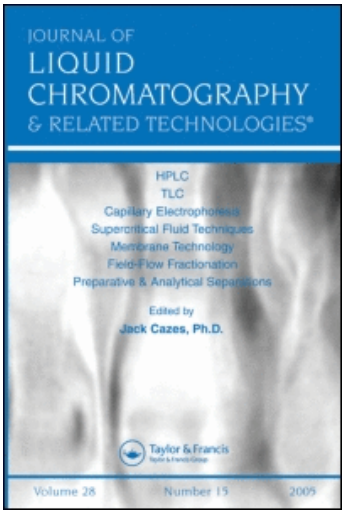

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Improving the Selectivity and Confidence in the HPLC Analysis of Microcystins in Lake Sediments

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Abstract: HPLC is a well established method for the congener selective analysis of microcystins (potent cyanobacterial hepatotoxins) in water samples or cyanobacterial cells. Its application to microcystin determination in sediments from aquatic ecosystems is still an emerging technique, often hampered by the inherent complexity of the matrix "sediment" that may lead to selectivity (and hence confidence) issues due to coeluting substances.

Using size exclusion chromatography cleanup, we could demonstrate that the confidence of microcystin analysis in sediments by HPLC with UV detection can be enhanced, yet the improvements are not always sufficient to yield unambiguous results. This drawback was avoided by employing mass spectrometric detection hyphenated to HPLC. The developed analysis protocol was applied to organic rich, silty sediments from Lake Federsee, a eutrophic lake in Southwestern Germany. Results from these experiments are presented, along with a discussion of artefacts arising from the esterification of the microcystins with methanol during sample handling.

Keywords: Electrospray ionisation mass spectrometry (ESI-MS), Eutrophic lakes, High performance liquid chromatography (HPLC), Microcystin methyl esters, Microcystins, Sediments

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INTRODUCTION

Aquatic cyanobacteria produce a wide range of bioactive compounds, amongst them different classes of highly potent toxins. Examples include neurotoxins like anatoxin-a, anatoxin-a(s), or the saxitoxins, and hepatotoxins such as the nodularins and microcystins.[1] Microcystins, named after the cyanobacteria genus *Microcystis*, are cyclic heptapeptides (Figure 1, cf.[2]).

The considerable acute toxicities of microcystins with LD_{50} values of down to approximately 50 μg kg^{-1} in the mouse,[3] along with their tumour promoting potential[4,5] have prompted extensive research in the field of their analysis. For individual congener identification and determination, high performance liquid chromatography (HPLC) certainly offers the greatest flexibility and selectivity. Often, HPLC methods for microcystin analysis use reversed phase (RP) chromatography with acetonitrile/water gradients, the mobile phase being acidified with trifluoroacetic acid (see, e.g.,[6,7]). With electrospray ionisation mass spectrometry (ESI-MS) becoming commercially available as an

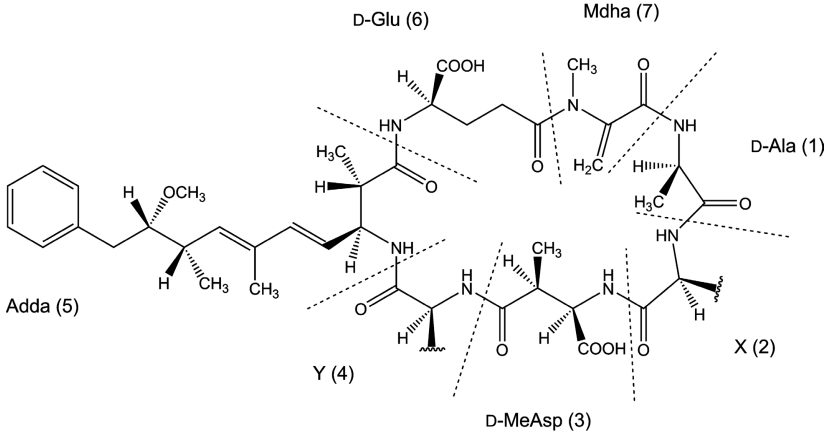

Figure 1. General structure of the microcystins. D-Ala = D-alanine, D-MeAsp = D-*erythro*-β-methyl-aspartic acid (*iso*-linked), Adda = (2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, D-Glu = D-glutamic acid (*iso*-linked), Mdha=*N*-methyldehydroalanine. X and Y are the variable L-amino acids, which are identified by their one-letter codes in the nomenclature of microcystins–microcystin-LR (abbreviated: MCYST-LR), for instance, features leucine and arginine in these positions, respectively. The numbers in parentheses are used to describe structural variations at other amino acid moieties.[2]

HPLC detector, many procedures employing this principle have been developed, e.g.[8–11]

Usually, method development has focused on the analysis of microcystins in water samples or cyanobacterial cells. Until recently, little attention has been paid to other environmental compartments such as lake sediments. Processes such as sedimentation of cyanobacteria or adsorption of dissolved microcystins can lead to the presence of the toxins in the lake bottom sediment. A method for the HPLC analysis of naturally occurring microcystins in lake sediments was first published by Tsuji et al.[12] The authors reported acceptable recoveries (between ca. 40% and 60%, depending on the microcystin congeners). However, accurate determination of the toxins was impossible due to severe coelution problems in HPLC analysis. Alternatively, Tsuji et al. presented a gas chromatographic method, based on ozonolysis of the Adda moiety and warranting excellent recoveries, but this screening method does not differentiate between congeners. Babica et al.[13] evaluated different extraction approaches and extensively investigated recoveries for different sediment types, toxin variants, and extraction solvents. They found the solvent systems recommended previously[12] to be most efficient. The recoveries reported in both papers[12,13] are quite similar and in good agreement with those found by Mohamed et al. after ELISA analysis of sediment extracts.[14] Yet, Babica and coworkers[13] also confirmed the problems caused by coeluting matrix impurities in HPLC, making identification and quantification of the toxins less reliable, especially at low concentrations. Lately, Chen and coworkers[15,16] introduced an improved extraction method based on aqueous solutions of strong chelators, which are thought to displace adsorbed toxins from metal ions at inorganic particle surfaces. Extraction efficiencies were reported to be around 90% for different sediment/soil types and toxin variants. Other recent articles deal with the determination of nodularins, pentapeptide cyanotoxins structurally related to the microcystins, in marine or prawn pond sediments.[17–20] Finally, a few more publications address microcystin analysis in sediments, but are rather oriented to biological or limnological questions and offer only restricted information on the performance of the analytical protocol itself.[18,21]

The goal of our research was to develop analytical strategies that enhance selectivity and confidence of microcystin analysis in limnic sediments with high organic carbon content. We especially investigated the effect of an orthogonal cleanup procedure prior to HPLC analysis, and the use of a mass selective detector hyphenated to HPLC. We herein report on our results obtained with sediments from a eutrophic German lake, and present some caveats that may be posed by artefacts from sample handling.

EXPERIMENTAL

Chemicals and Standards

All solvents (methanol and acetonitrile) were of HPLC gradient grade (LiChrosolv®, Merck, Darmstadt, Germany). Trifluoroacetic acid (TFA), acetic acid, and formic acid were also purchased from Merck, and were at least analytical reagent grade. Demineralised water was purified to HPLC grade quality with an Elgastat UHQ PS system (Elga, Celle, Germany). Microcystin standards were from Calbiochem (La Jolla, CA, USA). Stock solutions of the individual toxins were prepared in methanol and stored at –20°C. Their concentration was determined by UV spectrometry[22] with a fibre optics spectrometer, Model S2000 (Ocean Optics, Dunedin, FL, USA). Sample solutions for HPLC analysis were prepared by diluting aliquots of the stock solutions with appropriate solvents; typically the solvent ratio in the sample solutions was adjusted to methanol/water = 20/80 (v/v). These solutions and all sediment extracts were also stored at –20°C.

Site Description

Lake Federsee is located in the administrative district of Biberach/Riß, Baden-Württemberg. It is the largest shallow lake in Southern Germany, with a surface area of about 1.4 km^2 and a maximum depth of just a little more than 3 m.[23] It has suffered from dramatic eutrophication processes in the second half of the 20th century, especially due to the input of communal wastewater. Remedial measures have had limited success so far, because abundant phosphorus reserves in the sediments can be remobilised easily, and seem to be largely recycled annually without any great losses.[24] Cyanobacterial blooms occur frequently in this lake, and the production of microcystins by cyanobacteria in Lake Federsee has been reported previously.[25]

Sampling and Sedimentology

Undisturbed sediment cores were collected with gravity corers built in house according to Meischner and Rumohr.[26] The cores were opened and the soft, water rich sediment was divided into a surface layer (0–8 cm) and a lower layer (8–39 cm). The sediment was rather homogenous with only gradual changes in sedimentological parameters. These were determined at the Institute for Lake Research in Langenargen (Table 1). The sediment samples were frozen before further processing.

Table 1. Sedimentological parameters of the samples from Lake Federsee. Organic carbon content was determined as the difference between total carbon and inorganic carbon (total carbon content was determined by infrared detection of CO_2 after sample combustion at >1500°C, and inorganic carbon (carbonates) by dissolution in 16% hydrochloric acid). Particle sizes were determined by laser diffractometry after a hydrogen peroxide digestion. Water content was calculated from the difference between wet and dry mass

Sediment layer	Water (%)	Organic carbon (%)	Particle size distribution		
			Sand (%)	Silt (%)	Clay (%)
0–8 cm	93.6	12.9	10.1	73.3	16.6
8–39 cm	85.9	11.7	20.1	69.4	10.5

(Freeze-thawing is a procedure that enhances lysing of cyanobacterial cells possibly present in the sediment, thus releasing intracellular toxins that would otherwise not be extractable).[27]

Sample Preparation and Extraction

After thawing, the sediments were dried *in vacuo*. The dry sediments were homogenised with a mortar and pestle and aliquots of 2–5 g were then extracted according to protocols published by Tsuji et al.[12] and Babica et al.[13] Briefly, 40–100 mL of a solvent mixture (5% acetic acid in 0.1% TFA–methanol) was added to the dry sediments, and extraction was aided by bath sonication at 0°C (Model 220, Bransonic, Danbury, CT, USA).

We conducted spiking experiments with sediment samples from the 8–39 cm layer. Prior to drying, microcystin standards were added to yield concentrations of 1.5 ppm microcystin-LR, 1.1 ppm microcystin-YR and 0.8 ppm microcystin-RR (all concentrations based on sediment dry mass). These concentrations are in the same order of magnitude as those used for spiking studies in previous investigations.[12,13]

After extraction, the suspensions were centrifuged and the supernatant was evaporated to dryness by rotary vacuum evaporation (40°C). The residue was reconstituted in 1000 µL of acetonitrile/water, 50/50 (v/v), which was found to afford better SEC peak forms than methanol/water.

Cleanup of the Extracts by Size Exclusion Chromatography (SEC)

The reconstituted extracts were purified by size exclusion chromatography, following a slightly modified literature procedure.[28] Of the

solutions 250 μL were injected onto a Superdex Peptide 10/300 GL column (30 × 1 cm, Amersham Biosciences, Uppsala, Sweden), and separation was achieved under isocratic conditions with a mobile phase of acetonitrile/water, 50/50, with 0.1% (v/v) TFA added to the water. The flow rate was set to 1.0 mL min^{-1}. The chromatography system we employed consisted of a solvent degassing module (model SCM 1000, Thermo Separation Products, Riviera Beach, FL, USA), a SpectraSYSTEM P4000 quaternary gradient pump (Thermo), a 6-port motor injection valve Model H (Besta, Wilhelmsfeld, Germany), and an SPD-6A UV detector set to 238 nm (Shimadzu, Kyoto, Japan). Chromatograms were recorded with a D-2500 chromato-integrator from Merck-Hitachi (Darmstadt, Germany). A second 6-port-2-position motor valve (Besta) placed after the detector enabled us to direct the eluate either to waste or to a fraction collector. The switching times for the microcystin fraction to be collected were determined by separate injections of microcystin standard solutions. The collected fractions were each subjected to rotary evaporation and the residues were redissolved in 600 μL of methanol/water, 20/80 (v/v), prior to HPLC analysis.

HPLC/UV Apparatus and Conditions

Our HPLC system included the following components: a Degasys DG-1300 degasser (Uniflows, Tokyo, Japan), an LG-980-02 ternary gradient unit and a PU-980 intelligent HPLC pump, both from Jasco (Tokyo, Japan), an M 491 dynamic mixer (Kontron, Milan, Italy), a Rheodyne 7125 injection valve with a 20 μL loop (Cotati, CA, USA), and an SP-4 UV spectrophotometer (238 nm) from Gynkotek (Munich, Germany). The column, operated at room temperature, was from Varian-Chrompack (ChromSpher C_{18}, 5 μm, 150 mm × 4.6 mm, Middelburg, The Netherlands). Data acquisition was done with a D-2500 chromato-integrator (Merck-Hitachi). We operated the system with a linear elution gradient of water–0.1% TFA (solvent A) and acetonitrile–0.04% TFA (solvent B[29]), at a flow rate of 0.8 mL min^{-1}. The gradient parameters were as follows: t=0 min, 25% B; t = 10 min, 33% B; t = 30 min, 50% B. The system was equilibrated at starting conditions for 12 min before each chromatographic run.

HPLC/ESI-MS Apparatus and Conditions

The chromatographic setup was basically the same as for the HPLC with UV detection. However, a column thermostat (STH 585, Dionex-Softron, Germering, Germany) was added to keep the column temperature

at 35°C. Further, a column with a smaller inner diameter was used (ReproSil-Pur ODS-3, C_{18}-modified silica gel, 3 μm, 150 × 2.0 mm, from Dr. Maisch, Ammerbuch-Entringen, Germany). Hence, the flow rate of the mobile phase was reduced to 0.2 mL min^{-1}, and an injection loop of 5 μL volume was used. Additionally, it is well known that TFA as an ion-pairing additive can cause significant ion suppression in ESI-MS.[30] Therefore, TFA was replaced by formic acid. The mobile phase was formed from two solvent systems, water–0.04% formic acid/acetonitrile–0.04% formic acid, 90/10 (v/v, eluent A), and acetonitrile–0.04% formic acid (eluent B). The linear gradient parameters were $t = 0$ min, 14% B; $t = 10$ min, 30% B; $t = 30$ min, 40% B.

The mass spectrometer was a Varian 1200 L triple quadrupole instrument equipped with an electrospray ion source (Varian, Walnut Creek, CA, USA), and an LCMS12-2 nitrogen generator from Domnick Hunter (Gateshead, Tyne and Wear, England/UK). The ion source was operated in the positive mode with a needle voltage of 5000 V and a shield voltage of 600 V, the drying gas pressure was 21 psi at a temperature of 335°C, while the nebulizing gas was set to 51 psi. The detector voltage was 1500 V. Chromatograms were recorded either in the selected ion monitoring (SIM) or in the scan mode. For further confirmation of peak identities, selected samples were also subjected to tandem MS measurements. Since analyte concentrations were often too low for the acquisition of informative full scan product ion spectra, the MS^2 data were recorded in the selected reaction monitoring (SRM) mode. The collision gas was argon (6.0, Westfalen, Münster, Germany) at a pressure of approximately 1 mTorr, while the collision offset voltage was set to –20 V for all ions investigated.

RESULTS AND DISCUSSION

Sedimentology

The results in Table 1 show that the sediments from Lake Federsee are relatively fine grained, with low to moderate sand content, high silt, and low to moderate clay content. Research has shown that a high silt and, especially, clay content causes stronger adsorption of microcystins to sediments and soils, and thus more difficult desorption, whereas a high sand content would cause only weak adsorption.[31,32] Hence, moderate to strong adsorptivity for microcystins was also to be expected for the Lake Federsee sediment samples. Further, the proportion of organic carbon is quite high in these sediments. The effect of this parameter on microcystin adsorption seems to be less clear, although limited evidence exists for a positive correlation between organic carbon and sorption to

sediments of the pentapeptide hepatotoxin nodularin.[32] Very recently, binding capacities (K_f values) of sediments for microcystins were shown to correlate with organic matter, but organic content, as well as particle sizes, did not seem to correlate clearly with extraction recovery rates.[14] Irrespective of that, high organic carbon percentage could lead to further problems, such as a great number of extractable UV active substances, which may interfere with HPLC determination.

HPLC with UV Detection

First, an HPLC method for the separation of three microcystin standards (MCYST-RR, -YR and -LR) was devised (cf. Experimental section). Figure 2a shows a chromatogram of a solution of these three standards, which are all baseline resolved. This method was then applied to raw sediment extracts (without prior purification by SEC), which led to

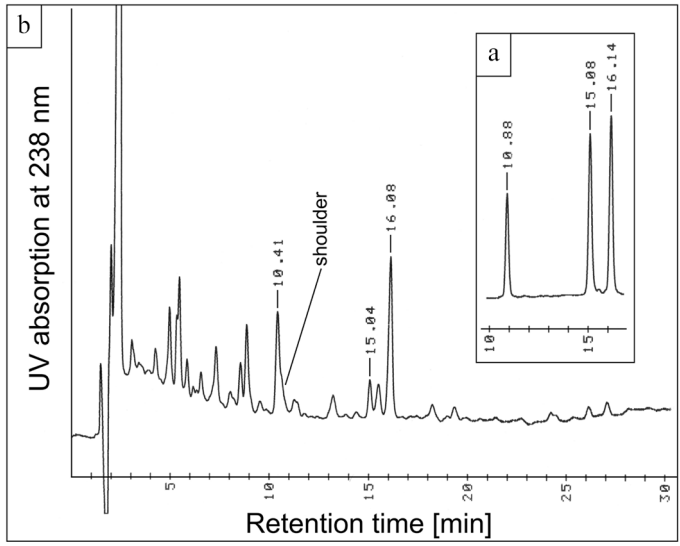

Figure 2. (a) HPLC/UV chromatogram of a solution of three microcystin standards in methanol/water, 20/80 (v/v). Conditions as described in the text. Peaks are labelled with their retention times in minutes. (b) HPLC/UV chromatogram obtained from an extract of microcystin spiked Lake Federsee sediments, after cleanup by SEC. Chromatographic conditions as for the standard solution. Peak assignments: (a) microcystin standards–MCYST-RR, $t_R = 10.88$ min; MCYST-YR, $t_R = 15.08$ min; MCYST-LR, $t_R = 16.14$ min. (b) sediment extract–MCYST-YR, $t_R = 15.04$ min; MCYST-LR, $t_R = 16.08$ min. The shoulder after the peak at $t_R = 10.41$ min is probably to be attributed to MCYST-RR, coeluting with unknown substances.

chromatograms heavily affected by matrix interferences (data not shown), confirming the results of Tsuji and coworkers.[12] We thus chose to purify the extracts before RP-HPLC analysis. C_{18} SPE is the most widely used enrichment and cleanup technique for microcystins, but while it is highly effective for concentrating microcystins from dilute samples, its cleanup efficiency is limited by the broad spectrum of the toxins' hydrophobicities.[28] On the other hand, SEC has been reported to be a straightforward and effective sample cleanup method, because the separation is based on molecular size, which is similar for all microcystins.[28] We, therefore, decided to implement an SEC step[28] prior to HPLC analysis. In our experiments, this measure permitted the generation of much cleaner RP-HPLC chromatograms, allowing the confident identification of toxins in sediment extracts in case of concentrations in the order of 1 ppm (based on sediment dry mass). Figure 2b shows a typical RP chromatogram obtained from a spiked sediment sample (see "Experimental") after SEC cleanup of the raw extract, allowing the identification of microcystin-YR and -LR. This task has been reported to be difficult by both Tsuji et al.[12] and Babica et al.,[13] who had not used any sample cleanup protocol beyond C_{18} SPE. Our RP chromatogram shows only a few peaks eluting in the same retention time window as the cyanotoxins and a nearly flat baseline in this time frame, and thus, little potential for interferences. Most of the remaining matrix components elute earlier ($t_R < 10$ min), hence not hindering toxin analysis. However, critical coelution problems remained in some cases, precluding reliable detection of the target analytes (for instance, in Figure 2b, microcystin-RR coelutes with unknown substances, only yielding a poorly separated shoulder peak).

This example demonstrates the limitations of UV detection in terms of selectivity. The SEC cleanup in our analytical protocol may add another chromatographic dimension, based on molecular size, and hence warrant some more confidence in the toxin identification, but it cannot dispel all possible doubts.

Therefore, we opted for mass spectrometry as a more selective detection principle to confirm peak identities with certainty.

HPLC with Electrospray Ionisation Mass Spectrometric Detection

Extracts from spiked sediments already characterised by HPLC/UV were investigated with mass selective detection. Two mass ranges were scanned: $m/z = 850$–1100 (singly protonated pseudo-molecular ions $[M+H]^+$ and fragment ions $[M + 2H–135]^+$), and $m/z = 480$–600 (doubly protonated pseudo-molecular ions $[M + 2H]^{2+}$ and corresponding Na or K adducts). The fragment ions $[M + 2H–135]^+$ arise from a bond cleavage on the Adda side chain due to in-source collision-induced

dissociation.[33,34] This loss of a $[PhCH_2CH(OMe)]^+$ group is typical for microcystins and, therefore, has great value for the identification of the toxins. The $[PhCH_2CH(OMe)]^+$ fragment at $m/z = 135$ can also be monitored. In our setup, the diagnostic quality of this ion was limited by the higher noise level at this m/z value, though. The total ion chromatogram (TIC) and extracted ion chromatograms (EIC) of the spiked sediment sample are shown in Figures 3a–d. For MCYST-RR, which features two basic arginine residues, the doubly protonated $[M + 2H]^{2+}$ ion is most abundant and because of this, it was selected for the EIC. MCYST-YR and -LR, carrying only one arginine residue, produce more abundant singly charged $[M+H]^+$ ions, which were chosen for the EICs. The retention times of the peaks corresponded to those of authentic microcystin standards. The mass spectra of the peaks (Figures 4a–c) were also found to be in good agreement with the spectra of toxin standards measured separately. In particular, the $[M+H]^+$ and $[M + 2H–135]^+$ ions offer good reliability for the detection of the LR and YR congeners. MCYST-RR does not produce singly charged $[M+H]^+$ and $[M + 2H–135]^+$ ions at a sufficient level and, therefore, only offers the $[M + 2H]^{2+}$ ion for identification (except if the toxin is present at a high concentration). Tandem MS experiments were carried out for further confirmation of the peak identities. For each toxin, the $[M + 2H]^{2+}$ ions were selected as precursor ions for the formation of the characteristic Adda fragment at $m/z = 135$, since the $[M + 2H]^{2+} > [PhCH_2CH(OMe)]^+$ transition is known to offer a better sensitivity than the $[M+H]^+ > [PhCH_2CH(OMe)]^+$ transition.[35] Daughter ion scans of microcystin standard solutions had previously shown that, under the conditions selected, the $m/z = 135$ ion was the base peak in the product ion spectra of all microcystin $[M + 2H]^{2+}$ ions investigated. The resulting chromatograms, corroborating our previous peak assignments, are shown in Figures 5a–c. To summarize, LC/MS(-MS) enabled us to clearly confirm the results of our preliminary HPLC/UV work, and to unequivocally identify microcystin-RR, which had not been possible with UV detection alone.

HPLC with mass selective detection in the scan mode not only offers the possibility to detect known substances for which standards are available, it also exhibits some potential to identify unknown toxins on the basis of retention characteristics combined with mass spectral information, especially the pseudo-molecular ions and typical fragmentation reactions. In order to determine whether further microcystins were present in the sample, we investigated the spectra of other peaks in the TIC of the single MS full scan chromatogram, putting particular emphasis on the presence of a $[M+H]^+$ ion and the characteristic fragment $[M + 2H–135]^+$, as well as the doubly charged $[M + 2H]^{2+}$ ions. We were able to find two peaks with such "microcystin like" mass spectral patterns, both eluting later than all three microcystin standards (RR,

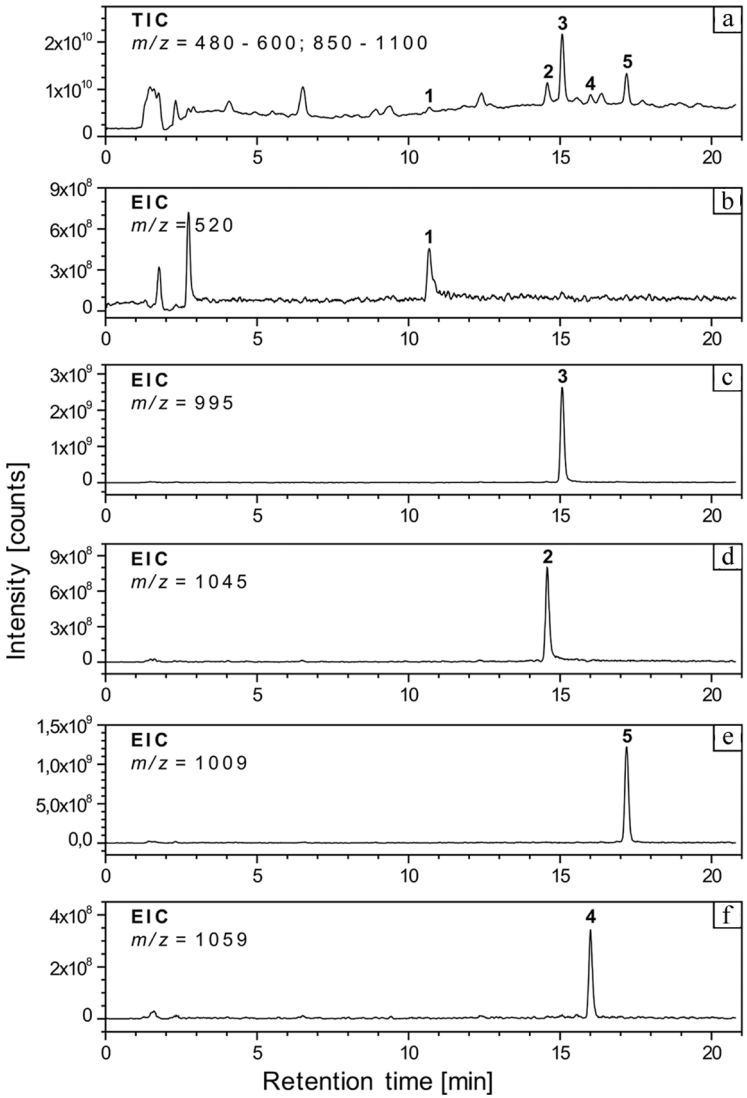

Figure 3. (a) TIC and (b–f) EICs obtained from the HPLC/MS analysis of the same sediment extract as in Figure 2. Conditions as described in the text. The most abundant ion of each microcystin was selected for the EICs: (b) MCYST-RR–$m/z = 520$ $[M + 2H]^{2+}$; (c) MCYST-LR–$m/z = 995$ $[M+H]^{+}$; (d) MCYST-YR–$m/z = 1045$ $[M+H]^{+}$; (e) MCYST-LR methyl ester–$m/z = 1009$ $[M+H]^{+}$; (f) MCYST-YR methyl ester–$m/z = 1059$ $[M+H]^{+}$. Peak assignments and retention times: 1, MCYST-RR, $t_R = 10.69$ min; 2, MCYST-YR, $t_R = 14.57$ min; 3, MCYST-LR, $t_R = 15.07$ min; 4, MCYST-YR methyl ester, $t_R = 16.01$ min; 5, MCYST-LR methyl ester, $t_R = 17.20$ min.

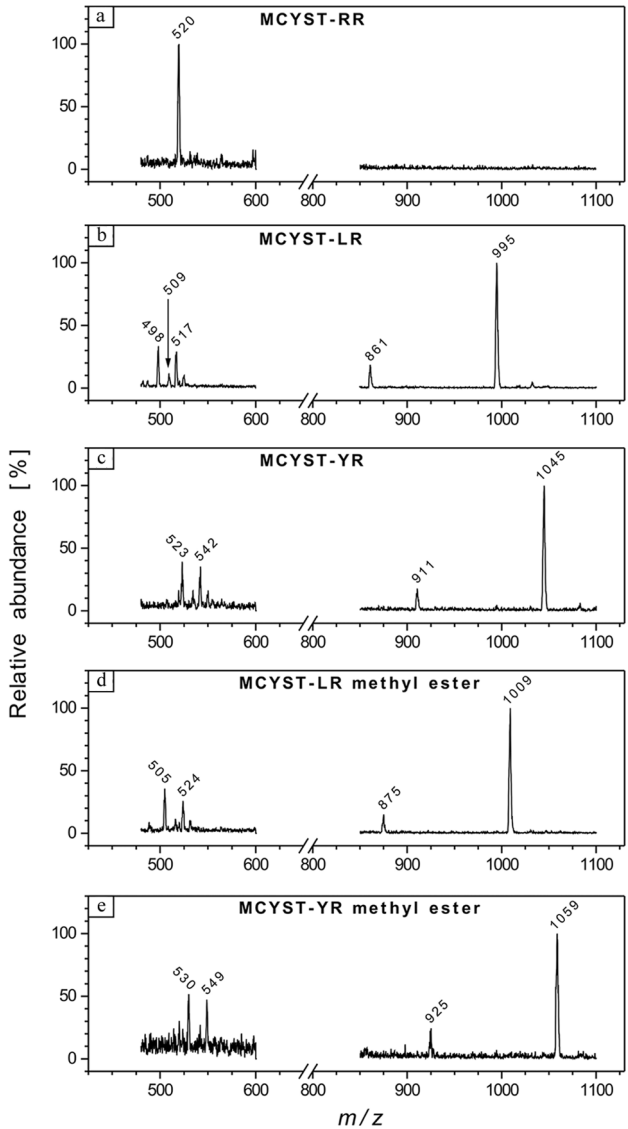

Figure 4. Mass spectra of the toxin peaks from Figure 3. Peak assignments: (a) MCYST-RR: $m/z = 520$ $[M + 2H]^{2+}$. (b) MCYST-LR: $m/z = 498$ $[M + 2H]^{2+}$; $m/z = 509$ $[M+H+Na]^{2+}$; $m/z = 517$ $[M+H+K]^{2+}$; $m/z = 861$ $[M + 2H–135]^{+}$; $m/z = 995$ $[M+H]^{+}$. (c) MCYST-YR: $m/z = 523$ $[M + 2H]^{2+}$; $m/z = 542$ $[M+H+K]^{2+}$; $m/z = 911$ $[M + 2H–135]^{+}$; $m/z = 1045$ $[M+H]^{+}$. (d) MCYST-LR methyl ester: $m/z = 505$ $[M + 2H]^{2+}$; $m/z = 524$ $[M+H+K]^{2+}$; $m/z = 875$ $[M + 2H–135]^{+}$; $m/z = 1009$ $[M+H]^{+}$. (e) MCYST-YR methyl ester: $m/z = 530$ $[M + 2H]^{2+}$; $m/z = 549$ $[M+H+K]^{2+}$; $m/z = 925$ $[M + 2H–135]^{+}$; $m/z = 1059$ $[M+H]^{+}$.

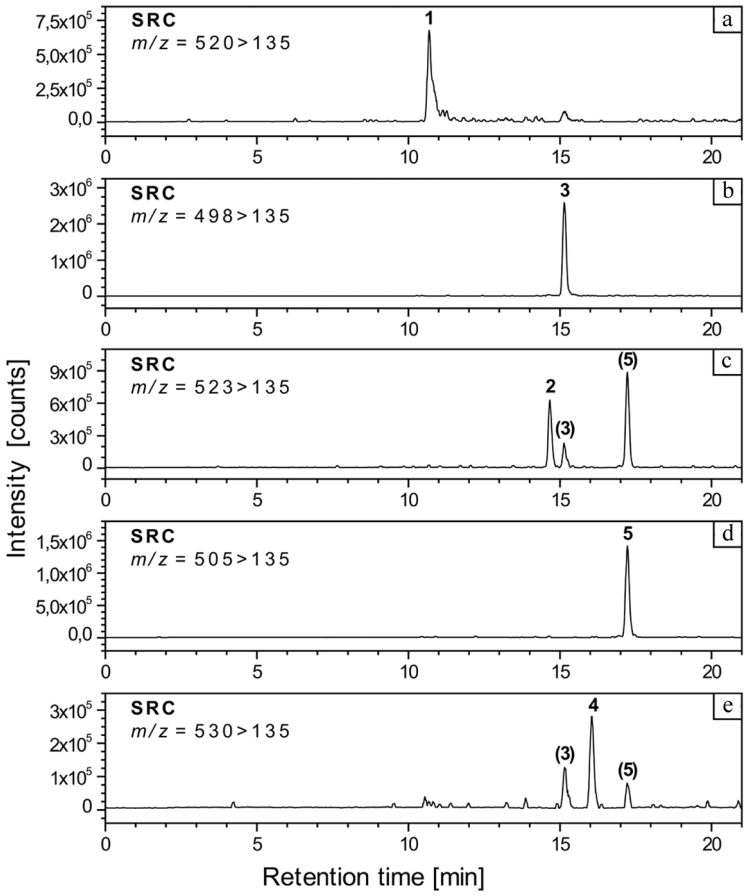

Figure 5. Selected reaction chromatograms (SRCs) obtained from the same sediment extract as in Figures 2 and 3. Conditions as described in the text. The transitions of the $[M+2H]^{2+}$ ions to the characteristic $[PhCH_2CH(OMe)]^+$ ion were recorded for each congener: (a) MCYST-RR; (b) MCYST-LR; (c) MCYST-YR; (d) MCYST-LR methyl ester; (e) MCYST-YR methyl ester. Peak assignments and retention times: 1, MCYST-RR, $t_R = 10.66$ min; 2, MCYST-YR, $t_R = 14.67$ min; 3, MCYST-LR, $t_R = 15.14$ min; 4, MCYST-YR methyl ester, $t_R = 16.04$ min; 5, MCYST-LR methyl ester, $t_R = 17.22$ min. It is assumed that the peaks marked with numbers in parentheses arise from the fragmentation of microcystin adduct ions that were transmitted by the first quadrupole to the collision cell.

YR, and LR). The EICs of the singly charged pseudo-molecular ions are shown in Figures 3e and f. The pseudo-molecular ions and typical fragment ions suggest a mass difference of 14 Da to MCYST-YR for the peak eluting at 16.01 min, and the same difference to MCYST-LR for the peak

eluting at 17.20 min (Figures 4d and e). This difference should arise from the presence of an additional methylene unit, which may indicate the substitution of a hydrogen atom in the toxin molecules by a methyl group. We conclude that these peaks are indeed to be attributed to other microcystins, an assumption supported by MS-MS results: both peaks yielded $[M+2H]^{2+}$ ions fragmenting to $m/z=135$ in an SRM experiment (Figure 5d,e). This clearly rules out the possibility that the spectral patterns observed in single MS measurements were in fact caused by coeluting substances and not by microcystins. In SIM and SRM experiments (but not in full-scan mode), we also found evidence for the presence of a microcystin that had a molecular mass 14 units higher than MCYST-RR. A peak eluting about 0.5 min after MCYST-RR could be noticed in the corresponding ion traces (SIM: $m/z=527$, $[M+2H]^{2+}$, SRM: $m/z=527>135$; chromatograms not shown).

These observations revealed an intriguing pattern: for each microcystin spiked into the sediment, another microcystin with a molecular mass difference of 14 Da had been detected, each one eluting after its "parent compound". While several microcystin congeners with appropriate masses have been described in the literature,[36] this regular pattern (which we also observed with other samples) appeared to hint at a common cause for the presence of these compounds. We think these substances were not naturally occurring microcystin congeners in the sediments, but rather derivatives of the toxins produced during storage of the sample solutions, or even earlier during extraction. Microcystins are known to form methyl esters in methanolic solutions, if acid catalysts are present.[37] As extraction was carried out with TFA and acetic acid in methanol, and the solutions were stored in methanol/water 20/80 prior to HPLC analysis (with the presence of acidic matrix components being likely), conditions for such an esterification would be fulfilled. Possible sites of methylation would be the free carboxylic acid functions in the D-glutamic acid or D-*erythro*-β-methyl-aspartic acid constituents (see Figure 1).[34,37–40] The retention times of the peaks in our chromatograms would be consistent with our tentative structural assignment, as methyl esters are expected to be more hydrophobic than their carboxylic acid analogues, hence eluting later in RP-HPLC.

Unspiked sediment samples were also analysed by LC/MS after SEC cleanup. In general, chromatograms were recorded in the SIM mode, which is more suitable than full scan mode for unspiked samples with toxins at trace levels, because it offers a better signal-to-noise ratio. It has even been shown superior to the SRM mode by some researchers.[41,42] As an example, selected ion chromatograms (SICs) of a sample from the uppermost sediment layer (0–8 cm) are shown in Figure 6. Again, peaks attributable to microcystin-RR and -LR can be seen in the corresponding ion traces

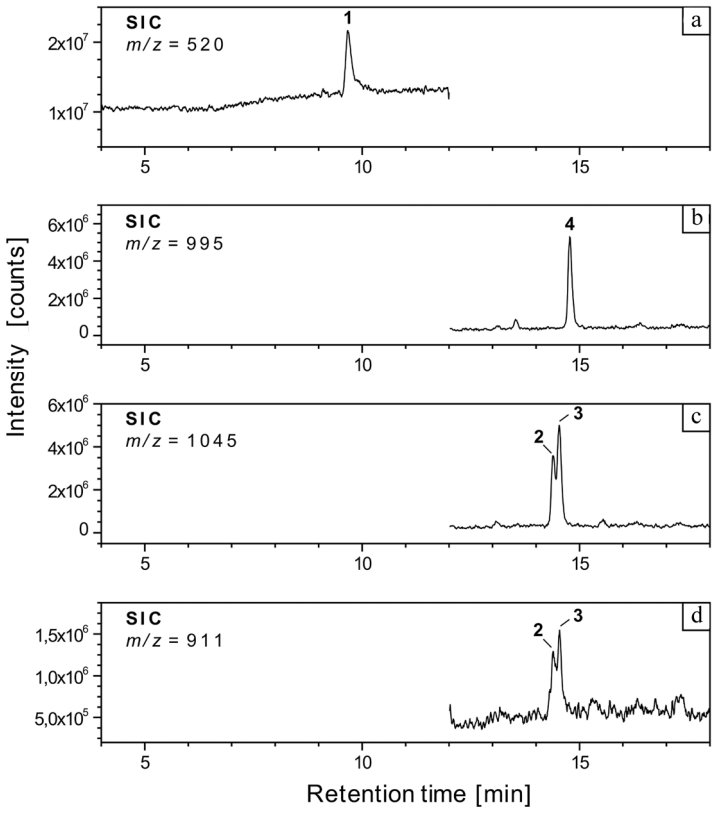

Figure 6. SICs obtained from the HPLC/MS analysis of an extract of Lake Federsee sediments (not spiked), after cleanup by SEC. The ions monitored correspond to $[M+2H]^{2+}$ for (a) MCYST-RR ($m/z=520$), and $[M+H]^{+}$ for (b) MCYST-LR ($m/z=995$) and (c) MCYST-YR ($m/z=1045$), respectively. (d) Additionally, the SIC of the MCYST-YR $[M+2H–135]^{+}$ fragment ion ($m/z=911$) is shown. Peak assignments and retention times: 1, MCYST-RR, $t_R=9.67$ min; 2, MCYST-YR, $t_R=14.39$ min; 3, MCYST-YR isomer, $t_R=14.54$ min; 4, MCYST-LR, $t_R=14.78$ min.

(Figure 6a and b). The toxin levels for each congener were in the range from 0.05 to 0.5 ppm in samples from this uppermost layer.

An interesting observation was made in the trace corresponding to the MCYST-YR $[M+H]^{+}$ ion (Figure 6c). A peak, eluting just after MCYST-YR, only partially separated and obviously representing a substance with the same molecular weight, was noticed. The $[M+H]^{+}$ ion alone is not sufficient to characterise this peak as arising from a microcystin congener. But the same elution profile could also be

observed in the SIC corresponding to the typical $[M + 2H–135]^+$ fragment ion of MCYST-YR (Figure 6d), thus strongly indicating that the unknown substance was indeed a microcystin variant with a molecular mass identical to MCYST-YR, and probably a quite similar structure, as retention properties are very similar to those of the YR congener. This putative microcystin was not detected in any samples from the lower sediment layer (8–39 cm), either spiked with toxins or unspiked (in an unspiked sample from this 8–39 cm layer, we could only clearly identify microcystin-YR and -LR at low levels (<0.05 ppm, thus with insufficient signal-to-noise ratios for quantitation, data not shown)). At this point, we cannot give any definitive structure assignment for this unknown toxin, since the amounts present in the sample were too small to yield valid MS-MS spectra. However, several microcystins with an adequate mass have been described in the literature, with homotyrosine substituted for tyrosine, and demethylation accordingly occurring at various other sites: e.g., [Dha^7]MCYST-HtyR,[43] [D-Asp^3, (*Z*)-Dhb^7]MCYST-HtyR,[44] [D-Asp^3, (*E*)-Dhb^7]MCYST-HtyR,[45] [D-Asp^3]MCYST-HtyR.[46]

CONCLUSIONS

High confidence in the analysis of microcystins in sediments was reached by combining the application of an SEC cleanup protocol, originally devised for cyanobacterial cell extracts and water samples, and the use of HPLC/MS(-MS) instead of HPLC/UV detection.

SEC on its own has the potential to significantly, though not always totally sufficiently, increase the reliability of results obtained by HPLC/UV. Mass selective detection permits the unambiguous identification of the toxins, particularly in critical cases.

The selectivity and confidence warranted by SEC cleanup and/or LC/MS analysis may prove beneficial for different applications of cyanotoxin analysis in sediments. For example, seasonal dynamics of toxin concentrations can be associated with the annual life cycle of *Microcystis* cyanobacteria and its pelagic and benthic phases, although the role of the toxins for benthic cyanobacteria remains somewhat speculative.[14,21] Also, the importance of sediments as a sink for the toxins may deserve more attention, possibly including the eventuality of microcystin remobilisation and bioaccumulation.[18,19] If sufficient persistence can be assumed, microcystins might even be of use in paleolimnology as marker substances for ancient toxigenic blooms, as suggested by Braun and Pfeiffer.[47] Furthermore, the potential of percolation through sediments to remove toxins from water has received some consideration.[48–50] Local populations in different

countries have traditionally been using such simple bankside filtration procedures since time immemorial.[51] Finally, sediment analysis might prove useful in lake monitoring, as a technique complementing water and cyanobacterial cell analysis.

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