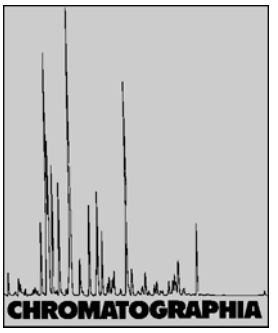
Liquid Chromatography – Mass Spectrometry Identification of Microcystins in *Microcystis aeruginosa* Strain from Lake Thanh Cong, Hanoi, Vietnam



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Key Words

Column liquid chromatography
MS and MS-MS detection
Microcystins
Microcystis aeruginosa

Summary

Various microcystins (MCs) were isolated from extracts of *Microcystis aeruginosa* grown in lake Thang Cong, Hanoi, Vietnam.

MC-RR, MC-YR, and MC-WR were determined by liquid chromatography with mass spectrometric detection (LC-MS). Besides these major microcystins five minor compounds were identified as microcystins using fragmentation mass spectrometry (LC-MS-MS). Two minor compounds were identified as desmethyl variants of MC-WR and MC-RR, respectively, and a third as an MC-LR variant. Two components showed characteristic fragmentation profiles of MC but could not be characterised exactly owing to their low concentrations. Three other components (900 – 1100 dalton) proved not to be microcystins.

Introduction

Some cyanobacterial species (often named blue-green algae) produce microcystins, a group of potent hepatotoxins. These toxins have been associated with toxic incidents affecting wildlife and domestic animals [1].

The first scientific report on the toxic effects of cyanobacteria dates from 1878, when in Australia a bloom of cyanobacteria in a freshwater lake was linked with fatalities among farm animals [2]. Problems with human health and even cases of death after exposure to water contaminated with cyanobacteria have also been reported [3, 4]. The most serious incident happened in Brazil in 1996 when about 60 patients died in a dialysis center after having been treated with MC contaminated water [5, 6].

The growth of cyanobacteria and the formation of blooms are controlled by physical, chemical, and biological factors, which have been described recently in detail [7, 8]. The toxin production varies over a broad range among MC producing species and is related to genetic and metabolic differences of cyanobacteria such as *Microcystis, Oscillatoria, Anabaena* or *Nostoc*. In addition, the ability to produce toxins can vary temporally and spatially at different sites or within a bloom itself, giving rise to characteristic toxin profiles [9, 10].

In the early 1980s, the chemical structure of one of these cyanobacterial hepatotoxins was determined [11]. The structure was elucidated as a cyclic peptide containing seven amino acids. Two amino acids (at positions 2 and 4 of the ring) are variable L-amino acids (x and y, Figure 1). The structural variations are indicated by suffix letters; e. g. MC-LR contains leucine (L) at position 2 and arginine (R) at position 4. Later, further MCs were identified. In these, the structural variations originate from side chain modifications others than at positions 2 or 4. In cases where the N-methyl-dehydroalanine (Mdha) at position 7 is demethylated (Dha)MCs result, whereas demethylation of D-*erythro*-β-methyl aspartic acid (D-MeAsp) at position 3 leads to (D-Asp) MCs. [12–14]. In addition, variations of the Adda (3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-deca-4(E),6(E)-dienoic acid) at position 5 have been described [15]. To date more than 60 MCs have been isolated and characterized [16, 17].

Cyanobacterial toxins are a major health concern, especially in areas where the ecological conditions in surface waters are optimal for the development of cyanobacterial blooms e. g. warm temperatures, calm wind conditions, sufficient nutrients. Frequently recurring cyanobacterial blooms have been reported from lakes "Hoan Kiem" and "Thanh Cong" in Hanoi, Vietnam [18]. The aim of this study was to identify potentially toxic *Microcystis* strains to obtain information for risk assessment to humans and animals.

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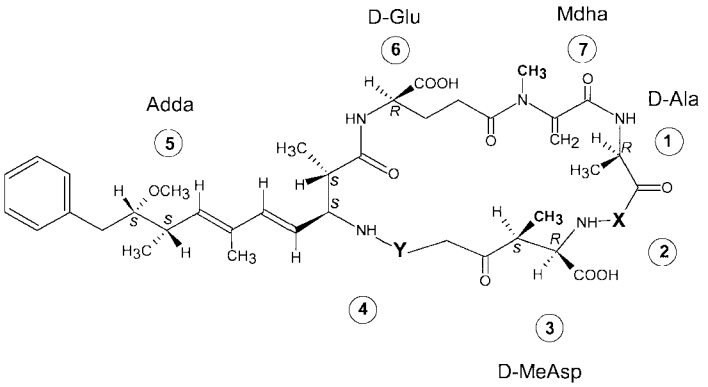

Figure 1. Structure of microcystins, with L-amino acids as X and Y, (D-*erythro*-β-methylaspartic acid = D-MeAsp, N-methyl-dehydroalanine = Mdha, 2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid = Adda, D-glutamic acid = D-Glu.

Samples of cyanobacteria were therefore, analyzed using an analytical procedure encompassing effective sample preparation, MC enrichment, and toxin determination by LC-MS. In addition, MS-MS experiments using characteristic MC fragments produced by collision-activated dissociation (CAD) were successfully carried out to elucidate the structure of MC compounds which had not been described so far in water samples from Vietnam.

Experimental

Standards

Standards of MC-LR, MC-YR, MC-RR, MC-LA and nodularin were from Calbiochem-Novabiochem, Bad Soden, Germany, MC-LW and MC-LF were from Alexis, Grünberg, Germany. The compounds were delivered as solid substances (MC-LR, -YR, -RR, and nodularin: 500 µg; MC-LA: 100 µg; MC-LW and MC-LF: 25 µg), dissolved in 1.0 mL methanol, and diluted with methanol-water (50:50, *v/v*). Commercially unavailable MCs such as (DAsp-Dha)MC-RR, MC-AR, (DAsp-Dha)MC-FR, (DAsp-Dha)MC-LR, (DAsp-Dha)MC-WR, MC-FR, and MC-WR were self-prepared from natural toxic phytoplankton.

Materials and Chemicals

Acetonitrile and methanol were HPLC-grade (Baker BV, Deventer, Netherlands). Water was purified to HPLC-grade quality with a Millipore-Q RG Ultra Pure Water system (Millipore, Milford, USA). Nitrogen for the turbo ion-spray (TIS) interface of the LC-MS system was generated using a Nitrox UHPLCMS nitrogen generator (Domnick Hunter, Krefeld, Germany). The quality of all other chemicals was at least analytical grade (p. a.). An analytical, LC column Luna C18(2) 3 µm (150 mm × 4.6 mm ID) with guard column (30 mm × 4.6 mm ID) (Phenomenex, Aschaffenburg, Germany) was used for chromatographic separation of the MCs.

Sample Material

Microcystis aeruginosa was isolated during a cyanobacterial bloom in lake Thanh Cong, Hanoi, Vietnam. *Microcystis* strains were cultivated at the Institute of Biotechnology of Hanoi, Vietnam (sample 1) using a procedure decribed elsewhere [19]. Sample material was lyophilized and sent to the Friedrich-Schiller-University, Jena, Germany for analysis. In addition, natural born biomass was also lyophilized during the bloom. This material was used as "reference material" to exclude influences of the cultivation on the MC profile (sample 2).

Extraction of Microcystins

The lyophilized sample material (ca. 20 mg, weighed exactly) was extracted twice (500 µL each time) with a mixture of water-methanol (50:50; *v/v*) by 10 min sonication in an ultrasonic bath and 2 min with an ultrasonic homogenizer Sonopuls GM 70 (Bandelin, Berlin, Germany). Combined extracts were centrifuged (14,000 rpm, ca. 2980 g) and the supernate filtered using 0.22 µm PTFE syringe filters (Roth, Karlsruhe, Germany).

Clean-up of Raw Extracts and Enrichment of Microcystins

The raw extracts were purified using size exclusion chromatography (SEC) as described recently [20]. Portions, 200 µL, were subjected to SEC. The MC-containing fractions were collected, and the combined fractions evaporated to dryness under a smooth stream of nitrogen. The residue was dissolved in 200 µL methanol-water (50:50, *v/v*).

LC-MS Determination of Microcystins

Liquid chromatography of MCs was on a PE Series 200 Quaternary Pump and a PE Series 200 autosampler (Perkin-Elmer, Rodgau-Jügesheim, Germany). The LC separation of MCs was carried out with a Luna C18 (2) 3 µm column (see above) using an aqueous eluent containing ammonia formate-formic acid. A gradient comprising 50 mM formic acid – 2 mM ammonia formate in water (eluent A) and 50 mM formic acid – 2 mM ammonia formate in water-acetonitrile (5:95, *v/v*) (eluent B). Eluent B was linearly increased from 32% to 50% within 8 min and then up to 95% for the following 7 min. Within 1 min the gradient was restored to the starting conditions (68% A and 32% B). After 10 min for re-equilibration the system was ready for the next injection. The flow rate was 0.8 mL min^{-1}, throughout.

Detection of MCs was by mass spectrometer. All MS measurements were on an API 165 PE Sciex mass spectrometer using an atmospheric pressure ionization (API) source operating in turbo ion-spray (TIS) mode (Applied Biosystems, Langen, Germany). Eluent from the LC: column was transferred to the mass spectrometer using a split ratio of 5:1 (volume wasted: volume transferred). Nitrogen at 400 °C (620 kPa, 7.5 L min^{-1}) was used to dry the ion-spray. The ionization voltage of the TIS interface was set to 4.8 kV. Nitrogen was also used as nebulizer gas at an operating pressure of approximately 520 kPa or 0.6 L min^{-1}.

When operating the mass spectrometer in scan mode two mass ranges were scanned, *m/z* 900–1150 for singly charged MCs $[M+H]^+$ and *m/z* 505–525 for doubly charged MCs $[M+2H]^{2+}$, respectively, as MCs with two basic arginine constituents such as MC-RR mainly form doubly charged ions under ion-spray conditions,

whereas other MCs are normally singly charged [21]. The step size within the scan range was set to 0.3 dalton. Significant masses found during scan experiments were also measured in selected ion monitoring (SIM) mode to increase sensitivity of detection. All ions monitored in SIM mode and the corresponding MCs are shown in Table I.

MS-MS Identification of Microcystins

Fragmentation spectra of MCs were obtained using an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Langen, Germany) equipped with the same TIS interface as above. Product ion spectra were measured in positive mode in the mass range *m/z* 100–1130 (step size set at 0.2 dalton) using collision activated dissociation (CAD) with nitrogen as target gas in the second quadrupole (approximately 60 eV collision energy). The triple quadrupole mass spectrometer was coupled to the LC system above.

Results and Discussion

LC-MS

Figure 2 shows chromatograms obtained after injection of an MC-containing standard solution into the LC-MS system, to demonstrate the benefits of the applied chromatographic separation. Mass detection was in SIM mode. The standard solution contained six commercially available MCs (marked with an asterisk) and some MCs, which were prepared from different cyanobacteria samples. This solution is routinely used to calibrate the LC-MS coupling with respect to retention times and signal intensity. The selectivity of mass detection instead of optical detection (e. g. UV at 238 nm) allows determination of different MCs even in those cases where identical retention times occur. This advantage is demonstrated by extracted, single ion masses in Figure 2.

The extraction of ion mass *m/z* 1045.6 gave a chromatogram with two peaks (Figure 2). The first peak at RT 5.9 min was identified as MC-YR by comparison with an MC-YR standard solution. Although the MC-like structure of the second peak (RT 8.0 min) was confirmed by MS-MS (spectrum not shown) its structure is still unknown. The peak with

Table I. Molecule ions measured in SIM mode and corresponding microcystins.

ion mass [*m/z*]	ion	mol weight [MW]	microcystin	reference
512.5*	$(M+2H)^{2+}$	1023.4	[D-Asp^3]MC-RR	[23]
			[Dha^7]MC-RR	[24]
			[D-Asp^3,Dhb^7]MC-RR	[25]
519.5*	$(M+2H)^{2+}$	1037.4	MC-RR#	[26]
825.5	$(M+H)^+$	824.5	Nodularin**	[27]
910.5	$(M+H)^+$	909.5	MC-LA#	[11]
939.0	$(M+H2O+H)^+$	956.2	Cyanopeptolin A	[28]
953.5	$(M+H)^+$	952.5	MC-AR	[15]
956.6*	$(M+H)^+$		Unknown	
973.6*	$(M+H)^+$		Unknown	
981.5	$(M+H)^+$	980.5	[Dha]MC-LR	[23]
			[DAsp]MC-LR	[12]
986.5	$(M+H)^+$	985.5	MC-LF#	[29]
995.5	$(M+H)^+$	994.5	MC-LR#	[30]
1007.8	$(M+H)^+$	1006.8	Unknown	
1015.6	$(M+H)^+$	1014.6	[D-Asp^3]MC-FR	[31]
			[Dha^7]MC-FR	this paper
1025.6	$(M+H)^+$	1024.6	MC-LW#	[32]
1029.6	$(M+H)^+$	1028.6	MC-FR	[15]
1031.6*	$(M+H)^+$	1030.6	[D-Asp^3]MC-YR	this paper
			[Dha^7]MC-YR	[33]
			[D-Asp^3,Dha^7]MC-HtyrR	[34]
1039.8*	$(M+H)^+$	1038.8	[D-Ser^{1}ADMAdda]MC-LR	[35]
1045.6*	$(M+H)^+$	1044.6	MC-YR#	[30]
			[D-Asp^3]MC-HtyrR	[36]
			[Dha^7]MC-HtyrR	[34]
			[D-Asp^3(Z)Dhb^7]MC-HtyR	[37]
			[D-Asp^3(*E*)Dhb^7]MC-HtyR	[38]
1054.6*	$(M+H)^+$	1053.6	[D-Asp^3]MC-WR	[31]
			[Dha^7]MC-WR	this paper
1068.9*	$(M+H)^+$	1067.9	MC-WR	[15]
1087.6	$(M+H)^+$		Unknown	
1100.6*	$(M+H)^+$		Unknown	

* ion found in sample material # commercially available; ** cyclic pentapeptide; abbreviations: D-Ser = D-serine; L-Htyr = L-homo tyrosine; Dhb = dehydro-α-amino butyric acid; D-Asp = D-aspartic acid; Dha = dehydro alanine; ADMAdda = O-acetyl-O-desmethyl-Adda.

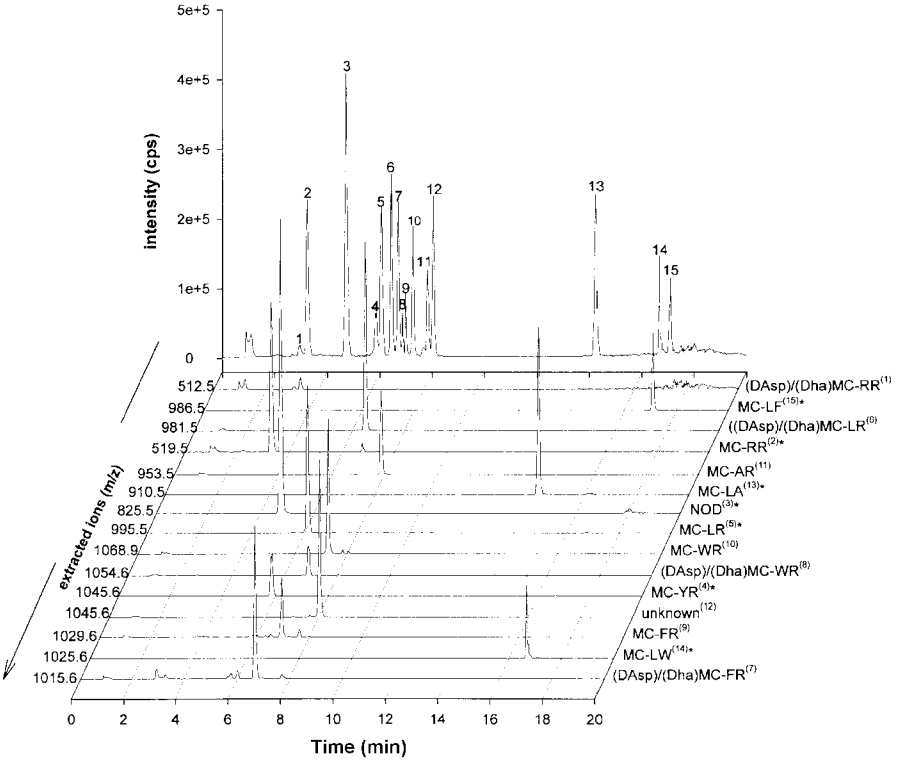

Figure 2. LC-MS chromatogram of microcystins-containing solution; * = available as standard (5 ng MC on column); (LC-MS conditions see text).

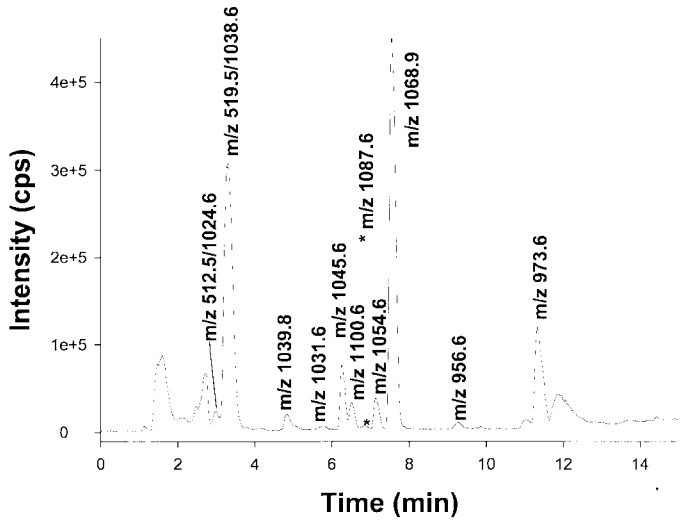

Figure 3. LC-MS TIC chromatogram of extract of cultivated *Microcystis aeruginosa* from lake Thanh Cong, Hanoi, Vietnam, (LC-MS conditions see text).

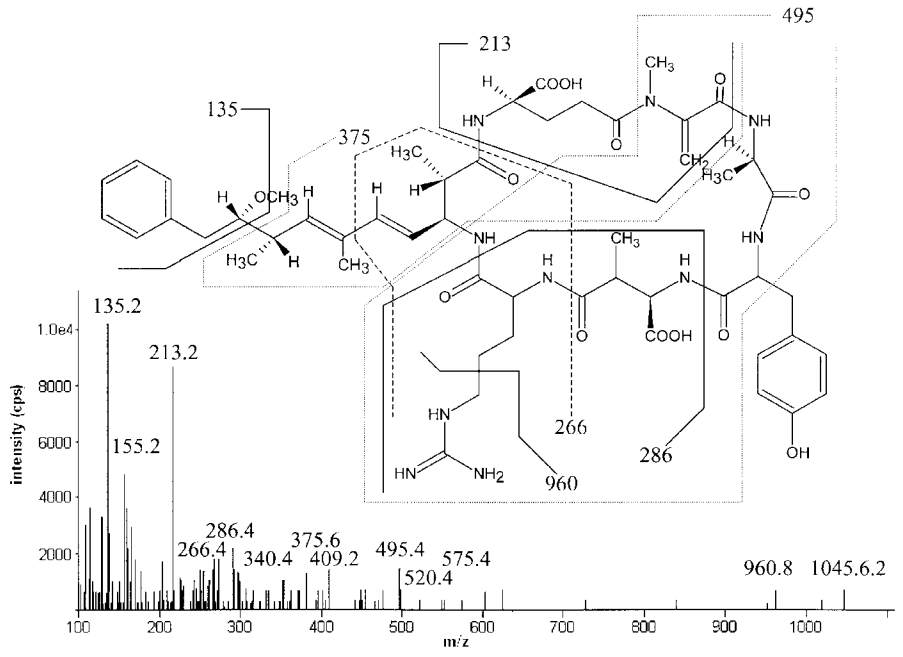

Figure 4. MS-MS spectrum of *m/z* 1045.6 from MC-YR and chemical structure of MC-YR (lines in structure indicate possibly corresponding ion-fragments), (LC-MS-MS conditions see text).

m/z 953.5 results from MC-AR, as confirmed by MS-MS experiments showing typical fragments for this MC (spectrum not shown).

In addition, (DAsp) or (Dha)MCs were discovered in the MC-containing solution (Table I). The molecular weight of compounds corresponding with some peaks in the LC-MS chromatogram (Figure 2) show mass differences of 14 dalton compared to other well known MCs. Therefore, it is not unlikely that, e. g. *m/z* 512.5 / 1024.6 (doubly charged / singly charged), represent (DAsp) or (Dha) structure variations of MC-RR (*m/z* 519.5 / 1038.6), *m/z* 981.5 would correspond to MC-LR (*m/z* 995.5), *m/z* 1015.6 to MC-FR (*m/z* 1029.5), and *m/z* 1054.8 to MC-WR (*m/z* 1068.9). The identity of (Dha)MC-LR (*m/z* 981.5) and of (DAsp) WR (*m/z* 1068.9) was indeed confirmed by MS-MS spectra (spectra not shown). However, it is still not clear whether 512.5 and 1024.6 represent Dha or DAsp structure variations of MC-RR.

In addition to the sample obtained from a cultivated *Microcystis aeruginosa* strain isolated from lake Thanh Cong (sample 1) a natural bloom sample from lake Thanh Cong (sample 2) was investigated. MC patterns of both samples did not significantly differ, the following discussion applies to both samples.

Figure 3 shows the chromatogram of an extract of cultivated *Microcystis aeruginosa* from lake Thanh Cong obtained with the MS in scan mode. To increase signal intensity additional measurements were carried out, in which only ions listed in table I were monitored (SIM mode, chromatogram not shown). Despite increased sensitivity in SIM mode no additional compounds were found. However, there were numerous peaks of compounds in the sample chromatograms suspected to be MCs – as described below.

The doubly charged ions *m/z* 519.5 and the singly charged ions *m/z* 1045.6 indicate the presence of MC-RR and MC-YR, respectively. In this context it is noteworthy, that the most common MC-LR, was not present in the samples. MC-LR absence and the finding that MC-WR was present in high concentration among several minor MCs demonstrates that the toxin profile from lake Thanh Cong is different to that known from European waterbodies. This finding is in agreement with former studies in which *Microcystis aeruginosa* strains (cyanobacteria from different geographical locations) were shown to produce strain-specific toxin profiles [8].

To the authors knowledge, all other significant ions monitored in the phytoplankton samples in the mass range 900–1150 dalton do not refer to MCs commercially available as standards. However, ions *m/z* 512.5 / 1024.6, 1031.6, 519.5 / 1038.6, 1039.8, 1054.6 and 1068.9 seem to be MCs, that have been described earlier (see Table I). Singly charged ions *m/z* 1024.6 and 1038.6 were observed at the same retention times as the corresponding doubly charged ions *m/z* 512.5 and 519.5, respectively.

The ions *m/z* 956.6, 973.6, 1087.6, and 1100.6 (Figure 3) did not correspond to known MCs and were therefore analyzed by MS-MS to decide whether or not they are MCs.

LC-MS-MS

MS-MS experiments were carried out for the unambiguous identification of MCs in both "standard mixture" and sample material from Vietnam. Here the same extracts as for the MS experiments were used.

MS-MS measurements showed no evidence of MC structures for compounds *m/z* 956.6, 973.6, and 1087.6, because no characteristic MC fragments such as those shown in Figure 4 were observed. Figure 4 shows the characteristic fragmentation spectrum obtained from an MC-YR containing standard solution together with the chemical structure of MC-YR. The fragment *m/z* 135 originates from the cleavage of the methoxy group of Adda and is, therefore, typical of MCs [15]. Fragments with *m/z* 213 resulting from the amino acids Glu-Mdha (position 6/7) and with *m/z* 375 that has been assigned to Glu-Mdha together with a partial structure from Adda (position 5) are also characteristic [21]. The loss of the guanidino group from L-Arg (position 4) results in an ion with *m/z* 960. The amino acids Arg-MeAsp (position 3/4) form the fragment with *m/z* 286, while *m/z* 495 originates from Ala-Tyr-MeAsp-Arg after losing hydroxyphenyl from the tyrosine residue. Since the MS-MS spectrum of the compound with *m/z* 1045.6 (RT 6.3 min, Figure 3) showed an identical fragmentation profile (spectrum not shown) the presence of MC-YR in the extract of sample material from Vietnam was evident.

The applicability of the LC-MS-MS method was demonstrated for the peak with *m/z* 1068.9 at RT 7.6 min in Figure 3 arising from the main compound in the *Microcystis* strain from Vietnam. LC-MS-MS experiments confirmed the results obtained with the LC-MS system.

Figure 5 shows the product ion MS-MS spectrum of the *m/z* 1068.9 precursor ions. Fragment ions with *m/z* 135, 213, and 375 were observed, where *m/z* 135 indicates that the compound is most probably an MC. Two more fragments were detected, which originated from Adda (*m/z* 163 and 176). An ion with *m/z* 347 consisting of Glu-Mdha (*m/z* 213) together with a smaller partial structure of Adda was also observed. Comparison of MS-MS spectra of compound *m/z* 1068.9 and of MC-WR together with their identical retention times clearly revealed MC-WR to be the main MC in *Microcystis aeruginosa* isolated from lake Thanh Cong.

Figure 6 shows the MS-MS spectrum of *m/z* 1054.8 in the extract of the phytoplankton samples (RT 7.2, Figure 3). The presence of *m/z* 135, 163, 213, and 375 identified this compound as an MC, while the mass difference of 14 dalton to *m/z* 1068.9 suggested that this MC could be a

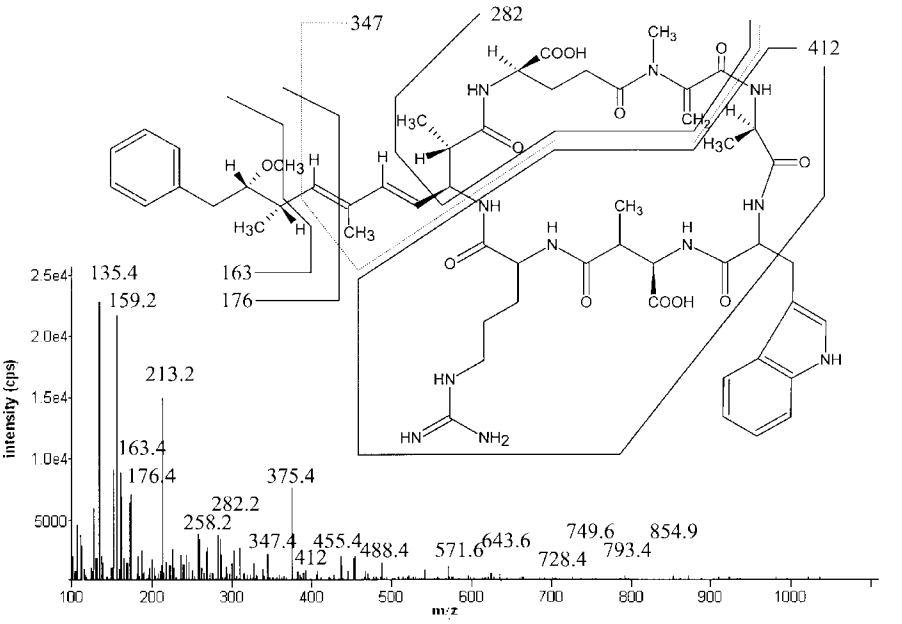

Figure 5. MS-MS spectrum of *m/z* 1068.9 of sample 1 and chemical structure of MC-WR. (lines in structure indicate possibly corresponding ion-fragments), (LC-MS-MS conditions see text).

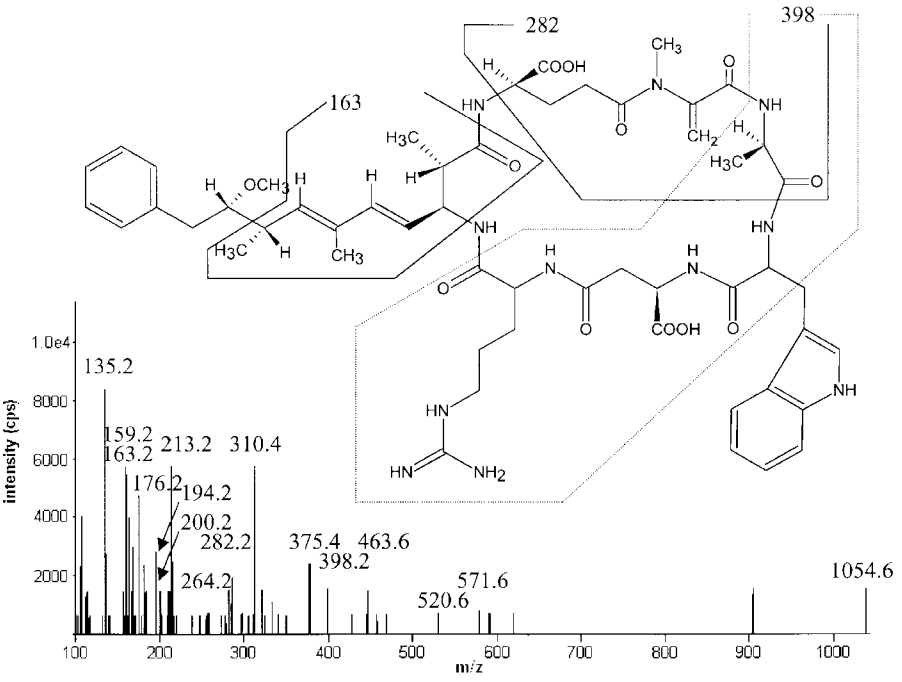

Figure 6. MS-MS spectrum of *m/z* 1054.6 and chemical structure of (DAsp)MC-WR (lines in the structure indicate possibly corresponding ion-fragments), (LC-MS-MS conditions see text).

(DAsp) or (Dha) variant of MC-WR (Table I). In the case of (Dha)MC-WR an ion with *m/z* 198 should appear instead of a fragment *m/z* 213, which was not the case. However, when comparing the MS-MS spectra from *m/z* 1068.9 (MC-WR, Figure 5) and from *m/z* 1054.6 (Figure 6) only one fragment pair was found showing a mass difference of 14 dalton. This fragment with *m/z* 398 in Figure 6 can be explained as Ala-Trp-Asp-Arg after loss of the aromatic ring from the Trp residue. In the case of MC-WR the same fragmentation mechanism would result in an Ala-Trp-MeAsp-Arg fragment with *m/z* 412 and indeed, such an ion is formed from MC-WR (Figure 5). This corroborates the proposal that ion *m/z* 1054.6 represents (DAsp)MC-WR and not (Dha)MC-WR.

Figure 7 shows the MS-MS spectrum from *m/z* 1039.8 (RT 4.9, Figure 3). The

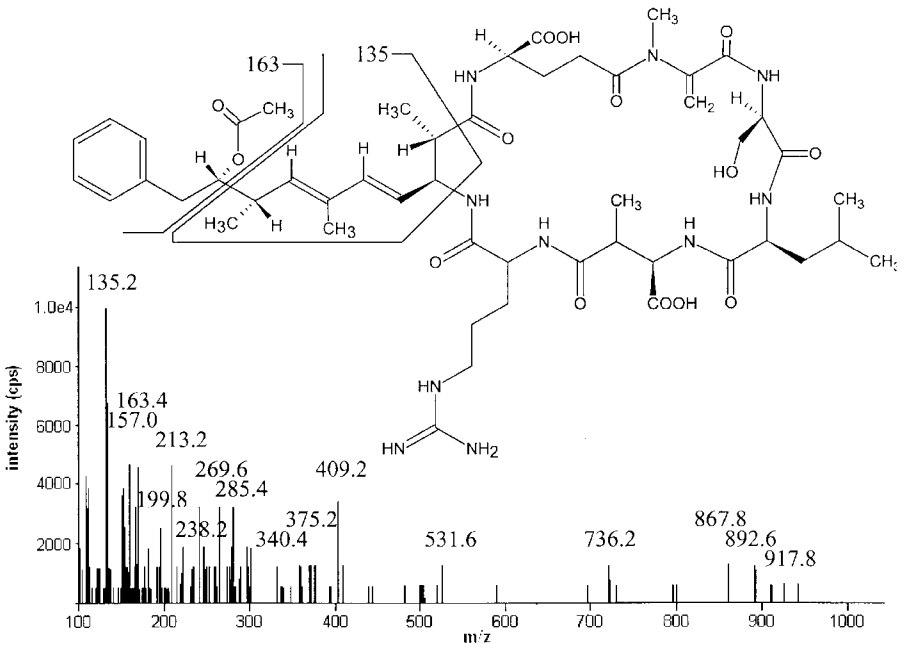

Figure 7. MS-MS spectrum of *m/z* 1039,8 and chemicalstructure of (D-Ser[1], ADMAdda)MC-LR. (lines in structure indicate possibly corresponding ion-fragments) (LC-MS-MS conditions see text).

Table II. Quantitative microcystin composition.

molecular weight	ion [*m/z*]	microcystin	concentration [$\mu g\,mg^{-1}$]
1030.6	1031.6	"MC-XY"	0.04 ***
1037.4	519.5	MC-RR	0.30 *
1044.6	1045.6	MC-YR	1.20 **
1053.6	1054.6	"(DAsp)MC-WR"	0.20 ***
1067.9	1068.9	MC-WR	2.40 ***
1099.6	1100.6	"MC-XY"	0.10 ***
		sum	4.24

* quantified against MC-RR standard; ** quantified against MC-YR standard; *** quantified against MC-LR standard; "MC-XY" microcystin, exact structure unknown.

ions *m/z* 135, 213, and 375 identified the compound as MC. However, so far only (D-Ser, ADMAdda)MC-LR has been reported as MC with the corresponding molecular weight 1038.8 (Table I). Therefore, the structure of this MC is shown together with the MS-MS spectrum. In contrast to MC-LR the (D-Ser, ADMAdda) MC-LR has D-Ser instead of D-Ala at position 1 and the O-acetyl-O-desmethyl derivative of Adda (ADMAdda) is located at position 5. According to the explanation for the origin of *m/z* 135 [15] the fragment with *m/z* 135 should not appear in mass spectra from this MC, because the cleavage of the acetyl group of ADMAdda leads to an ion *m/z* 163 (see Figure 7). However, both fragments *m/z* 135 and *m/z* 163 were observed. In figures 4 and 7 two different mechanisms for the formation of *m/z* 135 are demonstrated, whereas Figures 5 and 6 show alternative fragmentation resulting in *m/z* 163. Since *m/z* 135 is regarded as the "key-fragment ion" for all MCs, a second source such as the proposed mechanism seems to be very likely. Theoretically, co-occurrence of *m/z* 135 and *m/z* 163 could also be caused by co-elution of two MCs with the same molecular weight. However, the existence of a second MC with molecular weight 1038.8 showing the same retention time is highly unlikely (see Table I).

The MS-MS spectrum obtained from the very low concentration compound with *m/z* 1031.6 (spectrum not shown) gives fragments with *m/z* 135, 163, and 213 although the signal intensities were rather poor. The MS-MS spectrum of the ion with *m/z* 1100.6 (not shown) also gives typical MC fragments *m/z* 135 and 375, too. Therefore, both compounds (*m/z* 1031.6 at RT 5.8 min and *m/z* 1100.6 at RT 6.5 min in Figure 3) are most probably MCs.

Quantitation

Quantitation of the MC-YR and MC-RR concentration in the sample material was carried out on the LC-MS system using an MC-YR and MC-RR calibration curve (peak area). The concentrations of all other MCs were calculated using MC-LR standard. Although most probably the response of different MCs varies, the lack of standards forced this compromise. The dominant MC in the samples is MC-WR ($2.4\,\mu g\,mg^{-1}$ dry weight) followed by MC-YR ($1.2\,\mu g\,mg^{-1}$ dry weight), and MC-RR ($0.3\,\mu g\,mg^{-1}$ dry weight). However, one should be aware that the quantitative results show only the relative composition of MCs and allowing an estimate of the overall MC content.

Table II shows the quantitative results.

Conclusions

Only a few MCs are commercially available as standards and the results concerning MCs in lake Thanh Cong revealed that other, more rare MCs can make the highest contribution to the MC burden of an ecosystem. Application of LC-MS-MS enabled identification of MCs in the phytoplankton samples from lake Thanh Cong. These MCs show characteristic fragmentation (e. g. *m/z* 135, 163, 213, and 375) which can be used for their unambiguous identification. Furthermore, comparison of fragmentation spectra of MCs can also be used to identify partial structures of unknown MCs.

It should be possible to create a data base with fragmentation spectra from different MCs obtained under standardized collision, activated dissociation (CAD) conditions. Such a data base might offer a fast and reliable tool to elucidate unknown MC structures by building up a complete MC ring from characteristic fragments. To date, the available MS-MS information is still insufficient for this aim.

However, the example of the MC with molecular weight 1100.6 indicates that even with LC-MS the structural information obtainable for very low concentration compounds is too limited for any speculation about their structures. The molecular weight of this new MC exceeds the molecular weight of the largest MC (D-Asp[3]ADMAdda[5]Dhb[7])MC-HtyR (MW = 1072) described so far [22] and additional experiments with larger quanti-

ties need to be carried out to identify its structure.

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