

Temporal Variabilities of the Concentrations of Intra- and Extracellular Microcystin and Toxic *Microcystis* Species in a Hypertrophic Lake, Lake Suwa, Japan (1991–1994)

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Received 3 July 1996; revised 19 October 1996; accepted 31 January 1997

ABSTRACT: Temporal variability in the concentration of toxic heptapeptide microcystin was studied during the warm season of four years (1991–1994) in a hypertrophic lake (Lake Suwa) in central Honshu, Japan. Lake water samples (ca. 5 L) were filtered to separate intracellular microcystin (cell fraction) from extracellular microcystin (filtered lake water fraction). These fractions were analyzed to measure the total quantity of microcystin in lake water. Total amounts of extra- and intracellular microcystin were measured with high performance liquid chromatography. Concentrations of intracellular microcystin usually exceeded concentrations of extracellular microcystin (24 out of 26 times). High concentrations of intracellular microcystin were found during the exponential growth phase of the blooms, whereas concentrations of extracellular microcystin were highest at the end of the blooms. However, concentrations of extracellular microcystin remained very small ($< 4 \mu\text{g/L}$) compared to the levels of intracellular microcystin. The relatively higher percentages of microcystin in filtered lake water ($> 20\%$) at the end of blooms suggests that release of microcystin from cells occurs during senescence and the decomposition period of *Microcystis* cells. © 1998 by John Wiley & Sons, Inc. *Environ Toxicol Water Qual* 13: 61–72, 1998

Keywords: extracellular microcystin; intracellular microcystin; hepatotoxin; Lake Suwa; microcystin; *Microcystis* species; temporal variation of microcystin

INTRODUCTION

Mass development of planktonic cyanobacteria is one of the consequences of worldwide acceleration of eutrophication in many freshwater bodies, including those in the temperate and subtropical regions. This phe-

nomenon is thought to be the result of increased exogenous nutrient loadings, coupled with temperature and light conditions. Toxic cyanobacterial blooms in eutrophic lakes and reservoirs have been reported in many countries (Skulberg et al., 1984; Carmichael, 1988a; Gorham and Carmichael, 1988). These toxic blooms have caused the death of livestock and wildlife in addition to cases of illness in humans (Billings, 1981; Falconer, 1989). Even though similar toxic cyanobacte-

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rial species present in freshwater sources in the countries where poisonous events have taken place also occur in Japan, no mortalities of wild or domestic animals have been reported. Aqueous extracts from the cyanobacteria were found to be lethal, however, upon intraperitoneal injection into mice (Watanabe and Oishi, 1980). Despite the presence of toxic cyanobacteria in many lakes, very little research has been done on the seasonal variation of toxins (Wicks and Theil, 1990; Lindholm and Meriluoto, 1991; Park et al., 1993b; Watanabe et al., 1994; Kotak et al., 1995).

Toxins from freshwater cyanobacteria are classified functionally into two groups: hepatotoxins and neurotoxins. *Microcystis aeruginosa* is the most common toxic cyanobacterium found worldwide, and it produces potent cyclic peptide hepatotoxins, termed microcystins (Carmichael, 1988a, b; Ohtake et al., 1989; Carmichael et al., 1990; Harada et al., 1991; Carmichael, 1992), of which more than 50 variants have been isolated. Microcystins are also found in *Microcystis viridis* (Watanabe et al., 1986; 1988; Kusumi et al., 1987), *Anabaena flos-aquae* (Krishnamurthy et al., 1986), *Oscillatoria agardhii* (Meriluoto et al., 1989), and *Nostoc* sp. (Sivonen et al., 1990). The chemical structures of the hepatotoxins contained in *M. aeruginosa* were elucidated by Botes et al. (1984, 1985). These cyclic heptapeptides are composed of five common amino acids with variations that combine a pair of L-amino acids. The structural differences among the toxins are related to the two L-amino acids (Fig. 1). Recently, desmethyl derivatives have been reported (Harada et al., 1991) in which methyl groups of *N*-methyldehydroalanine and *N*-methyl aspartic acid are replaced by hydrogen atoms. Microcystins and nodularin inhibit protein phosphatase activity, especially type 1 and 2A, in a manner similar to that of okadaic acid (Matsushima et al., 1990; Yoshizawa et al., 1990; Mackintosh et al., 1990). Microcystin-YR and -LR were purified from a Japanese strain of *Microcystis aeruginosa* isolated from Lake Suwa

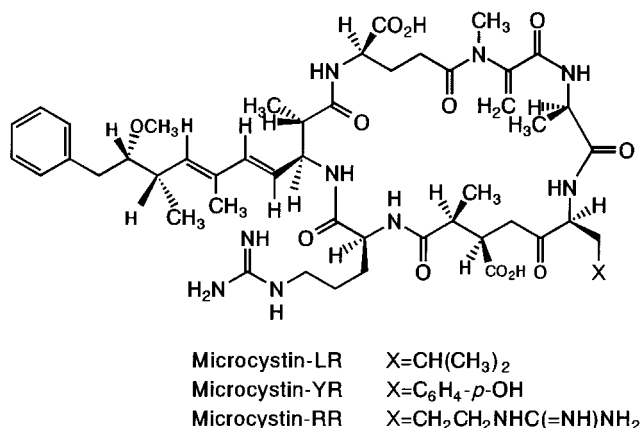


Fig. 1. Structure of three heptapeptide toxins: microcystin-LR, -YR, and -RR.

by Kungswan et al. (1987). Thereafter, three toxins (microcystin-RR, -YR, and -LR) were detected in two strains of *M. aeruginosa* and four strains of *M. viridis* (Watanabe et al., 1988), plus natural samples of *Microcystis* spp. obtained from lakes in Japan (Watanabe et al., 1989; Park et al., 1993a).

In spite of the frequent occurrence of toxic *Microcystis* species, very little research has been done on the dynamics or fate of the toxins in lake ecosystems. Temporal variability of intra- and extracellular microcystin and the seasonal change of *Microcystis* species were investigated in the present study to establish a quantitative assessment of the toxins in a hypertrophic lake.

MATERIALS AND METHODS

Lake Suwa (36°3'N, 138°5'E), a typical hypertrophic shallow lake, is located in the Japanese prefecture of Nagano, in central Honshu, at an altitude of about 760 m. The surface area of the lake is 13.3 km², with a maximum depth of 6.8 m and an average depth of about 5 m (Fig. 2). A single stream, the Tenryu River, drains the lake water into the Pacific Ocean. Because of its shallowness, vertical mixing by the wind does occur, even during the summer. Although Lake Suwa is located in a mountainous area, the surrounding cities, with a combined population of over 170,000 inhabitants, contribute a large anthropogenic input. A dense bloom of *Microcystis* is regularly observed during the summer season.

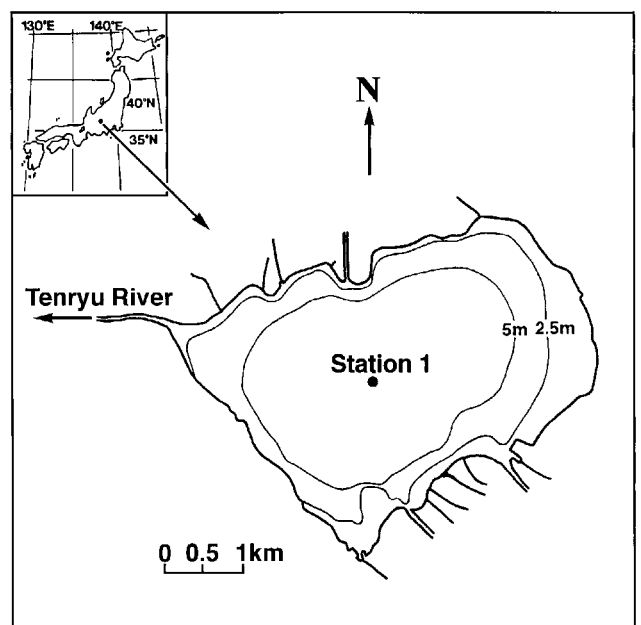


Fig. 2. Map of Lake Suwa showing the sampling station.

Microcystis cells for determination of microcystin content and cell concentration were collected from the center of Lake Suwa at 10 day intervals from June to October in the years 1991–1994, using a plankton net (40 μm mesh size). The *Microcystis* cell concentration was estimated by using a hemocytometer (Fuchs-Rosenthal, Kayagaki) under a microscope (Olympus BH-2).

Water temperatures were measured using a thermistor-type underwater thermometer (Takara thermistor type 600). To measure chlorophyll a concentration, water samples were filtered through glass fiber filters (Whatman GF/C) filters and the filters plus their contents were ground in a glass mortar with 10 mL of 90% acetone solution and centrifuged to remove turbidity. The concentration of chlorophyll a was determined by the spectrophotometric method of SCOR-UNESCO (1966).

Measurements of microcystin concentrations and cleanup methods for analysis of trace amounts of microcystins in lake water were done according to Harada et al. (1988) and Tsuji et al. (1994), respectively (Fig. 3). A lake water sample (about 5 L) was filtered through a glass microfiber filter (Whatman GF/C) and adjusted to pH 7. The filter and its content were homogenized and extracted with 5% aqueous acetic acid and the supernatant was applied to an ODS (octadecylsilance)

cartridge (0.5 g, Bakerbond SPE 7020-03) after centrifugation. The 90% methanol-extracted eluate from the cartridge was applied to an high performance liquid chromatography (HPLC) system equipped with an ODS column (Cosmosil 5C18-AR, 4.6×150 mm, Nacalai, Japan). The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump coupled to a SPD-10A set at 238 nm and an SPD-M10A photodiode array detector, and a C-R6A integrator. Microcystin concentrations were quantified by comparing the absorbance peak area of test samples, at 238 nm, after separation with a methanol:0.05 M phosphate buffer (pH 3.0, 58:42) with those of standards. The flow rate was 1 mL/min. The filtered water was applied directly to an ODS silica gel cartridge (5 g; Chromatorex ODS, 100–200 mesh, which was packed into a polypropylene cartridge). The cartridge was rinsed with water and 20% methanol–water. The eluate from the cartridge with 90% methanol–water was evaporated to dryness, and the residue was dissolved in methanol. The methanol solution was applied to a silica gel cartridge (2 g; SepPak), which was preconditioned with methanol, and the cartridge was rinsed with methanol. The eluate from the cartridge with 50% methanol–water was evaporated to dryness; the residue was then dissolved in methanol. The methanol solution was subjected to HPLC analysis to resolve the microcystins.

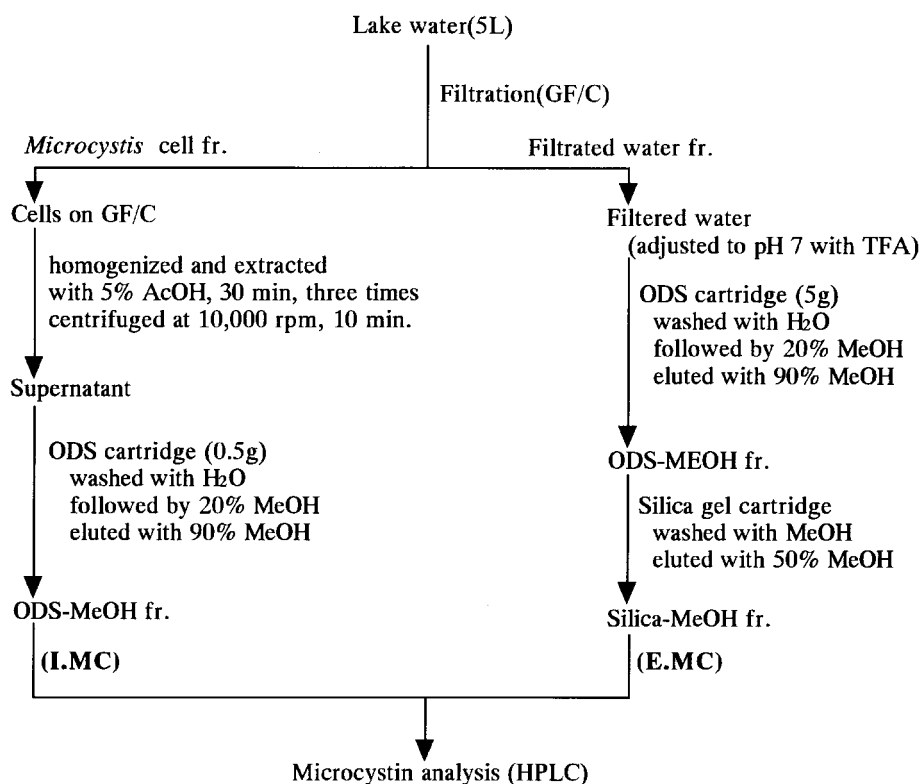


Fig. 3. Procedure for analysis of microcystins in lake water. I.M.C, intracellular microcystin; E.M.C, extracellular microcystin.

RESULTS

Seasonal Changes of *Microcystis* Species Composition and Cell Concentration

Blooms of *Microcystis* in Lake Suwa were composed of five species: *M. aeruginosa*, *M. viridis*, *M. wesenbergii*, *M. ichthyoblabe*, and *M. novacekii*. These five species were not identified until 1993. Figure 4 shows the relative abundances of the *Microcystis* species for the years 1991–1994 in Lake Suwa (June–October).

In 1991 and 1992, three *Microcystis* species and four groups were identified; in 1993 and 1994, five *Microcystis* species were identified. In 1991 and 1992 two groups of cells—small and large—were recognized in *M. aeruginosa*, which was tentatively identified as a species without hyaline layers under a ordinary microscope (Watanabe et al., 1991).

In 1991, although *M. aeruginosa* predominated in June, *M. viridis* began to increase in July and showed a remarkable dominance ranging from 41 to 93% in August and October. Although *M. wesenbergii* showed no dominance in any period, this species was about 30% of all observed species in September and October except at the end of October. *M. aeruginosa* began to increase gradually again in September.

M. viridis showed a remarkable dominance from June to September in 1992, whereas the *M. aeruginosa* small cell size group predominated in October.

In 1993, *M. ichthyoblabe*, *M. viridis*, *M. wesenbergii*, and *M. aeruginosa* codominated in June and July; thereafter *M. aeruginosa* predominated until October.

In 1994, *M. aeruginosa* and *M. ichthyoblabe* dominated in June and July and on October 30, but *M. aeruginosa* and *M. novacekii* or *M. viridis* were present after August. *M. ichthyoblabe* was not present in significant amounts between August and October, but increased again to over 50% between October 20 and 30.

Figure 5 shows the seasonal changes of *Microcystis* cell concentration for the years 1991–1994 in Lake Suwa (June–October; samples taken at 10 day intervals). In 1991 and in 1992, three *Microcystis* species and four groups were identified; in 1993 and 1994, five *Microcystis* species were identified.

In 1991, the cell concentration of *Microcystis* increased from June 11 to August 10, and dropped suddenly on August 20. Thereafter, the cell concentration gradually increased until September, but progressively decreased after September 30. A similar fluctuation of cell concentration was apparent in 1992, but the maximum cell concentration was about one and half times higher than in 1991. In 1992 the cell concentration was also about three times higher than in 1993. The highest cell concentration of *Microcystis* was 8×10^5 cells/mL on October 10, 1994.

Figure 6 shows the seasonal changes of chlorophyll a concentration and water temperature at the surface of Lake Suwa from June to October in the years 1991 to 1994. Water temperatures increased gradually from June to August except in 1993 (this corresponded with *Microcystis* population increase). The water temperature then decreased rapidly from September to October. The summer of 1993 was unusually cool, with unusual amounts of rain. In 1993 chlorophyll a concentrations were therefore much lower than is usual during the warm season in this lake (Park et al., 1993b). The highest concentration of chlorophyll occurred in August in 1991 and 1992. In 1993 and 1994, chlorophyll a concentrations peaked in October. The concentration of chlorophyll (1661 $\mu\text{g/L}$) on October 10, 1994, was the highest on record (records go back to 1969).

Seasonal Changes of Intra- and Extracellular Microcystin

The toxins of *Microcystis* collected from Lake Suwa were identified as microcystin-RR, -YR, and -LR (Fig. 1). Microcystin-RR and -LR were the main components of the toxins contained in Lake Suwa's *Microcystis* blooms; microcystin-YR was either present in very small quantities or undetectable (Park et al., 1993a, b). Figure 7 shows the seasonal changes of microcystin-RR and -LR in dried cells collected at 10 day intervals with a phytoplankton net from June to October in the years 1991–1994. Microcystin-RR and -LR were detected in all samples. Microcystin-YR was not included in the figure because it was detected either in quantities too small to be represented at scale or not detected at all.

In 1991, microcystin content increased gradually from June until July 20 and then rapidly decreased until July 30. Thereafter, no remarkable change in the amounts of these toxins was observed. The highest amount of microcystins in 1991 was 202 μg per 100 mg dried cells on July 20. The fluctuations of microcystin-RR and -LR showed almost the same pattern in 1994 as in 1991. In 1992, microcystin-RR was produced in greater quantities than microcystin-LR, but in lesser quantities in 1993. In 1993, the levels of microcystin were very low. The maximum and minimum values of microcystin throughout the observation period were 226 and 1.8 μg per 100 mg dried cells, respectively. The minimum amounts of the toxins were measured on October 20, 1993.

Figure 8 shows seasonal changes of the microcystin concentrations in Lake Suwa's water. Microcystin concentrations calculated include intracellular microcystin and extracellular microcystin. Table I shows lake water concentration of microcystin at 10 day intervals between June and October from 1992 to 1994. These concentrations include the *Microcystis* cell fraction (in-

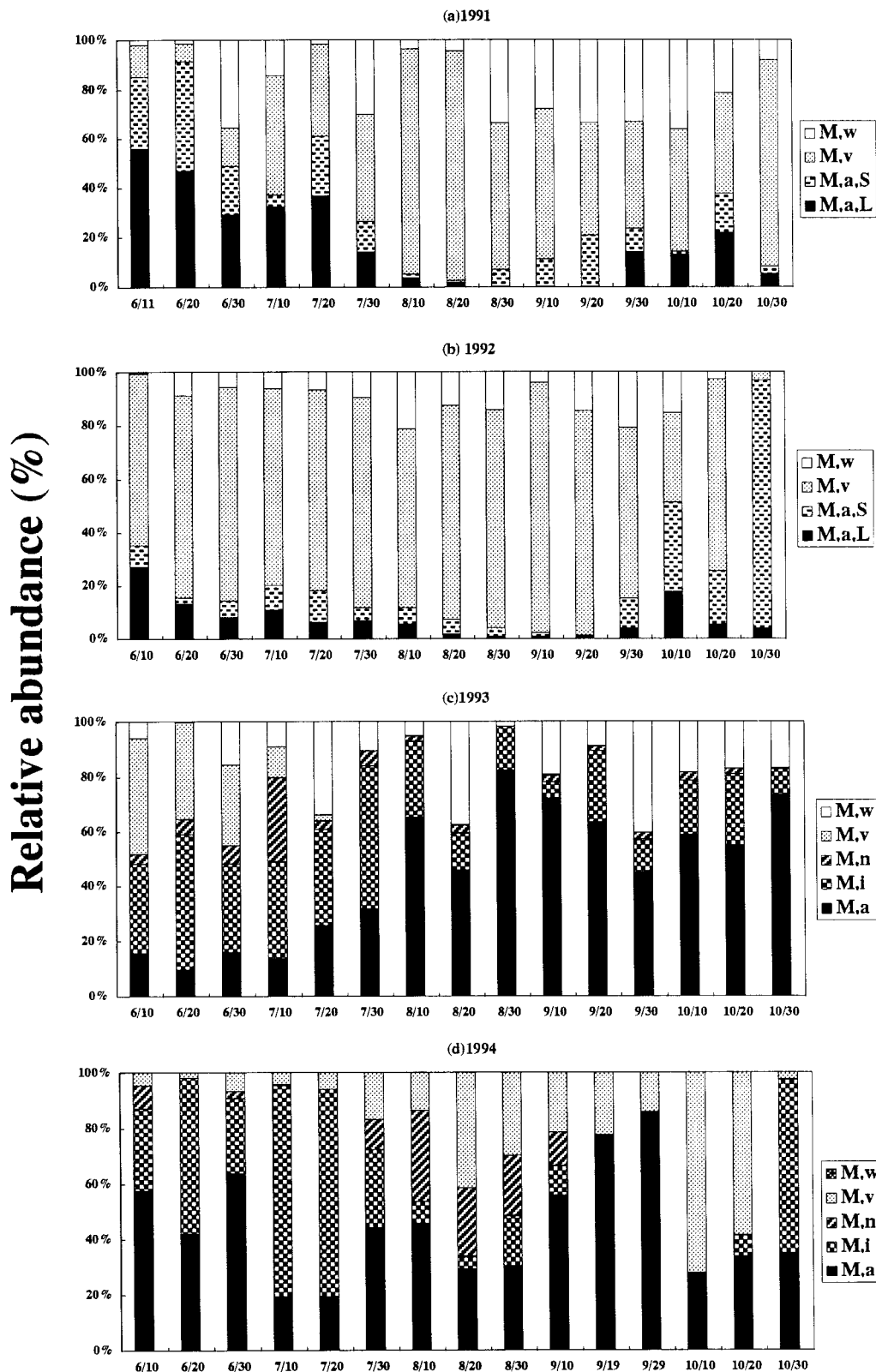


Fig. 4. Relative abundance of *Microcystis* species in Lake Suwa from June to October in (a) 1991, (b) 1992, (c) 1993, and (d) 1994. M.a.L, large cell size group of *M. aeruginosa*; M.a.S, small cell size group of *M. aeruginosa*; M.a, *M. aeruginosa*; M.i, *M. ichthyoblabe*; M.n, *M. novacekii*; M.v, *M. viridis*; M.w, *M. wesenbergii*.

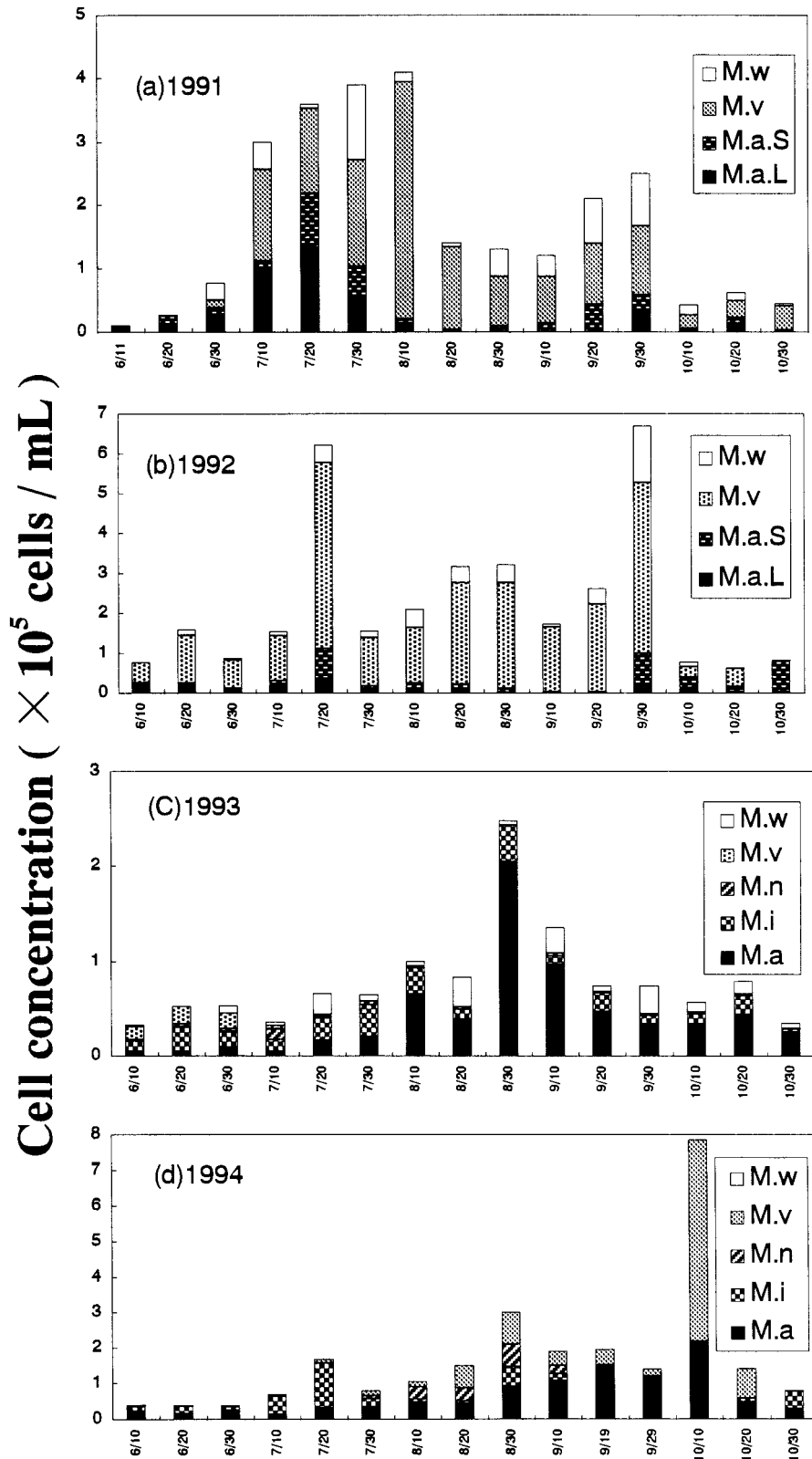


Fig. 5. *Microcystis* species cell concentration in Lake Suwa from June to October in (a) 1991, (b) 1992, (c) 1993, and (d) 1994. M.a.L, large cell size group of *M. aeruginosa*; M.a.S, small cell size group of *M. aeruginosa*; M.a, *M. aeruginosa*; M.i, *M. ichthyoblabe*; M.n, *M. novacekii*; M.v, *M. viridis*; M.w, *M. wesenbergii*.

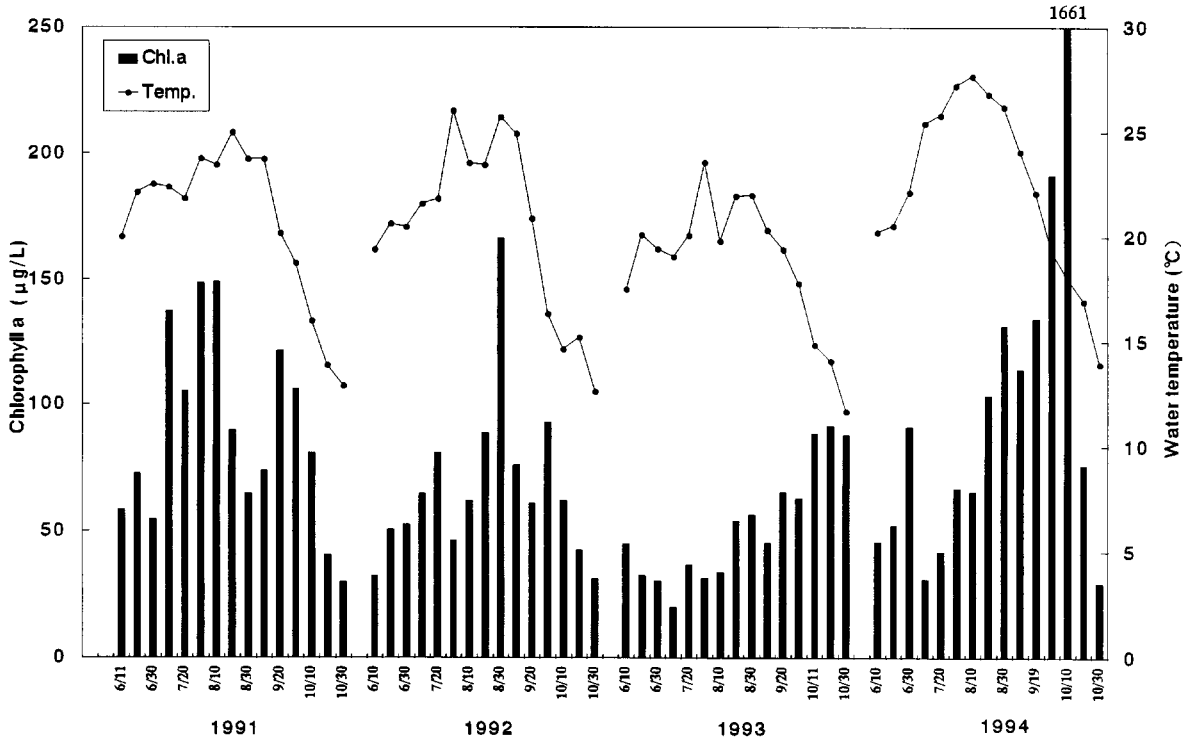


Fig. 6. Seasonal changes of chlorophyll a concentration and water temperature at the surface of Lake Suwa from June to October (1991–1994).

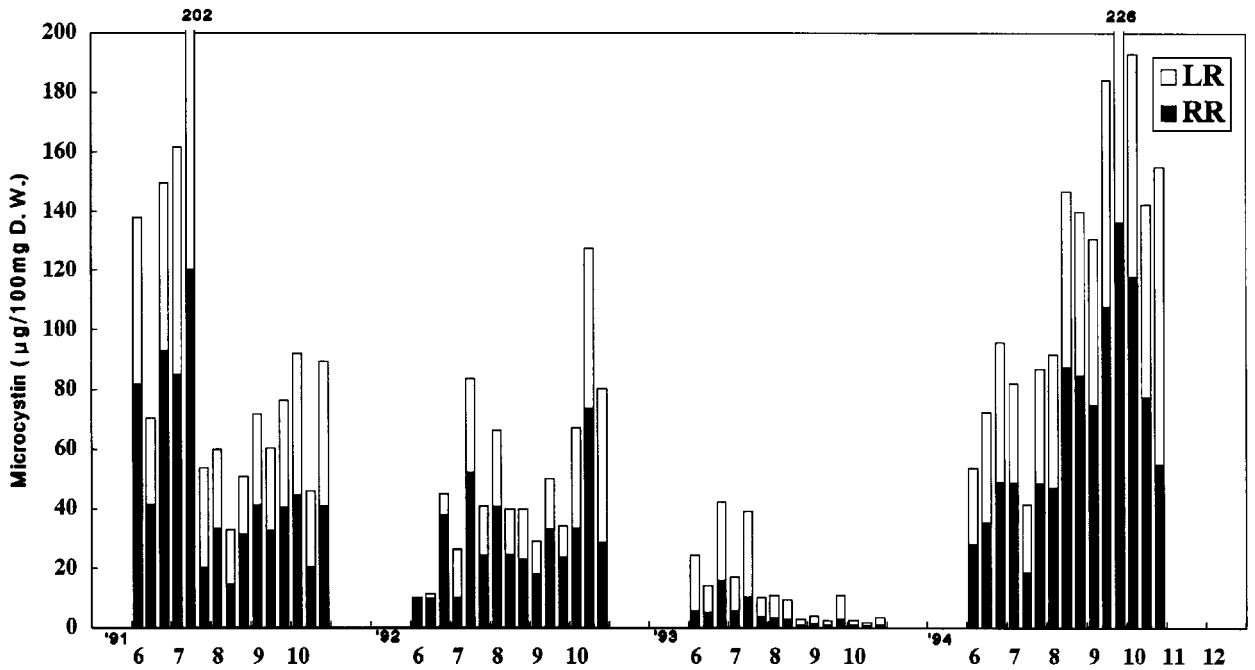


Fig. 7. Amounts of microcystins in *Microcystis* cells (100 mg dry weight) collected from Lake Suwa at 10 day intervals from June to October (1991–1994).

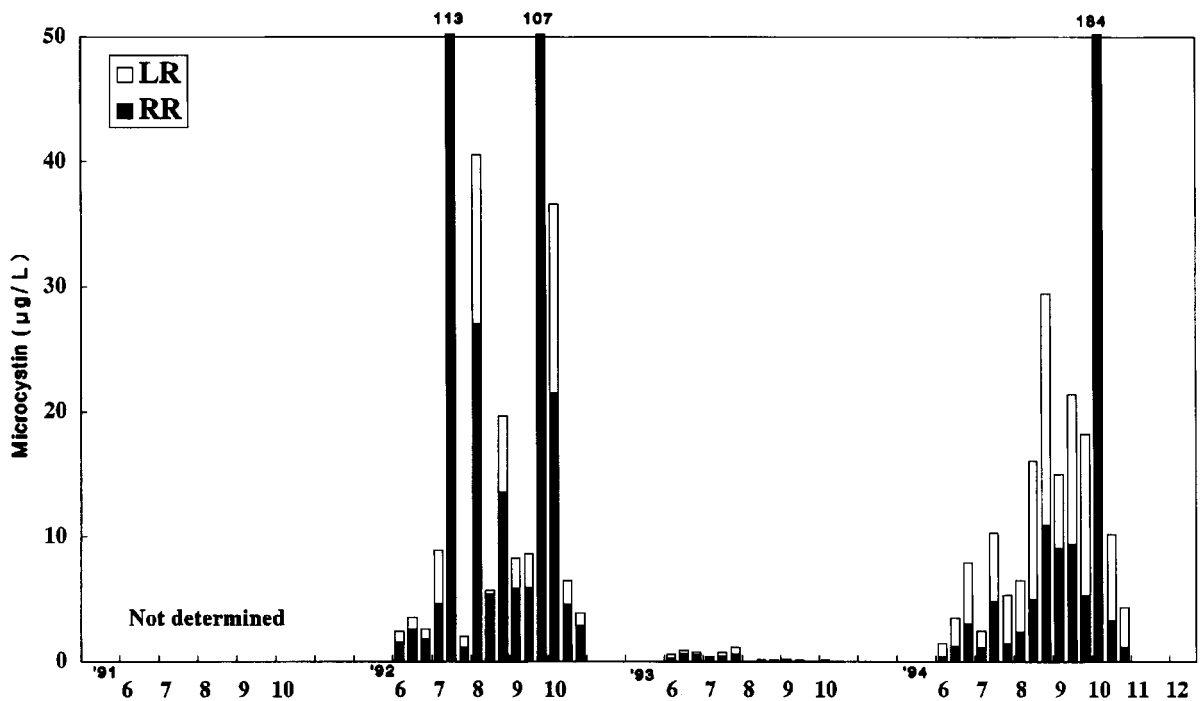


Fig. 8. Seasonal changes of microcystin concentrations in lake water containing *Microcystis* cells collected from Lake Suwa at 10 days intervals from June to October (1991–1994).

tracellular microcystin) and the filtered lake water fraction (extracellular microcystin). These concentrations fluctuated remarkably in 1992. High values of microcystin were observed on July 20 and September 30, 1992. In 1992 the microcystin concentration increased abruptly between July 10 and July 20 and then rapidly decreased before July 30. Thereafter, remarkable changes in the concentrations of these microcystins were further observed between August and October. In 1993, lake water microcystin concentrations were much lower than in other years ($< 1 \mu\text{g/L}$). In 1994, microcystin levels increased gradually from June to October 10 and then rapidly decreased until October 30. The maximum concentration of intracellular microcystin was $184 \mu\text{g/L}$ on October 10, 1994; the maximum concentration of extracellular microcystin was $3.61 \mu\text{g/L}$ on September 20, 1994.

DISCUSSION

Microcystin Content and *Microcystis* Species

In general, in Japan, natural cyanobacterial blooms containing microcystins are dominated by *M. aeruginosa* and *M. viridis* (Watanabe et al., 1989; Park et al., 1993a). As is often observed in *Microcystis* populations, there are considerable variations in cell size, and shape

and cell arrangement of the colony. According to Komárek's (1958) classification system for *Microcystis*, *M. viridis* and *M. wesenbergii* can be clearly distinguished from *M. aeruginosa*. However, some morphological variations are observed in colonies of *M. aeruginosa*, *M. ichthyoblabe*, *M. flos-aquae*, and *M. novacekii* which do not have a clearly defined visible layer (Komárek, 1991). In Lake Suwa, *Microcystis* blooms consisted of up to five species: *M. aeruginosa*, *M. viridis*, *M. wesenbergii*, *M. ichthyoblabe*, and *M. novacekii*. In 1991 and 1992, three species and four groups in the *Microcystis* population were identified. Two groups of cells—small and large—were recognized in *M. aeruginosa* (Watanabe et al., 1991). The large cell size group was later identified as corresponding to *M. aeruginosa*; the small cell size group was later discovered to be composed of at least two species—*M. ichthyoblabe* and *M. novacekii*. In Japanese waters, the production of microcystins has been reported to be related to the species of *Microcystis*. Although the strains belonging to the *Microcystis aeruginosa* large cell size group produced three toxins, the *M. aeruginosa* small cell size group showed toxin production variations (Watanabe et al., 1991). All strains of *M. viridis* contained lower amounts of these three toxins, and *M. wesenbergii* was found to be nontoxic (Watanabe et al., 1988). In 1991, the amounts of microcystins of

TABLE I. Intracellular microcystin (cell fraction) and extracellular microcystin (filtered water fraction) concentrations in Lake Suwa's surface water during the warm seasons of 1992, 1993, and 1994

Date	Microcystins ^a ($\mu\text{g/L}$)			Lake Water
	I.MC	E.MC	Percent ^b	
10/06/92	1.90	0.50	(20.8)	2.40
20/06/92	2.80	0.70	(20.0)	3.50
30/06/92	2.30	0.27	(10.5)	2.57
10/07/92	6.60	2.31	(25.9)	8.91
20/07/92	112	0.99	(0.88)	113
30/07/92	1.80	0.21	(10.4)	2.01
10/08/92	40.5	N.D.	N.D.	40.5
20/08/92	5.40	0.28	(4.93)	5.68
30/08/92	19.2	0.48	(2.44)	19.7
10/09/92	8.10	0.16	(1.94)	8.26
20/09/92	5.00	3.61	(41.9)	8.61
30/09/92	107	N.D.	N.D.	107
10/10/92	34.1	2.48	(6.78)	36.6
20/10/92	4.40	2.07	(32.0)	6.47
30/10/92	2.50	1.39	(35.7)	3.89
20/06/93	0.18	0.25	(58.1)	0.43
20/07/93	0.69	N.D.	N.D.	0.69
20/08/93	0.05	0.09	(64.3)	0.14
20/09/93	0.06	0.05	(45.5)	0.11
20/10/93	N.D.	N.D.	N.D.	N.D.
20/06/94	3.20	0.22	(6.47)	3.4
20/07/94	10.2	0.11	(1.07)	10.3
20/08/94	15.9	0.12	(0.75)	16.0
19/09/94	21.1	0.21	(0.99)	21.3
10/10/94	184	0.14	(0.08)	184
20/10/94	10.1	0.17	(1.70)	10.3

^aI.MC, intracellular microcystin; E.MC, extracellular microcystin; N.D., microcystin not detected ($< 0.02 \mu\text{g/L}$).

^bE.MC/(I.MC + E.MC).

Microcystis species found from June to July 20 were about threefold higher than those found in August in Lake Suwa (Fig. 7). The higher level of microcystins was measured during the exponential growth phase of the bloom, from June 11 to July 20 (Fig. 5). At this period, when the higher amounts of microcystins were determined, *M. aeruginosa* predominated, except on July 10. Even though *M. viridis* was predominant, the amounts of microcystins it produced were lower (Figs. 4 and 7). *M. viridis* showed a remarkable dominance in 1992 between June and September, whereas the small cell size group of *M. aeruginosa* predominated in October. Contents of microcystin at the period when *M. viridis* was predominant were lower than those when *M. aeruginosa* dominated. In 1993, cell concentration amounts were much lower because the summer was very cool and levels of precipitation were unusually high. Toxin concentrations were therefore also much lower than in the other 3 years of the study. In 1994, *M. aeruginosa* and *M. ichthyoblabe* dominated in June

and July and on October 30, and *M. aeruginosa* codominated with *M. novacekii* or *M. viridis* after August. During the four years of the study, the high amounts of microcystin concurred with the exponential growth phase of the bloom and codominance of *M. aeruginosa* and *M. viridis*, except in 1993. In this lake, the temporal variation of microcystins may be closely related to species composition of *Microcystis*.

Intra- and Extracellular Microcystin

Lake water microcystin was present in the *Microcystis* cells (intracellular microcystin) and free floating in the filtered lake water (extracellular microcystin). The total microcystin concentration in lake water was calculated by adding intracellular microcystin and extracellular microcystin (Fig. 8). Amounts of toxin usually have been expressed in units of weight per unit of weight (e.g., micrograms per gram). Volumetric units (e.g., micrograms per liter) are more appropriate for the

estimation of risk levels for aquatic biota. Few researchers, however, have reported microcystin concentrations in volumetric units (Lindholm et al., 1989; Lindholm and Meriluoto 1991; Kotak et al., 1995). Lindholm and Meriluoto (1991) reported that the highest toxin (desmethyl-microcystin-RR) levels in the metalimnion were 20–40 $\mu\text{g/L}$ in the summers of 1988–1990, in a Finish lake. *Microcystis* made up almost all of the total phytoplankton in Lake Suwa's surface water on 20 July 1991. The amounts of microcystin in surface water corresponded to 50.5 $\mu\text{g/L}$. This value was calculated from the dry weight of the seston (25 mg/L) in surface water. The highest amount of microcystins in 1992 was about a hundredfold higher than the highest amount in 1993. This difference may be due to differences in growth or to differences in *Microcystis* species composition. Microcystin amounts in Lake Suwa during the exponential growth phase of the bloom (from June 11 to July 20, 1991) were about threefold higher than in August. This supports the

hypothesis that a higher amount of microcystins was produced during the exponential phase than during the stationary or senescent phases (Watanabe et al., 1989). In addition high concentrations of microcystin were found during the exponential growth phase of the bloom; the highest concentration of microcystin was 184 $\mu\text{g/L}$ on October 10, 1994. However, the amount of microcystin in the filtered lake water was highest at the end of the bloom; this amount was very small ($< 4 \mu\text{g/L}$) during the period of the study. The high percentage of extracellular microcystin in filtered lake water ($> 20\%$) at the end of blooms suggests that release of microcystin from cells occurs during senescence and the decomposition periods of *Microcystis* cells (Fig. 8 and Table I).

Table II shows the amounts of microcystins contained in natural cells of *Microcystis* species obtained from Lake Suwa from 1971 to 1995. The amounts of these toxins are very low compared with the maximum value of the toxins since 1991. A complete understand-

TABLE II. Concentrations of microcystins in samples of *Microcystis* species obtained from Lake Suwa in the years 1971–1995

Date	Microcystins ^a ($\mu\text{g}/100 \text{ mg cells}$)		
	RR	YR	LR
15/08/71	35	N.D.	8
24/07/78	3	N.D.	4
12/08/80 ^b	38	N.D.	44
28/08/81 ^b	23	N.D.	6
10/08/82 ^b	13	N.D.	25
23/08/82 ^b	17	N.D.	29
08/08/84 ^b	12	N.D.	13
13/08/85	63	N.D.	25
28/06/86 ^b	20	9	29
31/07/86	45	N.D.	24
25/07/89 ^c	46	N.D.	33
03/07/90 ^c	51.4	3.4	34.1
14/08/90 ^c	1.2	N.D.	1.3
11/08/90 ^c	3.6	N.D.	3.1
20/07/91 ^c	121	9.8	81.6
20/08/91 ^c	14.8	N.D.	18.2
10/09/91 ^c	41.3	N.D.	30.4
20/10/92	74	N.D.	54.0
03/08/93	N.D.	N.D.	N.D.
18/07/94	N.D.	N.D.	3.9
02/08/94	N.D.	N.D.	N.D.
29/09/94	136	N.D.	89.8
21/06/95	20.3	N.D.	45.4
16/09/95	9.1	N.D.	18.2
03/10/95	N.D.	N.D.	5.3

^aN.D., toxins not detected.

^bConcentration of toxin already reported by Watanabe et al. (1989).

^cConcentration of toxin already reported by Park et al. (1993b).

ing of toxins in lakes cannot be obtained from only a few water samples. Temporal and spatial measurements of microcystin and toxic species composition in lakes and reservoirs are necessary to assess the risks for humans health, aquatic animals, livestock, and wildlife.

This work was partly supported by a research grant from the Nippon Life Insurance Foundation.

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