Simultaneous Quantification of Cyanobacteria and *Microcystis* spp. Using Real-Time PCR

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In order to develop a protocol to quantify cyanobacteria and *Microcystis* simultaneously, the primers and probe were designed from the conserved regions of 16S rRNA gene sequences of cyanobacteria and *Microcystis*, respectively. Probe match analysis of the Ribosomal Database Project showed that the primers matched with over 97% of cyanobacterial 16S rRNA genes, indicating these can be used to amplify cyanobacteria specifically. The TaqMan probe, which is located between two primers, matched with 98.2% of sequences in genus GpXI, in which most *Microcystis* strains are included. The numbers of cyanobacterial genes were estimated with the emission of SYBR Green from the amplicons with two primers, whereas those of *Microcystis* spp. were measured from the fluorescence of CAL Fluor Gold 540 emitted by exonuclease activity of Taq DNA polymerase in amplification. It is expected that this method enhances the accuracy and reduces the time to count cyanobacteria and potential toxigenic *Microcystis* spp. in aquatic environmental samples.

**Keywords:** Cyanobacteria, *Microcystis*, real-time PCR, simultaneous quantification

Heavy blooms of cyanobacteria are mainly caused by eutrophication of water bodies, which is thought to be the result of exogenous or endogenous nutrient loadings [26, 38]. Global climate warming has the potential to stimulate cyanobacterial growth and maintenance by increasing summer temperatures and the stability of the water column [25]. A massive cyanobacterial bloom induces potentially negative effects on environmental water quality, such as reduced transparency, decreased biodiversity, release of malodorous and bad-tasting compounds, and production of toxins [8, 26]. Moreover, oxygen depletion and ammonia release as the cyanobacteria decay can induce ecological distortion such as fish kills [32].

Cyanobacterial blooms have been observed in most reservoirs and rivers used as sources of drinking water supply in Korea, especially Daechung and Paldang Reservoirs and Nakdong River [1, 36]. Thereby, an algal alert system has been operated for large reservoirs by measurement of cyanobacterial abundance since 1996. In this system, alert is given based on chlorophyll *a* concentration and numbers of cyanobacteria counted by visual examination.

*Microcystis* is one of the best studied cyanobacterial genera because it is representative of microcystin-producing cyanobacteria threatening the health of humans and animals, and commonly appears in aquatic ecosystems around the world [7]. *Microcystis*, *Planktothrix*, and *Anabaena* are the major bloom-forming cyanobacteria in Korean freshwaters [2, 3, 33]. Quantification of potentially toxic cyanobacteria in the environmental samples is one of the most important data in studies for monitoring of harmful algal blooms (HABs) and for constructing remediation strategies. The proportion of *Microcystis* spp. to cyanobacteria is also an important parameter to predict the productive potential of cyanotoxins produced by the toxigenic *Microcystis*.

Direct microscopic count used ordinarily for enumerating cyanobacteria is rapid and sensitive. However, even skilled and experienced operators are occasionally unable to identify species and count the numbers of these organisms in complex environmental samples. In particular, *Microcystis* strains usually form scum, making quantification harder. Recently, molecular techniques such as quantitative real-time PCR (qPCR) had been developed to estimate the abundance of cells carrying specific target genes in natural samples. A qPCR technique known as Taq-nuclease assay (or TaqMan PCR) was firstly introduced in a study of *Synechococcus* ecotypes in deep lakes [6]. This technique has been
subsequently applied for the detection and quantification of *mcy* genes in toxigenic *Microcystis* and *Anabaena* in cultures as well as in natural samples [13, 19, 35].

In this study, new methods for simultaneous quantification of cyanobacteria and *Microcystis* using qPCR in the environmental samples were developed and optimized. For these purposes, new primers that are specific to the corresponding organisms were designed, and their specificities to target organisms were examined.

**MATERIALS AND METHODS**

**Cyanobacterial Strains and Cultivation**

*Microcystis viridis* NIER 10020 and *M. novacekii* NIER 10022, isolated from the Nakdong River in Korea, were courteously provided from the National Institute of Environmental Research, Korea [22]. *M. aeruginosa* UTEX 2388 and *M. viridis* NIES 102 were purchased from the Korean Collection for Type Cultures. Three cyanobacteria, *Anabaena flos-aquae* UTEX LB2557, *Anabaena circinalis* NIES 1645, and *Aphanizomenon flos-aquae* NIES 1258, were provided from the Korean Water Resources Corporation. The culture was maintained in CB medium in an incubation room at 25°C with fluorescent light at ~40 µE/m²/s on a 10:14 h dark/light cycle [14, 37].

**DNA Extraction and Quantification**

The cyanobacterial cells were centrifuged or filtration with GF/C filters for culture or environmental samples, respectively. Cells were resuspended in lysozyme solution (0.15 M NaCl, 0.1 M EDTA, 15 mg/ml of lysozyme, pH 8.0), and incubated for 1 h at 37°C with gentle mixing at 15 min intervals. An SDS buffer [0.1 M NaCl, 0.5 M Tris-HCl, 6% (w/v) SDS, pH 8.0] was added after incubation, and then the mixture was ultrasonicated for 5 min using an ultrasonic cleaner (150 W; Daihan Science, Korea). For complete lysis of cyanobacterial cell, the samples were incubated 5 min using an ultrasonic cleaner (150 W; Daihan Science, Korea). The cell suspension was centrifuged at 14,000 rpm for 3 min. The DNA pellet was air-dried, and subsequently dissolved in sterile distilled water.

Purified DNAs were quantified using Quant-IT PicoGreen dsDNA Reagent and Kits (Molecular Probes, Inc., Eugene, OR, USA) and Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) following the manufacturers’ instructions.

**Primers and Probe**

In order to quantify cyanobacteria with qPCR, 16SCF (5'-GGC AGC AGT GGG GAA TTT TC-3') and 16SUR (5'-GTM TTA CCG CGG CTG CTG G-3') were used, which were designed from the conserved regions of 16S rRNA gene sequences of cyanobacteria deposited in the Ribosomal Database Project (RDP) database [10]. To quantify *Microcystis* in the same sample simultaneously, a TaqMan probe (16SMT; 5'-CGG CTG CTG G-3') designed from 16S rRNA gene sequences of *Microcystis* strains was used. At the 5' and 3' ends of the probe, fluorochromes, CAL Fluor Gold 540 and BHQ-1, were attached, respectively. To confirm that the primers and probe will not form secondary structures, the sequences were checked using the OligoCalc program [15].

**Quantitative Real-Time PCR**

The PCR was conducted with a Rotor-Gene 6000 using the manufacturer’s software. The qPCR assay was performed in a volume of 10 µl containing 5 µl of 2× SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), 1 µl of each primer (5 pmol), 1 µl of TaqMan probe (5 pmol), and 2 µl of template DNA suspension. Two negative controls without template DNA were included for each PCR run. Amplification was performed as follows: The first step was an initial preheating to activate polymerase for 15 min at 95°C. This step was followed by 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. A 10-fold dilution series of the DNA from cultured cyanobacteria and environmental samples was prepared and amplified. The melting temperature for the real-time PCR products was determined using the manufacturer’s software. All tests were performed in triplicate.

The qPCR data were analyzed using Rotor-Gene 6000 Series Software version 1.7. Calculations of cycle threshold (Ct) values were completed automatically for each real-time PCR assay by the software using the maximum correlation coefficient approach. In this approach, the threshold is automatically determined to obtain the highest possible correlation coefficient (r) for the standard curve, which was obtained by the plotting between Ct values and the known DNA copy numbers of external standards. If required, manual manipulation was conducted to find the appropriate Ct value for the samples with low copy number (<100 copies/ml). The amplification efficiency of qPCR was calculated with the slope of a standard curve divided by the ideal value (1/log2=3.32), which indicates an exact doubling occurs per every polymerization cycle [21].

Gene copies per sample were calculated using the linear regression equations of the standard curves for each assay. The concentration of target genes (copy/µl of environmental samples) was calculated by Eq. (1).

\[
\text{Concentration of target gene (copy/µl)} = \frac{C_t \times d_f \times V_o}{V_w}
\]  

where \(C_t\) is the copy number (copy/µl) calculated by the \(C_t\) value from the standard curve, and \(d_f\) is the dilution factor of DNA samples. \(V_o\) and \(V_w\) indicate the volume of extracted DNA sample and volume of filtered water to extract DNA, respectively.

External standards used to determine the copy numbers were prepared using the genomic DNA from *M. aeruginosa* UTEX 2388. The single-copy plasmid standard was also used as the external standard, which was prepared by cloning of the amplicon of the 16S rRNA gene of *M. aeruginosa* UTEX 2388 with T-blunt vector (Solgent, Daejeon, Korea). The amplicon was prepared with cyanobacteria-specific primers of the 16S rRNA gene, 27F1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1494Rc (5'-TAC GGC TAC CTT GTT ACG AC-3') [29].

**Sampling and Analysis**

Water samples from five reservoirs in Korea were collected from July 1 to September 17, 2008, from the surface in a sterile bottle (Table 1). Those were transported to the laboratory, and the concentrations of chlorophyll *a* and microcystins were analyzed.

The chlorophyll *a* concentration was measured by the method represented in Standard Methods [4]. Briefly, samples were filtered through a 0.45 µm pore-sized cellulose nitrate membrane filter (Advantec MFS, Inc., Dublin, CA, USA). Pigment on the filter was extracted with 90% of acetone overnight, and then the concentration
rRNA gene sequences of cyanobacteria and
The primers for qPCR were designed on the basis of 16S
Evaluation of Molecular Markers
condition with 32% acetonitrile and 68% water.
both containing 0.05% (v/v) trifluoroacetic acid, under isocratic
at a flow-rate of 0.8 ml/min. The mobile phase was acetonitrile/water,
Xterra RP18 column (5 µm particle size, 15 cm×3.9 mm I.D.; Waters),
with a UV detector at 238 nm. Separations were carried out on an
HPLC with UV absorbance detector (UV 725S; Younglin Instrument
Milford, MA, USA) [24]. HPLC analyses were conducted with seven strains of cyanobacteria including four of Microcystis strains. The agarose gel analyses showed that single amplicons were observed (Fig. 1). On the other hand, the DNAs from the non-cyanobacterial taxa yielded
was calculated with the absorbances of extract at 750, 664, 647, and
630 nm.
In order to measure the concentration of microcystins, water
samples were filtered through a GF/C filter paper. The microcystins
in filters were extracted with methanol, and then the extract was
purified with a C18 column cartridge (Sep-Pak Vac 3 cc; Waters, Milford, MA, USA) [24]. HPLC analyses were carried out using a
HPLC with UV absorbance detector (UV 725S; Younglin Instrument
Co., Korea) and data acquisition program (Autochro 2000). The
injected sample volume was 20 µl, and the detection was performed
with a UV detector at 238 nm. Separations were carried out on an
Xterra RP18 column (5 µm particle size, 15 cm×3.9 mm I.D.; Waters),
at a flow-rate of 0.8 ml/min. The mobile phase was acetonitrile/water,
both containing 0.05% (v/v) trifluoroacetic acid, under isocratic condition with 32% acetonitrile and 68% water.

RESULTS AND DISCUSSION
Evaluation of Molecular Markers
The primers for qPCR were designed on the basis of 16S
rRNA gene sequences of cyanobacteria and Microcystis
from the RDP database [10]. Probe match analysis for 16SCF
showed that 82.5% of sequences in class Cyanobacteria
matched with this primer (Table 2). If the sequences of
family Chloroplast belonging in class Cyanobacteria were
excluded, 97.3% of sequences in class Cyanobacteria fitted
with 16SCF. The 16SCF also fitted with 351 sequences of
non-cyanobacterial bacteria belonging to phyla Acidobacteria,
Firmicutes, Proteobacteria, Tenericutes, TM7, and unclassified
bacteria. These sequences leading to mismatch can be
neglectable because most of these came from unidentified
soil bacteria [11, 23]. These results indicated that 16SCF is
suitable as a primer to amplify the 16S rRNA gene of
cyanobacteria specifically. 16SUR was designed to bacterial
16S rRNA sequences, because the appropriate reverse primer that specifically binds to cyanobacteria and make
about 200 bp of amplicon with the forward primer for the
reproducible quantification using qPCR could not be
found. This primer was matched with most sequences of
cyanobacteria (97.9%) based on the probe match analysis
of the RDP database, indicating that 16SUR can be used as
a reverse primer to amplify cyanobacteria. To confirm the
specificity of 16SCF and 16SUR empirically, PCRs were
conducted with seven strains of cyanobacteria including
four of Microcystis strains. The agarose gel analyses showed
that single amplicons were observed (Fig. 1). On the other
hand, the DNAs from the non-cyanobacterial taxa yielded

Table 1. Environmental parameters in the samples taken at the cyanobacterial blooming locations in five Korean reservoirs.

<table>
<thead>
<tr>
<th>Reservoir</th>
<th>Location</th>
<th>Date</th>
<th>Chl a (mg/m²)</th>
<th>Microcystins (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RR</td>
<td>YR</td>
</tr>
<tr>
<td>Daechung</td>
<td>36°21'34.17&quot; N 127°34'10.38&quot; E</td>
<td>Jul. 01, 2008</td>
<td>33.7</td>
<td>46.9</td>
</tr>
<tr>
<td>Yongdam</td>
<td>35°52'33.15&quot; N 127°30'31.95&quot; E</td>
<td>Jun. 27, 2008</td>
<td>40.6</td>
<td>11.0</td>
</tr>
<tr>
<td>Chungju</td>
<td>37°01'50.98&quot; N 128°02'16.82&quot; E</td>
<td>Sep. 02, 2008</td>
<td>64.2</td>
<td>14.2</td>
</tr>
<tr>
<td>Soyang</td>
<td>37°31'33.94&quot; N 127°51'14.08&quot; E</td>
<td>Sep. 05, 2008</td>
<td>4.6</td>
<td>1.34</td>
</tr>
<tr>
<td>Eum</td>
<td>37°52'29.82&quot; N 127°42'28.30&quot; E</td>
<td>Sep. 17, 2008</td>
<td>41.0</td>
<td>6.85</td>
</tr>
</tbody>
</table>

*aNot detected.

Table 2. Specificity of primers for quantification of cyanobacteria and Microcystis.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>No. of sequences</th>
<th>16SCF</th>
<th>16SUR</th>
<th>16SMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain Bacteria</td>
<td>962,279</td>
<td>8,792</td>
<td>859,240</td>
<td>284</td>
</tr>
<tr>
<td>Phylum Cyanobacteria</td>
<td>10,230</td>
<td>8,441</td>
<td>10,020</td>
<td>279</td>
</tr>
<tr>
<td>Class Cyanobacteria</td>
<td>10,230</td>
<td>8,441</td>
<td>10,020</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>(82.5)*</td>
<td></td>
<td>(97.9)</td>
<td></td>
</tr>
<tr>
<td>Family Family II</td>
<td>2,112</td>
<td>2,083</td>
<td>2,088</td>
<td>1*</td>
</tr>
<tr>
<td>Family Family IV</td>
<td>252</td>
<td>244</td>
<td>248</td>
<td>7</td>
</tr>
<tr>
<td>Family Family XI</td>
<td>276</td>
<td>267</td>
<td>268</td>
<td>271</td>
</tr>
<tr>
<td>Genus GpXI</td>
<td>276</td>
<td>267</td>
<td>268</td>
<td>271</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(98.2)</td>
</tr>
<tr>
<td>Family Chloroplast</td>
<td>3,782</td>
<td>2,167</td>
<td>3,680</td>
<td>0</td>
</tr>
<tr>
<td>Other Families</td>
<td>3,808</td>
<td>3,680</td>
<td>3,736</td>
<td>0</td>
</tr>
<tr>
<td>Other Phyla</td>
<td>952,049</td>
<td>351</td>
<td>849,220</td>
<td>5</td>
</tr>
<tr>
<td>Domain Archaea</td>
<td>11,489</td>
<td>0</td>
<td>66</td>
<td>0</td>
</tr>
</tbody>
</table>

*The sequences showing good quality and with >1,200 bp length were
analyzed out of 1,921,179 sequences of domain Bacteria that were available
in the Ribosomal Database Project database on Nov. 2011.
*Indicates the number of sequences that match with the corresponding
primers.
*Numbers in parentheses indicate percent coverage of corresponding primers.
*Most Microcystis strains belong to genus GpXI except two strains, M. elbens NIES 42(U40335) and M. holsatica NIES 43(U40336), which are in
genus GpIIb.
*Sequence of M. holsatica NIES 43(U40336) in genus GpIIb.
no product, indicating the primers, 16SCF and 16SUR, can amplify the 16S rRNA gene of cyanobacteria specifically.

In order to quantify the copy number of the 16S rRNA gene of cyanobacteria and Microcystis simultaneously, a TaqMan probe was designed based on the conserved sequences of the 16S rRNA gene of Microcystis strains. The 16SMT matched with 271 out of 276 sequences in genus GpXI, in which most Microcystis strains are included, except for two strains in genus GpIIb isolated from Japanese lakes, *M. elabens* NIES42 (U40335) and *M. holsatica* NIES43 (U40336) [29].

The mismatched sequences in genus GpXI were *M. aeruginosa* NIES 87 (D89031), *M. wesenbergii* NIES 111 (D89034), *M. ichthyoblabe* (AB012339), *M. wesenbergii* NIES 112 (U40334), and unidentified cyanobacterium Ni3-C1 (AB275352), found in Japanese lakes and lichen [18, 29, 31]. Unexpected matches were also found in seven sequences of non-Microcystis cyanobacteria. These sequences, however, came from salt water (uncultured cyanobacterium DPC044, DQ269094; uncultured bacterium OTU44, GU451375; uncultured bacterium OTU45, GU451376; uncultured bacterium OTU46, GU451377; uncultured bacterium OTU101, GU451320) and coral (cyanobacterium SC-1, EF372582; filamentous cyanobacterium FLK9, EU196364) [20, 27, 28], having no effect on the analysis of *Microcystis* sp. in freshwater. Five sequences of non-cyanobacterial eubacteria were also fitted with 16SMT from gut microbiota (uncultured bacterium aab54c08, DQ814841) [34] and thermophilic anaerobic solid waste (uncultured bacterium G55_D25_M_B_H05, DQ887947; uncultured bacterium B55_K_B_H08, DQ887957), and feces (uncultured bacterium C1-156, GQ896693; uncultured bacterium N15, FJ951846). These results indicate that the 16SMT probe can specifically detect the cyanobacteria in genus Microcystis in freshwaters.

The specificity of 16SMT to Microcystis strains was confirmed using qPCR (Fig. 2). Genomic DNAs from four strains of *Microcystis*, *Anabaena flos-aquae*, *Anabaena circinalis*, and *Aphanizomenon flos-aquae* were used as templates. In all samples, except for the no-template negative control, the amount of fluorescence emission was increased with cycle (Fig. 2A). This result indicated that the primers successfully bind to cyanobacterial 16S rRNA genes and amplification of these genes occurs. On the other hand, the fluorescence from the reporter dye, CAL Fluor Gold 540, was only increased in the samples with genomic DNA from Microcystis strains, not from non-Microcystis strains, indicating that 16SMT specifically anneals to the genomic DNAs of *Microcystis* strains (Fig. 2B). From these results, it was concluded that the primers and TaqMan probe can be used to quantify the copy numbers of 16S rRNA genes of cyanobacteria and *Microcystis* strains, respectively.

![Fig. 1. Gel image of results from PCR amplification with the 16S rRNA gene of cyanobacteria.](image1)


![Fig. 2. Change of fluorescence caused by amplification of 16S rRNA genes of cyanobacteria (A) and *Microcystis* (B).](image2)

Simultaneous Quantification of Cyanobacteria and Microcystis

In order to quantify cyanobacteria and Microcystis simultaneously in the environmental samples, qPCR with a thermal cycler with two channels of excitation/detection wavelengths was conducted. In this study, two primers, 16SCF and 16SUR, designed to amplify a part of the 16S rRNA gene of cyanobacteria specifically were used to quantify cyanobacteria. A TaqMan probe, 16SMT, was used to quantify Microcystis, the annealing site of which is inside of 16SCF and 16SUR. Using the amount of amplified DNA with 16SCF and 16SUR, which was measured with fluorescence emitted from SYBR Green 1, the copy number of the cyanobacterial 16S rRNA gene was quantified. The copy number of the Microcystis 16S rRNA gene was estimated with fluorescence from CAL Fluor Gold 540 generated by 5’ nuclease activity of Taq DNA polymerase.

The genomic DNA extracted from M. aeruginosa UTEX 2388 or a plasmid, which was prepared with the PCR product of the 16S rRNA gene of M. aeruginosa UTEX 2388 with 27F and 1494Rc as primers, was used for PCR template. A standard curve was generated using five dilutions of template DNA that ranged from $1.2 \times 10^5$ – $1.2 \times 10^8$ cells per reaction or $1.3 \times 10^5$ – $1.3 \times 10^8$ copies per reaction for M. aeruginosa UTEX 2388 or plasmid DNA, respectively. When the 16SCF and 16SUR primer set was used, highly significant linear curves between the amount of starting DNA (in cell or copy numbers) and the C<sub>t</sub> were obtained (Table 3). The regression equation was $Y=30.17 - 3.546X$ ($R^2=0.9946$, $p<0.0001$) and $Y=32.09 - 3.672X$ ($R^2=0.9994$, $p<0.0001$) for chromosomal DNA of M. aeruginosa UTEX 2388 and plasmid DNA, respectively, where $Y$ is the C<sub>t</sub> and $X$ is the amount of starting DNA (represented as logarithmic scale of cell or copy numbers). The absolute values of the slopes, 3.55 and 3.67, were very close to the ideal value, indicating the primers anneal to the target gene with yielding products in almost every polymerization cycle. Melting curve analysis showed that no primer dimers were formed, and all the product peaks were found at a temperature of 83°C.

In the same samples for PCR amplification with 16SCF/16SUR primers, the fluorescence generated from CAL Fluor Gold 540 by 5’ nuclease activity, which was attached on 16SMT, was measured at 72°C in the polymerization cycles. The standard curves of copy numbers of the 16S rRNA gene against C<sub>t</sub> values were plotted for M. aeruginosa UTEX 2388 and plasmid DNA. Highly significant correlations were found between two parameters, yielding the regression equations $Y=36.60 - 3.551X$ ($R^2=0.9964$, $p<0.0001$) and $Y=37.09 - 3.458X$ ($R^2=0.9974$, $p<0.0001$) for M. aeruginosa UTEX 2388 and plasmid DNA, respectively (Table 3). These reliable results indicated that the numbers of cyanobacteria and Microcystis strains can be counted simultaneously in the environmental samples by using qPCR with organism-specific primers or probe.

Cyanobacteria and Microcystins in the Environmental Samples

The microcystin concentrations in the environmental samples were in the range of 1.34–48.6 µg/l, which were much higher than the previous reports (Table 1) [16]. Only microcystin-RR was found in the sample taken from four reservoirs, except Daechung Reservoir, in which microcystins consisted with RR (97%) and LR (3%). The microcystin

Table 3. Amplification Efficiencies and standard curve parameters from quantitative real-time PCR analysis for the cyanobacteria and Microcystis.

<table>
<thead>
<tr>
<th>Target organism</th>
<th>Primers or probe</th>
<th>Standard&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amplification efficiency</th>
<th>Slope</th>
<th>Y-intercept</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td>16SCF/16SUR</td>
<td>UTEX 2388</td>
<td>0.937</td>
<td>-3.546</td>
<td>30.17</td>
<td>0.9946</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasmid&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.905</td>
<td>-3.672</td>
<td>32.09</td>
<td>0.9994</td>
</tr>
<tr>
<td>Microcystis</td>
<td>16SMT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>UTEX 2388</td>
<td>0.935</td>
<td>-3.551</td>
<td>36.60</td>
<td>0.9964</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasmid&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.961</td>
<td>-3.458</td>
<td>37.09</td>
<td>0.9974</td>
</tr>
</tbody>
</table>

<sup>a</sup>TaqMan probe.<br>
<sup>b</sup>The chromosomal or plasmid DNA used to generate the standard curve, which is needed to convert C<sub>t</sub> values to copy numbers of target gene.<br>
<sup>c</sup>T-blunt vector cloned with corresponding target genes.
analog, RR, was announced as the major component in the Korean lakes [30].

The WHO guideline for drinking water is 1.0 µg microcystin-LR equivalent per liter for free plus cell-bound [12]. Although the values found in the analyzed samples exceeded 1.0 µg/l, it is hard to apply these values to the WHO guidelines. The reasons are as follows; all sampling locations were located at the tributary of reservoirs and far from the intake sites of drinking water, and most microcystin variants found in samples were microcystin-RR, which is less toxic than microcystin-LR [39]. Despite these limitations, the values are enough to give warning because the targeted reservoirs are used as sources of drinking water.

The abundances of cyanobacteria in water samples were in the range of 1.1 – 7.4×10<sup>6</sup> copies of 16S rRNA gene/l, with the maximum value in the Chungju Reservoir. It has been reported that the number of cyanobacteria is linearly correlated with the concentration of chlorophyll a [35]. In this study, however, no correlation of the two parameters was found. This discrepancy was probably caused by the heterogeneity of the phytoplankton community; the cyanobacteria were dominant in the samples of Daechung, Yongdam, and Chungju Reservoirs, whereas the eukaryotic algae were in the samples of Soyang and Euam Reservoirs (data not shown).

In all studied reservoirs, except Yongdam Reservoir, the copy numbers of the 16S rRNA gene of *Microcystis* strains represented over 50% of those of cyanobacteria (Table 4). Because the copy numbers of the 16S rRNA gene in cyanobacteria are variable, those found in the environmental samples cannot be transformed to the numbers of cyanobacteria [17]. Thus, this result does not mean the number of *Microcystis* strains occupied over half of cyanobacteria. However, it is definite that *Microcystis* strains are dominant in these reservoirs. Depending on the microscopical examination, a lot of filamentous and/or heterocyst-forming cyanobacteria such as *Planktothrix, Nodularia,* and *Anabaena,* and diatoms were found together with *Microcystis* in the samples from the Yongdam Reservoir. This observation is one of the reasonable explanations for the less abundance of *Microcystis* to cyanobacteria in this sample than others.

When the specific microcystin production in *Microcystis* calculated from the concentration of microcystins and the copy number of the 16S rRNA gene of *Microcystis* were examined in the investigated reservoirs, the values in the samples from the Chungju, Soyang, and Euam Reservoirs were relatively lower than those from the Daechung and Yongdam Reservoirs. There are three possibilities to explain these results. (i) The physicochemical parameters in these reservoirs were not adequate to produce microcystins, because microcystin production is affected by physicochemical parameters such as pH, light intensity, nitrogen and phosphorus concentration, ratio of nitrogen and phosphorus, iron concentration, and so on [35, 38, 39]. (ii) It is attributed to the difference of cyanobacterial populations derived from geographical features; Daechung and Yongdam Reservoirs belong to the Geum River basin, and Chungju, Euam, and Soyang Reservoirs belong to the Han River basin with different headsprings. (iii) The activity of microcystin production by *Microcystis* in each area can be quite different by the sequence diversity of microcystin synthase genes from different locations [5]. In order to find the reason for the discrepancy of microcystin concentrations and the copy number of the *mcy* gene, more elaborate research is required, such as quantification of mRNAs of *mcy* genes using reverse transcription–real-time PCR (qRT-PCR); this method allows measurement of the activity of structural genes [9].

In this study, an advanced method was proposed for simultaneous quantification of cyanobacteria and potentially toxic *Microcystis* by using primers and TaqMan probe that specifically amplify cyanobacteria and *Microcystis,* respectively. This method can be used to replace the counting method under a microscope that is considered to be inaccurate and time-consuming one.

### Acknowledgments

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### References


<table>
<thead>
<tr>
<th>Reservoir</th>
<th>Cyanobacterial copy number (10&lt;sup&gt;6&lt;/sup&gt; copies/l)</th>
<th>Microcystis copy number (10&lt;sup&gt;6&lt;/sup&gt; copies/l)</th>
<th>Microcystins (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daechung</td>
<td>2.828.9 ± 78.2</td>
<td>1.654.4 ± 140</td>
<td>48.6</td>
</tr>
<tr>
<td>Yongdam</td>
<td>1.053.1 ± 79.7</td>
<td>350.6 ± 5.60</td>
<td>11.0</td>
</tr>
<tr>
<td>Chungju</td>
<td>7.351.6 ± 312</td>
<td>5.409.4 ± 677</td>
<td>14.2</td>
</tr>
<tr>
<td>Soyang</td>
<td>1.199.4 ± 64.2</td>
<td>581.7 ± 113</td>
<td>1.34</td>
</tr>
<tr>
<td>Euam</td>
<td>1.321.5 ± 168</td>
<td>924.1 ± 169</td>
<td>6.85</td>
</tr>
</tbody>
</table>


