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Identification and Determination of Microcystins in Source Water and Waterbloom Sample From Meiliang Bay, Taihu Lake, China[1]

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Objective To identify and determine the congener and level of microcystins in the source water of Taihu Lake. **Methods** Improved method of SPE combined with HPLC was employed to detect the concentration and varieties of microcystins in source water and bloom samples collected from Meiliang Bay, Taihu Lake. **Results** The contents of two predominant microcystin components, MC-RR, and MC-LR, were relatively high in samples during warm months and correlated with the phase of algae growth. The maximum concentrations of MC-RR and MC-LR in water sample reached 3.09 ±0.53 μg/L and 2.39±0.41 μg/L during the period of water bloom in September 2004, respectively. Even without waterbloom, the concentration of MC-LR in source water sample was still higher than the guideline value. **Conclusion** The status of microcystin pollution in this region is serious and measures to monitor and control the growth of cyanobacteria are urgently needed.

Key words: Microcystin; Determination; Source water; HPLC; Taihu Lake

INTRODUCTION

The toxic water bloom of cyanobacteria algae, widely reported in eutrophic freshwater, has been a serious pollution problem worldwide[1]. Microcysin (MCs), the most common cyanobacterial toxin, is a group of extremely hepatotoxic compounds produced continuously by species of cyanobacteria belonging to the genera Microcystis, Anabaena and Oscillatoria. All MCs possess a cyclic heptapeptide structure consisting of D-Ala-L-X-D-methylAsp-L-Y-Adda-D-Glu-N-methyldehydroAla, where Adda is the amino acid: 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and X and Y are two variable L-amino acids (Fig. 1). Different MCs are distinguished by two variable amino acids and a number of minor modifications, e.g. demethylation to the generic structure[2]. For example, MC-LR is a kind of MCs with leucine (L) and arginine (R) in the variable positions. Approximately 60 isolated MC congeners are known to exist[3]. Because of special molecule structure, MCs are very durable and do not readily undergo proteolytic or hydrolytic attack in nature[4], react neither with acids nor with alkali. It has been proven unreliable to remove them by conventional water treatments[5].

As a potent tumor promoter, MCs possess high hepatotoxicity due to their inhibition of protein phosphatases[6], acute MC poisoning is the leading cause of death in a few hours, longer-term exposure to lower levels in drinking water is thought to be a contributing factor in primary liver cancer (PLC). MCs in water body are responsible for a significant health hazard to humans. The most severe health case attributed to MCs has been emphasized by the deaths of 60 haemodialysis patients in Brazil in 1996[7]. For its acute and chronic toxicity, the World Health Organization has proposed a guideline value of 1.0 μg/L for MC-LR in drinking water[8].

Taihu, the third largest freshwater lake in China, is a major source of drinking water for Jiangsu and Zhejiang Provinces. In recent years, cyanobacteria blooms caused by eutrophication often occur in warm seasons and have become increasingly serious in this region. Using such water supplies for domestic purposes, particularly drinking water, may constitute a risk to public health. Harada *et al.*[9] have reported the high incidence of PLC in Qidong and Haimen

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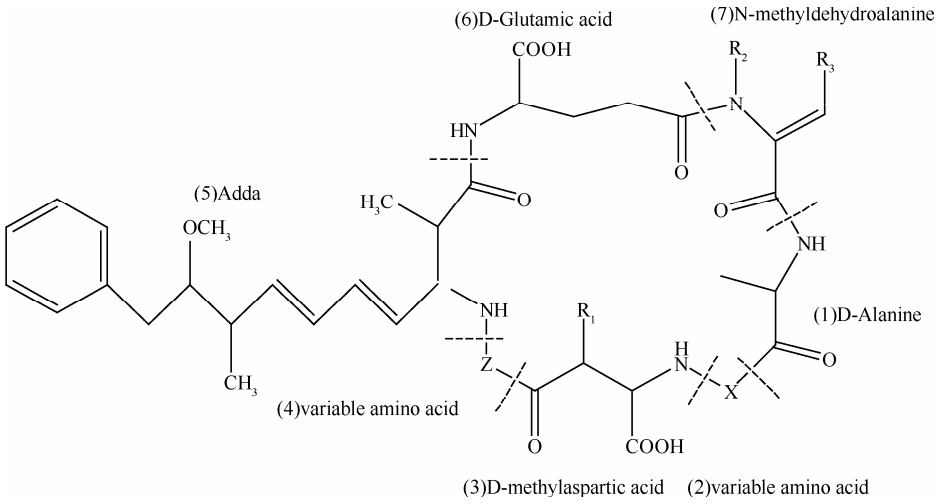

FIG. 1. General structure of MCs (R_1, R_2, R_3 stand for CH_3 or H)

Counties near Shanghai. Recent epidemiological studies have suggested that the high incidence of PLC in this area is related to the contamination of MCs in drinking water[10-11].

Due to the complex appearance of MCs in nature, as well as the high cost and scarcity of standard toxins, it is difficult to analyze MCs qualitatively and quantitatively at such low concentration in water. In previous reports on MC pollution in domestic and foreign areas, MC-LR is the most frequently observed. So far, however, the situation of MC pollution in Taihu Lake region may be distinctive because of different environment conditions from other areas. It is necessary to investigate the congeners and levels of MCs in Taihu Lake during algae bloom period. In this study, we set up a determination method, solid-phase extraction (SPE) combined with high-performance liquid chromatography (HPLC), for two main MC congeners at trace level[12], and then further analyzed the congeners and concentration of MCs extracted from both bloom and water samples collected in Meiliang Bay, Taihu Lake.

MATERIALS AND METHODS

Materials

Standard MC-LR, MC-RR were purchased from Alexis Co (Switz). The purity was 98%. HPLC-grade methanol and HPLC-grade trifluoroacetic acid (TFA) were obtained from Merck Co. (USA). Deionized-distilled water was obtained from ultra-pure water system (Aquapro, AVP-2-35G-01). All other chemicals were of analytical grade.

Sample Collection

From July to October, 2004, when waterbloom took place, bloom and subsurface water samples were collected monthly from source water site in Meiliang Bay, water sample was also collected at random time points in August without waterbloom.

After harvested by 40 μm-mesh net, cyanobacterial field samples were examined for species composition. The examination revealed that the blooms consisted of Microcystis aeruginosa, Microcystis wesenbergii and Microcystis marginata. M aeruginosa was the dominating species (more than 70%). Bloom samples were washed by deionized-distilled water and reconcentrated. Surface water samples were collected via vertical water sampler. All samples were stored at -20℃ until thawed for subsequent treatment.

Sample Purification and Concentration

Before HPLC analysis, MCs extracted from bloom and water samples were purified and Trace-enriched by SPE.

Thirty mL of concentrated cyanobacterial cell pellet in beaker was extracted and 80 mL of methanol-water (75:25) plus 0.1% TFA was added. The samples were sonicated for 20 min and centrifuged at 12 000 rpm for 15 min. The extraction procedure for residue was repeated once with 40 mL of the same solvent. The supernatants resulting from both steps were pooled and reduced to approximately one-fourth of its initial volume by rotary evaporation. After filtrated through the 0.45 μm glass microfibre filter (Whatman GF/C), 40 mL aliquot of the MCs-rich concentration was applied to a

preconditioned *insolutable* C_{18} cartridge (Argonaut, USA) by AutoTrace SPE equipments (Zymark, USA) at 2.0 mL/min. The preconditioning step included washing with 10 mL each of 100% methanol and deionized-distilled water. The loaded cartridge was washed with 10 mL of 20%, 30%, and 5 mL of 40% aqueous methanol. Then the MCs were eluted by 5 mL of 80% methanol plus 0.02% TFA, and the effluent was evaporated to dry under nitrogen stream, and the residue was taken up in 0.5 mL 100% methanol for HPLC analysis.

As for water sample, 500 mL aliquot of subsurface water sample, filtered though 0.45 μm microfibre filter, was introduced to preconditioned C_{18} cartridge with the similar solid-phase extraction manner as mentioned above, except that the cartridge was washed with only 10 mL of 20% aqueous methanol.

Analyzed by HPLC

MCs in methanol were analyzed by Alliance HPLC system (Waters, USA) equipped with 2996 photodiode array detector. The optimal chromatogram conditions were mobile phase: 62% methanol, 38% water (0.1%TFA); separation column: reverse phase ODS_{18} (4.6 mm×250 mm, Kromasil, Swe); flux: 1.0 mL/min; sample volume: 10 μL; wavelength of detector: UV239 nm. The system was controlled by Waters Millennium software.

Recovery Experiments

To validate the accuracy and reliability of the procedure, especially in concentration step by C_{18} SPE cartridges, standard recovery experiments were performed using two kinds of C_{18} SPE cartridges, insolutable glass C_{18} and plastic C_{18}. The known mass of MC standard spiked into 500 mL deionized-distilled water, the expected concentration of covered solution was calculated (assuming 100% recovery), and then the actual concentration of recovered solution was obtained from the regression equation and chromatogram, finally, the percentage recovery was calculated on the basis of expected and actual concentration.

RESULTS

Chromatogram of MC Standard and Regression Equation of Calibration Curve

Under the conditions as mentioned above, separation chromatogram of a mixture of MC standard at the concentration of 25 μg/mL is shown in Fig. 2. Peak 1 was MC-RR and its retention time was 9.2 min; peak 2 was MC-LR and its retention time was 18.8 min. It was evident that MC-RR and MC-LR were separated distinctively from the system. Within the concentration range from 0.25 to 50 μg/mL, good linearity of the calibration curve was obtained. The regression equation and correlation coefficient are shown in Table 1. This optimal condition enabled the detection limit of common MCs at levels as low as 0.25 μg/mL (s/n=3).

TABLE 1

Regression Equations of Calibration Curves and Correlation Coefficients

MCs	MC-LR	MC-RR
Regression Equations	$Y=2.09\times10^4X+1.03\times10^3$	$Y=1.80\times10^4X-4.01\times10^3$
Correlation Coefficient	0.999881	0.998998

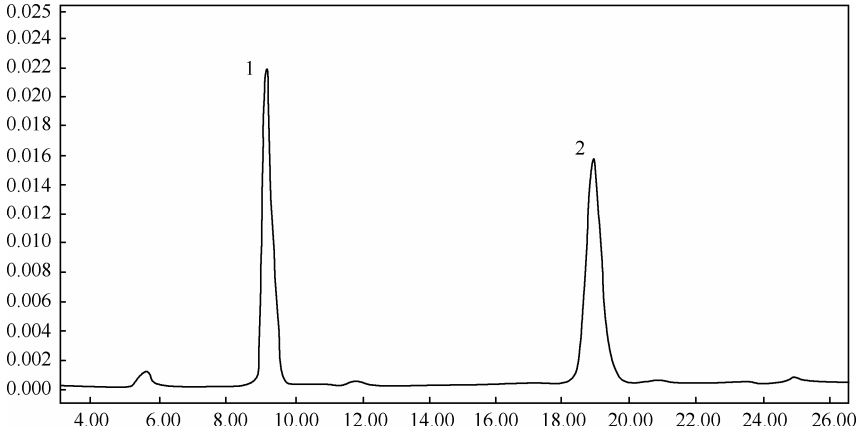

FIG. 2. HPLC trace of Microcystin standards (25 μg/mL).

Recovery Experiments

A series of solutions, prepared by spiking MC standard into deionized-distilled water, were extracted and concentrated by C_{18} SPE cartridges as described above. The recovery rate by plastic C_{18} cartridges was relatively poor (about 50%-60%, data not shown), while the result of recovery by glass C_{18} cartridges was satisfactory. Expected concentration (assuming 100% recovery) and actual concentration of covered solution obtained by glass C_{18} cartridges and HPLC analyses are shown in Table 2.

Sample Determination

The Separation chromatogram of MCs extracted from cyanobacterial bloom in August and water sample in September, is shown in Figs. 3 and 4. Compared the retention time with that in Fig. 2, peaks1 and 2 in Figs. 3 and 4 were consider to be MC-RR and MC-LR initially, this conclusion was proved to be exact by the UV spectra of peaks 1 and 2 in Fig. 4 shown in Fig. 5 where the maximum adsorption lies in 239.2 nm, the same UV spectra were obtained for corresponding peak in Fig. 3. According to the areas of peaks 1 and 2 in the chromatogram in Fig. 4, regression equation and the corresponding percentage recovery, the concentration of MC-LR and MC-RR in source water sample from July to October, 2004 were detected (Table 3).

TABLE 2

Recovery of Mcs Spiked Sample by Insolute Glass C_{18} Cartridges

MCs	Expected Concentration (μg/L)				Actual Concentration (μg/L)				Recovery (%)				Average Recovery (%)
	1.00	2.00	6.00	10.00	0.97	1.92	5.60	9.60					
	1.00	2.00	6.00	10.00	0.88	1.85	5.51	9.48					
MC-LR	1.00	2.00	6.00	10.00	0.96	1.88	5.53	9.12	94.80	95.50	92.27	94.20	94.18
	1.00	2.00	6.00	10.00	0.98	1.94	5.49	9.70					
	1.00	2.00	6.00	10.00	0.95	1.96	5.55	9.20					
	1.00	2.00	6.00	10.00	0.78	1.59	4.42	7.82					
	1.00	2.00	6.00	10.00	0.77	1.62	4.65	7.90					
MC-RR	1.00	2.00	6.00	10.00	0.80	1.60	4.26	8.36	76.20	80.10	73.74	79.20	77.30
	1.00	2.00	6.00	10.00	0.76	1.56	4.10	8.10					
	1.00	2.00	6.00	10.00	0.74	1.64	4.69	7.42					

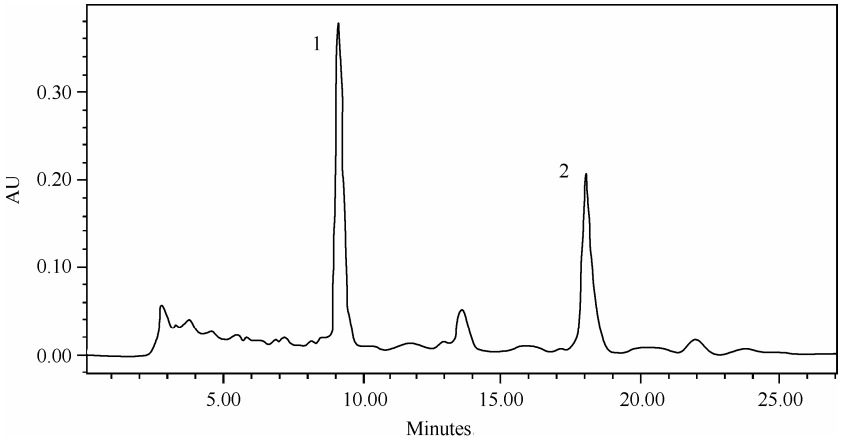

FIG. 3. HPLC chromatogram of MCs from waterbloom sample in August.

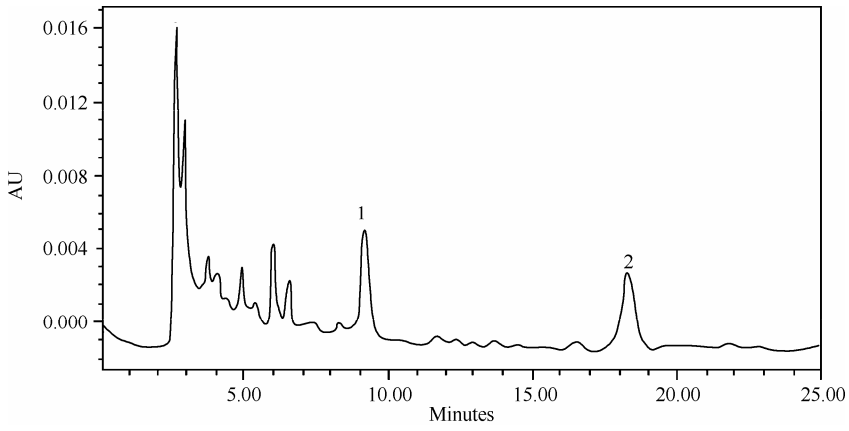

FIG. 4. HPLC chromatogram of MCs from source water sample in September.

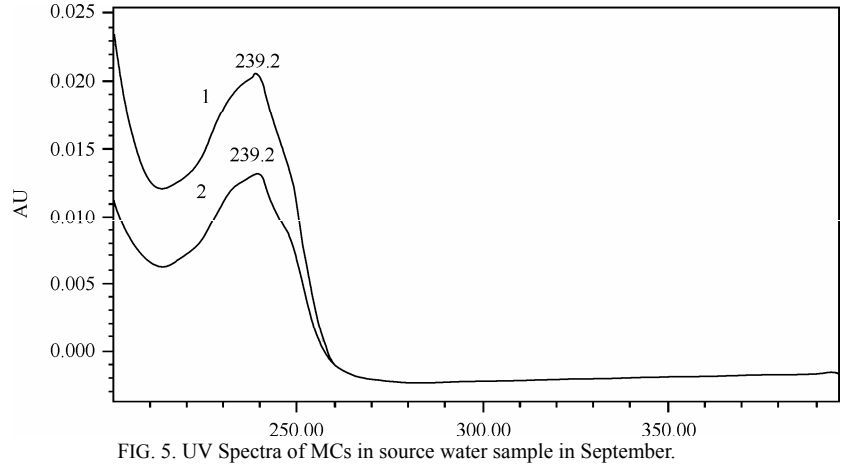

FIG. 5. UV Spectra of MCs in source water sample in September.

TABLE 3

Concentration of Mcs in Source Water Sample From July to October, 2004 ($\bar{x} \pm s$) μg/L (n=5)

MCs	July	August	August*	September	October
MC-LR	1.11±0.28	1.45±0.24	0.74±0.11	2.39±0.41	2.12±0.56
MC-RR	1.64±0.19	2.28±0.35	1.05±0.17	3.09±0.53	2.58±0.37

Note. *No water bloom.

DISCUSSION

SPE combined with HPLC is a conventional method for MC determination, compared with enzyme-linked immunosorbent assay (ELISA)[13-15] for the determination of a variety of MCs. In our experiment, improvements were made for qualitative analysis of bloom sample and quantitative analysis of water sample. The employment of Auto TraceSPE equipments with glass SPE cartridges not only provides the excellent reappearance, but also avoids low recovery caused by common plastic cartridge[16-18]. The different rinse manner for SPE cartridges is introduced for bloom and water samples. Satisfactory results were obtained on the basis of optimization for this method.

The data shown in Table 3 indicate that the significant variation of MC content lies in water sample collected in different periods, the concentrations of MCs maintain about 1-3 μg/L in water sample during summer without bloom. Once bloom occurs, the concentration of MC-LR and MC-RR increases rapidly with the maximum reached 2.39±0.41 μg/L and 3.09±0.53 μg/L in September. As for MCs content in bloom sample, the results of determination except for the maximum content obtained in August had no marked change during the whole monitoring period, indicating that this

tendency is correlated with the phase of algae growth. In general, the toxin content of algae cells is the maximum at the late exponential growth phase. After cell lysis and death, the leakage of toxins makes the concentration of toxins in liquid medium increase sharply.

It was reported that the growth conditions have important effect on microcystin variant production, the temperature above 25℃ favors MC-RR production, otherwise MC-LR is a predominant algae toxin[19]. For this reason, MC-LR is the most frequently investigated toxin in Europe. However, some distinctive aspects in algae toxin pollution are exhibited in TaiHu Lake due to great difference in climate, hydrological and environment conditions.

Taihu lake basin is a complex natural-social ecosystem. In recent years, the cumulative increase in population and the development of industry, agriculture, large amounts of wastewater discharged into Lake has led to an increase of total nitrogen and phosphorus concentration[20]. According to the report of Vezie *et al.*[21], nitrogen and phosphorus levels influence the growth of Microcystis and the production of MC. High levels of nitrogen and phosphorus in freshwater favor the growth of toxic Microcystis strains over nontoxic ones.

Previous studies on MC pollution in Taihu lake have seldom dealt with other MC congeners than MC-LR. In fact, many kinds of MC congeners have toxicity and can cause poisonings. Blom *et al.*[22] reported that MC-RR has nearly the same LC_{50} values as MC-LR and MC-YR in grazer toxicity experiment, and [D-Asp^3, Dha^7] MC-RR, a kind of special MC-RR with demethyl-cyclic heptapeptide, is the most toxic microcystin tested. In this study, the major toxins in source water of Meiliang Bay determined by SPE and HPLC were MC-RR and MC-LR, the content of MC-RR in water sample was higher than that of MC-LR during the whole monitoring period. The same result was also obtained from waterbloom samples. This result is consistent with previous reports[23-25]. It is regretful that our study did not detect other MC variants for short of standards, but MC-RR and MC-LR are no doubt the predominant MCs according to chromatogram.

The result of this study ascertained the presence of MCs in samples from Meiliang Bay, Taihu Lake, indicating that the source water system in this area has been contaminated with high levels of MCs. In view of their stability and resistant to degradation, higher than the guideline value for MCs has been detected in the drinking water in this area after traditional water treatments in summer[15]. Since these toxins enter into human body and constitute potential hazard to public health, measures must be taken to make the MC pollution under control and minimize its harm to human health as soon as possible.

CONCLUSIONS

The results of this survey represent a MC pollution in Taihu Lake. According to the present results for the analysis on MCs from source water and bloom samples during warm months, the main MC components are MC-RR and MC-LR.

In view of their potential hazard to human health, it is necessary to monitor the concentration of MCs in Taihu Lake, and to control the eutrophication rate and growth of cyanobacteria blooms. Meanwhile, measures should be taken to remove toxins from source water.

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