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Field Studies on the Environmental Factors in Controlling Microcystin Production in the Subtropical Shallow Lakes of the Yangtze River

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Abstract Microcystin (MC) problem made more and more care about in China, intercellular MC (Int-MC) and cellular MC (Cel-MC) were important contents to reflect the producing-MC ability by cyanobacteria and by lakes. To study the correlations between Int-MC, Cel-MC concentration and biological and environmental factors, eight cyanobacterial blooming lakes were studied in the middle and lower reaches of the Yangtze River. Microcystin-RR (MC-RR) and Microcystin-LR (MC-LR) were the primary toxin variants in our data. From the linear correlations between MC and environmental factors, cellular-YR had significant correlation with most of chemical factors except total nitrogen (TN) and the ratio of total nitrogen and total phosphorus (TN/TP), most intracellular MC analogues had significant correlations with total dissolved nitrogen (TDN), ammonium (NH_4^+), nitrite (NO_2^-), TP, total dissolved phosphorus (TDP), *Microcystis*. From the canonal correspondence analysis, Int-MC concentrations were closely related with the chemical and biological factors, such as TP, total organic carbon (TOC), chlorophyll *a* (Chl *a*), *Microcystis* biomass, et al. While Cel-MC contents, especially Cel-RR and Cel-LR, were closely related with light environmental in the lakes such as water depth and transparence.

Keywords Microcystin · *Microcystis* · Shallow lakes · The Yangtze River

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Bloom-forming cyanobacteria have been observed in water bodies including drinking water reservoirs all over the world. Several strains of these microorganisms have the ability to produce potent toxins as secondary metabolites, the so-called cyanotoxins. These have caused many animal deaths and have also been implicated in cases of human illness, e.g. in U.S.A., Australia, China and Brasil (Tisdale 1931; Ueno et al. 1996; Jochimsen et al. 1998).

Two biotic factors influence microcystin concentration in the field: cellular microcystin production and content (Orr and Jones 1998; Long et al. 2001), and species composition of cyanobacteria in lakes (Chorus et al. 2001; Vézie et al. 2002). Environmental conditions also indirectly influence microcystins through their effects on these two factors. Numerous laboratory studies and investigations in specific lakes have examined the effects of various environmental factors (e.g., nutrients, light, temperature) on MC production (Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Utkilen and Gjølme 1992; Jacoby et al. 2000; Sekadende et al. 2005). Regional studies on the interactions between microcystins and physicochemical variables have been conducted in Canada (Kotak et al. 2000), America (Graham et al. 2004), and Spain (Aboal and Puig 2005), but such studies have been lacking in Asia, even though lake water there is generally more eutrophic and toxic cyanobacteria are more common (Jin 2003). Many of the numerous shallow lakes in the middle and lower reaches of the Yangtze River area in subtropical China, and many of these lakes have cyanobacterial blooms in the warm season due to high nutrient levels and high water temperature. Contamination of lake water by microcystins is becoming a serious concern in this region.

We investigate eight cyanobacterial blooming lakes in the middle and lower reaches of the Yangtze River area during 2003–2004. The main purposes were: (1) to

determine concentration and distributions of the variations of the intracellular MC concentrations (ng/L) and cellular MC concentration (μg/g); (2) to explore relationships between intracellular MC, cellular MC (μg/g) and environmental factors and dominate cyanobacteria; and (3) to propose the possible mechanism underlying MC production in these subtropical lakes.

Materials and Methods

Water samples in all lakes were collected with Tygon tubing fitted with a one-way valve. For analysis of TN, TP, NO_3^-, NO_2^- and chlorophyll *a* (Chl *a*), a composite sample was taken from the surface (0–1 m), middle and bottom layers at each site. Samples were kept dark in a refrigerator before laboratory analysis. The lake water was filtered through a membrane filter (0.45-μm-pore-diameter) for analysis of NO_3^-, NH_4^+ and NO_2^-. Total nitrogen (TN) was determined by the alkaline potassium persulfate digestion-UV spectrophotometric method (Nydahl 1978). Total phosphorus (TP) was determined by the ammonium molybdate method after potassium persulfate digestion (Prepas and Rigler 1982). Ammonium (NH_4^+–N) was measured by the Nessler method, nitrite by the α-naphthylamine method and nitrate by the UV spectrophotometry method (Eaton et al. 1995). Chl *a* was measured spectrophotometrically (Zhang and Huang 1991). Mean water temperature was measured in situ using a thermometer and Secchi depth was recorded at one central site.

Phytoplankton and zooplankton were preserved in Lugol's solution from the combined water samples. Phytoplankton and zooplankton were identified based on descriptions of Prescott (1978) and enumerated with a microscope equipped with a calibrated micrometer (Kotak et al. 1995).

MC was fractionated to MC in phytoplankton >64 μm (called as cellular toxin) (μg/g) and toxins in seston >0.7–1 μm (called as intracellular MC) (ng/L), to collect cyanobacteria for cellular toxin analysis, a 64-μm-mesh size plankton net was skimmed across the water surface. Samples were then transported to the laboratory, frozen at −25°C and freeze-dried for subsequent toxin analysis. For intracellular MC analysis, 1 L of the composite lake water was filtered through a glass fibre filter (Whatman GF/C, UK) using a vacuum pump. The filters were frozen immediately and returned to the laboratory. Both kinds of samples were extracted thrice in 75% methanol. The supernatant was diluted 1:5 with distilled water and were directly concentrated on solid phase extraction (SPE) cartridges (10 mL, C18, 500 mg), which were previously activated with 10 mL of methanol (100%) followed by 10 mL distilled water. The cartridges were then washed with 10 mL of 10% methanol followed by 10 mL distilled water. Microcystins were eluted from the cartridges with 10 mL 100% methanol and then evaporated to dryness. The residue was dissolved with 100 μL distilled water and used for the final detection and identification of MCs by LC-MS.

MC concentration was measured using a Finnigan LC-MS system consisting of a thermo surveyor auto sampler, a surveyor MS pump, a surveyor PDA system, and a Finnigan LCQ-Advantage MAX ion trap mass spectrometer equipped with an atmospheric pressure ionization fitted with an electro spray ionization source (ESI). The sample was separated on a Hypersil GOLD 5 μm column (2.1 mm i.d. × 150 mm) with a linear gradient run of acetonitrile (30–100%) and acidified water. Both water and acetonitrile were acidified with 0.05% formic acid. Data acquisition was in the positive ionization centroid mode with full mass mode at a mass range between 400 and 1400. MS tuning and optimization were achieved by infusing microcystin-RR and monitoring the $[M + 2H]^{2+}$ ion at m/z 520 (Chen and Xie 2005). Microcystins in cyanobacterial extracts were identified against standards of MC-RR, MC-LR and MC-YR.

To examine the correlation between MC-LR, -YR and -RR concentration, biomass of *M. aeruginosa*, limnological variables, and cyanobacterial species composition, STATISTIC for Windows statistical software (version 6.0) and Canoco software (version 4.5) were used for correlation analyses and canonal correspondence analyses.

Results and Discussion

This is first report about microcystin was detected in Eastdongting, Poyang, Junshan and Longgan Lakes in China. Among the eight lakes, Eastdongting and Poyang lakes were lakes naturally connected with the Yangtze River throughout the year while other lakes were seasonally connected with the river. These lakes were in eutrophic and hyper-eutrophic states in terms of TP level, with a maximum TP in Dianshan Lake, a minimum TP in Poyang Lake, a maximal TN/TP in Poyang Lake and a minimal TN/TP in Junshan Lake (Table 1).

The values of phytoplankton biomass (PB) and cyanobacterial biomass were maximal in Yangcheng Lake (4.98 and 3.78 mg/L) (Fig. 1). The highest percent of cyanobacteria in total phytoplankton was 90.4% in Junshan Lake where cyanobacteria were mainly composed of *Anabaena* and *Microcystis* (Table 2), while the lowest percent was 23.0% in Poyang lake where *Microcystis* were the dominant cyanobacteria.

The results showed that cyanobacteria (mainly *Anabaena* and *Microcystis*) dominated in these lakes, while *Pediastrum*, *Melosira* and *Caloneis* spp. were dominant species in the no-cyanobacterial community (Table 2).

Table 1 Limnological characteristics and mean MC values of the eight study lakes in the Yangtze River area

Lake Name	Longitude E	Latitude N	Area (km^2)	Mean depth (m)	Sechii depth (cm)	pH	Temp. (°C)	TN (mg/L)	$NO_3 + NO_2$ (mg/L)	NH_4 (mg/L)	TP (mg/L)	TN/TP	TC (mg/L)	TOC (mg/L)
East Dongting Lake	E113°02′	N29°15′	1478.2	6.1	83	8.4	28.8	0.79	0.86	0.14	0.05	15.8	46.27	10.09
Poyang Lake	E115°58′	N29°16′	3583.7	2.5	133	6.4	30.8	1.21	0.45	0.19	0.02	60.5	9.17	2.85
Hong Lake	E113°22′	N29°49′	348.4	1.9	58	8.0	30.5	1.69	0.39	0.09	0.08	21.1	21.49	11.25
Shijiu Lake	E118°53′	N31°30′	210.4	2.3	113	7.8	26.0	0.69	0.20	0.49	0.07	9.9	14.84	7.68
Yangcheng Lake	E120°49′	N31°25′	119.1	1.6	78	8.3	30.0	0.63	0.43	0.13	0.09	7.0	29.51	7.69
Dianshan Lake	E120°58′	N31°10′	63.7	2.2	33	8.5	30.5	1.52	1.53	0.76	0.25	6.1	30.54	10.50
Longgan Lake	E116°10′	N29°57′	316.2	2.1	67	7.8	30.7	0.52	0.40	0.10	0.03	17.3	20.78	6.70
Junshan Lake	E116°18′	N28°37′	192.5	3.7	221	7.5	30.5	0.62	0.13	0.02	0.05	12.4	30.54	10.50

Area, lake area; Temp, water temperature

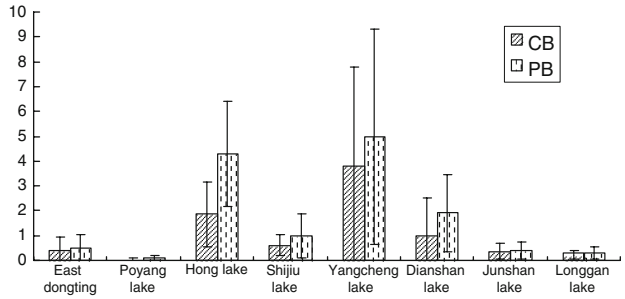

Fig. 1 Biomass of zooplankton and phytoplankton (mg/L)

Table 2 Main algal species in the specific sample site among the studied lakes

Yangcheng Lake	*Anabaena* (42.31%), *Microcystis* (19.03%), *Nostoc* (13.77%)
Dianshan Lake	*Microcystis* (85.78%)
East Dongting	*Microcystis* (89.36%)
Jun Shan Lake	*Microcystis* (83.33%)
Longgan Lake	*Anabaena* (58.94%), *Caloneis* (9.49%)
Shijiu Lake	*Microcystis* (51.34%), *Pediastrum* (33.98%)
Poyang Lake	*Microcystis* (37.22%), *Fragilaria* (8.54%)
Honghu Lake	*Microcystis* (36.17%), *Melosira* (22.27%)

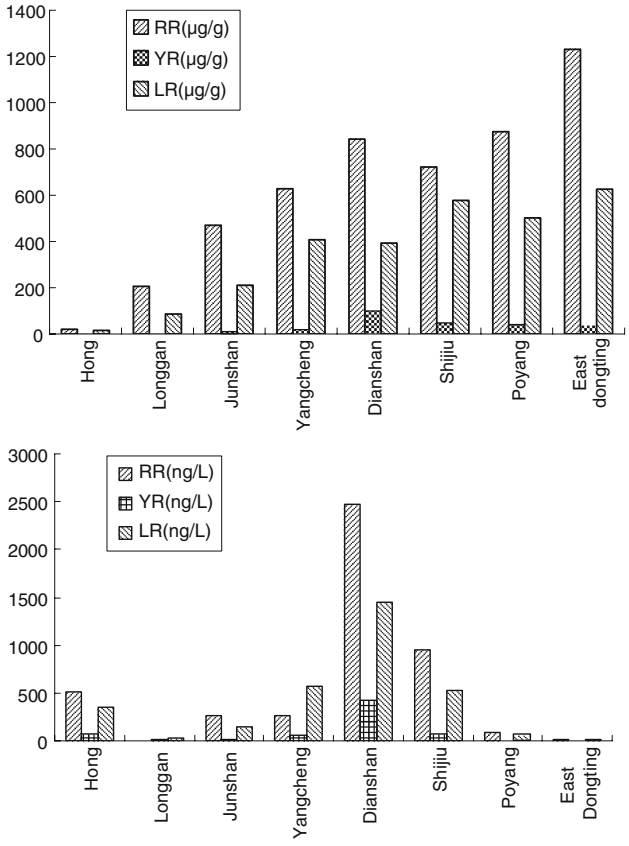

Fig. 2 Intracellular MC in lakes (ng/L) and cellular MC in cells (μg/g) in various sampling sites

The toxins of the eight lakes were identified as MC-LR, -YR and -RR, with MC-LR and -RR as the main components. Cellular MC-RR and -LR contents were the highest in East Dongting Lake (Fig. 2), and MC-YR was the highest in Dianshan Lake. However, the intracellular MC concentrations were not in accordance with the cellular MC content. Dianshan Lake has the highest intracellular MC (-RR, -LR and -YR) values in these lakes (Fig. 2).

Table 3 Correlations between intracellular MC and cellular MC concentrations

	Cel-RR	Cel-YR	Cel-LR
Int-RR	0.14	0.84**	0.10
Int-YR	0.14	0.82*	0.04
Int-LR	0.11	0.78*	0.10

Cel - means cellular - (LR, YR, RR, MC) content (μg/g) and Int - means intracellular - (LR, YR, RR, MC) content (ng/L)

* $p < 0.05$; ** $p < 0.01$

Both cellular MC and intracellular MC analogues had significant correlations with each other ($p < 0.01$), except correlations between cellular-YR and cellular-LR, -RR (Table 3). However, the cellular-RR and -LR did not have significant correlation with any of the intracellular MC ($p > 0.05$). These results suggest that the MC analogues might have some similar physiological interactions in cyanobacteria.

The correlation coefficients between cellular microcystin and environmental factors showed the MC and their three analogues were positively correlated with all nitrogen, phosphorus' parameters and TN/TP, but only YR in cellular MC has the very significant correlated with most of nitrogen and phosphorus parameters (Table 4).

Wicks and Thiel (1990) studied the variation of microcystin content in cyanobacteria of a hypereutrophic reservoir in South Africa. They reported that total microcystin production was positively correlated with Chl *a* and solar radiation, and the microcystins-YR, -LR, -YA and -LA were negatively correlated with Chl *a* and the concentration of soluble reactive phosphorus. Kotak et al. (1995) reported that microcystin-LR content of cyanobacteria in three Canadian lakes was positively correlated with the concentrations of total phosphorus, dissolved phosphorus and Chl *a*; and negatively correlated with that of nitrate. Vézie et al. (1997) reported the total amount of microcystin was positively correlated with the concentration of Chl *a* in water, and negatively correlated with solar radiation.

In the present study, total MC showed very significant correlations with RR, YR and LR, suggesting that the appearance of RR, YR and LR is concurring in a consistent way. However, there are also distinct differences in these correlations between the three MC variants and environmental factors. For example, in our results, although RR and LR had significant correlations with NO_3^-, YR did not have a significant correlation with NO_3^-. Similarly, some of the three MC variants had significant correlations with water depth, NH_4^+ and TP. Compared with studies in Canada (Kotak et al. 2000) and America (Graham et al. 2004), the relationships between MC and environmental factors were not always consistent. For example, MC was strongly correlated with TN in America, but not in Canada, German (Chorus et al. 2001) and our results. As expected, microcystin concentrations have better correlations with biological factors than with environmental factors. For example, RR, YR and LR all have significant correlations with Chl *a*, Cyanophyta, *Microcystis* and *Anabaena*. Because MC is produced by cyanobacteria, it is easy to understand why MC has better correlations with cyanobacteria, such as *Microcystis* and *Anabaena*.

The significant correlations with TP agree with other studies in Canada (Kotak et al. 2000) and America (Jacoby et al. 2000; Graham et al. 2004). It is not surprised that MC did not have significant correlation with TN, because some cyanobaterial species are able to do nitrogen-fixing.

Canonal correspondence analysis (CCA) offered us a good way to study the correlations between MC and environmental factors. It produced an ordination in which the first axe was statistically significant ($p < 0.01$), with eigen value of 0.348, and cumulative percentage of variance in the toxin-environment relationship explained by this axe was 92.4%. Intracellular MC concentrations were closely related with the chemical and biological factors, such as TP, TOC, Chl *a*,

Table 4 Linear correlations between cellular MC and intracellular MCs' analogues and environmental factors

	TN	TDN	NO_3^-	NH_4^+	NO_2^-	TP	TDP	TN/TP	Chl *a*	Trans.	Cyan.	*Mic.*
Cel-RR	0.07	0.49	0.53	0.22	0.32	0.04	0.12	0.44	−0.42	−0.14	−0.03	0.05
Cel-YR	0.28	0.86**	0.71*	0.76*	0.85**	0.71*	0.78*	0.03	−0.05	−0.38	0.03	0.48
Cel-LR	0.03	0.45	0.30	0.36	0.14	0.00	0.00	0.39	−0.39	−0.13	0.03	0.01
Cel-MC	0.07	0.51	0.48	0.31	0.29	0.06	0.12	0.41	−0.41	−0.15	−0.01	0.06
Int-RR	0.28	0.77*	0.58	0.82*	0.89***	0.92***	0.92***	−0.40	0.28	−0.38	0.14	0.69
Int-YR	0.28	0.72*	0.69	0.70	0.95***	0.95***	0.98***	−0.38	0.32	−0.47	0.24	0.73*
Int-LR	0.19	0.65	0.55	0.77*	0.84**	0.99***	0.94***	−0.49	0.38	−0.45	0.43	0.78*
Int-MC	0.25	0.73*	0.59	0.80*	0.89***	0.96***	0.94***	−0.43	0.32	−0.42	0.25	0.73*

Cel - means cellular - (LR, YR, RR, MC) content (μg/g) and Int - means intercellular - (LR, YR, RR, MC) content (ng/L)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$

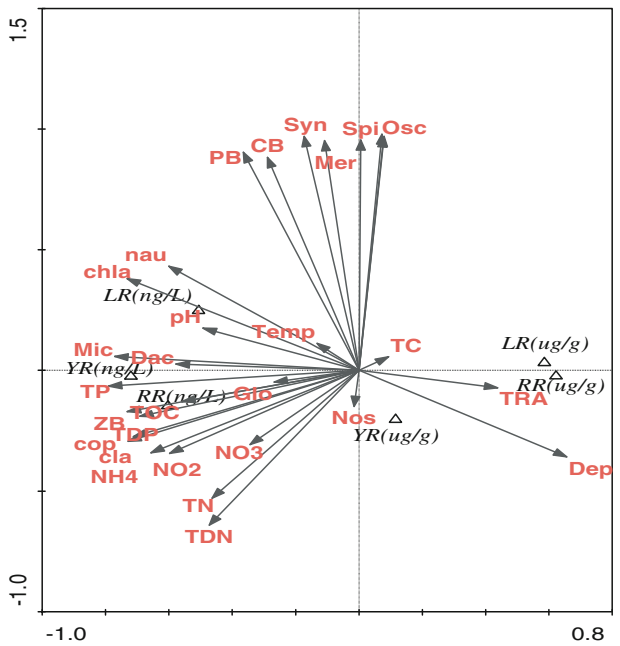

Fig. 3 CCA biplot of MC and physical, chemical and biological factors in the eight lakes. PB, phytoplankton biomass; ZB, zooplankton biomass; CB, cyanobacterial biomass; Temp, temperature; TRA, transparence; DEP, depth; Mic, *Microcystis*; Nos, *Nostic*; Spi, *Spirulina*; Mer, *Merismopedia*; Syn, *Synechocystis*; Dac, *Dactylococcopsis*; nau, *nauplius*; cop, *Copepoda*

Microcystis biomass, et al. (Fig. 3). While cellular MC contents, especially -RR and -LR, were closely related with light environmental in the lakes such as water depth and transparence. Compared with intercellular MC, cellular MC in lakes was less studied, our result showed the cellular was more closely related with light in waters, that may due to the cyanobacteria toxin-producing ability was mainly restrict by light intensity. The intercellular MCs reflect toxin-producing ability of all cyanobacteria in the whole lake, so they more related with the lakes nutrient level, pH, the biomass of cyanobacteria and zooplankton, et al.

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