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Quantification of Toxigenic *Microcystis* spp. in Freshwaters by Quantitative Real-time PCR Based on the Microcystin Synthetase A Gene

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A method to estimate the abundance of toxigenic *Microcystis* in environmental samples by using quantitative real-time PCR was developed and optimized. The basis of this method is the amplification of a highly conserved region of the *mcyA* gene within the microcystin synthetase gene cluster. Using this method, the average copy number of *mcyA* gene per cell in toxigenic *Microcystis* strains was estimated. The molecular markers and method developed in this study can be used to monitor toxigenic strains of *Microcystis* in Korean freshwaters, in which harmful cyanobacterial blooms are routinely found.

***Keywords*:** toxic *Microcystis*, microcystins, quantitative real-time PCR, microcystin synthetase gene

Introduction

Many bloom-forming freshwater cyanobacterial genera include strains capable of producing cyanotoxins. Contamination of freshwater supplies by harmful cyanobacterial blooms can be a source of morbidity and mortality in livestock, wildlife, and also humans (Kuiper-Goodman *et al.*, 1999). Microcystins (also known as cyanoginosins) are the best-studied class of cyanotoxins due to their global distribution in freshwater supplies (Falconer and Humpage, 2005). Microcystins are produced by a number of cyanobacterial species within the genera *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*), *Nostoc*, and *Phormidium* (Vieira *et al.*, 2003; Carmichael and Li, 2006; Jungblut and Neilan, 2006). Quantification of potentially toxic cyanobacteria in environmental samples is essential for the monitoring of harmful cyanobacterial blooms and for constructing remediation strategies.

An algal alert system that uses measurements of cyanobacterial abundance in environmental samples to monitor large artificial reservoirs, mostly used as the sources of drinking water, for harmful algal blooms has been in operation in Korea since 1996 (Ahn *et al.*, 2007). In this system, the Caution, Warning, and Outbreak Levels are determined by the concentration of chlorophyll *a* and the number of total cyanobacteria, which is estimated by direct enumeration under the microscope. Although light microscopy is often the method of choice to count cyanobacteria in environmental samples, it cannot differentiate toxigenic from non-toxigenic cyanobacteria (Ouellette and Wilhelm, 2003). Because the purpose of the algal alert system is to reduce the risk of cyanobacterial toxins in the water supply reservoirs, the concentrations of microcystins should be measured together with the number of toxigenic cyanobacteria to predict the potential of toxin production.

Recently, molecular technique such as quantitative real-time PCR (qPCR) has been developed and widely used to estimate cyanobacterial abundance based on the levels of cyanobacteria-specific target genes in environmental samples (Pearson and Neilan, 2008; Martins and Vasconcelos, 2011). A qPCR technique known as the Taq-nuclease assay was firstly used to quantify *Synechococcus* ecotypes in deep lakes (Becker *et al.*, 2002). This technique using PCR primers which amplify genes involved in toxin synthesis has subsequently been used for the detection and quantification of microcystin-producing *Microcystis* and *Anabaena* in culture and in environmental samples (Vaitomaa *et al.*, 2003; Rinta-Kanto *et al.*, 2005; Furukawa *et al.*, 2006; Fortin *et al.*, 2010). qPCR was also used to find out the role of microcystins in the toxigenic cyanobacteria and the environmental parameters affecting microcystin synthesis (Alexova *et al.*, 2011; Ha *et al.*, 2011). The reliability of such assays requires the design of specific qPCR primers to amplify highly conserved sequences within the target genes. For example, using non-specific primers targeting the *mcyB* gene resulted in discrepancies between the quantity of toxin gene expression and toxin production due to the high sequence similarity between the *mcyB* gene and genetic loci within other peptide synthetase genes (Dittman *et al.*, 1997). In this study we used *mcyA* gene to design primers for qPCR, because the sequences of the *mcyA* gene are less diverse compared to other genes in the microcystin synthetase (*mcy*) gene cluster (Hisbergues *et al.*, 2003; Rantala *et al.*, 2004). Moreover, sequence analysis of the *mcyA* gene in samples taken from 5 representative freshwater reservoirs showed a high degree of sequence conservation between cyanobacterial species, indicating that the *mcyA* gene is suitable for quantification of *mcy* gene expression by using qPCR (Oh *et al.*, 2010). The aim of this study was to develop a re-

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Table 1. Calculated copy number of 16S rRNA and *mcyA* genes in genome of *Microcystis* spp.

Strains	16S rRNA		*mcyA*	
	Ratio	Integer	Ratio	Integer
Microcystis sp. NIER 10004	2.090	2	1.045	1
Microcystis aeruginosa NIER 10038	1.938	2	0.969	1
Microcystis aeruginosa NIER 10111	1.919	2	0.960	1
Microcystis ichthyoblabe NIER 10112	2.675	3	1.338	1
Microcystis viridis NIER 10113	1.752	2	0.876	1
Microcystis viridis NIER 10020	1.922	2	0.961	1
Microcystis aeruginosa UTEX 2388	2.090	2	1.041	1
Microcystis viridis NIES 102	1.716	2	0.858	1
Microcystis aeruginosa NIES 107	2.097	2	1.048	1
Microcystis aeruginosa NIES 1058	2.196	2	1.098	1

liable and adequate method for quantification of toxigenic *Microcystis* by using primers designed to amplify the *mcyA* gene in environmental samples.

Materials and Methods

Cyanobacterial strains and cultivation

The *Microcystis* strains *M. aeruginosa* UTEX 2388, *M. viridis* NIES 102, *M. aeruginosa* NIES 107, *M. aeruginosa* NIES 1058, and *M. aeruginosa* Mi 0601 were purchased from Korean Collection for Type Cultures. An additional 7 strains that were isolated from Korean freshwater (denoted as NIER in Table 1) were generously provided by the National Institute of Environmental Research in Korea (Lee *et al*., 2007). NIER 10022 and Mi 0601 are non-toxigenic, whereas the others produce the microcystins LR, RR, and YR (Oh *et al*., 2010). Cyanobacteria were grown at 25°C cultured in CB medium in an incubation room at 25°C under fluorescent light at ~40 μE/(m^2·s) on a 10 h/14 h dark/light cycle (Shirai *et al*., 1989; Han *et al*., 2010).

Sampling and analysis

Surface water samples were collected from five Korean reservoirs between July 1 and September 17, 2008 (Table 2; Oh *et al*., 2012). These reservoirs are the representative ones in Korea, used as the sources of water supply. Surface water within 0.5 m of depth was collected with the Van Dorn water sampler and transferred to sterile bottles. The bottles were kept cold during transport to the laboratory. To prevent the decomposition of DNA, the samples were filtered through sterile GF/C filters to concentrate at the date of sampling, and then the filters were kept in a deep-freezer until DNA extraction.

In order to measure the concentrations of chlorophyll *a* and microcystins, water samples were filtered through a GF/C filter paper. The chlorophyll *a* concentration was measured by the method represented in Standard Methods (American Public Health Association, 2005). Microcystins were extracted from the filters with 75% methanol and then purified with a C18 column cartridge (Sep-Pak Vac 3 cc; Waters, USA) (Maatouk *et al*., 2002). The concentration and identification of microcystins was determined using HPLC equipped with UV absorbance detector, and with the microcystins LR, YR, and RR as the authentic standards (Wako Pure Chemical Ind., Japan) (Oh *et al*., 2012).

DNA extraction and quantification

Cyanobacterial DNA was extracted as described previously (Oh *et al*., 2012). Briefly, cells that were collected on the GF/C filter papers were lysed by the addition of buffer containing lysozyme followed by freeze-thaw cycles. DNA was then purified by phenol:chloroform extraction.

The concentration of DNA was measured using Quant-iT PicoGreen dsDNA Reagents and Kits (Molecular Probes, Inc., USA) following the manufacturer's instructions. Briefly,

Table 2. Sampling locations, environmental parameters, and real-time PCR-based quantification of abundances of *Microcystis* and toxigenic *Microcystis* in the water samples

Reservoir	Sampling location	Sampling date	Water temp. (°C)	Chl *a* (mg/m^3)	Cell number ($\times 10^6$ cells/L)[a]		Microcystins (μg/L)
					Microcystis	Toxigenic *Microcystis*	
Daechung	36°21′34.17″ N 127°34′10.38″ E	Jul. 01, 2008	27.0	33.7	827.2 (±70.0)	281.0 (±52.9)	48.6
Yongdam	35°52′33.15″ N 127°30′31.95″ E	Jun. 27, 2008	28.5	40.6	350.6 (±5.6)	254.1 (±63.6)	11.0
Chungju	37°01′50.98″ N 128°02′16.82″ E	Sep. 02, 2008	27.5	64.2	2704.7 (±338.7)	81.1 (±37.6)	14.2
Soyang	37°31′33.94″ N 127°51′14.08″ E	Sep. 05, 2008	27.0	4.6	290.8 (±56.2)	0.88 (±0.82)	1.34
Euam	37°52′29.82″ N 127°42′28.30″ E	Sep. 17, 2008	26.5	41.0	462.0 (±84.7)	129.8 (±60.1)	6.85

[a] Numbers in parentheses indicate standard deviation.

Table 3. PCR primers used in this study

Target gene	Name	Sequence (5′→3′)	Amplicon size (bp)[a]	Reference
16S rRNA	16SCF	GGCAGCAGTGGGGAATTTTC	164	Oh *et al.* (2012)
	16SUR	GTMTTACCGCGGCTGCTGG		
	16SMT[b]	CGCCGCGTGAGGGAGGAAGGTC		
mcyA	mcyAMF2	CTCCCAGAATACATGATACC	188	This study
	mcyAMR2	CCCAAAACTTCAGCCCAAAT		

[a] Based on the sequence of *Microcystis aeruginosa* NIES-843 (GenBank Accession No. NC_010296).
[b] TaqMan probe. Fluorochromes, CAL Fluor Gold 540 and BHQ-1, were attached at 5′ and 3′ end, respectively.

diluted DNA sample in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was mixed with PicoGreen dye. Fluorescence was measured with Rotor-Gene 6000 (Corbett Life Science, Australia). The DNA concentration was calculated from the regression equation of a standard curve in which fluorescence was plotted against the concentration of serially diluted plasmid lambda DNA.

Quantitative real-time PCR

To quantify the total *Microcystis* in samples, primers were designed to amplify the 16S rRNA gene of *Microcystis* (Table 3; Oh *et al.*, 2012). The primers, mcyAMF2 and mcyAMR2, were designed in the current study using the conserved region of *mcyA* gene sequences of *Microcystis* spp. from the GenBank database. All qPCR reaction mixtures contained 2× SyBr Green Real-time PCR Master Mix (Toyobo, Japan), primers, and template DNA. A 10-fold dilution series of the template DNA was prepared and amplified. Two negative controls without template DNA were included for each PCR run. Reaction conditions for qPCR were as follows: activation of the hot-start polymerase by incubation at 95°C for 15 min followed by 45 cycles of denaturation (95°C for 10 sec), annealing (56°C for 15 sec), and extension (72°C for 20 sec). Reaction conditions were controlled with a Rotor-Gene 6000 using the manufacturer's software. DNA melting curve analysis of the resulted amplicon was performed at the end of amplification by cooling the samples at 2°C/sec to 60°C and then increasing the temperature to 95°C at 0.03°C/sec. All tests were performed in triplicate.

The qPCR data were analyzed using Rotor-Gene 6000 Series Software version 1.7. For each real-time PCR assay, cycle threshold (C_t) values were computed automatically by the software using the maximum correlation coefficient approach. In this approach, the threshold is automatically determined to obtain the highest possible correlation (as determined by the coefficient of determination, r^2) between C_t values and the known DNA copy numbers of external standards. For samples with low copy number (<100 copies/ml), manual manipulation was sometimes required to find the appropriate C_t value, and was performed as necessary. Gene copies per sample were calculated using the linear regression equations for the standard curves from each assay. External standards used to determine the copy numbers were prepared using genomic DNA from *M. aeruginosa* UTEX 2388 or a single-copy plasmid standard, which was prepared by cloning the amplicon of the *mcyA* gene of *M. aeruginosa* UTEX 2388 into the T-blunt vector (Solgent, Korea).

The copy number of *mcyA* gene per cell (N_{PC}) was calculated using equation 1, which was modified from an equation to calculate the copy number of specific genes in the environmental samples by using the DNA concentration and real-time PCR data (Oh *et al.*, 2012).

$$N^{PC} = \frac{C_T \times A_N}{S_A \times MW_{bp} \times 10^6} \quad (1)$$

where C_T is the number of copies of the target gene in the DNA sample (copy/μg) estimated by qPCR using the single-copy plasmid as the external standard. A_N and MW_{bp} are Avogadro's number and the average molecular weight of a base pair (660 g per mole of bp; Vaitomaa *et al.*, 2003), respectively. S_A is the genome size (bp) of *Microcystis*. The value resulting from this calculation was rounded to the nearest whole number, and used as a conversion for calculating the total number of toxigenic cyanobacteria in environmental samples from qPCR data.

Results and Discussion

Specificity of primers for the *mcyA* gene

The *mcy* gene cluster in toxigenic *Microcystis* consists of 10 genes from *mcyA* to *mcyJ* (Dittmann and Börner, 2005). Six genes (*mcyA*, *mcyB*, *mcyD*, *mcyE*, *mcyH*, and *mcyJ*) have been used to enumerate toxigenic *Microcystis* using qPCR (Vaitomaa *et al.*, 2003; Kim *et al.*, 2010; Alexova *et al.*, 2011; Martins and Vasconcelos, 2011). We evaluated whether primers designed to target *mcyE*, mcyE-F2, and MicmcyE-R8, were suitable to quantify toxigenic *Microcystis* (Vaitomaa *et al.*, 2003). When PCRs were performed with DNA from several toxigenic *Microcystis* isolates from Korean reservoirs, no amplicon was found with two toxigenic strains, *Microcystis* sp. NIER 10004 and *M. aeruginosa* NIER 10038, isolated from Soyang and Paldang Reservoirs, respectively (Oh *et al.*, 2010). This result is likely due to the highly diverse sequences of *mcyD* gene in the water samples of Korean Reservoirs (data not shown).

The *mcyA* gene contains two adenylation and thiolation domains, a condensation domain, an *N*-methyltransferase domain, and an epimerization domain that encodes enzymes needed for the transformation of l-serine to *N*-methyl-dehydroalanine through *N*-methylation and dehydration (Tillett *et al.*, 2000). Owing to its conserved sequences, the *mcyA* gene is a suitable target for PCR-based quantification of toxigenic *Microcystis* (Hisbergues *et al.*, 2003; Rantala *et al.*, 2004; Oh *et al.*, 2010).

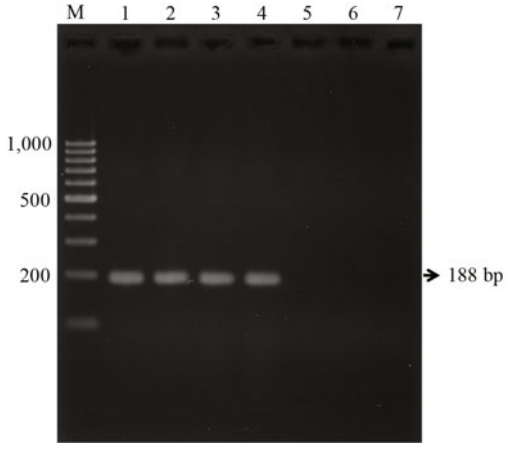

Fig. 1. Gel image of results from PCR amplification with *mcyA*-specific primers. Lanes: M for size marker (GeneRuler 100 bp DNA ladder; Fermentas); 1–4 for toxic *Microcystis*; 5–6 for non-toxic *Microcystis* strains; 7 for no template negative control. Lanes: 1, *Microcystis* sp. NIER 10004; 2, *M. viridis* NIER 10020; 3, *M. aeruginosa* UTEX 2388; 4, *M. viridis* NIES 102; 5, *M. novacekii* NIER 10022; 6, *M. aeruginosa* Mi 0601.

Furukawa and colleagues first used primers designed to target the *mcyA* gene, MSF and MSR-2R, to quantify toxigenic *Microcystis* by using qPCR (Furukawa *et al.*, 2006). Unfortunately, a recent examination of *mcyA* sequences in the GenBank database revealed mismatches in the reverse primer, MSR-2R. Furthermore, there were 59 sequences of isolated *Microcystis* that matched the region of the *mcyA* gene targeted with this primer in the GenBank database as of November 2012. Of these, 21 sequences (36%) were mismatched with MSR-2R at two bases. This mismatch may be due to the recent updating of *mcyA* gene sequences in the database. Another possibility is that the primer design was based on a cyanobacterial strain, *Microcystis* sp. UWOCC001, and validated with 8 toxigenic *Microcystis* isolated from four Japanese lakes (Furukawa *et al.*, 2006). This result indicates that the primer is not suitable for specific amplification of the *mcyA* gene. To address this problem and allow more accurate quantification of toxigenic *Microcystis*, we used *mcyA* gene sequences from the GenBank database and from cyanobacteria isolated from Korean freshwater reservoirs to develop and validate new PCR primers specific for the 3′ end of the condensation region in the *mcyA* gene.

To verify the specificity of our *mcyA* primers, we used the primers to amplify DNA from 10 microcystin-producing *Microcystis* strains and 2 non-producing strains. Gel electrophoresis of the resulting PCR products revealed single-amplification products when genomic DNA from microcystin-producing strains was used as a template in the PCR (Fig. 1). By contrast, DNA from the non-toxic strains yielded no reaction products. Thus, the primers designed in this study are specific for microcystin-producing strains of *Microcystis* spp.

The two most commonly used fluorescent reporters in qPCR assays are intercalating dyes such as SYBR green and Taq-nuclease assays (Martins and Vasconcelos, 2011). The SYBR green assay is simple, but it lacks specificity since the dye binds to all double-stranded DNA and not only to the target DNA. However, the melting curve analysis after the completion of PCR can overcome this drawback (Madani *et al.*, 2005). The Taq-nuclease assay requires an additional conserved region within the target amplicon sequence in order to make a probe (Livak *et al.*, 1995). Although this method provides highly sensitive and specific quantification, the design and production of the probe indicate that it is more expensive and complex to use when compared to the SYBR green assay (Martins and Vasconcelos, 2011). In this study, SYBR green was used for quantification because a conserved region could not be found in the product generated by the two primers. To ensure product specificity and to test for primer dimer formation, we performed a melting curve analysis on qPCR products from several different concentrations of target DNA. These analyses revealed uniform melting curves and no primer dimer peaks for all qPCR products tested. For all products, we observed a single peak at 80.9°C ±0.4°C, indicating that the mcyAMF2/mcyAMR2 primers amplified only the *mcyA* gene. Furthermore, samples containing no template yielded negative results. These results demonstrate that the qPCR method developed in this study may be applied for estimating the proportion of microcystin-producing *Microcystis* spp. within a mixed cyanobacterial population.

Quantification of the *mcyA* gene

We optimized our qPCR-based method for counting toxigenic *Microcystis* by using genomic DNA extracted from *M. aeruginosa* UTEX 2388 or a plasmid with one copy of the *mcyA* gene as external standards. Standard curves were developed using dilutions of template DNA ranging from 1.2×10^5 to 1.2×10^1 cells per reaction for *M. aeruginosa* UTEX 2388 or from 1.3×10^6 to 1.3×10^2 copies per reaction for plasmid DNA. Quantification using our method reliably produced highly significant linear relationships between the amount of starting material (cells or plasmid) and C_t values (Fig. 2). The resulting regression equations were Y= 28.52-3.341X (R^2=0.9976, p<0.0001) and Y=26.23-3.218X

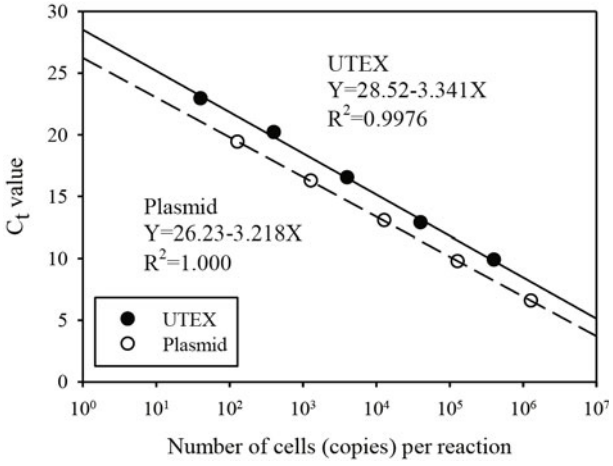

Fig. 2. Standard curves for *mcyA* gene by relating the number of cells (or copies) per reaction to the C_t values. Template DNAs were DNA extracts from *M. aeruginosa* UTEX 2388 or plasmid cloned with *mcyA* gene of UTEX 2388. All plots represent the mean of triplicate determinations.

(R^2=1.0000, p<0.0001) for plasmid DNA, where Y is the C_t and X is the amount of starting DNA (represented as logarithmic scale of cell or copy numbers). The amplification efficiencies for chromosomal and plasmid DNA were 99.4% and 103%, respectively. The dynamic range of detection for our method was 10^1–10^6 *mcyA* copies per 10 μl of reaction mixture.

Copy number of *mcyA* gene per cell of toxigenic *Microcystis*

The number of copies of a specific gene in a sample can be converted to the number of cells in the sample if the number of copies (copy number) of the gene per cell is known. To calculate the copy number of the *mcyA* gene in a *Microcystis* cell, the approximate genome size of *Microcystis* spp. has to be determined. In a previous study, a value of 4.70 Mb, estimated from the genome size of *Microcystis* sp. strain PCC 7941, was used to calculate the copy number of the *mcyE* gene in toxigenic *Microcystis* (Vaitomaa *et al*., 2003). However, the genome sizes of 6 *Microcystis* strains, *M. aeruginosa* NIES-843 (Kaneko *et al*., 2007) and 5 *Microcystis* spp. deposited in the Pasteur Culture Collection of Cyanobacteria (PCC 7005, PCC 7806, PCC 7813, PCC 7820, and PCC 7941; http://www.pasteur.fr/recherche/banques/PCC), ranged from 4.73 to 5.84 Mb. In this study, the genome size of *Microcystis* was considered to be 5.05 Mb, which was estimated from the average sizes of six *Microcystis* strains.

The number of copies of 16S rRNA and *mcyA* genes per genome were calculated by dividing the number of copies of each target gene as determined by qPCR by the number of cells in the reaction tube (Table 1). For amplification of the *mcyA* gene cluster, a high degree of correspondence was observed between input cell number (for genomic DNA) and input copy number (for plasmid DNA), suggesting the presence of one copy of the *mcyA* gene per UTEX 2388 genome. This correspondence was observed for all *Microcystis* strains investigated here, and the number of *mcyA* genes per cell was estimated as one in all cases (Table 1), which is in agreement with the number of *mcyA* genes reported for *M. aeruginosa* NIES-843 (Kaneko *et al*., 2007).

Some cyanobacterial strains with the *mcy* gene cluster cannot produce microcystins because they have defective genes due to mutation or evolutional loss (Tillett *et al*., 2001). However, the proportion of *Microcystis* with a defective *mcy* gene cluster is low in environmental samples (Vaitomaa *et al*., 2003). Thus, the number of *mcyA* gene copies found in environmental samples corresponds to the number of toxigenic *Microcystis* cyanobacteria in the sample.

Based on the amplification of the 16S rRNA gene, we estimated that there are two copies of this gene present in the genome of most *Microcystis* strains as *M. aeruginosa* NIES-843 (Table 1; Kaneko *et al*., 2007). The highest number of copies of the 16S rRNA gene, 2.7, was observed in *M. ichthyoblabe* NIER 10112. However, it is unclear whether this strain harbors 3 copies of the 16S rRNA gene, or whether this value represents an overestimation of the true copy number because of a high growth rate or incomplete replication of the bacterial genome (Zyskind and Smith, 1992; Koskenniemi *et al*., 2007). Notably, we also found the highest number of copies of the *mcyA* gene in this strain (Table 1). Based on these results, we estimated a copy number of two 16S rRNA genes per cell in the *Microcystis* strains studied here. It should be noted that the accuracy of this calculation as performed here assumes a homogeneous population of cyanobacteria, and this method may be less accurate for mixed cyanobacteria in environmental samples where the number of genomes per cell varies, depending on the growth phase.

Estimation of toxigenic *Microcystis* in environmental samples

Samples taken from five representative Korean freshwater reservoirs contained concentrations of microcystins ranging from 1.34 to 48.6 μg/L (Table 2). Using our qPCR-based assay, we estimated that the number of toxigenic *Microcystis* in those same samples ranged from 8.8×10^5 to 2.81×10^8 cells/L. We did not observe a linear correlation between the number of toxigenic *Microcystis* and the concentration of microcystins in these samples (regression analysis, P>0.17), although such a relationship had been previously reported in samples taken from Finnish and Japanese lakes (Vaitomaa *et al*., 2003; Furukawa *et al*., 2006). There are three hypotheses to explain the discrepancy between the number of toxigenic *Microcystis* and the concentrations of microcystins in the environmental samples. Firstly, the samples were taken from several reservoirs with quite different environmental conditions affecting microcystin production. Notably, in three samples containing high concentrations of microcystins (taken from Daechung, Yongdam, and Chungju Reservoirs), the specific production of microcystins per toxigenic *Microcystis* in the Daechung and Chungju Reservoir samples was almost 4 times higher than that in the Yongdam Reservoir sample. This difference may reflect the influence of environmental factors on microcystin production, because microcystin production is sensitive to a number of physicochemical parameters, including pH, light intensity, nitrogen and phosphorus concentrations, and iron concentration (Kaebernick and Neilan, 2001). Second, it is possible that other genera, such as *Anabaena* and *Oscillatoria*, produce microcystins in the samples. A Taq-nuclease assay with a *mcyD* probe highly specific to *Microcystis* spp. failed to detect the *mcyD* gene in some environmental samples taken from Lake Erie, in which a detectable concentration of microcystins was measured. This result was caused by presence of other microcystin-producers such as *Anabaena* and *Oscillatoria* (Rinta-Kanto *et al*., 2009). Under microscopic examination, *Oscillatoria* spp. was not found, whereas cyanobacterial strains belonging to the genera *Microcystis* and/or *Anabaena* were dominant in the samples (data not shown). Moreover, when PCR was conducted with primers designed to detect the *mcyA* and *mcyB* genes of *Anabaena* spp., no amplicon was observed (Oh *et al*., 2010). These results make it unlikely that other genera that produce microcystins against *Microcystis* are present. Finally, the activity of the *mcy* gene in microcystin production varies among strains harboring these genes. In order to estimate the activity of *mcy* genes, a more detailed examination of cyanobacterial physiology is needed. This should include quantification of the *mcy* gene mRNA using reverse transcription-real-time PCR, because this method allows the measurement of activity of structural genes (Church *et al*., 2005).

Harmful cyanobacterial blooms become a global threat

because of their production of cyanotoxins. Many countries set up guidelines for the concentration of microcystins in recreational and drinking water (Burch, 2008). If the number of toxigenic cyanobacteria can be measured simply and accurately, it could be a good indicator of the potential amount of toxin production. In this study, we developed and evaluated a qPCR-based method that uses new molecular markers from the conserved region of the *mcyA* gene to quantify toxigenic *Microcystis* in environmental samples. One of the pitfalls of the qPCR method is the difficulty of converting the copy number of specific gene into the number of cells harboring this gene (Rinta-Kanto *et al*., 2009). In this study, we found conversion factors to convert the copy number of 16S rRNA and *mcyA* genes to the cell number of both total and toxigenic *Microcystis* in the environmental samples, respectively. Quantification of potentially toxigenic cyanobacteria using qPCR in the environmental samples may help determine the physiological and ecological parameters that regulate cyanotoxin production (Pearson and Neilan, 2008). Moreover, the qPCR method allows the detection of toxigenic *Microcystis* at an earlier stage than that which can be achieved by the detection of the toxin via chemical analyses (Fortin *et al*., 2010). Thus, this method can be used to manage the risk from harmful cyanobacterial blooms caused by toxigenic *Microcystis* through early and efficient monitoring of the most at-risk water bodies.

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