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Fast, rugged and sensitive ultra high pressure liquid chromatography tandem mass spectrometry method for analysis of cyanotoxins in raw water and drinking water—First findings of anatoxins, cylindrospermopsins and microcystin variants in Swedish source waters and infiltration ponds

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ABSTRACT

Freshwater blooms of cyanobacteria (blue–green algae) in source waters are generally composed of several different strains with the capability to produce a variety of toxins. The major exposure routes for humans are direct contact with recreational waters and ingestion of drinking water not efficiently treated. The ultra high pressure liquid chromatography tandem mass spectrometry based analytical method presented here allows simultaneous analysis of 22 cyanotoxins from different toxin groups, including anatoxins, cylindrospermopsins, nodularin and microcystins in raw water and drinking water. The use of reference standards enables correct identification of toxins as well as precision of the quantification and due to matrix effects, recovery correction is required. The multi-toxin group method presented here, does not compromise sensitivity, despite the large number of analytes. The limit of quantification was set to 0.1 μg/L for 75% of the cyanotoxins in drinking water and 0.5 μg/L for all cyanotoxins in raw water, which is compliant with the WHO guidance value for microcystin-LR. The matrix effects experienced during analysis were reasonable for most analytes, considering the large volume injected into the mass spectrometer. The time of analysis, including lysing of cell bound toxins, is less than three hours. Furthermore, the method was tested in Swedish source waters and infiltration ponds resulting in evidence of presence of anatoxin, homo-anatoxin, cylindrospermopsin and several variants of microcystins for the first time in Sweden, proving its usefulness.

1. Introduction

Toxin producing cyanobacteria can be a hazard for drinking water treatment plants (WTPs) using surface waters as source water [1,2]. The World Health Organization (WHO) has established a guideline value of 1 μg/L microcystin-LR in drinking water, including both the free and cell bound forms [1]. In addition, other national authorities have implemented guidelines for anatoxin at 3 μg/L and cylindrospermopsin at 1–15 μg/L [1,3]. Potentially toxin producing cyanobacteria have been identified in 45% of the Swedish source waters [4,5] and microcystins and nodularin have been frequently detected in fresh water and brackish waters, respectively [5,6].

The major human exposure routes are considered to be direct contact with recreational waters and ingestion of drinking water, not sufficiently treated [1]. Acute poisoning of cyanotoxins may lead to gastroenteritis, liver damage, jaundice and neurotoxic effects [1]. At worst, exposure to contaminated water can cause

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severe intoxication such as in Caruaru, Brazil, where 52 patients died after receiving renal dialysis with contaminated water [7].

During drinking water treatment, the removal efficiency of cyanotoxins depends on the degree of toxins present in intracellular and extracellular forms [1,2]. Intracellular toxins are commonly removed by separation processes such as coagulation, sedimentation/flocculation and filtration [2]. Extracellular toxins are removed by adsorption on active carbon, membrane filtration, or destruction using free chlorine, ozone or potassium permanganate [2,8]. Rupture of cell membranes may cause release of cyanotoxins from cells during the drinking water treatment processing steps [9,10]. Since drinking water treatment steps are different for removing intra- and extra cellular cyanotoxins, fast analytical methods are needed that are capable of distinguishing and quantifying toxins in the both states. Furthermore, fast analysis is crucial for WTPs experiencing cyanobacterial blooms at their water intake, to prevent toxic water from reaching the consumers.

Studies of microcystins in surface waters suggest that at high concentrations, the toxin is present almost solely in intracellular form [1]. Despite this, many recently developed analytical methods fail to address the analysis of intracellular cyanotoxins in source water to WTPs, since the validated methods lack a cell lysing step [11,12]. Cells from cyanobacteria in water samples can be lysed using ultrasonication, lyophilization, freeze–thaw cycles, bead beating or by chemicals treatments such as using surfactants or commercial lysing agents [13,14].

For the first time, an in-house validated fast, rugged and sensitive ultra high pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for analysis of 22 intra- and extracellular cyanotoxins in raw water and drinking water is presented. The toxins include anatoxins, cylindrospermospins, nodularin, and microcystins. The quantification is based on pure reference standards, using external calibration and delivers results on intra- and extra-cellular basis for all 22 toxins within three hours. Direct analysis of a large volume of water after cell lysing using UPLC-MS/MS, as proposed in this method, is fast and provides the advantage that several toxin groups can be analyzed simultaneously. Compared to UPLC-MS/MS methods developed solely for microcystins using advanced sample clean-up, the proposed multi-toxin group method presented here does not compromise sensitivity [11,15]. Earlier methods that have been developed for multi-toxin group analyses did not evaluate the cell lysing step, were less sensitive and covered significantly fewer cyanotoxins [16,17]. Direct analysis of a large volume of water has also been performed previously for anatoxin-a and cylindrospermopsin but not in the context of multi-toxin analysis [18,19]. The validation study, presented here covers all steps of the analysis. The functionality of the method is proven by the identification of anatoxins, cylindrospermopsins, nodularin and variants of microcystin for the first time in Swedish source waters and infiltration ponds.

2. Material and methods

2.1. Chemicals and consumables

Methanol (LiChrosolve) and formic acid (98%) was purchased from Merck (Darmstadt, Germany), and LC–MS grade acetonitrile from Fisher Scientific (Loughborough, United Kingdom). LC–MS grade water was produced by a Milli-q purification system from Millipore (Billerica, MA, USA). Discardit II 10 mL polypropylene syringes were ordered from Beckton, Dickinson and Company (Franklin Lakes, NJ, USA) and Ø 13 mm PVDF 0.20 μm filters from Whatman (Maidstone, United Kingdom).

2.2. Reference standards

The cyanotoxins included in this method are listed in Table 1. Standards of anatoxins, cylindrospermopsins, nodularin, and microcystins were ordered from several sources as described in supplementary material (Table S1). When possible, standards were purchased as solution (concentrations ranging from 2.0 to 12 μg/mL). However, some of the substances were only available as solid standards. Stock solutions of 5000 μg/L in methanol were therefore prepared in-house. All stock solutions were stored in darkness at −20 °C. Three separate mixed standard solutions were prepared, and the cyanotoxins were divided between the solutions as described in supplementary material (Table S1). The three mixed standard solutions had a concentration of 625 μg/L, and were prepared in methanol (MeOH). Next, the three mixed standard solutions were pooled and diluted to 62,5 μg/L using Milli-q water/MeOH 97/3 (v/v). The calibration standards were prepared from this mix and the calibration points were 0.1, 0.5, 1.0, 5.0 and 10.0 μg/L. All calibration standards were diluted in 97/3 (v/v) Milli-q/MeOH.

2.3. UPLC-MS/MS method

Chromatography was performed on an ACQUITY UPLC system (Waters, Manchester, United Kingdom). Separation was achieved with an ACQUITY BEH C_{18} UPLC column, 2.1 × 100 mm fitted with a VanGuard ACQUITY BEH C_{18} UPLC pre-column, 2.1 × 5 mm, both having a particle size of 1.7 μm (Waters, Manchester, United Kingdom). The columns were kept at 35 °C during analysis, and injection volume was 100 μL. Mobile phase A was 0.1% formic acid (FA) in Milli-q and mobile phase B 0.1% FA in acetonitrile (ACN). The gradient elution was performed as follows: 0–0.7 min, 2% B, flow 0.3 mL/min; 0.80 min, 2% B, starting from here, flow increased to 0.45 mL/min; 9.0 min, 70% B; 9.1 min, 90% B; 10.0 min, 90% B; 10.1 min, 2% B; 12.0 min, 2% B.

Quantification of cyanotoxins was performed in dynamic Multiple Reaction Monitoring (MRM) mode using a triple quadrupole mass spectrometer (MS/MS), Xevo TQ-S from Waters (Manchester, United Kingdom). The mass spectrometer was used in positive electrospray mode (ES +), with a capillary voltage of 3.0 kV. The source offset was 50 V and the source temperature 150 °C. Nitrogen was generated from pressurized air, and used as desolvation and cone gas at flows of 650 and 150 L/Hr, respectively. The desolvation gas temperature was 350 °C. The nebulizing gas was also N_2 at a pressure of 7.0 bars. Argon (Alphagas™, Malmö, Sweden) was used as collision gas at a flow of 0.15 mL/min. The compound specific mass spectrometric parameters such as cone voltage (CV), collision energy (CE) and mass transitions are presented in Table 1. The mass spectrometric parameters of individual cyanotoxins were optimized using 1 μg/mL solutions in methanol with a constant flow of mobile phase. The individual solutions of microcystin-[D-Asp3]-LR, microcystin-[D-Asp3]-RR, microcystin-[D-Asp3, (*E*)-Dhb7]-77 and microcystin-[Dha7]-RR at the concentration 1 μg/mL was also utilized to perform product ion scans at 10 eV, 30 eV, 60 eV and 90 eV in an attempt to find unique fragments for quantification of the co-eluting isomers. The scan range was *m*/*z* 70–*m*/*z* 1250.

2.4. Identification and quantification

Criteria for positive identification and quantification were set in accordance with guidance for analytical methods used in food control [20]. Compounds were considered positively identified when the relative intensities of the quantification product ion in relation to the qualifier product ion, expressed as a ratio, corresponded to those of the calibration standard. For positive identification, the ion ratios differed no more than ±30% between specific

Table 1
Mass spectrometric parameters (including mass transitions, cone voltage and collision energy), retention time, molecular formula and monoisotopic mass.

Compound name	Mass transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)	Mono-isotopic mass (*m*/*z*)
Anatoxin-A	166 > 149[a] 166 > 43[b] 166 > 91[c]	10	20 20 10	2.42	$C_{10}H_{15}NO$ 165.1
Homo-anatoxin	180 > 57[a] 180 > 163[b]	10	20 10	2.97	$C_{11}H_{17}NO$ 179.1
Cylindrospermopsin	416 > 194[a] 416 > 336[b] 416 > 176[c]	40	40 30 20	1.82	$C_{15}H_{21}N_5O_7S$ 415.1
Deoxy-cylindrospermopsin	400 > 194[a] 400 > 176[b]	60	30 30	2.49	$C_{15}H_{21}N_5O_6S$ 399.1
Nodularin	825.5 > 135[a] 825.5 > 103[b] 825.5 > 163[c]	60	50 50 100	5.97	$C_{41}H_{60}N_8O_{10}$ 824.4
Microcystin-LR	995.5 > 135[a] 995.5 > 213[b] 995.5 > 70[c]	60	90 60 50	6.29	$C_{49}H_{74}N_{10}O_{12}$ 994.5
Microcystin-RR	520 > 135[a] 520 > 127[b] 520 > 103[c]	30	40 30 60	5.65	$C_{49}H_{75}N_{13}O_{12}$ 1037.6
Microcystin-YR	1045.5 > 135[a] 1045.5 > 70.0[b] 1045.5 > 213[c]	40	70 60 90	6.19	$C_{52}H_{72}N_{10}O_{13}$ 1044.5
Microcystin-HtyR	1059.5 > 135[a] 1059.5 > 107[b] 1059.5 > 213[c]	40	90 70 60	6.23	$C_{53}H_{74}N_{10}O_{13}$ 1058.5
Microcystin-LA	910.5 > 776.5[a] 910.5 > 135[b] 910.5 > 213[c]	30	60 40 20	7.50	$C_{46}H_{67}N_7O_{12}$ 909.5
Microcystin-LF	986.5 > 135[a] 986.5 > 544[b] 986.5 > 478[c]	40	60 20 30	8.44	$C_{52}H_{71}N_7O_{12}$ 985.5
Microcystin-LW	1025.5 > 135[a] 1025.5 > 213[b] 1025.5 > 107[c]	90	90 70 60	8.27	$C_{54}H_{72}N_8O_{12}$ 1024.5
Microcystin-LY	1002.5 > 135[a] 1002.5 > 868.5[b] 1002.5 > 107[c]	50	20 60 90	7.66	$C_{52}H_{71}N_7O_{13}$ 1001.5
Microcystin-WR	1068.5 > 135[a] 1068.5 > 213[b] 1068.5 > 107[c]	20	70 60 70	6.53	$C_{54}H_{73}N_{11}O_{12}$ 1067.5
Microcystin-[D-Asp3]-LR and Microcystin-[Dha7]-LR	981.5 > 135[a] 981.5 > 213[b] 981.5 > 70[c]	60	80 70 60	6.33	$C_{48}H_{72}N_{10}O_{12}$ 980.5
Microcystin-[D-Asp3]-RR and Microcystin-[D-Asp3, (*E*)-Dhb7]-RR	513 > 135[a] 513 > 70[b] 513 > 103[c]	60	50 30 50	5.55	$C_{48}H_{73}N_{13}O_{12}$ 1023.6
Microcystin-HilR	505 > 135[a] 505 > 213[b] 505 > 163[c]	20	10 33 29	6.44	$C_{50}H_{76}N_{10}O_{12}$ 1008.6
Microcystin-(*N*-methyl-L)-R	505 > 135[a] 505 > 163[b] 505 > 127[c]	30	30 40 30	6.44	$C_{50}H_{76}N_{10}O_{12}$ 1008.6
Microcystin-[D-Asp3, (*E*)-Dhb7]-HphR	515 > 135[a] 515 > 117[b] 515 > 105[c]	30	30 40 10	6.44	$C_{52}H_{72}N_{10}O_{12}$ 1028.5
Microcystin-[D-Asp3, (*E*)-Dhb7]-HtyR	523 > 150[a] 523 > 135[b] 523 > 195[c]	20	10 30 40	6.18	$C_{52}H_{72}N_{10}O_{13}$ 1044.5

[a] Quantification ion.
[b] Primary confirmatory ion.
[c] Secondary confirmatory ion.

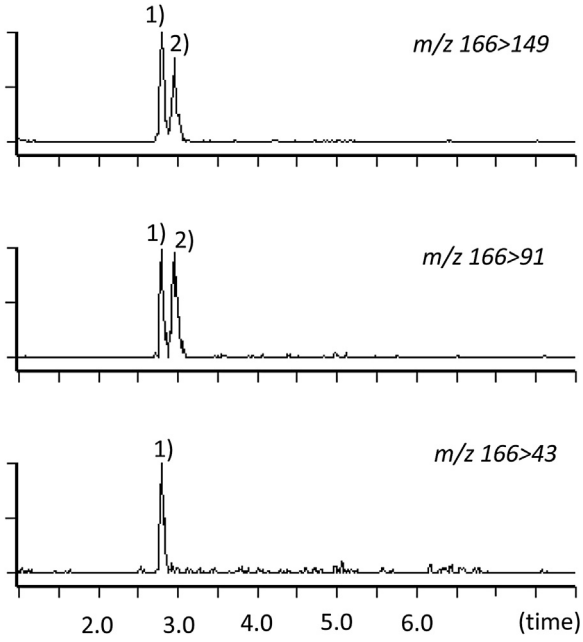

Fig. 1. Identification of anatoxin-a in an infiltration pond at Sydvatten 05/08/13. Peak (1) was identified as anatoxin-a with isotope ratios compliant to reference standard solution. Peak (2) is phenylalanine which is chromatographically separated from anatoxin-a. In addition, the transition *m/z* 166 > 43 is specific for anatoxin-a.

compound in sample and calibration standard [20]. Anatoxin-A elutes approximately 0.1 min before phenyl-alanine which frequently is present in water samples. Positive identification of anatoxin-a should therefore be based on ion ratios using transition 166 > 143 since phenylalanine does not fragment on that transition (Fig. 1). Retention times for the compounds in the sample were required to be within in ±0.2 min agreement to the corresponding compounds in the calibration standard [20].

A blank and a pooled raw water control sample spiked with the 22 cyanotoxins, each at the concentration of 1 μg/L, were analyzed within all sample batches. Since no ^{13}C or ^{15}N labeled standards are available for cyanotoxins, external calibration was performed. The calibration standards were analyzed before and after every set of samples (i.e. "bracketing"). Samples in this study were quantified by interpolation using weighed linear regression ($1/x$) and a minimum of three calibration points with multiple injections of calibration standards. The calibration curve residuals differed no more than ±20% when the experiments required quantification [20]. Field samples exceeding 10 μg/L were diluted with blank raw water or drinking water until falling into the calibration range of the specific compound (Table 2). To correct for matrix effects, recovery correction of the quantitative result was performed using the control sample analyzed in each batch.

2.5. Samples

Raw water and drinking water samples were collected from different WTPs to perform spiking experiments and samples were stored at −20 °C upon arrival to the laboratory. The cyanobacterial bloom samples from Swedish raw water sources, which were used to investigate lysing efficiency, are presented in Table 3. These samples were analyzed directly or stored at +4 °C in darkness for maximum three days. The storage conditions were enough to impede bio degradation of wild type cyanobacteria in the surface water samples to the extent that lysing experiments could be performed. Additional field samples were collected in raw water sources and infiltration ponds and also stored at −20 °C upon arrival to the laboratory (Tables 4 and 5). They were analyzed to ensure that the developed method was fit for purpose and had the ability to identify and quantify cyanotoxins in real water samples.

2.6. Lysing efficiency

The experiments to determine lysing efficiency were performed on samples A–D further described in Table 3. For each sample, the lysing experiments were performed during the same forenoon to ensure that results would be comparable. All experiments were performed in five replicates. After the lysing step, samples were always filtered into 1.5 mL HPLC vials using the syringe and filter described in Section 2.1.

2.6.1. Extracellular toxins

1 mL of the cyanobacterial bloom samples A–D (Table 3) were filtered into separate 1.5 mL HPLC vials using the syringe and filter described in Section 2.1.

2.6.2. Lysing of intracellular toxins using bead beating

600 μL of the cyanobacterial bloom samples A–D (Table 3) were pipetted into separate 1.5 mL Eppendorf tubes and 0.2 g of Zirconium Oxide Beads with Ø 0.5 mm were added. Samples were beaten in an air-cooled Bullet Blender, model BBX24BWCE, from Next Advance (Averill Park, NY, USA). Speed 8 was applied for 3 min. After checking that the samples were not significantly heated, speed 8 was applied another 2 min.

2.6.3. Lysing of intracellular toxins using freeze–thaw cycles

1.5 mL of the cyanobacterial bloom samples A–D (Table 3) were pipetted into separate 4 mL glass vials. Samples were frozen in −80 °C for 20 min and then thawed in a water bath at ambient temperature. The cycle was repeated two times more.

2.6.4. Lysing of intracellular toxins using QuickLyse™

QuickLyse™ is a registered trade mark and the composition of the lysing solution has not been published. QuickLyse™ conducts chemical lysing of the cyanobacteria. 1000 μL of the cyanobacterial bloom samples A–D (Table 3) were pipetted into separate 1.5 mL glass vials. The lysing solution QuickLyse™ (Abraxis, Warminster, PA, USA) was then applied. At first, 100 μL of reagent solution A was added to the samples. Samples were shaken for 2 min and then incubated for 8 min. Thereafter, reagent solution B was added to the samples. Samples were again shaken for 2 min and incubated for 8 min.

2.7. Linearity and matrix effects

The calibration points were 0.1, 0.5, 1.0, 5.0 and 10.0 μg/L (Section 2.2). Three injections were made at each calibration point to determine the linearity. Matrix effects were investigated in the full calibration range for raw water (i.e. at 0.5 μg/L, 1 μg/L, 5 μg/L and 10 μg/L). Five different raw water samples, originating from different lakes, were prepared by lysing raw water according to 2.6.3 and thereafter filtering samples through 0.2 μm PVDF syringe filters (Section 2.1). The samples were then spiked with the cyanotoxins to concentrations of 0.5 μg/L, 1 μg/L, 5 μg/L and 10 μg/L, respectively. The spiked samples were analyzed together with a calibration curve ranging from 0.1 μg/L to 10 μg/L diluted in Milli-q/MeOH 97/3 (v/v). Matrix effects for drinking water was studied at the concentration 1 μg/L. Five different drinking waters and raw waters were prepared by lysing raw water according to 2.6.3 and thereafter filtering samples through 0.2 μm PVDF filters (Section 2.1). The samples were then spiked to a concentration of 1 μg/L for each cyanotoxin. The spiked samples were analyzed together with

Table 2
Linear range and regression for the cyanotoxins in Milli-q/MeOH 97/3 (v/v). Matrix effects in raw water at 0.5 μg/L, 1.0 μg/L, 5 μg/L and 10 μg/L.

Compound name	Linear range (μg/L)	Regression (r^2)	Matrix effects Raw water 0.5 μg/L (%)	Matrix effects Raw water 1.0 μg/L (%)	Matrix effects Raw water 5.0 μg/L (%)	Matrix effects Raw water 10.0 μg/L (%)	Average matrix matched slope[a] Raw water (%)	Average matrix matched slope[a] Raw water RSD (%)
Anatoxin-a	0.1–10	0.998	−4.1 ± 2.9	1.3 ± 3.3	1.5 ± 4.0	−1.4 ± 2.9	100	1.8
Homo-anatoxin	0.1–10	0.997	12.1 ± 12.3	10.6 ± 7.3	4.0 ± 4.0	8.5 ± 4.6	107	3.0
Cylinderospermopsin	0.1–10	0.999	0.7 ± 4.5	−2.3 ± 5.1	−5.5 ± 5.3	−2.9 ± 4.9	95	4.5
Deoxy-cylinderospermopsin	0.5–10	0.998	N/A	N/A	N/A	N/A	N/A	N/A
Nodularin	0.1–10	0.998	2.0 ± 7.0	1.1 ± 5.8	4.4 ± 5.0	10.1 ± 5.7	107	5.4
Microcystin-LR	0.1–10	0.999	−1.4 ± 8.3	−0.25 ± 6.2	−3.5 ± 2.6	2.5 ± 3.1	99	2.7
Microcystin-RR	0.1–10	0.999	2.5 ± 4.2	−5.8 ± 3.5	−4.4 ± 6.1	−1.8 ± 4.5	97	2.3
Microcystin-YR	0.5–10	0.999	−3.0 ± 9.0	−2.5 ± 8.1	−4.8 ± 6.4	−4.0 ± 6.3	95	2.0
Microcystin-HtyR	0.1–10	0.999	35.7 ± 15.5	14.0 ± 9.6	13.8 ± 6.8	23.7 ± 6.5	120	3.4
Microcystin-LA	0.1–10	0.999	−8.4 ± 3.7	−7.3 ± 3.4	−5.2 ± 3.9	−5.4 ± 2.7	95	2.3
Microcystin-LF	0.1–10	0.998	−3.4 ± 9.4	−5.3 ± 8.1	−0.1 ± 7.7	−0.3 ± 7.3	99	1.3
Microcystin-LW	0.5–10	0.997	−6.9 ± 5.5	−7.9 ± 7.5	−3.4 ± 3.9	−3.9 ± 4.6	101	1.2
Microcystin-LY	0.5–10	0.995	−7.2 ± 2.5	−6.2 ± 3.0	−5.1 ± 2.2	−5.7 ± 1.9	95	1.1
Microcystin-WR	0.1–10	0.999	−4.7 ± 10.9	−6.6 ± 13.6	−6.5 ± 8.9	0.1 ± 8.3	99	3.7
Microcystin-[D-Asp3]-LR	0.1–10	0.999	22.4 ± 9.3	1.6 ± 8.2	−3.5 ± 4.0	7.8 ± 5.9	104	3.1
Microcystin-[D-Asp3]-RR	0.1–10	0.999	5.3 ± 4.1	−6.5 ± 3.1	−6.4 ± 3.5	−2.8 ± 3.5	95	2.5
Microcystin-HilR	0.1–10	0.995	−14.6 ± 10.8	−12.3 ± 7.3	−8.8 ± 6.7	−10.8 ± 8.3	91	4.3
Microcystin-(*N*-methyl-L)-R	0.1–10	0.990	−11.4 ± 11.1	−8.5 ± 8.0	−7.3 ± 7.9	−9.2 ± 9.5	93	3.5
Microcystin-[D-Asp3, (*E*)-Dhb7]-HphR	0.1–10	0.996	−13.2 ± 10.1	−10.2 ± 7.8	−6.9 ± 7.4	−9.6 ± 9.0	93	4.6
microcystin-[D-Asp3, (*E*)-Dhb7]-HtyR	0.1–10	0.992	−8.8 ± 13.4	−8.4 ± 11.3	−6.5 ± 9.0	−7.2 ± 9.5	95	3.1
Microcystin-[D-Asp3, (*E*)-Dhb7]-RR	0.1–10	0.999	−11.4 ± 7.2	−9.3 ± 4.8	−5.3 ± 5.6	−4.9 ± 5.0	93	3.0
Microcystin-[Dha7]-LR	0.1–10	0.994	1.2 ± 8.7	−2.8 ± 6.8	−2.4 ± 6.9	2.5 ± 5.4	100	0.9

[a] Calibration slopes were evaluated individually for the five different raw waters in comparison to cyanotoxins in Milli-q/MeOH 97/3 (v/v).

Table 3
Lysing efficiency on five individual water samples using bead beating, freeze–thaw cycles and lysing solution QuickLyse™. Five replicates were made for each sample.

Sample	Sampling date	Sample type	Extracellular toxins (μg/L)	Bead beating (μg/L)	Freeze–thaw cycles (μg/L)	QuickLyse™ (μg/L)
A) St. Hällungen—*microcystins*	30/11/11	Surface water	0.9 ± 0.1	1.5 ± 0.2	2.2 ± 0.2	0.9 ± 0.1
B) Mälaren—*microcystins*	22/08/12	Scum	4.7 ± 0.5	77 ± 17	90 ± 6.1	61 ± 6.2
C) Vombsjön—*microcystins*	31/08/12	Scum	3.4 ± 0.3	397 ± 50	424 ± 81	301 ± 34
D) Mälaren—*microcystins*	31/07/13	Surface water	<0.5	6.2 ± 6.6	8.1 ± 1.2	10.5 ± 4.7
D) Mälaren—*anatoxins*	31/07/13	Surface water	2.6 ± 0.1	2.9 ± 0.1	7.9 ± 1.3	6.2 ± 0.6
E) Baltic Sea—*nodularins*	16/08/12	Surface water	5.0 ± 0.3	5.1 ± 0.2	4.8 ± 0.2	4.5 ± 0.3

Table 4
Repeatability and recovery at four different concentrations of drinking water and raw water.

Compound name	Recovery drinking water 0.1 μg/L (%)	Recovery drinking water 0.5 μg/L (%)	Recovery drinking water 1.0 μg/L (%)	Recovery drinking water 2.0 μg/L (%)	Recovery raw water 0.5 μg/L (%)	Recovery raw water 1.0 μg/L (%)	Recovery raw water 5.0 μg/L (%)	Recovery raw water 10.0 μg/L (%)
Anatoxin-a	89 ± 17	112 ± 23	103 ± 13	101 ± 4	117 ± 41	112 ± 34	138 ± 3	125 ± 2
Homo-anatoxin	94 ± 11	119 ± 20	99 ± 4.2	105 ± 3	133 ± 23	135 ± 17	137 ± 5	128 ± 4
Cylinderospermopsin	100 ± 6	70 ± 10	87 ± 3.3	80 ± 12	93 ± 16	97 ± 5	97 ± 6	94 ± 5
Deoxy-cylinderospermopsin	70 ± 7	90 ± 8	84 ± 13	80 ± 12	92 ± 11	91 ± 3	95 ± 2	94 ± 2
Nodularin	<LOQ	69 ± 13	111 ± 7.4	108 ± 4	102 ± 20	107 ± 14	92 ± 8	101 ± 5
Microcystin-LR	104 ± 16	96 ± 5	103 ± 4.9	106 ± 14	98 ± 14	107 ± 14	106 ± 10	94 ± 11
Microcystin-RR	80 ± 3	85 ± 4	79 ± 2.4	103 ± 17	91 ± 6	91 ± 4	100 ± 4	94 ± 4
Microcystin-YR	<LOQ	91 ± 9	98 ± 6.2	99 ± 12	91 ± 17	101 ± 19	107 ± 10	96 ± 10
Microcystin-HtyR	107 ± 12	70 ± 21	89 ± 3.7	90 ± 1	118 ± 15	113 ± 18	79 ± 8	95 ± 8
Microcystin-LA	74 ± 4	82 ± 4	73 ± 3.4	91 ± 10	67± 5	70 ± 6	68 ± 1	71 ± 5
Microcystin-LF	<LOQ	92 ± 7	84 ± 3.1	101 ± 17	62 ± 27	65 ± 20	63 ± 14	65 ± 23
Microcystin-LW	<LOQ	87 ± 7	85 ± 6.7	99 ± 17	74 ± 20	72 ± 22	70 ± 12	75 ± 16
Microcystin-LY	<LOQ	77 ± 9	84 ± 4.0	86 ± 12	76 ± 8	80 ± 6	72 ± 5	75 ± 5
Microcystin-WR	94 ± 3.0	88 ± 5	83 ± 3.8	103 ± 17	89 ± 18	81 ± 5	93 ± 4	89 ± 6
Microcystin-[D-Asp3]-LR	103 ± 11	70 ± 21	82 ± 4.8	84 ± 2	104 ± 11	95 ± 17	83 ± 9	96 ± 9
Microcystin-[D-Asp3]-RR	123 ± 11	91 ± 9	98 ± 3.4	104 ± 2	115 ± 20	112 ± 13	98 ± 5	98 ± 2
Microcystin-HilR	<LOQ	81 ± 13	67 ± 18	65 ± 6	89 ± 19	80 ± 9	87 ± 4	84 ± 2
Microcystin-(*N*-methyl-L)-R	114 ± 14	78 ± 7	60 ± 6.7	63 ± 4	92 ± 11	81 ± 13	97 ± 6	92 ± 4
Microcystin-[D-Asp3, (*E*)-Dhb7]-HphR	132 ± 21	93 ± 12	81 ± 5.1	88 ± 2	88 ± 8	82 ± 7	88 ± 12	85 ± 12
Microcystin-[D-Asp3, (*E*)-Dhb7]-HtyR	95 ± 8	102 ± 6	92 ± 3.9	98 ± 3	94 ± 6	92 ± 5	91 ± 6	85 ± 5
Microcystin-[D-Asp3, (*E*)-Dhb7]-RR	112 ± 4	97 ± 5	89 ± 2.6	92 ± 1	104 ± 3	102 ± 3	89 ± 3	87 ± 3
Microcystin-[Dha7]-LR	142 ± 9	118 ± 5	104 ± 2.8	107 ± 2	116 ± 4	114 ± 4	93 ± 3	91 ± 3

a standard of the same concentration containing the cyanotoxins, diluted in Milli-q/MeOH 97/3 (v/v).

2.8. Validation study

In Sweden, drinking water is considered a food product and the regulatory authority is the National Food Agency. Therefore a validation protocol from the food sector was chosen as a basis for this study. The validation protocol followed was SANCO/12571/2013 Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed since it is suitable for multi-methods covering several substance classes [20]. Raw waters from five different WTPs were spiked using the mixed 62.5 μg/L standard solution (Section 2.2) to concentrations of 0.5 and 1.0 μg/L, while the 625 μg/L solution (Section 2.2) was used for concentrations 5.0 and 10.0 μg/L. All samples were lysed using the freeze–thaw cycles described in Section 2.6.3, in order to enable analysis of total cyanotoxins content. Samples were then filtered into 1.5 mL HPLC vials using the syringe and filter described in Section 2.1. For finished drinking water analysis, only extracellular toxins were considered. Drinking water from five different WTPs were spiked using the mixed 62.5 μg/L standard solution (Section 2.2) for concentrations of 0.1, 0.5, 1.0 and 2.0 μg/L. Samples were then filtered into 1.5 mL HPLC vials using the syringe and filter described in Section 2.1.

Lowest limit of quantification (LOQ) was set to the lowest spiked level meeting the performance criteria for trueness and precision i.e. average recovery for spike levels tested should fall between 70 and 120% and the repeatability for the spiked levels tested should be ≤20% [20].

2.9. Application to source water samples—a survey

Samples from Swedish source waters were prepared and lysed using the freeze–thaw method described in Section 2.6.3. Since the samples had been stored frozen, no analysis of extracellular toxins could be performed, so the total concentration was determined instead. Samples were analyzed according to Section 2.3 and identification and quantification was performed as described in Section 2.4.

3. Results and discussion

3.1. Reference standards and UPLC-MS/MS method

The UPLC-MS/MS method was developed using pure reference standards. The UPLC mobile phase and elution gradient were inspired by earlier reports on comprehensive separation of microcystins [21]. However, to yield retention also for the hydrophilic anatoxins and cylindrospermopsins a longer UPLC column was selected that had previously been shown to perform good separation for microcystins [11]. In addition, the elution gradient was modified to start at 2% mobile phase B. Retention was achieved

Table 5
Application of the method to field samples from source waters and infiltration ponds used for drinking water production. Results are reported as quantitative when exceeding LOQ (0.5 μg/L) and as identified (but not quantified) when the MRM ratios are compliant but the concentration is lower than LOQ. Estimated concentrations are given for the identified compounds.

	Quantified toxins (μg/L)	Identified toxins < LOQ, estimated concentration (μg/L)
Mälaren 30/07/13 Surface water	Homo-anatoxin: 10.60[a] Microcystin-LR: 1.87 Microcystin-RR: 0.52 Microcystin-YR: 0.70 Mikrocystin-[D-Asp3]-LR and microcystin-[Dha7]-LR: 0.57 Mikrocystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR: 0.87	
Ülmiste 09/07/14 Surface water	Microcystin-LR: 9.13 Microcystin-RR: 2.97 Microcystin-YR: 11.70 Microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR: 4.66 Microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR: 6.82 Microcystin-[D-Asp3, (*E*)-Dhb7]-HphR: 0.59	Microcystin-LY: ~0.1 Microcystin-LW: ~0.2 Microcystin-LF: ~0.1
Långasjön 21/08/13 Surface water	Microcystin-LR: 6.90 Microcystin-LF: 0.57	Microcystin-RR: ~0.3 Microcystin-LY: ~0.4 Microcystin-LW: ~0.2 Microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR: ~0.4 Microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR: ~0.2
Vombsjön 19/06/13 Surface water	Microcystin-LR: 0.68 Microcystin-RR: 0.83	Microcystin-YR: ~0.2 Microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR: ~0.2
Immeln 30/11/12 Surface water	Microcystin-LR: 16.8 Microcystin-RR: 40.35 Microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR: 97.01 Microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR: 660,80	
Sydvatten 29/07/13 Infiltration pond	Microcystin-RR: 1.78	Anatoxin-a: ~0.1[a] Homo-anatoxin: ~0.2[a] Microcystin-YR: ~0.3 Microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR: ~0.2 Microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR: ~0.2 Microcystin HtyR-[D-Asp3, (*E*)-Dhb7]: ~0.2
Sydvatten Infiltration pond 05/08/13	Anatoxin-a: 1.30[a] Microcystin-LR: 0.69 Microcystin-RR: 0.86	Homo-anatoxin: ~0.5[a] Cylindrospermopsin: ~0.2 Microcystin-YR: ~0.1 Microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR: ~0.3 Microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR: ~0.2

[a] Result not recovery corrected.

for all toxins, but at the expense of complete separation of some microcystin variants (Table 1).

All compounds were detected with MS/MS. Some of the MRM transitions for anatoxin-a, homoanatoxin-a, cylindrospermopsin, deoxy-cylindrospermopsin and microcystins have been described previously [15,22,23,24]. In this study, additional MRM transitions were optimized for the compounds since quantitative analysis requires, apart from quantifier MRM, also one or two confirmatory MRM transitions (Table 1). In Table 1 quantifying ion, primary confirmatory ion and secondary confirmatory ions are listed for the 22 cyanotoxins. Full scan mass spectra optimization of the microcystin variants revealed single charged parent ions for the anatoxins, cylindrospermopsins, nodularin, microcystin-LR, microcystin-YR, microcystin-HtyR, microcystin-LA, microcystin-LF, microcystin-LW, microcystin-LY, microcystin-WR, microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR and double charged parent ions for microcystin-RR, microcystin-[D-Asp-3]-RR, microcystin-HilR, microcystin-(*N*-methyl-L)-R, microcystin-[D-Asp3, (*E*)-Dhb7]-HphR, microcystin-[D-Asp3, (*E*)-Dhb7]-HtyR, and microcystin-[D-Asp3, (*E*)-Dhb7]-RR. These results are in agreement with earlier reports [15,22]. When fragmented in the collision cell, the microcystin variants produced well known daughter ions such as *m*/*z* 135 derived from the ADDA moiety and *m*/*z* 213 corresponding to fragment ion ([Glu-Mdha+H]+) [25]. Full information about the MRM transitions is presented in Table 1.

Microcystin-[D-Asp3]-LR co-eluted with microcystin-[Dha7]-LR and the variants was also not distinguished from one another in their fragmentation patterns produced by triple quadrupole MS/MS although collision energies ranging from 10 eV to 90 eV were investigated. Product ion mass spectra for microcystin-[D-Asp3]-LR and microcystin-[Dha7] is displayed in Supplementary material, Fig. S2. These two desmethylated variants of microcystin-LR were therefore quantified together in the samples using the average response factor. Similarly, microcystin-[D-Asp3]-RR, co-eluted with microcystin-[D-Asp3, (*E*)-Dhb7]-RR and their fragmentation patterns also resembled each other to a great extent. The product ion mass spectrum for microcystin-[D-Asp3]-RR and microcystin-[D-Asp3, (*E*)-Dhb7]-RR are also shown in Supplementary material (Fig. S2). The two desmethylated variants of microcystin-RR were therefore also quantified together in the samples using the average response factor. Despite efforts, the triple quadruople MS/MS product ion scans did not yield abundant heavier mass fragments

which differentiate the two desmethylated microcystin-LR and the two desmethylated microcystin-RR variants from each other, respectively. Since the primary focus for this method is quantitative analysis highly abundant fragment ions were chosen to increase sensitivity. Unique fragments for the different variants of desmethylated microcystin-RR and desmethylated microcystin-LR have been reported previously using other mass spectrometric techniques such as MALDI-TOF and Orbitrap MS^n [26,27].

Since toxicity data for microcystin-[D-Asp3]-LR and microcystin-[Dha7]-LR indicates a LD_{50} of 160–300 μg/kg and 250 μg/kg respectively, quantification of the two isomers together using an average response factor will not impact risk assessment significantly [28]. Similarly, the LD_{50} for microcystin-[D-Asp3]-RR and microcystin-[D-Asp3, (*E*)-Dhb7]-RR are both determined to 250 μg/kg [28].

3.2. Sample preparation and lysing efficiency

3.2.1. Sample preparation

The sample preparation consists of a lysing step and a syringe filtration through PVDF filters. Compared with SPE purification, it gives the advantage that toxins from several toxin groups such as anatoxins, cylindrospermopsins, nodularin and microcystins can be analyzed simultaneously. The drawback is that the sample preparation has no step of pre-concentration and therefore requires a large volume to be injected into the LC–MS/MS. Other common sample clean-up strategies for microcystin analysis involve SPE or liquid–liquid partitioning, yielding cleaner and more concentrated extracts, enabling smaller volumes to be injected into the LC–MS/MS [11,29]. However, such methods are in general not more sensitive than the method developed and validated in this study, and methods with extensive sample clean-up still suffers the drawback of being time consuming and limited to analysis of one toxin group.

3.2.2. Lysing efficiency—extracellular toxins

The water samples, A–D (Table 3), were collected from surface water from Swedish lakes used for drinking water production. In sample A and C from Lake St. Hällungen and Lake Vombsjön, different variants of microcystins were identified. Both samples collected from Lake Mälaren B and D contained different variants of microcystins and sample D also contained homo-anatoxin. Sample E was collected in the Baltic Sea and contained nodularin.

The amount of extracellular toxins in the samples varied, and the percentage reported stands in relation to the total amount of toxin in each sample (intra- and extracellular toxins). Sample A from Lake St. Hällungen contained 39% (0.85 ± 0.12 μg/L) extracellular toxins, sample B from Lake Mälaren 120822 contained 5% (4.7 ± 0.47 μg/L) and sample C from Lake Vombsjön <1% (3.4 ± 0.29 μg/L). Interestingly, sample D from Lake Mälaren 31/07/13 contained no quantifiable levels of extracellular microcystins (<0.5 μg/L) and 33% (2.6 ± 0.10 μg/L) of extracellular homo-anatoxin-a. Unfortunately, the sample from the Baltic Sea contained 100% (5.0 ± 0.30 μg/L) extracellular nodularin and it was therefore not suitable for testing lysing efficiency. Results are presented in detail in Table 3.

3.2.3. Lysing efficiency—intra cellular toxins using bead beating, freeze–thaw cycles and QuickLyse™

Freeze–thaw was the most efficient lysing technique for microcystins in sample A from Lake St. Hällungen (2.2 ± 0.19 μg/L), sample B from Lake Mälaren 22/08/12 (90 ± 6.1 μg/L) and sample C from Lake Vombsjön (424 ± 81 μg/L). The homo-anatoxin-a in sample D Lake Mälaren 31/07/13 (7.9 ± 1.3 μg/L) was also most efficiently lysed by freeze–thaw cycles while the microcystins in the same sample was most efficiently lysed by the lysing agent QuickLyse™, implying that homo-anatoxin-a and the microcystins are produced by different species of cyanobacteria blooming together. Bead beating and use of lysing agent QuickLyse™ was faster than lysing cells with freeze–thaw cycles and the methods gave reasonable results for most samples (Table 3). The results for the lysing agent QuickLyse™ were in agreement with earlier reported studies for wild blooms of cyanobacteria [30].

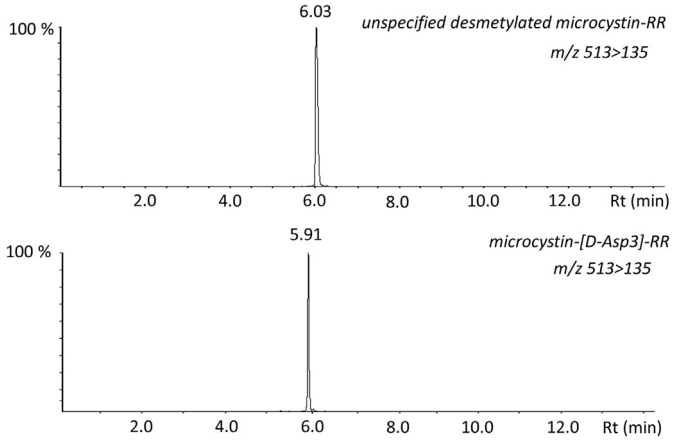

Fig. 2. The unspecified desmethylated microcystin-RR is chromatographically resolved from microcystin-[D-Asp3]-RR and also microcystin-[D-Asp3, (*E*)-Dhb7]-RR (not in the figure).

3.3. Results of linearity test and matrix effects

The linear range for the method was determined to 0.1–10 μg/L for all substances except deoxy-cylindrospermopsin, microcystin-YR, microcystin-LW and microcystin-LY which had the linear range 0.5–10 μg/L (Table 2). The linearity of the calibration curves (R^2) were between 0.990–0.999 (Table 2). The residuals were examined for calibration points 0.1 μg/L, 0.5 μg/L, 1.0 μg/L, 5.0 μg/L and 10 μg/L and they were all between 0 and ±20%. The exceptions were microcystin-(*N*-methyl-L)-R at calibration point 1 μg/L which resulted in a residual of −28 ± 9.7%. Detailed data for the residuals in each calibration point is reported in supplementary material (Table S2).

In general, the matrix effects in raw water were similar for all compounds. During the evaluation of matrix effects over the full calibration range in raw water, microcystin-[D-Asp3]-LR and microcystin-HtyR showed enhancement of >20% in at least one calibration point (Table 2). All other cyanotoxins showed less matrix effects than ±20% (Table 2). Calibration slopes were established for all cyantoxins in the five different raw waters and compared to a calibration slope of cyanotoxins in Milli-q/MeOH 97/3 (v/v). The average slope for cyanotoxins in raw water matrix varied between 91 and 120% compared to the calibration slope of cyanotoxins in Milli-q/MeOH 97/3 (v/v) (Table 2). The standard deviation of the five slopes in raw water matrix had standard deviations of 0.9–4.3% (Table 2). Conclusively, the matrix effects in raw water were not concentration dependent and the matrix effects were similar for the five waters tested. Evaluation of matrix effects for natural toxins over a full calibration range has previously been evaluated according to this principle [31].

The matrix effects in drinking water and raw water was also studied at the concentration 1 μg/L in a separate experiment. There anatoxins were least affected by matrix effects, showing suppression ≤16% (Table S3). The most severe suppression was registered for the cylindrospermopsins; up to 58%. However, the standard deviation was low, indicating that the different drinking- and raw waters affected the ionization similarly (Table S3). Most microcystins suffered suppressions up to 30%, with the

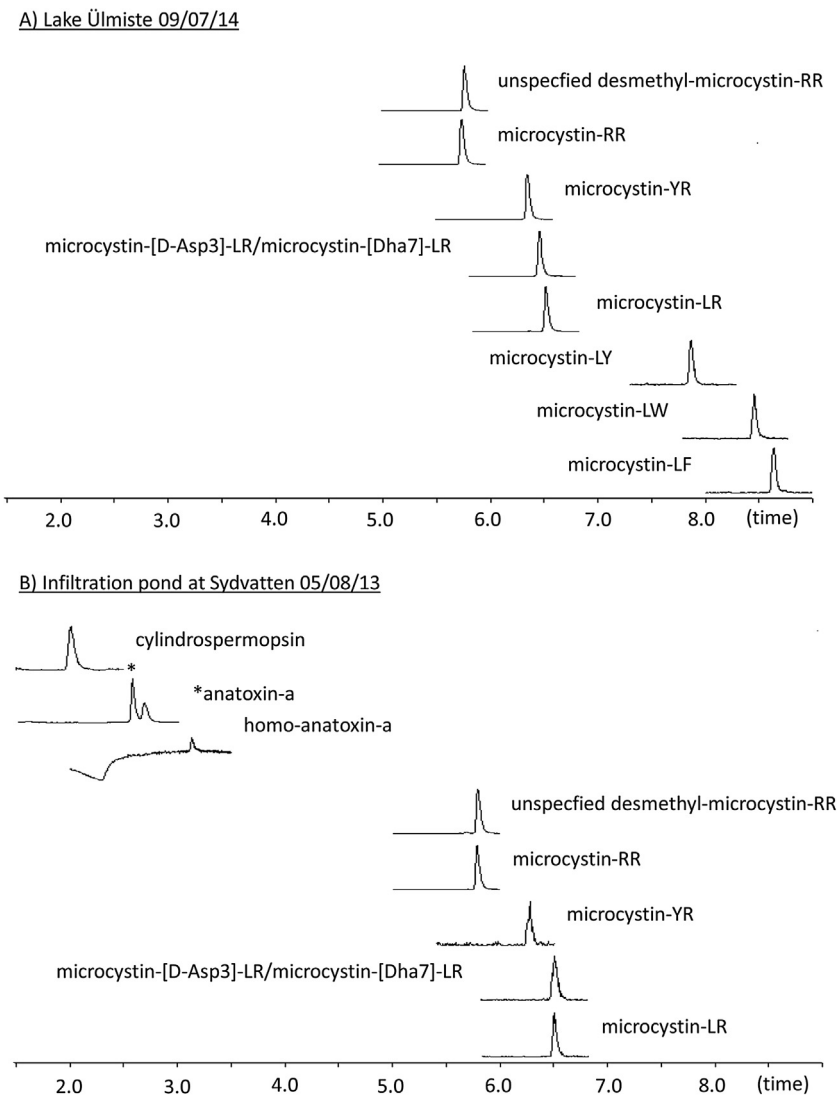

Fig. 3. Multiple reaction monitoring (MRM) chromatograms of quantified and/or identified cyanotoxins from (A) Lake Ülmiste 09/07/14 and (B) infiltration pond at Sydvatten 05/08/13. Quantitative results are reported in Table 4.

exception of microcystin-HiLR, microcystin-(*N*-methyl-L)-R and microcystin-[Dha7]-LR that showed enhancement of 58%, 22% and 12%, respectively (Table S3).

Due to that significant matrix effects may occur during analysis, recovery correction is recommended. The experiments shows that the matrix effects are not biased by concentration to any significant extent for the cyanotoxins investigated, which is essential for successful recovery correction. Moreover, for some cyanotoxins the matrix effects changed significantly between two experiments indicating that recovery correction should be performed using a spiked drinking or raw water sample analyzed in the same batch as the samples.

3.4. Results from method validation

The validation study of drinking water and raw water consisted of five replicates at 0.1 μg/L, 0.5 μg/L, 1.0 μg/L and 2.0 μg/L for drinking water and at 0.5 μg/L, 1.0 μg/L, 5.0 μg/L and 10.0 μg/L in raw water. The validation comprised 22 substances validated at four concentration levels in raw water and four concentration levels in drinking water, resulting in 176 validation points (Table 4). 92% of all validation points were giving recoveries from 70–120%. Some compounds deviated in different aspects. Anatoxin gave recoveries of 138 ± 2.8 and 125 ± 1.7% in raw water at concentrations 5.0 μg/L and 10 μg/L. Phenylalanine have previously been reported to cause interference in LC–MS/MS analysis of anatoxin [32]. In the present method, phenylanine is chromatographically separated from anatoxin, and therefore contribution from phenylalanine cannot be the cause for the high recoveries (Fig. 1). In raw water, homo-anatoxin consistently gave too high recoveries ranging from 128% to 137%, while the recoveries in all four validation points for drinking water were compliant. However, standard deviations were mostly smaller than ±20% indicating that the error is rather related to accuracy than precision. Microcystin-LA and microcystin-LF were compliant in all drinking water validation points, but yielded somewhat low recoveries in raw water, ranging from 67% to 71% for microcystin-LA and 62% to 65% for microcystin-LF. Similarly, microcystin-HiLR and microcystin-(*N*-methyl-L)-R had low recoveries in drinking water ranging from 65% to 81% and 60% to 114%, respectively (Table 4). The low recoveries cannot be justified if compared to the matrix effects for the same compounds (Table 2). The sample preparation only involves filtration and the lower recoveries obtained can therefore either be contributed to loss of cyanotoxin during filtration or the fact that the matrix effects in this method are variable over time. Similar effects can be observed for microcystin-HtyR and microcystin-[D-Asp3]-LR

although here the matrix effects exceed the recoveries in some validation points indicating that variable matrix effects are the cause (Tables 2 and 4, Table S3). It is therefore imperative to apply recovery correction using a spiked sample analyzed in the same batch as the samples. The lowest level of quantification (LOQ) in drinking water was set to to 0.1 μg/L for all substances except nodularin, microcystin-YR, microcystin-LF, microcystin-LW and microcystin-LY, for which the LOQ was set to 0.5 μg/L (Table 4). The LOQ for raw water was set to 0.5 μg/L for all substances (Table 4).

3.5. Performance in proficiency tests

The method was tested in ielab Proficiency Testing Schemes Raw Water 2013, single round October 2013 [33]. Twelve labs took part and the consensus value for total microcystins in raw water was 1.68 μg/L. The UPLC-MS/MS based method presented here gave a quantitative result of 1.40 μg/L, generating a *Z*-score of −0.7. In food analysis, *Z*-score of ±2 is considered compliant. The results are indeed satisfactory considering that six labs used immunoassay, one used PPIA inhibition and five labs used LC–MS or LC–MS/MS based techniques [23]. The result reported to the proficiency test was not corrected for recovery.

3.6. Results from field samples from Swedish lakes and infiltration ponds

Water samples from five different source waters were used, along with two infiltration ponds. The cyanotoxins in the samples were considered identified and quantified when exceeding LOQ (0.5 μg/L). However, the cyanotoxins were considered identified – but not quantified – when the MRM ratio and the retention time fitted the reference standard solution, but the concentration was below LOQ. All identified cyanotoxins had a signal to noise ratio ≥3. The quantitative results are reported in Table 5. In addition, for the identified toxins, estimated concentrations were also reported (Table 5).

Interestingly, the sample from Lake Mälaren 30/07/13 contained homo-anatoxin, which thereby was identified for the first time in Swedish waters. Homo-anatoxin has previously been found in other European lakes such as in the Netherlands and on Ireland [34,35]. The sample from Mälaren also contained microcystin-LR, microcystin-RR, microcystin-YR, mikrocystin-[D-Asp3]-LR and/or mikrocystin-[Dha7]-LR and an unspecified desmethylated microcystin-RR. The unspecified desmethylated microcystin-RR eluted slightly later than the reference standards of microcystin-[D-Asp3]-RR and microcystin-[Asp3, (*E*)-Dhb7]-RR (Fig. 2). In the present method the unspecified desmethylated microcystin-RR is chromatographically resolved from microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR but the fragmentation patterns are very similar. Since no reference standard is available for that variant it was quantified using the average response factor of microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR.

The surface water from Lake Ülemiste in Estonia and Lake Långasjön contained the same microcystin variants as the sample from Lake Mälaren, however small amounts of microcystin-LY, microcystin-LW and microcytin-LF were also present. No microcystin-YR was identified in Lake Långasjön. Lake Immeln was dominated by the unspecified desmethylated microcystin-RR, with small amounts of microcystin-LR and microcystin-RR. In general, the profile of the microcystin variants in Lake Ülemiste in Estonia, Lake Långasjön and Lake Immeln were consistent with observations from Finnish lakes [36,37].

The raw water sample from Lake Vombsjön contained microcystin-LR and microcystin-LF in quantifiable concentrations, and microcystin-YR, microcystin-[D-Asp3]-LR and/or microcystin-[Dha7]-LR and the unspecified desmethylated microcystin-RR were also identified (but below LOQ). The infiltration ponds studied are recharged from Lake Vombsjön. The two samples from the infiltration ponds showed very interesting results. Anatoxin-a and homo-anatoxin-a were quantified or identified in both samples. In addition, cylindrospermopsin was identified in the sample from the infiltration pond 05/08/13. Cylindrospermopsin has not previously been reported from Swedish waters. The infiltration ponds also contained quantifiable amounts of microcytin-LR and microcystin-RR and identified was also microcystin-YR and microcystin-[D-Asp3]-LR and/or mikrocystin-[Dha7]-LR, similarly to Lake Vomb. However, the infiltration ponds also contained microcystin HtyR-[D-asp3, (*E*)-Dhb7].

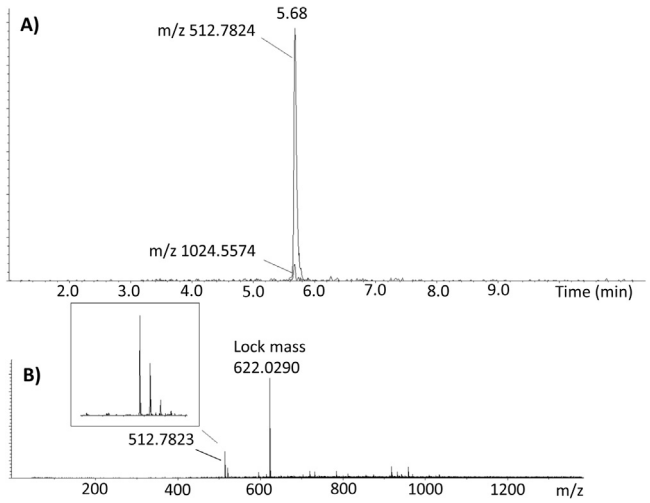

Fig. 4. (A) UPLC-TOF-MS extracted ion chromatogram *m*/*z* 512.7824 and *m*/*z* 1024.5574 representing the doubly and singly charged ion from the unspecified desmethylated microcystin-RR in the Lake Mälaren 30/07/15 sample. (B) High resolution mass spectrum from the unspecified desmethylated microcystin-RR in the Lake Mälaren 30/07/13 sample.

Chromatograms of quantified and identified toxins for Lake Mälaren 30/07/13, Lake Ülemiste 09/07/14 and an infiltration pond at Sydvatten 05/08/13 are shown in Fig. 3. Drinking water samples were collected simultaneously with the surface water and infiltration pond samples. None of the drinking water samples contained the cyanotoxins included in this method.

3.7. Unspecified desmethylated microcystin-RR

Structure elucidation of the unspecified microcystin-RR found in the sample from Mälaren 30/07/13 and Lake Immeln was beyond the scope of this study. However, in the sample from Lake Mälaren the presence of a desmethylated microcystin-RR was confirmed by UPLC-TOF-MS showing a singly charged ion at *m*/*z* 1024.5574 and a doubly charged ion at *m*/*z* 512.7824 (Fig. 4). Similarly, the unspecified desmethylated microcystin-RR in the sample from Lake Immeln was also confirmed with UPLC-TOF-MS. A brief method description for the UPLC-TOF-MS method is available in supplementary material (Table S4). Previous studies have shown the presence of another variant, microcystin-[Dha7]-RR, in addition to microcystin-[D-Asp3]-RR and microcystin-[Asp3 (*E*)-Dhb7]-RR in strains of *Microcystis Aeruginosa* [36]. Furthermore, two desmetylated microcystin-RR isomers were identified in Lake Albano but the structures of the isomers were not elucidated further [19].

The bloom sample from Lake Mälaren 30/07/13 was dominated by *Microcystis flos-aquae* but also contained significant amounts of other species of Microcystis, among others *Microcystis Aeruginosa*. The potentially toxins producing strains *Dolichospermum* was also present in the sample from Lake Mälaren 30/07/13. A complete list

of cyanobacterial species in sample 30/07/13 from Lake Mälaren is found in supplementary material (Table S5). Analysis of cyanobacteria was also performed for the sample from Lake Immeln and the dominating species was *Microcystis Botrys* but significant amounts of *Woronichinia Naegeliana* were also present. A complete list of cyanobacterial species in the sample from Lake Immeln is found in supplementary material (Table S5). The two blooms that produced the unspecified desmethylated microcystin-RR were both dominated by the species *Microcystis* but the strains were different. It is well known that the toxin profiles produced by *Microcystis* are strain dependent. Therefore, there were no conclusive evidence that could link the unspecified desmethylated microcystin-RR to a certain species and strain of cyanobacteria.

4. Conclusions

A fast, rugged and sensitive UPLC-MS/MS method has been developed, enabling simultaneous analysis of anatoxins, cylindrospermopsins, nodularin and microcystins, both intracellular and extracellular, in raw water and drinking water. The method is designed to monitor the guidance values for cyanotoxins in drinking water and to provide early warning for the WTPs that struggles with cyanobacterial blooms in their source waters. The method is accurate and has good precision, as shown in the validation and in the proficiency tests. Furthermore, analysis of the field samples from source waters and infiltration ponds identified anatoxin-a, homo-anatoxin-a, cylindrospermopsin and several variants of microcystins in Swedish source waters for the first time. Traditionally in Sweden, only microcystins are monitored during cyanobacterial blooms. As shown in this study, there is a need to expand the analytical scope and include the anatoxins, cylindrospermopsins and nodularins. The presented method has been proven to be fit for such a purpose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.12.049.

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