RESEARCH ARTICLE

Annual variation of *Microcystis* genotypes and their potential toxicity in water and sediment from a eutrophic reservoir

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Abstract

The relative genetic diversity of microcystin-producing *Microcystis* in the water and sediment of the Daechung Reservoir, Korea, was investigated over an entire year, including the cyanobacterial bloom season. The cells of potentially toxic *Microcystis* strains containing *mcyJ* genotypes and cells containing the genus-specific *cpcBA* gene were quantified by a real-time PCR. The ratio of cells with *mcyJ* genotypes to the total *Microcystis* population in the water body was the highest (68.3%) in August when the cyanobacterial bloom reached its peak and the microcystin concentration in the water began to increase. A denaturing gradient gel electrophoresis profile analysis of the *mcyJ* genotypes performed to monitor any changes in the toxic *Microcystis* population showed the appearance of new genotypes and the disappearance of existing genotypes in the reservoir water collected during the summer months, when compared with the profile for the samples collected in spring and autumn. However, very little change was observed over the course of the year as regards the population diversity of the sediment samples.

Introduction

The assessment of cyanobacterial toxins in water is heavily dependent on understanding the population dynamics of the cyanobacteria from which they are produced. *Microcystis* is one of the most well-known freshwater, bloom-forming cyanobacteria that produce hepatotoxic microcystins. In *Microcystis* blooms, the population is often composed of both nontoxic and toxic strains (Via-Ordorika *et al.*, 2004; Welker *et al.*, 2004). Thus, understanding the growth dynamics of *Microcystis* spp. in terms of their toxicities and genetic properties is very important for water quality control.

Microcystis blooms are thought to be linked to the cyanobacterial annual life cycle, as the blooms regularly occur at a particular place and at a specific time of the year. The relationship between the pelagic and benthic populations, particularly as a recruitment source for *Microcystis* spp. bloom, has already been the focus of many studies. In spring, it is already known that the sediment in which cells overwinter from the previous autumn represents the main recruitment source for *Microcystis* (Ihle *et al.*, 2005). Verspagen *et al.* (2004) suggested that the overwintering pelagic and benthic *Microcystis* populations both have an influence on the occurrence of bloom in the next year. In another study, the recruitment of *Microcystis* from the sediment in a shallow lake was concluded to be a passive process that was mainly dependent on resuspension (Verspagen *et al.*, 2005). However, a more precise understanding of the role of benthic and pelagic *Microcystis* spp. in recruitment and bloom formation, especially in terms of microcystin production, is needed to aid and improve water quality control.

To determine the diversity and for a quantitative analysis of *Microcystis* spp. in environment samples, the 16S rRNA

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gene-internal transcribed spacer (rRNA-ITS) gene is often used as a molecular probe (Janse *et al.*, 2003; Humbert *et al.*, 2005). Although a denaturing gradient gel electrophoresis (DGGE) analysis of the rRNA-ITS can be used to screen the toxic and nontoxic *Microcystis* diversity, this method still requires a further confirmation analysis of the toxicity, such as MS of the microcystins (Janse *et al.*, 2004), and is unable to differentiate all the strains in the environment according to their toxicity, due to limited database information on the toxicity and the rRNA-ITS sequence of each strain (Kardinaal *et al.*, 2007).

In another approach, the gene sequences of *cpcB* and *cpcA* and the associated intergenic space (*cpcBA*), which code for phycocyanin, an accessory pigment of the photosynthetic apparatus in cyanobacteria, have been used for a phylogenetic analysis of the cyanobacterial diversity (Neilan *et al.*, 1995; Bittencourt-Oliveira *et al.*, 2001; Kim *et al.*, 2006). While the *cpc* gene has been used as a probe for the general cyanobacterial diversity and detection of toxic *Microcystis* cells, recent advances in the analysis of microcystin-synthesizing genes have presented the opportunity to design molecular probes for the detection of microcystin-producing cyanobacteria in environment samples. Microcystins are produced by the enzyme complex synthetase, namely *mcy* genes, which code for both nonribosomal peptide synthetase and polyketide synthase. The six genes, *mcyA*, *mcyB*, *mcyC*, *mcyD*, *mcyE*, and *mcyG*, encode a multienzyme synthase/synthetase complex, while the remaining genes, *mcyF*, *mcyI*, and *mcyJ*, encode proteins that function in epimerization, dehydration, and *O*-methylation, respectively (Tillett *et al.*, 2000). The idea of using the *mcy* genes as molecular probes for the detection of toxic *Microcystis* strains was first applied by Tillett *et al.* (2001). In subsequent studies, *mcyA* (Furukawa *et al.*, 2006), *mcyB* (Kurmayer *et al.*, 2003), *mcyD* (Rinta-Kanto *et al.*, 2005), and *mcyE* (Vaitomaa *et al.*, 2003) have also been used for a quantitative analysis of toxin-producing cyanobacteria from the environment based on a real-time PCR. In addition, the *mcyA* gene has been targeted to analyze the diversity of microcystin-producing cyanobacteria in lakes by constructing a phylogenetic tree based on the amino acid sequence (Rinta-Kanto & Wilhelm, 2006) or using the banding pattern of a highly iterated palindromic PCR (Wilson *et al.*, 2005). However, as yet, there has been no report on a DGGE analysis using the *mcy* genes, possibly due to the high degree of sequence dissimilarity that exists between the species and subspecies within *Microcystis*.

Accordingly, the studies reported here were aimed at assessing the changes in the *Microcystis* population diversity in reservoir water and sediment over the course of a year, by determining the percentage of toxic *Microcystis* spp. using a real-time PCR assay of the *cpcBA* and *mcyJ* genes. In addition, the diversity of the *cpcBA* and *mcyJ* genotypes in the reservoir water and sediment samples was investigated by DGGE profile fingerprinting the *Microcystis*.

Materials and methods

Sample collection

Surface water and sediment samples were collected periodically at a point in the Daechung Reservoir (36°20′54″N, 127°33′43″E), Chungcheongbuk-do, Korea (Fig. 1). The Daechung Reservoir is a branch-type lake, formed by the construction of a 72-m-high dam upstream on the Geum River in Korea (Oh *et al.*, 2001). The sampling site was located at one of the mouths of the lake where a constant inflow occurred. The site was dominated by *Microcystis*, and cyanobacterial blooming was observed to occur there almost every year. The water depth at the site was influenced by the amount of rainfall and the season, and showed changes within a range of 2–8 m. A 20-L sample of surface water (0–20 cm) was collected monthly, from March 2005 to February 2006. To monitor the microcystin concentration during the bloom season, weekly sampling was conducted from June to November 2005. At the same site and time as the water samples were taken, sediment samples were collected from material within 1 cm of the surface of the submerged sediment using a tailor-made core sampler

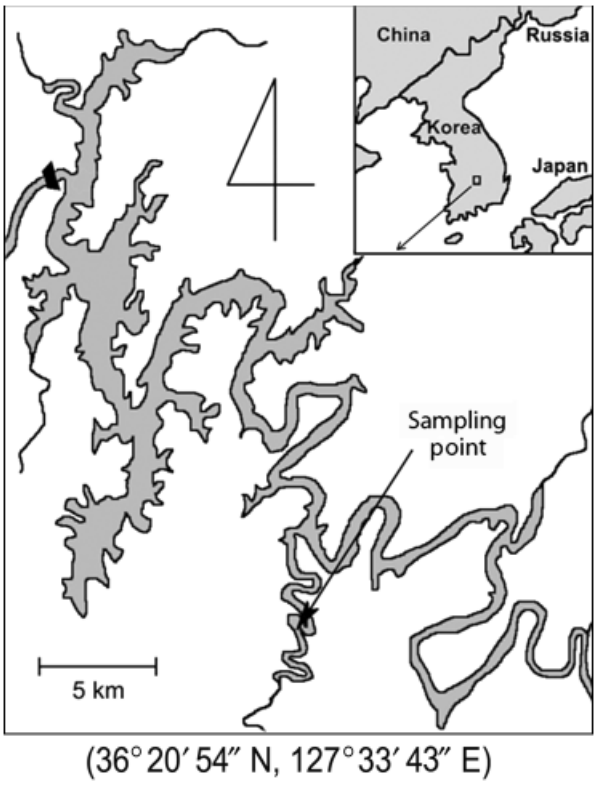

Fig. 1. Sampling site and location of Daechung Reservoir in Korea.

(length 533 mm, diameter 54 mm). The collected water and sediment samples were maintained at 4 °C and transported to the laboratory. Upon arrival at the laboratory, the samples were stored for subsequent DNA extraction. The water samples were first filtered using a 0.45-μm nitrocellulose filter (Millipore System, Bedford, MA), and the filter paper and sediment samples were then stored at −70 °C until use.

DNA extraction

The cells were removed from the filter paper by brief sonication in 10 mL of a TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, pH 7.6) for 20–30 s, and the cells were then harvested by centrifugation (6300 ***g***, 10 min) (Ko *et al.*, 2004). The genomic DNA of the concentrated cells was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The genomic DNA from the sediment samples was extracted using a Power Soil DNA Kit (MO BIO Laboratories Inc., Carlsbad, CA), according to the protocol of the manufacturer. The purity and quantity of genomic DNA were estimated using a spectrophotometer (ND-1000 UV/Vis, NanoDrop Technologies, Wilmington, DE).

Environmental factors and cell count of *Microcystis*

The chlorophyll *a* from the cells obtained by filtration with a filter paper (Whatman GF/C) was extracted using a chloroform–methanol mixture (2 : 1, v/v), and the concentration was measured using a fluorometer (Turner 450, Barnstead/Thermolyne, Dubuque, IA) (Wood, 1985). The number of *Microcystis* cells in the water and sediment samples was determined using a hemocytometer (Fuchs-Rosenthal, Paul Marienfeld GmbH & Co., Lauda-Königshofen, Germany) and a phase-contrast microscope (Microphot-FXA, Nikon, Tokyo, Japan). The cyanobacteria in the water samples were first fixed with Lugol's solution, while the sediment samples were filtered using three consecutive testing sieves with decreasing pore sizes (200, 100, and 25 μm) to remove the suspended particles, and the filtered samples were then used to determine the cell number. The microcystin concentration dissolved in the water samples was determined using the HPLC method (Oh *et al.*, 2001).

Primer design

The sequences of the *cpcBA* genes of 138 *Microcystis* strains and 226 other cyanobacterial strains, representing all five cyanobacterial classifications (Rippka *et al.*, 1979), were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and aligned using the LASERGENE program (DNASTAR Inc. ver. 6.0) to identify a uniquely conserved sequence among the *Microcystis* genes. As a result, two conserved regions in the *cpcBA* gene were selected for the primer set design: cpc57F (5′-AACCTATGTAGCTTTAGGAGTACC-3′) and cpc356R (5′-CTTAAGAAACGACCTTGAGAATC-3′). The primer set for the detection of toxic *Microcystis* was designed based on the alignment of the sequences of various microcystin synthetase genes (*mcyA*, 56 cyanobacterial strains; *mcyB*, 30 cyanobacterial strains; *mcyD*, 25 cyanobacterial strains; *mcyE*, 57 cyanobacterial strains; *mcyJ*, 11 cyanobacterial strains). In addition, a sequence conserved in the *mcyA, B, D, E*, and *J* regions of the microcystin synthetase genes was used to design the primers for targeting the microcystin-producing genotypes. Only the primers targeting *mcyJ*, mcyJMF (5′-TAGCTAAAGCAGGGTTATCG-3′) and mcyJMR (5′-TCTTACTATTAACCCGCAGC-3′) were suitable for generating a DGGE profile. The theoretical melting temperatures (T_m) of the primers were determined using a program available on the Internet (http://www.bioneer.com). The T_m values for cpc57F and cpc356R were 49.3 and 50.9 °C, respectively, and those for mcyJMF and mcyJMR were 49.1 and 48.9, respectively. The newly developed *mcyJ* primer sets were tested using 29 reference strains of representative cyanobacteria, which included both toxic and nontoxic *Microcystis* spp., to determine whether the primers demonstrated an adequate sensitivity to discriminate toxic strains of *Microcystis* spp. The specificity of the primer set was also compared with the *mcyE* gene-specific primer sets for mcyE-F2-MicmcyE-R8 (Vaitomaa *et al.*, 2003) and mcyE-R4 (Rantala *et al.*, 2004) using the above reference strains.

PCR, DGGE, and real-time PCR

Separate PCR reactions were performed for each water and sediment sample: one to detect the percentage of *Microcystis* cells in the total bacterial population and one to determine, from among these *Microcystis* cells, the percentage of potentially toxic *Microcystis* spp. To detect the total *Microcystis* population, a portion of the *cpcBA* gene was PCR amplified using the cpc57F–cpc356R primer set, to detect only potentially toxic *Microcystis*, while the *Microcystis* spp. *mcyJ* gene fragment was amplified using the mcyJMF–mcyJMR primer set. The reaction was conducted in a 50 μL (total volume) reaction mixture containing 5 μL of 10 × the PCR buffer, 4 μL of a 2.5 mM mixture of each dNTP, 20 pmol of the respective primer set, 2.5 U DNA polymerase (Takara, Japan), and 10 ng of template DNA. The PCR protocol consisted of preincubation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, plus a final extension step at 72 °C for 5 min. The PCR was performed using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA), and the amplified PCR products were visualized on 1.5% agarose

gels stained with ethidium bromide under UV light. For the DGGE analysis, the PCR reactions were performed with modified primer sets of cpc57F–cpc356R or mcyJMF–mcyJMR, each with a GC clamp (5′-CGCCCGCCGCGC CCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) attached at the 5′ end of the forward primer (Ishii & Fukui, 2001). The same PCR protocol was used as for the primers lacking the GC clamp. The amplification products were separated in a denaturing gradient gel of an 8% polyacrylamide gel [8% acrylamide–bisacrylamide (37.5 : 1, v/v), 40% (v/v) formamide, 7 M urea] containing a linear denaturant concentration gradient of 40–60% for the *cpcBA* primers, or 30–50% for the *mcyJ* primers. The gels were run for 4.5 h at 60 °C and 200 V, using a BioRad DCode System (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, the DGGE gels were stained with ethidium bromide and photographed under UV transillumination (Muyzer *et al.*, 1993). Thereafter, the bands were excised from the DGGE gel and incubated in distilled water (30 μL) for 24 h at 4 °C. The eluent was then reamplified for sequencing using the same primers as those used in the PCR reaction. The real-time PCR analysis (qPCR) was performed using TaqMan probes for *cpcBA*, 5′-AGCTACTTCGACCGCGCCG-3′, and *mcyJ*, 5′-TCGAGTTTTGCAGCCCGGTG-3′. The TaqMan probes included a fluorescent reporter dye (6-carboxyfluorescein) and Black Hole quencher dye attached to the 5′ and 3′ ends, respectively. The real-time PCR was performed using a DNA Engine Opticon 2 system (CFB-3220, Bio-Rad Laboratories) with 20 μL (total volume) of the reaction mixture containing 10 μL of a DyNAmo^{TM} Probe qPCR kit (Finnzymes Inc., Espoo, Finland), 5 pmol of each primer, 5 pmol of a TaqMan probe, and 1 μL of template DNA. The qPCR programs for both *cpcBA* and *mcyJ* consisted of 15 min at 95 °C, followed by 50 cycles at 94 °C for 20 s and 60 °C for 1 min.

Standard curve of qPCR

A linear equation was determined to estimate the cell number in the sample using the DNA of *Microcystis aeruginosa* PCC 7806 as the standard. The DNA of this standard strain was extracted from cell dilutions that ranged from 10^7 to 10^8 cells mL^{-1} for which the exact cell number was determined using a hemocytometer under an optical microscope. The DNA concentration of the extract was then measured using a spectrophotometer. Finally, the correlation of the DNA concentration and the cell number, based on five dilutions of the standard strain, was used to calculate the cell number in the environmental samples. Individual standard curves were established for the *cpcBA* and *mcyJ* probes, based on the correlation between the DNA concentration and the threshold cycles (C_t) of the diluted standard samples, where C_t was automatically determined using OPTICON MONITOR software (ver. 3.0) during the real-time PCR reaction. The DNA concentration in the environmental samples was then calculated using the correlation between the C_t value of the *cpcBA* and *mcyJ* probes and the DNA concentration of the standard DNA. Finally, the proportion of potentially toxic *Microcystis* to the total number of *Microcystis* cells was determined by dividing the *mcyJ* gene number by the *Microcystis*-specific *cpcBA* gene number.

Nucleotide sequence accession numbers

The nucleotide sequences obtained from the environmental samples using the DGGE analysis were deposited in the GenBank under accession numbers EF579623–EF579628 (*cpcBA* sequences from water samples), EF579629–EF579636 (*cpcBA* sequences from sediment samples), EF579637–EF579640 (*mcyJ* sequences from water samples), and EF579641–EF579645 (*mcyJ* sequences from sediment samples).

Results

General conditions in Daechung Reservoir

The water temperature in the Daechung Reservoir reached its highest point, 30 °C, in summer, and declined to its lowest point, 5 °C, in December. The water temperature remained low through the winter, until February. The majority of cyanobacteria that cause massive blooms and produce microcystins in the Daechung Reservoir during the summer belong to the species *Microcystis* (Oh *et al.*, 2001). To monitor the changes in the *Microcystis* concentration in both the water and the sediment over the course of a year, the chlorophyll *a* concentration and the direct microscopic cell count were determined for samples collected monthly from March 2005 to February 2006. After the month of May, the concentration of cyanobacteria in the reservoir water began to increase, and the highest *Microcystis* cell count, determined by microscopic enumeration, was recorded at about 10^7 cell mL^{-1} in the samples collected in August and September (Fig. 2a, b), values positively correlated with the chlorophyll *a* concentration (Fig. 2a). The highest number of *Microcystis* in the sediment was recorded in November (Fig. 2b).

Microcystin concentration in reservoir water and cyanobacterial diversity

The dissolved microcystin concentration in the water was measured using an HPLC analysis of the water samples collected at the same site each week from June to November 2005. The concentration was low until early June, then increased gradually and reached its highest point, 613 ng L^{-1}, on August 30, a time corresponding to cyanobacterial bloom in the Daechung Reservoir (Fig. 2a, b). The

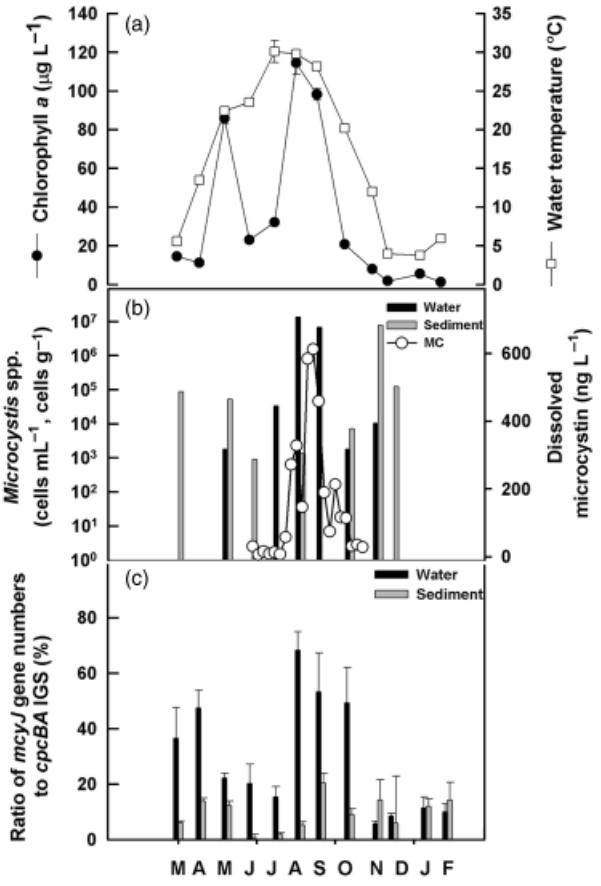

Fig. 2. (a) Chlorophyll *a* and water temperature for each month during the study. (b) Number of *Microcystis* cells in water and sediment, as determined by microscopic observation and the dissolved microcystin concentration in water. (c) Proportion of the *mcyJ* gene number against the *cpcBA* gene number for the total *Microcystis* population in water and sediment samples.

microcystin concentration decreased along with a decline in the temperature and chlorophyll *a*.

Ratio of *mcyJ* and *cpcBA* genotypes in reservoir water and sediment

A melting temperature curve analysis using syber-green dye and the primer sets of *cpcBA* and *mcyJ* genes showed that the PCR produced only one product (data not shown). Standard curves were developed to determine the number of total *Microcystis* and toxic *Microcystis* cells in the environmental samples (Fig. 3). The number of toxic *Microcystis* cells represented by the *mcyJ* genotype was determined by performing a real-time PCR with primers specific for the *mcyJ* gene of *Microcystis*; the total number of *Microcystis* cells was determined by performing a real-time PCR with a primer set targeting the *cpcBA* gene, which is specific for the *Microcystis* genus. The ratio of the *mcyJ* gene number to the *Microcystis cpcBA* gene number was determined for the

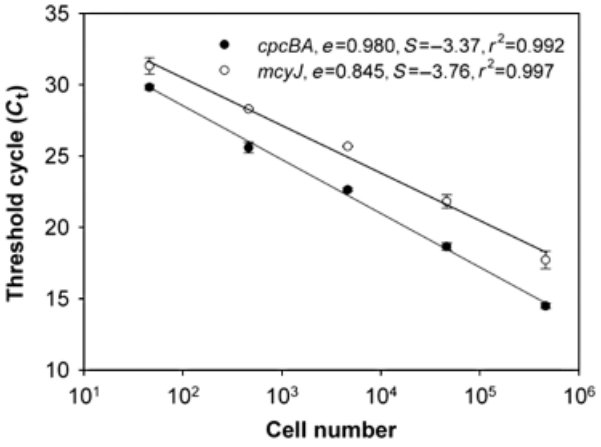

Fig. 3. Standard curve for cell number to C_t values of *cpcBA* or *mcyJ* genes by relating known DNA concentrations of *Microcystis aeruginosa* PCC 7806 to those obtained by a real-time PCR assay of DNA extracted from predetermined cell number. The experiment was repeated in triplicate. All plots represent the mean of triplicate tests, with the error bars showing the SD. The amplification efficiency was calculated as follows: $e = 10^{-1/S} - 1$, where S is the slope.

samples collected from the water and sediment. The ratio of *mcyJ* genes to *cpcBA* genes was the highest (68.3%) for the water samples collected in August (Fig. 2c), indicating that about two-thirds of the *Microcystis* population found in the reservoir water during the bloom season was composed of *Microcystis* with the *mcyJ* genotype, representing the potential to produce microcystins. Meanwhile, the percentage was low from November until the next spring, during which time the total *Microcystis* cell number was low and the water temperature was below 15 °C. In contrast, the cells found in the sediment were mainly composed of those without the *mcyJ* gene, and the population did not exhibit any appreciable seasonal changes.

DGGE profile of *mcyJ* and *cpcBA* from reservoir water and sediment

Twenty-four samples of water and sediment from the Daechung Reservoir were obtained each month for 1 year, and analyzed for changes in the relative and proportional diversity of *Microcystis cpcBA* and *mcyJ* genes. The dynamics of the total *Microcystis* population were analyzed by DGGE with a PCR primer set targeting the *cpcBA* gene of all *Microcystis* spp. This PCR primer set was shown to detect *cpcBA* genes representative of *Microcystis* spp., and yet not those of other cyanobacteria (Table 1). From the DGGE *cpcBA* gene profile, the sequences of six and eight major bands were determined from the water and the sediment, respectively (Fig. 4). A greater dynamic change in the *Microcystis cpcBA* genotype was observed in the water samples than in the sediment. In general, the number of *cpcBA* bands observed in the DGGE profile was low for the

Table 1. Test of PCR primers for the specificity and detection of microcystin production using reference strains

Cyanobacterial strains*		Microcystin production tested by†		Specificity of primers‡	
		HPLC	PPIA	*cpcBA*	*mcyJ*
Microcystis aeruginosa	UTEX 2388	+	+	+	+
Microcystis aeruginosa	PCC 7806	+	+	+	+
Microcystis aeruginosa	UTEX 2666	+	+	+	+
Microcystis aeruginosa	NIES 90	+	+	+	+
Microcystis viridis	NIES 102	+	+	+	+
Microcystis wesenbergii	NIES 107	+	+	+	+
Microcystis viridis	NIES 1058	+	+	+	+
Microcystis wesenbergii	NIES 1067	−	−	+	−
Microcystis aeruginosa	NIES 1075	−	−	+	−
Microcystis ichthyoblabe	NIES 1182	−	−	+	−
Oscillatoria tenuis	NIES 33	−	−	−	−
Planktothrix agardhii	NIVA-CYA 126	+	+	−	−
Planktothrix agardhii	NIVA-CYA 127	+	+	−	−
Planktothrix sp.	49	NT	NT	−	−
Planktothrix sp.	97	NT	NT	−	−
Anabaena sp.	202A1	NT	NT	−	−
Anabaena sp.	90	NT	NT	−	−
Anabaena flos-aquae	UTEX 2577	−	−	−	−
Anabaena flos-aquae	NIVA-CYA 83/1	+	+	−	−
Nodularia sp.	HEM	NT	NT	−	−
Nodularia sp.	AV1	NT	NT	−	−
Nodularia spumigena	UTEX 2092	−	−	−	−
Nostoc sp.	152	NT	NT	−	−
Nostoc sp.	PCC 7120	−	−	−	−
Synechocystis sp.	PCC 6803	−	−	−	−
Lyngbya hieronymusii	KCTC-AG10199	−	−	−	−
Merismopedia tenuissima	NIES 230	−	−	−	−
Synechococcus leopoliensis	UTEX 625	−	−	−	−
Spirulina platensis	NIES 39	−	−	−	−

*Some strain designations include culture collection abbreviations. Culture collections: UTEX, The Culture Collection of Algae at University of Texas, Austin, TX; PCC, Pasteur Culture Collection, Paris, France; NIES, National Institute for Environmental Studies, Tsukuba, Japan; NIVA-CYA, Norwegian Institute for Water Research, Oslo, Norway; KCTC, Korean Collection for Type Cultures, Daejeon, Korea; Strains 49, 97, 202A1, 90, HEM, AV1, and 152, from the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland.

†The presence (+) or lack (−) of microcystins by the cyanobacterial culture was analyzed by HPLC and a protein phosphatase inhibition assay (PPIA).

‡Specificity of *Microcystis cpcBA* primers (cpc57F and cpc356R) and *mcyJ* primers (this study). The presence (+) or absence (−) of PCR amplification.

NT, not tested.

water samples collected in the months when the water temperature was $< 15\,^{\circ}C$ (from December to April), while the diversity and complexity of the *Microcystis* population increased, as evidenced by the greater number of DGGE profile bands when the temperature was $> 15\,^{\circ}C$ (from May to November). Two genotypes (DGGE bands C-W-3 and C-W-5) were observed in all the water samples throughout the year, whereas two other bands (DGGE bands C-W-4 and C-W-6) were only observed in summer. While the DGGE profiling of the sediment samples showed no significant temporal changes in the *Microcystis* population (Fig. 4b), the number of total recovered bands was greater than that for the water samples.

To monitor the population dynamics of potentially toxic *Microcystis* spp., the DGGE profile of the *mcyJ* gene was analyzed (Fig. 4c, d). In contrast to the sediment samples, the potentially toxic *mcyJ* genotypes in the water samples underwent a dynamic temporal change during the summer (Fig. 4). Notably, the diversity of the toxic genotypes (*mcyJ*) in the water samples was reduced during the cyanobacterial bloom season (August to October), and then returned to a broader diversity in the spring, whereas the banding pattern for the sediment samples showed very little change over the course of the entire year.

Phylogenetic analysis of *cpcBA* genotypes from DGGE bands

A phylogenetic tree to show the positional relationship of the DGGE-retrieved sequences with those of other strains in

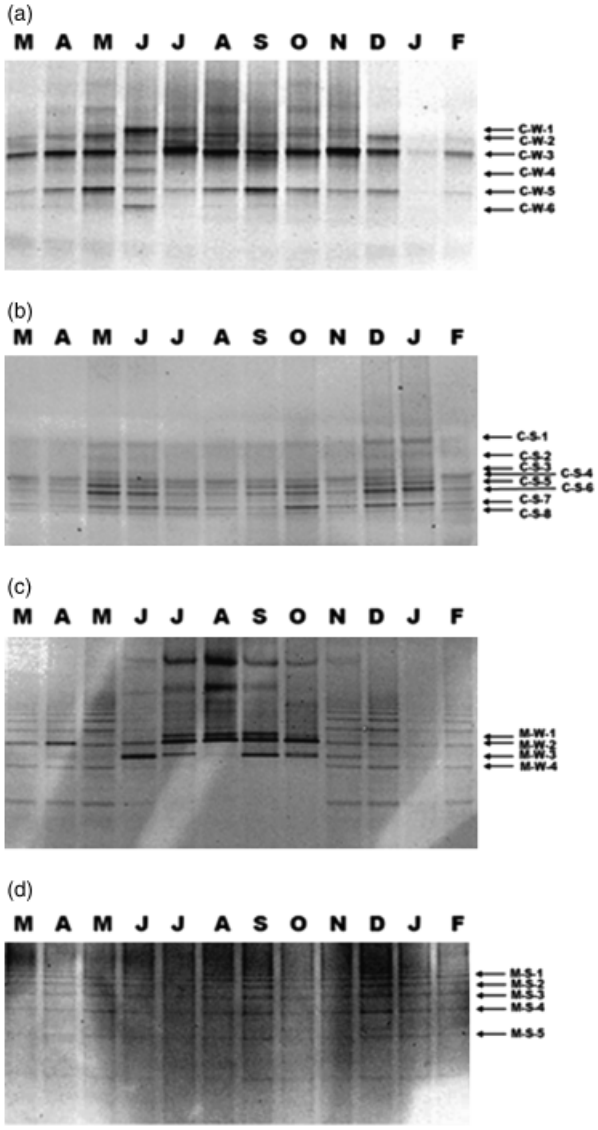

Fig. 4. DGGE profile of the *cpcBA* gene fragment in (a) water and (b) sediment, plus the *mcyJ* gene fragment in (c) water and (d) sediment. The bands with retrieved sequence information were designated as follows: C-W-n, the *cpcBA* gene from the water body; C-S-n, the *cpcBA* gene from the sediment; M-W-n, the *mcyJ* gene from the water body; M-S-n, the *mcyJ* gene from the sediment (*n* is an integer number).

the *Microcystis* genus was constructed using the neighbor-joining algorithm. The tree showed that all the bands retrieved from DGGE analysis belonged to the genus *Microcystis* (Fig. 5). Despite the lack of significant bootstrap support, it was confirmed that some bands from the water and sediment samples, such as the sequences of C-W-2 and C-S-4, were clustered together. The *mcyJ* genotypes sequenced in this study were almost identical to each other (> 99% similarity) and to other known sequences from the genus *Microcystis* (data not shown).

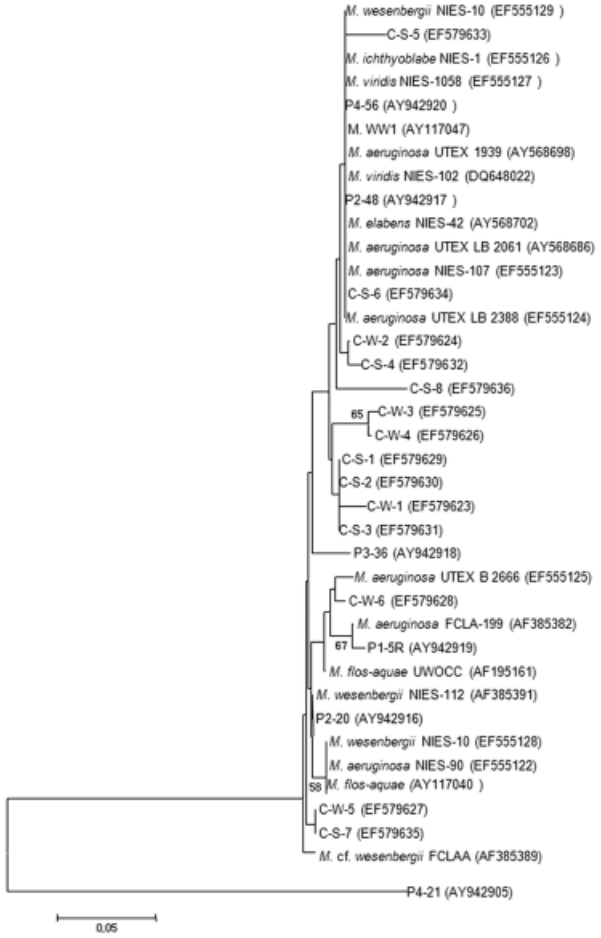

Fig. 5. Phylogenetic tree based on the neighbor-joining algorithm of *cpcBA* genes isolated from water and sediment samples. DGGE bands and other representative strains in the genus *Microcystis*. P1-5R, P2-20, P2-48, P3-36, and P4-56 are sequences from Daechung Reservoir samples (Kim *et al.*, 2006). The sequences were aligned using the BIOEDIT program, and the tree was constructed using the CLUSTAL W program. Bootstrap values > 50% are indicated at the nodes. The bar indicates 0.05 substitutions per nucleotide position.

Discussion

For the detection and quantitative analysis of microcystin-producing *Microcystis* spp. in environment samples, a number of molecular probes targeting genes involved in microcystin synthesis have been used: *mcyA*, *mcyB*, *mcyD*, and *mcyE*. In this study, the *mcyJ* gene was selected as the target, because among the primer sets tested, only the PCR primers targeted against this gene were competent to produce a DGGE profile of the *Microcystis* population. To the best of our knowledge, there has been no previous report on the DGGE profile of other *mcy* gene fragments for analyzing the diversity of microcystin-producing cyanobacteria from the environment. To validate the *mcyJ* gene as a

toxicity probe for *Microcystis*, various template DNAs were tested from various cyanobacterial reference strains, including 10 *Microcystis* strains and 19 strains from other genera. The results showed that the *mcyJ* gene fragment could be used as a surrogate to detect bacteria capable of producing microcystins (Table 1). Tooming-Klunderud *et al.* (2008) analyzed the genetic structure of the *mcy* genes from 17 *Microcystis* strains collected from the field. Although not the main purpose of their study, all the strains containing a *mcyJ* gene also happened to be microcystin producers.

Not all *mcy* gene-harboring *Microcystis* strains are toxic. Tillett *et al.* (2001) analyzed the genetic variation of 37 strains of *Microcystis*, and showed that two of 20 strains tested contained the *mcyA* gene and yet were not toxic. There are strains with a *mcyABC* gene cluster and yet no other *mcy* genes, which can explain the faulty PCR results when using *mcyA* (Tooming-Klunderud *et al.*, 2008). The toxic strains of *Microcystis* whose genomes have been entirely sequenced (NIES-843 and PCC 7806) have one copy of the *mcyJ* gene within the *mcy* cluster (Kaneko *et al.*, 2007; Frangeul *et al.*, 2008). In the report of Tanabe *et al.* (2004), sequencing *mcyJ* and the other three genes in the *mcy* gene cluster for 10 toxic *Microcystis* strains showed that all the strains had the *mcyJ* gene. In the comprehensive study performed by Tanabe *et al.* (2009), a multilocus sequence typing analysis of the *mcy* genes of *Microcystis* strains found that 113 of 118 strains with a positive PCR result targeting the *mcyJ* gene were also toxic (i.e. capable of producing microcystins). Thus, a clear interpretation of the relation between the presence of the *mcyJ* gene and the production of microcystins by *Microcystis* requires more intense study.

In the samples collected from the Daechung Reservoir, the ratio of *mcyJ*-positive to *mcyJ*-negative *Microcystis* cells was comparable to that reported in other studies. In a similar study that counted the number of potentially toxic microcystin-producing *Microcystis* spp. in water samples collected from a lake in Japan, the ratio of the *mcyA* gene to the *cpcBA* gene varied from 0.3% to 35% (Yoshida *et al.*, 2007). Another study reported that the *mcyB* gene was found in 2–28% of *Microcystis* cells containing the *cpcBA* gene (Schober *et al.*, 2007). In this study, the highest percentage was 68.3%, which was higher than that reported previously.

The higher percentage of *mcyJ*-positive cells determined from the present data may have resulted from using a PCR primer set with a greater sensitivity than those used previously. It is also possible that the Daechung Reservoir inherently comprises a greater percentage of *mcyJ*-positive genotype *Microcystis* spp. than that found in other waters. Notably, the highest ratio appeared in August, a time coincident with the peak of *Microcystis* bloom in the Daechung Reservoir and the initial increase in the microcystin concentration.

DGGE profiling provided a detailed analysis of the dynamic changes in the *Microcystis* spp. population in the water and sediment samples. As the *mcyJ* gene sequence isolated from the DGGE analysis was short and showed little diversity, a phylogenetic analysis of the *mcyJ* genotypes was not possible. The DGGE profile of the *mcyJ* gene-harboring population in the water was substantially changed during the summer months (July to September). The DGGE *cpcBA* gene profile showed that some *Microcystis* strains in the reservoir water were present only perennially or temporarily. It was reported previously that recruitment from the sediment in the Quitzdorf Reservoir during the spring season was $< 3\%$, as the majority of the cells in the sediment were prone to degradation (Ihle *et al.*, 2005). The sources potentially responsible for population changes in reservoir water include inflows and sediment. Verspagen *et al.* (2004) also proposed that resuspension by wind is the driving force for the recruitment of *Microcystis* from the sediment in the case of shallow water. The present study site is at the mouth of the reservoir with a shallow depth, and resuspension occurred due to constant inflows as well as wind. Thus, the recruitment of benthic resting cells may have occurred all year round, playing a significant role in the formation of the cyanobacterial bloom in summer.

Most DGGE bands of the *cpcBA* and *mcyJ* genotypes in the sediment samples were observed to be independent of the month or the season of the sample collection. The sediment represents one inoculation source for the Daechung Reservoir and, based on the river-like property of the reservoir, another source may be located upstream. After rainfall, the depth of the water at the collection site increased as a result of heavy inflows that occurred upstream. Moreover, the inflows were shown to contain an abundance of nutrients suitable for cyanobacterial growth, with concentrations of nitrate and phosphate as high as 2.56 and 154 $\mu g\, L^{-1}$, respectively (Kim *et al.*, 2006).

Welker *et al.* (2007) compared the dynamics and diversity of the benthic and planktonic *Microcystis* populations in a reservoir based on the oligopeptide chemotype composition. In their study, a few dominant chemotypes with microcystin-producing ability emerged in the water body at the time of blooming, and these chemotypes also appeared in the sediment after deposition. Meanwhile, Briand *et al.* (2009) found that a non-microcystin-producing genotype became dominant at the time of blooming. In the present study, the mcy-positive genotype dominated during the bloom season. The diversity in the sediment was different from that in the water column in July, and yet became similar to that in the water column due to the deposition of cyanobacteria in November. In contrast to the sediment samples, the potentially toxic *mcyJ* genotypes in the water samples underwent a dynamic temporal change during the summer (Fig. 4). Notably, the diversity of the toxic

genotypes (*mcyJ*) in the water samples was reduced during the cyanobacterial bloom season (August to October), and then returned to a broader diversity in the spring, whereas the banding pattern for the sediment samples showed very little change over the course of the entire year.

Verspagen *et al*. (2005) reported that changes in the benthic population were reflected in the pelagic population after 3–12 weeks, and that the time positively reflected the depth of the water column. Here, we argue that the broader diversity during spring and autumn in the Daechung Reservoir was due to consistent recruitment from the sediment, whereas during summer, the environmental and nutritional conditions favored a restricted genotype to be dominant over the other genotypes and form a bloom. Still, it was impossible to determine whether the presence of the *mcy* gene in a *Microcystis* strain provided any advantage over a nonproducing strain for dominant growth in summer.

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Authors' contributions

S.-G. K and S.-H. J. contributed equally to this study.

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