

# Seasonal Production and Molecular Characterization of Microcystins in Oneida Lake, New York, USA

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**ABSTRACT:** Oneida Lake, northeast of Syracuse, New York, in the United States, is a shallow eutrophic lake with a well-established toxic cyanobacterial population. Samples for DNA, toxin, and phycological analyses were collected from six stations throughout the summers of 2002 (78 samples) and 2003 (95 samples). DNA was amplified by PCR using primer sets specific to the nonribosomal microcystin synthetase complex (*mcyB* and *mcyD*). PCR analysis in 2002 indicated that the microcystin genes were present in the water column from mid-June through October, as 88% of the samples tested positive for *mcyB* and 79% of the samples tested positive for *mcyD*. In both years the onset of microcystin production was detected as early as mid-July by the protein phosphatase inhibition assay, reaching a maximum in 2002 of  $2.9 \mu\text{g L}^{-1}$  and in 2003 of  $3.4 \mu\text{g L}^{-1}$ . Beginning in mid- to late August of both years the microcystin level at all six stations was in excess of the World Health Organization (WHO) advisory level of  $1.0 \mu\text{g L}^{-1}$ . In the present study we compared microcystin occurrence and potential production at the six stations using protein phosphatase inhibition assay, high-performance liquid chromatography, and polymerase chain reaction analyses. © 2005 Wiley Periodicals, Inc. *Environ Toxicol* 20: 243–248, 2005.

**Keywords:** cyanobacteria; microcystin; Oneida Lake; PCR; PPIA; toxin

## INTRODUCTION

Freshwater systems represent a source of drinking and recreational water for people worldwide. In recent years the rate of nutrient input to lakes and rivers has increased because of fertilizer runoff, wastewater treatment plants, and city drainage systems synonymous with industrialization and shoreline urban development. This eutrophication has amplified the frequency and size of algal blooms in slow-moving water bodies. Often these algal blooms occur during the late summer months at peak temperatures and

can be accompanied by toxin production from various species of cyanobacteria.

Cyanobacteria have a competitive advantage over other phytoplankton through their ability to exploit environmental conditions. They can produce biologically active secondary metabolites and toxins that may inhibit the growth of grazers and have detrimental effects on other organisms (Chorus, 2001). Environmental awareness of cyanotoxins has increased in recent years as reports of human, domestic animal, and wildlife poisonings have become more frequent (Falconer, 2001). In the United States there have been reports of dogs dying from exposure to cyanotoxins after swimming in waters containing a thick surface scum (Yang et al., 2001; Mihuc et al., in press). Although it is unlikely that humans will consume water with high microcystin content, there is concern over low-level chronic exposure, the exact consequences of which have not been adequately studied.

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It has been shown that microcystins, a family of potent hepatotoxins, are produced by a wide range of cyanobacteria including *Microcystis*, *Anabaena*, *Nostoc*, *Planktothrix/Oscillatoria*, and *Nodularia* species. These peptides are synthesized nonribosomally via a combination peptide–polyketide synthesis. The enzyme complex, as elucidated in *Microcystis*, is comprised of two operons transcribed bidirectionally from an internal promoter region. The first operon contains the *mcyD–J* genes for the biosynthesis of the nonstandard amino acid Adda and the incorporation of glutamic acid. The second operon, *mcyA–C*, contains the peptide synthetase genes that code for the incorporation of the remaining amino acids, cyclizes and releases the final microcystin (Tillett et al., 2000). The nonribosomal mechanism of synthesis has led to a microcystin family composed of over 65 known forms, making monitoring techniques difficult due to their structural variability.

Complications with current microcystin detection methods, as well as the limited information they supply, have increased the desire for molecular-based methods of detection using the microcystin gene cluster. In contrast to the activity-based protein phosphatase inhibition assay (PPIA) and the structure-based high-performance liquid chromatography (HPLC) method for microcystin detection, molecular techniques provide information on the potential for toxin production. The polymerase chain reaction (PCR) previously has been used to identify microcystin-producing colonies on the basis of the rRNA internal transcribed spacer (ITS) (Janse et al., 2004) and to correlate microcystin production with *Microcystis* colony size (Kurmayer et al., 2003). Real-time PCR has been used to identify the species responsible for microcystin production in two Finnish lakes (Vaitomaa et al., 2003) and to study the distribution and abundance of *Microcystis* species in the western basin of Lake Erie (Rinta-Kanto et al., in press). The cyanobacterial history of Oneida Lake makes it an ideal environmental system to study microcystin production using a number of methods. In the present study we compared microcystin occurrence and production potential in Oneida Lake using PPIA, HPLC, and PCR analyses.

## MATERIALS AND METHODS

### Sample Site

Oneida Lake is a 20,700-ha, east–west oriented lake 18 km north of Syracuse, New York, with a maximum depth of 16.8 m (Greeson, 1971). It is a shallow (average depth of 6.8 m), well-mixed eutrophic lake that is isothermal during the summer months. Oneida Lake lies in a fertile drainage basin (drainage area 3579 km<sup>2</sup>) and has a surface area of 206.7 km<sup>2</sup> (Greeson, 1971). It is valued for its sportfishing, boating, and recreational swimming and is part of the New York State Barge Canal system connecting the Great Lakes

**TABLE I. Locations of the six sampling stations on Oneida Lake**

Station	Latitude (°N)	Longitude (°W)
Muskrat Bay	43.229	76.104
3 Mile Bay	43.221	76.046
Shackelton Station	43.183	75.927
Station 125	43.213	75.924
Station 117	43.194	75.853
Station 109	43.186	75.770

with the Hudson River and New York City. Historically, by midsummer of each year Oneida Lake has formed algal blooms dominated by the cyanobacterial species *Anabaena*, *Aphanizomenon*, *Microcystis*, *Gleotrichia*, and *Lyngbya* (Greeson, 1971).

### Sample Collection

During the summers of 2002 and 2003, six stations were sampled along the long axis of Oneida Lake beginning in early June and continuing until October (Table I). At each station water samples were collected from a depth of 1.0 m, filtered, and immediately stored on dry ice for lab analysis. Toxin samples (20 L) were filtered through a 90-mm glass-fiber filter (Whatman 934-AH). DNA and chlorophyll samples (1.0 L) were filtered onto 47-mm glass-fiber filters (Whatman 934-AH). Surface samples of cyanobacteria were collected with a 10- $\mu$ m plankton net and stored in 0.2% v/v glutaraldehyde for phycollogical analysis.

### Toxin Analysis

Toxin filters were extracted by sonication in 10 mL of 50% aqueous methanol acidified to 1% with acetic acid. Samples were clarified by centrifugation, followed by filtration through a 0.45- $\mu$ m nylon syringe filter. Extracts were stored at –20°C and used directly in the following analyses. Microcystin was detected using PPIA and HPLC with PDA and MS detection. The PPIA assays, modified from Carmichael and An (1999), were run in 96-well plates containing 0.1 mU enzyme, 1.05 mg of pNPP, and 10  $\mu$ L of sample or microcystin standard (MC-LR). Absorbance (405 nm) was read after the addition of substrate and again after a 60-min incubation at 37°C. The 60-min rate was determined and standardized as a percentage of the control (+ enzyme, – toxin). Microcystin content in unknown samples was quantified by comparing the control percentages with those of the MC-LR standards.

For HPLC analysis, samples were separated on a 250  $\times$  4.6 mm Ace<sup>®</sup> 5 C18 column using a two-step gradient of 30%:70% acetonitrile to water, both containing 0.1% trifluoroacetic acid, at a flow rate of 0.8 mL min<sup>–1</sup>. Detection

TABLE II. PCR primer sequences

Target	Primer	Direction	Primer Sequence (5' → 3')
Cyanobacteria	CYA <sup>a</sup>	F	ACGGGTGAGTAACRCGTRA
16S rRNA		R	CTTCAYGYAGGCGAGTTGCAGC
<i>Microcystis</i>	MIC <sup>b</sup>	F	ATGTGCCGCGAGGTGAAACCTAAT
16S rRNA		R	TTACAAYCCAARRRCCCTCCTCCC
<i>Microcystis</i>	<i>mcyD</i> <sup>c</sup>	F	GGTTCGCCTGGTCAAAGTAA
<i>mcyD</i>		R	CCTCGCTAAAGAAGGGTTGA
<i>Microcystis</i>	<i>mcyB</i> <sup>d</sup>	F	TGGGAAGATGTTCTTCAGGTATCCAA
<i>mcyB</i>		R	AGAGTGGAAACAATATGATAAGCTAC

<sup>a</sup>Primer sequence obtained from Urbach et al., 1992;

<sup>b</sup>Primer sequence obtained from Neilan et al., 1997;

<sup>c</sup>Primer sequence obtained from Kaebnick et al., 2000;

<sup>d</sup>Primer sequence obtained from Nonneman and Zimba, 2002.

was at 239 nm (PDA) and by MS with electrospray ionization (Harada, 1996). For LC-MS, molecular ions from 900 to 1150 mass units were extracted from the total ion current. For both detection methods, unknown peaks that fell between the retention times of our most polar (MC-RR) and nonpolar (MC-LF) standards were considered putative MC variants, and compared to the list of MC variants published by Lawton and Edwards (2001).

### Chlorophyll Determination

Chlorophyll filters were extracted by sonication in 15 mL of 90% acetone. Samples were clarified by centrifugation in a swinging bucket rotor at  $1000 \times g$ . Chlorophyll-*a* was quantitated by UV/Vis spectrometry (Parson et al., 1984) and by fluorescence using a Turner Designs TD-700 fluorometer equipped with a chlorophyll excitation (436 nm) and emission (680 nm) filter set and a mercury blue light source.

### DNA Analysis

For DNA analysis, a 1.1-cm-diameter subsample was taken from the original filter with a cork borer. DNA was extracted using a modified protocol from Rudi et al. (1998). Filters were submerged in a buffer containing 10 mM EDTA and 12.5 mM Tris (pH 8) and digested with lysozyme ( $1.5 \text{ mg mL}^{-1}$ ) and RNase A ( $0.5 \text{ mg mL}^{-1}$ ) for 30 min at 37°C. Proteinase K ( $0.5 \text{ mg mL}^{-1}$ ) and sodium dodecyl sulfate (0.5%) were added, and the samples were incubated for 60 min at 65°C. The filters were removed and the supernatant extracted twice using phenol:chloroform:isoamyl alcohol (25:24:1), with a final extraction using chloroform:isoamyl alcohol (24:1). DNA was precipitated overnight in cold 95% ethanol at  $-20^\circ\text{C}$ , rinsed with 80% ethanol, and dried under vacuum. The final sample was rehydrated in 75  $\mu\text{L}$  of  $1 \times$  TE buffer [10 mM Tris and 1 mM EDTA (pH 8)]. Quantitation was done by UV/Vis absorbance at 260 nm using a submicro quartz cuvette. DNA was amplified by PCR using four primer sets, sepa-

rated by 1.5% agarose gel electrophoresis, and visualized using ethidium bromide. Primer sequences used for PCR were specific to the cyanobacterial 16S rRNA (CYA), *Microcystis* spp. 16S rRNA (MIC), and two toxin biosynthetic genes (*mcyB* and *mcyD*; see Table II and Fig. 1). The PCR reaction conditions were: 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  each dNTP, 0.04 U  $\mu\text{L}^{-1}$  Taq polymerase (Applied Biosystems), 300 ng  $\mu\text{L}^{-1}$  bovine serum albumin, 5 ng  $\mu\text{L}^{-1}$  of the DNA sample, and 400 nM of each primer. Amplification was performed in an MJ Research PTC-100 thermocycler using a protocol of initial denaturation at 94°C for 2 min; followed by 20 cycles of 94°C for 30 s, 65°C for 45 s (decreasing by 0.5°C each cycle), and 72°C for 1 min; then 15 additional cycles with a steady annealing temperature of 55°C and a final extension of 72°C for 8 min.

### Phycological Analysis

Species identification, based on Whitford and Schumacher (1969), was done using a phase-contrast light microscope

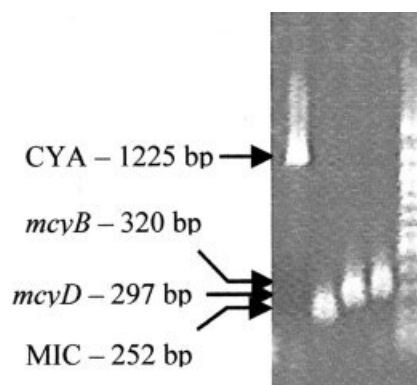
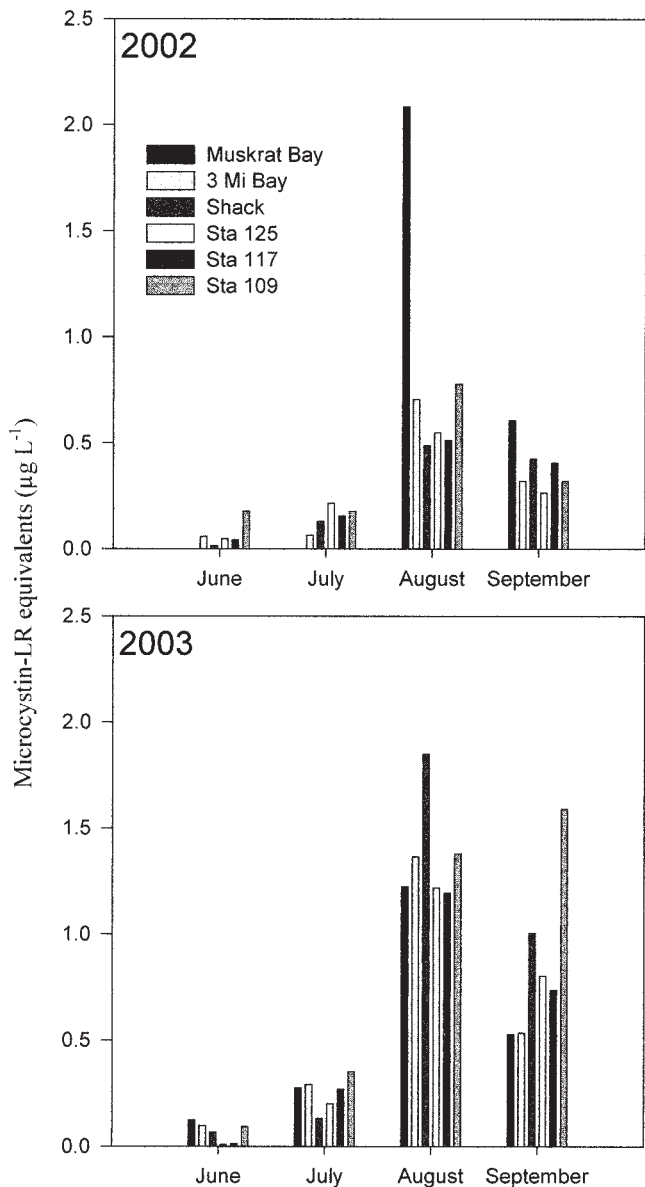


Fig. 1. A 1.5% agarose gel of the PCR products amplified from a microcystin-producing *Microcystis aeruginosa* culture. Primer sets (defined in Table II) are CYA (lane 1), MIC (lane 2), *mcyD* (lane 3), and *mcyB* (lane 4). Lane 5 contains a 100-bp DNA ladder.



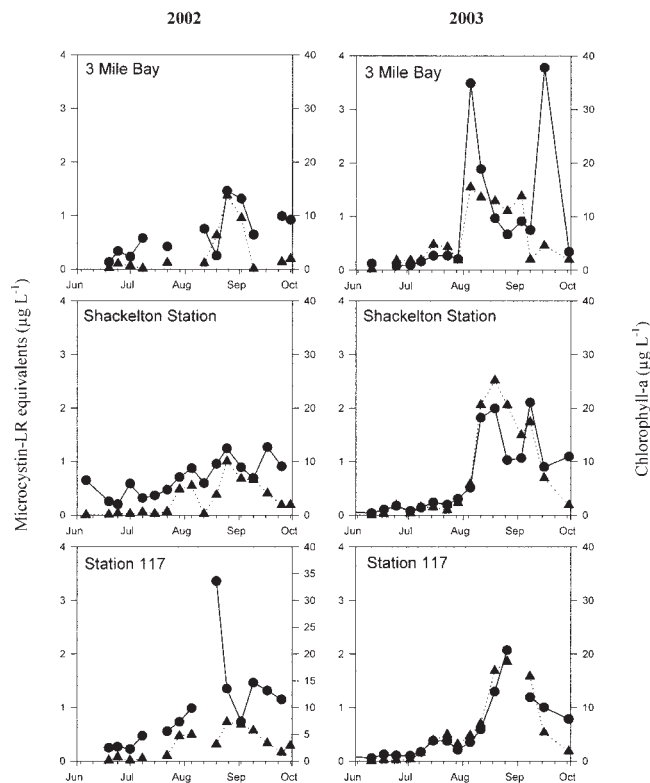
**Fig. 2.** Average microcystin concentrations (PPIA) per station per month in 2002 and 2003. Stations are oriented from west to east on Oneida Lake.

under 100×–250× magnification. Cyanobacterial colonies and filaments were estimated using a Palmer–Malony counting chamber.

**RESULTS**

Average monthly microcystin production in the eastern (Stations 109 and 117), central (Shackelton Station and Station 125), and western (3-Mile and Muskrat bays) basins of Oneida Lake are shown in Figure 2. For both years lake-wide toxin levels were low early in the season, reaching

elevated concentrations in August and September. These levels dropped by mid-September and stayed low for the rest of the sampling season. No clear east–west trend was observed between the six stations despite the strong prevailing westerly winds. Seasonal changes in microcystin and chlorophyll-*a* concentrations at Station 117, Shackelton Station, and 3-Mile Bay Station are shown in Figure 3. Shackelton Station and Station 117 had two separate toxin events in August and September 2002; however, this was not observed at all stations (e.g., 3-Mile Bay). From August through mid-September 2003 toxin levels lakewide were consistently high, exceeding the World Health Organization (WHO) advisory level for drinking water of 1 µg L<sup>-1</sup> for 15 consecutive days. Toxin production in 2002, as measured by PPIA, reached a maximum of 2.9 µg L<sup>-1</sup> (0.22 µg µg<sup>-1</sup> chlorophyll-*a*) in the western basin (Muskrat Bay) on August 25. By contrast, in 2003 toxin production reached a maximum of 3.4 µg L<sup>-1</sup> (0.21 µg µg<sup>-1</sup> chlorophyll-*a*) in the eastern basin (Station 109) on September 8 (Figs. 2 and 3). A linear correlation of chlorophyll-*a* concentration with toxin concentration was not observed ( $r^2 = 0.09$ ). The maximum chlorophyll-*a* concentrations were observed in the lake center in 2002 (Station 125, 40.2 µg L<sup>-1</sup>) and at the western end of the lake in 2003 (3-Mile Bay, 37.8 µg L<sup>-1</sup>).



**Fig. 3.** Seasonal concentrations of microcystin (PPIA; triangle with dotted line) and chlorophyll-*a* (circle with solid line) in 2002 and 2003 at three Oneida Lake stations representing the western (3-Mile Bay), central (Shackelton), and eastern (117) basins.

**TABLE III. Relative abundance of cyanobacterial genera from Oneida Lake during 2002 expressed as a percentage of the total number of colonies plus filaments counted**

	<i>Microcystis</i>	<i>Lyngbya</i>	<i>Anabaena</i>	<i>Aphan</i> <sup>a</sup>	<i>Gleotrichia</i>
June	21.9	0.0	75.0	0.0	3.1
July	14.7	0.3	64.4	19.1	0.7
August	39.3	2.0	11.7	46.9	0.0
September	68.6	1.1	15.4	14.6	0.1

<sup>a</sup>*Aphanizomenon*

In mid-June 2002 DNA specific to the microcystin biosynthetic gene cluster was detected, at a time when particulate toxin was undetectable by PPIA. The potential for toxin production was observed lakewide, with more than 88% of the samples testing positive for *mcyB* and 79% of the samples testing positive for *mcyD*. At Shackelton Station, the *mcyD* primer set did not detect the microcystin biosynthetic genes until August, whereas the *mcyB* primer set indicated the *mcyB* biosynthetic gene was present in late June (data not shown).

The samples from Shackelton Station collected in 2002 also were analyzed by LC-MS and HPLC-PDA. The predominate microcystin variants at this station were microcystin-LR and -RR. In 2003 MC, variants MC-LR, -RR, and -YR were detected lakewide from various samples. Both chromatographic analyses detected microcystin variants as early as mid-June (data not shown).

Visual examination of samples collected from Oneida Lake in 2002 indicated a trimodal trend in cyanobacterial dominance with *Anabaena* dominant early in the season, *Aphanizomenon* dominating midseason, and *Microcystis* dominating in the latter part of the summer at all six sample stations (Table III). Other common cyanobacteria noted were *Gleotrichia* and *Lyngbya*.

## DISCUSSION

Oneida Lake is a eutrophic lake, receiving nutrients from a large drainage area, and well mixed by strong westerly winds throughout the summer. Massive cyanobacterial blooms have been reported in the past for Oneida Lake (Greeson, 1971); however, they have never been tested for toxicity. Here we report the first results for cyanobacterial toxins. Oneida Lake consistently produced microcystins during the summers of 2002 and 2003, exhibiting peaks in both years during late summer. The lake contained a large number of potentially toxic species, including *Microcystis*, *Anabaena*, *Aphanizomenon*, and *Lyngbya* (Sivonen and Jones, 1999). Genetic analysis of samples collected from the lake indicated that the potential for toxin production was present early in the season and persisted throughout the

summer. Further information is needed to determine which cyanobacterial species have been producing microcystins.

The distribution of toxin production as measured by PPIA and toxin potential as detected by the *mcyB* and *mcyD* genetic assays was lakewide. No clear separation was observed between enclosed bays and open waters in Oneida Lake, reflecting its well-mixed nature. Distribution of peak chlorophyll-*a* abundance did not correlate with the distribution of peak toxin abundance, indicating the bloom events contained both toxic and nontoxic organisms.

Probes against the microcystin peptide synthetase-polyketide synthase complex have been used to test for microcystin biosynthetic potential in catfish ponds (Nonneman and Zimba, 2002), Lake Erie (Ouellette et al., submitted), and Finnish lakes (Vaitomaa et al., 2003). There are several potential gene targets for the biosynthetic gene cluster. The *mcyD* gene codes for part of polyketide synthase, involved in the biosynthesis of the Adda amino acid, and the *mcyB* gene codes for peptide synthetase, which helps to incorporate D-methyl aspartate into the cyclic structure. Both sites are conserved in most microcystins. We observed that the *mcyD* and *mcyB* primer sets were different in being able to predict toxin potential, which could have several explanations. The toxin biosynthetic gene cluster can be significantly different between genera. This is illustrated in the organization of the gene clusters in *Anabaena* strain 90, *Microcystis aeruginosa* PCC 7806, and *Planktothrix agardhii* CYA126 (Christiansen et al., 2003; Rouhiainen et al., 2004). The *Planktothrix* gene cluster is transcribed unidirectionally, except for *mcyT*, which is unique to *Planktothrix* (Christiansen et al., 2003). The *Anabaena* gene cluster contains three putative operons (*mcyA-C*, *mcyD-J*, and *mcyH*; Rouhiainen et al., 2004), as opposed to the two operons in *Microcystis* (Tillett et al., 2000). PCR primers designed against one organism, *Microcystis aeruginosa* in our case, may not effectively amplify the target sequence in a different organism. The observed difference between the two primer sets also could reflect slight changes in the gene sequence that affects the annealing of the primers. To determine this, isolation of the organisms responsible for microcystin production in Oneida Lake and the screening of their microcystin biosynthetic operons is currently in progress.

A combination of PPIA and PCR provides an excellent indicator of lake health in terms of microcystins. The PPIA assay measures actual toxicity, whereas PCR provides an estimate of potential toxin production. Because Oneida Lake is commonly used for recreational activities, it is important to monitor both actual and potential microcystin levels. The information gained from monitoring can be used to protect the public well-being, as well as to alert health and regulatory agencies of potential problems before they reach critical levels. The increase in lakewide distribution of toxin between 2002 and 2003 emphasizes the need for continued monitoring efforts. As Oneida Lake exhibits consistent cyanobacterial blooms, it offers an ideal system

for studying the onset of microcystin-producing blooms, testing new techniques, and analyzing the conditions necessary for toxin formation.

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