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# Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatography–electrospray ionisation mass spectrometry

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**Abstract**

Water samples taken from 93 freshwater and brackish water locations in Åland (SW Finland) in 2001 were analysed for biomass-bound microcystins and nodularin, cyanobacterial peptide hepatotoxins, by liquid chromatography–mass spectrometry (LC–MS) in selected ion recording (SIR) and multiple reaction monitoring modes, HPLC–UV, and enzyme-linked immunosorbent assay (ELISA). The extracted toxins were separated on a short $C_{18}$ column with a gradient of acetonitrile and 0.5% formic acid, and quantified on a Micromass Quattro Micro triple-quadrupole mass spectrometer with an electrospray ion source operated in the positive SIR or scan mode. An injection of 50 pg of microcystin-LR, *m/z* 995.5, on column gave a signal-to-noise ratio of 17 (peak-to-peak) at the chosen SIR conditions. In-source or MS–MS fragmentation to *m/z* 135.1, a fragment common to most microcystins and nodularin, was used for confirmatory purposes. Microcystins with a total toxin concentration equal to or higher than 0.2 $\mu g\ l^{-1}$ were confirmed by all three methods in water samples from 14 locations. The highest toxin concentration in a water sample was 42 $\mu g\ l^{-1}$. The most common toxins found were microcystins RR, LR and YR with different degrees of demethylation (non-, mono- or didemethylated). Parallel results achieved with ELISA and HPLC–UV were generally in good agreement with the LC–MS SIR results.


*Keywords:* Water analysis; Environmental analysis; Bacteria; Hepatotoxins; Toxins; Microcystins; Nodularin

## 1. Introduction

Cyanobacteria, also known as blue-green algae, are widely distributed in fresh, brackish and marine environments, in soil and on moist surfaces. Cyanobacterial metabolites can be lethally toxic to wildlife, domestic livestock and even to humans [1]. Noteworthy is that not all cyanobacterial blooms are toxic, neither are all strains toxic within one species. Toxic and non-toxic strains show no predictable difference in appearance and, therefore, physico-chemical, biochemical and biological methods are essential for the detection of cyanobacterial toxins [2].

The most frequently reported cyanobacterial toxins are cyclic heptapeptide hepatotoxins, microcystins (>70), isolated from several species of the fresh-

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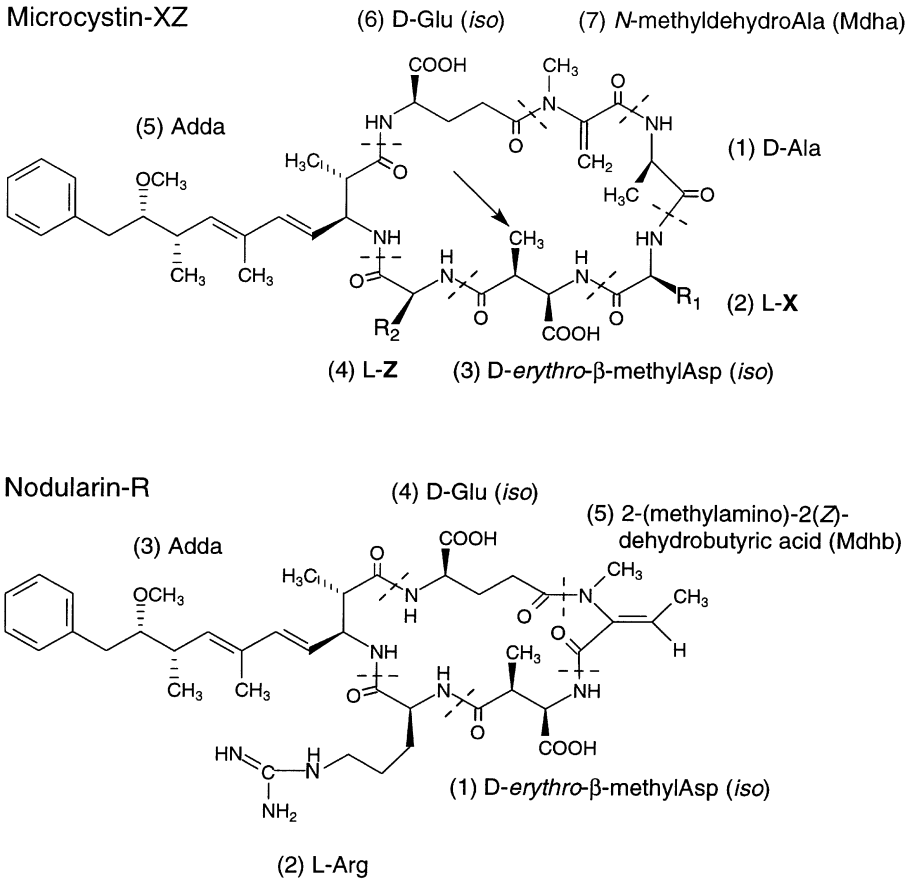


Fig. 1. Structures of microcystin-XZ (the 3-demethylation site is marked with an arrow) and nodularin-R.

water genera *Microcystis*, *Planktothrix* (*Oscillatoria*), *Anabaena* and *Nostoc*. Nodularins (<10), cyclic pentapeptide hepatotoxins, are found in the brackish water cyanobacterium *Nodularia* [3]. In addition, general cytotoxins such as cylindrospermopsin and various neurotoxins such as anatoxin-a, anatoxin-a(s) and saxitoxin analogues have been detected in cyanobacteria. The common structure of microcystins (Fig. 1) is cyclo(–D-Ala–L-*X*–D-*erythro* -β -methylAsp (iso-linkage)–L-*Z*– Adda–D- Glu(iso- linkage)– *N*-methyldehydroAla) where Adda stands for the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4 (*E*),6(*E*)-dienoic acid [4]. The main structural variation in microcystins is observed in the L-amino acid residues 2 (*X*) and 4 (*Z*), which are indicated by a two-letter suffix; for example, the common microcystin-LR contains leucine (L) in position 2 and arginine (R) in position 4. Nodularins are cyclic pentapeptides with the general structure cyclo(–D-*erythro*-β-methylAsp(iso-linkage) –L-*Z*–Adda–D-Glu(iso-linkage)–2-(methylamino)-2(*Z*)-dehydrobutyric acid) (Fig. 1). Nodularin-R (commonly known as nodularin) contains arginine in position 2.

Most studied microcystins and nodularins have been shown to be potent acute liver toxins with an $LD_{50}$ of about 50–500 μg kg $^{-1}$ (mouse, i.p.) [3,5]. The toxins can also act as tumour promoters [6–8]. The molecular basis of the tumour promotion is the inhibition of protein phosphatases 1 and 2A, two key enzymes in cellular regulation [9,10]. Nodularin has also been identified as a direct environmental carcinogen [11].

Monitoring of water bodies for toxins is often difficult since cyanobacterial blooms may contain complex mixtures of microcystins and sometimes several classes of toxins. Biological methods of toxin detection, e.g. the mouse bioassay, detect different classes of toxic compounds and also novel toxins. However, due to the low sensitivity and ethical problems associated with the mouse bioassay, alternative methods have been developed. Enzyme-linked immunosorbent assays (ELISAs) [12–14] and other immunoassays such as time-resolved fluoroimmunoassays (TR-FIA) [15] for microcystins, are very useful as a first screen of microcystins in a water sample. Usually the quantitative detection ranges of immunoassays are within microcystin concentrations in natural waters [16] and no sample concentration is necessary to reach the provisional guideline value of 1 $\mu g\ l^{-1}$ microcystin-LR in drinking water [17].

In order to elucidate the toxin profile and thus indirectly estimate the total toxicity of a complex sample, physico-chemical methods are required. These methods should be able to separate and quantify individual microcystins because microcystin variants have different toxicities. High-performance liquid chromatogrphy (HPLC) coupled with photodiode-array UV detection (DAD) [18–20] has been advocated for the identification of microcystins and nodularins based on the characteristic UV spectra with a maximum at 238 nm. Nevertheless, the use of diode-array detector has its drawbacks as it lacks specificity and the microcystin spectra are prone to interferences from the matrix and other analytes. Mass spectrometric detection offers unsurpassed specificity, especially when MS–MS techniques such as multiple reaction monitoring (MRM) are used.

The advent of atmospheric pressure ionisation (API) provided a facile method of ionising labile and/or involatile substances for mass spectrometry. Poon and coworkers pioneered the use of liquid chromatography–mass spectrometry (LC–MS) with electrospray ionisation in the quantitative analyses of microcystins, anatoxin-a and saxitoxin [21]. Edwards et al. developed an improved LC–MS method (with ionspray interface) for the identification of microcystins from cyanobacteria and water samples [22]. They also described the possibility of incorporating a scan of *m/z* 135, a fragment ion formed from the Adda-side chain, into an LC–MS–MS screening method. The same cleavage had been earlier described by Namikoshi et al. [23] and Kondo et al. [24] under fast atom bombardment (FAB) conditions. Mass spectra obtained by collisionally activated decomposition of microcystins in electrospray ionisation (ESI) MS have been later reported by e.g. Yuan et al. [25]. Bateman et al. found both LC and capillary electrophoresis (CE) combined with electrospray mass spectrometry useful in the identification and quantitation of microcystins [26].

Recent advances in instrumentation have resulted in lowered detection limits. Zweigenbaum et al. applied microbore LC coupled to an ion-trap mass spectrometer in the analysis of microcystins in environmental samples. A full-scan mass spectrum could be obtained when 250 pg of microcystin-LR was injected into the HPLC column [27]. Pietsch et al. designed an analytical procedure which combined quantification of cyanobacterial hepato- and neurotoxins in a single method based on reversed-phase LC with tandem MS detection. They reported a limit-of-detection of 27 $ng\ l^{-1}$ for microcystin-LR in water. The total detection limit for microcystin-LR was 330 fg when the signal-to-noise ratio was 3. The quantification was done in MRM mode (transition from the protonated molecule to the product ion *m/z* 135) via external calibration [28]. Lawrence et al. analysed several blue-green algal health food products for microcystins with different methods, ELISA, protein phosphatase inhibition assay, LC–MS and LC–MS–MS, at concentrations down to 0.1 $\mu g\ g^{-1}$ [29]. LC–MS–MS operated in MRM was considered to be the preferred approach to quantitation for regulatory purposes. They concluded that the results obtained by ELISA and LC–MS–MS agreed well with each other. Hummert et al. demonstrated the usefulness of LC–MS–MS in identifying unknown compounds (lacking commercial standards) as microcystins in environmental cyanobacterial samples [30]. Barco et al. used microbore columns for the analysis of hepatotoxins by LC–ESI-MS. Detection limits, calculated by using a signal-to-noise ratio of 3, were 60–340 pg in full scan and 6–72 pg in the selected ion monitoring (SIM) mode when using a 1 mm I.D. column [31].

As different analytical methods for microcystins based on different detection principles complement each other, the use of combined instrumental tech-

niques, i.e. HPLC–UV or ELISA for quantitative analyses and LC–MS(–MS) for analyte identification, has been advocated in several recent papers [32–36]. It is expected that UV techniques based on the detection of common chromophores (such as the conjugated diene in Adda absorbing at 238 nm) can perform relatively well in the preliminary quantification of unknown microcystins. In contrast, mass spectrometric quantification is prone to errors arising from variable ionisation degrees of different analytes. In some cases these errors can be eliminated by the use of (isotope-labelled) internal standards.

The study area of this paper, Åland, is situated in SW Finland in the northern Baltic Sea. On the Åland mainland, there are more than 100 lakes larger than one hectare, representing a large variety of lake types. Problems with toxic cyanobacteria have been recorded earlier in several lakes in Åland. For example, Östra Kyrksundet, the largest lake in Åland, has a history of subsurface maxima of toxic *Planktothrix* (*Oscillatoria*) *agardhii* and problems in drinking water quality [37,38]. Another lake, Lake Vargsundet, suffered from fish kills in connection with simultaneous blooms of the ichtyotoxic haptophyte *Prymnesium* and the microcystin-containing cyanobacterium *Planktothrix* [39]. Many of the lakes in Åland are currently used for drinking water abstraction or crop irrigation purposes, or for recreational use.

The aim of this work was to screen waterbodies in Åland for microcystins and nodularin in 2001 and compare LC–MS (selected ion recording; SIR), LC–MS–MS (MRM), HPLC–UV and ELISA techniques in microcystin detection and quantitation.

## 2. Experimental

### 2.1. Chemicals

Acetonitrile (HPLC S quality) and methanol (HPLC quality) were purchased from Rathburn (Walkerburn, UK). Trifluoroacetic acid (protein sequence analysis grade) was from Fluka (Buchs, Switzerland) and formic acid (analytical reagent grade) was from Riedel-de Haën (Seelze, Germany). Water was purified to 18.2 MΩ cm on a Milli-Q plus PF system from Millipore (Molsheim, France). GF/A and GF/C glass fibre filters were from Whatman (Maidstone, UK). Envirogard Microcystins Plate Kit was from Strategic Diagnostics (Newark, DE, USA).

### 2.2. Test of sonication efficiency in microcystin extraction

Two types of ultrasonic equipment were tested in sample extraction: a Branson (Danbury, CT) 2510E-MT bath sonicator and a Branson Sonifier II W-250 ultrasonic disruptor equipped with a microtip probe. The effect of bath sonication versus the combined use of bath sonication and probe sonication, on microcystin extraction, was studied with cyanobacterial material filtered on GF/A filters (diameter 25 mm). Five filters contained cells harvested from cultured *Microcystis* PCC7820 (deposited at Institut Pasteur, Paris, France) and five filters from *Anabaena* strain 90 (from culture collection of Professor Kaarina Sivonen, University of Helsinki, Finland). The filters were air-dried, freeze–thawed twice and extracted with 1 ml of 75% aqueous methanol in the bath sonicator for 15 min, after which a 200 μl aliquot was removed and centrifuged at 10 000 *g* for 10 min. The rest of the sample (800 μl) was further sonicated for 1 min with the probe sonicator (30% duty cycle, output setting 2) and centrifuged. The samples were then analysed with HPLC.

The effect of freeze-drying on microcystin extraction was also examined. A set of filters containing cyanobacterial material from *Microcystis* PCC7820 or *Anabaena* strain 90 were freeze-dried in a HETO CT60E apparatus (Birkerød, Denmark). The extraction procedure with combined bath and probe sonication was as above. The results were compared to those from a second set of freeze–thawed and sonicated filters.

The recovery of microcystins in extraction processes was studied by spiking GF/C filters (25 mm in diameter) with 1.2 ml of 75% aqueous methanolic extracts of *Anabaena* strain 90. The extracts contained approximately 1.3 μg $ml^{-1}$ of microcystin-RR and 1.0 μg $ml^{-1}$ of microcystin-LR. The samples were sonicated as earlier in the bath sonicator and thereafter with the probe sonicator. After centrifugation an aliquot of the supernatant was analysed on HPLC. Furthermore, 500 μl of the supernatant was evaporated to dryness and reconstituted in 100 μl of 75% aqueous methanol and finally analysed with HPLC.

### 2.3. Sample preparation of field samples

Samples were taken from the 0–1 m layer, filtered on GF/C filters (diameter 25 mm), air-dried and frozen. The frozen GF/C filters were freeze–thawed twice after which they were extracted with 1.2 ml of 75% aqueous methanol in the bath sonicator for 15 min (typically 15–20 tubes in the bath simultaneously). Some GF/C filters with a diameter of 47 mm were extracted with 2 ml of 75% aqueous methanol. In other aspects the procedure was the same as for the 25 mm filters. After the ultrasonic bath treatment the samples were sonicated further with the ultrasonic disruptor for 1 min. The sonifier was operated in a pulsed mode with a 30% duty cycle and with an output setting of 2. The extracts were then centrifuged at 10 000 *g* for 10 min and divided in aliquots. For ELISA: 50 μl of the extract was evaporated to dryness at 50 °C under argon, kept frozen at −20 °C until reconstitution in 300 μl of 75% aqueous methanol. Further dilutions were made with water to give a methanol percentage usually below 4% (but with some samples up to 9.5%) and these dilutions were then analysed by ELISA. For HPLC–UV and LC–MS: 500 μl of the supernatant were evaporated to dryness at 50 °C under argon. The residue was reconstituted in 100 μl of 75% aqueous methanol and an aliquot was analysed with HPLC–UV. A volume of 50 μl of the remaining extract was transferred to a new vial and evaporated to dryness under argon. The samples were kept frozen at −20 °C until reconstitution in 50 μl of 75% aqueous methanol prior to analysis by LC–MS.

### 2.4. High-performance liquid chromatography

The HPLC system consisted of a Degasys DG-2410 degasser from Uniflows (Tokyo, Japan), and an FCV-10AL gradient mixer, an LC-10AT pump and a SIL-9A autosampler from Shimadzu (Kyoto, Japan). Column temperature was regulated with a laboratory-constructed heater (based on water circulation around a dry column compartment made of aluminium). UV detection was performed with a Merck-Hitachi L-7450A photodiode-array detector (Darmstadt, Germany) operated at 238 nm or 200–300 nm. The methanolic extracts were separated on a Discovery RP-Amide $C_{16}$ HPLC column, 150 mm × 2.1 mm I.D., from Supelco (Bellefonte, PA, USA) [40]. The mobile phase consisted of a gradient of 0.05% aqueous trifluoroacetic acid (TFA; solvent A) and 0.05% TFA in acetonitrile (solvent B) with the following linear gradient programme: 20% B at 0 min, 65% B at 25 min, 65% B at 27 min, 20% B at 28 min, 20% B at 45 min. Sample volume was 10 μl, flow-rate 0.3 ml $min^{-1}$ and column temperature 40 °C. Microcystins in the samples were identified by retention time and UV spectrum comparisons with those of nodularin, microcystin-LR, -RR, -YR, -LY, -LW, -LF and the 3-demethylated variants of microcystin-LR and -RR. The absorbance data were analysed with Hitachi D-7000 HPLC System Manager (HSM) software, version 3.1.1.

### 2.5. LC–ESI-MS

The LC–MS experiments were carried out on an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) comprising of a vacuum degasser, binary pump, autosampler and thermostatted column compartment, coupled to a Micromass (Manchester, UK) Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray interface. Separation of the toxins was achieved on a Purospher STAR RP-18 endcapped (3 μm particles, 30 mm×4 mm I.D.) column from Merck (Darmstadt, Germany) at 40 °C. Injection volumes were 10 μl. The mobile phase consisted of 0.5% formic acid (solvent A) and acetonitrile (solvent B) with the following linear gradient programme: 0 min 25% B, 10 min 70% B, 11 min 70% B, 11.1 min 25% B; run interval 17 min; flow-rate was 0.5 ml $min^{-1}$.

The capillary voltage was set at 3.8 kV and the cone voltage at 80 V. The desolvation gas (nitrogen) temperature and flow-rate were set at 350 °C and 615 l/h, respectively. The ion source temperature was set at 120 °C. The instrument was operated in the positive ion mode.

#### 2.5.1. Selected ion recording

A selection of 12 *m/z* values corresponding to some important microcystins and nodularin-R were monitored by using the instrument in selective ion recording (SIR) mode (Table 1). Furthermore, the Adda-derived fragment of *m/z* 135.1 and the demethyl-Adda-derived fragment of 121.1 formed by in-source fragmentation were monitored. A dwell time of 0.1 s was employed

Table 1
Ions monitored in LC–MS with SIR mode

| Toxin or toxin fragment | *m/z* $[M+H]^+$ | *m/z* $[M+2H]^{2+}$ |
|---|---|---|
| Microcystin-LR | 995.5 | |
| Demethylmicrocystin-LR | 981.5 | |
| Didemethylmicrocystin-LR | 967.5 | |
| Microcystin-YR | 1045.5 | |
| Demethylmicrocystin-YR | 1031.5 | |
| Didemethylmicrocystin-YR | 1017.5 | |
| Nodularin | 825.5 | |
| Microcystin-RR | | 519.7 |
| Demethylmicrocystin-RR | | 512.7 |
| Didemethylmicrocystin-RR | | 505.7 |
| Adda fragment | 135.1 | |
| Demethyl-Adda fragment | 121.1 | |

for all masses except for *m/z* 135.1 for which the dwell time was 0.5 s.

In addition to the SIR channels, a scan function covering *m/z* 900–1100 was employed. Microcystin-LY, -LW and -LF were monitored as sodium adducts, *m/z* 1024.4, 1047.1 and 1008.3, respectively, with the scan function.

### 2.5.2. Multiple reaction monitoring

Following analyses in SIR mode, the same samples were also run in MRM mode utilising the specific fragmentation reaction of single or doubly protonated arginine-containing microcystins and nodularin to *m/z* 135, a fragment from Adda (Table 2). Collision energy was set to 50 eV and the argon gas cell pressure was approximately $3.4\times10^{-3}$ mbar. The transitions were monitored in two retention windows; 1 min–4.0 min and 4.01 min–6.0 min. The dwell time for each fragmentation was 0.1 s.

The chromatographic separation was again achieved on Purospher STAR with the same mobile phases as stated earlier. To reduce the total analysis time a new gradient programme was set: 0 min 25% B, 5 min 47.5% B, 5.1 min 70% B, 6 min 70% B, 6.1 min 25% B, 8.9 min 25% B; run interval 9 min; flow-rate 0.5 ml $min^{-1}$.

## 2.6. Quantification of microcystins and nodularin in chromatographic techniques

Methanolic extracts prepared from samples of *Microcystis* PCC7820 and *Anabaena* strain 90 were used as standards for qualitative and quantitative purposes. *Microcystis* PCC7820 has been previously shown to produce microcystin-LR, -LY, -LW and -LF [18] and *Anabaena* strain 90 microcystin-LR and -RR and 3-demethyl variants of microcystin-LR and -RR [41]. Our own mass spectrometric analyses and amino acid analyses have verified the identities of the main toxins. Furthermore, the *Microcystis* PCC7820 contained some 3-demethylmicrocystin-LR. The standard extracts were diluted with water to 20% aqueous methanol after which they were concentrated on Oasis HLB solid-phase extraction cartridges. The toxins were eluted in methanol and evaporated to dryness at 50 °C under argon. The residues were reconstituted in 1 ml of 75% aqueous methanol and aliquots were diluted to 1:10, 1:100 and 1:1000 with 75% aqueous methanol. *Anabaena* strain 90 extract was also spiked with a commercial standard of microcystin-YR (Calbiochem, La Jolla, CA, USA).

The concentrations of microcystins in the extracts were determined with HPLC using established techniques [20,40,42]. Microcystin-RR, -YR, -LY, -LW,

Table 2
Transitions monitored in LC–MS–MS with MRM mode

| Toxin | Retention window 1.0min–4.0min, *m/z* | Toxin | Retention window 4.01min–6.0min, *m/z* |
|---|---|---|---|
| Didemethylmicrocystin-RR | 505.7 → 135 | Didemethylmicrocystin-LR | 967.5 → 135 |
| Demethylmicrocystin-RR | 512.7 → 135 | Demethylmicrocystin-LR | 981.5 → 135 |
| Microcystin-RR | 519.7 → 135 | Microcystin-LR | 995.5 → 135 |
| Nodularin | 825.5 → 135 | Didemethylmicrocystin-YR | 1017.5 → 135 |
| Demethylmicrocystin-LR | 981.5 → 135 | Demethylmicrocystin-YR | 1031.5 → 135 |
| Microcystin-LR | 995.5 → 135 | Microcystin-YR | 1045.5 → 135 |

-LF and nodularin were quantified using the same molar response as for microcystin-LR.

The LC–MS technique for quantitation was the SIR mode. The methanolic extracts with different dilutions were analysed within the series of field samples (usually one standard after five unknowns) and standard curves were drawn for microcystins demethyl-RR, RR, YR, demethyl-LR, LR, LY, LW and LF. The concentrations of nodularin were based on the molar standard curve for microcystin-LR. As there were big variations in the MS response corresponding to the doubly charged microcystin-RR (*m/z* 519.7, Fig. 6) the concentrations of microcystin-RR and its demethylated counterparts were determined on the basis of the standard curve for *m/z* 135.1 generated in-source. The areas were then corrected with daily response factors.

The MRM mode was used only qualitatively due to three reasons: (a) the RR-type microcystins gave a very low signal in MRM with the used settings which excluded the detection of low amounts of RR-type microcystins that were dominant in some samples, (b) there was a 3-day delay before the MRM analyses and, therefore, the toxin concentrations might have changed due to solvent evaporation, adsorption etc. and (c) there is no transition which could function as a general microcystin marker (parent scan is too insensitive), cf. in-source generation of *m/z* 135.1 in the SIR mode.

### 2.7. Enzyme-linked immunosorbent assays

Samples were analysed by the Envirogard ELISA kit using the protocol from the manufacturer.

### 2.8. Chlorophyll a analyses

Additional samples were filtered on GF/C filters, frozen, homogenised, extracted with 90% acetone and analysed spectrophotometrically for chlorophyll *a* as described earlier [38].

## 3. Results and discussion

### 3.1. Microcystin extraction

Microcystin extraction was improved by a factor of 1.32–1.44 when a probe sonication step was added after the bath sonication (Table 3). Freeze-drying of the samples (Table 3) also improved the extraction in the case of the *Anabaena* sample (factor of 1.25–1.30) but the advantages were only marginal with the *Microcystis* sample (1.01–1.07). The recovery of microcystin-RR and -LR in the recovery test was practically 100% after the sonication step, and about 96% for microcystin-RR and 97% for microcystin-LR after the five-fold concentration step.

The extraction solvent used in this study, 75% aqueous methanol, has been recommended by Fastner et al. as the preferred solvent for (lyophilised) cyanobacterial samples [43]. We have good experience of 75% aqueous methanol as an effective solvent also for freeze–thawed samples. The need of probe sonication in microcystin extraction has been discussed lately by Rapala et al. [16]. Their work involved water samples with cyanobacterial cells (sample volume not reported) and they concluded that freeze–thawing complemented with bath sonication is insufficient for the effective extraction of microcystins in water

Table 3
Effects of probe sonication and free drying on toxin extraction

| | *Anabaena* | | *Microcystis* | | |
|---|---|---|---|---|---|
| | Microcystin-LR | Microcystin-RR | Microcystin-LR | Microcystin-LW | Microcystin-LF |
| Effect of probe sonication[a] | 1.44 ± 0.11 | 1.38 ± 0.11 | 1.33 ± 0.13 | 1.32 ± 0.13 | 1.32 ± 0.12 |
| Effect of freeze-drying[b] | 1.25 | 1.30 | 1.01 | 1.07 | 1.05 |

[a] The values were calculated as the following ratio: peak area after combined bath/probe sonication divided by peak area after bath sonication. The values indicate means ± SD ($n = 5$).

[b] The values were calculated as the following ratio: the sum of peak areas from five freeze-dried and sonicated samples divided by the sum of peak areas from five identical but freeze–thawed and sonicated samples. Values indicate mean ($n = 5$).

samples. Our results obtained with filtered material and 75% aqueous methanol as the extraction solvent corroborate their findings. However, we advise against prolonged probe sonication for two reasons: (a) small samples might reach high temperatures and evaporate or degrade, and (b) glass-fibre filters disintegrate and release fibres which can clog HPLC systems. A dual sonication approach (bath sonication + probe sonication) was also adopted by Hummert et al. [30].

The improved extraction after freeze-drying found in our experiments could also be an artefact due to residual moisture in the freeze–thawed filters which gives some additional volume to the extraction solvent resulting in dilution of analytes. We did not include the freeze-drying step in our field sample protocol. This was mainly due to difficulties in handling large sets of small amounts of cyanobacterial material on filters without the risk for thawing while being inserted in the freeze-drier.

Table 4
Total biomass-bound microcystin/nodularin and chlorophyll *a* concentrations (μg $l^{-1}$) in samples from Åland in 2001

| Sample number | Date | Location | HPLC–UV (μg $l^{-1}$) | LC–MS-SIR (μg $l^{-1}$) | ELISA (μg $l^{-1}$) | Main toxins identified by HPLC–UV | Main toxins identified by LC–MS-SIR | Chlorophyll *a* (μg $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | 6-Jun-01 | Högbolstad | 0.07 | 0.22 | 0.15 | RR | dmRR, RR | 18 |
| 2 | 7-Jun-01 | Godby träsk | 1.40 | 0.48 | 0.20 | LR, RR | RR, LR, dmRR | 35 |
| 3 | 7-Jun-01 | Prästträsket | 12.10 | 25.70 | 8.00 | RR, LR, dmLR | dmRR, dmLR, didmRR | 106 |
| 15 | 18-Jun-01 | Basttjärnan | 0.13 | 0.34 | 0.00 | RR, LR | LR, dmLR, dmRR | 7 |
| 17 | 19-Jun-01 | Brantsböle träsk | 0.26 | 0.47 | 0.50 | dmLR, LR* | LR, dmLR, RR | 60 |
| 26 | 25-Jun-01 | Nåtö Hemviken | 6.40 | 7.40 | 7.60 | RR, LR*, dmRR* | RR, LR, YR | 229 |
| 27 | 26-Jun-01 | Gloskärs träsk | 0.20 | 0.10 | 0.10 | YR | LR, YR, dmLR | n.a. |
| 28 | 26-Jun-01 | Gloskärs träsk | 0.25 | 0.11 | 0.10 | YR | LR, YR, dmLR | n.a. |
| 32 | 27-Jun-01 | Brantsböle träsk | 0.86 | 1.00 | 0.40 | YR, LR, dmLR | YR, LR, dmLR | 66 |
| 33 | 27-Jun-01 | Brantsböle träsk | 0.62 | 0.49 | 0.40 | YR, dmLR*, LR* | YR, LR, dmLR | n.a. |
| 34 | 27-Jun-01 | Brantsböle träsk | 0.67 | 0.74 | 0.40 | YR, LR* | YR, LR, dmLR | n.a. |
| 38 | 28-Jun-01 | Strömma träsk | 1.00 | 1.50 | 0.80 | RR, dmRR | dmRR, didmRR, RR | 20 |
| 41 | 28-Jun-01 | Kaldersfjärden | 0.70 | <0.10 | 0.10 | YR, dmRR, dmLR* | YR*, RR*, dmRR* | 169 |
| 42 | 29-Jun-01 | Prästträsket | 33.90 | 42.00 | 16.00 | RR, LR, YR | dmRR, RR, didmRR | 380 |
| 48 | 6-Jul-01 | Lemböte Byträsk | 2.30 | 3.50 | 3.00 | YR, LR, dmLR* | YR, LR, dmLR | 81 |
| 57 | 21-Jul-01 | Lemböte Byträsk | 6.00 | 8.10 | 4.00 | YR, LR, RR | LR, YR, dmLR | 23 |
| 58 | 13-Jul-01 | Vargata träsk | 5.40 | 3.90 | 6.80 | RR, LR | RR, LR, YR | 1128 |
| 60 | 17-Jul-01 | Vargata träsk | 4.60 | 4.70 | 4.80 | RR, LR | RR, LR, YR | 477 |
| 61 | 14-Jul-01 | Godby träsk | 5.10 | 3.60 | 4.80 | YR, RR, LR | RR, LR, dmRR | 110 |
| 66 | 23-Jul-01 | Norra Långsjön, Saltvik | 0.10 | 0.12 | 0.20 | LR | LR, dmLR, nodularin* | 2 |
| 73 | 24-Jul-01 | Överby insjö | 0.94 | 0.97 | 0.50 | LR | LR, dmLR* | 27 |
| 76 | 25-Jul-01 | Mösjön | 0.00 | 6.30 | 0.00 | None | dmLR, YR | 58 |
| 81 | 26-Jul-01 | Södra Slemmern | 0.24 | 0.30 | 0.20 | Nodularin | Nodularin, YR*, LR* | 10 |
| 82 | 26-Jul-01 | Högskär | 0.61 | 1.20 | 0.50 | Nodularin | Nodularin, LR*, YR* | 4 |
| 85 | 29-Jul-01 | Lillfjärden | 0.16 | 0.21 | 0.10 | LR | LR, dmLR* | 8 |
| 90 | 30-Jul-01 | Katthavet | 1.70 | 1.80 | 0.90 | LR, LY, LF | LR, LF, LY | 8 |
| 92 | 30-Jul-01 | Prästträsket | 3.10 | 3.40 | 3.00 | RR, YR, LR | RR, YR, LR | n.a. |
| 113 | 8-Aug-01 | Hägnträsk | 0.44 | <0.10 | 0.00 | dmLR, RR | YR*, dmLR* | 5 |
| 116 | 9-Aug-01 | Vargata träsk, SW | 42.30 | 38.60 | 30.00 | RR, LR, YR | RR, LR, YR | 537 |
| 117 | 9-Aug-01 | Vargata träsk, NE | 36.80 | 32.80 | 30.00 | RR, LR, YR | RR, LR, YR | 547 |
| 118 | 9-Aug-01 | Prästträsket | 7.50 | 8.50 | 9.10 | RR, LR, YR* | RR, LR, YR | 115 |
| 119 | 9-Aug-01 | Brantsböle träsk | 3.10 | 1.40 | 1.50 | dmLR, LR* | LR, YR, dmLR | 123 |
| 130 | 7-Oct-01 | Brantsböle träsk | 0.21 | 0.37 | 0.10 | LR | YR, LR, dmLR | n.a. |
| 131 | 7-Oct-01 | Strömma träsk | 0.00 | <0.10 | 0.30 | None | didmRR*, dmRR*, dmLR* | n.a. |

Water bodies with microcystin/nodularin concentrations equal to or above 0.2 μg $l^{-1}$ (by any method) have been included in the Table. Three main toxins in each sample identified by the chromatographic techniques are indicated; * denotes trace level (below 500 pg per injection in HPLC–UV and below 50 pg per injection in LC–MS). ELISA results are given in microcystin-LR equivalents. dm = demethyl, n.a. = not analysed.

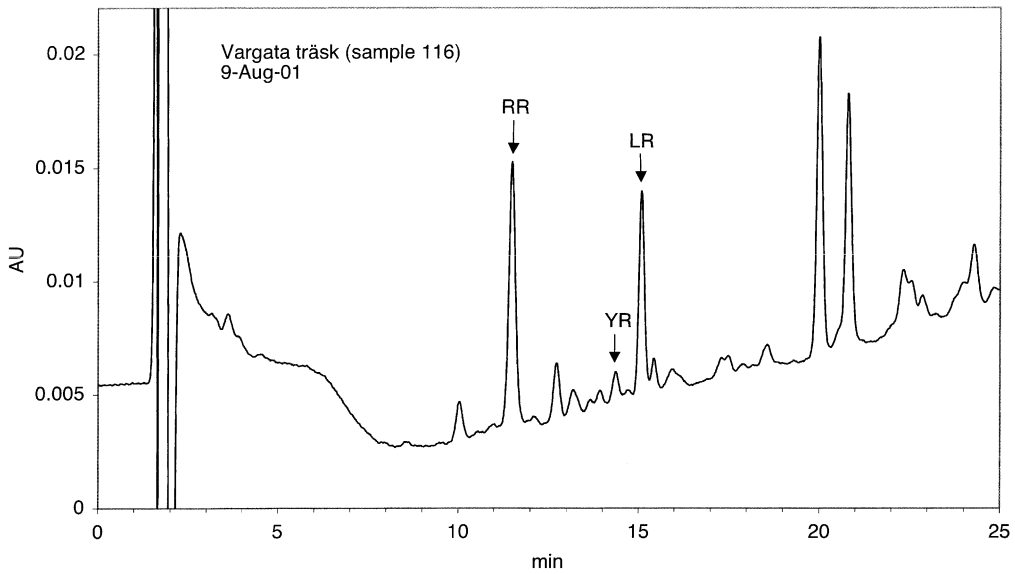


Fig. 2. HPLC–UV elution profile at 238 nm of the sample from Vargata träsk (number 116). See Section 2.4 for HPLC details.

### 3.2. Instrumental analyses

All three analytical methods tested in this study were found useful in the microcystin analyses of authentic environmental water samples. There was some variation in the results (Table 4) but generally speaking the found microcystin concentrations were in good or reasonable agreement. Linear regression analyses of the observed total toxin concentrations revealed the following correlations: 1. HPLC–UV ($x$) vs. LC–MS-SIR ($y$), $y = 1.0084x + 0.5866$, correlation coefficient $R^2 = 0.9216$; 2. HPLC–UV ($x$) vs. ELISA ($y$), $y = 0.6828x + 0.3608$, $R^2 = 0.9374$; 3. LC–MS-SIR ($x$) vs. ELISA ($y$), $y = 0.6026x + 0.4030$, $R^2 = 0.8057$. If one assumes that the ELISA technique is able to detect most microcystin variants present in the sample, neither of the chromatographic methods gave false negative results with samples exceeding the proposed guideline value of 1 μg microcystin-LR per litre. The sum of the total microcystin concentrations of the positive samples (Table 4) was 179.1 (μg $l^{-1}$) for HPLC–UV, 200.2 for LC–MS-SIR, and 134.6 for ELISA. This suggests that the ELISA method tended to give somewhat lower analytical results than the chromatographic methods. The cross-reactivity of ELISA towards different microcystin analogues is known to vary but is fairly similar towards most common microcystins. The microcystin concentrations leading to 50% inhibition in the ELISA assay as reported by the kit manufacturer are the following: microcystin-LR 0.31 μg $l^{-1}$, RR 0.32 μg $l^{-1}$, YR 0.38 μg $l^{-1}$. Corresponding data is not available for most other microcystins.

Examples of HPLC–UV elution profiles are seen in Figs. 2 and 3. The microcystin-LR content per injection was 14.5 ng (in water 13.9 μg $l^{-1}$) in the sample from Vargata träsk (sample number 116, filtered lake water volume 25 ml) and 1.3 ng (in water 0.2 μg $l^{-1}$) in a sample from Brantsböle träsk (sample number 130, filtered lake water volume 150 ml). Other major microcystins in the Vargata träsk sample were microcystin-RR (25.3 ng per injection, in water 24.3 μg $l^{-1}$) and microcystin-YR (3.9 ng per injection, in water 3.7 μg $l^{-1}$). Figs. 4 and 5 show examples of different reconstructed ion chromatograms of the same samples. For example, sample 116, Vargata träsk, exhibited the $[M+2H]^{2+}$ ion of microcystin-RR at $m/z$ 519.7, the $[M+H]^{+}$ ion of microcystin-YR at $m/z$ 1045.5, and the $[M+H]^{+}$ ion of microcystin-LR at $m/z$ 995.5.

An injection of 50 pg of microcystin-LR, $m/z$ 995.5, on column gave a signal-to-noise ratio of 17 (peak-to-

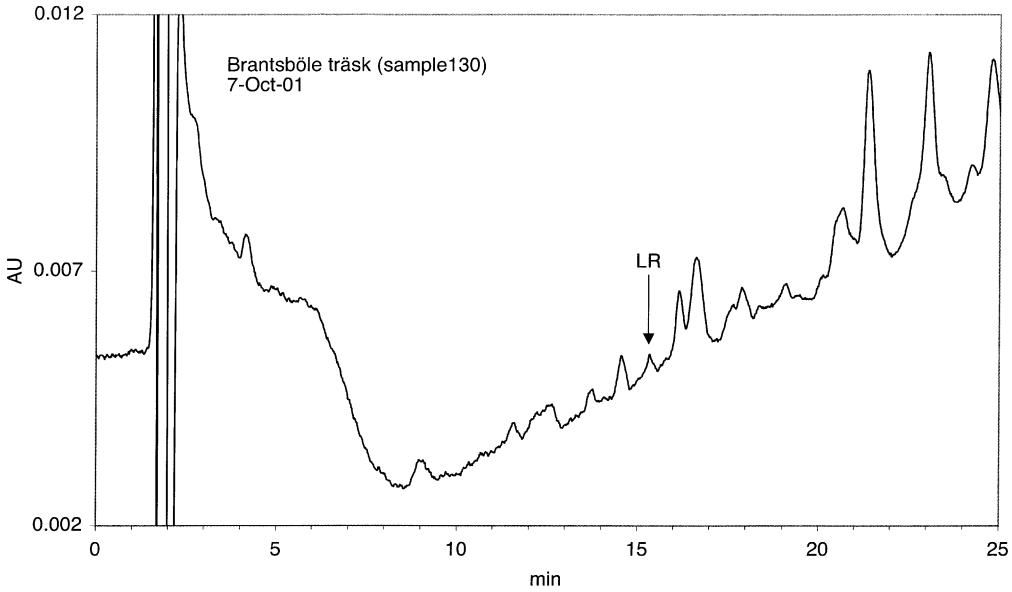


Fig. 3. HPLC–UV elution profile at 238 nm of the sample from Brantsböle träsk (number 130). See Section 2.4 for HPLC details.

peak) at the chosen LC–MS-SIR conditions. LC–MS–MS in the MRM mode was used for confirmatory purposes only and the MRM mode showed generally lower concentrations of microcystins as compared to the SIR mode. Especially the weakness in detecting microcystin-RR and its demethylated forms in MRM was apparent at lower concentrations. When the samples contained mostly microcystin-LR and/or -YR all the detection methods were generally in good agreement with each other. Furthermore, the microcystin-RR response (including the demethyl variants) on the LC–MS-SIR system was unstable, and the *m/z* 519.7 associated with the doubly protonated ion of microcystin-RR weakened as the sample analyses progressed. Fig. 6 shows how the peak areas for the *m/z* 519.7 from a standard sample were decreasing with the simultaneous increase in the area of *m/z* 135.1 during the sample series. The sudden drop in peak area at 23 h is supposed to be due to an inaccurate injection.

The response of microcystin-RR seems to be difficult to control in the mass spectrometer as the ionisation is sensitive to the ionisation environment. For example, in our case, the gradual contamination of the cone from the samples and solvents led to an obvious decrease in the abundance of *m/z* 519.7. The charge states of microcystin-RR and other microcystins have been investigated by Yuan et al. [44]. Arginine-containing microcystins are known to produce doubly charged ions $[M+2H]^{2+}$ in addition to the singly charged ions $[M+H]^{+}$. Yuan et al. studied the relative abundances of $[M+2H]^{2+}/[M+H]^{+}$of microcystins in electrospray MS and observed that the abundance ratio of these ions increased with the number of Arg residues in microcystins [44]. The absence of such basic amino acids led to the formation of $[M+Na]^{+}$ ions. Such cationised species of microcystin ions are derived from traces of alkali metal salts in the sample or the container. Since the sodium adduct ions yielded no structurally informative fragment ions via collision-induced dissociation Yuan et al. controlled the charge states of microcystins by oxalic acid in order to promote the formation of $[M+H]^{+}$ ions. They also noted that the intensity of doubly charged molecular ion species of microcystin-LR became predominant at lower sample concentrations (direct infusion of the sample in methanol–water mobile phase containing 5% acetic acid). As a general method for the electrospray-MS quantitative analysis of microcystins, Yuan et al. advocated the SIR mode [44].

The ion *m/z* 135 has been shown previously to be characteristic of most microcystins, and it corresponds

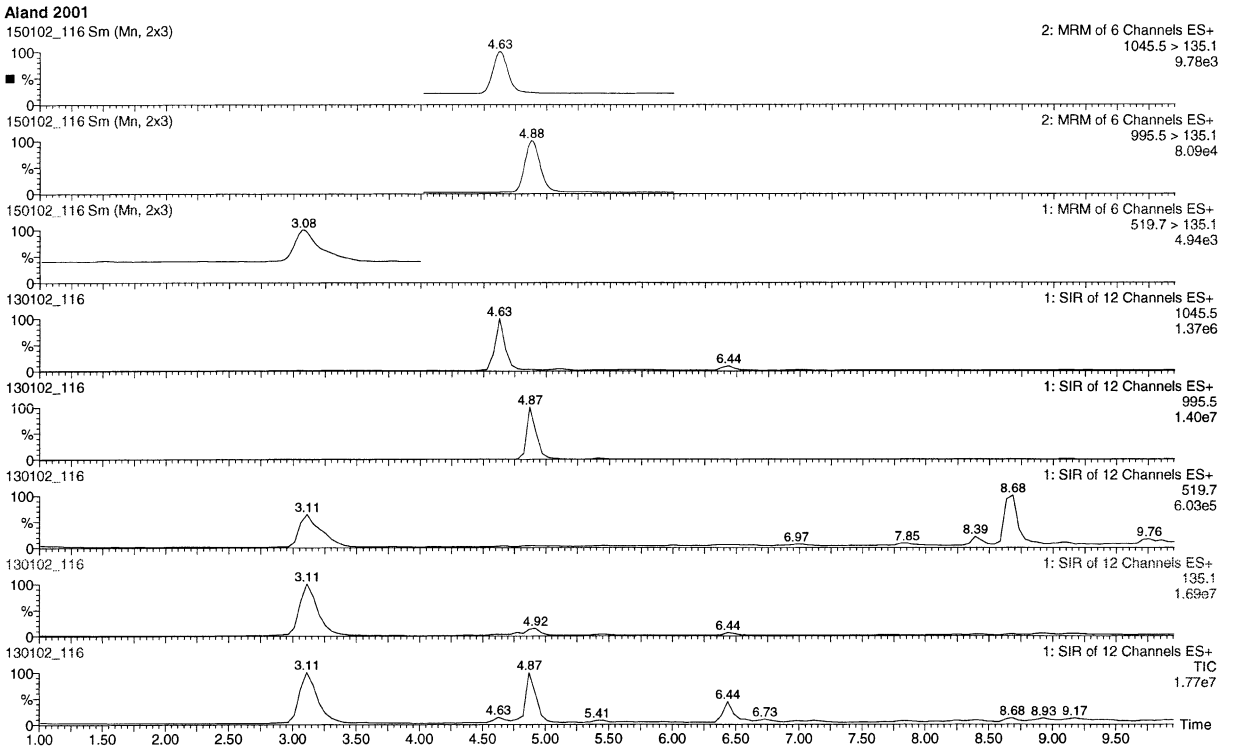


Fig. 4. Reconstructed ion chromatograms of the sample from Vargata träsk (number 116, with high toxin content). See Section 2.5 for instrumental details. MS traces from bottom to top: 1, total ion chromatogram (TIC) of the 12 SIR channels; 2, SIR *m/z* 135.1; 3, SIR *m/z* 519.7; 4, SIR *m/z* 995.5; 5, SIR *m/z* 1045.5; 6, MRM *m/z* 519.7 >135.1; 7, MRM *m/z* 995.5 >135.1; 8, MRM *m/z* 1045.5 >135.1. Time scale in min.

to the $PhCH_2CH(OCH_3)$ fragment, resulting from the α-cleavage of the methoxy group of the Adda residue [23]. The generation of the ion of *m/z* 135 is of course not unique for microcystins. In spite of this, we tried to draw some conclusions about the appearance of the in-source generated *m/z* 135.1 and its possible usefulness as a diagnostic indicator of microcystins in a sample. Fig. 7 shows the rather weak correlation between the sum of peak areas for the fragment ion *m/z* 135.1 in a retention time window (2.5–6 min) and the found total toxin concentration. We conclude that peak areas over a certain threshold value (10 000–20 000 in our case) could indicate microcystin presence in the sample. The cleavage of microcystin-RR in the source resulted in a strong ion of *m/z* 135.1 (this was observed also by Edwards et al. [22]) and, therefore, samples containing predominantly microcystin-RR were more easily detected in this experiment.

One obvious false positive result was found among the LC–MS-SIR results (Mösjön, sample number 76). “Demethylmicrocystin-LR” and “microcystin-YR” in this sample had good retention time matches with the standards and there were co-occurrences of the diagnostic ion *m/z* 135.1 with the “demethylmicrocystin-LR” and “microcystin-YR”. The LC–MS–MS MRM analysis of this sample revealed no microcystins in this sample which was consistent with the results from the HPLC–UV and ELISA analyses. We have currently no explanation for the LC–MS-SIR behaviour of the sample. At the time of the sampling there was a dense bloom of *Gloeotrichia echinulata* in Lake Mösjön. As also demonstrated by the trace of SIR *m/z* 995.5 in Fig. 5, identification of microcystins should be based on both *m/z* match and retention time match: a strong ion of *m/z* 995.5 eluting at 5.36 min must not be identified as microcystin-LR

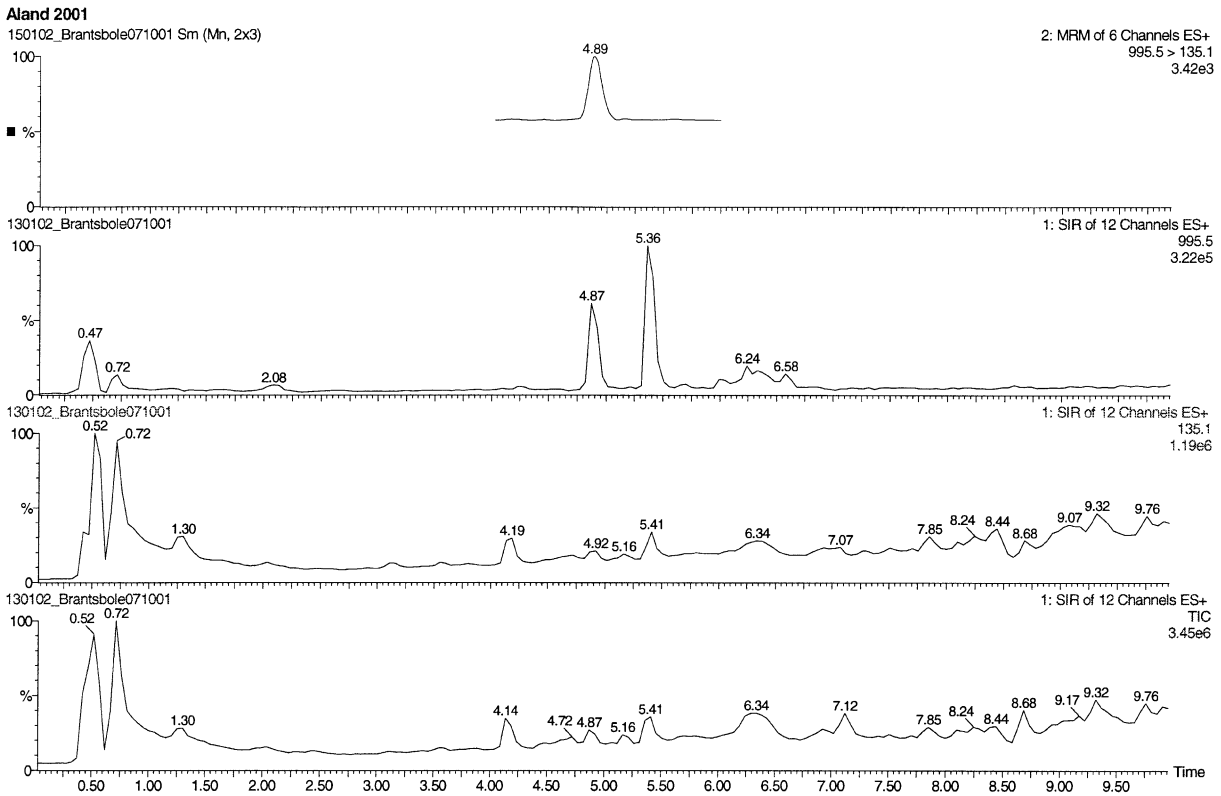


Fig. 5. Reconstructed ion chromatograms of the sample from Brantsböle träsk (number 130, with low toxin content). See Section 2.5 for instrumental details. MS traces from bottom to top: 1, total ion chromatogram (TIC) of the 12 SIR channels; 2, SIR *m/z* 135.1; 3, SIR *m/z* 995.5; 4, MRM *m/z* 995.5 >135.1. Time scale in min.

as the correct retention time of microcystin-LR is 4.87 min.

### *3.3. Microcystin content in the water samples from Åland*

Table 4 shows the total biomass-bound microcystin concentrations in the water samples analysed by HPLC–UV, LC–MS-SIR and ELISA. One hundred and thirteen samples collected from 93 different locations in Åland were analysed. Some locations were sampled only once, some several times during the summer. Microcystins with a total toxin concentration equal to or higher than 0.2 $\mu g\ l^{-1}$ were detected by at least one of the techniques in water samples from 17 locations, in 14 locations microcystins could be confirmed by all three techniques. LC–MS-SIR showed the highest toxin concentration of 42.0 $\mu g\ l^{-1}$ in the water sample collected from Lake Prästträsket (29-June-2001, sample number 42). The plankton of this lake was dominated by the cyanobacterium *Planktothrix agardhii*. The lake was resampled 1 month later showing a much lower toxin concentration. Another lake with high microcystin concentrations was Vargata träsk where up to 38.6 $\mu g\ l^{-1}$ microcystin was detected by LC–MS-SIR. This lake was dominated by *Anabaena spiroides*-like cyanobacteria in July but by *Planktothrix agardhii* in August when the highest toxin concentrations were recorded.

The most common toxins found were microcystins RR, LR and YR with different degrees of demethylation (non-, mono- or didemethylation). The only sample where more hydrophobic microcystin variants (LY, LF, traces of LW) were detected was the sample from Katthavet (30-July-2001, sample number 90), dominated by *Microcystis aeruginosa* and *Snowella*

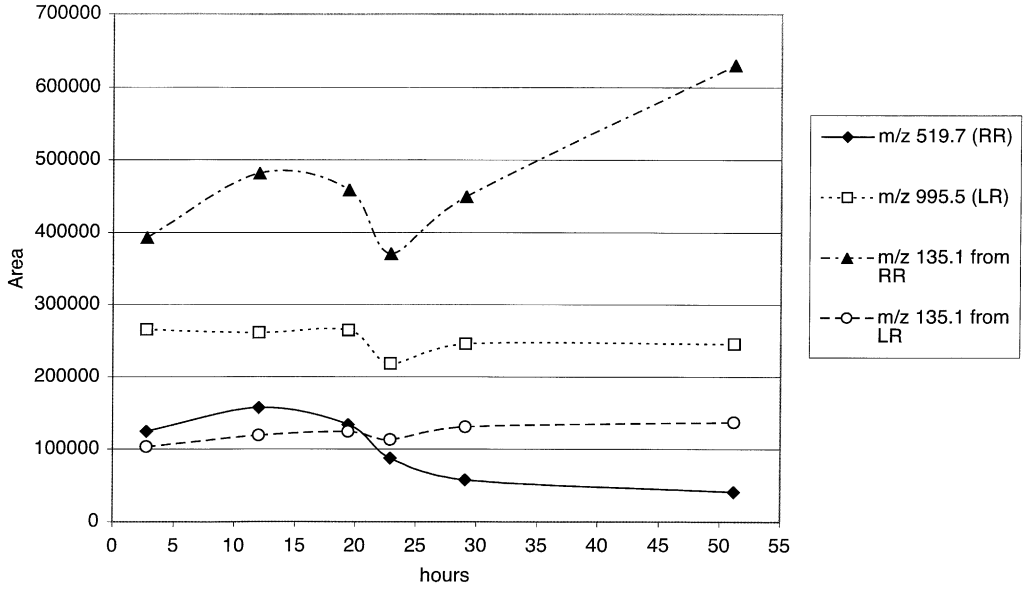


Fig. 6. Change in LC–MS-SIR peak areas of ions corresponding to microcystin-LR (*m/z* 995.5), -RR (*m/z* 519.7) and their in-source fragmentation products, *m/z* 135.1, over time.

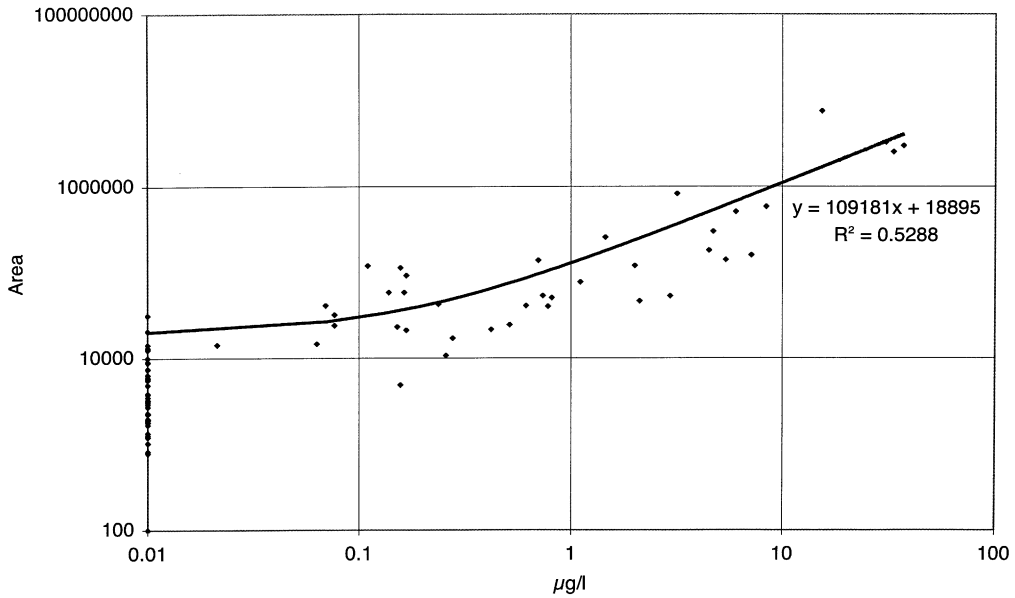


Fig. 7. Relation between the summarised area of *m/z* 135.1 ions in LC–MS-SIR traces and sample toxin concentration calculated as the average of the toxin concentrations obtained by HPLC–UV, LC–MS and ELISA. Only the *m/z* 135.1 peaks appearing in the time interval of 2.5 to 6 min were included. In order to be able to make logarithmic scales 0.01 μg $l^{-1}$ and 100 area units have been added to the *x*- and *y*-axis values, respectively.

spp. However, the concentrations of these more hydrophobic microcystins was much lower than that of microcystin-LR. The arginine-containing microcystins LR and RR, and their demethylated variants have also earlier been shown to be the most common ones found in Finland [45]. Nodularin with a concentration of 0.3–1.2 μg $l^{-1}$ (LC–MS-SIR) was detected in samples from two brackish near-shore locations in the Baltic Sea.

We also analysed the chlorophyll *a* concentration in most of the water samples and it varied in the microcystin-positive samples from a few μg $l^{-1}$ to more than 1100 μg $l^{-1}$ (Table 4). The ratio of microcystin (average of three methods) to chlorophyll *a* varied from less than 0.01 to 0.26. In a German study by Fastner et al., the microcystin-to-chlorophyll *a* ratio in the biomass mostly varied between 0.1 and 0.5, with maxima of 1–2 [46]. We have in our earlier studies concerning toxic *Planktothrix agardhii* in Lake Östra Kyrksundet in Åland [37,38] recorded microcystin-to-chlorophyll *a* ratios close to 1 but such high ratios were not found in the present study. In the German study, the biomass-bound microcystin concentration in pelagic waters was usually below 10 μg $l^{-1}$ and our results from the lakes of Åland agree with their findings. In the present study, toxins were detected in both eutrophic and fairly oligotrophic waters. High microcystin concentrations were, however, recorded only in eutrophic lakes with high chlorophyll concentrations (>20 μg $l^{-1}$).

## 4. Conclusions

The efficiency of 75% aqueous methanol as the extraction solvent for microcystins was verified in this study, and the combined use of bath and probe sonication was found necessary for adequate toxin recovery.

Parallel toxin quantitation results achieved with HPLC–UV, LC–MS-SIR and ELISA were usually in good agreement. Correlation coefficients from linear regression analyses, $R^2$, were 0.92 and 0.94 for HPLC–UV vs. LC–MS-SIR and HPLC–UV vs. ELISA results, respectively.

SIR was the preferred mode of quantitation in MS. Microcystin-RR and its demethylated analogues showed variations in their MS response.

Biomass-bound microcystins equal to or exceeding 0.2 μg $l^{-1}$ were confirmed by all three techniques in water samples from 14 out of 93 locations. The highest microcystin concentration recorded was 42.0 μg $l^{-1}$ in a lake dominated by *Planktothrix agardhii*. The most common toxins found in this study were microcystins RR, LR and YR with different degrees of demethylation.

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