Environ. Sci. Technol. **2009,** *43,* 812–818

Quantification of Toxic *Microcystis* and Evaluation of Its Dominance Ratio in Blooms Using Real-Time PCR

JOO H. HA,* TAIRA HIDAKA, AND HIROSHI TSUNO

Department of Urban and Environmental Engineering, Kyoto University, Kyoto-Daigaku-Katsura, Nishikyo-ku, Kyoto 615-8540, Japan

Received May 8, 2008. Revised manuscript received August 8, 2008. Accepted October 29, 2008.

Microcystins are produced by cyanobacteria carrying microcystin synthetase (*mcy*) genes in blooms. The present study investigated seasonal variations in concentrations of *mcy* A and 16S rRNA genes, encoded in the genus *Microcystis* using real-time PCR, and measured the water quality variables and microcystin concentrations in a hypereutrophic fish pond between June and November 2007. Prior to field survey, the relationship between axenic *Microcystis aeruginosa* NIES 102 cell abundance and concentrations of *mcy* A and 16S rRNA genes was determined by real-time PCR in the laboratory. Based on the quantitative relationship, microcystin-producing and total *Microcystis* cell numbers in the study site were estimated. The average dominance ratio of microcystin-producing strains in the total *Microcystis* population was approximately 80% and microcystin concentration was highly associated with toxic *Microcystis* cell numbers equivalent of *mcy* A gene. It was also observed that total nitrogen concentration was associated with development of *Microcystis* blooms, and high concentrations of NO_x and NH_4 increased microcystin production by promoting growth of microcystin-producing *Microcystis* strains.

Introduction

Microcystins, mainly produced by the genus *Microcystis*, are potentially lethal toxins reported to cause disruption of the liver cytoskeleton by inhibiting protein phosphatases 1 and 2A and have been linked to liver cancer (*1, 2*). Many studies have analyzed the relationships between microcystin production and environmental factors, including temperature (*3, 4*), nutrients (*5*−*9*), pH (*10*), and light intensity (*3, 11*−*14*). The findings, however, were not conclusive because the effects of external conditions on presence or growth of toxic and nontoxic strains were not elucidated in these studies (*9*).

Microcystins are synthesized by peptide synthetases through nonribosomal pathways (*15*). It has been reported that microcystin-producing *Microcystis* strains have different gene sequences involved in the formation of the enzymes (*16*). Dittmann et al. (1997) (*17*) verified that a nonidentical gene encodes the multifunctional peptide synthetase necessary for microcystin biosynthesis, and named it microcystin synthetase (*mcy*) gene (*17*). Recently, real-time PCR has been applied to measure *mcy* gene concentrations to monitor microcystin-producing cyanobacteria in bloom (*18, 19*). Real-time PCR has been used to quantify microcystin-producing *Microcystis* cells in natural samples using standard curves for predetermined cell numbers and threshold cycles (Ct) of *mcy* B (*18*) and A genes (*19*). Cell numbers of microcystin-producing and total *Microcystis* in Lake Erie using the quantitative relation between the counted cell number and concentration of *mcy* D gene have been estimated in a similar way (*20*). These studies presented a new approach to approximating microcystin-producing cells in cyanobacterial blooms; however, to date, there has been little discussion on the effects of environmental conditions on microcystin concentrations and microcystin-producing *Microcystis* behavior.

The present study analyzed the cell numbers of axenic *Microcystis aeruginosa* NIES 102 cultivated under various laboratory culture conditions. Also, copy numbers of the *mcy* A and 16S rRNA genes in those cells were assessed with real-time PCR before investigation in natural water. During field survey at the Hirosawa-no-ike fish pond, Kyoto, Japan, the water quality, microcystin levels, and concentrations of the two genes were determined.

Using ratios to determine the copy numbers of the two genes in one *Microcystis* cell, the quantity of microcystin-producing and total *Microcystis* cells, and the dominance of microcystin-producing *Microcystis* strains in the total *Microcystis* population were estimated. The correlation between the microcystin-producing *Microcystis* cell number equivalent of *mcy* A gene and microcystin concentration was examined. Finally, the environmental factors associated with the growth of toxic *Microcystis* and with the microcystin production were investigated.

Materials and Methods

Axenic culture of *Microcystis aeruginosa* NIES 102. The axenic toxic strain *Microcystis aeruginosa* NIES-102 (Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan) was cultured for 28 days to establish standard curves between cell numbers and concentrations of *mcy* A and 16S rRNA genes determined by real-time PCR.

The cells were cultivated in 50 mL of MA media (Ca $(NO_3)_2 \cdot 4H_2O$, 5 mg; KNO_3, 10 mg; $NaNO_3$, 5 mg; Na_2SO_4, 4 mg; $MgCl_2 \cdot 6H_2O$, 5 mg; β-Na_2 glycerophosphate$\cdot 5H_2O$, 10 mg; Na_2-EDTA, 0.5 mg; $FeCl_3 \cdot 6H_2O$, 0.05 mg; $MnCl_2 \cdot 4H_2O$, 0.5 mg; $ZnCl_2$, 0.05 mg; $CoCl_2 \cdot 6H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.08 mg; H_3BO_3, 2 mg; and Bicine, 50 mg, in distilled water of 100 mL, pH 8.6 as described at http://mcc.nies.go.jp/), and half-diluted MA media. Incubation temperatures were 25 and 30 °C and irradiance was 1000 lx (light:dark, 12:12 h). Cell numbers were counted three times a week with a compound microscope and the samples on days 7, 14, 21, and 28 were stored at −80 °C for DNA extraction.

Field Survey. From June to November 2007, water samples were collected once a week or twice a month (in late autumn) from the Hirosawa-no-ike fish pond to monitor the development of microcystin-producing *Microcystis* blooms and microcystin production. The Hirosawa-no-ike pond is generally used for carp fishery, and has a circumference of 1.3 km, surface area of 1.4×10^5 m^2, maximum depth of 1.8 m, and a water volume of about 10^5 m^3. Water temperature, dissolved oxygen (DO), turbidity, conductivity, pH, and irradiance were measured in situ.

Water quality variables were determined as follows: suspended solids and PO_4 according to the standard methods (*21*), chlorophyll-a by SCOR/UNESCO equation (*22*), total nitrogen (TN) and total phosphorus (TP) by a two-channel

* Corresponding author phone: +81-75-383-3352; fax: +81-75-383-3351; e-mail: jhha@water.mbox.media.kyoto-u.ac.jp.

10.1021/es801265f CCC: $40.75
Published on Web 12/30/2008

continuous flow system AACS II (BRAN+LUEBBE, Norderstedt, Germany), NH_4 and NO_x by a AA II (BRAN+LUEBBE). Phytoplankton samples were immediately preserved in Lugol's solution, and species composition was identified using a compound microscope. Water quality samples were stored at −20 °C and microcystin measurement and DNA extraction samples were stored at −80 °C.

Microcystin Measurement. Total microcystin (both cell-bound and extracellular microcystin) was measured in triplicate using a commercial ELISA kit (manufactured by Tokiwa, Japan, and supplied by Mitsubuishi, Japan).

Intracellular microcystin extraction was required because microcystins are usually found within the cells and extracellular concentrations are often very low in open water (*23*). To release microcystins from the frozen samples, three cycles of freezing, thawing, and sonication for 5 min, followed by shaking for 30 min were carried out. The ELISA kit used a microcystin-monoclonal antibody that reacts with most microcystin variants as well as with microcystin-LR (*24*), and its detection limit is 0.05 μg/L.

DNA Extraction and Real-Time PCR. DNA template was extracted from the frozen samples in triplicate with the DNeasy Tissue Reagents (Qiagen, Valencia, CA) according to the manufacturer's instructions. In real-time PCR, a Light Cycler and a Light Cycler FastStart DNA Master SYBR Green I (Hot-Start reaction mixture for PCR containing FastStart *Taq* DNA polymerase) (Roche, Mannheim, Germany) were used. Extracted DNA templates were amplified using two primer sets: MSF (5′-ATCCAGCAGTTGAGCAAGC-3′)/MSR (5′-TGCAGATAACTCCGCAGTTG-3′) for *mcy* A gene detection in a microcystin-producing *Microcystis* strain (*19, 25*), and 209F (5′- ATGTGCCGCGAGGTGAAACCTAAT-3′)/409R (5′-TTACAATCCAAAGACCTTCCTCCC-3′) for 16S rRNA gene selective detection in the genus *Microcystis* (*26*). The MSF/MSR primer set was verified to effectively distinguish the *Microcystis* strains containing the *mcy* A gene from the nontoxic *Microcystis* strains (*25*). Among 37 *Microcystis* strains, positive results were obtained for all 18 toxic strains and negative results for 17 out of 19 nontoxic strains. The 209F/409R primer set was reported to amplify the 16S rRNA gene in 12 *Microcystis* strains, including both toxic and nontoxic strains, and showed negative results in a PCR screening test of other cyanobacteria (26).

To obtain 1300 bp of PCR product from *mcy* A gene, preheating at 95 °C for 10 min, and 25 cycles comprising 95 °C for 10 s, 62 °C for 10s, and 72 °C for 30s were performed. With 209F/409R primer, 200 bp of 16S rRNA gene products were obtained by the process of preheating at 95 °C for 10 min, and 25 cycles of 95 °C for 10 s, 50 °C for 30 s, and 70 °C for 60 s. Real-time PCR products amplified by the procedures described above were added to 2% agarose gel electrophoresis to make internal standard solutions of *mcy* A and 16S rRNA genes. The obtained bands were extracted and purified by QIAquick Gel Extraction and QIAquick PCR Purification kits (Quiagen). Weights of DNA fragments in each of the extracted solution were measured by ND 1000 spectrophotometer (Nanodrop, Wilmington, DE). To determine the DNA concentrations in the field samples, a series of 10-fold diluted plasmid standard solutions were used to develop internal standard curves. Sample gene concentrations were automatically calculated during real-time PCR by comparing their Ct at a set level of fluorescence intensity with the plasmid standard Cts. Comparisons were abandoned when the regression coefficient of internal standard curve was less than 0.99. Triplicate data were determined by real-time PCR, and the gene concentrations within the median ±0.5 cycle number were accepted and averaged. DNA copy number was determined by

$$\text{copies} \times \mu\text{L}^{-1} = (A \times B \times 10^{-9})/(C \times \text{MW} \times 2) \quad (1)$$

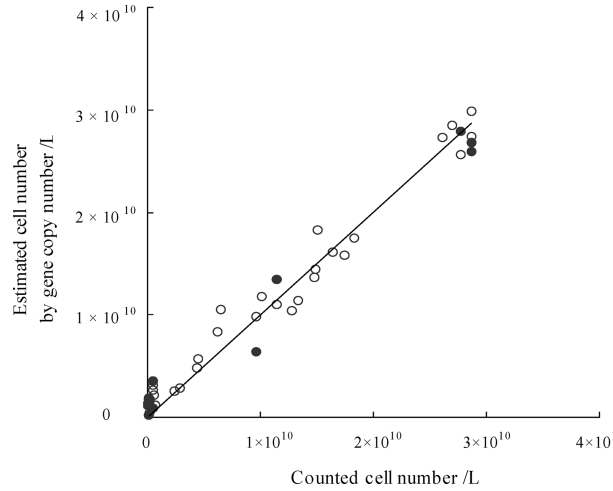

FIGURE 1. Comparison between the counted cell number by microscope and the estimated *Microcystis* cell number equivalent of *mcy* A gene (empty circle) ($y = x$, $R^2 = 0.97$) and 16S rRNA gene (solid circle) ($y = x$, $R^2 = 0.95$) by quantitative relations.

where A is the Avogadro number (6.02×10^{23}), B is DNA concentration (ng/L), C is base pair (bp) length of amplified DNA, and MW is bp DNA molecular weight (331 g/mol).

Results

Standard Cell Number and Gene Concentration Curves Using Real-Time PCR. The concentrations of *mcy* A and 16S rRNA genes in an axenic microcystin-producing *Microcystis aeruginosa* NIES 102 cell were determined by real-time PCR. The relationship between the counted cell number (cell concentrations between 4×10^4 to 3×10^7/mL) by microscope and the copy numbers of the two DNA fragments are shown as follows: The relationship in the microcystin-producing *Microcystis* cells was cells $\times$ L^{-1} = 0.07 $\times$ *mcy* A gene [copies $\times$ L^{-1}] ($R^2 = 0.98$, real-time PCR efficiency 52%, slope = −5.46), while among the total *Microcystis* cells the relationship was cells $\times$ L^{-1} = 0.004 $\times$ 16S rRNA gene [copies $\times$ L^{-1}] ($R^2 = 0.97$, real-time PCR efficiency 103%, slope = −3.25)

Though Figure 1 indicates some overestimation at low cell concentrations, which were harvested in the initial or the early exponential growth phase, strong relationships were observed at high cell concentrations, both in the exponential and stationary growth phases.

Study Site. The monthly averages for the various water quality variables are presented in Table 1. The pond was determined to be in an extremely hypereutrophic state by calculation of its trophic state index (TSI) (*27*).

$$\text{TSI(chlorophyll-a)} = 10[6\text{-}\{2.04\text{-}\ln(\text{chlorophyll-a}[\mu\text{g/L}])\}/\ln 2] \quad (2)$$

The chlorophyll-a TSI remained > 70 for more than six months. The seasonal variation in blooms show three chlorophyll-a peaks of over 3 mg/L, in June, August, and November with extremely high TN:TP ratios of 78, 40, and 92, respectively (Figure 2). Among these observations, the highest chlorophyll-a level of 14 mg/L appeared in November (microcystin:chlorophyll-a ratio of 0.4:1.0 μg), while the maximum microcystin concentration was 6.4 mg/L in August (microcystin:chlorophyll-a ratio of 1.4:1.0 μg).

The samples were predominantly *Microcystis* sp. and the relative dominance of *Microcystis* sp. and *Anabaena* sp. increased as the bloom became massive (Table 1). Colony numbers of *Anabaena* sp. and *Oscillatoria* sp. were relatively small and the periods of their occurrence were relatively short compared to those of *Microcystis* sp.

TABLE 1. Seasonal Variations in Water Quality of Hirosawa-no-ike Pond (*1*) % of Colony Number of *Microcystis* sp. among Total Microcystin-Producing Cyanobacteria, *Anabaena* sp. *Oscillatoria* sp., and *Microcystis* sp.)

	June	July	August	September	October	November
frequency of sampling	3	4	4	4	3	2
water temperature (°C)	26.0	26.4	29.7	27.5	21.4	14.5
DO (mg/L)	12.8	10.0	10.6	10.0	8.8	9.0
pH	8.4	8.2	9.0	9.0	8.0	7.7
irradiance (lux)	6.5×10^3	5.2×10^4	6.4×10^4	4.5×10^4	5.0×10^4	4.2×10^4
SS (g/L)	0.57	0.049	0.19	0.10	0.62	1.5
chlorophyll-a (mg/L)	2.8	0.2	1.5	0.3	0.2	6.8
TSI (Chl-a)	87	79	96	86	82	102
T-N (mg/L)	11.7	5.0	10.1	4.1	2.8	23.8
T-P (mg/L)	0.4	1.3	0.7	0.5	0.6	2.0
TN:TP (mass)	28	6	15	8	5	48
NH_4−N (μg/L)	22.7	17.9	30.0	18.4	24.2	62.5
NO_x-N (mg/L)	0.11	0.16	0.12	0.11	0.14	0.25
PO_4−P (μg/L)	39.7	17.8	65.0	78.0	35.3	26.5
TOC (mg/L)	198	16.1	81.8	54.0	17.4	475
DOC (mg/L)	8.1	4.5	12.7	6.9	6.0	7.1
microcystin (μg/L)	412	473	2040	378	30	2480
microcystis sp. (colony/ml)	8.5×10^4	6.9×10^3	1.3×10^4	7.8×10^3	5.4×10^3	6.6×10^4
Anabaena sp. (colony/mL)	5.4×10^4	2.8×10^3	1.7×10^4	4.2×10^3	2.5×10^3	3.9×10^3
Oscillatoria sp. (colony/mL)	4.0×10^2	0	2.0×10^2	7.5×10^2	6.8×10^2	2.6×10^3
% of *microcystis* sp. in Microcystin-producing cyanobacteria (colony/colony)(*1*))	77	74	52	63	61	83
total phytoplankton (colony/ml)	1.4×10^5	2.7×10^4	5.7×10^4	4.5×10^4	4.1×10^4	9.9×10^4
number of species identified by microscope	13	19	14	14	17	17

Quantification of Microcystin-Producing *Microcystis* Strains and Their Dominance in Blooms. The cell concentrations of microcystin-producing *Microcystis* as well as total *Microcystis* in the Hirosawa-no-ike pond were estimated by two quantitative relations between cell number and concentrations of *mcy* A and 16S rRNA genes.

The cell concentrations of toxic *Microcystis* equivalent of *mcy* A gene were 3.12×10^4/mL to 5.72×10^8/mL over the six month study period.

The dominance of microcystin-producing *Microcystis* to total *Microcystis* was determined by dividing the estimated cell concentrations equivalent of *mcy* A gene by the cell concentrations equivalent of 16S rRNA gene. However, before applying this method to determine the toxicity of the blooms at the study site, we confirmed that the estimated cell numbers by two gene concentrations were consistent with each other, in amplifying axenic culture samples with identical cell concentrations (cell concentrations from 4×10^4/mL to 3×10^7/mL; $n = 10$). The calculated ratios varied, and showed a log-normal distribution (confirmed by Shapiro-Wilk W test (*28*)) with an average of 0.075 ($10^{0.075} = 120\%$) and standard deviation of 0.38 at log scale. One estimate showed the cell number equivalent of *mcy* A gene to be 8 times more than that of the 16S rRNA gene. Figures 2 and 3 show the variations in the estimated microcystins-producing *Microcystis* cell number equivalent of *mcy* A gene and total *Microcystis* cell number equivalent of 16S rRNA gene at Hirosawa-no-ike pond.

The field data also showed log-normality and the average was −0.022 ($10^{-0.022} = 95\%$). Considering that the average dominance ratio from culture data was 0.075 ($10^{0.075} = 120\%$), this method was inclined to overestimate the proportion of microcystin-producing cells, and there is a possibility that the actual dominance of microcystin-producing *Microcystis* cells to total *Microcystis* cells at the study site was less than 95%. Based on the assumption that the calculated dominance

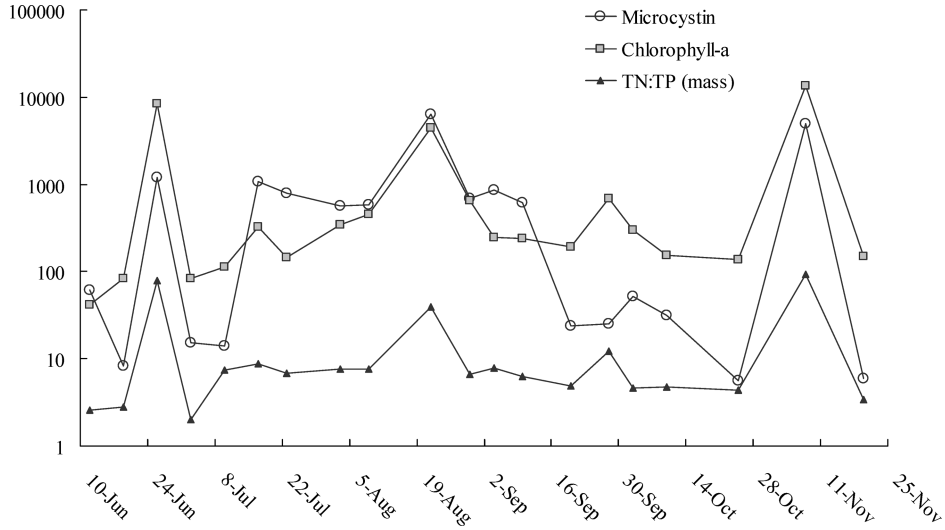

FIGURE 2. Seasonal variation in TN:TP ratio (mass), chlorophyll-a (μg/L) and microcystin (μg/L) at the study site.

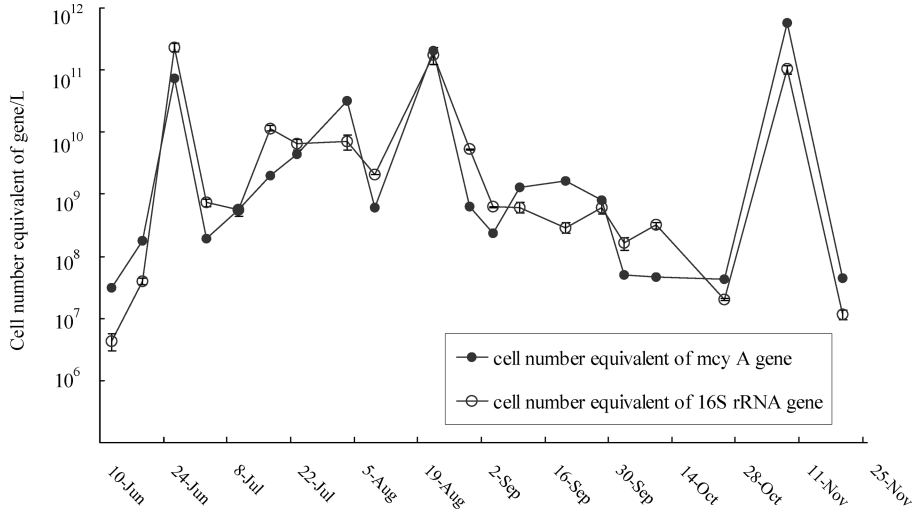

FIGURE 3. Variations of *Microcystis* cell amounts equivalent of *mcy* A gene and 16S rRNA gene during the six month survey.

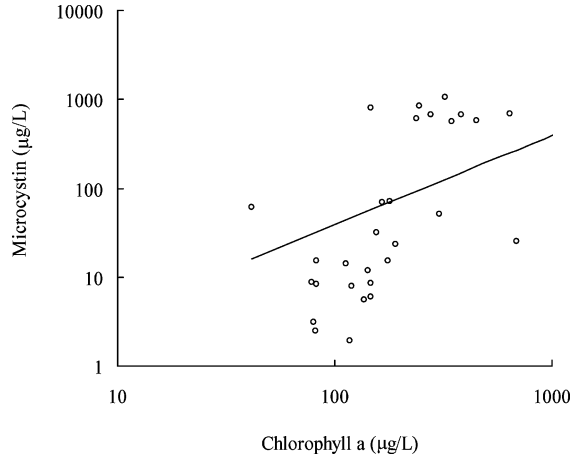

FIGURE 4. Correlation between microcystin concentration (μg/L) and chlorophyll-a concentration (μg/L) ($y = 0.36\ x$, $R^2 = 0.54$, the equation, and R^2 were obtained by regression analysis).

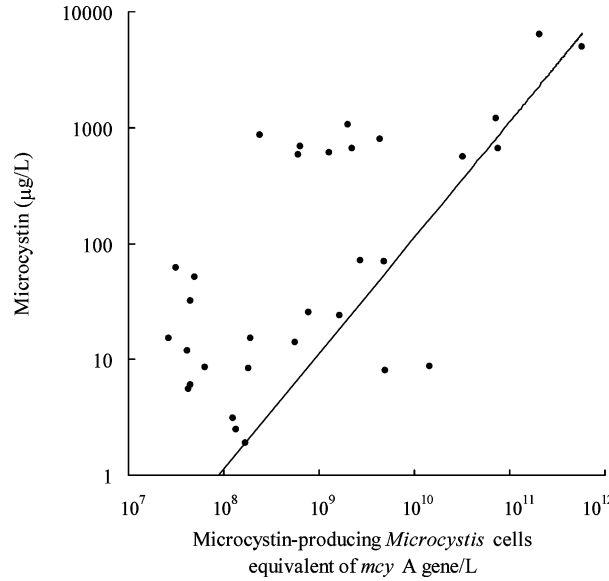

FIGURE 5. Correlation between microcystin concentration (μg/L) and microcystin-producing *Microcystis* cells equivalent of *mcy* A gene/L ($y = (1.05 \times 10^{-8})\ x$, $R^2 = 0.66$, the equation, and R^2 were obtained by regression analysis).

ratio of 120% in the culture samples actually reflects 100%, the average dominance ratio in the Hirosawa-no-ike pond would be ~80% (95:120% = field dominance ratio:laboratory dominance ratio). The standard deviation in the field data was 0.60 at log-scale, and the variance was statistically the same as that in the laboratory experiment (F-test; $\alpha = 0.01$). Consequently, the variations of toxicity within each bloom might have resulted from the methodology's internal error. It was concluded that the differences in the ratios of microcystin-producing *Microcystis* cells to total *Microcystis* cell numbers were not large enough to indicate seasonal variability of toxicity during the six month survey.

Is Toxic Strain Amount Deterministic for Microcystin Production? In the *Microcystis*-dominant study site, microcystin concentration was correlated with chlorophyll-a concentrations ($p < 10^{-5}$, $R^2 = 0.5$) (Figure 4). In a comparison of coefficients of determination (R^2), the estimated toxic *Microcystis* cell concentrations were found to be more highly correlated with microcystin ($p < 10^{-7}$, $R^2 = 0.66$) (Figure 5) than with chlorophyll-a. The ratio of microcystin-producing *Microcystis* cell number equivalent of *mcy* A gene, to chlorophyll-a was correlated with microcystin concentration ($p < 0.05$, $R^2 = 0.25$).

Environment, Microcystin-Producing Microcystis, and Microcystin. The effects of environmental conditions on the growth of microcystin-producing *Microcystis* and on microcystin production were examined by regression analysis (Table 2). The results show that an increase in nitrogen (N) increased microcystin levels, whereas a change in phosphorus (P) was irrelevant. TN enhanced the growth of both microcystin-producing and total *Microcystis*, which might be related to the absence of N_2 fixing function in *Microcystis* (*29*). NH_4 and NO_x promoted microcystin-producing *Microcystis*, whereas total *Microcystis* was not influenced by them. Our results indicate that a dissolved inorganic N (DIN) source promoted growth of microcystin-producing *Microcystis* strains and lead to higher microcystin concentrations in the hypereutrophic water body.

Discussion

Quantifying toxic cells in blooms using DNA concentrations determined by real-time PCR includes the possibilities that intrinsic error rates might affect the results. Such errors might be derived from a loss of yield through DNA extraction, inefficient amplification during real-time PCR, increased DNA amounts in cells during some growth phases, and DNA being released from dead cells. In this study, higher ratios of the *mcy* A and 16S rRNA genes in an existing cell number were observed during the early

TABLE 2. Coefficient of Determination (R^2) between Parameters Associated with Growth of Microcystin-Producing Cyanobacterial Blooms and Environmental Conditions[a]

	chlorophyll-a (μg/L)	TMCY[1]) (cells/L)	MCY[2]) (cells/L)	TMCY/Chl-a	microcystin (μg/L)
water temperature (°C)	0.012	0.036	0.058	0.019	0.033
irradiance (lux)	0.012	0.050	0.011	0.023	0.19**
pH	0.027	0.010	0.01	0.0020	0.093
T-N (mg/L)	0.87*	0.62*	0.78*	0.091	0.55*
NH_4−N (mg/L)	0.43*	0.55*	0.12	0.30*	0.28*
NO_x-N (mg/L)	0.62*	0.61*	0.12	0.037	0.21*
T-P (mg/L)	0.035	0.024	0.030	0.041	0.037
PO_4−P (mg/L)	0.025	0.032	0.015	0.081	0.0058

[a] TMCY[1], microcystin-producing microcystis cell numbers equivalent of mcy A gene/L; MCY[2], microcystis cell numbers equivalent of 16S rRNA gene/L) (*$p < 0.05$, **$p < 0.1$).

growth phase. This observation agrees with a report indicating that genomic copy numbers per *Synechococcus* sp. cell were higher in a 1-week-old culture than in an 8-week-old culture (*30*).

This study assumed that one *Microcystis aeruginosa* NIES 102 cell has a constant copy number of 250 16S rRNA gene and 14 *mcy* A gene. This assumption was based on the linear regression equations obtained from the quantified relationships between cell number and DNA concentration determined by real-time PCR. It has been reported that *Microcystis* strains have one *mcy* gene cluster (*31*), whereas genome sequencing analysis has revealed that a single *Microcystis aeruginosa* cell contains two copies of the rRNA gene cluster (*32*). Ideally, copy numbers per cell measured by real-time PCR should be the same as the actual copy numbers per cell, or the relative ratio of the *mcy* A gene to the 16S rRNA gene in one cell should be 1:2. DNA copy numbers have been shown by others to be about two-orders of magnitude higher than estimated cell numbers (*20*); thus, copy number per cell determined by real-time PCR does not exactly reflect the results through sequencing. However, high R^2 values for estimated cell numbers and microscopically counted cell numbers imply that an approximation of cell number with the ratios of 250 16S rRNA gene copies and 14 *mcy* A gene copies per *Microcystic* cell can represent the cell concentrations satisfactorily.

In determining the toxicity in cyanobacterial blooms with the method presented in this study, portions higher than 100% of microcystin-producing cell number, equivalent to the concentration of the *mcy* A gene, to the total *Microcystis* cell number, equivalent to the concentration of the 16S rRNA gene, were observed. While the ratios of copy numbers of the *mcy* A gene to those of the 16S rRNA gene ranged from 0.7 to 41% (average 12%), estimation of cell number with the standard curves developed in the laboratory sometimes leads to a toxic cell number higher than the total *Microcystis* cell number. Overestimation of toxic cell numbers in the total cell number was also shown in Rinta-Kanto et al. (*20*). Because *Microcystis* sp. has an average of 1000 cells per colony (*33*) and is both temporally and spatially variable, with differences of up to 3 orders of magnitude (*34*), the observed degree of overestimation, i.e., less than 1.0 at log-scale, is acceptably small. However, overestimation should be reduced in order to discriminate subtle seasonal variations of toxicity, thereby resulting in more accurate monitoring of toxic blooms.

In previous studies, more than 80% of *Microcystis* blooms were toxic (*35*), and the ratios of toxic to nontoxic *Microcystis* strains were reported to be 15% (*36*), 16% (*37*), and in the range of 6−68% (*38*) by isolating different strains from blooms. In other studies, which determined toxicity of bloom in a manner similar to that in our study, the results were 0−11% in the Great Lakes (*39*) and 0−37% at Oneida Lake (*40*). Because toxic strains have been reported to utilize higher nutrient levels and grow better in eutrophic states than nontoxic strains (*7*), the higher proportion of toxic strains in our study might have resulted from the hypereutrophic characteristics of the study site.

Past studies have recommended chlorophyll-a concentration as a predictor of microcystin despite arguments that large blooms result in high microcystin levels (*7, 8*). Rogalus and Watzin (2008) (*41*) suggested a chlorophyll-a concentration of about 5 μg/L as a threshold value for a toxin screening test. The microcystin to chlorophyll-a ratio of 0.4:1.0 μg in our study agrees with a previous study, in which microcystin in *Microcystis* culture was about half the chlorophyll-a concentration (*7*). In another study, 94% of 295 samples showed a ratio lower than 0.5:1.0 μg (*23*). The high correlation ($R^2 = 0.66$) between the estimated toxic *Microcystis* cell concentration, as an equivalent of the *mcy* A gene, and microcystin concentration observed in our study was in accord with the result ($R^2 = 0.65$) of another study that employed the *mcy* D gene (*40*). This suggests that the measurement of the *mcy* gene with real-time PCR is effective in predicting microcystin concentrations, and might be applicable regardless of the type of target gene within the *mcy* gene cluster.

Finally, we analyzed the relationships between the growth of microcystin-producing *Microcystis* equivalent of *mcy* A gene quantified by real-time PCR and environmental conditions. While many previous studies suggested that a P source, such as TP (*42*), total dissolved phosphorus (*43*), and PO_4 (*8*), was the main factor in increasing microcystin levels, the P source in the present study was irrelevant. On the other hand, TN showed significant positive correlations with concentrations of chlorophyll-a, microcystin-producing and total *Microcystis* cell numbers as equivalent of *mcy* A and 16S rRNA genes, respectively, and microcystin. These results agreed with those of Vézie et al. (*9*). In particular, NH_4, and NO_x were found to enhance toxic *Microcystis* growth and microcystin production. This agrees with a laboratory experiment and statistical analysis report that NO_3^- increases growth of toxic *Microcystis aeruginosa* cells and microcystin production (*44*), and supports Gobler et al. (2007) that suggested high levels of DIN and TN were promoters of lake toxicity (*45*).

Our study demonstrated that quantifying microcystin-producing *Microcystis* strains with real-time PCR can be utilized as an efficient method for monitoring microcystin outbreaks and can contribute to a more comprehensive understanding of the development of toxic strains in cyanobacterial blooms than the currently used measurement systems.

Acknowledgments

This work was supported by KAKENHI (19760370).

Literature Cited

(1) Ito, E.; Kondo, F.; Terao, K.; Harada, K. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. *Toxicon* **1997**, *35* (9), 1453–1457.

(2) Mackintosh, C.; Beattie, K. A.; Klumpp, S.; Cohen, P.; Codd, G. A. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **1990**, *264*, 187–192.

(3) Rapala, J.; Sivonen, K.; Luukkanien, R.; Niemelä, S. Anatoxin-a concentration in *Anabaena* and *Aphanizomenon* under different environmental conditions and comparison of growth by toxic and non-toxic *Anabaena* strains - a laboratory study. *J. Appl. Phycol.* **1993**, *5*, 581–591.

(4) Amé, M. V.; Díaz, M. P.; Wunderlin, D. A. Occurrence of toxic cyanobacterial blooms in San Roque Reservoir (Córdoba, Argentina): A field and chemometric study. *Environ. Toxicol.* **2003**, *18*, 192–201.

(5) Sivonen, K. Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. *Appl. Environ. Microbiol.* **1990**, *56* (9), 2658–2666.

(6) Orr, P. T.; Jones, G. J. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. *Limnol. Oceanogr.* **1998**, *43*, 1604–1614.

(7) Lee, S. J.; Jang, M. H.; Kim, H. S.; Yoon, B. D.; Oh, H. M. Variation of microcystin content of *Microcystis aeruginosa* relative to medium N:P ratio and growth stage. *J. Appl. Microbiol.* **2000**, *89*, 323–329.

(8) Oh, H. M.; Lee, S. J.; Kim, J. H.; Kim, H. S.; Yoon, B. D. Seasonal variation and indirect monitoring of microcystin concentrations in Daechung Reservoir, Korea. *Appl. Environ. Microbiol.* **2001**, *67* (4), 1484–1489.

(9) Vézie, C.; Rapla, J.; Vaitomaa, J.; Seitsonen, J.; Sivonen, K. Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentration. *Microb. Ecol.* **2002**, *43*, 443–454.

(10) Song, L.; Sano, T.; Li, R.; Watanabe, M. H.; Liu, Y.; Kaya, K. Microcystin production of *Microcystis viridis* (cyanobacteria) under different culture conditions. *Phycol. Res.* **1998**, *46* (suppl), 19–23.

(11) van der Westhuizen, A. J.; Eloff, J. N. Effect of temperature and light on the toxicity and growth of the blue-green alga *Microcystis aeruginosa* (UV-006). *Planta.* **1985**, *163*, 55–59.

(12) Watanabe, M. F.; Oishi, S. Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. *Appl. Environ. Microbiol.* **1985**, *49* (5), 1342–1344.

(13) Utkilen, H.; Gjølme, N. Toxin production by *Microcystis aeruginosa* as a function of light in continuous cultures and its ecological significance. *Appl. Environ. Microbiol.* **1992**, *58* (4), 1321–1325.

(14) Wiedner, C.; Visser, P. M.; Fastner, J.; Codd, G. A.; Mur, L. R.; Metcalf, J. Effects of light on the microcystin content of *Microcystis* Strain PCC 7806. *Appl. Environ. Microbiol.* **2003**, *69* (3), 1475–1481.

(15) Marahiel, M. A. Multidomain enzymes involved in peptide synthesis. *FEBS Lett.* **1992**, *307*, 40–43.

(16) Meiβner, K.; Dittmann, E.; Börner, T. Toxic and non-toxic strains of the cyanobacterium *Microcystis aeruginosa* contain sequences homologous to peptide synthetase genes. *FEMS Microbiol. Lett.* **1996**, *135*, 295–303.

(17) Dittmann, E.; Neilan, B.; Erhard, M.; Döhren, H. V.; Börner, T. Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. *Mol. Microbiol.* **1997**, *26* (4), 779–787.

(18) Kurmayer, R.; Kutzenberger, T. Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. *Appl. Environ. Microbiol.* **2003**, *69* (11), 6723–6730.

(19) Furukawa, K.; Noda, N.; Tsuneda, S.; Saito, T.; Itayama, T.; Inamori, Y. Highly sensitive real-time PCR assay for quantification of toxic cyanobacteria based on microcystin synthetase A gene. *J. Biosci. Bioeng.* **2006**, *102* (2), 90–96.

(20) Rinta-Kanto, J. M.; Ouellette, A. J. A.; Boyer, G. L.; Twiss, M. R.; Bridgeman, T. B.; Wilhelm, S. W. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Eire using quantitative real time PCR. *Environ. Sci. Technol.* **2005**, *39*, 4198–4205.

(21) APHA, AWWA, WEF. *Standard Methods for the Examination of Water and Wastewater*; APHA: MD, 2005.

(22) Vörös, L.; Padisák, J. Phytoplankton biomass and chlorophyll-a in some shallow lakes in central Europe. *Hydrobiologia.* **1991**, *215*, 111–119.

(23) Fastner, J.; Wirsing, B.; Wiedner, C.; Heinze, R.; Neumann, U.; Chorus, I. Microcystin and hepatocyte toxicity. In *Cyanotoxins; Occurrence, Causes, Consequences*; Chorus, I., Ed.; Springer: Berlin, 2001.

(24) Nagata, S.; Soutome, H.; Tsutsumi, T.; Hasegawa, A.; Sekijima, M.; Sugamata, M.; Harada, K.; Suganuma, M.; Ueno, Y. Novel monoclonal antibodies against microcystin and their protective activity for hepatotoxicity. *Nat. Toxins* **1995**, *3*, 78–86.

(25) Tillett, D.; Parker, D. L.; Neilan, B. A. Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcy*A) of the cyanobacterial genus *Microcystis*: Comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. *Appl. Environ. Microbiol.* **2001**, *67* (6), 2810–2818.

(26) Neilan, B.; Jacobs, D.; Deldot, T.; Blackall, L. L.; Hawkins, P. R.; Cox, P. T.; Goodman, A. E. rRNA sequences and evolutionary relationships among toxic and non-toxic cyanobacteria of the genus. *Microcyst. Int. J. Syst. Bacteriol.* **1997**, *47* (3), 693–697.

(27) Carlson, R. E. A trophic state index for lakes. *Limnol. Oceanogr.* **1977**, *22* (2), 361–369.

(28) Takizawa, S. *Statistical Methods for Environmental Engineering*; Saiensu: Tokyo, 2004; (in Japanese).

(29) Cuvin-Aralar, M. L.; Fastner, J.; Focken, U.; Becker, K.; Aralar, E. V. Microcystins in natural blooms and laboratory cultured *Microcystis aeruginosa* from Laguna de Bay, Philippines. *Syst. Appl. Microbiol.* **2002**, *25*, 179–182.

(30) Becker, S.; Fahrbach, M.; Böger, P.; Ernst, A. Quantitative tracing, by Taq nuclease assays, of a *Synechococcus ecotype* in a highly diversified natural population. *Appl. Environ. Microbiol.* **2002**, *68* (9), 4486–4494.

(31) Tanabe, Y.; Kaya, K.; Watanabe, M. M. Evidence for recombination in the microcystin synthetase (*mcy*) genes of toxic cyanobacteria *Microcystis* spp. *J. Mol. Evol.* **2004**, *58*, 633–641.

(32) Kaneko, T.; Nakajima, N.; Okamoto, S.; Suzuki, I.; Tanabe, Y.; Tamaoki, M.; Nakamura, Y.; Kansai, F.; Watanabe, A.; Kawashima, K.; Kishida, Y.; Ono, A.; Shimizu, Y.; Takahashi, C.; Minami, C.; Fujishiro, T.; Kohara, M.; Katoh, M.; Nakazaki, N.; Nakayama, S.; Yamada, M.; Tabata, S.; Watanabe, M. M. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. *DNA Res.* **2007**, *14*, 247–256.

(33) Ichise, S.; Tanaka, K.; Sono, T.; Wakabayashi, T.; Yamanaka, S.; Kaneda, E.; Yada, M.; Naitoh, M.; Kawabe, K.; Aoki, K.; Sanuki, N.; Fujiwara, N.; Nomura, K. Unusual prolification and distribution of picoplankton: distribution of picoplankton. *Bull. J. Soc. Sci. Fish.* **1995**, *61*, 932–933.

(34) Kotak, B. G.; Lam, A. K. Y.; Prepas, E. E.; Kenefick, S. L.; Hrudey, S. E. Variability of the hepatotoxin microcystin-LR in hypereutrophic drinking-water lakes. *J. Phycol.* **1995**, *31*, 248–263.

(35) Sedmak, B.; Kosi, G. The role of microcystins in heavy cyanobacterial bloom formation. *J. Plankton. Res.* **1998**, *20* (4)), 691–708.

(36) Ohtake, A.; Shirai, M.; Aida, T.; Mori, N.; Harada, K.-I.; Matsuura, K.; Suzuki, M.; Nakano, M. Toxicity of *Microcystis* species isolated from natural blooms and purification of the toxin. *Appl. Environ. Microbiol.* **1989**, *55*, 3202–3207.

(37) Vézie, C.; Brient, L.; Sivonen, K.; Bertru, G.; Lefeuvre, J. C.; Salkinoja-Salonen, M. Variation of microcystin content of cyanobacterial blooms and isolated strains in Lake Grand-Lieu (France). *Microb. Ecol.* **1998**, *35*, 126–135.

(38) Shirai, M.; Ohtake, A.; Sano, T.; Matsumoto, S.; Sakamoto, T.; Sato, A.; Aida, T.; Harada, K.; Shimada, T.; Suzuki, M.; Nakano, M. Toxicity and toxins of natural blooms and isolated strains of *Microcystis* spp. (Cyanobacteria) and improved procedure for purification of cultures. *Appl. Environ. Microbiol.* **1991**, *57* (4), 1241–1245.

(39) Doblin, M. A.; Coyne, K. J.; Rinta-Kanto, J. M.; Wilhelm, S. W.; Dobbs, F. C. Dynamics and short-term survival of toxic cyanobacteria species in ballast water from NOBOB vessels transiting the Great Lakes—implications for HAB invasions. *Harmful Algae.* **2007**, *6* (4), 519–530.

(40) Hotto, A. M.; Satchwell, M. F.; Berry, D. L.; Gobler, C. J.; Boyer, G. L. Spatial and temporal diversity of microcystins and microcystin-producing genotypes in Oneida Lake, NY. *Harmful Algae* **2008**, *7* (5), 671–681.

(41) Rogalus, M. K.; Watzin, M. C. Evaluation of sampling and screening techniques for tiered monitoring of toxic cyanobacteria in lakes. *Harmful Algae.* **2008**, *7* (4), 504–514.

(42) Chorus, I.; Niesel, V.; Fastner, J.; Wiedner, C.; Nixdorf, B.; Lindenschmidt, K. E. Environmental factors of microcystin levels in waterbodies. In *Cyanotoxins; Occurrence, Causes, Consequences*; Chorus, I., Eds.; Springer: Berlin, 2001.
(43) Gupta, S.; Giddings, M.; Sheffer, M. Cyanobacterial toxins in drinking water: A Canadian perspective. In *Cyanotoxins; Occurrence, Causes, Consequences*; Chorus, I., Eds.; Springer: Berlin, 2001.
(44) Jiang, Y.; Ji, B.; Wong, R. N. S.; Wong, M. H. Statistical study on the effects of environmental factors on the growth and microcystins production of bloom-forming cyanobacterium—*Microcystis aeruginosa*. *Harmful Algae*. **2008**, *7* (2), 127–136.
(45) Gobler, C. J.; Davis, T. W.; Coyne, K. J.; Boyer, G. L. Interactive influences of nutrient loading, zooplankton grazing, and microcystin synthetase gene expression on cyanobacterial bloom dynamics in a eutrophic New York lake. *Harmful Algae*. **2007**, *6* (1), 119–133.

ES801265F