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Quantification of Toxic *Microcystis* spp. during the 2003 and 2004 Blooms in Western Lake Erie using Quantitative Real-Time PCR

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In August of 2003 and August of 2004, blooms of potentially toxic cyanobacteria *Microcystis* spp. persisted in western Lake Erie. Samples collected from the bloom were analyzed for the cyanobacterial toxin microcystin and the presence of *Microcystis* spp. cells. Estimates of microcystin toxicity exceeding 1 μg L^{-1} (microcystin−LR activity equivalents), the safety limit set by the World Health Organization, were found from the samples in both 2003 and 2004. The presence of *Microcystis* spp. in water samples was confirmed through standard polymerase chain reaction (PCR) using a combination of four primer sets. Quantification of *Microcystis* was accomplished by a real-time PCR assay utilizing specific primer-Taq-man probe sets targeted on a conserved, *Microcystis*-specific 16S rDNA fragment and a microcystin toxin synthetase gene *mcy*D. This approach allowed us to specifically study the distribution and abundance of toxic *Microcystis* in the lake in contrast to previous studies that have assessed *Microcystis* populations with less refined methods. On the basis of quantification by quantitative real-time PCR analysis, the total abundance of *Microcystis* cells in the bloom area varied from 4×10^8 to 2×10^3 cells L^{-1}. The results of this study provide novel insight regarding the distribution and abundance of *Microcystis* spp. in the western basin of Lake Erie, a region plagued in recent years by large-scale (>20 km^2) blooms. Our results suggest that the Maumee River and Bay may serve as a source for *Microcystis* to western and central Lake Erie.

Introduction

Recurring blooms of toxic cyanobacteria in the western basin of Lake Erie have been a nuisance during the past decade. *Microcystis* spp. has been commonly found in samples collected from the bloom areas during the summer months since 1996 (*1*). *Microcystis* blooms have often been associated with varying concentrations of the cyanotoxin microcystin in the surrounding water. Observations in the regions suggest that it is common for microcystin concentrations to exceed the provisional guideline concentration of 1.0 μg L^{-1} set by the World Health Organization (*2*) in the western basin of Lake Erie. Our goal in this study was to utilize quantitative PCR (qPCR) for a single-step detection and quantification of target genes for assessment of the total abundance of cyanobacteria, *Microcystis*, and potentially toxic *Microcystis* in natural water samples. Use of this method improves the resolution at which mixed populations of toxigenic and nontoxic *Microcystis* can be analyzed. Use of qPCR also expedites the analysis of bloom samples, reducing the number of time-consuming steps involved in the analysis of samples by microscopy and chemical toxin assays.

Microcystins are nonribosomally synthesized cyclic heptapeptides produced by *Microcystis* spp., as well as other species of cyanobacteria belonging to the genera *Anabaena*, *Nostoc*, and *Oscillatoria* (*3*, *4*). The large nonribosomal peptide synthetase gene cluster involved in microcystin synthesis (*mcy* A-J) has been identified and sequenced, and its involvement in microcystin synthesis has been confirmed in mutagenesis studies (*5*). The peptide synthetase gene cluster has been shown to be present in toxin-producing and potentially toxic *Microcystis* spp. (referred to as toxic and potentially toxic cells, respectively) (*5*, *12*).

Microcystins are among the most cosmopolitan cyanobacterial toxins to be found in lakes and in brackish water (*3*). Blooms of toxin producing *Microcystis* cause severe aesthetic water quality problems (*6*) and pose a health risk to humans and animals upon ingestion of contaminated water (*6*−*9*). Trophic transfer of microcystins in the food web (from phytoplankton to planktivorous fish) has also been hypothesized (*1*, *8*). The onset of these cyanobacterial blooms may be influenced by numerous factors, including nutrient availability and environmental factors that affect population size and dispersal (*3*, *6*). In field studies, the production of microcystin has been shown to be correlated to concentrations of carbon, dissolved phosphorus, pH, nitrate, chlorophyll *a*, and light conditions (*10*, *11*). However, the combination of environmental conditions responsible for inducing a toxin-producing bloom remains unknown in the Lake Erie ecosystem as well as in other freshwater systems where toxic *Microcystis* blooms occur.

In the past, detection of *Microcystis* in water samples has commonly been based on microscopic techniques combined with the chemical detection of microcystin in the water samples (*1*). However, discerning the ability to produce toxins among cyanobacterial strains solely based on cellular morphology is difficult or even impossible (*12*, *13*). PCR-based techniques allow for the detection of specific DNA sequences, which can make a distinction between toxic and nontoxic strains of *Microcystis* spp. (*4*). This approach subsequently facilitates an analysis of the distribution of genotypes based on the presence or absence of a combination of target genes in the samples (*13*). Detection and quantification of cyanobacteria and the cells belonging to the genus *Microcystis* are possible based on the design of PCR primers targeted on the regions of conserved 16S rDNA sequences among these groups (*11*, *12*, *13*). In the current study, an initial screening of samples was completed by multiplex PCR using a combination of previously published primer sets (Table 1) (*13*). A quantitative real-time PCR technique, the Taq nuclease assay (also known as the 5′ nuclease assay),

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TABLE 1. PCR Primer Sets and Taq Nuclease Assay Probes (Taq) Used in This Study

primer	sequence (5′-3′)	ref
*mcy*B 2959F	TGGGAAGATGTTCTTCAGGTATCCAA	9
*mcy*B 3278R	AGAGTGGAAACAATATGATAAGCTAC	9
*mcy*D F2	GGTTCGCCTGGTCAAAGTAA	11
*mcy*D R2	CCTCGCTAAAGAAGGGTTGA	11
*mcy*D F2 (Taq)	FAM[a]-ATGCTCTAATGCAGCAACGGCAAA-BHQ-1[b]	this paper
MICR 184F	GCCGCRAGGTGAAAMCTAA	32
MICR 431R	AATCCAAARACCTTCCTCCC	32
MICR 228F (Taq)	FAM[a]-AAGAGCTTGCGTCTGATTAGCTAGT-BHQ-1[b]	this paper
CYAN108F (PLG1.3)[c]	ACGGGTGAGTAACRCGTRA	33
16SCYR[a]	CTTCAYGYAGGCGAGTTGCAGC	modified from Urbach et al. (*33*)
CYAN 108F (PLG1.3)[d]	ACGGGTGAGTAACRCGTRA	33
CYAN 377R[b]	CCATGGCGGAAAATTCCCC	34
CYAN 328R (Taq)	FAM[a]-CTCAGTCCCAGTGTGGCTGNTC-BHQ-1[b]	this paper

[a] 6-Carboxyfluorescein. [b] Black Hole Quencher-1 (quenching range 480−580 nm) (Biosearch Technologies, Inc., Novato, CA). [c] CYAN 108F and 16S CYR are used in conventional PCR assay. [d] CYAN 108F and CYAN 377R are used in Taq nuclease assay.

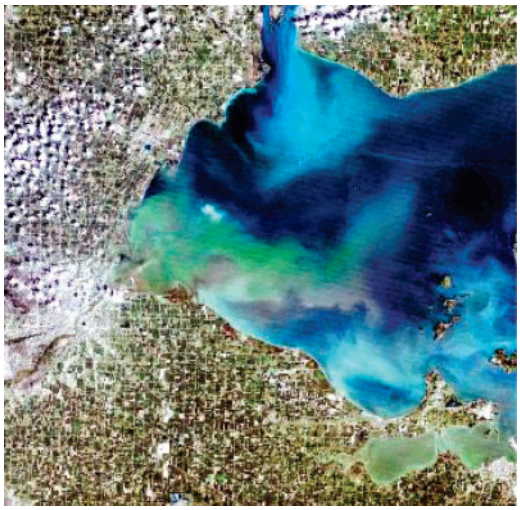

FIGURE 1. LANDSAT 7 image taken August 18, 2003. The true color composite image demonstrates the presence of a significant bloom of phytoplankton in the surface waters (image from LANDSAT 7 server courtesy of OhioView).

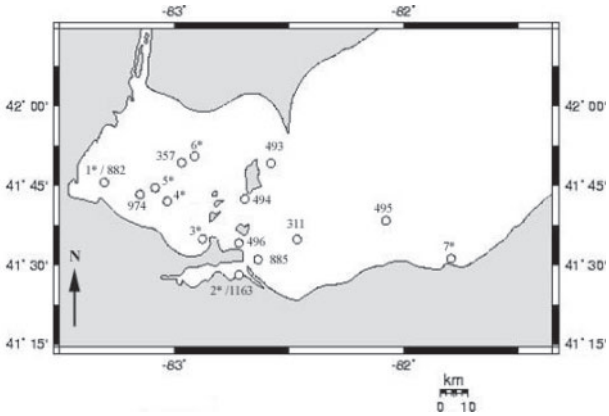

FIGURE 2. Sampling sites in 2003 are indicated by numbers 1−7 and an asterisk. Stations were renumbered consecutively because different groups collecting samples used different coding systems for the sampling sites. The sampling sites used in August 2004 are numbered according to the Environment Canada station coding system consisting of 3- and 4-digit station numbers as indicated on the map. Sandusky Bay, area of sampling station 1163 and mouth of Maumee River, area of sampling station 882.

was used to quantify cells carrying specific target genes in our samples. The Taq nuclease assay has been recently applied successfully to the analysis of microbial components of natural water samples (*14*−*18*).

During our initial screening, the distribution of cyanobacteria was studied using a cyanobacterial-specific 16S ribosomal RNA gene fragment as a target. At the same time, the occurrence of toxigenic *Microcystis* spp. was studied through the detection of a *Microcystis*-specific 16S rRNA gene fragment and toxin synthetase genes *mcy*B and *mcy*D in a multiplex PCR assay (*13*). Subsequently, Taq nuclease assays were employed for the quantification of cyanobacterial and *Microcystis* 16S rDNA and *mcy*D gene copies and the total abundance of cells carrying these target genes in all samples. In all cases, we were able to estimate *Microcystis* abundance through the use of standard model organisms (*Microcystis aeruginosa* LE-3).

In this study, we report observations that were concurrent with two toxic cyanobacterial blooms that occurred in the western basin of Lake Erie in late summer of 2003 and late summer of 2004. The greenish biomass of a large algal bloom in summer 2003 at the mouth of Maumee River was visible in true-color LANDSAT images of this area (Figure 1). The aim of this study was to provide further information about the spatial distribution and abundance of *Microcystis* in the western basin of Lake Erie. The results of this study provide more detailed information about the structure of the cyanobacterial community on Lake Erie than previous studies, while also raising questions about the presence of other microcystin-producing cyanobacteria in the western basin of Lake Erie.

Materials and Methods

Sample Collection. Because of logistical constraints, water samples were collected by three independent groups who were concurrently working in Lake Erie at different locations in the western basin of the Lake on August 15, 2003. Researchers on the R/V *Lake Guardian* (U.S. EPA), C.C.G.S. *Limnos* (Canada Centre for Inland Waters), and several research support crafts of the Lake Erie Center (Toledo, OH) all collected samples and observations. In addition, samples were collected during field work in August 2004 onboard the C.C.G.S. *Limnos*. All sampling sites in 2003 and 2004 are indicated in the map in Figure 2. In all cases, water samples were collected from a 1 m depth using a surface water pump (C.C.G.S. *Limnos*) or Niskin bottles (R/V *Lake Guardian*). Cells used to extract DNA for PCR analysis were collected by filtering onto 47 mm diameter, 0.2 μm nominal pore-size polycarbonate membrane filters (Millipore), which were immediately frozen (−20 °C) until processing.

Phytoplankton Biomass. Chlorophyll *a* (a proxy for phytoplankton biomass) was collected on 0.2 μm nominal pore-size polycarbonate filters (47 mm diameter, Millipore)

and quantified after extraction (ca. 24 h, 4 °C) in 90% acetone. Chlorophyll *a* retained on the filters was quantified with either an AU-10 or a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using the nonacidification protocol (*19*).

Microcystin Concentration. Cyanotoxin activity in water samples was determined with the protein phosphatase inhibition assays (PPIA) normalized to microcystin−LR standards. Samples were collected on GF/F (Whatman) filters. The PPIA assays were run in 96-well plates containing 0.1 mU enzyme (recombinant protein phosphatase 1A, catalytic subunit, Roche Applied Science), 1.05 mg of para-nitrophenyl phosphate (Sigma), and 10 μL of sample or microcystin−LR (Sigma Biochemical) using the method of Carmichael and An (*20*). The rate of phosphate hydrolysis was calculated from the change in absorbance at 405 nm over 1 h and compared to the control (no added microcystin−LR) and standards containing between 6 and 40 μg L^{-1} microcystin−LR. All toxin results as such are expressed as microcystin−LR equivalents. Blanks (no enzyme, no toxin), unknowns, standards, and controls were all run in duplicate. The sensitivity of the assay was dependent on the volume of water (1−20 L) filtered by the individual sampling groups.

Extraction of DNA from Natural Samples. High molecular weight nucleic acids were isolated using a modification of the protocol of Giovannoni et al. (*21*). Cells collected onto filters were suspended from the filter in lysis buffer (40 mM EDTA, 400 mM NaCl, 50 mM Tris-hydrochloride, pH 9.0). Cells were disrupted by adding lysozyme to a final concentration of 1 mg mL^{-1} followed by incubation at 37 °C for 20 min. After incubation, proteinase K was added to a final concentration of 50 μg mL^{-1} and sodium dodecyl sulfate to a final concentration of 0.5%. The cell suspension was then incubated at 50 °C for 2 h. DNA was extracted by first adding a phenol/chloroform/isoamyl alcohol (25:24:1) volume equal to the aqueous phase, with a subsequent extraction of the aqueous phase using an equal volume of chloroform isoamyl alcohol (24:1). DNA was precipitated overnight (−20 °C) after the addition of absolute ethanol (2× aqueous volume) and 10 M ammonium acetate (0.1× the volume of the aqueous phase). DNA was collected the next day by centrifugation at 11 900*g* for 25 min (Beckman J2-21 centrifuge equipped with Fiber Lite F21B rotor; Piramoon Technologies, Santa Clara, CA). DNA pellets were air-dried and subsequently resuspended in sterile 1× TE buffer, pH 8. The concentration and purity of extracted DNA was measured spectrophotometrically (BioMate5, Thermo Spectronics) as previously described (*22*).

Multiplex PCR. The initial sample screening was carried out using a combination of the four primer sets described by Ouellette and Wilhelm (*13*). All reactions were performed in 50 μL volumes in 96-well plates (Eppendorf). For each sample, two separate PCR reactions were set up: one reaction to detect cyanobacteria using primers CYAN 108F and 16S CYR (Table 1) and a second multiplex reaction to detect *Microcystis*-specific 16S rDNA fragments and the microcystin toxin synthetase genes *mcy*B and *mcy*D using three primer sets (MICR 185F and MICR 431R, *mcy*B 2959F and *mcy*B 3278R, and *mcy*D F2 and *mcy*D R2 (Table 1)). All reactions contained 400 nM of each primer, 200 nM dNTPs, 1× Mg-free PCR buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 300 ng μL^{-1} (final concentration) bovine serum albumin (Sigma cat# A-7030 (*23*)), 0.04 U μL^{-1} (final concentration) Taq polymerase (Promega, Madison, WI), and 20−200 ng of DNA template. Bovine serum albumin was added into the reactions because it has been shown to enhance the sensitivity of the PCR-based detection of target genes in natural samples (*23*). The PCR protocol consisted of an initial denaturation step at 95 °C for 5 min, 50 cycles at 94 °C for 30 s, 56 °C for 60 s, 72 °C for 30 s, and a final single step at 72 °C for 15 min. Each PCR reaction was subjected to electrophoresis in 6% polyacrylamide gels. DNA bands were visualized under UV illumination after staining the gel with 0.01% SYBR green I (Molecular Probes, Eugene, OR) in TBE (90 mM Tris-borate, 1 mM EDTA, pH 8.0).

Real-Time Quantitative PCR. To provide quantitative information on cyanobacterial, specifically *Microcystis* spp., populations in Lake Erie, all samples were subjected to real-time PCR analysis to quantify gene copy numbers of cyanobacteria-specific 16S rRNA genes, *Microcystis*-specific 16S rRNA genes, and *mcy*D genes. These results were used to infer the abundance of cells carrying these target genes in the original samples.

Dual labeled probes CYAN 328R, MICR 228F, and *mcy*DF2 (Table 1) were designed to accompany each primer set in qPCR. Briefly, the probes were designed according to guidelines from Applied Biosystems (Foster City, CA) and from Bustin et al. (*24*). To confirm that probes will not form secondary structures, the probe sequences were checked using the mfold web server (*25*). The functionality and sensitivity of the probes were confirmed by assaying different pure cyanobacterial cultures in the laboratory (including *Synechococcus* spp., *Synechocystis* spp., *Anabaena* spp., *Planktothrix* sp., and 12 different *Microcystis* spp.) prior to analysis of natural samples (data not shown).

Amplifications and quantifications were performed using a BioRad iCycler equipped with a iQ real time fluorescence detection system and software, version 3.0 (Bio-Rad, Hercules, CA). Triplicate Taq nuclease assays were performed to quantify the gene copies for each sample. All reactions were carried out in a total volume of 25 μL. Three separate assays were performed to detect and quantify cyanobacterial 16S rDNA, *Microcystis* 16S rDNA, and *mcy*D in the samples. For cyanobacterial 16S and *Microcystis* 16S assays, each PCR reaction contained 10 μL of Eppendorf HotMasterMix (Brinkmann Instruments, Inc., Westbury, NY). For *mcy*D assays, each PCR reaction contained 12.5 μL of Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). In addition, all three assays contained 10 μM each primer (Sigma-Genosys, Inc., The Woodlands, TX), 10 μM Taq probe (Biosearch Technologies, Inc., Novato, CA), 300 ng μL^{-1} bovine serum albumin (Sigma cat# A-7030) (*20*), and 5 μL of undiluted or 10-fold diluted template DNA suspension. Each PCR reaction was run in triplicate on a 96-well plate (Bio-Rad, Hercules, CA) sealed with optical quality sealing tape (Bio-Rad, Hercules, CA). Two negative controls without DNA were included for each PCR run. The PCR program for cyanobacterial 16S rDNA and *Microcystis*-specific 16S rDNA primers consisted of 1.5 min at 95 °C, 55 cycles at 95 °C for 30 s, 56 °C for 1 min, and 65 °C for 20 s. The PCR program for the *mcy*D assay consisted of 3 min at 50 °C, 10 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 1 min at 61 °C, and 20 s at 72 °C. High cycle numbers were required so that the most dilute standards (discussed next), as well as samples with low concentrations of target DNA, could be quantified.

Threshold cycle (C_t) calculations were completed automatically for each real-time PCR assay by the iCycler software using the maximum correlation coefficient approach. In this approach, the threshold is automatically determined to obtain the highest possible correlation coefficient (r^2) for the standard curve (Table 2). Gene copies per sample were calculated using a standard curve (target gene copy number vs C_t) determined for each assay. The cell abundance was inferred from a standard curve (cell abundance vs C_t) determined in each assay.

Standards for Real-Time PCR: Preparation of Single Copy Plasmid Standard. A cyanobacterial 16S rDNA fragment was amplified by PCR using primers CYAN 108F and 16S CYR (Table 1) from *M. aeruginosa* LE-3 (*1*) as described previously. The DNA fragment was cloned into the PCR 2.1 vector using TOPO-TA cloning kit (Invitrogen, Carlsbad, CA)

TABLE 2. Efficiencies and Standard Curve Parameters of from Real-Time PCR Analysis for the Cyanobacterial, *Microcystis*, and *mcy*D Specific Primer Sets

target gene	standard	efficiency (%)	slope	*y*-intercept	r^2
Cyan 16S	plasmid DNA	92.1	−3.526	45.877	0.999
Cyan 16S	*M. aeruginosa* LE-3 genomic DNA	95.4	−3.437	37.628	0.997
Micr 16S	plasmid DNA	97.9	−3.373	42.815	0.997
Micr 16S	*M. aeruginosa* LE-3 genomic DNA	104.7	−3.213	35.037	0.999
*mcy*D	plasmid DNA	98.5	−3.359	41.138	0.997
*mcy*D	*M. aeruginosa* LE-3 genomic DNA	94.2	−3.470	36.864	0.998

following the manufacturer's instructions. Plasmid DNA was purified using the Wizard Plus Minipreps kit (Promega, Madison, WI) following the manufacturer's instructions. Inserts in the clones were confirmed by PCR using primers CYAN 108F and 16S CYR and subsequent electrophoresis. The DNA concentration (ABS_{260}) and purity (ABS_{260}/ABS_{280}) of the plasmid preparation were determined spectrophotometrically (BioMate5, Thermo Spectronics), as previously described (*22*). The molecular weight of the double-stranded plasmid and the double-stranded PCR product was determined using Biopolymer Calculator, version 4.4.1 (*26*). Using Avogadro's number, 6.022×10^{23} plasmid copies mol^{-1}, the plasmid copy number of the stock was determined. A plasmid standard, in which each plasmid contains one copy of the target gene, for the *mcy*D assays was prepared as described previously, but the PCR amplicon was obtained using *mcy*DF2 and *mcy*DR2 primers (Table 1). Dilutions containing $1 \times 10^6 - 5$ plasmid copies μL^{-1} were prepared to establish a linear standard curve for real-time PCR assays.

***M. aeruginosa* LE-3 Genomic DNA Standard.** *M. aeruginosa* LE-3 (*1*) was grown in batch cultures in BG11 medium (*27*) at 25 °C and ca. 80 μmol of photons m^{-2} s^{-1}. Cells from a known volume of the LE-3 culture were harvested onto a GF/F filter (Whatman), lysed, and DNA isolated as described previously. A subsample (2 mL) of the cell culture was obtained immediately before the cells were harvested to determine the cell density of the culture by direct counts. For microscopic enumeration of the *M. aeruginosa* LE-3 cultures, cells were harvested on a 0.22 μm nominal pore-size black polycarbonate membrane filter (Poretics). The filter was mounted on a glass slide (Fisher Scientific), a drop of immersion oil (Type FF) (R. G. Chargille Laboratories, Inc., Cedar Grove, NJ) was added on top of the filter, and the filter was covered with a glass cover slip. Autofluorescent cyanobacterial cells were enumerated under 1000× magnification with a *Leica DMRXA* epifluorescence microscope (excitation filter $\lambda = 530-595$ nm; dichroic mirror $\lambda = 600$ nm; barrier filter $\lambda = 615$ nm) equipped with an ocular grid. A minimum of 20 fields or 200 cells was counted from each sample. The cell abundance of the original cell culture was related to the total yield of extracted genomic DNA by dividing the total DNA yield (ng of DNA) by the total number of cells contained in the original volume of liquid culture. This gave us a way to relate how much DNA corresponded to one cell in the *Microcystis* LE-3 sample. We used 100-fold dilutions of the DNA sample to establish the genomic DNA standard curve (see next discussion).

Quantitative PCR—Detection Limits. Standard curves were established using four serial dilutions of standard plasmid DNA and genomic DNA isolated from *M. aeruginosa* LE-3 pure culture. For all real-time PCR assays, the dilutions of the plasmid standard ranged from 5×10^6 to 25 plasmid copies per reaction (DNA concentrations for the 16S standards ranged from 2.6×10^6 to 1.3 ng of plasmid DNA per reaction, and for *mcy*D, DNA concentrations of the plasmid standards ranged from 2.2×10^{-2} to 1.1×10^{-7} ng of plasmid DNA per reaction). Using these standards, the lower detection limit of our assay is 25 target gene copies per reaction, which corresponds roughly to 5000 gene copies per liter of lake water. Genomic DNA from *M. aeruginosa* LE-3 culture was serially diluted to correspond to cell densities from 1.31×10^6 to 1.31 LE-3 cells per reaction (corresponding DNA concentrations $308-3.1 \times 10^{-4}$ ng of genomic DNA per reaction). Thus, the results based on standardization using *M. aeruginosa* LE-3 are expressed in LE-3 equivalents. The lowest detection limit was 1.31 LE-3 equivalents per 5 μL of subsample, corresponding roughly to 262 LE-3 equivalents per liter of lake water.

TABLE 3. Chlorophyll *a* and Toxin Concentrations in Samples Collected in the Western Basin of Lake Erie in August 2003 (Sample Stations Numbered 1—7) and 2004 (Sampling Stations Numbered with 3 or 4 Digits)[a]

sampling station	chlorophyll *a* ($\mu g\ L^{-1}$)	microcystin (μg of microcystin LR equiv L^{-1})
1	40.0	15.4
2	5.2	<0.3
3	6.4	0.3
4	4.0	<0.3
5	15.3	0.4
6	26.0	1.8
7	6.5	<0.3
493	8.8	0.1
311	14.1	0.3
1163	20.1	2.6
885	19.1	0.1
496	21.7	0.4
495	15.5	0.4
494	12.8	0.3
357	7.4	0.1
974	7.8	1.0
882	8.3	0.04

[a] Microcystin concentrations are in microcystin—LR activity equivalents per liter. Detection limits are controlled by the volume of water filtered (2003, ~1 L; 2004, ~20 L).

Results

Station Descriptions. During the bloom event in 2003, chlorophyll *a* concentrations ranged from 4 to 40 $\mu g\ L^{-1}$ in the western basin; in 2004, concentrations varied from 7 to 20 $\mu g\ L^{-1}$ (Table 3). In 2003, the highest chlorophyll concentrations were found at sites 1, 5, and 6, which are located in the proximity of the tip of the green algal mass visible in the LANDSAT image (Figure 1).

In 2003 and 2004, samples from all stations were toxic (Table 3). The microcystin concentration exceeded the safety limit (1.0 $\mu g\ L^{-1}$) set by the World Health Organization (*2*) at two sampling locations (sites 1 and 6) in 2003 and at one location in 2004 (site 1163). In 2003, the highest level of toxicity was detected at the mouth of Maumee River (sampling site 1), where the toxicity of the samples varied from 14.3 to 20.0 $\mu g\ L^{-1}$. At sampling site 6, 1.8 $\mu g\ L^{-1}$ microcystin was found in the sample. In 2004, the highest concentrations of microcystins were found at stations 974 and 1163.

Multiplex PCR Analysis. Results from the initial multiplex PCR analysis of the 2003 and 2004 samples are presented in

TABLE 4. Initial Screening of Water Samples using Multiplex PCR Assays[a]

sampling site	Cyan 16S	Micr 16S	*mcy*B	*mcy*D
1	+	+	+	+
2	+	+	−	+
3	+	+	−	−
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
493	+	+	+	+
311	+	+	+	+
1163	+	+	+	+
885	+	+	+	+
496	+	+	+	+
495	+	+	+	+
494	+	+	+	+
357	+	+	+	+
974	+	+	+	+
882	+	+	+	+

[a] The columns are labeled with the PCR primers used for the analysis. The presence or absence of a visible band in the gel after staining with SYBR green I is indicated by + or −.

Table 4. PCR analysis indicated the presence of cyanobacteria and *Microcystis* spp. in all sampling sites. In 2003, toxigenic *Microcystis* spp. were present in six out of seven sampling sites, indicated by the presence of a *Microcystis*-specific 16S rRNA gene fragment and either one or both microcystin toxin synthetase genes *mcy*B and *mcy*D (Figure 3). Interestingly, at site 3 (in 2003), neither of the toxin synthetase genes *mcy*B or *mcy*D were detected by PCR, despite detectable microcystin concentrations in the water. In 2004, all target genes were detected by PCR in all samples analyzed, indicating the presence of toxic *Microcystis* spp. at all sampling sites.

Gene Copy Numbers and Cell Abundance in Lake Water Samples. In 2003, the highest abundance of cyanobacterial 16S rDNA target genes was detected at the mouth of the Maumee River (Table 5). At other sampling sites, the abundance was 1−3 orders of magnitude lower. The abundance of *Microcystis spp.* 16S rDNA genes was highest at the mouth of Maumee River, and the quantities decreased as the distance increased from the mouth of the river. A similar trend was found in the abundance of *mcy*D copies, with abundances 2−3 orders of magnitude lower at sites 4 and 5 relative to site 1. At sites 3 and 6, abundances of *Microcystis* 16S genes and *mcy*D were below quantifiable. In 2004, the quantities of all target genes in all samples were within quantifiable limits of the real-time PCR assay. On the basis of the percentages calculated using the quantities of cyanobacterial 16S and *Microcystis* 16S in the samples (Table 5), *Microcystis* dominated (>50%) the cyanobacterial population at site 1 in 2003 and at stations 357 and 882 in 2004.

At the mouth of the Maumee River, we estimated the abundance of cyanobacteria and *Microcystis* spp. to be about 10^8 LE-3 equiv L^{-1}. The abundance of all cell types decreased toward sampling sites 4 and 5, which are located approximately on the tip of the blooming mass originating from the mouth of the Maumee River as seen on the LANDSAT image (Figure 1). A low, but still detectable, abundance of *Microcystis* was found at sites 2 and 7. Although *Microcystis* was also found to be present at sites 3 and 6, the abundances of *Microcystis* cells were too low to be quantified in our assay. The highest abundance of cells carrying the *mcy*D gene was found at sites 1, 4, and 5. In 2004, the abundances of *Microcystis* and toxic *Microcystis* were within quantifiable limits at all sampling sites, and the total *Microcystis* abundance varied from 10^3 to 10^6 LE-3 equiv L^{-1} with the highest abundance of cells found at station 496. At station 974, a notably high concentration of microcystin LR was detected (Table 3); however, the abundance of toxic *Microcystis* was only approximately 800 cells per liter (Table 5).

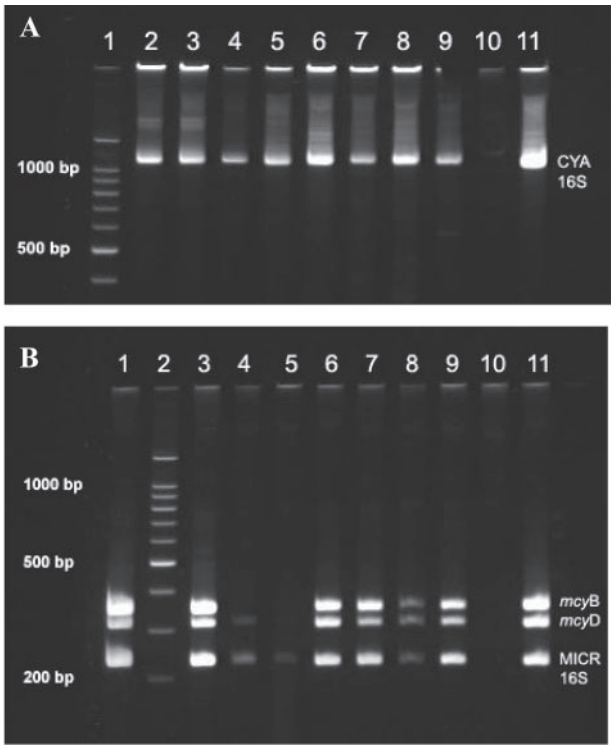

FIGURE 3. Gel image of multiplex PCR results. Gel A: detection of cyanobacterial 16S rDNA fragment. Lane 1: 100 bp molecular weight marker; lane 2: site 1a; lane 3: site 1b; lane 4: site 2; lane 5: site 3; lane 6: site 4; lane 7: site 5; lane 8: site 6; lane 9: site 7; lane 10: negative control, no template DNA; lane 11: positive control (*M. aeruginosa* LE-3 genomic DNA). Gel B: detection of *Microcystis* spp. 16S rDNA fragment and microcystin synthetase genes *mcy*B and *mcy*D. Lane 1: site 1a; lane 2: 100 bp molecular weight marker; lane 3: site 1b; lane 4: site 2; lane 5: site 3; lane 6: site 4; lane 7: site 5; lane 8: site 6; lane 9: site 7; lane 10: negative control, no template DNA; and lane 11: positive control (*M. aeruginosa* LE-3 genomic DNA).

Discussion

Three important implications arise from the results of this study. First, a tiered response to a potentially toxic cyanobacterial bloom was demonstrated to be a practical approach to monitoring these events. The combined use of satellite, conventional PCR, and then quantitative PCR allowed us to rapidly and reliably detect and characterize this bloom event. Second, the results of this study confirm previous research that not all strains of *Microcystis* found in natural samples are capable of producing toxins (*13*, *28*). Finally, the results demonstrate the limits of molecular approaches, as microcystin-producing cyanobacteria not detected by the probes used in this study (which were developed from our current knowledge of the *Microcystis* gene system) appear to have persisted in some areas we obtained our samples. As such, this work highlights the strengths of these tools as well as our continuing need for the development of a better understanding of the causative agents of freshwater cyanotoxin production.

At the time of sampling on August 15 2003, a bloom of cyanobacteria was persisting in the western basin of Lake Erie. The bloom area (region of station 882 in Figure 2) was well visible as a green mass in the water column in the LANDSAT image (Figure 1) taken 3 days after sampling. In addition, ground level observations (by M. R. Twiss and T.

TABLE 5. Real-Time PCR-Based Quantification of Abundances of Three Target Genes in Water Samples and Cell Abundance as LE-3 Equivalents of Total Cyanobacteria, Total *Microcystis*, and Toxic *Microcystis* (Cells Carrying *mcyD* Gene) in Samples Collected in August 2003 (Sampling Sites Numbered 1−7) and August 2004[a]

sampling site	Cyan 16S copies L^{-1}	Micr 16S copies L^{-1}	*mcy*D copies L^{-1}	total cyanobacteria (LE-3 equiv) L^{-1}	total *Microcystis* (LE-3 equiv) L^{-1}	toxic *Microcystis* (LE-3 equiv) L^{-1}
1	$3.9\ (\pm3.8) \times 10^{10}$	$3.4\ (\pm0.5) \times 10^{10}$	$3.2\ (\pm0.6) \times 10^{8}$	$9.9\ (\pm1.1) \times 10^{8}$	$3.9\ (\pm1.1) \times 10^{8}$	$1.1\ (\pm0.3) \times 10^{6}$
2	$1.7\ (\pm0.5) \times 10^{7}$	$6.2\ (\pm1.8) \times 10^{4}$	BQL	$3.2\ (\pm0.6) \times 10^{5}$	$1.8\ (\pm0.5) \times 10^{3}$	BQL
3	$1.5\ (\pm0.1) \times 10^{9}$	BQL	ND	$3.1\ (\pm0.3) \times 10^{7}$	BQL	ND
4	$2.1\ (\pm0.6) \times 10^{8}$	$1.1\ (\pm0.7) \times 10^{7}$	$2.8\ (\pm0.6) \times 10^{6}$	$4.7\ (\pm0.5) \times 10^{6}$	$6.6\ (\pm4.3) \times 10^{4}$	$9.0\ (\pm4.0) \times 10^{4}$
5	$1.0\ (\pm0.1) \times 10^{8}$	$1.7\ (\pm0.0) \times 10^{7}$	$7.0\ (\pm4.2) \times 10^{5}$	$1.9\ (\pm0.1) \times 10^{6}$	$1.0\ (\pm0.0) \times 10^{5}$	$3.4\ (\pm2.0) \times 10^{4}$
6	$2.9\ (\pm0.3) \times 10^{8}$	BQL	BQL	$5.0\ (\pm0.6) \times 10^{6}$	BQL	$4.2\ (\pm1.2) \times 10^{3}$
7	$8.7\ (\pm0.2) \times 10^{7}$	$2.4\ (\pm6.9) \times 10^{5}$	$7.7\ (\pm2.7) \times 10^{4}$	$1.6\ (\pm0.1) \times 10^{6}$	$7.5\ (\pm0.5) \times 10^{3}$	$8.6\ (\pm2.8) \times 10^{3}$
493	$4.3\ (\pm1.0) \times 10^{8}$	$5.1\ (\pm0.8) \times 10^{7}$	$4.2\ (\pm2.8) \times 10^{5}$	$5.5\ (\pm1.4) \times 10^{6}$	$7.0\ (\pm1.1) \times 10^{5}$	$2.0\ (\pm1.3) \times 10^{4}$
311	$6.6\ (\pm0.3) \times 10^{8}$	$1.3\ (\pm0.7) \times 10^{8}$	$3.9\ (\pm0.5) \times 10^{6}$	$8.7\ (\pm0.4) \times 10^{5}$	$1.8\ (\pm1.0) \times 10^{6}$	$1.8\ (\pm0.2) \times 10^{5}$
1163	$5.6\ (\pm0.4) \times 10^{9}$	$5.6\ (\pm0.4) \times 10^{7}$	$1.5\ (\pm1.0) \times 10^{6}$	$7.9\ (\pm0.2) \times 10^{7}$	$7.4\ (\pm0.6) \times 10^{5}$	$6.8\ (\pm4.5) \times 10^{4}$
885	$2.8\ (\pm0.3) \times 10^{8}$	$4.0\ (\pm0.8) \times 10^{7}$	$3.5\ (\pm0.3) \times 10^{6}$	$3.9\ (\pm0.4) \times 10^{6}$	$5.4\ (\pm1.1) \times 10^{5}$	$1.6\ (\pm1.6) \times 10^{5}$
496	$8.0\ (\pm2.2) \times 10^{8}$	$1.7\ (\pm0.1) \times 10^{8}$	$6.0\ (\pm0.3) \times 10^{6}$	$1.6\ (\pm7.8) \times 10^{7}$	$3.2\ (\pm1.5) \times 10^{6}$	$2.8\ (\pm0.1) \times 10^{5}$
495	$1.2\ (\pm0.4) \times 10^{8}$	$8.3\ (\pm0.3) \times 10^{6}$	$1.5\ (\pm0.7) \times 10^{5}$	$1.3\ (\pm0.8) \times 10^{6}$	$8.4\ (\pm2.9) \times 10^{4}$	$7.1\ (\pm3.4) \times 10^{3}$
494	$2.3\ (\pm0.7) \times 10^{8}$	$1.6\ (\pm0.2) \times 10^{7}$	$1.4\ (\pm1.0) \times 10^{6}$	$3.3\ (\pm1.1) \times 10^{6}$	$2.3\ (\pm0.3) \times 10^{5}$	$6.6\ (\pm4.7) \times 10^{4}$
357	$2.5\ (\pm1.3) \times 10^{7}$	$0.8\ (\pm1.1) \times 10^{7}$	$3.5\ (\pm0.3) \times 10^{4}$	$2.7\ (\pm1.9) \times 10^{5}$	$5.4\ (\pm6.4) \times 10^{4}$	$1.7\ (\pm0.2) \times 10^{3}$
974	$1.2\ (\pm0.05) \times 10^{7}$	$4.0\ (\pm1.3) \times 10^{5}$	$1.5\ (\pm1.0) \times 10^{4}$	$1.4\ (\pm0.1) \times 10^{5}$	$4.6\ (\pm1.5) \times 10^{3}$	$8.0\ (\pm\text{ND}) \times 10^{2}$
882	$6.2\ (\pm1.0) \times 10^{7}$	$3.5\ (\pm0.1) \times 10^{7}$	$7.4\ (\pm1.5) \times 10^{5}$	$7.5\ (\pm1.3) \times 10^{5}$	$4.8\ (\pm0.2) \times 10^{5}$	$3.4\ (\pm0.7) \times 10^{4}$

[a] Description for all samples $n = 3$ (±standard deviation) except sampling site 1, where $n = 5$ (±standard deviation) and sampling site 974 toxic *Microcystis*, where $n = 2$. ND = not detected, and BQL = detected but below quantifiable limit.

B. Bridgeman) noted thick green slicks on the surface. This observation was confirmed through measuring high chlorophyll *a* concentrations at the mouth of the Maumee River at that time (Table 3). At the same time, another cyanobacterial bloom was located at Sandusky Bay (station 1163 in Figure 2), distinguishable by a turbid, greenish water mass in the bay on the LANDSAT image (Figure 1). *Microcystis* was abundant in the western basin of Lake Erie also in August 2004. In both years, various concentrations of microcystin, determined using the protein phosphatase inhibition assay and expressed as microcystin−LR equivalents, were detected at all sampling sites; however, it is notable that toxin-producing *Microcystis* spp. were not present at every sampling location in 2003.

Chlorophyll *a* concentrations measured in the western basin of Lake Erie in 2003 and 2004 were similar to those measured in a 2002 lake-wide survey (1.5 ± 0.6 to 75.2 ± 7.9 $\mu g\ L^{-1}$) (*29*). In Sandusky Bay, the chlorophyll concentrations measured in this study were lower than in 2002, when a chlorophyll concentration as high as 75.2 $\mu g\ L^{-1}$ was reported. In 2003 and 2004, the chlorophyll concentrations in the western basin were notably higher than chlorophyll *a* concentrations reported for the eastern and central basins in the lake wide survey in 2002. In 2002, the chlorophyll concentrations in the eastern basin varied from 0.7 ± 0.6 to 22.3 ± 2.7 $\mu g\ L^{-1}$ and in the central basin from 0.7 ± 0.0 to 3.6 ± 0.0 $\mu g\ L^{-1}$ (*29*).

On the basis of real-time PCR analysis of the samples, the abundance of total *Microcystis* spp. cells varied from 4×10^8 to 2×10^3 *M. aeruginosa* LE-3 equivalents per liter among the sites where the abundance of *Microcystis* spp. was within the quantifiable limits. These results agree with a previous survey of the abundance of *Microcystis* in the western basin of Lake Erie. In the summer months (June to August) of 1995−1997, the total *Microcystis* abundance was reported to vary between 2×10^2 and 3×10^9 cells per liter (*1*). Thus, the real-time PCR based method used in this study provides data that are comparable to earlier results obtained through direct microscopic examination.

There are possible explanations for finding microcystin in the samples but no toxic *Microcystis*. One is that the abundance of *Microcystis* spp. producing microcystin was extremely low in these samples, and it was not detectable through conventional PCR due to a collapsed or senescent bloom in the sampling area since naked DNA remains in the PCR detectable form for only approximately 10 days in lake water (*30*). The samples for this study were collected at 1 m at all sampling sites, which leaves open the possibility of not detecting cells that may have migrated to deeper water. The other possible explanation for finding no toxigenic *Microcystis* but finding microcystin is the production of microcystins by other cyanobacteria. Other cyanobacterial species, in addition to the *Microcystis* species, belonging to genera *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc*, and *Anabaenopsis* are known producers of microcystins (*3*), and at least *Anabaena* and *Planktothrix* were observed via light microscopy of samples collected during a visit to Sandusky Bay in July 2003 (S. W. Wilhelm and G. L. Boyer, unpublished data). As the molecular probes used in this study are highly specific for *Microcystis* spp. (*13*), the presence of other microcystin producers in water samples could not be detected.

Estimates of the density of cells carrying the specific 16S rDNA target genes from *Microcystis* spp. are presented here using genomic DNA from a *Microcystis aeruginosa* isolate originally collected in Lake Erie as a standard. Implicit in this work is that variations in the copy number of 16S rDNA genes occur within the genomes of different prokaryotes—a brief survey of the literature suggests that these copies can range from one to four or more per genome. As such, any estimates of cell density using this approach are built around the caveat that variations in this copy number per genome within the natural population will be a source of error. In the case of the *mcy*D gene, it appears that cells carry only one copy per genome (*31*). Estimates of the percentage of the total *Microcystis* population that are toxic are therefore sensitive to this ratio: in the case of the current study, this conversion alludes to more toxic *Microcystis* cells than total *Microcystis* cells. Although this obviously cannot be the case, the results nonetheless provide a snapshot of the approximate dominance of the potentially toxigenic strains within the community.

It is important to note that the toxin data presented here represent estimates from the particulate fraction of the water samples (i.e., cyanotoxin bound within cells). Given the dogma that most toxins are maintained within cells, the toxin content within the dissolved phase (i.e., extracellular) was not determined in this study. As such, estimates of microcystin toxicity in this paper represent the minimum estimate

of the total toxicity in the dissolved and particulate fractions of water.

By employing a combination of ground level observation, satellite images, toxin data, and qualitative and quantitative PCR data, we have outlined a stepwise approach that allows for a precise evaluation of the composition of cyanobacterial blooms within this lake. The data presented here suggest that the composition of cyanobacterial communities varies spatially in the western basin of Lake Erie and suggest that in some regions (e.g., Sandusky Bay), novel toxigenic organisms may persist. Indeed, the application of these novel molecular tools is currently only limited by the availability of skilled researchers and funds—developments in the field of molecular biology will no doubt make applications of this work both feasibly and fiscally practical soon. These tools will also be augmented by ongoing studies, which include sequence analysis of toxin genes from *Microcystis* isolated from different locations within this system. It is anticipated that these results will provide resource managers and researchers with a tool set to study *Microcystis* bloom events as well as to develop monitoring approaches for future use with *Microcystis* as well as other toxic cyanobacteria (*18*).

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