogenetic cluster, indicating the importance of picoplankton in this large lake system.

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Introduction

The Laurentian (aka North American) Great Lakes, composed of Lakes Superior, Huron, Michigan, Erie, and Ontario, contain 17% of the world's available freshwater and are important to the economic and recreational activity of millions of people [41]. Like many major waterways, the Lake Erie ecosystem has changed considerably because of anthropogenic influences that include an influx of pollutants, the introduction of exotic organisms, and system-wide nutrient loading. Algae blooms were common in the 1970s and early 1980s because of "cultural eutrophication." In 1972, Canada and the USA agreed to an aggressive phosphorus abatement plan as laid out in the Great Lakes Water Quality Agreement, setting annual limits on the discharge of phosphorus to this lake [12]. Following the decrease in phosphorus, algal blooms became less frequent, and the water quality of the lake improved considerably [26, 30]. However, in 1995, a bloom of toxic *Microcystis* occurred in the western basin of Lake Erie, and a toxic *Microcystis* isolate (LE-3) was obtained [5]. This bloom was unexpected because of the nutrient reductions and overall improved health of the lake.

Around 1986, the nonnative zebra mussel *Dreissena polymorpha* was accidentally introduced into the Great Lakes, in Lake St. Clair or Lake Michigan, and, by 1988, had spread to Lake Erie [24]. Zebra mussels have been found to exhibit selective feeding, expelling toxic *Microcystis* (strain LE-3 and seston from Lakes Erie and Huron) in the pseudofeces [48]. A survey of 61 Michigan lakes found that the presence of *Dreissena* correlated with *Microcystis* success in lakes with <25 $\mu g\ L^{-1}$ total phosphorus. Overall, phytoplankton biomass and chlorophyll *a* were reduced dramatically following the introduction of zebra mussels into Lake Erie [29, 34].

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Water quality and biogeochemistry in Lake Erie have been altered in dramatic ways that include an increased water transparency, lower total phosphorus concentrations (through nutrient reduction programs), and overall lower nitrogen-to-phosphorus ratios, presumably because of zebra mussels [2, 28]). Because phytoplanktons respond differently to these parameters, observed shifts in phytoplankton community structure are not surprising [29, 34, 51].

Microcystis spp. and a variety of microcystins have been reported in Lakes Erie [5, 31], Huron [48], and Ontario [31]. Microcystins are cyclic hepatotoxins that can pose health risks and are produced by a variety of freshwater cyanobacteria throughout the world [7]. Many strains of *Microcystis* produce microcystins and, as such, are among the most prevalent of the toxic cyanobacteria. Detection of microcystins is routinely carried out by mass spectrometry, enzyme-linked immunosorbent assay, and phosphatase inhibition assays, with each of these methods offering different levels of sensitivity and specificity [7].

To detect cyanobacterial cells, microscopic identification based on morphology is commonly utilized. However, microcystins are synthesized via multifunctional enzymes that include polypeptide synthetase and polyketide synthase modules, and as such, potentially toxic cells have no unique morphometric traits. DNA sequences for the gene clusters encoding these enzymes have been published from two *Microcystis* strains [35, 36, 44] and one *Planktothrix* strain [8]. These sequences enable the genes to be targeted by DNA-based detection systems. As such, the polymerase chain reaction (PCR) is rapidly becoming the basis for the detection of toxigenic microorganisms, including *Microcystis* [3, 4, 11, 16, 32, 37–39, 43, 46, 49]. Analysis of PCR-amplified microcystin genes from size-fractionated *Microcystis* colonies suggests that microcystin diversity is not necessarily driven by genotypic diversity, but instead may occur from promiscuous amino acid activation [23]. Furthermore, a positive correlation was found between colony size and the presence of toxin gene *mcy*B and microcystin production [22, 23].

As ecosystems change (naturally or because of human influences), knowledge concerning the response(s) of the microbial community is required to fully understand and develop predictive capabilities for ecosystem dynamics. Surveys of Lake Erie phytoplankton include microscopic analysis [17, 27, 29, 34] and measurements of size-class fractionated chlorophyll *a* [10, 51]. These approaches offer information concerning the morphotypes and abundances of phytoplankton. However, the genetic diversity of cyanobacteria in Lake Erie has not been investigated. Because cyanobacteria contribute significantly to nutrient and carbon flow in freshwater aquatic ecosystems, and we are concerned with an important member (*Microcystis*) of the Lake Erie cyanobacterial population, we have begun to genetically characterize the cyanobacterial component of Lake Erie.

Based on a multiplex approach to detect *Microcystis* 16S rRNA genes and the toxin biosynthesis genes *mcy*B and *mcy*D, we present the first study employing PCR-based mapping of toxic *Microcystis* within Lake Erie. Furthermore, as part of this study, we have assessed cyanobacterial community structure at several locations within the lake using 16S rRNA gene analysis as a beginning to our efforts to understand the *Microcystis*-associated cyanobacterial communities.

Methods

Lake Erie Field Sampling. Sampling was conducted during July in 1999–2002 during research cruises aboard the CCGS *Limnos*. Water samples were collected into acid-washed polycarbonate bottles with a submersible pump lowered to about 1 m below the surface. The samples were pressure-filtered (about <5 psi) onto glass fiber filters (GFF, Whatman). Samples were immediately frozen (−20°C) until processing.

Isolation and Evaluation of Genomic DNA. For genomic DNA isolation, we compared two procedures to assess biases in the specificity and/or efficiency of extraction. To yield ecologically relevant results, this comparison was conducted using samples from two different aquatic ecosystems, Sandusky Bay in Lake Erie and a pond in Knoxville, TN. The GFF were cut into equal dimensions, and DNA isolation was conducted in duplicate for each method. For method A, filters were manipulated in 100 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, and 3 mg mL^{-1} lysozyme to dislodge material, and the entire mixture was shaken for 2 h at 37°C. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1% and was then shaken for 1 h at 37°C. The samples were then incubated at 95, 80, and 37°C for 15 min each. The samples were then extracted with phenol/chloroform and phenol/chloroform/isoamyl alcohol, and DNA was precipitated with ethanol and ammonium acetate [42]. Method B was a xanthogenate-based method [45], where the filters were incubated in the xanthogenate buffer for 2 h, and then the supernatant was decanted and vortexed before proceeding to the ice incubation step. For both methods, DNA concentration was measured by fluorescence (Hoefer Dynaquant 200, Amersham Biosciences).

To assess any bias in the cyanobacterial DNA extracted, we conducted PCR terminal restriction fragment length polymorphism (T-RFLP) analysis on the genomic DNA. PCR was conducted using cyanobacterial 16S rDNA-targeted primers (Table 1). The forward and

Table 1. Primers used for PCR detection of cyanobacteria (CYA), *Microcystis* (MIC), and microcystin biosynthesis genes B (*mcy*B) and D (*mcy*D)

Gene target	*Amplicon size*	*Primer name*	*Sequence (5′? 3)*	*Source (original name)*
Cyanobacteria	~1200 bp	CYA 108F	ACGGGTGAGTAACRCGTRA	Urbach *et al.* [47] (PLG1.3)
16S rRNA		CYA 16SCYR	CTTCAYGYAGGCGAGTTGCAGC	Modified from Urbach *et al.* [47] (PLG2.4)
Microcystis	230 bp	MIC 184F	GCCGCRAGGTGAAAMCTAA	Modified from Neilan *et al.* [33] (209F)
16S rRNA		MIC 431R	AATCCAAARACCTTCCTCCC	Modified from Neilan *et al.* [33] (409R)
Microcystis	320 bp	*mcy*B 2959F	TGGGAAGATGTTCTTCAGGTATCCAA	Nonneman and Zimba [37] (MCY F1)
*mcy*B		*mcy*B 3278R	AGAGTGGAAACAATATGATAAGCTAC	Nonneman and Zimba [37] (MCY R1)
Microcystis	298 bp	*mcy*D F2	GGTTCGCCTGGTCAAAGTAA	Kaebernick *et al.* [19] (*mcy*D F2)
*mcy*D		*mcy*D R2	CCTCGCTAAAGAAGGGTTGA	Kaebernick *et al.* [19] (*mcy*D R2)

reverse primers were labeled with 6-carboxyfluorescein and hexachlorofluorescein, respectively (Sigma-Genosys). PCR amplicons were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia); aliquots were separately digested with *Dde*I and *Mnl*I, and the fragments precipitated with ethanol. The samples were then denatured with formamide and analyzed using an ABI PRISM 310 Genetic Analyzer.

Detection of Microcystis by Multiplex PCR Amplification. To determine the presence of cyanobacteria, *Microcystis*, and the toxin genes *mcy*B and *mcy*D, four primer sets (Sigma-Genosys) were used for this study (Table 1). For each sample, two separate PCRs were conducted: tube A was "cyanobacterial specific", where CYA primers were utilized, and tube B was "*Microcystis* specific", a multiplex using the primer sets for MIC (*Microcystis* 16S rDNA), *mcy*B, and *mcy*D. Initially, we utilized a "touchdown" protocol for these reactions [annealing temperatures: 65°C (initial) to 55°C (final)]. However, we found that for environmental samples, nonreproducible amplification of the toxin genes was not uncommon. Our subsequent protocol (initial denaturation at 95°C for 5 min followed by 50 cycles at 94°C for 30 s, 56°C for 60 s, 72°C for 30 s, and a final single step of 72°C for 15 min) yielded more reliable results and is the method used in these studies. Reactions were 50 μL using either Easy start tubes (Molecular BioProducts) or in 96-well plates when processing large numbers of samples. Reactions contained 400 nM each primer, 300 ng μL^{-1} bovine serum albumin (BSA; Sigma cat # A-7030; [20]), 0.04 U μL^{-1} *Taq* polymerase (Promega), 0.1% Triton® X-100 (Molecular BioProducts), 200 nM dNTPs, 2 mM $MgCl_2$, and 1–80 ng DNA. Five microliters of each PCR was subjected to electrophoresis in 6% polyacrylamide gels (37.5:1) in TBE (90 mM Tris–borate, 1 mM EDTA, pH 8.0). The gels were stained with 0.01% SYBR green (in TBE).

Clone Library Generation and Analysis. Clone libraries were created using gel-purified (QIAquick) CYA amplicons from PCRs using 66°C as the annealing temperature. Clone libraries were generated using the TOPO-TA cloning kit (Invitrogen), and plasmids were isolated with Wizard® Plus miniprep system (Promega). For samples of low transformation efficiency, the PCRs were repeated to include a final 15-min, 72°C step with 200 μM dATP, and the resulting amplicons were desalted using a Microcon®-PCR centrifuge filter (Amicon) and cloned as above.

For RFLP analysis, clones were PCR-amplified using the same CYA primers. Aliquots of each sample were independently restriction-digested (overnight at 37°C) with *Dde*I and *Mnl*I (New England Biolabs). Fragments were separated by gel electrophoresis and were visualized as above. Fragmentation patterns from the *Dde*I and *Mnl*I digests were assigned separately and then were combined to give a binomial operational taxonomic assignment (OTU) system for each sample. Sequencing was conducted (The University of Tennessee Molecular Biology Resource Facility) using the primer 700R (CTACGCATTTCACYGCTMCAC) [47] or M13 Forward (Invitrogen). The sequences were trimmed to remove uncertain nucleotides at the beginning of the data file, as well as primer sequences as necessary. Finally, the sequences generated from 700R were reversed and complemented, and the sequences were deposited in GenBank with accession numbers AY857989–AY858034.

Phylogenetic Analysis. Sequences were initially aligned using PileUp via the Wisconsin Package (version 10.3, Accelrys Inc., San Diego, CA) and then were manually edited in BioEdit [15]. Basic local alignment search tool (BLAST) analyses were conducted through the NCBI Web site [1]. For each sequence, the BLAST result with the highest sequence identity to the cloned sequence was noted (Table 3). Chimera Check at the Ribosomal Database Project II [9] and Bellerophon [18] were employed for detecting potential chimeric PCR products. Neighbor-joining analysis was conducted using Kimura two-parameter distance and complete deletion using the Mega2 software package [21]. Only positions that were unambiguous and present for all sequences

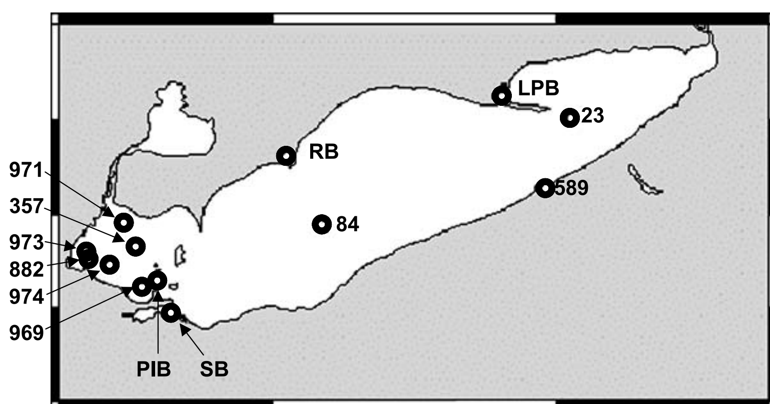

Figure 1. Sampling locations within Lake Erie. LPB, Long Point Bay; PIB, Put in Bay; RB, Rondeau Bay; SB, Sandusky Bay.

were utilized, resulting in 461 nucleotide positions. Bootstrapping employed 5000 replications.

Results

DNA Isolation Comparison. For the Knoxville pond sample, the yield of DNA was 6.3 (±1.3) μg L^{-1} water and 6.6 (±1.9) μg L^{-1} water using methods A and B, re-spectively, and for Sandusky Bay (Fig. 1), the DNA yield was 17.3 (±1.4) μg L^{-1} water and 18.5 (±1.4) μg L^{-1} water for methods A and B, respectively. Thus, the DNA yields for the two methods are essentially the same. For the Sandusky Bay samples, multiple filters were required to filter a large volume, so the volume of water per filter is estimated.

For a quick assessment of extraction bias, T-RFLP analysis (see [38] and references therein) was conducted on the DNA samples. In T-RFLP, PCR is conducted

Table 2. PCR analysis of cultures

Organism	*CYA*	*MIC*	*mcyD*	*mcyB*	*MCY*[a]
Vibrio sp.	−	−	−	−	NR
Unicellular/noncolonial cyanobacteria					
Synechocystis sp. UTEX 2470	+	−	−	−	NR
Synechococcus sp. WH 7803	+	−	−	−	NR
Filamentous cyanobacteria					
Nodularia sp.	+	−	−	−	NR
Oscillatoria luteus	+	−	−	−	NR
Plectonema boryanum 594	+	−	−	−	NR
Nostoc muscorum UTEX 1037	+	−	−	−	NR
Anabaena flos-aquae UTEX 2383	+	−	−	−	NR
Anabaena sp. UTEX 2391	+	−	−	−	NR
Anabaena sp. UTEX 2558	+	−	−	−	NR
Anabaena sp. UTEX 2576	+	−	−	−	NR
Planktothrix sp. PCC 7811	+	−	−	−	NR
Planktothrix sp. PCC 7821	+	−	−	−	NR
Microcystis spp.					
M. aeruginosa LE-3	+	+	+	+	Y
Microcystis sp. PCC 7813	+	+	+	+	Y
Microcystis sp. PCC 7820	+	+	+	+	Y
Microcystis sp. PCC 7941	+	+	+	+	Y
M. aeruginosa UTCC 124	+	+	+	+	NR
M. aeruginosa UTCC 459	+	+	+	+	Y
M. aeruginosa UTEX 2385	+	+	+	+	NR
M. aeruginosa UTEX 2386	+	+	−	−	N
M. aeruginosa UTEX 2661	+	+	−	−	NR
M. aeruginosa UTEX 2663	+	+	+	+	Y
M. aeruginosa UTEX 2666	+	+	+	+	Y
Microcystis sp. PCC 7806	+	+	+	+	Y

Detection of cyanobacteria 16S rRNA (CYA), *Microcystis* 16S rRNA (MIC), microcystin polyketide synthase D gene (*mcy*D), and microcystin peptide synthetase B gene (*mcy*B).
[a]Reportedly microcystin producer or not: NR, not reported; Y, reportedly produces microcystin; N, reportedly does not produce microcystin.

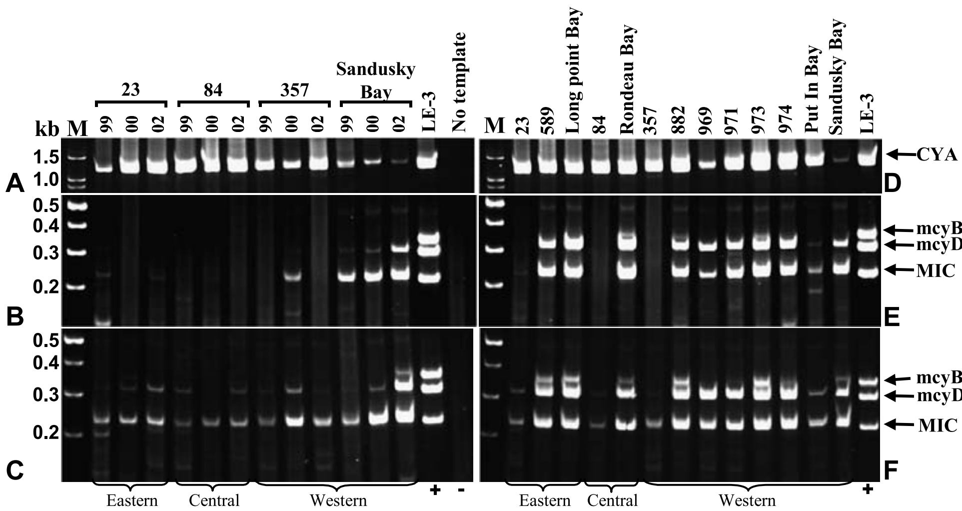

Figure 2. PCR results for detection of toxic *Microcystis* in Lake Erie. Reactions for A and D contained CYA primers; B, C, E, and F contained *mcy*B, *mcy*D, and MIC primers. PCRs contained 8 ng of DNA in A, B, D, and E and 80 ng of DNA for C and F. Molecular weight markers are in lanes M, with the size of the markers labeled as kb (kilobase pairs). Basin locations of the stations (eastern, central, and western) are noted at the bottom. A *Microcystis* strain isolated from Lake Erie [5] was used as a positive control (+), and water was used as a negative control (no template; −).

using at least one fluorescently labeled primer. Following restriction digestion of the resulting amplicons, the fragments are separated by capillary electrophoresis, and the labeled fragments are monitored. The resulting electropherograms display peaks that correspond to terminal restriction fragments. In this way, a measure of the gene diversity is obtained. The T-RFLP analysis from the two different DNA isolation procedures yielded electropherograms within which the majority of peaks were essentially the same (data not shown). Because the DNA extraction efficiency and the overall gene diversity were very similar, we concluded that either genomic DNA isolation method was valid for this study, and we used the xanthogenate–SDS method (method B).

Multiplex PCR Detection Validation Using Cultures. Polymerase-chain-reaction-based presence/absence assays for cyanobacteria, *Microcystis*, and the toxin genes *mcy*D and *mcy*B were conducted on laboratory cultures (Table 2 and see Ouellette and Wilhelm [38]) using the primers in Table 1. Two separate PCRs were conducted for each sample, one "cyanobacteria and chloroplast specific" and the other "*Microcystis* specific". To detect the presence of cyanobacteria, a portion of the cyanobacterial 16S rRNA gene was targeted by PCR, which yields an amplicon of approximately 1200 base pairs (labeled CYA, Fig. 2). To detect *Microcystis*, primers were utilized that specifically target sequences of the 16S rRNA gene that are highly conserved and unique among known *Microcystis* strains [33]. These primers detect *Microcystis* strains that are reportedly toxic and nontoxic and yield the amplicons labeled MIC in Fig. 2 [33, 38]. To detect *Microcystis* strains that contain microcystin biosynthesis genes (i.e., toxigenic), we employed primers to PCR amplify portions of the *mcy*B [37] and *mcy*D [19] genes (amplicons labeled in Fig. 2). Table 2 summarizes the results of these assays for a variety of laboratory cultures to include the heterotrophic *Vibrio* as a negative control. All of the cyanobacteria tested positive using the CYA primers, and the MIC primers are specific to *Microcystis*. The eight reportedly toxic *Microcystis* test positive for *mcy*D and *mcy*B, whereas the strain reportedly nontoxic (UTEX 2386) tests negative for these microcystin biosynthesis genes.

Multiplexing Lake Erie Samples. To understand the spatial distribution of *Microcystis* within Lake Erie, we have used these PCR protocols on genomic DNA isolated from various locations (Fig. 1). Data from four sampling stations are presented from 3 years: 1999, 2000, and 2002 (Figs. 2A–C). Data for 13 stations throughout the lake are presented from the year 2002 (Figs. 2D–F). To assess the ability of the genomic DNA to be amplified, CYA primers were used as positive controls for each station, as it is a basic assumption that cyanobacteria are ubiquitously present. All samples tested positive for cyanobacterial 16S rRNA genes (Figs. 2A, D). Some samples that tested positive for one or more of the *Microcystis*-specific genes (MIC, *mcy*D, *mcy*B) using 80 ng DNA tested negative when 8 ng genomic DNA was assayed (Fig. 2, compare C to B and F to E). Without the addition of BSA to the reactions, many samples failed to yield *any* amplicons when 80 ng of DNA was used (data not shown). However, when the samples contained BSA, all tested positive for cyanobacteria and *Microcystis*, and all but stations 23 (1999), 84 (2000), and 357 (1999) reliably tested positive for one or both of *mcy*D and *mcy*B (Fig. 2).

Amplicons for MIC, *mcy*B, and *mcy*D were sequenced from two stations and searched against the nonredundant database (blastn). These sequences are 95 and 97% (MIC), 92 and 96% (*mcy*B), and 99 and 100% (*mcy*D) identical to sequences in the databank that correspond to the targeted genes. These results confirm the

validity of the PCR-based detection. These PCR-based results indicate the widespread presence of toxic *Microcystis* throughout Lake Erie in 1999, 2000, and 2002.

Lake Erie Clone Library/Phylogenetic Analysis. To begin characterization of cyanobacterial communities, we have constructed and analyzed 16S rRNA gene clonal libraries. Clone libraries were generated for seven stations, and 50 clones from each library were selected for RFLP analysis using two different restriction enzymes. Using RFLP analysis, we were able to assign 57 unique OTUs from 295 plasmids based on two separate digests by *Dde*I and *Mnl*I (some samples were not assigned OTUs because of lack of clone growth, difficulties in restriction, etc.). Of the 50 plasmids submitted for sequencing, 2 had no insert, 1 did not sequence, 1 had more than one sequence, and 46 usable sequences were generated. These 46 sequences represent 40 of the 57

Table 3. Clone library OTUs: distribution in Lake Erie and BLAST results

Stations and OTU frequency								*BLAST results*		
OTU	*23*	*LPB*	*589*	*84*	*RB*	*357*	*SB*	*Identity*	*Accession number*	*% ID*
1a			1	22	36	1	3	*Synechococcus* PS723	AF216955	98
1b								*Cyanobium* (*Synechococcus*) LB03	AY83115	96
2a, b	10	25	17	8	4	22		*Synechococcus* LBG2	AF330249	96[a]
3					1			*Synechococcus* BS5	AF330253	96
4					1			*Synechococcus* BS4	AF330253	96
5				1				*Synechococcus* LBG2	AF330249	94
6				1				Uncultured cyanobacterium	AY100325	81
7				1				*Synechococcus* PCC 7920	AF216948	97
8				1				Uncultured cyanobacterium	AB060934	97
9						2		*Synechococcus* PS723	AF216955	98
10a								*Synechococcus* BS5	AF330253	96
10b	18	11	2			6	4	Uncultured cyanobacterium	AB060934	99
10c								*Synechococcus* PS680	AF216951	97
11						2		*Synechococcus* PCC 9005	AF216950	97
12						1		*Synechococcus* LBG2	AF330249	98
13						1		*Synechococcus* PCC 7920	AF216948	98
14						1		*Synechococcus* LBB3	AF330250	97
15		6						*Synechococcus* BS5	AF330253	94
16		1						Uncultured cyanobacterium	AB060934	96
17			11					*Synechococcus* LBG2	AF330249	94
18			2					*Synechococcus* PS723	AF216955	96
19							1	*Spirulina laxissma* SAG B256.80	Y18798	90
20							2	*Spirulina laxissma* SAG B256.80	Y18798	90
21							2	*Oscillatoria*	AJ133106	94
22a, b							4	*Synechococcus* PCC 7920	AF216948	97[a]
23							1	*Synechococcus* BS5	AF330253	95
24			1					*Lauderia borealis* P125	AJ536459	95
25						1		Uncultured eukaryote	AB060935	92
26						1		Uncultured diatom HT2B7	AF418974	98
27				2				Environmental clone OCS20	AF041468	90
28				1		1		Uncultured eukaryote	AB060935	92
29					1			Uncultured earthworm cast	AY037734	94
30					1			Uncultured bacterium Tc57	AF445119	75
31a, b			1			1		Uncultured bacterium B2	AB060931	95[a]
32				2				Uncultured bacterium MB-A2-100	AY093455	89
33				1				Uncultured bacterium 120-145	AF316677	93
34							3	Uncultured bacterium LD29	AF009975	97
35						1		Uncultured bacterium CL120-145	AF316677	93
36		1						Uncultured bacterium DEV005	AJ401105	87
37			1					Uncultured bacterium CL120-145	AF316677	93
38							2	Uncultured bacterium LD29	AF009975	97
39							8	Uncultured bacterium LD29	AF009975	95
40	6							Uncultured bacterium LD29	AF009975	98
Other	5	1	1	1	0	6	7	Not sequenced		
Total	39	45	37	41	44	47	37			

The identity (provided by the depositor) for the best sequence match is reported, along with the corresponding accession number and the percent identity (% ID) as compared to the Lake Erie sequence.
[a]Two individual clones with the same OTU gave the same results; the lowest % ID for the two is reported.

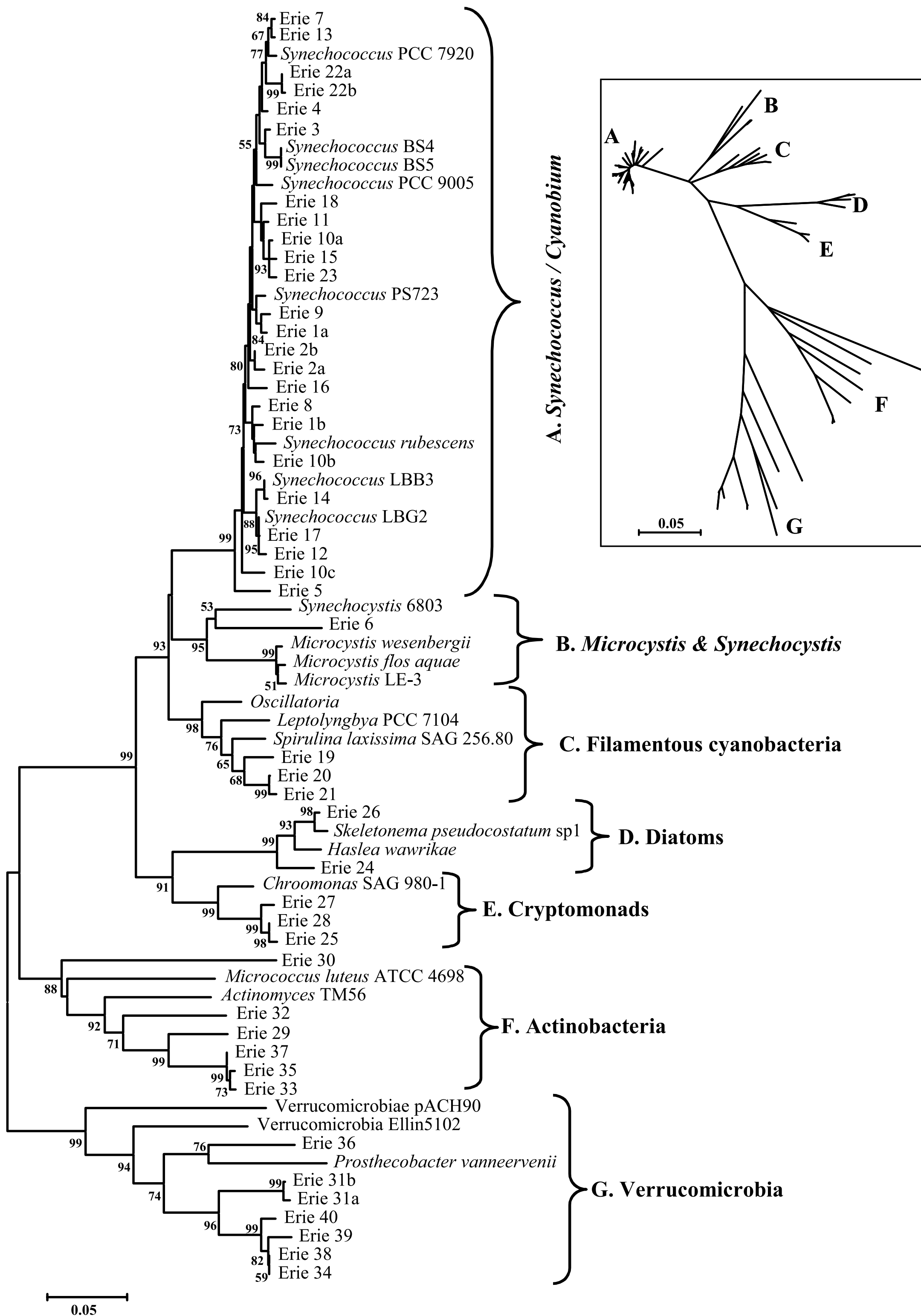

Figure 3. Neighbor-joining phylogenetic tree for Lake Erie 16S rRNA genes and sequences obtained from GenBank. A–C, cyanobacteria; D + E, eukaryotic algae; F + G, heterotrophic bacteria. Inset depicts the same tree, but displayed in radiation format.

OTUs (72%), thereby representing 269 clones (92%). The occurrences of the OTUs and the resultant best nucleotide sequence match from the BLAST analyses were compiled (Table 3). Sequences from the clone library, BLAST results, and other cultivated organisms were included in a sequence alignment, and a neighbor-joining tree was constructed (Fig. 3). Phylogenetic analysis (Table 3 and Fig. 3) reveals that 86% of the sequenced OTUs represent cyanobacteria (Figs. 3A–C), 3% are from plastids (Figs. 3D, E), and the remaining 11% are from heterotrophic bacteria (Figs. 3F, G). Thus, whereas the CYA primers target 16S rRNA genes of cyanobacteria, a small percentage of chloroplast and heterotroph sequences are in the clone library, allowing a partial characterization of the microbial community aside from the cyanobacterial members. None of the sequences were identified as chimeras. Comparable results from the BLAST analysis and the phylogenetic tree were obtained, although the tree was constructed from a smaller number of nucleotides (because of masking).

The BLAST results (Table 3) were dominated by sequences from the *Synechococcus*/*Cyanobium* cyanobacterial cluster (which is a diverse, cosmopolitan, polyphyletic group) and included sequences related to phycocyanin- and phycoerythrin-rich isolates. The shallowness of the *Synechococcus*/*Cyanobium* cluster (Fig. 3) reflects the high sequence identity for members of this group.

Discussion

This study presents molecular data that identify the geographical distribution of *Microcystis* and members of the oxygenic photosynthetic community in Lake Erie. PCR-based approaches are gaining popularity for use in the detection of toxic algae and have been used extensively in marine and freshwater systems to assess microbial diversity. This is the first report that we know of that employs PCR detection methods for toxic *Microcystis* in the Laurentian Great Lakes and only the third report that we know of within North America [37, 38]. Furthermore, the partial characterization of microbial community structure by 16S rRNA gene clone libraries is also the first that we know of for Lake Erie. The results of this study are discussed below in consideration of the re-occurring toxic cyanobacterial blooms as well as the importance of picoplankton in this Laurentian Great Lake.

Influential Factors on Successful Amplification. Initially, we assayed aliquots of 4 and 40 ng of genomic DNA from environmental samples (data not shown). In many samples, amplification was not detected for any of the genes using the 40ng samples, and *Microcystis*-specific genes (*mcy*B, *mcy*D, and MIC) were not detected in many of the 4ng samples. The subsequent addition of BSA [20] in the PCRs resulted in the amplification of cyanobacterial 16S rRNA gene (CYA, a positive control) in all of the samples and *Microcystis*-specific genes in some. The BSA presumably aids by binding PCR inhibitors in the samples. However, the generation of some *Microcystis*-specific amplicons was not reproducible even when 40 ng of genomic DNA was used.

Subsequently, we found that 80 ng of DNA per reaction increased the reproducibility of amplification and gene detection from all basins (Figs. 2C, F). If the positive control for the sample (CYA) does not yield an amplicon when 80 ng is employed, then analyzing less DNA may be necessary. It is important to keep in mind that dilution of genomic DNA to decrease the concentration of PCR inhibitors also decreases the number of target genes. As is evident, numerous samples containing 8 ng of genomic DNA did not yield *Microcystis*-specific amplicons (Figs. 2B, E), whereas amplicons were obtained when 80 ng of genomic DNA was used (Figs. 2C, F). As with any PCR-based approach, the accumulation of gene sequences in the literature and databases will help in the refinement and design of primer sequences. Excellent progress identifying toxin gene sequence diversity has been made [23]. However, the specificities of current primer sets based on known sequences should be considered limiting factors. Therefore, utilizing two primer sets (*mcy*D and *mcy*B) for the assessment of potential toxicity is recommended as are anticipated (and ongoing) refinements of these techniques.

Mapping Potentially Toxigenic Microcystis spp. in Lake Erie. The detection of toxic *Microcystis* is a requisite to the understanding of the dynamics of *Microcystis* and toxin production in the Lake Erie ecosystem. Whereas analysis and identification by microscopy are indispensable, alternate detection methods are required that can detect very small cell densities as well as work when *Microcystis* is a small percentage of the entire community. The polymerase chain reaction is employed because of the exponential amplification of targets by this detection method, as well as the potentially high specificity. These methods allow the mapping of toxic and nontoxic *Microcystis* (Table 1 and Fig. 2).

Microscopic/morphologically based descriptions of abundance and identification of phytoplankton members within Lake Erie give considerable insight into the phytoplankton communities in Lake Erie [17, 27, 29]. One long-term study (1969–1978) of phytoplankton diversity and biomass in Long Point Bay reports the presence of *Microcystis* [17]. Our molecular data for 2002 also detect *Microcystis* in this bay. For the period 1983–1987, *Microcystis* was microscopically identified in the western and central basins but was not found in the eastern basin [27]. In part, this illustrates the limitations of microscopy

and the increased sensitivity provided by molecular tools [28]. Indeed, we have detected toxigenic *Microcystis* in the eastern basin of Lake Erie at station 23 from more recent years (Fig. 2), although there is no doubt that the detection of *Microcystis* at pelagic sites (23, 84, and 357) is more sporadic. Taking the data as a whole, the amplicons produced from *Microcystis* 16S rDNA, *mcy*B, and *mcy*D genes in Lake Erie's three basins indicate that toxigenic *Microcystis* is spatially and temporally widespread.

Verification of mcyB, mcyD, and MIC Amplicons from Lake Erie. None of the sequences retrieved from the cyanobacterial 16S rDNA clone libraries are predicted to have come from *Microcystis* (Table 3 and Fig. 3). However, the multiplex data (Fig. 2) yield positive results for the genes encoding *Microcystis* 16S rRNA, *mcy*B, and *mcy*D from the majority of the samples. Sequencing of MIC, *mcy*B, and *mcy*D amplicons from several samples confirmed the identity of these genes. Therefore, toxic *Microcystis* are indeed found in these samples, and the absence of these sequences in our clone library analysis may reflect the low abundance of *Microcystis* in the samples relative to other potential targets or the bias of the techniques utilized for the cloning (see below). This illustrates limitations of clone libraries, and that the absence of a sequence does not imply the absence of a particular organism.

Lake Erie Clone Library/Phylogenetic Analysis. PCR amplification of cyanobacterial 16S rRNA genes and subsequent cloning, sequencing, and phylogenetic analysis of the amplicons were undertaken to identify members of the cyanobacterial community associated with *Micro-cystis*. As expected, we also obtained some sequences predicted to originate from plastids. Furthermore, the clone library also contains a number of sequences that appear to be most closely related to heterotrophic bacteria.

Synechococcus- and *Cyanobium*-like sequences dominate the Lake Erie 16S rDNA clone library (227 of 291 clones) for the seven stations analyzed (Table 3). In contrast, a Lake Erie phytoplankton study 20 years ago (1983–1987) reports neither *Synechococcus* nor *Cyanobium*. Furthermore, a long-term study (1969–1978) of phytoplankton diversity and biomass in Long Point Bay does not report the presence of *Synechococcus* [17]. Over 90% of our 16S rRNA gene sequences from Long Point Bay (2002) are ascribed to *Synechococcus* (Fig. 3 and Table 3). The fact that *Synechococcus* sequences dominate the Lake Erie 2002 clone libraries presented here but are absent from reports spanning 1969–1978 and 1983–1987 warrants more attention. It remains possible that the small size and relatively recent discovery [50] of *Synechococcus* as a ubiquitous component of aquatic environments may have led to their omission from previous studies.

Synechococcus and *Cyanobium* frequently dominate within the picoplankton size class of oligotrophic freshwater lakes [6, 40]. Historical shifts in phytoplankton community structure have been reported for Lake Erie, and in the 1990s, the picoplankton played a significant role in primary productivity [30]. During 1986–1988, an average of 10% of the total primary productivity in Lakes Michigan and Huron were attributed to *Synechococcus*, with up to 50,000 cells mL detected [13]. In July 2002 (during the same cruise that samples for the clone libraries presented here were obtained), the picoplankton size class (0.2–2.0 μM) contributed 33, 19, and 24% of the total chlorophyll *a* in the eastern, central, and western basins of Lake Erie, respectively (excluding data from bays and rivers) [10]. The picoplankton contribution of chlorophyll *a* for stations specifically represented by the 2002 clone libraries (Table 3) are 68, 43, 37, 16, 61, and 16% for stations 23, Long Point Bay, 589, 84, 357, and Sandusky Bay, respectively [10]. Thus, the picoplankton contributed significantly to the phytoplankton community in these samples, and the clone library results indicate that *Synechococcus* was prevalent. In 2001, 4, 57, and 48% of the total chlorophyll *a* at stations 23, 84, and 357 was produced by picoplankton [51].

Whether the current populations of *Synechococcus*/*Cyanobium* in Lake Erie reflect a recent phytoplankton community shift remains to be seen, but because Lake Erie has shifted from eutrophic to oligo-mesotrophic, the dominance of these gene sequences in the clone libraries is not surprising. Furthermore, changes in the N/P ratio and selective feeding by zebra mussels are probably influencing the phytoplankton composition of Lake Erie. We have demonstrated here that PCR can be used to characterize members of the community and, as such, has the potential of assessing any such shift in archived samples as long as the stored DNA is intact.

Caveats [52] regarding the clone libraries include potential bias in DNA extraction, PCR, and/or cloning steps that might favor the ultimate dominance of particular sequences (e.g., the *Synechococcus*-like sequences in this study). We stress that clone library results are not quantitative. However, we do favor the notion that *Synechococcus* comprised a significant fraction of the cyanobacterial community in the water samples because (1) the picoplankton contributed a significant fraction of the total chlorophyll *a* (see above); (2) two very different DNA extraction methods yielded the same results in DNA yield (by quantification) and diversity of 16S rRNA genes (assessed by T-RFLP); (3) the primers utilized have amplified 16S rRNA genes that are closely related to numerous cyanobacteria, chloroplasts, and heterotrophic bacteria (unpublished data) [38]; and (4) the cloning

ligation relies on A-overhangs added to the 3′ ends of the PCR amplicons (essentially the 5′ ends of the primers) and, as such, is independent of the target DNA sequences. Furthermore, having used different cyanobacterial-specific primers, three of the five sequences reported from a Lake Michigan 16S rRNA clone library grouped with *Synechococcus* [25].

The Sandusky Bay clone library was the only library to reveal sequences that correspond to filamentous cyanobacteria (Oscillatoriales: *Spirulina* and *Oscillatoria*). Furthermore, this is the only station that was not dominated by *Synechococcus*/*Cyanobium* sequences (because of a significant abundance of amplicons that appear most closely related to heterotrophic bacteria). In some respects, it is not surprising that the DNA library from Sandusky provided markedly different results than the libraries from other stations. In their comparative study of Lake Erie and Sandusky Bay, Garono *et al.* [14] concluded that the phytoplankton communities were different, primarily because of spatial and seasonal variations in cyanophytes and diatom abundance. Given the extreme concentrations of bacteria that can be found in this bay [10], it is not surprising that some heterotrophic bacterial sequences were found.

Another interesting result from this study is the significant abundance of sequences associated with phycoerythrin-rich *Synechococcus* spp. Whereas phycocyanin-rich *Synechococcus* are frequently identified in freshwater systems, the presence of phycoerythrin-rich isolates in North American Great Lakes (and their availability as clonal cultures for laboratory studies) is much rarer. As for the *Microcystis* populations, the results of this study demonstrate our previous lack of knowledge concerning the ubiquitous presence of many of these cyanobacteria throughout Lake Erie. We realize that a comprehensive cyanobacterial community analysis based on gene sequences would entail more sampling (more clones) and more nucleotides; however, the data as presented here constitute an important first step for Lake Erie.

The rapid detection of *Microcystis*, toxic and not, is important for water managers, as well as for those interested in the geographical distribution of these organisms. Microscopic detection of *Microcystis* cannot discern between potentially toxigenic and nontoxic cells. Previous studies have identified *Microcystis* in the western and central basins of Lake Erie, but not in the eastern basin. Here we report the PCR-based detection of toxic *Microcystis* throughout Lake Erie, including the eastern basin. *Synechococcus*- and *Cyanobium*-like sequences dominate the clone libraries of 16S rRNA genes generated from Lake Erie, with sequences very similar to members of the Oscillatoriales and to plastids from diatoms and cryptomonads also present. Sequences most closely associated with Actinobacteria and Verricumicrobia were also observed. Therefore, whereas the *Vibrio* isolate functioned as a negative control for the PCR methods (Table 2), the cyanobacterial primers do amplify 16S rRNA genes from other bacteria. This is the first study of its kind for the Great Lakes. We suggest that this approach may be valuable in the future, particularly in combination with quantitative estimates of the abundance of toxin genes and concentrations of toxin, and can be used to determine correlations between water column chemistry, cyanobacterial community structure, and the presence of toxic *Microcystis*.

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References

1. Altschul, S, Gish, W, Miller, W, Myers, EW, Lipman, D (1990) Basic local alignment search tool. J Mol Biol 215: 403–410
2. Arnott, DL, Vanni, MJ (1996) Nitrogen and phosphorus cycling by the zebra mussel (*Dreissena polymorpha*) in the western basin of Lake Erie. Can J Fish Aquat Sci 53: 646–659
3. Baker, JA, Neilan, BA, Entsch, B, McKay, DB (2001) Identification of cyanobacteria and their toxigenicity in environmental samples by rapid molecular analysis. Environ Toxicol 16(6): 472–482
4. Bittencourt-Oliveira, MDC (2003) Detection of potential microcystin-producing cyanobacteria in Brazilian reservoirs with a mcyB molecular marker. Harmful Algae 2: 51–56
5. Brittain, SM, Wang, J, Babcock-Jackson, L, Carmichael, WW, Rinehart, KL, Culver, DA (2000) Isolation and characterization of microcystins, cyclic heptapeptide hepatotoxins from a Lake Erie strain of *Microcystis aeruginosa*. J Great Lakes Res 26(3): 241–249
6. Callieri, C, Stockner, JG (2002) Freshwater autotrophic picoplankton: a review. J Limnol 61(1): 1–14
7. Chorus, I, Bartram J (Eds.) (1999) Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring, and Management. E & FN Spon, London p. 416
8. Christiansen, G, Fastner, J, Ernhard, M, Börner, T, Dittmann, E (2003) Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. J Bacteriol 182(2): 564–572
9. Cole, JR, Chai, B, Marsh, TL, Farris, RJ, Wang, Q, Kulam, SA, Chandra, S, McGarrell, DM, Schmidt, TM, Garrity, GM, et. al. (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31(1): 442–443
10. DeBruyn, JM, Leigh-Bell, JA, McKay, RM, Bourbonniere, RA, Wilhelm, SW (2004) Microbial distributions and the impact of phosphorus on bacterial activity in Lake Erie. J Great Lakes Res 30(1): 166–183

11. Dittman, E, Börner, T (2005) Genetic contributions to the risk assessment of microcystin in the environment. Toxicol Appl Pharmacol 3: 192–200
12. Dolan, DM (1993) Point source loadings of phosphorus to Lake Erie: 1986–1990. J Great Lakes Res 19(2): 212–223
13. Fahnenstiel, GL, Carrick, HJ, Iturriaga, R (1991) Physiological characteristics and food-web dynamics of *Synechococcus* in Lakes Huron and Michigan. Limnol Oceanogr 36(2): 219–234
14. Garano, RJ, Heath, RT, Hwang, SJ (1996) Detrended correspondence analysis of phytoplankton abundance and distribution in Sandusky Bay and Lake Erie. J Great Lakes Res 22(4): 818–829
15. Hall, TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98
16. Hisbergues, M, Christiansen, G, Rouhiainen, L, Sivonen, K, Borner, T (2003) PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. Arch Microbiol 180: 402–410
17. Hopkins, GJ, Lea, C (1982) A ten-year study of phytoplankton biomass and composition in the Nanticoke region of Long Point Bay, Lake Erie. J Great Lakes Res 8(3): 428–438
18. Huber, T, Faulkner, G, Hugenholtz, P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20(14): 2317–2319
19. Kaebernick, M, Neilan, BA, Börner, T, Dittmann, E (2000) Light and the transcriptional response of the microcystin biosynthesis gene cluster. Appl Environ Microbiol 66(8): 3387–3392
20. Kirchman, DL, Yu, L, Fuchs, BM, Amann, R (2000) Structure of bacterial communities in aquatic systems as revealed by filter PCR. Aquat Microb Ecol 26: 13–22
21. Kumar, S, Tamura, K, Nei, M (1994) MEGA: molecular evolutionary genetics analysis software for microcomputers. Comput Appl Biosci 10: 189–191
22. Kurmayer, R, Christiansen, G, Chorus, I (2003) The abundance of microcystin producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. Appl Environ Microbiol 69: 787–795
23. Kurmayer, R, Dittmann, E, Fastner, J, Chorus, I (2002) Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). Microb Ecol 43: 107–118
24. Leach, JH (1993) Impacts of the zebra mussel (*Dreissena polymorpha*) on water quality and fish spawning reefs in western Lake Erie. In: Nalepa, TF, Schloesser, DW (Eds.) Zebra Mussels: Biology, Impacts and Control. Lewis Publishers/CRC Press, Boca Raton, pp 381–397
25. MacGregor, BJ, Van Mooy, B, Baker, BJ, Mellon, M, Moisander, HW, Paerl, HW, Zehr, J, Hollander, D, Stahl, DA (2001) Microbiological, molecular biological and stable isotopic evidence for nitrogen fixation in the open waters of Lake Michigan. Environ Microbiol 3(3): 205–219
26. Makarawicz, JC, Bertram, P (1991) Evidence for the restoration of Lake Erie. Bioscience 41(4): 216–223
27. Makarewicz, JC (1993) Phytoplankton biomass and species composition in Lake Erie, 1970 to 1987. J Great Lakes Res 19(2): 258–274
28. Makarewicz, JC, Bertram, P, Lewis, TW (2000) Chemistry of the offshore surface waters of Lake Erie: pre- and post-*Dreissena* introduction (1983–1993). J Great Lakes Res 26: 82–93
29. Makarewicz, JC, Lewis, TW, Bertram, P (1999) Phytoplankton composition and biomass in the offshore waters of Lake Erie: pre- and post-*Dreissena* introduction (1983–1993). J Great Lakes Res 25(1): 135–148
30. Munawar, M, Munawar, IF, Dermott, R, Niblock, H, Carou, S (2002) Is Lake Erie a resilient ecosystem? Aquat Ecosyst Health Manag 5(1): 79–93
31. Murphy, TP, Irvine, K, Guo, J, Davies, J, Murkin, H, Charlton, M, Watson, SB (2003) New microcystin concerns in the lower Great Lakes. Water Qual Res J Can 38(1): 127–140
32. Neilan, BA, Dittman, E, Rouhiainen, L, Bass, RA, Schaub, V, Sivonen, K, Börner, T (1999) Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. J Bacteriol 181(13): 4089–4097
33. Neilan, BA, Jacobs, D, DelDot, T, Blackall, LL, Hawkins, PR, Cox, PT, Goodman, PT (1997) rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. Int J Syst Bacteriol 47(3): 693–697
34. Nichols, KH, Hopkins, GJ (1993) Recent changes in Lake Erie (north shore) phytoplankton: cumulative impacts of phosphorus loading reductions and the zebra mussel introduction. J Great Lakes Res 19: 637–647
35. Nishizawa, T, Asayama, M, Fujii, K, Harada, K, Shirai, M (1999) Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. J Biochem (Tokyo) 126(3): 520–529
36. Nishizawa, T, Ueda, A, Asayama, M, Fujii, K, Harada, K-I, Ochi, M, Shirai, M (2000) Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. J Biochem 127: 779–789
37. Nonneman, D, Zimba, PV (2002) A PCR-based test to assess the potential for microcystin occurrence in channel catfish production ponds. J Phycol 38: 230–233
38. Ouellette, AJA, Wilhelm, SW (2003) Toxic cyanobacteria: the evolving molecular toolbox. Front Ecol Environ 1: 359–366
39. Pan, H, Song, L, Liu, Y, Börner, T (2002) Detection of hepatotoxic *Microcystis* strains by PCR with intact cells from both culture and environmental samples. Arch Microbiol 178: 421–427
40. Postius, C, Ernst, A (1999) Mechanisms of dominance: coexistence of picocyanobacterial genotypes in a freshwater ecosystem. Arch Microbiol 172: 69–75
41. Reynolds, CS (1996) Foreward. In: Munawar, M, Munawar, IF (Eds.) Phytoplankton dynamics in North American Great Lakes. Volume 1, Ecovision World Monograph Series. SPB Academic Publishing. Amsterdam, pp xvii–xix
42. Sambrook, J, Russel, DW (2001) Molecular Cloning: A Laboratory Manual. Argentine J (Eds.) Cold Spring Harbor Laboratory Press. New York
43. Schatz, D, Eshkol, R, Kaplan, A, Hadas, O, Sukenik, A (2000) Molecular monitoring of toxic cyanobacteria. Adv Limnol 55: 45–54
44. Tillett, D, Dittmann, E, Erhard, M, von Dohren, H, Börner, T, Neilan, BA (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide–polyketide synthetase system. Chem Biol 7(10): 753–764
45. Tillett, D, Neilan, BA (2000) Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. J Phycol 36(1): 251–258
46. Tillett, D, Parker, DL, Neilan, BA (2001) Detection of toxigenicity by a probe for the microcystin synthetase A gene (mcyA) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (Phycocyanin Intergenic Spacer) phylogenies. Appl Environ Microbiol 67(6): 2810–2818
47. Urbach, E, Vergin, KL, Young, L, Morse, A, Larson, GL, Giovannoni, SJ (2001) Unusual bacterioplankton community structure in the ultra-oligotrophic Crater Lake. Limnol Oceanogr 46(3): 5557–5572
48. Vanderploeg, HA, Liebig, JR, Carmichael, WW, Agy, MA, Johengen, TH, Fahnenstiel, GL, Nalepa, TF (2001) Zebra mussel (*Dreissena polymorpha*) selective filtration promoted toxic *Microcystis* blooms in Saginaw Bay (Lake Huron) and Lake Erie. Can J Fish Aquat Sci 58: 1208–1221

49. Via-Ordorika, L, Fastner, J, Kurmayer, R, Hisbergues, M, Dittman, J, Komarek, J, Erhard, M, Chorus, I (2004) Distribution of *Microcystin*-producing and non-microcystin-producing *Microcystis* sp. in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies. Syst Appl Microbiol 27: 592–602
50. Waterbury, JB, Watson, SW, Guillard, RRL, Brand, LE (1979) Widespread occurrence of a unicellular, marine, planktonic cyanobacterium. Nature 277: 293–294
51. Wilhelm, SW, DeBruyn, JM, Gillor, O, Twiss, MR, Livingston, K, Bourbonniere, RA, Pickell, L, Trick, CG, Dean, AL, McKay, RML (2003) Effect of phosphorus loading on present day plankton communities in pelagic Lake Erie. Aquat Microb Ecol 32: 275–285
52. Wintzingerode, Fv, Gobel, UB, Stackebrandt, E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21: 213–229