

Spatial Distribution and Temporal Variation of *Microcystis* Species Composition and Microcystin Concentration in Lake Biwa

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ABSTRACT: Spatial and temporal variation in *Microcystis* species composition and microcystin concentration, quantified by enzyme-linked immunosorbent assay and high-performance liquid chromatography, were investigated during a 3-year period (1998–2000) in the Northern Basin of Lake Biwa. The Northern Basin generally had a concentration of $5 \mu\text{g L}^{-1}$ or less, except at station 1 (Nagahama Bay) from July to October during the study period. The maximum concentration at station 1 was 22.7, 35.9, and $22.0 \mu\text{g L}^{-1}$ in October of 1998, 1999, and 2000, respectively. Eleven species of cyanobacteria were observed: *Microcystis aeruginosa*, *M. ichthyoblabe*, *M. novacekii*, *M. wesenbergii*, *Oscillatoria raciborskii*, *Anabaena oumiana*, *A. affinis*, *A. flos-aquae*, *A. ucrainica*, *A. smithii*, and *A. crassa*. Of these, *M. aeruginosa* and *M. wesenbergii* were the main components observed. A high concentration of microcystin in the lake water was mostly a result of variation in the relative amount of toxic *M. aeruginosa* rather than of the total *Microcystis* cell number. This was supported by the analytical results for isolated strains. *Microcystis* spp. cell density in the Northern Basin appeared to increase gradually over the course of the study. This is the first study to have surveyed the Northern Basin of Lake Biwa, which supplies drinking water to 14 million people and is the largest lake in Japan. © 2005 Wiley Periodicals, Inc. *Environ Toxicol* 20: 270–276, 2005.

Keywords: horizontal distribution of microcystin; Lake Biwa; microcystin concentration; *Microcystis* strain; seasonal variation of microcystin

INTRODUCTION

Cyanobacterial blooms in lakes, rivers, and artificial water bodies, including drinking water reservoirs, have been

reported in different regions of the world and have sometimes caused severe problems for wildlife, livestock, and humans (Carmichael, 1994; Christoffersen, 1996). Cyanobacteria are known to produce a variety of metabolites including toxins, among which microcystins (MCs) are globally the most frequent. MCs have caused many animal deaths and have been implicated in cases of human illness (Ueno et al., 1996; Jochimsen et al., 1998). Because MCs have been found in drinking water (Ueno et al., 1996), the World Health Organization

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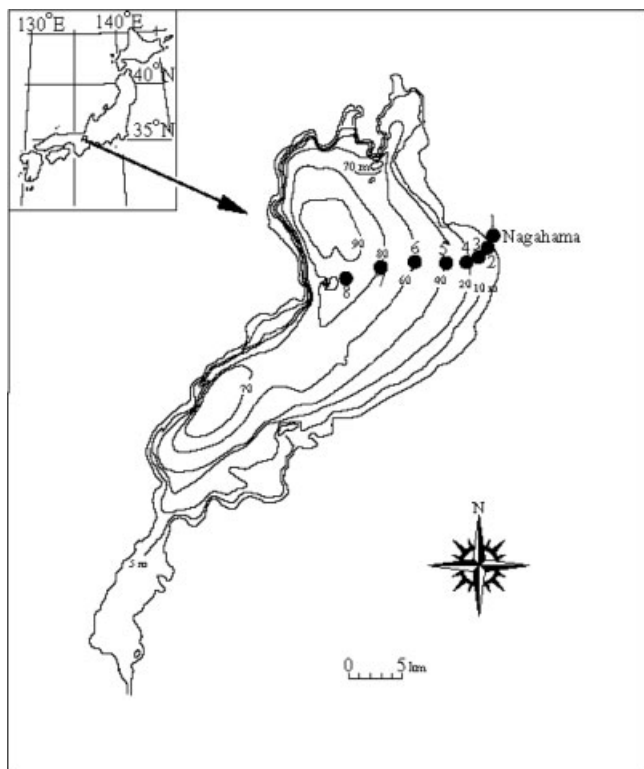


Fig. 1. Map of Lake Biwa with sampling stations.

(WHO, 1998) proposed as a guideline that the MC-LR concentration in drinking water no greater than $1.0 \mu\text{g L}^{-1}$.

The chemical structure of MC is composed of seven amino acids including a unique amino acid called Adda [3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Botes et al., 1984], and 62 variants of MCs have been reported to date (Rinehart et al., 1994; Park et al., 2001). MCs are produced by *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc*, and *Microcystis* spp., commonly *M. aeruginosa*, has been linked to toxic blooms worldwide (Sivonen, 1996). In aquatic environments MCs usually remain within cyanobacterial cells but are released naturally by the biological process of lysis and artificially by inducing cell destruction with copper sulfate or other treatments. In view of the high chemical stability (Harada and Tsuji, 1998) and water solubility of MCs, contamination by toxic blooms has important implications for their environmental persistence and for exposure to humans in surface water bodies (Chorus and Bartram, 1999).

Lake Biwa, in central Honshu Island, is the largest lake in Japan (Fig. 1). It is characterized by two morphologically distinct basins: the deep Northern Basin (616 km^2 ; average depth = 43 m) and the shallow Southern Basin (58 km^2 ; average depth = 4 m). Lake Biwa supplies drinking water to 14 million people living around the lake. In addition, the lake is a tourist attraction and is used for various recreational purposes. Since 1950 the lake has been subject to eutrophication, and blooms of *Anabaena* and *Microcystis* have occurred

since 1983, especially in the Southern Basin (Kumagai and Roberts, 1996). Since 1993 blooms caused by *Microcystis* also have occurred in inshore areas of the Northern Basin with poor water circulation, such as Nagahama Harbor (station 1 in Fig. 1). More recently, cyanobacterial blooms have been observed from late summer to autumn, even in the mesotrophic Northern Basin (Watanabe et al., 2000).

In this article we describe the horizontal distribution and seasonal variation of *Microcystis* cell density and MC concentration in the Northern Basin of Lake Biwa, quantified by enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC). We also report the characteristics of MC production by a strain of *Microcystis* isolated from this lake. The overall objective of the present study therefore was to determine the quantity of the MCs in the lake and to elucidate the relationship between the dominant cyanobacterial species and MC concentration.

MATERIALS AND METHODS

Chlorophyll-*a* Concentration and Cyanobacteria Count

Eight sampling stations were set up along an east–west transect starting at Nagahama Harbor (station 1 in Fig. 1). An investigation was carried out from April to October in 1998 and from July to October in 1999 and 2000.

At each station a 10-L sample of surface water was collected with a sampling bucket (Fig. 1). Phytoplankton biomass was estimated as chlorophyll, which was determined with a water sample filtered through a glass-fiber filter (GF/C, Whatman, UK). The filter was frozen and extracted in methanol overnight in a dark freezer. After centrifugation of the extract, the concentration of chlorophyll-*a* was determined spectrophotometrically according to Marker et al. (1980). Samples for enumerating the *Microcystis* cells were fixed with Lugol's iodine solution and estimated by using a Sedgwick–Rafter counting chamber under an epifluorescence microscope.

Microcystin Analysis

To estimate MC concentration in cyanobacterial cells, a plankton sample was collected in 1998 with a $40\text{-}\mu\text{m}$ -mesh plankton net from surface water. The net was hauled horizontally through the surface water. The phytoplankton sample was then transported to the laboratory and stood to permit separation of cyanobacteria from other phytoplankton. Floating cyanobacterial cells were concentrated on a GF/C filter, and the filter was immediately frozen at -40°C .

MC was analyzed quantitatively according to Park et al. (1998). Briefly, frozen cyanobacterial cells on GF/C were lyophilized and extracted 3 times with 10 mL of 5% aqueous acetic acid for 30 min while being stirred. The

extract was centrifuged at approximately $6000 \times g$ for 30 min, and the supernatant was applied directly to an ODS (octadecylsilane) cartridge (0.5 g) preconditioned with methanol and water. The cartridge was rinsed with distilled water, followed by 20% methanol, and the toxin was finally eluted from the cartridge with a trifluoroacetic acid (TFA)-methanol solution. The eluate was completely evaporated to dryness, and the residue was suspended in methanol. The methanol solution was subjected to reversed-phase HPLC using a Cosmosil (Nakarai, Japan) 5C18-AR 4.6×150 mm ODS column; The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump coupled to an SPD-10A set at 238 nm, an SPD-M10A photodiode array detector, and a C-R6A integrator. The sample was separated with a mobile phase consisting of methanol:0.05 M phosphate buffer (pH 3.0, 58:42) at a flow rate of 1 mL min^{-1} .

Indirect Competitive ELISA for Detection of Microcystin

The preparation of bloom and tap water samples and their analysis by ELISA were performed using the method of Nagata et al. (1995). About 20 mL of sample was collected directly into a double-capped plastic bottle, then 0.2 mL of 10% sodium azide was added and maintained at -20°C . The samples were freeze-thawed twice, then filtered through Whatman GF/C glass-fiber filters (25 mm in diameter) and used for the ELISA. The water samples and MC-LR standard were mixed with the appropriate dilution of anti-MC-LR MAb M8H5 and added to a 96-well microtiter previously coated with MC-LR-bovine serum albumin conjugate. After washing, the bound MAb was detected with horseradish peroxidase-labeled goat antimouse IgG (TAGO 4550) plus substrate (0.1 mg mL^{-1} of 3',3',-5,5'-tetramethyl benzidine, 0.05% H_2O_2 in 0.1 M acetate buffer, pH 5), and absorbance was measured at 450 nm. The mean of two triplicate estimates of the ELISA data was calculated, expressed as picograms per milliliter of MC-LR, with a detection limit of 50 pg mL^{-1} .

Isolation and Incubation of *Microcystis*

Colonies of *Microcystis* from the Northern Basin were collected with a needle, and single cells were picked up using a capillary pipette under an inverted microscope and inoculated to a test tube with 10 mL of CT medium (Watanabe and Ichimura, 1977). All together 22 *Microcystis* strains were isolated from the colonies. The isolated cells were incubated at 28°C under a 14:10 LD cycle with a light intensity of approximately $100 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. After incubation the cells were collected by centrifugation and lyophilized for MC analysis as described above.

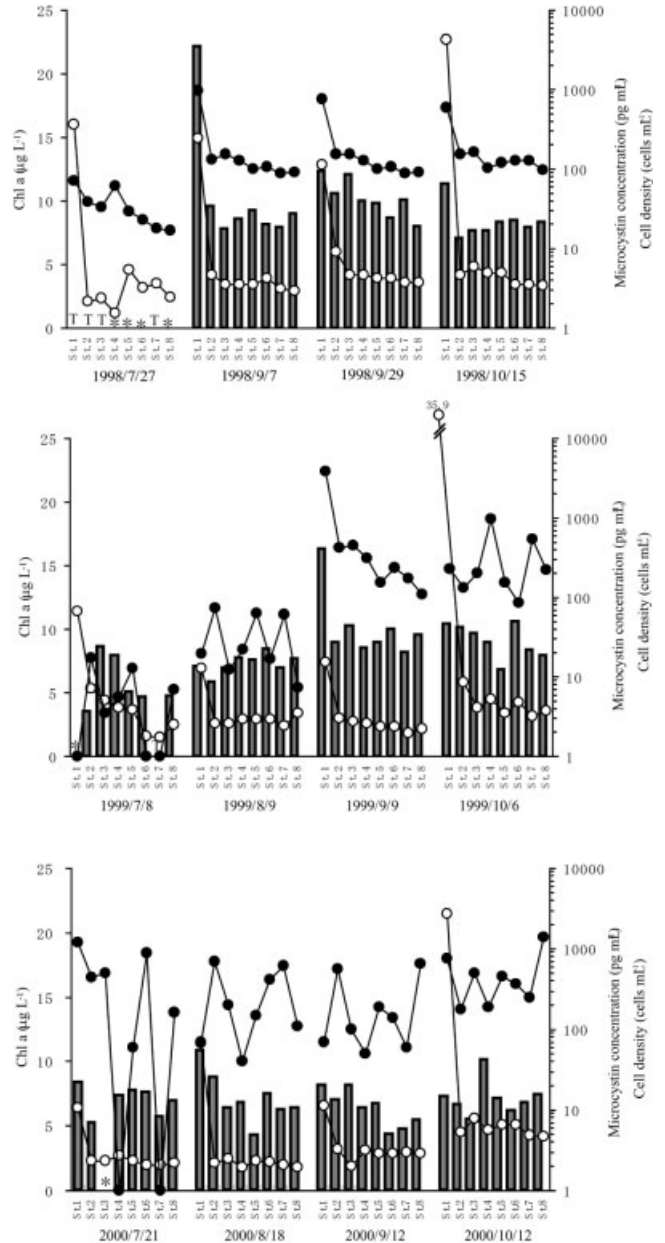


Fig. 2. Horizontal distribution and seasonal variation of chlorophyll a concentration (open circles), microcystin concentration quantified by ELISA (solid bars), and *Microcystis* cell density (solid circles) from 1998 to 2000 [*microcystin not detected; T, traces (less than quantitative limit)].

RESULTS

Horizontal Distribution and Seasonal Variation of *Microcystis*

Figure 2 shows the horizontal distribution and seasonal variation of chlorophyll-a concentration during the 3-year investigation. The Northern Basin generally had a concentration of about $5 \mu\text{g L}^{-1}$ or less, except for station 1 from July to October. The maximum concentration at station 1

was 22.7, 35.9 and 22.0 $\mu\text{g L}^{-1}$ in October 1998, 1999, and 2000, respectively (Fig. 2).

Eleven species of cyanobacteria were observed: *Microcystis aeruginosa*, *M. ichthyoblabe*, *M. novacekii*, *M. wesenbergii*, *Oscillatoria raciborskii*, *Anabaena oumiana*, *A. affinis*, *A. flos-aquae*, *A. ucrainica*, *A. smithii*, and *A. crassa*. The main components of the cyanobacteria were *M. aeruginosa* and *M. wesenbergii*. *M. viridis* was not found in this investigation, although it was seen in a previous study (Watanabe et al., 2000), but only at station 1 in 1995, as this was found to be a rare species.

Microcystis cell density differed markedly in the patterns of horizontal distribution and seasonal variation during the study period (Fig. 2). The lowest density was observed in 1998 (data not shown). In that year *Microcystis* was not observed at any station from April to June, appearing for the first time in July, but at low density (less than 100 cells mL^{-1}). The density at station 1 increased on September 7, with a maximum density of 960 cells mL^{-1} , and remained relatively high until October. However, the density at stations 2–8 was relatively constant, ranging from 75 to 160 cells mL^{-1} from September to October, and tended to be much lower than density at inshore station 1. In 1999 *Microcystis* was distributed widely throughout the lake from the end of summer to autumn but was hardly observed at any sites in July. Density increased between July and August, with the highest value recorded at station 1 in September (3900 cells mL^{-1}), which subsequently had decreased to 220 cells mL^{-1} in October. The highest and second highest density values were observed at the offshore stations, stations 4 and 7, respectively. In 2000 *Microcystis* had the widest and earliest distribution throughout the Northern Basin during the study period. Density varied markedly, with some peaks at the offshore stations. *Microcystis* was observed from July on except at stations 4 and 7. The highest density was recorded in October, not at the inshore station, station 1, but instead at station 8 (1.4×10^3 cells mL^{-1}).

The relative abundance of cyanobacteria during this study is shown in Figure 3. The relative abundance of the various species of *Microcystis* dominant at each station changed markedly during the study. In 1998 the dominant cyanobacteria were *M. aeruginosa* and *M. wesenbergii*. The relative abundance of *M. aeruginosa* and *M. wesenbergii* ranged from 0% to 92.8% (average = 48.9%) and from 5.1% to 100% (average = 45.5%), respectively (Fig. 3). The relative abundance of *M. aeruginosa* tended to decrease gradually from station 1 to station 8. In 1999 the relative abundance of *M. aeruginosa* and *M. wesenbergii* ranged from 0% to 88.6% (average = 28.2%) and from 11.4% to 100% (average = 57.5%), respectively. *M. aeruginosa* and *M. wesenbergii* were dominant in August and September, but only the latter in October. In 2000 the relative abundance of *M. aeruginosa* and *M. wesenbergii* ranged from 0% to 90.9% (average = 22.1%) and 9.1% to

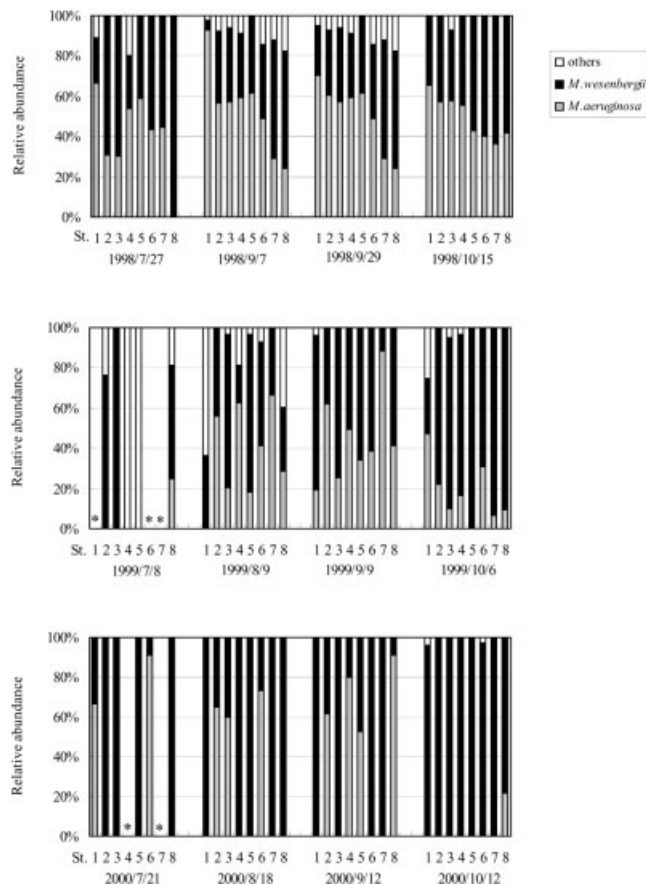


Fig. 3. Seasonal changes and relative abundance of cyanobacteria in an east–west transect of Lake Biwa during 1998–2000 (*no cyanobacteria).

100% (average = 77.7%), respectively. *M. wesenbergii* showed a marked dominance at all stations in October.

Horizontal Distribution and Seasonal Variation of Microcystin

The concentration of MC differed substantially between years. It was most pronounced in 1998, although *Microcystis* cell density was the least among the three years. A small quantity of MC was detected at stations 1–3 and 7 in July. The MC concentration in surface water increased corresponding to when *Microcystis* cell density was increasing. The maximum MC concentration in the surface water was recorded on September 7 (3622 pg mL^{-1}), when that in the GF/C filtrate fraction (extracellular MC) was 68 pg mL^{-1} . This indicates that approximately 2% of MC was dissolved in the lake water. The MC concentration in the surface water then decreased at station 1. In 1999 the MC concentration in the surface water increased gradually from July to September in accord with the increase in the number of *Microcystis* cells. The maximum concentration was recorded at station 1 on September 9 (401 pg mL^{-1}). In

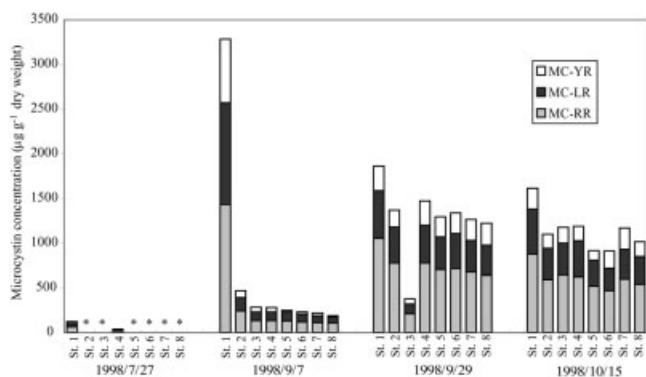


Fig. 4. Horizontal distribution and seasonal variation of microcystin concentrations in cyanobacterial cells in 1998 (*not detected).

2000 MC concentrations were lower overall (maximum was $56.5 \mu\text{g mL}^{-1}$ at station 1 on August 18), whereas *Microcystis* cell density was the highest. The changes in MC concentration were not always consistent with those in *Microcystis* cell density.

Toxicity of Cyanobacterial Cells

In 1998, the only year for which samples of cyanobacterial cells were collected for MC analysis, MC-RR, -LR, and -YR

were detected by HPLC analysis in cyanobacterial cells collected with a $40\text{-}\mu\text{m}$ plankton net (Fig. 4). MC-RR and -LR were the main toxins but MC-YR was also detected in almost all samples. Total MC concentration in cyanobacteria ranged from 39.0 to $3284 \mu\text{g g}^{-1}$ dry weight. The average values for MC-RR, -LR, and -YR were 53.0%, 32.0%, and 15.0% of total microcystin content, respectively. There was a seasonal change in the peak concentration of MC in cyanobacterial cells at each station. At station 1 the concentration increased on September 7 and decreased thereafter. In contrast, the concentration generally increased on September 29 and remained constant until October at stations 2–8. There was a tendency for values to decrease from station 1 to station 8.

Toxicity of Isolated Strains

The toxicity of 22 strains isolated from Lake Biwa was analyzed by reversed-phase HPLC (Table I). The characteristics of MC production by the various strains differed markedly. MC content of strains of *M. aeruginosa* showed wide variation. Eight of the 10 *M. aeruginosa* strains contained a large amount of MCs, ranging from 1078 to $3715 \mu\text{g g}^{-1}$ dry weight, whereas the other two had small amounts (153 and $78 \mu\text{g g}^{-1}$ dry weight, respectively). The main components of the MCs produced by *M. aeruginosa* were MC-RR and -LR. Three strains (M12, M21, and M23) had high production of MC-YR but not of MC-RR. Two of the three

TABLE I. Characteristics of microcystin production by *Microcystis* strains isolated from Lake Biwa

Strain Number	Species	Microcystin Concentration ($\mu\text{g g}^{-1}$ dw)			
		RR	YR	LR	Total
M1	<i>Microcystis aeruginosa</i>	153	ND	ND	153
M12	<i>Microcystis aeruginosa</i>	7	1959	1214	3180
M15	<i>Microcystis aeruginosa</i>	1199	29	265	1493
M17	<i>Microcystis aeruginosa</i>	495	ND	962	1457
M18	<i>Microcystis aeruginosa</i>	1361	ND	374	1735
M20	<i>Microcystis aeruginosa</i>	78	ND	ND	78
M21	<i>Microcystis aeruginosa</i>	ND	2001	1714	3715
M23	<i>Microcystis aeruginosa</i>	ND	1306	1746	3053
M27	<i>Microcystis aeruginosa</i>	ND	ND	3647	3647
M35	<i>Microcystis aeruginosa</i>	604	ND	474	1078
M13	<i>Microcystis ichthyoblabe</i>	94	ND	25	119
M26	<i>Microcystis ichthyoblabe</i>	T	ND	T	T
M29	<i>Microcystis ichthyoblabe</i>	T	ND	T	T
M5	<i>Microcystis novacekii</i>	5120	ND	2030	7150
M10	<i>Microcystis novacekii</i>	3137	ND	320	3457
M22	<i>Microcystis novacekii</i>	251	39	237	528
M4	<i>Microcystis wesenbergii</i>	T	ND	T	T
M8	<i>Microcystis wesenbergii</i>	T	ND	ND	T
M24	<i>Microcystis wesenbergii</i>	T	ND	T	T
M36	<i>Microcystis wesenbergii</i>	T	ND	T	T
M37	<i>Microcystis wesenbergii</i>	T	ND	ND	T
KOY3	<i>Microcystis wesenbergii</i>	T	ND	ND	T

T: traces (less than quantitative limit); ND: not detected.

M. novacekii strains contained a large amount of MCs, with strain M5 having the greatest ability to produce MC (7150 $\mu\text{g g}^{-1}$ dry weight) of the 22 strains isolated. The three strains of *M. ichthyoblabe* were almost nontoxic, with only strain M13 containing a small amount of MCs. Very small quantities of MC-RR and/or -LR were detected by ELISA from HPLC fractions from six strains of *M. wesenbergii*, but the concentrations were below the detection limit for HPLC.

DISCUSSION

Although toxic cyanobacteria occur in a large number of lakes, reservoirs, and rivers, quantitative reports on horizontal distribution of cyanobacterial species composition and toxin concentration are very few. Our study showed considerable differences in *Microcystis* cell density within and between years. Typically, although there were exceptions (Fig. 2), *Microcystis* cell density at inshore stations (stations 1–3) was higher than that at offshore stations (stations 4–8). This is because levels of nutrients such as ammonium ($\text{NH}_4\text{—N}$) and nitrate ($\text{NO}_3\text{—N}$) are higher at inshore than at offshore stations (Ishikawa et al., 2002). Dilution associated with greater lake depth and counterclockwise circulation during thermal stratification may contribute to low *Microcystis* density. Tsujimura et al. (2000) found that *Microcystis* colonies do not return to the water column from the lake sediment, because no seasonal changes in *Microcystis* colony density were observed in the deep Northern Basin of Lake Biwa. Furthermore, Ishikawa et al. (2002) addressed the hypothesis that buoyant, nutrient-replete colonies of cyanobacteria generated inshore are advected to offshore by large-scale horizontal transport processes. It can be concluded that *Microcystis* was widely distributed throughout the inshore parts of the Northern Basin.

Our study showed that peaks of *Microcystis* cell density did not necessarily coincide with those of MC concentration. There are at least two possible reasons for this. First, high concentrations of MC occurred concurrently with the exponential growth phase of the bloom (Park et al., 1998), and large amounts of MC were detected when *Microcystis* cell density increased exponentially (station 1 on September 7, 1998, and September 9, 1999). Sampling on the equivalent day in the previous month recorded a high concentration of MC, but a much lower cell density. Second, temporal variation of the toxin may be related to *Microcystis* species composition (Park et al., 1993, 1998). Moreover, Carmichael and Gorham (1977, 1981) suggested that variation in the toxicity of blooms may be partly a result of changes in the relative proportion of toxic and nontoxic strains of the toxin-producing species. The MC concentration in 1998, when *M. aeruginosa* was dominant, was the highest during the 3 years, whereas that in 2000, when *M. wesenbergii* was dominant, was the lowest. This suggests that changes in MC concentration are related to changes in dominant *Microcystis* spp. The

properties of MC production by isolates from Lake Biwa also support this hypothesis. Almost all the strains identified as *M. aeruginosa* and *M. novacekii* had large quantities of MCs, but *M. ichthyoblabe* and *M. wesenbergii* were nontoxic. These results coincide with the reports by Watanabe et al. (1988, 1991). However, there were reports of MCs being detected from *M. wesenbergii* (Yasuno et al., 1998). The cyanobacterial cell fraction collected in 1998 showed that the amounts of MC-YR contained in this fraction were high compared to those from other Japanese lakes. The main components of cyanobacterial toxins are known to be MC-RR and MC-LR, and MC-YR has been reported in small quantities or as undetectable in Japanese lakes (Park et al., 1993, 1998). In natural water bodies with toxic cyanobacteria, a number of MCs may be present. For example, in a *Microcystis* bloom from Homer Lake, in the United States, 12 MCs were characterized (Namikoshi et al., 1992), and in a *M. ichthyoblabe* bloom from Lake Oued Mellah, 11 microcystin variants were detected by HPLC (Sabour et al., 2002). It has been reported that almost all toxic strains of *Microcystis* produce several MCs, but generally one or two are dominant (Watanabe et al., 1988, 1991). The results reported from these studies coincide with our results for isolated strains, including that three strains of *M. aeruginosa* could produce high amounts of MC-YR (Table I). The characteristic MC composition and production by a cyanobacterial bloom may be attributed to the dominance of a particular species or strain.

In conclusion, the results obtained from our 3-year investigation indicate that the high concentration of MC in Lake Biwa was mostly a result of the relative amount of toxic *M. aeruginosa* rather than of total *Microcystis* cell density. This is supported by the analytical results for isolated strains (Table I). It seems that the cell density of *Microcystis* spp. is gradually increasing in the Northern Basin. This is the first study to survey the Northern Basin of Lake Biwa. Given that buoyant cyanobacterial species are accumulated by physical processes and sometimes have high amounts of toxin, it is necessary to monitor the dynamics of *Microcystis* spp. blooms continuously in the interest of public health.

We dedicate this article to the late Professor Mariyo F. Watanabe, who initiated cyanotoxin studies in Japan. We express our gratitude to the captain, engineer, and crew of the R/V *Hakken* for their help in the field investigation. We also thank many colleagues at Shinshu University for supporting our investigation and analyses.

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