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Relations Between DNA- and RNA-Based Molecular Methods for Cyanobacteria and Microcystin Concentration at Maumee Bay State Park Lakeside Beach, Oregon, Ohio, 2012

Scientific Investigations Report 2013–5189

U.S. Department of the Interior
U.S. Geological Survey

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By Erin A. Stelzer, Keith A. Loftin, and Pamela Struffolino

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Conversion Factors

Inch/Pound

Multiply	By	To obtain
	Length	
mile (mi)	1.609	kilometer (km)
	Volume	
liter (L)	0.2642	gallon (gal)
milliliter (mL)	0.03381	fluid ounce (oz)
microliter (µL)	1,000	milliliter (mL)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)
milligram (mg)	1,000	gram (g)
microgram (µg)	1,000	milligram (mg)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows: °F=(1.8×°C)+32

Relations Between DNA- and RNA-Based Molecular Methods for Cyanobacteria and Microcystin Concentration at Maumee Bay State Park Lakeside Beach, Oregon, Ohio, 2012

By Erin A. Stelzer,[1] Keith A. Loftin[1], and Pamela Struffolino[2]

Abstract

Water samples were collected from Maumee Bay State Park Lakeside Beach, Oregon, Ohio, during the 2012 recreational season and analyzed for selected cyanobacteria gene sequences by DNA-based quantitative polymerase chain reaction (qPCR) and RNA-based quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Results from the four DNA assays (for quantifying total cyanobacteria, total *Microcystis*, and *Microcystis* and *Planktothrix* strains that possess the microcystin synthetase E (*mcyE*) gene) and two RNA assays (for quantifying *Microcystis* and *Planktothrix* genera that are expressing the microcystin synthetase E (*mcyE*) gene) were compared to microcystin concentration results determined by an enzyme-linked immunosorbent assay (ELISA).

Concentrations of the target in replicate analyses were $\log_{10}$ transformed. The average value of differences in $\log_{10}$ concentrations for the replicates that had at least one detection were found to range from 0.05 to >0.37 copy per 100 milliliters (copy/100 mL) for DNA-based methods and from >0.04 to >0.17 copy/100 mL for RNA-based methods.

RNA has a shorter half-life than DNA; consequently, a 24-hour holding-time study was done to determine the effects of holding time on RNA concentrations. Holding-time comparisons for the RNA-based *Microcystis* toxin *mcyE* assay showed reductions in the number of copies per 100 milliliters over 24 hours. The log difference between time 2 hours and time 24 hours was >0.37 copy/100 mL, which was higher than the analytical variability (log difference of >0.17 copy/100 mL).

Spearman's correlation analysis indicated that microcystin toxin concentrations were moderately to highly related to DNA-based assay results for total cyanobacteria (rho=0.69), total *Microcystis* (rho=0.74), and *Microcystis* strains that possess the *mcyE* gene (rho=0.81). Microcystin toxin concentrations were strongly related with RNA-based assay results for *Microcystis mcyE* gene expression (rho=0.95). Correlation analysis could not be done for *Planktothrix mcyE* gene expression because of too few detections.

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Introduction

Toxic freshwater cyanobacterial blooms are of concern in many parts of the world because of their effects on drinking water, water-based recreation, and watershed ecology. Among the most common cyanobacterial toxins are the hepatotoxins (microcystins and nodularins). Microcystins in freshwaters are frequently produced by cyanobacteria of the genera *Microcystis*, *Planktothrix*, and *Anabaena* (Sivonen, 2008); however, these genera also include strains that lack the ability to produce the microcystin toxin. The toxin-producing strains cannot be differentiated from the nonproducing strains by traditional microscopy. Chemical methods and assays can be used to measure toxin concentration, but they cannot determine which genera produced the toxins. Knowledge of toxin-producing genera is helpful for mitigating bloom formation and evaluating factors that affect cyanobacterial growth and toxin production.

The structure of the microcystin toxin gene is known, which has made possible the development of quantitative polymerase chain reaction (qPCR) assays for rapid detection of toxic strains. The commonly used DNA-based qPCR assays determine concentrations of toxin genes present and identify which genera contain these toxin genes. These assays can only determine whether the toxin gene is present, which means they only measure the potential for toxin production. A cyanobacteria cell must transcribe its DNA encoded toxin gene into messenger RNA (mRNA) to initiate the biosynthetic process responsible for toxin production. RNA-based quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays are able not only to identify toxin-producing genera but also to determine whether the toxin gene is being expressed, which means the cell is involved or is about to be involved in active toxin production. These RNA methods have not been extensively tested in the United States.

Past studies have found elevated levels of microcystins in Lake Erie, and the western basin has seen an increase in the number and size of cyanobacterial blooms over the last several years (Ouellette and others, 2006; Rinta-Kanto and others, 2009). The State of Ohio established a recreational advisory program whereby two microcystin concentration thresholds were established: (1) a "public health" advisory

at 6 micrograms per liter (µg/L) of microcystin and (2) a “no contact” advisory at 20 µg/L in surface waters of Ohio (Ohio Environmental Protection Agency, 2013a). For posting of a “no contact” advisory sign at the beach, toxin levels must exceed the 20-µg/L threshold and one or more probable cases of human illness or pet death must be attributable to the bloom. Since the state of Ohio enacted its Harmful Algal Bloom Advisory program, 12 cyanobacterial blooms have been sampled at Maumee Bay State Park Lakeside Beach; among these samples Ohio’s “public health” advisory threshold for microcystin has been exceeded six times (50 percent) and the “no contact” advisory threshold for microcystin exceeded three times (25 percent) between August 19, 2011, and August 5, 2013 (Ohio Environmental Protection Agency, 2013b).

This report describes the results of research by the U.S. Geological Survey (USGS) and the University of Toledo (UT), in cooperation with the Ohio Lake Erie Commission, to evaluate relations between results from DNA- and RNA-based molecular methods and actual microcystin concentrations. Eleven samples were collected from Maumee Bay State Park Lakeside Beach, Ohio, and analyzed by using DNA-based general cyanobacteria and *Microcystis* qPCR assays, DNA-based genus-specific toxin gene qPCR assays, RNA-based genus-specific toxin gene qRT-PCR assays, and microcystin toxin concentrations by enzyme-linked immunosorbent assay (ELISA). Also, a sample holding-time comparison was completed for the RNA-based molecular methods to compare concentrations obtained by filtering after 2 hours to those obtained by filtering after 24 hours. These molecular assays may be useful in future large-scale research projects to better understand cyanobacterial blooms and to potentially create an early warning system that can be used at recreational beaches.

Methods of Study

Eleven samples were collected by UT from the swimming area in cove 3 at Maumee Bay State Park Lakeside Beach in Oregon, Ohio (fig. 1), from June to August 2012. To prepare for sampling and reduce contamination, 500-milliliter (mL) polypropylene sample bottles and labware were treated with 10 percent hydrochloric acid, then rinsed with copious amounts of deionized water prior to being sterilized in an autoclave. All other supplies were purchased DNA- and RNA-free. Samples were collected by using standard grab-sampling techniques (Graham and others, 2010) in the decontaminated 500-mL bottles. Samples were placed on ice immediately after sampling. For microcystin concentrations by ELISA, an 80-mL aliquot of each sample was frozen at −70° C. At the end of the sampling period, all samples were shipped on ice to the USGS Kansas Organic Geochemistry Research Laboratory (OGRL) for microcystin analysis. Procedures for processing of samples for qPCR and qRT-PCR analyses are described below.

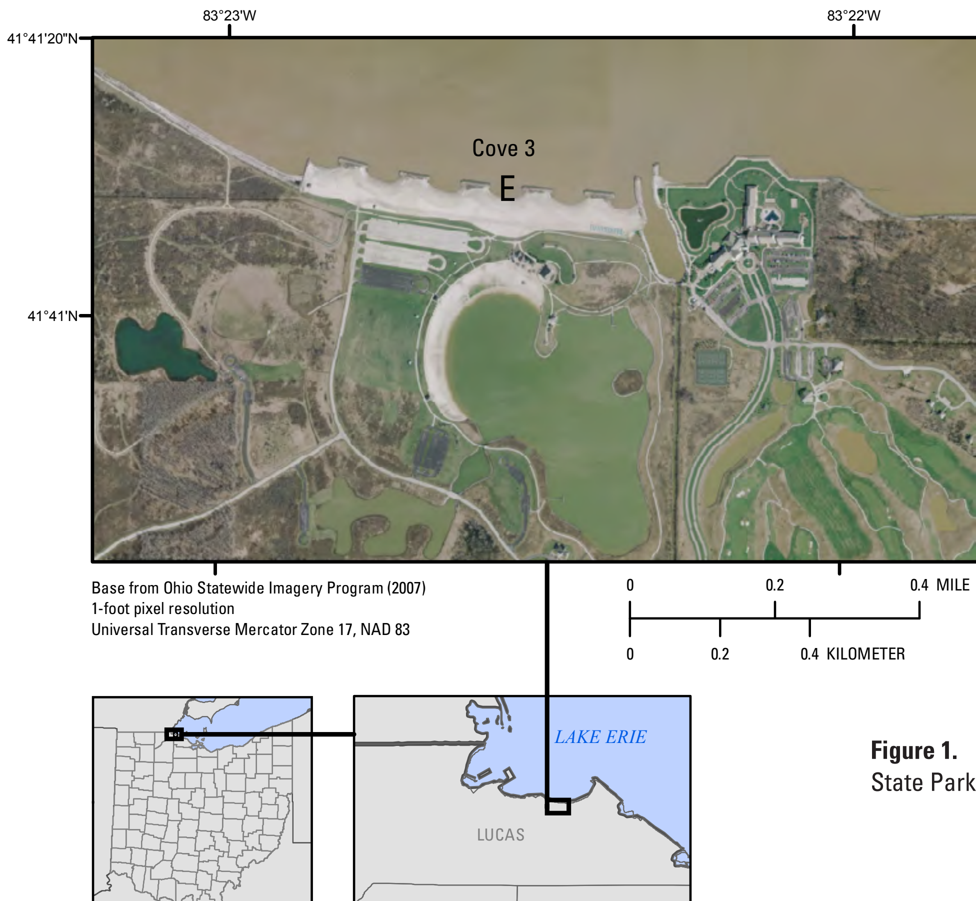

Figure 1. Study area, Maumee Bay State Park, Lake Erie, Ohio.

Table 1. Primers and probes for cyanobacteria assays.

[°C, degrees Celsius; s, seconds]

Assay	Primer or probe	Sequence (5′ - 3′)	Concentration used (nanomoles)	Assay run conditions[a]	Reference
Total cyanobacteria	Forward primer	ACGGGTGAGTAACRCGTRA	400	(95 °C for 30s, 56 °C for 60s, 65 ° C for 20s) × 40 cycles	Rinta-Kanto and others, 2005
	Reverse primer	CCATGGCGGAAAATTCCCC	400		
	TaqMan probe	CTCAGTCCCAGTGTGGCTGNTC	200		
Total *Microcystis*	Forward primer	GCCGCRAGGTGAAAMCTAA	400	(95 °C for 30s, 56 °C for 60s, 65 °C for 20s) × 40 cycles	Rinta-Kanto and others, 2005
	Reverse primer	AATCCAAARACCTTCCTCCC	400		
	TaqMan probe	AAGAGCTTGCGTCTGATTAGCTAGT	200		
Microcystis toxin *mcyE*	Forward primer	AAGCAAACTGCTCCCGGTATC	300	(95 °C for 30s, 62 °C for 60s) × 40 cycles	Sipari and others, 2010
	Reverse primer	CAATGGGAGCATAACGAGTCAA	300		
	TaqMan probe	CAATGGTTATCGAATTGACCCCGGAGAAAT	200		
Planktothrix toxin *mcyE*	Forward primer	GAAATTTGTGTAGAAGGTGC	500	(94 °C for 30s, 57 °C for 30s, 68 °C for 60s) × 40 cycles	Vaitomaa and others, 2003; Rantala and others, 2006
	Reverse primer	CTCAATCTGAGGATAACGAT	500		

[a] A required TaqMan hotstart activation of 95 °C for 10 minutes was applied before the listed run conditions for each assay.

Sample Concentration for Molecular Methods

Three aliquots of each water sample were filtered onto Nucleopore polycarbonate filters (Whatman/GE Healthcare, Piscataway, N.J.) and stored in screw-cap vials with 0.3 g of acid-washed glass beads (Sigma-Aldrich, St. Louis, Mo.). One aliquot of each sample was filtered at the UT laboratory approximately 2 hours after sampling and preserved at −20° C for use in an RNA holding-time comparison. Volumes filtered ranged from 10 to 30 mL. The remaining sample was shipped on ice overnight to the USGS Ohio Water Microbiology Laboratory (OWML), where two more aliquots were filtered approximately 24 hours after sampling and preserved at −70° C (one aliquot was used for DNA-based assays, the other aliquot for an RNA holding-time comparison). Volumes filtered ranged from 25 to 50 mL. After sampling was completed, UT shipped all preserved filters to the USGS OWML on dry ice for batch analysis.

DNA Extraction and qPCR Analyses

One of the USGS OWML filtered aliquots for each sample was extracted by use of a DNA-EZ extraction kit (GeneRite, North Brunswick, N.J.) according to manufacturer's instructions, except that no prefilter was used and the final elution volume was 100 microliters (µL). From this extract, 5 µL was analyzed by qPCR in duplicate for total cyanobacteria (Rinta-Kanto and others, 2005), total *Microcystis* (Rinta-Kanto and others, 2005), *Microcystis* toxin *mcyE* (Sipari and others, 2010), and *Planktothrix* toxin *mcyE* (Vaitomaa and others, 2003; Rantala and others, 2006). Primer and probe sequences and run conditions for each assay are listed in table 1. All assays were run on either an Applied Biosystems 7500 or a StepOne Plus (Foster City, Calif.) thermal cycler. Depending on the assay, either TaqMan Universal PCR Master Mix or SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif.) was used.

Sample inhibition was determined by seeding an aliquot of the sample extract with an extracted positive control target in a duplicate qPCR reaction. The concentration of target in the sample was then compared to the concentration of target in a clean matrix control (molecular-grade water) that was seeded with the same extracted positive control target. Sample extracts were considered inhibited and were diluted if the seeded test sample was more than 2 threshold cycles (CT) higher than that of the seeded clean matrix control.

RNA Extraction and qRT-PCR Analyses

Two aliquots—one of the USGS OWML filtered aliquots and the UT filtered aliquot for each sample—were extracted by using an Ultraclean Plant RNA extraction kit (MoBio Laboratories, Carlsbad, Calif.) (Sipari and others, 2010) according to manufacturer's instructions. A DNase treatment was included during extraction, and a DNA *Microcystis mcyE* qPCR was analyzed to verify that the RNA samples were completely DNA free.

Extracted mRNA was reverse transcribed to complementary DNA (cDNA) as a two-step process by using 6.7 µL of RNA extract. In brief, RNA extract was mixed with random primers (concentration 10 nanograms per microliter; Promega, Madison, Wis.) and nuclease-free water (VWR International, Radnor, Penn.), heated for 4 minutes at 99° C, then placed on ice, and finally supplemented with 16.8 µL of a reverse

transcription (RT) reaction mixture. The mixture components and their final concentrations were as follows: 10 millimolar (mM) Tris-HCl (pH 8.3) (Applied Biosystems, Foster City, Calif.), 50 mM KCl (Applied Biosystems, Foster City, Calif.), 3 mM $MgCl_2$ (Applied Biosystems, Foster City, Calif.), 10 mM dithiothreitol (Promega, Madison, Wis.), 0.8 mM deoxynucleotide triphosphates (Promega, Madison, Wis.), 20 units of RNase Inhibitor (Promega, Madison, Wis.), and 64 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif.). Reaction tubes were inserted into a thermal cycler (Applied Biosystems, Foster City, Calif.), and the following thermal profile was run: 25° C for 15 minutes, 42° C for 60 minutes, and 99° C for 5 minutes and then holding at 4° C until qPCR amplification.

After the RT reaction, 6 µL of cDNA sample was analyzed by qPCR for *Microcystis* toxin *mcyE* and *Planktothrix* toxin *mcyE* by using the primer and probe sets as well as the conditions supplied in the references mentioned above, with the exception that only a 14-µL master mix was used.

Inhibition of the qRT-PCR reaction was checked for every sample by seeding the reverse transcription reaction with 1 µL of armored RNA Hepatitis G virus (Asuragen, Inc., Austin, Tex.) as described by Lambertini and others (2008). If the seeded test sample was more than 2 C_T higher than the seeded clean matrix control, RNA extracts were diluted and the RT reaction repeated with the diluted extracts.

Quantifying Cyanobacteria by qPCR and qRT-PCR

Plasmid standards for each assay were used to establish standard curves for quantification. Plasmids were constructed by insertion of PCR-amplified target sequences into a pCR4 TOPO® *E. coli* plasmid vector (Invitrogen, Carlsbad, Calif.). The plasmid DNA was extracted and purified from *E. coli* cells by using the QuickLyse Miniprep Kit (Qiagen, Valencia, Calif.). Plasmid sequences were verified by DNA sequencing at The Ohio State University Plant-Microbe Genomics Facility. Copy number was calculated from DNA concentration measured by use of the PicoGreen® assay (Invitrogen, Carlsbad, Calif.) and the molecular weight of the plasmid. Sample results were reported as copies per 100 milliliters (copies/100 mL).

Guidelines for interpreting standard-curve data are available in the Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems Reagent Guide (Applied Biosystems, 2010). Standard curve characteristics are listed in table 2. The amplification efficiency of the qPCR should be 90–110 percent; an efficiency of 100 percent means an exact doubling of the target DNA sequence at each cycle. The dynamic range describes the lowest and highest standards analyzed by the laboratory for each assay in copies per qPCR reaction. The assay limit of quantification is the lowest concentration that can reasonably be quantified with some certainty and is the lowest standard indicated in the dynamic range. The limit of detection is the lowest value that can reasonably be detected with some certainty. The detection limit is 1 copy per reaction unless there are detections in blanks; in that case, the detection limit is determined by taking the 95th percentile of all blank detections, and samples are reported as less than the sample reporting limit (described below). All sample results lower than the limit of quantification but above the limit of detection are reported as estimated values. The regression coefficient (R^2) is used to assess the fit of the standard curve to the plotted data points. The closer the R^2 value is to 1, the better the fit.

Sample reporting limits (SRLs) are the “less-than values” for each sample and assay. They are sample specific because original sample volumes were sometimes different; also, a sample may have been diluted before being analyzed if it was found to be inhibited. To determine sample reporting limits, the assay’s limit of detection was divided by the actual amount of sample that was analyzed.

Sample Holding Time Comparison

At the OWML, DNA qPCR samples have a holding time of 24–48 hours before processing. Because RNA is known to have a shorter half-life than DNA, a sample holding-time

Table 2. Standard curve characteristics for DNA- and RNA-based molecular methods for cyanobacteria.

[Dynamic range and limit of detection are reported in copies per reaction]

DNA assay	Dynamic range	Amplification efficiency (percent)	R^2 value	Limit of detection[a]
Total cyanobacteria	13.2-1.32E+06	85	0.997	34
Total *Microcystis*	94.5-9.45E+06	91	0.999	1.0
Microcystis toxin *mcyE*	6.85-6.85E+06	93	0.999	1.0
Planktothrix toxin *mcyE*	90.4-9.04E+06	98	0.998	1.0
RNA assay	**Dynamic range**	**Amplification efficiency (percent)**	**R^2 value**	**Limit of detection**
Microcystis toxin *mcyE*	2.19-2.19E+05	95	0.996	1.0
Planktothrix toxin *mcyE*	1.83-1.83E+05	98	0.991	1.0

[a] Limit of detection is based on detections found in blanks.

comparison was done by using one RNA qRT-PCR assay (*Microcystis* toxin *mcyE*) to test whether RNA qRT-PCR samples can be shipped overnight or whether they need to be filtered and frozen immediately after sampling.

Microcystin by ELISA

Samples for microcystin concentration by use of the enzyme-linked immunosorbent assay (ELISA) were lysed, filtered, and analyzed according to procedures in Graham and others (2010). Briefly, frozen samples were lysed by three sequential-freeze/thaw cycles followed by syringe filtration with a 0.7-micrometer glass fiber syringe filter. Analysis of filtered aliquots was conducted by using the Abraxis microcystins/nodularins-ADDA ELISA kit (Abraxis LLC, Warminster, Pa.) with four-parameter calibration curve fit and a minimum reporting level of 0.10 µg/L. All samples that exceeded the upper calibration level of the kit (5 µg/L) were diluted so that the nondilution-corrected concentration was near the middle of the calibration curve, where the semisigmoidal curve is linear and most accurate and precise.

Quality Control

Laboratory quality-control samples for all qPCR and qRT-PCR assays included processing blanks (phosphate-buffered water processed with the water samples when filtered), extraction blanks (extraction elution buffer processed with filtered samples during each batch of extraction), no-template controls (molecular-grade water included on each qPCR and RT plate), and positive controls (plasmid-based standard curves as described above). All qPCR and qRT-PCR reactions were analyzed in duplicate.

ELISA field replicates were analyzed for all samples, and laboratory sample replicates and kit control standards (0.75 µg/L) were analyzed on approximately 10 percent of samples. Concentrations were acceptable if replicates were within 28.3 percent relative standard deviation (RSD). Blanks for the ELISA assay were analyzed on approximately 10–20 percent of samples by using both deionized water (used for sample dilutions) and the Abraxis ELISA kit diluent.

Quality-Control Considerations for Assessing Molecular Methods for Cyanobacteria

Quality-control samples were analyzed to aid in the assessment of the methods and interpretation of data. All blanks and no-template controls for both the DNA- and RNA-based assays were below the limit of detection except for one processing blank, which was estimated at 300 copies/100 mL for the total cyanobacteria DNA-based assay. Blanks for the ELISA assay were all found to be negative.

Analytical Variability of Molecular Methods

All qPCR and qRT-PCR reactions were analyzed in duplicate, and the absolute value $\log_{10}$ differences (AVLD) for each sample were calculated; data are listed in table 3. The AVLD was determined by calculating the absolute value of the difference between concentration results for two replicate samples that were $\log_{10}$ transformed. Average AVLDs for the assays ranged from >0.04 to >0.37 copy/100 mL. The DNA-based *Planktothrix* toxin *mcyE* assay had the largest average AVLD at >0.37 copy/100 mL, followed by the RNA-based *Microcystis* toxin *mcyE* assay at >0.17 copy/100 mL. Because concentrations of the *mcyE* gene by use of DNA- or RNA-based assays for *Planktothrix* were either low or below the sample reporting limit, it is hypothesized that the genus *Planktothrix* was not a major contributor of microcystin in Lakeside Beach Cove 3 during the sampling period in 2012. Therefore, the AVLDs for the DNA- and RNA-based *Planktothrix* toxin *mcyE* assays are probably not accurate representations of the variability of these assays. The RNA-based *Microcystis* toxin *mcyE* assay's average AVLD may be higher than the DNA-based assays because of error and (or) loss during the extra two-step reverse transcription procedure needed for all RNA assays.

Sample Holding-Time Comparison for an RNA-Based Molecular Method

The results of the holding-time comparison are listed in table 4. Filtering the day of sampling (time 2 hours) yielded higher RNA concentrations in all but one of the test samples. The log difference between time 2 hours and time 24 hours for each sample was calculated, and the average log difference for all samples was >0.37 copy/100 mL. The average log difference between time 2 hours and time 24 hours is more than twice the analytical variability for the RNA-based *Microcystis* toxin *mcyE* assay (table 3, >0.17 copy/100 mL). Therefore, the differences between the sample processing times cannot be entirely attributed to variability of the method, and the general trend suggests that RNA recovery declined over time. Because of these findings, only the filters processed on the same day they were sampled were used for the remainder of the RNA-based methods data analysis.

Concentrations of Cyanobacteria by DNA- and RNA-Based Molecular Methods and Their Relations to Toxin Production

A total of 11 samples were analyzed for the DNA qPCR assays, for RNA qRT-PCR assays, and for microcystin by ELISA. Table 5 lists the results for all samples.

Table 3. Quality-control replicate for DNA- and RNA based molecular methods for cyanobacteria.

[AVLD is absolute value $\log_{10}$ difference beween qPCR and qRT-PCR replicate reactions A and B; molecular assays reported in copies per 100 milliliters (copies/100 mL); <, less than (below sample reporting limit); ND, not determined because there were two values below detection]

Sample date	Total cyanobacteria (DNA-based) (copies/100 mL)			Total *Microcystis* (DNA-based) (copies/100 mL)			*Microcystis* toxin *mcyE* (DNA-based) (copies/100 mL)		
	A	B	AVLD	A	B	AVLD	A	B	AVLD
6/19	380,000	350,000	0.04	5,000	4,100	0.09	7,500	8,700	0.06
6/26	1,600,000	1,100,000	0.16	56,000	53,000	0.02	66,000	82,000	0.09
7/2	1,000,000	1,000,000	0.00	1,100	500	0.34	680	560	0.08
7/10	910,000	780,000	0.07	140,000	150,000	0.03	250,000	240,000	0.02
7/17	2,000,000	1,800,000	0.05	240,000	230,000	0.02	120,000	110,000	0.04
7/24	1,400,000	1,300,000	0.03	110,000	100,000	0.04	73,000	77,000	0.02
7/31	8,800,000	8,800,000	0.07	1,700,000	1,900,000	0.05	1,100,000	1,500,000	0.13
8/9	6,000,000	5,500,000	0.04	1,600,000	1,700,000	0.03	220,000	220,000	0.00
8/14	2,500,000	2,400,000	0.04	600,000	630,000	0.02	28,000	32,000	0.06
8/22	5,000,000	4,600,000	0.04	2,300,000	2,300,000	0.00	660,000	680,000	0.01
8/29	17,000,000	18,000,000	0.02	14,000,000	13,000,000	0.03	4,300,000	3,200,000	0.13
Average AVLD			**0.05**			**0.06**			**0.06**
Sample date	***Planktothrix* toxin *mcyE* (DNA-based) (copies/100 mL)**			***Microcystis* toxin *mcyE* (RNA-based) (copies/100 mL)**			***Planktothrix* toxin *mcyE* (RNA-based) (copies/100 mL)**		
	A	**B**	**AVLD**	**A**	**B**	**AVLD**	**A**	**B**	**AVLD**
6/19	<33	47 E	>0.15	<166	<166	ND	<166	<166	ND
6/26	<28	<28	ND	<166	<166	ND	<166	<166	ND
7/2	<20	<20	ND	190 E	<166	>0.06	<166	<166	ND
7/10	140	<40	>0.54	1,500	900	0.22	<166	<166	ND
7/17	<33	150	>0.66	180 E	<166	>0.04	<166	<166	ND
7/24	<28	70 E	>0.40	930	810	0.06	<166	<166	ND
7/31	<33	<33	ND	3,300	1,500	0.34	<166	<166	ND
8/9	<33	<33	ND	440	180 E	0.39	<166	<166	ND
8/14	<33	<33	ND	<500	1,200	0.38	<166	180 E	>0.04
8/22	57 E	120	0.32	1,200	1,200	0.00	<166	<166	ND
8/29	190	270	0.15	7,100	8,100	0.06	<166	<166	ND
Average AVLD			**>0.37**			**>0.17**			**>0.04**

Detectable levels of microcystin toxin were found in all samples and ranged from 0.1 to 16 micrograms per liter (µg/L). There were two peaks in toxin concentration, one at the end of July and the other at the end of August 2012. The last sample on August 29 had a microcystin concentration of 16 µg/L, making it the only sample during this study period that exceeded the State of Ohio Microcystin Public Health Advisory of 6 µg/L.

Three DNA-based qPCR assays, total cyanobacteria, total *Microcystis*, and *Microcystis* toxin *mcyE* yielded quantifiable detections for every sample (table 5). Total cyanobacteria concentrations ranged from 360,000 to 17,000,000 copies/100 mL, total *Microcystis* ranged from 800 to 13,000,000 copies/100 mL, and DNA-based *Microcystis* toxin *mcyE* ranged from 620 to 3,800,000 copies/100 mL. Only for the last sample date, August 29, was reported DNA-based *Planktothrix* toxin *mcyE* above the limit of quantification, at 230 copies/100 mL. All other DNA-based *Planktothrix* toxin *mcyE* detections were either estimated with an "E" designation or not detected and given a less-than value. The highest concentrations obtained from each of the DNA-based qPCR assays occurred from late July through August. Microcystin concentrations by ELISA were moderately to highly correlated with three of the DNA-based assays by using Spearman's correlation analysis: total cyanobacteria (rho=0.69, p=0.0185), total *Microcystis* (rho=0.74, p=0.0089), and *Microcystis* toxin *mcyE* (rho=0.81, p=0.0027).

The RNA-based *Microcystis* and *Planktothrix* toxin *mcyE* genes were not detected in the two June samples. RNA-based *Planktothrix* toxin *mcyE* was detected in only one sample, at an estimated 180 copies/100 mL on August 14. RNA-based *Microcystis* toxin *mcyE* was detected in nine samples and

ranged from an estimated 180 to 7,600 copies/100 mL. The two days with the highest concentration of microcystin toxin also had the highest concentrations of RNA-based *Microcystis* toxin *mcyE* (July 31 and August 29). Using Spearman's correlation analysis, the RNA-based *Microcystis* toxin *mcyE* assay was significantly related to microcystin concentrations by ELISA (r=0.95, p<0.0001). Correlation analysis could not be done for the *Planktothrix* toxin *mcyE* assay results because of too few detections.

Table 4. Sample holding-time comparison for an RNA-based molecular method.

[Log difference is the $\log_{10}$ difference between time 2 hours and time 24 hours; time 2 hours and time 24 hours results are reported in copies per 100 milliliters; E, estimated value; <, less than (below sample reporting limit); ND, not determined because there were two values below detection]

Sample date	*Microcystis* toxin *mcyE* at time 2 hours	*Microcystis* toxin *mcyE* at time 24 hours	Log difference
6/26/12	<166	<166	ND
7/10/12	1,200	<166	>0.86
7/24/12	870	450	0.29
7/31/12	2,400	1,400	0.23
8/9/12	310	760	–0.39
8/14/12	780	180 E	0.64
8/22/12	1,200	<166	>0.86
8/29/12	7,600	6,300	0.08
Average log difference			**>0.37**

Implications for Future Studies

The results showed that the DNA- and RNA-based molecular methods can be useful for studying the potential for and the expression of toxins. It should be emphasized that these qPCR and qRT-PCR assays for cyanobacteria are in an experimental and developmental phase as a field of science specialization. These assays will need to be tested on samples collected from a variety of sites and under different hydrological, spatial, and environmental conditions for each site before their usefulness in creating some type of early warning system for toxic cyanobacterial blooms can be developed.

Summary and Conclusions

Toxic cyanobacterial blooms are of concern because they can affect drinking water, water-based recreation, and watershed ecology. Microcystin toxin is one of the most commonly found cyanotoxins and is frequently produced by the genera *Microcystis*, *Planktothrix*, and *Anabaena*. Because these genera include not only strains that have the ability to produce toxins but also strains that lack the ability to produce toxins, a method that can differentiate between toxin producers and toxin nonproducers is needed. DNA-based molecular methods have the ability to determine the concentration of toxin genes present and can identify which genera contain these toxin genes. The RNA-based molecular methods can determine which toxic genera are actively expressing their toxin genes and nearing or already involved in toxin production.

To study relations between DNA- and RNA-based molecular methods and actual microcystin toxin production,

Table 5. Microcystin and DNA and RNA-based molecular method results for cyanobacteria in samples collected at Maumee Bay State Park Lakeside Beach, 2012.

[qPCR and qRT-PCR results are the average of two replicate reactions and are reported in copies per 100 milliliters; E, estimated value (below limit of quantification); <, less than (below sample reporting limit); † duplicate analyses did not agree and only the detected value was reported]

Date	DNA-based qPCR assays				Microcystin concentration (micrograms per liter)	RNA-based qRT-PCR assays	
	Total cyano-bacteria	Total *Microcystis*	*Microcystis* toxin *mcyE*	*Planktothrix* toxin *mcyE*		*Microcystis* toxin *mcyE*	*Planktothrix* toxin *mcyE*
6/19	360,000	4,600	8,100	47 E †	0.1	<166	<166
6/26	1,400,000	55,000	74,000	<28	0.1	<166	<166
7/2	1,000,000	800	620	<20	0.2	190 E †	<166
7/10	850,000	140,000	240,000	140 †	0.7	1,200	<166
7/17	1,900,000	230,000	120,000	150 †	0.2	180 †	<166
7/24	1,300,000	110,000	75,000	70 E †	1.6	870	<166
7/31	8,100,000	1,800,000	1,300,000	<33	2.2	2,400	<166
8/9	5,800,000	1,700,000	220,000	<33	0.4	310	<166
8/14	2,500,000	620,000	30,000	<33	0.2	780	180 E
8/22	4,800,000	2,300,000	670,000	89 E	1.6	1,200	<166
8/29	17,000,000	13,000,000	3,800,000	230	16	7,600	<166

11 samples were collected at Maumee Bay State Park Lakeside Beach, Oregon, Ohio during the recreational season in 2012. Three separate aliquots of each sample were filtered for use with the molecular methods: one approximately 2 hours after sampling at the University of Toledo (UT) laboratory and used in an RNA-based methods sample holding-time comparison, one approximately 24 hours after sampling at the Ohio Water Microbiology Laboratory (OWML) and used in an RNA-based methods sample holding-time comparison, and one approximately 24 hours after sampling at the OWML and used for DNA-based methods. An 80-mL aliquot of each sample was preserved for microcystin concentrations by ELISA analysis.

Quality-control samples for the molecular methods provided insights into analytical variability. The absolute value log differences (AVLDs) between replicate samples were calculated to identify the analytical variability associated with each assay. The AVLD results indicated that the DNA-based total cyanobacteria assay had the least amount of analytical variability, with an average AVLD of 0.05 copy/100 mL. The *Planktothrix* toxin *mcyE* DNA-based assay had the most analytical variability with an average AVLD of >0.37 copy/100 mL. Concentrations for both the DNA- and the RNA-based *Planktothrix* toxin *mcyE* assays were either low or below the sample reporting limit; therefore these AVLD measurements are insufficient to evaluate the analytical variability of these assays.

A sample holding-time comparison was done for the RNA-based *Microcystin* toxin *mcyE* assay because RNA is known to have a shorter half-life than DNA. Filtering approximately 2 hours after sampling yielded higher RNA concentrations than filtering after 24 hours in all but one test sample. The average log difference between time 2 hours and time 24 hours (>0.37 copy/100 mL) was more than twice the analytical variability for the assay (average AVLD of >0.17 copy/100 mL).

The highest concentrations for the DNA-based assays occurred from late July through August. DNA-based *Planktothrix* toxin *mcyE* was the exception, being detected in only six samples and at low concentrations. Three of the DNA-based assays were correlated with microcystin concentration by ELISA using Spearman's correlation analysis: total cyanobacteria, total *Microcystis*, and *Microcystis* toxin *mcyE*.

RNA-based Microcystin toxin *mcyE* genes were detected in 9 of the 11 samples. On the two days when actual toxin concentration in the water was highest, the RNA-based Microcystin toxin *mcyE* assay also measured the greatest expression of toxin genes. Using Spearman's correlation analysis, the RNA-based Microcystin toxin *mcyE* assay was significantly related to microcystin concentration by ELISA.

In this study, DNA- and RNA-based molecular methods have shown they can be useful for studying the potential for and the expression of toxins. These molecular assays may be used in future large-scale research projects to better understand cyanobacterial blooms and to potentially create an early warning system that can be used at recreational beaches.

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