Sulfide oxidations for LC-MS analysis of methionine-containing microcystins in dolichospermum flos-aquae NIVA-CYA 656

Miles, Christopher O.; Melanson, Jeremy E.; Ballot, Andreas

Publisher's version / Version de l'éditeur:
https://doi.org/10.1021/es5029102
Environmental Science and Technology, 48, 22, pp. 13307-13315, 2014-10-21

Environmental Science & Technology

Article

pubs.acs.org/est

Sulfide Oxidations for LC-MS Analysis of Methionine-Containing Microcystins in *Dolichospermum flos-aquae* NIVA-CYA 656

Christopher O. Miles,*[,†,‡] Jeremy E. Melanson,[§] and Andreas Ballot[†,▽]

[†]Norwegian Veterinary Institute, P.O. Box 750 Sentrum, N-0106 Oslo, Norway

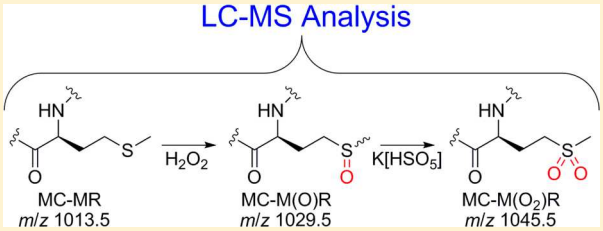
[‡]Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, N-0316 Oslo, Norway

[§]Measurement Science and Standards, National Research Council Canada, 1200 Montreal Road, Ottawa, Ontario K1A 0R6 Canada

[▽]Norwegian Institute for Water Research, Gaustadalléen 21, N-0349 Oslo, Norway

Supporting Information

ABSTRACT: Microcystins are cyclic heptapeptides produced by a range of cyanobacteria. More than 150 microcystin analogues have been reported from cultures, algal blooms, or other contaminated samples. Relatively few analytical standards are available, making identification and quantitation of these toxins a challenge, even with LC-MS technology. We developed a two-step oxidative procedure that allows LC-MS identification of microcystins containing methionine and methionine sulfoxide, and reveals the oxidation state of the methionyl sulfur atom. The procedure was used in parallel with mercaptoethanol derivatization and LC-MS^2 analysis to demonstrate the presence of [Asp^3]MC-MR (**12**) and MC-MR (**17**) in a culture of *Dolichospermum flos-aquae*, together with low levels of [Asp^3]MC-M(O)R (**5**) and MC-M(O)R (**7**), as well as 20 other microcystins. Fresh culture contained only traces of sulfoxides **5** and **7**, but these increased during storage or sample extraction and preparation. This suggests that microcystins containing methionine sulfoxide are primarily postextraction oxidation artifacts, rather than being produced by biosynthesis in cyanobacteria. A simple, rapid extraction under inert gas followed promptly by LC-MS analysis minimized oxidation artifacts for *D. flos-aquae*.



INTRODUCTION

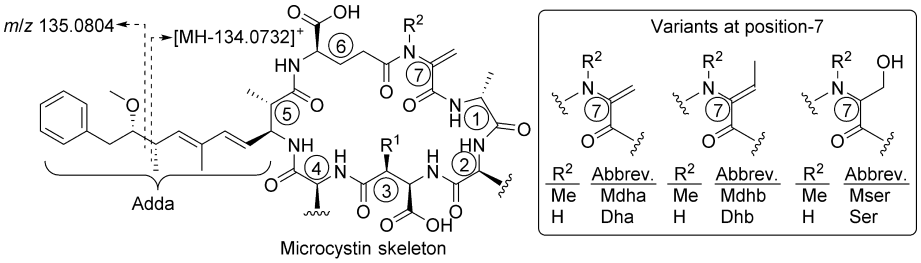
Dolichospermum flos-aquae (Brébisson ex Bornet et Flahault) P.Wacklin, L.Hoffmann & J.Komárek belongs to the cyanobacterial family Nostocaceae and is able to fix nitrogen through special cells called heterocytes.[1] Formerly known as *Anabaena flos-aquae* ([Lyngbye] Brébisson ex Bornet et Flahault), it is a planktonic species possessing gas vesicles and was therefore assigned to the new genus *Dolichospermum*.[2] *D. flos-aquae* forms blooms in lakes and reservoirs worldwide and has caused mortalities of domestic animals and wildlife.[3] Several strains produce the neurotoxins anatoxin-a and anatoxin-a(s).[3] Production of hepatotoxic microcystins, including [Asp^3]MC-HtyR (**15**), [Asp^3]MC-LR (**18**), MC-HtyR (**19**), and MC-LR (**20**) (Figure 1), has been demonstrated in strains of *D. flos-aquae* and unidentified *Dolichospermum* and *Anabaena* spp.[4−6]

Microcystins (MCs) are cyclic heptapeptides produced by a range of cyanobacteria, including *Microcystis*, *Nostoc*, and *Planktothrix* spp.[7] Microcystins inhibit protein phosphatase-1 and -2A, and have been responsible for numerous poisonings of livestock, wild animals, and, occasionally, human beings.[7] In addition to acute toxic effects, microcystins are suspected of chronic toxicity through consumption of contaminated water used for drinking and food preparation.[7]

The World Health Organization has issued a guideline level of 1 μg/L for MC-LR in drinking water.[8,9] However, more than 150 microcystin congeners have been reported in the literature,[10,11] making measurement of such low concentrations in water challenging. For example, a recent analysis from a *Microcystis* bloom in Hartbeespoort Dam, South Africa, identified 41 microcystins in the water.[12] A further problem is that analytical standards are available for only a very few MC congeners, and the quantitation of some of these commercial standards appears to be unreliable.[13]

LC-MS analytical methods have, to a degree, alleviated the problem of lack of standards, because tentative identification can often be obtained from characteristic ions in the MS/MS spectra[14,15] although quantitation without standards remains problematic. For example, the majority of reported microcystins contain the unusual β-amino acid Adda (Figure 1), which produces a characteristic product ion during MS fragmentation.[14] Recently, we described a method using thiol derivatization together with LC-MS/MS analysis to identify microcystins in complex matrices, based on the reactivity of the Mdha and Dha groups (Figure 1) present in most MCs.[12,16,17] This method also discriminates between microcystins containing the thiol-reactive Mdha and Dha and the unreactive Mdhb

Received: June 16, 2014
Revised: October 15, 2014
Accepted: October 21, 2014
Published: October 21, 2014



		②	④	⑦	R^1	$[MH]^+$ *m/z*
5	[Asp^3]MC-M(O)R	Met(O)	Arg	Mdha	H	1015.4917
6	[Asp^3]MC-M(O_2)R	Met(O_2)	Arg	Mdha	H	1031.4866
7	MC-M(O)R	Met(O)	Arg	Mdha	Me	1029.5074
8	[Ser^1,Asp^3]MC-HtyR	Hty	Arg	Mdha	H	1061.5302
9	[Asp^3, Mser^7]MC-HtyR	Hty	Arg	Mser	H	1063.5459
10	MC-M(O_2)R	Met(O_2)	Arg	Mdha	Me	1045.5023
11	[Mser^7]MC-HtyR	Hty	Arg	Mser	Me	1077.5615
12	[Asp^3]MC-MR	Met	Arg	Mdha	H	999.4968
13	[Asp^3, Mser^7]MC-LR	Leu	Arg	Mser	H	999.5510
14	[Mser^7]MC-LR	Leu	Arg	Mser	Me	1013.5666
15	[Asp^3]MC-HtyR	Leu	Arg	Mdha	H	1045.5353
16	[Asp^3]MC-Hty(OMe)R	Hty(OMe)	Arg	Mdha	H	1075.5459
17	MC-MR	Met	Arg	Mdha	Me	1013.5125
18	[Asp^3]MC-LR	Leu	Arg	Mdha	H	981.5404
19	MC-HtyR	Hty	Arg	Mdha	Me	1059.5510
20	MC-LR	Leu	Arg	Mdha	Me	995.5560
21	[Asp^3]MC-FR	Phe	Arg	Mdha	H	1015.5247
22	MC-HilR	Hil	Arg	Mdha	Me	1009.5717
23	[Asp^3]MC-HilR	Hil	Arg	Mdha	H	995.5560
24	MC-FR	Phe	Arg	Mdha	Me	1029.5404
25	MC-HphR	Hph	Arg	Mdha	Me	1043.5560
26	[Asp^3]MC-HphR	Hph	Arg	Mdha	H	1029.5404

Figure 1. Microcystins discussed in the text, with standard amino acid abbreviations, calculated *m/z* values, and showing the characteristic fragmentation pathway in the Adda-moiety. Note that Met(O) denotes methionine sulfoxide as a mixture of diastereoisomers, while Met(O_2) denotes methionine sulfone.

and Dhb (Figure 1) residues, which are otherwise difficult to differentiate by LC-MS.[18]

Microcystins containing methionine are relatively uncommon in the literature, but MC-M(O)R (**7**),[19] MC-YM(O),[20,21] MC-YM[19,20] and MC-LM[22] ($[MH]^+$ *m/z* 1029.5, 1036.5, 1020.5, and 970.5, respectively) have been reported (M, methionine; M(O), methionine sulfoxide).

While analyzing an extract from *D. flos-aquae* by LC-MS^2, we observed peaks at *m/z* 1015.5, 1029.5, 999.5, and 1013.5 whose thiol reactivities (Table 1, Supporting Information Figure S41) and MS^2 spectra (Supporting Information Figures S25 and S27−29) were consistent with Met-containing microcystins, but in the absence of high-resolution MS, these masses also corresponded to plausible non-Met-containing microcystins.

Here, we report a two-step oxidation procedure with hydrogen peroxide and potassium peroxymonosulfate that, together with unit-mass-resolution LC-MS^2 and the recently reported[16,18] thiol-derivatization procedure, provides a high level of certainty in the identification of Met-containing microcystins. Subsequent application of high-resolution LC-MS before and after oxidation was used to confirm their identities and to validate the procedure.

MATERIALS AND METHODS

Chemicals and Cyanobacteria. Mercaptoethanol and potassium peroxymonosulfate (Oxone) (technical grade) were from Sigma−Aldrich, Oslo, Norway. A mixed standard (MC-RR, MC-LR (**20**), MC-YR, MC-LA, MC-LY, MC-LF, MC-LW; ca. 0.4−1 μg/mL in MeOH−H_2O (1:1)) was prepared from microcystins from Alexis Biochemicals (Grünberg, Germany) as described previously,[16] while standards of MC-HilR (**22**) and MC-HtyR (**19**) were from AH Diagnostics (Oslo, Norway). Certified reference materials of MC-LR, [Dha^7]MC-LR, MC-RR and NOD, and a reference material of [Asp^3]MC-LR (**18**) quantified by LC with a chemiluminscence nitrogen detector, were from NRC Measurement Science and Standards, Halifax, NS, Canada. Hydrogen peroxide (30%) was from Merck (Darmstadt, Germany). Solvents for LC and extraction were of gradient (Romil, Oslo, Norway) or LC/MS quality (Fisher Scientific, Fair Lawn, NJ, or Loughborough, United Kingdom). DIAION HP-20 was from Mitsubishi Chemical Corporation (Tokyo, Japan).

Cyanobacterial Culture. *D. flos-aquae* strain NIVA-CYA 656 (from the culture collection of the Norwegian Institute for Water Research) was originally isolated as strain AB2008/17 from Lake Scharmützelsee, Germany, in 2008.[23] The culture was maintained in 50 mL flasks containing 20 mL Z8 medium[24] at 22 °C and a photon flux density of 80 mol m^{-2} s^{-1}. Because of the recent taxonomic revision of *Anabaena*, and 6 years of regular subculturing including 2 interlaboratory transfers, the species identity of this culture was confirmed by isolation of genomic DNA and a 16S rRNA gene analysis (Supporting Information). The strain was also classified based on standard morphological traits[1,25] using a Leica DM2500 light microscope, Leica DFC450 camera and Leica Application Suite software (Leica, Oslo, Norway) (Supporting Information).

Sample Preparation. *Culture 1.* Aliquots of culture (1 mL) were frozen and thawed three times then ultrasonicated for 5 min. MeOH (1 mL) was then added and the samples filtered (0.2 μm, Costar Spin-X Microcentrifuge, Corning, NY).

Table 1. Microcystin Analogues Identified in Extracts from a Culture of *D. flos-aquae* (NIVA-CYA 656)

						culture 2				
						R_t (min)		HP-20 extract	quantitative extract[c]	
microcystin		$[MH]^+$ formula	measured (m/z)	Δ (ppm)	status[a]	grad.[b]	isocrat.[c]	% of total[d]	conc. (ng/ml)	% of total[d]
[seco-4/5][Asp^3]MC-HtyR	1	$C_{52}H_{75}O_{14}N_{10}$	1063.5466	0.7	tentative	2.41	nd[e]			
[seco-4/5]MC-HtyR	2	$C_{53}H_{77}O_{14}N_{10}$	1077.5636	1.9	tentative	2.49	nd			
[seco-4/5][Asp^3]MC-LR	3	$C_{48}H_{75}O_{13}N_{10}$	999.5517	0.8	tentative	2.60	nd			
[seco-4/5]MC-LR	4	$C_{49}H_{77}O_{13}N_{10}$	1013.5675	0.9	tentative	2.67	nd			
[Asp^3]MC-M(O)R	5	$C_{47}H_{71}O_{13}N_{10}S$	1015.4905	−1.2	tentative	3.19	2.84	0.3	trace	trace
[Asp^3]MC-M(O_2)R	6	$C_{47}H_{71}O_{14}N_{10}S$	1031.4851	−1.4	tentative	nd	3.43[f]	nd	nd	nd
MC-M(O)R	7	$C_{48}H_{73}O_{13}N_{10}S$	1029.5056	−1.7	tentative	3.47	3.69	0.3	trace	trace
[Ser^1,Asp^3]MC-HtyR[g]	8	$C_{52}H_{73}O_{14}N_{10}$	1061.5289	−1.2	uncertain	3.37	4.05	trace	nd	nd
[Asp^3,Mser^7]MC-HtyR	9	$C_{52}H_{75}O_{14}N_{10}$	1063.5432	−2.7	tentative	3.51	4.07	0.1	trace	trace
MC-M(O_2)R	10	$C_{48}H_{73}O_{14}N_{10}S$	1045.5005	−1.6	tentative	nd	4.31[f]	nd	nd	nd
[Mser^7]MC-HtyR	11	$C_{53}H_{77}O_{14}N_{10}$	1077.5591	−2.3	tentative	3.60	4.56	nd	nd	nd
[Asp^3]MC-MR	12	$C_{47}H_{71}O_{12}N_{10}S$	999.4963	−0.5	tentative	3.63	4.58	1.5	4.7	1.8
[Asp^3, Mser^7]MC-LR	13	$C_{48}H_{75}O_{13}N_{10}$	999.5499	−1.0	tentative	3.63	4.64	0.1	trace	trace
[Mser^7]MC-LR	14	$C_{49}H_{77}O_{13}N_{10}$	1013.5645	−2.1	tentative	3.66	4.71	0.1	trace	trace
[Asp^3]MC-HtyR	15	$C_{52}H_{73}O_{13}N_{10}$	1045.5341	−1.2	tentative	3.64	4.75	14	36	14
[Asp^3]MC-Hty(OMe)R	16	$C_{53}H_{75}O_{14}N_{10}$	1075.5474	0.6	tentative	3.73	nd	trace		
MC-MR	17	$C_{48}H_{73}O_{12}N_{10}S$	1013.5113	−1.1	tentative	3.75	5.33	2.0	6.4	2.5
[Asp^3]MC-LR	18	$C_{48}H_{73}O_{12}N_{10}$	981.5399	−0.5	confirmed	3.78	5.63	23	60	23
MC-HtyR	19	$C_{53}H_{75}O_{13}N_{10}$	1059.5507	−0.2	confirmed	3.82	5.63	19	53	20
MC-LR	20	$C_{49}H_{75}O_{12}N_{10}$	995.5546	−1.4	confirmed	3.88	6.00	33	87	34
[Asp^3]MC-FR	21	$C_{51}H_{71}O_{12}N_{10}$	1015.5233	−1.4	tentative	3.98	7.71	1.5	3.9	1.5
MC-Hilr	22	$C_{50}H_{77}O_{12}N_{10}$	1009.5705	−1.1	confirmed	4.06	7.78	0.8	1.4	0.5
[Asp^3]MC-HilR	23	$C_{49}H_{75}O_{12}N_{10}$	995.5547	−1.4	tentative	3.99	7.85	0.8	1.0	0.4
MC-FR	24	$C_{52}H_{73}O_{12}N_{10}$	1029.5392	−1.1	tentative	4.13	8.35	1.9	5.1	2.0
MC-HphR	25	$C_{53}H_{75}O_{12}N_{10}$	1043.5550	−1.0	tentative	4.28	10.50	0.5	0.8	0.3
[Asp^3]MC-HphR	26	$C_{52}H_{73}O_{12}N_{10}$	1029.5389	−1.4	tentative	4.19	10.79	0.3	0.5	0.2

[a]Compounds were considered confirmed when they had the same R_t, MS, and MS^2 spectra, and thiol reactivity as authentic standards. Analogues were considered tentatively identified if the R_t, MS, and MS^2 spectra, and thiol reactivity were consistent with the proposed structure. All analogues reacted rapidly with mercaptoethanol (except for Mser^7-congeners **9**, **11**, **13**, and **14**), showed prominent loss of m/z 134.0732 in LC-HRMS methods B(i) or C(i), and LC-MS^2 spectra are presented in the Supporting Information, together with the LC-MS AIF chromatogram showing product ions at m/z 135.0804 (Adda fragmentation). [b]LC-HRMS method B [c]LC-HRMS method C(i) [d]Expressed as percent of total microcystins **5**−**26**. [e]Not detected. [f]Detected only after treatment with K[HSO_5]. [g]LC-MS^2 data is consistent with an extra oxygen atom on either amino acid 2 or, more probably, amino acid 1 (Supporting Information).

For thiol derivatization, sodium carbonate buffer (0.2 M, pH 9.7) was added to filtrate at 1:4 v/v, and to aliquots (200 μL) of the buffered filtrates was added 1 μL mercaptoethanol or *O*-(2-mercaptoethyl)-*O*′-methyl-hexa(ethylene glycol) (MEM-HEG)[16] with vortex-mixing followed by LC-MS^2 analysis (method A) 2−3 h later. Underivatized (i.e., no thiol) buffered filtrates were used as controls. Remaining culture material was extracted with HP-20 as described previously[16] and analyzed by LC-MS^2 (method A), then stored at −20 °C and analyzed periodically by LC-MS^2, and after 3 years storage by LC-HRMS (methods B−D).

Culture 2. A second culture was extracted as above, except that containers used for freeze−thawing, extraction, sample storage/analysis, and filtration were, wherever possible, flushed with argon to hinder autoxidation. The methanol−water extract was used for quantitative analysis. The eluate from the HP-20 was evaporated to dryness under a stream of nitrogen and dissolved in 1:1 MeOH−H_2O (10 mL). Aliquots of the quantitative and HP-20 extracts were analyzed immediately by LC-MS^2 (method A) and LC-HRMS (methods B−C) and the remainder stored under argon at −20 °C for further analysis (method D).

Oxidations. Aliquots of HP-20 extract (1 mL) were filtered (Spin-X, 0.22 μm, Costar, Corning Inc., NY). To each sample was added 50 μL of water (control), 30% hydrogen peroxide, or potassium peroxymonosulfate (5.1 mg/mL), and the progress of the reactions was followed by LC-MS methods A, B, or C starting from ∼1−2 min after addition of oxidant and at ∼15 min intervals thereafter.

LC-MS Analyses. *LC-MS^2 (Method A).* Liquid chromatography was performed on a Symmetry C18 column (3.5 μm, 100 × 2.1 mm; Waters, Milford, MA) as described previously,[16] eluted with a linear gradient (0.3 mL/min) of acetonitrile (A) and water (B) each containing 0.1% formic acid. The gradient was from 22−75% A over 10 min, to 95% A at 11 min (1 min hold), followed be a return to 22% A with a 3 min hold to equilibrate the column. The HPLC system was coupled to a Finnigan LTQ ion trap mass spectrometer (Finnigan Thermo Electron Corp., San Jose, CA) operated as described previously.[16]

LC-HRMS (Method B). A Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) was used as detector, with spray voltage 3.5 kV, capillary temperature 350 °C, probe heater 300 °C, S-lens RF level 50, with sheath and auxiliary gas 35 and 10, respectively. Chromatography was as for method A,

except that a Waters Acquity UPLC pump and autosampler were used. The spectrometer was operated in either: (i) full MS SIM mode scanned m/z 970−1150 with automatic gain control (AGC) target 3×10^3, resolution 70,000, and maximum injection time (max IT) 200 ms, or; (ii) all-ion-fragmentation (AIF) mode (full scan: scanned m/z 450−1150, AGC target 5×10^6, resolution 70 000, and max IT 200 ms; AIF scanned m/z 110−1500, AGC target 3×10^6, resolution 35 000, max IT 200 ms, and normalized collision energy 50).

LC-HRMS (Method C). Analysis was as for method B, except that the LC was eluted isocratically with 29% eluent A in (i) full MS SIM mode or (ii) AIF mode but with full scan m/z 840−1070.

LC-HRMS (Method D). Chromatography was performed as for method A, except that an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA) and 20 μL injections were used. The mass spectrometer was an LTQ-Orbitrap with a heated electrospray (HESI-II) probe (Thermo Scientific, Bremen, Germany). Source parameters were: spray voltage 5 kV, vaporizer temperature 350 °C, capillary temperature 300 °C, capillary voltage 45 V, tube lens voltage 120 V, sheath gas 55, and auxiliary gas 15. Fragmentation in the LTQ was performed as per method A, except that a 1000 ms maximum fill time was employed. A resolution setting of 15 000 was used for detection in the Orbitrap to maximize sensitivity.

RESULTS AND DISCUSSION

In a polyphasic approach using morphological and genetic criteria, cyanobacterial strain NIVA-CYA 656 was confirmed as *D. flos-aquae* (Supporting Information Figure S58). The filaments showed a high variability of coiling. Vegetative cells were characterized by a hemispherical to spherical form (4.8−8.2 μm), heterocysts by an ellipsoid to spherical form (6.1−8.3 μm), and akinets by a kidney-shaped form with a cell size of up to 24 μm. The morphological traits corresponded to those described for *D. flos-aquae*.[1] The morphological determination of NIVA-CYA 656 as *D. flos-aquae* was supported by the 16S rRNA gene analysis. NIVA-CYA 656 clustered with other *A. flos-aquae* strains (Supporting Information Figure S59). The *Dolichospermum/Anabaena flos-aquae* cluster is supported by a bootstrap value of 99%. The sequence similarity of NIVA-CYA 656 to *A. flos-aquae* sequences AJ630419, AJ630422, and AJ630423 was 100%.

LC-MS^2 analysis of an extract of *D. flos-aquae* (NIVA-CYA 656) revealed numerous putative microcystins. LC-MS^2 analysis after derivatization[16] with mercaptoethanol and MEMHEG identified [Asp^3]MC-LR (**18**), MC-HtyR (**19**), MC-LR (**20**), and MC-HilR (**22**) by comparison with authentic standards, and tentatively identified 20 other candidate microcystins (**1**−**5**, **7**−**9**, **11**−**17**, **21**, and **23**−**26**) based on their thiol reactivity and MS^2 spectra (Supporting Information). Among the tentatively identified analogues were the methionine-containing [Asp^3]MC-MR (**12**) and MC-MR (**17**) and their sulfoxides (**5** and **7**, respectively). No standards were available for confirmation of these analogs, and only MC-M(O)R (**7**) has been reported in the literature.[19] Several microcystins with the same nominal $[MH]^+$ m/z values as these Met-containing analogues are known,[10] so it is not possible to identify these compounds solely from their nominal masses. MS^2 spectra can be used to identify the masses of the amino acids at most positions in the macrocyclic ring system,[15] and this approach could be used to distinguish many of the candidate microcystins (e.g., [$Mser^7$]MC-LR (**14**) from MC-MR (**17**)) provided the candidate peaks do not overlap. However, not all mass spectrometers are suitable for obtaining such spectra, especially during routine analysis, and the sample must be fairly concentrated to ensure adequate signal-to-noise in the product-ion spectra. Furthermore, phenylalanine (F) is often found in microcystins and has the same nominal mass as methionine sulfoxide (M(O)). Thus, identifying the amino acid masses in the macrocycle will not necessarily afford unambiguous tentative structural assignments. The mass spectral isotopic envelope can, in principle, reveal the presence of sulfur-containing microcystins because of the increased intensities of their $[MH + 2]^+$ and $[MH + 3]^+$ peaks resulting from the presence of ^{34}S.[20] However, with an increase in the relative abundance of the $[MH + 2]^+$ peak of less than 3% compared to an MC of the same nominal mass without sulfur, as determined for **7** versus **24** by isotopic pattern simulation (Supporting Information, Figure S1), this minor difference in isotopic distribution cannot be measured reliably by conventional mass spectrometry. We therefore set out to develop simple approaches for identifying methionine-containing microcystins without high-resolution mass spectrometry (which is capable of resolving the masses of, e.g., **7** and **24**). High resolution LC-MS systems subsequently became available, and were used to confirm results of the methodologies initially developed for lower resolution instruments.

Sulfides, such as Met, are easily oxidized to sulfoxides by a range of oxidants, and more powerful oxidants can oxidize sulfides and sulfoxides to their sulfones, leading to increases in mass of either 16 or 32 Da. Among the rapid and relatively selective reagents for oxidation of sulfides to sulfoxides,[26] we investigated hydrogen peroxide because it is compatible with many of the amino acids commonly found in microcystins (although H_2O_2 slowly oxidizes tryptophan-containing MCs).[27] Sulfides and sulfoxides are oxidized to sulfones by excess potassium peroxymonosulfate,[26] and these reagents were used to develop a two-step oxidation procedure (Figure 2) for confirming the presence of M and M(O) in microcystins via LC-MS without recourse to high resolution MS.

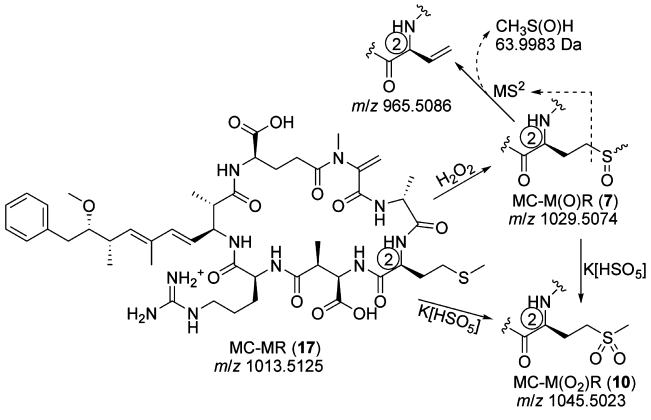


Figure 2. Stepwise oxidation of methionine-containing microcystins for LC-MS^2 analysis, as exemplified by MC-MR (**17**). Treatment with hydrogen peroxide efficiently and selectively oxidizes the sulfide group to the sulfoxide to form MC-M(O)R (**7**), which shows a characteristic loss of 64 Da ($CH_3S(O)H$) during LC-MS^2 analysis. Treatment of **7** or **17** with potassium peroxymonosulfate rapidly oxidizes the methionyl residue to the corresponding sulfone, MC-M(O_2)R (**10**). Note that sulfone **10**, as well as all the other microcystins present, also slowly degrade in the presence of the peroxymonosulfate.

Oxidation to Sulfoxides. Addition of hydrogen peroxide to extracts of NIVA-CYA 656 completely oxidized the putative Met-containing microcystins **12** and **17** (Figure 3) within 1−2 min. There was no overoxidation to sulfones, nor was there any oxidation of other amino acids in any of the microcystins, even after storage for several days. The only new products in the chromatograms possessed the same retention times, MS, and MS^2 spectra as putative sulfoxides **5** and **7** detected at low levels prior to oxidation. Sulfoxides **5** and **7** fragmented very differently to their corresponding sulfides (**12** and **17**) and most other microcystins (Figure 4 and Supporting Information), in that their MS^2 spectra consisted almost exclusively of a peak at $[MH - 63.9983]^+$. The next biggest peaks in the spectra, including the characteristic fragment at m/z 599, were less than 5% relative intensity (Figure 4B). This fragmentation is attributable to elimination of $HS(O)CH_3$ from the M(O) moiety to yield an olefin (Figure 2), and has been observed in other M(O)-containing peptides.[28] It should be noted that the $[M - HS(O)CH_3]^+$ ion was not prominent in the MS^2 spectra of **5** and **7** using LC-HRMS methods B or C because of the elevated energy of fragmentation in the HCD cell of the Q Exactive under the conditions used in the present study. However, LC-HRMS method D on an LTQ−Orbitrap offered MS^2 spectra similar to those obtained on the LTQ (method A), but with accurate masses. The measured masses of the $[M - HS(O)CH_3]^+$ ions for **5** and **7** were m/z 951.4930 and 965.5086, respectively (Supporting Information). While use of multiple LC-HRMS platforms is generally not necessary, the Q Exactive and LTQ−Orbitrap provided complementary data in this case, as the LTQ-Orbitrap was unable to detect the diagnostic Adda-cleavage fragments of the MCs at m/z 135.0804 because of the fundamental low-mass limitation of ion traps operating in MS^n-mode. By contrast, these diagnostic ions were readily detected on the Q Exactive.

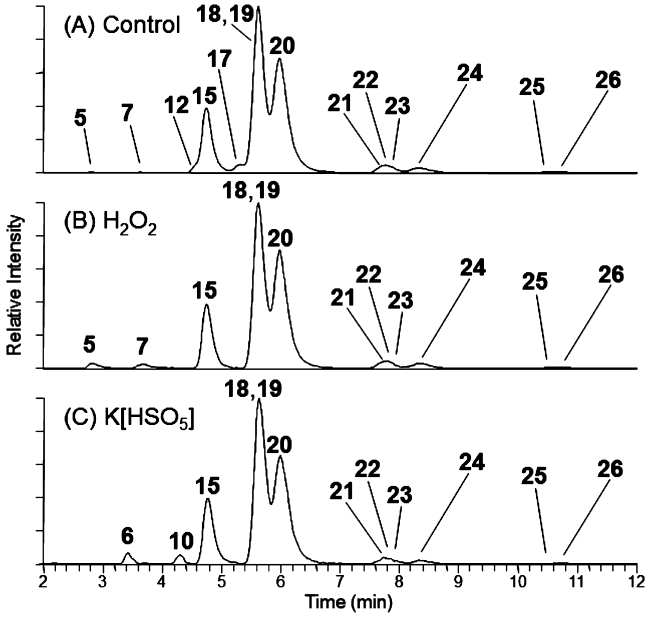


Figure 3. LC-HRMS chromatograms (method C(i)) of the preparative HP-20 extract from culture 2 (NIVA-CYA 656) before (A), and after treatment with hydrogen peroxide (B) or potassium peroxymonosulfate (C). The two oxidants converted [Asp^3]MC-MR (**12**) and MC-MR (**17**) to the corresponding sulfoxides (**5** and **7**), and sulfones (**6** and **10**), respectively, whereas other microcystin congeners (**8**, **9**, **11**, **13**−**16**, and **18**−**26**) were unaffected (except that all microcystin congeners were also slowly destroyed by peroxymonosulfate).

The product ion spectra of the $[MH - 64]^+$ ions (Figure 4C) were consistent with this, being essentially identical to the MS^2 spectrum of the original Met-containing congener, but with all Met-containing fragments occurring at lower mass by m/z 48 (i.e., addition of O to Met followed by loss of $HS(O)CH_3$). This facile elimination did not appear to significantly affect the responses of the sulfoxide and sulfide forms in LC-HRMS method C, because the measured total contributions of **5** + **12** and **7** + **17** were essentially the same in the quantitative and partially oxidized HP-20 extracts (Table 1).

Analysis by LC-HRMS was consistent with the LC-MS^2 analysis. Peaks attributable to **12** and **17** disappeared completely upon treatment with H_2O_2, being replaced by peaks with m/z values corresponding to sulfoxides **5** and **7**. Close overlap of peaks attributable to [Asp^3,$Mser^7$]MC-LR (**13**) and [Asp^3]MC-MR (**12**) (both m/z 999) was observed in the LC-MS chromatograms, even using isocratic elution, but with LC-HRMS the $[MH]^+$ ions were resolved (Supporting Information, Figure S38). Nevertheless, even with low resolution LC-MS^2 method A, the presence of these two compounds at essentially the same retention time was readily detected by their chemical reactivity; **12** reacted rapidly with thiols (at position 7) and with H_2O_2 (at position 2), whereas [Asp^3,$Mser^7$]MC-LR (**13**) was unreactive toward both reagents. Furthermore, oxidation converted **12** to sulfoxide **5**, which eluted much earlier, allowing the MS^2 spectrum of **13** to be acquired without interference from overlapping **12** (Supporting Information Figure S39). Comparison of chromatograms before and after oxidation (Figure 3) revealed diagnostic changes in peak intensities, masses, and retention times of the peaks of Met-containing microcystins which, together with thiol derivatization, clearly reveal the presence of many of the microcystins and, taken alongside the MS^2 spectra, provide strong indications as to their structural identities (Supporting Information).

Oxidation to Sulfones. Addition of potassium peroxymonosulfate to extracts of NIVA-CYA 656 (Figure 3) caused complete oxidation of the putative Met-containing microcystins **12** and **17**, as well as of sulfoxides **5** and **7** (present at low levels in HP-20 extracts and at higher levels in the stored sample) within 1−2 min. The only new products detected by LC-MS possessed masses (Table 1) and MS^2 spectra (Figure 4, and Supporting Information) consistent with sulfones **6** and **10**.

All microcystin congeners slowly degraded at approximately the same rate with peroxymonosulfate, so that their ratios remained similar over time after treatment, although after 2−3 days microcystins were usually no longer detectable by LC-MS. This overoxidation could probably be prevented by using lower concentrations of peroxymonosulfate and/or by quenching the reaction (e.g., by adding a small volume of DMSO to consume excess oxidant) shortly after adding the peroxymonosulfate. Nevertheless, provided the sample is analyzed within a short time, the results appear to be nearly quantitative and the relative intensities of the peaks approximately reflect their relative abundances in the sample.

Microcystin Profile. Assessment of the microcystin profile (Table 1) of the culture was greatly assisted by the stepwise oxidation (Figure 3, and Supporting Information) procedure, together with thiol-derivatization (Supporting Information).[16] The latter method identifies peaks containing thiol-reactive α,β-

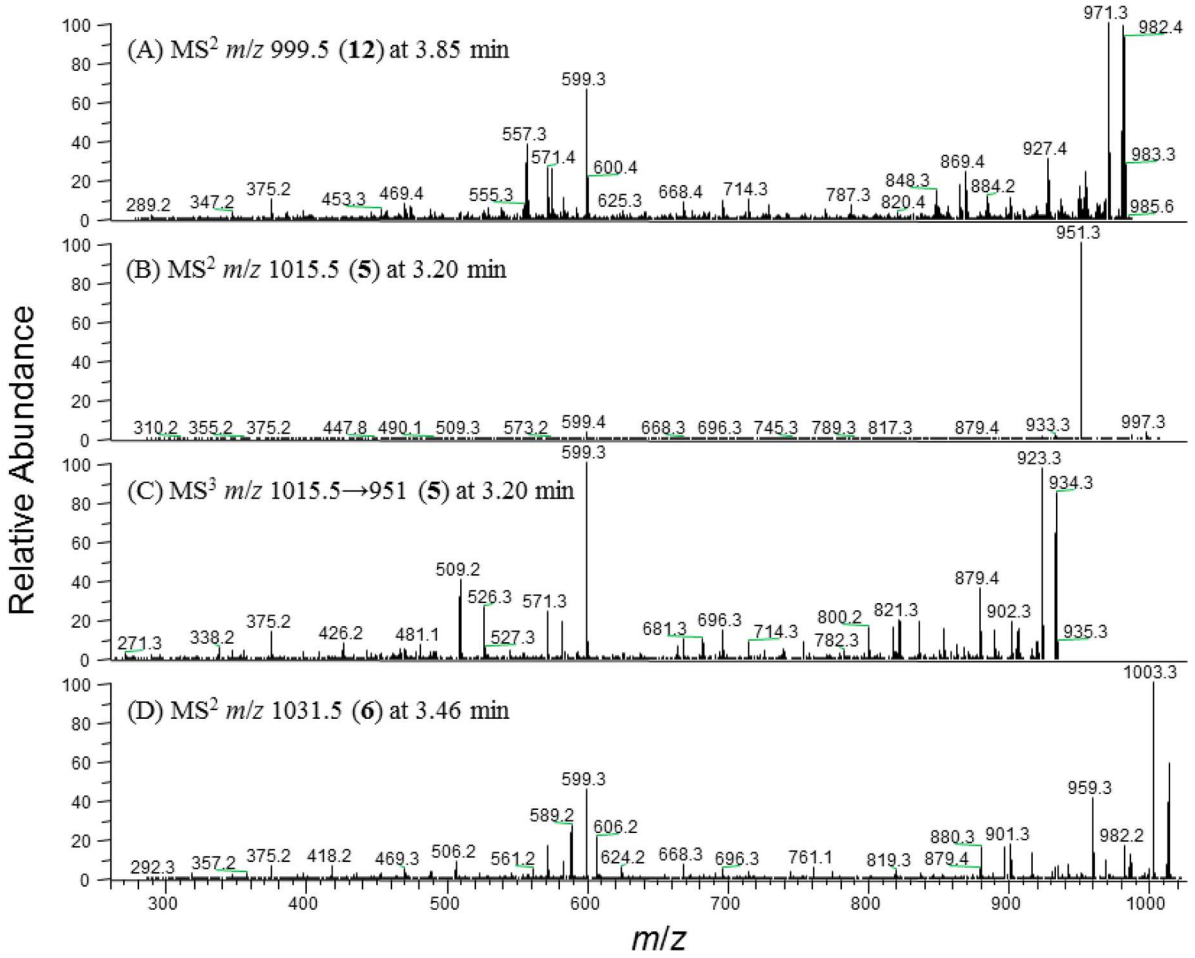


Figure 4. MS^n spectra (obtained via LC-MS^2 method A) of selected Met-containing microcystins. (A) MS^2 spectrum of [Asp^3]MC-MR (**12**) (NB partially overlapped with [Asp^3,$Mser^7$]MC-LR (**13**)); (B) MS^2 spectrum of the corresponding, sulfoxide, [Asp^3]MC-M(O)R (**5**); (C) MS^3 spectrum of **5** using the abundant $[MH - 64]^+$ product ion from MS^2; (D) MS^2 spectrum of the corresponding sulfone, [Asp^3]MC-M(O_2)R (**6**).

unsaturated moieties such as the $Mdha^7$- and Dha^7-units present in most microcystins, but no appreciable reaction occurs with Mdhb-^{7}, Dhb^7-, $Mser^7$, or Ser^7-containing microcystins.[16,18] All compounds listed in Table 1 either reacted rapidly with the thiols and showed MS^2 fragment ions (e.g., *m*/*z* 375 and 599) consistent with $Mdha^7$-microcystins, or else did not react with thiols and showed MS^2 fragment ions (e.g., *m*/*z* 393 and 599) consistent with $Mser^7$-microcystins (Supporting Information). The microcystins were present as pairs of $Masp^3$- and Asp^3-congeners (Figure 1, R^1 = Me or H, respectively) in a 3:2 ratio, with only trace amounts of the $Mser^7$-congeners of the most abundant microcystins being detectable. Identities of **18**−**20** and **22** were confirmed by comparison with authentic standards, while putative **13**−**15**, **19**, and **21**−**24** displayed the same retention times, MS and MS^2 spectra as tentatively identified specimens in extracts of cultures and algal blooms from previous studies.[12,16−18] Microcystins **15** and **18**−**20** have been reported in *Dolichospermum*/*Anabaena* spp.,[4−6,29] while the LC-MS properties of **21** would be consistent with "toxin 3" of Harada et al.,[6] and a homophenylalanine congener similar to **25** and **26** has been reported in an *Anabaena* culture.[30] Low levels (ca. 0.2% of **15**) of a putative methoxyhomotyrosine congener (**16**) were also detected, paralleling reports of putative methoxytyrosine-containing microcystins.[12,16,17]

Early eluting peaks (**1**−**4**) were observed during gradient LC-MS^2 method A, with masses corresponding to addition of water to the most abundant MCs (**15**, **18**−**20**), and which reacted rapidly with mercaptoethanol. Their MS^2 spectra (method A) showed prominent losses of 17 and 151 Da (Supporting Information), and resembled the MS/MS spectrum of [seco-4/5]MC-LR (**4**).[31] LC-HRMS method B showed that **1**−**4** gave fragment ions at *m*/*z* 135.0804 (Adda cleavage), $[MH - NH_3]^+$, and $[MH - C_9H_{13}ON]^+$ (Adda cleavage with loss of NH_3; Supporting Information) which, together with the *m*/*z* of their $[MH]^+$ ions (Table 1), was consistent with the [seco-4/5]-congeners of **15** and **18**−**20** (Table 1). [Seco-4/5]MC-LR (**4**) was first identified in a *Microcystis* bloom and considered to be a biosynthetic precursor to MC-LR (**20**),[32] but is also produced by bacterial degradation of **20**.[31] **1**−**4** eluted too early to be detected with isocratic LC-HRMS method C(i) used for quantitation (Table 1), but LC-MS^2 method A indicated levels ∼2−4% of their parent congeners (**15**, **19**, **18**, and **20**, respectively).

The Met-containing microcystins identified in NIVA-CYA 656 were relatively minor components of the microcystin profile. **12** and **17** were detected in fresh extracts of cultures 1 and 2, but HP-20 extracts contained small amounts of the corresponding sulfoxides **5** and **7**. For culture 2, this was despite flushing with argon during storage and extraction. Presumably aerial oxidation occurred during filtration and

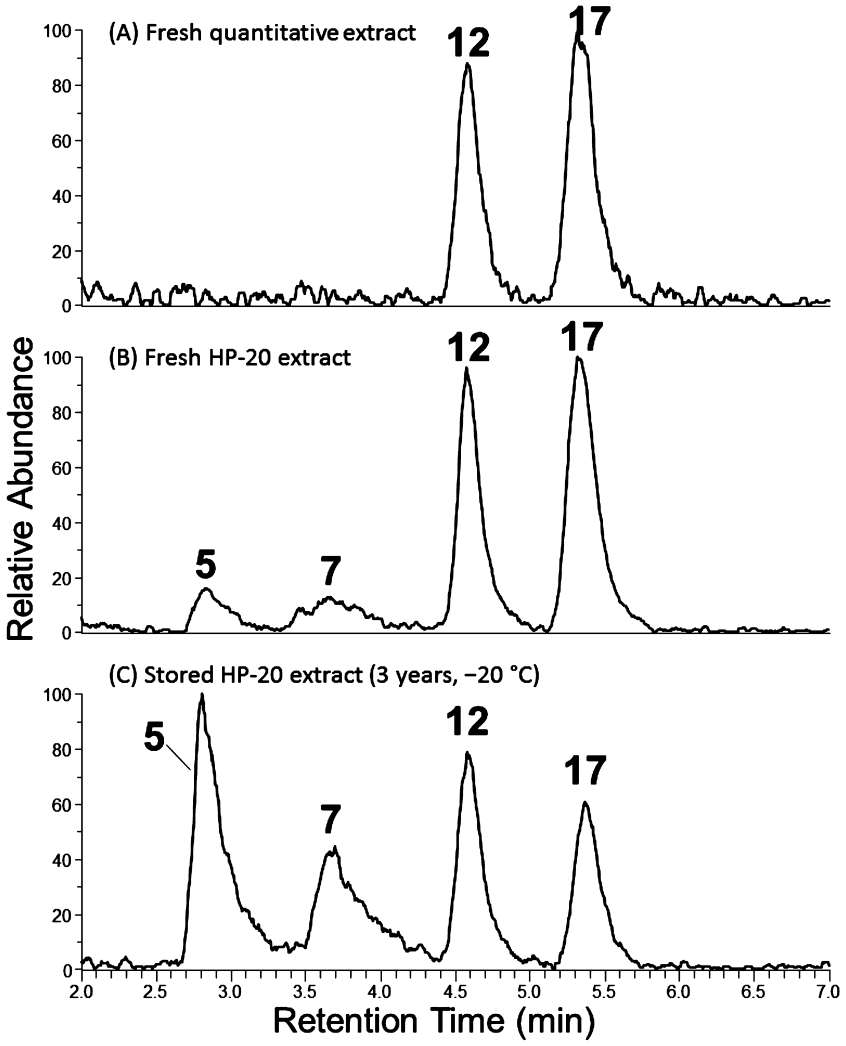


Figure 5. Effect of extraction and sample storage on the relative levels of [Asp^3]MC-M(O)R (**5**), MC-M(O)R (**7**), [Asp^3]MC-MR (**12**), and MC-MR (**17**) in an HP-20 extract of NIVA-CYA 656. The Figure shows LC-MS chromatograms (method B) extracted for the exact masses (±5 ppm) of the $[M + H]^+$ ions of **5**, **7**, **12**, and **17**. Chromatograms A and B are from culture 2, chromatogram C is from culture 1. Note the peaks for sulfoxides **5** and **7** are broader than those for the corresponding sulfides **12** and **17** (and also for sulfones **6** and **10** produced by oxidation with $K[HSO_5]$, Supporting Information), possibly due to their being diastereoisomers.

elution from the HP-20. A subsample of the HP-20 extract was evaporated to dryness and stored on the bench overnight, and another subsample was similarly held in MeOH. Neither treatment increased the proportion of sulfoxide, suggesting the toxins may be more susceptible to oxidation when bound to the adsorbent. Nevertheless, during long-term storage (1:1 MeOH−H_2O, −20 °C), sulfides **12** and **17** were slowly oxidized in solution to sulfoxides **5** and **7**, and within 3 years the sulfoxides were more abundant than the sulfides (Figure 5).

Several Met-containing microcystins have been isolated, including MC-YM, MC-LM, as well as the Met-sulfoxide-containing MC-M(O)R and MC-YM(O), raising the possibility that there might be an effect on the susceptibility of Met to aerial oxidation arising from its location in the macrocycle (position 2 vs 4) or from the presence or absence of Arg in the structure. The present study suggests that the sulfoxides are postextraction artifacts from aerial oxidation, rather than being the products of biosynthesis or intracellular oxidation.

In summary, stepwise oxidation is effective at identifying methionine-containing microcystins and their sulfoxides by LC-MS, even without high resolution MS, in the absence of authentic standards. The procedure is simple, rapid, reveals the oxidation state of the sulfur atom, and the reagents are cheap and readily available. The characteristic fragmentation pathway for the sulfoxides provides additional confirmation, as do the fragmentation patterns for the sulfide, sulfoxide elimination product ($[MH - HS(O)CH_3]^+$), and sulfone. The oxidation reactions are so rapid that the much slower oxidation of tryptophan-containing microcystins[27] should not interfere with analysis. The analytical procedure showed that for NIVA-CYA 656, M(O)-containing microcystins were artifacts produced during extraction and storage. Although other methods are available for analyzing the proportion of methionine sulfoxides in peptide samples (e.g., LC-MS analysis after oxidation with ^{18}O-labeled peroxide[33]), the two-step oxidation procedure is suitable for routine microcystin analysis. Improvements to the oxidation procedure, to prevent overoxidation during peroxymonosulfate-mediated oxidation to the sulfone, would increase the method's utility further.

■ ASSOCIATED CONTENT

Supporting Information

LC-MS^2, LC-HRMS-AIF, and LC-$HRMS^2$ chromatograms, MS and MS^2 spectra, structures of [seco-4/5]microcystins (**1**–**4**), and tabulated retention times for LC-MS methods A–D, and photomicrograph and details of morphological and phylogenetic analysis of *D. flos-aquae* NIVA-CYA 656. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +47 2321 6228. Fax: +47 2321 6201. E-mail: chris.miles@vetinst.no.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Norwegian Institute for Water Research (NIVA) for *D. flos-aquae* culture NIVA-CYA 656, and Michael A. Quilliam and Krista M. Thomas of NRC Measurement Science and Standards, Halifax, NS, Canada for reference materials. This study was supported by grant 196085/V10 (Monitoring of Cyanotoxins in Southern Africa) from The Research Council of Norway, and by the Norwegian Institute for Water Research.

■ ABBREVIATIONS

Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid
AGC	automatic gain control
AIF	all-ion fragmentation
Dha	dehydroalanine
Dhb	dehydrobutyrine
ESI	electrospray ionization
Hph	homophenylalanine
Hty	homotyrosine
Hil	homoisoleucine
Max	IT maximum injection time
MC	microcystin
MEMHEG	*O*-(2-mercaptoethyl)-*O*′-methyl-hexa(ethylene glycol)
ML	maximum likelihood
M(O)	methionine sulfoxide
$M(O)_2$	methionine sulfone
Mdha	*N*-methyldehydroalanine
Mdhb	*N*-methyldehydrobutyrine
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SIM	selected ion monitoring

■ REFERENCES

(1) Komárek, J. *Cyanoprokaryota Part 3: Heterocytous Genera*; Springer Verlag: Berlin, 2013; Vol. *19/3*, p 484 pp.

(2) Wacklin, P.; Hoffmann, L.; Komárek, J. Nomenclatural validation of the genetically revised cyanobacterial genus *Dolichospermum* (Ralfs ex Bornet et Flahault) comb, nova. *Fottea* **2009**, *9*, 59–64.

(3) Backer, L. C.; Landsberg, J. H.; Miller, M.; Keel, K.; Taylor, T. K. Canine cyanotoxin poisonings in the United States (1920s–2012): Review of suspected and confirmed cases from three data sources. *Toxins* **2013**, *5*, 1597–1628.

(4) Halinen, K.; Jokela, J.; Fewer, D. P.; Wahlsten, M.; Sivonen, K. Direct evidence for production of microcystins by *Anabaena* strains from the Baltic Sea. *Appl. Environ. Microbiol.* **2007**, *73*, 6543–6550.

(5) Sivonen, K.; Namikoshi, M.; Evans, W. R.; Carmichael, W. W.; Sun, F.; Rouhiainen, L.; Luukkainen, R.; Rinehart, K. L. Isolation and characterization of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. *Appl. Environ. Microbiol.* **1992**, *58*, 2495–2500.

(6) Harada, K.; Ogawa, K.; Kimura, Y.; Murata, H.; Suzuki, M.; Thorn, P. M.; Evans, W. R.; Carmichael, W. W. Microcystins from *Anabaena flos-aquae* NRC 525–17. *Chem. Res. Toxicol.* **1991**, *4*, 535–540.

(7) Chorus, I.; Bartram, J. *Toxic Cyanobacteria in Water. A Guide to their Public Health Consequences, Monitoring, and Management*; WHO: London, 1999; 400 pp.

(8) WHO. *Guidelines for Drinking-Water Quality*; World Health Organisation: Malta, 2011; p 541.

(9) WHO. Addendum to volume 2—Health criteria and other supporting information. *Guidelines for Drinking-Water Quality*, 2nd ed.; World Health Organization: Geneva, Switzerland, 1998; p 127.

(10) Stirling, D. J.; Miles, C. O. Marine algal toxin and cyanobacterial toxin mass lists. http://www.toxinology.no/Portals/94/media/Services/Toxin%20mass%20list_COM_v5.xls (accessed 7 Mar 2014).

(11) Neffling, M.-R. Fast LC-MS detection of cyanobacterial peptide hepatotoxins—method development for determination of total contamination levels in biological materials. PhD thesis, Åbo Akademi University, Turku, Finland, 2010.

(12) Ballot, A.; Sandvik, M.; Rundberget, T.; Botha, C. J.; Miles, C. O. Diversity of cyanobacteria and cyanotoxins in Hartbeespoort Dam, South Africa. *Mar. Freshwater Res.* **2014**, *65*, 175–189.

(13) Samdal, I. A.; Ballot, A.; Løvberg, K. E.; Miles, C. O. Multihapten approach leading to a sensitive ELISA with broad cross-reactivity to microcystins and nodularin. *Environ. Sci. Technol.* **2014**, *48*, 8035–8043.

(14) Diehnelt, C. W.; Dugan, N. R.; Peterman, S. M.; Budde, W. L. Identification of microcystin toxins from a strain of *Microcystis aeruginosa* by liquid chromatography introduction into a hybrid linear ion trap–Fourier transform ion cyclotron resonance mass spectrometer. *Anal. Chem.* **2006**, *78*, 501–512.

(15) Mayumi, T.; Kato, H.; Imanishi, S.; Kawasaki, Y.; Hasegawa, M.; Harada, K.-I. Structural characterization of microcystins by LC/MS/MS under ion trap conditions. *J. Antibiot.* **2006**, *59*, 710–719.

(16) Miles, C. O.; Sandvik, M.; Nonga, H. E.; Rundberget, T.; Wilkins, A. L.; Rise, F.; Ballot, A. Thiol derivatization for LC-MS identification of microcystins in complex matrices. *Environ. Sci. Technol.* **2012**, *46*, 8937–8944.

(17) Miles, C. O.; Sandvik, M.; Nonga, H. E.; Rundberget, T.; Wilkins, A. L.; Rise, F.; Ballot, A. Identification of microcystins in a Lake Victoria cyanobacterial bloom using LC-MS with thiol derivatization. *Toxicon* **2013**, *70*, 21–31.

(18) Miles, C. O.; Sandvik, M.; Haande, S.; Nonga, H.; Ballot, A. LC-MS analysis with thiol derivatization to differentiate [Dhb^7]- from [$Mdha^7$]-microcystins: analysis of cyanobacterial blooms, *Planktothrix* cultures and European crayfish from Lake Steinsfjorden, Norway. *Environ. Sci. Technol.* **2013**, *47*, 4080–4087.

(19) Namikoshi, M.; Rinehart, K. L.; Sakai, R.; Stotts, R. R.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W.; Evans, W. R. Identification of 12 hepatotoxins from a Homer Lake bloom of the cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. *J. Org. Chem.* **1992**, *57*, 866–872.

(20) del Campo, F. F.; Ouahid, Y. Identification of microcystins from three collection strains of *Microcystis aeruginosa*. *Environ. Pollut.* **2010**, *158*, 2906–2914.

(21) Botes, D. P.; Wessels, P. L.; Kruger, H.; Runnegar, M. T. C.; Santikarn, S.; Smith, R. J.; Barna, J. C. J.; Williams, D. H. Structural studies on cyanoginosins-LR, -YR, -YA, and -YM, peptide toxins from *Microcystis aeruginosa*. *J. Chem. Soc. Perkin Trans. 1* **1985**, 2747–2748.

(22) Craig, M.; McCready, T. L.; Luu, H. A.; Smillie, M. A.; Dubord, P.; Holmes, C. F. B. Identification and characterization of hydrophobic microcystins in Canadian freshwater cyanobacteria. *Toxicon* **1993**, *31*, 1541–1549.

(23) Ballot, A.; Fastner, J.; Wiedner, C. Paralytic shellfish poisoning toxin-producing cyanobacterium *Aphanizomenon gracile* in Northeast Germany. *Appl. Environ. Microbiol.* **2010**, *76*, 1173−1180.
(24) Kotai, J. *Instructions for Preparation of Modified Nutrient Solution Z8 for Algae*, Publication B-11/69; Norwegian Institute for Water Research: Oslo, Norway, 1972.
(25) Komárková-Legnerová, J.; Eloranta, P. Planktic blue−green algae (Cyanophyta) from central Finland (Jyväskylä region) with special reference to the genus. *Anabaena. Archiv. Hydrobiol./Algol. Stud.* **1992**, *67*, 103−133.
(26) Roy, K.-M. Sulfones and Sulfoxides. In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2000.
(27) Puddick, J.; Prinsep, M. R.; Wood, S. A.; Miles, C. O.; Rise, F.; Cary, S. C.; Hamilton, D. P.; Wilkins, A. L. Structural characterization of new microcystins containing tryptophan and oxidized tryptophan residues. *Mar. Drugs* **2013**, *11*, 3025−3045.
(28) Jiang, X.; Smith, J. B.; Abraham, E. C. Identification of a MS−MS fragment diagnostic for methionine sulfoxide. *J. Mass Spectrom.* **1996**, *31*, 1309−1310.
(29) Sivonen, K.; Skulberg, O. M.; Namikoshi, M.; Evans, W. R.; Carmichael, W. W.; Rinehart, K. L. Two methyl ester derivatives of microcystins, cyclic heptapeptide hepatotoxins, isolated from *Anabaena flos-aquae* strain CYA 83/1. *Toxicon* **1992**, *30*, 1465−1471.
(30) Namikoshi, M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Rouhiainen, L.; Luukkainen, R.; Rinehart, K. L. Structures of three new homotyrosine-containing microcystins and a new homophenylalanine variant from *Anabaena* sp. strain 66. *Chem. Res. Toxicol.* **1992**, *5*, 661−666.
(31) Yan, H.; Wang, H.; Wang, J.; Yin, C.; Ma, S.; Liu, X.; Yin, X. Cloning and expression of the first gene for biodegrading microcystin LR by *Sphingopyxis* sp. USTB-05. *J. Environ. Sci. (China)* **2012**, *24*, 1816−1822.
(32) Choi, B. W.; Namikoshi, M.; Sun, F.; Rinehart, K. L.; Carmichael, W. W.; Kaup, A. M.; Evans, W. R.; Beasley, V. R. Isolation of linear peptides related to the hepatotoxins nodularin and microcystins. *Tetrahedron Lett.* **1993**, *34*, 7881−7884.
(33) Liu, H.; Ponniah, G.; Neill, A.; Patel, R.; Andrien, B. Accurate determination of protein methionine oxidation by stable isotope labeling and LC-MS analysis. *Anal. Chem.* **2013**, *85*, 11705−11709.