SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 36, No. 3, pp. 645–654, 2017

Printed in the USA

Environmental Chemistry

MONITORING OF FRESHWATER TOXINS IN EUROPEAN ENVIRONMENTAL WATERS BY USING NOVEL MULTI-DETECTION METHODS

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(Submitted 7 March 2016; Returned for Revision 4 June 2016; Accepted 7 August 2016)

Abstract: Monitoring the quality of freshwater is an important issue for public health. In the context of the European project μAqua, 150 samples were collected from several waters in France, Germany, Ireland, Italy, and Turkey for 2 yr. These samples were analyzed using 2 multitoxin detection methods previously developed: a microsphere-based method coupled to flow-cytometry, and an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method. The presence of microcystins, nodularin, domoic acid, cylindrospermopsin, and several analogues of anatoxin-a (ATX-a) was monitored. No traces of cylindrospermopsin or domoic acid were found in any of the environmental samples. Microcystin-LR and microcystin-RR were detected in 2 samples from Turkey and Germany. In the case of ATX-a derivatives, 75% of samples contained mainly H_2-ATX-a and small amounts of H_2-homoanatoxin-a, whereas ATX-a and homoanatoxin-a were found in only 1 sample. These results confirm the presence and wide distribution of dihydro derivatives of ATX-a toxins in European freshwaters. *Environ Toxicol Chem* 2017;36:645–654. © 2016 SETAC

Keywords: Freshwater toxins Microcystins Anatoxin-a Homo-anatoxin-a Dihydro-anatoxin-a

INTRODUCTION

Aquatic ecosystems represent important natural resources essential for supplying drinking water and constitute the economic base for fisheries and industrial, agricultural, and recreational activities. Increased levels of 1 or more limiting nutrients can enhance the biological production of the ecosystem, resulting in a dangerous status known as eutrophication. The appearance of this phenomenon has increased in relation to human activities, although it has occurred naturally for centuries [1]. Eutrophication can result in excessive growth of harmful microorganisms, such as some cyanobacteria or even diatoms, posing a threat to human health when toxic species proliferate. In freshwater, cyanobacteria, or blue-green algae, are the most common organisms present during microalgal proliferations. The excessive growth episodes are known as cyanobacterial harmful algal blooms. Increasing numbers of these microorganisms produce toxic metabolites called cyanotoxins. Three groups of compounds with different effects and structures are included within the term "cyanotoxins": hepatotoxins, as microcystins (MCs), nodularins (NODs), and cylindrospermopsins (CYNs); neurotoxins, as anatoxin-a (ATX-a) and analogues, saxitotins, and ß-N-methylamino-L-alanine; and dermatotoxins, as aplysiatoins and lyngbyatoxins [2]. These compounds can produce severe effects and have been associated with numerous animal and human poisonings [3]. As consequence, cyanotoxins are considered as an environmental and public health concern. The World Health Organization has established a provisional upper limit of 1 μg microcystin-LR (MC-LR)/L drinking water [4]; however, other microalgal toxins are becoming increasingly recognized as potential risks to both human and animal health, resulting in their inclusion in legal regulations regarding the safety of waters of several countries [2,5,6]. Recently, US Environmental Protection Agency (USEPA) recommended limits below 1.6 μg/L and 0.3 μg/L of MC-LR for adults and children, respectively, and a limit of 3 μg/L of CYN [7].

Microcystins have been isolated from microorganisms such as *Microcystis*, *Anabaena*, *Oscillatoria*, *Planktothrix*, *Chroococcus*, *Nostoc*, and *Anabaenopsis* [8]. Their cyclic heptapeptide scaffold (D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha) has approximately 90 structural variants though amino-acidic modifications (as representative examples, the modifications for 2 common analogs, MC-RR and MC-YR, are shown in the Figure 1). Once ingested, MCs are concentrated in hepatic tissues, where they bind to the catalytic subunit of protein phosphatases, triggering hepatotoxicity. It has been suggested that this biological target is also involved in tumor development [2,9,10]. The NODs, mostly synthesized by the cyanobacteria *Nodularia spumigena*, are chemically similar to

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Published online 8 July 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3577

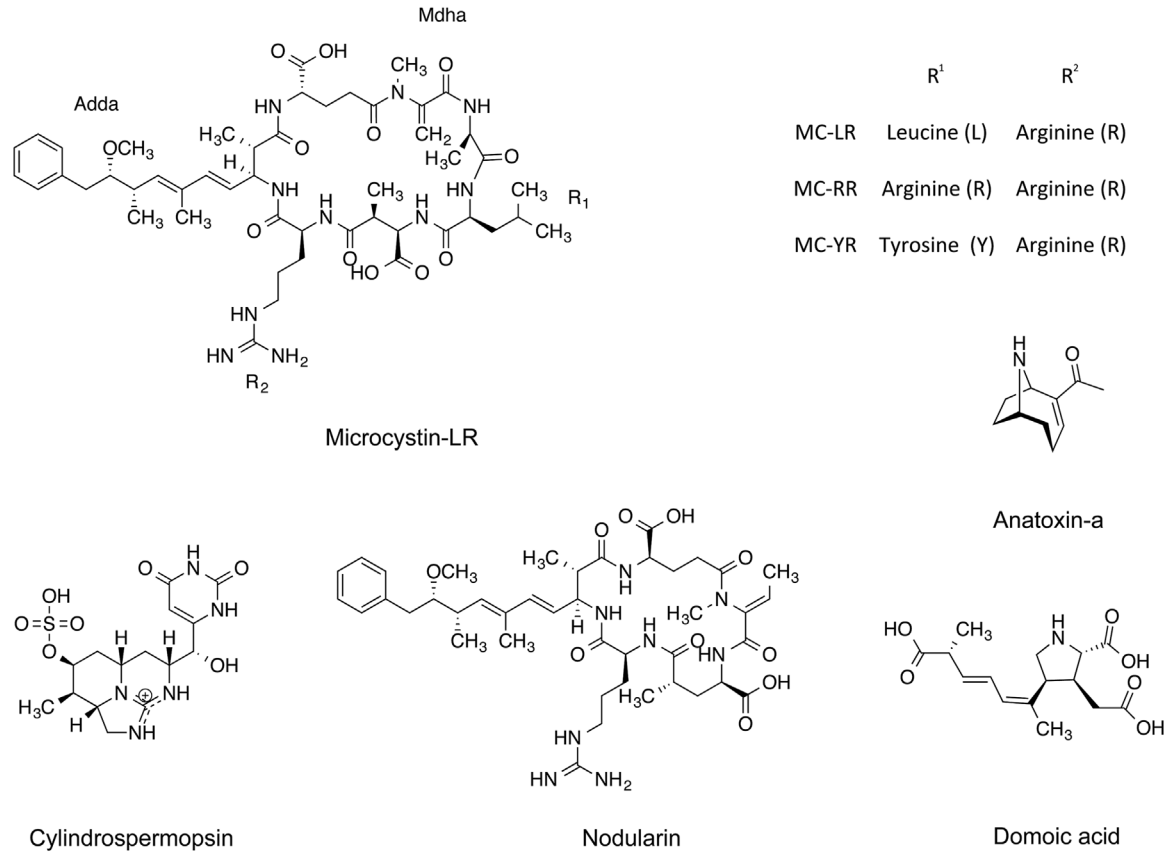

Figure 1. Structures of the representative toxins analyzed in the present study: microcystin-LR, cylindrospermopsin, anatoxin-a, domoic acid, and nodularin. MC = microcystin.

MCs because they are also cyclic pentapeptides (Adda-D-Glu-MeDhb-D-MeAsp-L-Arg); however, fewer than 10 analogs have been described to date. The main mechanism of action for NODs also involves protein phosphates and can lead to high tumorogenic activity [11,12]. Different CYNs producing microorganisms have been identified, including *Cylindrospermopsis*, *Aphanizomenon*, *Umezakia, Raphidiopsis*, *Anabaena*, and *Lygnbya* [13]. The CYN is a tricyclic alkaloid, and modifications of this structure have resulted in an epimer and an analog (7-deoxy-CYN) [14,15]. Hepatotoxicity, potential carcinogenicity, and protein synthesis inhibition have been associated with CYN [9,16,17]. Several species of *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Phormidium*, *Cylindrospermum*, *Raphidiopsis*, and *Planktothrix* have been described as ATX-a toxins producers [18,19]. The main representatives compounds of this group of toxins are the alkaloid ATX-a and its analog homoanatoxin-a (homoATX-a), 2 potent agonists of nicotinic acetylcholine receptors (nAChR) [20–23]. The chemical instability of both toxins leads to the occurrence of epoxy and dihydro products [24,25], although some of these compounds can occur naturally [18,26]. In addition to the above toxins, the present study also analyzed the group of amnesic shellfish toxins (ASTs) because the production of ASTs by diatoms belonging the genera *Pseudo-nitzschia* and *Nitzschia* has been reported in brackish waters [27]. The ASTs include domoic acid (DA) and its 9 isomers (iso-domoic-A to iso-domoic-H and epi-domoic acid), which bind to and activate kainate receptors, causing neurological symptoms such as amnesia as well as gastrointestinal effects [28,29]. Therefore, microalgal toxins represent a global sanitary threat, as their occurrence has been reported worldwide. In this sense, a recent study established a correlation between the presence of cyanobacteria and MC-LR and gastrointestinal symptoms, which underscores the importance of the control of freshwater toxins to protect human and wildlife health [30].

Several methods for detecting cyanotoxins in the environment have been developed [31], including the use of microspheres coupled to flow-cytometry system [32] and ultra-performance liquid chromatography–tandem mass spectrometry, UPLC-MS/MS [33]. The flow-cytometry technology coupled to microspheres with different spectral properties and surface carboxyl groups allows to the covalent immobilization of ligands. Each type of microsphere is specific for an analyte (i.e., toxin), which recognizes through the specific ligand covalently attached on its surface. The flow cytometry system separates individual microspheres with red and green lasers and allows the quantification of attached compound. Multiplexing is provided by the simultaneous incubation of a sample with multiple types of analyte-specific microspheres. This technology has been widely employed in clinical and research fields [34]. The UPLC-MS/MS method is a fast analytical technology used to detect and quantify cyanobacterial toxins. For toxin quantification, the mass spectrometric methods are based on detection in multiple-reaction monitoring mode. In this way, high selectivity and sensitivity are possible because at least 2 product ions by compound are identified. An efficient detection system can be done using first a screening method

Table 1. Details of source, dates, and types of water from samples during the 2-yr monitoring campaign

Country	Source	Sample ID	Geographical coordinates	Sampling depth (m)	Collection date	Water type
France						
	Tap Water	F1–F6	—		Jan–Nov 2013	Tap
	Seine River	F7–F12	48°46.100, 02°25.117	0.5–1.0	Jan–Nov 2013	Fresh
	Oise River	F13–F19	40°04.000, 02°12.000	0.5–1.0	Jan–Nov 2013	Fresh
	Marne River	F20–F25	48°50.999, 02°31.999	0.5–1.0	Jan–Nov 2013	Fresh
	Canet Lagoon	F26–F39	42°65.976, 03°02.996	0.5	Oct 2011–May 2013	Brackish
	Mondony River	F40–F53	42°47.084, 02°66.912	0.5	Oct 2011–May 2013	Fresh
Germany						
	Schlei/Kappeln	G1	54°39.610, 09°56.280	0.75	Jun 2013	Brackish
	Wittensee/Sande	G2	54°22.882, 09°43.475	0.5	Jun 2013	Fresh
	Kieler Innenförde/Neumühlen-Dietrichsdorf	G3	54°20.633, 10°10.508	1.2	Jun 2013	Brackish
	Orther Bucht	G4	54°25.669, 11°04.063	0.5	Jul 2013	Brackish
	Kieler Innenförde/Heikendorf	G5	54°22.404, 10°11.538	0.5	Aug 2013	Brackish
	Kellersee/Malente	G6	54°10.620, 10°34.822	0.4	Aug 2013	Fresh
	Schlei/Maasholmer Breite	G7	54°40 480, 10°01.647	0.3	Aug 2013	Brackish
	Eckernförder Bucht/Eckernförde	G8	54°28.179, 09°52.721	0.5	Aug 2013	Brackish
	Neukirchener See	G9, G15	54°12.935, 10°33.653	0.5, 1.2	Aug 2013, May 2014	Fresh
	Selenter See/Fargau	G10	54°19.202, 10°23.137	0.5	Aug 2013	Fresh
	Schwentine/Rosensee	G11	54°16.852, 10°15.936	1.0	Aug 2013	Fresh
	Fehmarnsund	G12	54°23.933, 11°06.285	1.0	Sept 2013	Brackish
	Schwentine/Wellingdorf	G13, G14	54°19.624, 10°11.230	1.0	Oct 2013	Fresh
Ireland						
	River Boyne	IR1–IR18	53°43.508, 06°25.025	1.0	Sept 2012–Mar 2014	Fresh
	River Dargle	IR19–IR37	53°12.325, 06°06.592	1.0	Sept 2012–Mar 2014	Fresh
	Stream near Slane	IR38–IR55	53°42.875, 06°34.525	0.5	Sept 2012–Mar 2014	Fresh
Italy						
	Albano Lake	I1–I3	41°44.692, 12°39.314	0.5	Oct 2012, Apr 2013, Jul 2013	Fresh
	Fiastrone Lake	I4–I5	43°02.284, 13°09.868	1.0	Nov 2012, Apr 2013	Fresh
	Mezzocamino (Tiber River)	I6	41°48.418, 12°25.113	0.5	Jun 2013	Fresh
Turkey						
	Lake Sapanca	T1–T20	40°43.938, 30°17.875	Samples between surface and 20 m	Sept 2012–Apr 2014	Fresh

based on the use of the flow-cytometry technology and toxin-specific microspheres, which would indicate the presence/absence of toxins, followed by an analytical method, such as UPLC-MS/MS, allowing quantification of the toxins and identification of different analogues. In the present study, these 2 technologies were applied to screen 150 samples collected during a 2-yr monitoring campaign as part of the European project (Universal Microarrays for the Evaluation of Fresh-Water Quality Based on Detection of Pathogens and Their Toxins (μAqua).

MATERIAL AND METHODS

Materials

Certified reference standard material of DA was obtained from CIFGA. Domoic acid for immobilization was purchased from Merck Millipore and CIFGA. Anatoxin-a, CYN, MC-LR, MC-YR, and MC-RR were obtained from ENZO. Biotin-α-bungarotoxin was from Molecular Probes. Analytical standard of MC-LR, MC-YR, MC-RR, N-hydroxysuccinimide, sodium tetraborate decahydrate, jeffamine (2,2′-[ethylenedioxy]

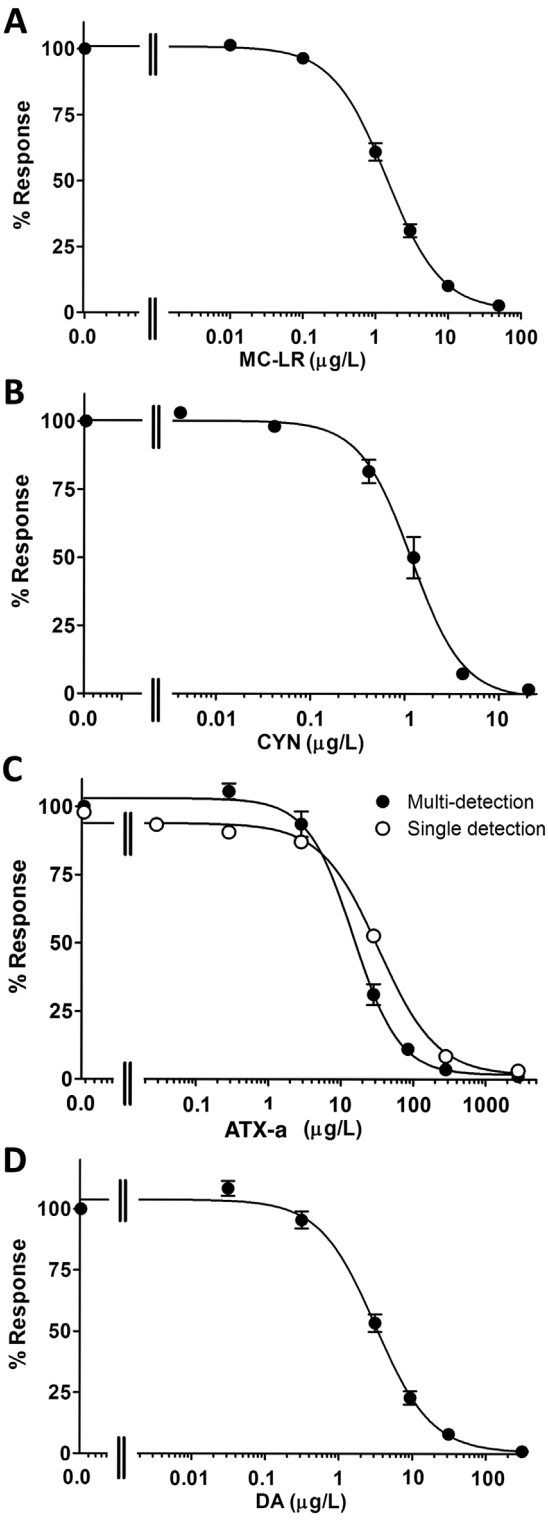

Figure 2. Microsphere-based detection method for (**A**) mycrocystin-LR (MC-LR), (**B**) cylindrospermopsin (CYN), (**C**) anatoxin-a (ATX-a), and (**D**) domoic acid (DA). Calibration curves for MC-LR, CYN, ATX-a, and DA were performed simultaneously in buffer solution (MC-LR, CYN, DA: mean ± standard error, $n = 7$; ATX-a: mean ± SEM, $n = 5$).

bis[ethylamine]), ethylenediamine, boric acid, sodium phosphate monobasic, ethanolamine, and Tween-20 were purchased from Sigma-Aldrich. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Pierce. The anti-MC-LR (MC-LR-Ab), anti-CYN (CYN-Ab), and anti-DA (DA-Ab) antibodies were obtained as previously described [35–37]. The acetylcholine binding protein from *Lymnaea stagnalis* (Ls-AChBP) was purified and characterized as previously described [38,39]. Phycoerythrin goat anti-mouse Ig (PE-Ab) was purchased from Invitrogen, phycoLink streptavidin-R phycoerythrin (PE-SA) from Prozyme, and sodium azide from Fluka. Carboxylated microspheres (LC10019-01, LC10027-01, LC10050-01, and LC10054-01) were from Luminex Corporation. Luminex sheath fluid, multiscreen 96-well filter plates, a 33-mm Millex filter with 0.45-μm pore size, and Ultrafree-MC (Durapore membrane) and Ultrafree-CL centrifugal filters (Low binding Durapore PVDF membrane) were purchased from Millipore. Hemoflow cartridges HF80S were from Fresenius Medical Care.

Formaldehyde (37%), dimethyl sulfoxide, sodium acetate anhydrous, disodium hydrogen phosphate anhydrous, and sodium chloride were obtained from reagent grade commercial sources (Sigma-Aldrich). Acetonitrile and methanol were supplied by Panreac. All solvents employed in the present study were HPLC grade or analytical grade, and the water was distilled and passed through a water purification system (Milli-Q; Millipore). Phosphate-buffered saline solution (PBS) was made from 130 mM NaCl, 1.5 mM NaH_2PO_4, 8.5 mM Na_2HPO_4 (pH 7.4). The PBS-BT solution was made as PBS supplemented with 0.1% w/v BSA and 0.1% v/v Tween-20.

Extraction of water samples

Water samples were collected from different countries in Europe (France, Germany, Ireland, Italy, and Turkey; see Table 1) during 2012, 2013, and 2014 as part of the μAQUA monitoring campaign. The selection of each sample collection station was done as representative of the country within the project scope, and it was identified according to geographical coordinates.

From each station, samples of 50 L of water were collected and concentrated to 1 L using a hemoflow cartridge (60 Da exclusion size), following manufacture specifications (Fresenius Care) [40]. Aliquots of 40 mL of the concentrate were separately frozen at −20 °C and sent to the laboratory of the Universidade de Santiago de Compostela in dry ice. On arrival, the samples were thawed and then sonicated 3 cycles of 30 s prior to use. Then the samples were centrifuged, and aliquots of 10 mL were separated. These aliquots were vacuum dried and dissolved in methanol (1 mL) and centrifuged 3700 rpm for 10 min at room temperature. The supernatants were filtered through 0.45-μm filters (Millipore), vacuum dried, and redissolved in water or PBS-BT for subsequent analyses. Concentration steps to detect small amounts of toxins were added when necessary. The final concentration of toxins was always expressed per liter of water.

Microsphere-based multidetection assay for MC-LR, CYN, ATX-a, and DA

In this method, MC-LR, CYN, DA, and Ls-AChBP were covalently attached to the carboxylated surface of 4 different types of microsphere (LC10050-01, LC10019-01, LC10054-01, and LC10027-01, respectively) and validated as previously described [32,41,42]. The detection method was designed and validated to include 4 simultaneous competition assays to be performed in the same well [32]. Briefly, during the first step, 60 μL of sample or calibration solution (containing a mixture of MC-LR, CYN, ATX-a, and DA) were incubated with 60 μL of the specific antibodies (MC-LR-Ab, CYN-Ab, and DA-Ab) and 2×10^3 of prewashed Ls-AChBP-coated microspheres in a

microtiter plate. After 1 h of incubation, 100 μL of this mixture were transferred to a second microtiter filter plate containing washed toxin-coated microspheres (2×10^3 MC-LR-microspheres, 2×10^3 CYN-microspheres, 2×10^3 DA-microspheres). After 1 h of incubation, 100 μL of α-BTX were added, and the mixture was incubated for 30 min and then washed. Finally, 100 μL of PE-labeled molecules (PE-Ab and PE-SA) were added, and the mixture was incubated for 1 h. After a washing step, the microspheres were suspended in 100 μL of PBS-BT and analyzed on a Luminex 200 analyzer (LuminexCorp). Microspheres were classified with a 635-nm wavelength laser, and PE fluorescence was quantified after excitation with a 532-nm wavelength laser. The acquisition volume was 75 μL, and the minimum number of bead counts was 100. All experiments were performed in duplicate. The incubations were performed at room temperature, and the washing buffer was always PBS-BT.

The calibration curves for the microsphere-based method were fitted by GraphPad Prism Ver 5.0 using a 4-parameter logistic equation obtained with a nonlinear regression fitting procedure:

$$Y = R_{hi} + (R_{lo} - R_{hi})/(1 + 10^{\wedge}[\{\text{LogIC50} - X\} \times \text{HillSlope}])$$

where R_{hi} is the bottom or the response at infinite concentration, R_{lo} is the top or the response at 0 concentration, IC50 is the half maximal inhibitory concentration, and X is the logarithm of concentration to base 10.

Limits of detection (LODs) for multiplexed calibration curves were estimated using the 20% inhibitory concentration [32,43–45]: 0.61 ± 0.21 μg/L for MCs, 0.67 ± 0.08 μg/L for CYNs, 6.59 ± 2.07 μg/L for ATX-a toxins, and 1.33 ± 0.32 μg/L for DA (Figure 2A–D).

For the microsphere-based method, methanol was evaporated to avoid interferences with organic solvents, as described in previously published immuno-detection methods [41,46]. After reconstitution in a buffer solution, the samples were checked for the presence of each analyte among the dynamic range of the detection curves done for each experiment (Figure 2).

UPLC-MS/MS analysis

The UPLC-MS/MS analyses were performed with a combination of UPLC with a mass detector. The UPLC system (Shimadzu) consisted of 2 pumps (LC-30AD), an autoinjector (SIL-10AC) with refrigerated rack, a degasser (DGU-20A), a column oven (CTO-10AS), and a system controller (SCL-10Avp). This system was coupled to a MS/MS system (model 8040; Shimadzu). The nitrogen generator was a Nitrocraft NCLC/MS from Air Liquide.

The Aquity HSS T3 column (100 mm × 2.1 mm inner diameter; Waters), 1.8 μm, was used for cyanotoxins separations. The temperature was set at 35 °C. The composition of the mobile phase was water (A) and acetonitrile (B), both containing 0.05% formic acid. Chromatographic separation was performed by gradient elution (12.5 min) starting with 2% of B for 4 min; then 70% B was held for 1 min, reduced to 2% B over 0.5 min, and held for 3 min until the next run. The mobile phase flow rate was 0.45 mL/min, and the injection volume was 5 μL. The electrospray ionization source of the 8040 mass spectrometer was operated with the following optimized values of source-dependent parameters: nebulizing gas flow, 2 L/min; Desolvation Line (DL) temperature, 300 °C; heat block temperature, 500 °C; and drying gas flow, 15 L/min. The mass spectrometer was operated in positive multiple-reaction monitoring mode, analyzing the transitions shown in Table 2. The collision energy was optimized for each transition [33].

Table 2. Precursor and transitions (mass-to-charge ratio [*m/z*]) of cyanotoxins and collision energy

Compound name	Molecular weight	Transitions (*m/z*)	Collision energy (eV)
H_2-homoATX-a	181	182 > 164	−18
		182 > 147	−20
H_2-ATX-a	167	168 > 150	−18
		168 > 133	−20
Epoxy-homoATX-a	195	196 > 178	−18
		196 > 138	−20
ATX-a	165	166 > 166	−5
		166 > 149	−18
		166 > 131	−20
		166 > 43	−24
Epoxy-ATX-a	181	182 > 164	−18
		182 > 138	−20
homoATX-a	179	180 > 163	−18
		180 > 145	−20
DA	311	312 > 266	−20
		312 > 161	−30
MC-RR	1038	1039 > 1039	−15
		1039 > 135	−74
		520 > 135	−35
MC-YR	1045	1046 > 1046	−15
		1046 > 135	−74
MC-LR	995	996 > 996	−15
		996 > 135	−74
MC-LA	910	911 > 911	−15
		911 > 135	−74
MC-LY	1002	1003 > 1003	−15
		1003 > 135	−74
MC-LW	1025	1026 > 1026	−15
		1026 > 135	−74
MC-LF	986	987 > 987	−15
		987 > 135	−74
NOD	825	826 > 826	−5
		826 > 135	−55
		826 > 70	−73
CYN	415	416 > 194	−36
		416 > 176	−40

homoATX-a = homoanatoxin-a; ATX-a = anatoxin-a; DA = domoic acid; MC = microcystin; NOD = nodularin; CYN = cylindrospermopsins.

Table 3. Typical losses and retention times of ATX-a toxins

Toxin	Retention time (min)	$[M+H]^+$	$[M-NH_3+H]^+$	$[M-NH_3-H_2O+H]^+$
ATX-a	2.8	166	149	131
H2-ATX-a	2.3	165	151	133
H2-homoATX-a	1.2	182.9	162.9	147

ATX-a = anatoxin-a; H_2-homoATX-a and H_2-ATX-a = dihydro derivatives of anatoxin-a.

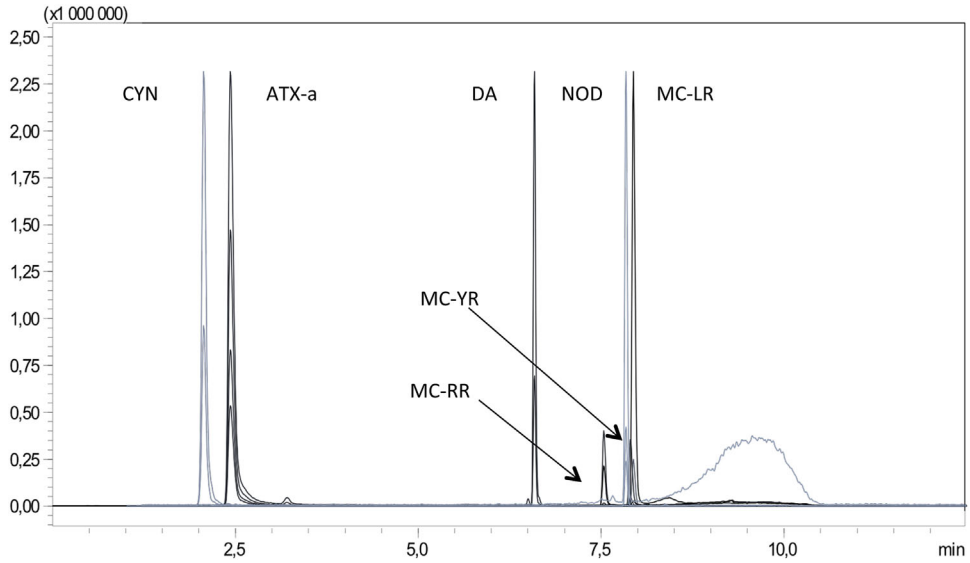

Figure 3. Chromatogram in positive multiple-reaction monitoring mode of standard solution in water: cylindrospermopsin (CYN; 500 ng/mL), anatoxin-a (ATX-a; 200 ng/mL), microcystin-LR (MC-LR; 500 ng/mL), microcystin-YR (MC-YR; 500 ng/mL), microcystin-RR (MC-RR; 500 ng/mL), nodularin (NOD; 500 ng/mL), and domoic acid (DA; 500 ng/mL).

The transitions of several toxins analogues, widely described in the literature, were added to the mass method, although no standards are available (Table 2). Similarly, following the fragmentation pathway of ATX-a, the analogues homoATX-a, H_2-ATX-a, and H_2-homoATX-a were added. Because no standards were available for some of these ATX-a analogues, in case of positive results, the product ion scan for each peak was done to confirm the identification, and the spectrums obtained showed the typical losses and structures of protonated compounds (Table 3). The identification of ATX-a analogues was done based on the molecular mass and the ionization pattern of each analogue, according to the bibliography. Given that the fragmentation pathway of each molecule is a tool often used to identify and confirm the presence of ATX-a analogues [33,47,48]. In addition to the fragmentation pathway, the chromatographic separation facilitated the differentiation of molecules. In this case, ATX-a, homoATX-a, H_2-ATX-a and H_2-homoATX-a have the same losses. First a molecule of NH_3 and then a molecule of water were lost; that is, $[M\text{-}NH_3+H]^+$ and $[M\text{-}NH_3\text{-}H_2O+H]^+$ ions appeared.

The LOD and limit of quantification (LOQ) for each toxin were: LOD (ATX-a), 0.0038 μg/L; LOD (CYN), 0.006 μg/L; LOD (DA), 0.005 μg/L; LOD (MCs and NOD), 0.0078 μg/L; LOQ (ATX-a), 0.01 μg/L; and LOQ (CYN, NOD, DA, and MCs), 0.02 μg/L. No matrix effect in water sample was observed. The percentage (%) of recovery in water higher than 90%.

RESULTS AND DISCUSSION

Field samples (150) from different countries, encompassing a wide range of different habitats, were screened for the presence of freshwater toxins using both the microsphere-based assay and the UPLC-MS/MS method (Table 1). Because the microsphere-based and the UPLC-MS/MS methods were already described and validated elsewhere as reported in the *Materials and Methods* section, we intend only to compare the toxin profile obtained by each method (screening and analytical), focusing on the toxin diversity of the samples rather than comparing the methods. Each sample was extracted following a simple protocol and dissolved in methanol before

Table 4. Samples from France[a]

	Microsphere assay	UPLC-MS/MS	
Code	ATX-a (μg/L)	H_2-homoATX-a (μg/L)	H_2-ATX-a (μg/L)
F7	0.928	0.0192	0.0525
F8	0.018	0.0283	0.0470
F9	0.618	0.0100	0.0439
F10	0.948		0.0612
F11	0.578		0.0525
F12	0.038	0.0149	0.0393
F13	0.448	0.0146	0.0493
F14		0.0116	0.0445
F15	0.038	0.0101	0.0498
F16	0.408	0.0100	0.0600
F18	1.098		0.0465
F19	0.038		0.0486
F20	0.058	0.0100	0.0657
F21	0.008	0.0247	0.0552
F22	0.348	0.0239	0.0461
F24	1.908	0.0100	0.0549
F25	0.028	0.0100	0.0329
F26	1.368		0.0478
F27	0.378		
F28	2.118		
F29	0.458	<LOQ	0.0294
F30	0.368		
F31	5.308	<LOQ	0.0261
F32	1.868		
F33	5.458		
F34	5.458		
F35	2.418		
F36	0.928		0.0460
F37	3.908	<LOQ	0.0651
F38	4.048	0.0112	0.0679
F39	3.218	<LOQ	0.0591
F43		<LOQ	0.0560
F45		<LOQ	0.0883
F49		<LOQ	0.0761
F50		<LOQ	0.0712
F52	0.498	<LOQ	0.0465
F53	0.988	0.0137	0.0549

[a]Positive results (μg toxin/L water collected) of samples collected in France in 2011 and 2013 analyzed by microsphere-based assay and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method.
ATX-a = anatoxin-a; H_2-homoATX-a and H_2-ATX-a = dihydro derivatives of anatoxin-a; <LOQ = traces below the limit of quantification.

Table 5. Samples from Germany[a]

	Microsphere assay		UPLC-MS/MS					
Code	ATX-a (μg/L)	MCs (μg/L)	ATX-a (μg/L)	H_2-ATX-a (μg/L)	homoATX-a (μg/L)	H_2-homoATX-a (μg/L)	MC-LR (μg/L)	MC-RR (μg/L)
G1	6.480			0.014	0.016	0.06		
G2				0.03				
G3	0.780			0.01				
G4	6.080			0.015				
G5	6.920			0.024				
G7	7.760					0.02		
G8	3.730			0.013				
G9	17.140	1.301	0.06	0.033	0.17	0.58	0.47	<LOQ
G10	0.230							
G11	1.630			0.03		0.01		
G12	9.420			0.011				
G13	0.570			0.026				
G14	0.470			0.016		0.01		
G15	1.630			0.016	0.027	0.10		

[a]Positive results (μg toxin/L water collected) of samples collected in Germany in 2013 and 2014 analyzed by microsphere-based assay and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method.
ATX-a = anatoxin-a; MCs = microcystins; H_2-homoATX-a, homoATX-a and H_2-ATX-a = dihydro derivatives of anatoxin-a; <LOQ = traces below the limit of quantification.

analysis, and the presence of MCs, NODs, CYNs, Das, and ATX-a toxins was tested from the same extracts.

The presence of freshwater toxins was also assessed by UPLC-MS/MS using a method previously developed [33]. Using this method, CYN, NOD, DA, ATX-a, MC-RR, MC-YR, and MC-LR could be quantified with good separation between peaks in 12.5 min (Figure 3). In addition, the amino acid phenylalanine was also separated to avoid misidentifications between ATX-a and this compound [33].

The environmental samples with positive toxin results by microsphere-based multidetection method or from UPLC-MS/MS are shown in Tables 4–8. No toxins were detected in samples F1 through F6 from France, which encompass 6 mo of municipal tap water samples collected from January to November 2013. As such, these samples were allocated as negative controls for both detection methods. No traces of CYN or DA were detected in any of the samples. In water bodies from Spain and Greece, CYN had been detected previously [49,50]. However, this toxin is not regularly identified in Europe. In the case of DA, although some species producing this toxin have been described in brackish waters (and are usually identified in estuaries and bay waters, where the salinity is not as high as in marine environments), to our knowledge the toxin has never been reported in fresh waters [27,51,52]. The present results suggest that these 2 toxins currently are not a risk in European waters sampled. Similarly, no traces of NOD were detected in any of the samples analyzed.

The same results were obtained for MCs in samples collected from France and Ireland. In samples collected in Germany, however, several MCs analogues were detected using the microspheres assay (Table 5). Within this assay, 1.30 μg/L of MCs were detected in sample G9 obtained from a lake in summer 2013, whereas no MCs were found in the remaining samples. When this sample was analyzed by UPLC-MS/MS, 0.47 μg/L of MC-LR and traces of MC-RR were detected. In general, MC levels were rather low, especially considering previous findings that show MCs in 24% of lakes in Germany, with up to 560 μg/L in extreme conditions [53]. These toxins were also detected in sample I4 from Italy, obtained from a lake in autumn 2012, measured by the microspheres assay; however no toxins were detected by UPLC-MS/MS when this sample was confirmed (Table 7). In several samples from Turkey, high levels of MCs were also detected by microspheres assay, but only MC-RR was found by UPLC-MS/MS in sample T7 (Table 8). Discrepancies between these methods can be attributed to antibody/assay cross-reactivity [32]. In samples from Turkey, some matrix interference in the microspheres assay could have taken place in addition to the cross-reactivity, since MCs were detected in 7 samples from 20, whereas no toxins were detected by UPLC-MS/MS. In the same way, matrix interferences have been described to produce false positives when commercial enzyme-linked immunosorbent assay kits for MCs and NODs were used to check tap and lake waters or when the salinity of the samples was increased [46,54].

Regarding ATX-a results, several conclusions can be drawn. From the microsphere-based assay data, ATX-a or some analogue seems to be present in a high number of samples (81 of 119). These observations were confirmed when the samples were analyzed by UPLC-MS/MS, because the presence of H_2-ATX-a and H_2-homoATX-a was observed in 113 of the 150 samples. Although the amount quantified was not exactly the same, in 96 of 119 samples analyzed by both methods, ATX-a analogues were detected; none of the target toxins were found in 23 of the samples. Interestingly, 15 samples were negative based on the microspheres method but were positive by UPLC-MS/MS; and 14 were negative by UPLC-MS/MS but were positive within the microspheres assay. Moreover, 31 samples were analyzed only by UPLC-MS/MS, and positive results for H_2-ATX-a and for H_2-homoATX-a were found. The discrepancies between the amounts quantified by both methods could be caused by a different affinity of ATX-a analogues for the protein involved in the microspheres assay. In addition, the higher number of these discrepancies occurred with samples from brackish waters, where the matrix interference has been described [46,54]. However the good equivalence between positives and negatives in samples analyzed by both methods (75%) supports the microspheres method as a reliable tool to detect the presence of ATX-a toxins as a prior screening, at least in fresh water. On the other hand, the detection of H_2-ATX-a and H_2-homoATX-a in all localities is a very important result

Table 6. Samples for Ireland[a]

Code	Microsphere assay	UPLC-MS/MS	
	ATX-a (μg/L)	H_2-homoATX-a (μg/L)	H_2-ATX-a (μg/L)
IR1	0.750	0.0414	0.1044
IR3	0.309	<LOQ	0.1134
IR5	0.174	<LOQ	0.1095
IR6	0.747	<LOQ	0.0896
IR7		<LOQ	0.0965
IR8	0.254	<LOQ	0.0905
IR9	0.245	<LOQ	0.1177
IR10	0.445	<LOQ	0.1137
IR11[b]		<LOQ	0.1024
IR12[b]		<LOQ	0.0782
IR13[b]		<LOQ	0.1083
IR14[b]		<LOQ	0.1375
IR15[b]		<LOQ	0.0794
IR16[b]			0.0987
IR17[b]			0.0951
IR18[b]			0.0802
IR19	0.401	<LOQ	0.1004
IR21		<LOQ	0.0796
IR23		<LOQ	0.0781
IR24			0.0792
IR25			0.0541
IR26			0.0575
IR27			0.0706
IR28			0.0858
IR29			0.0780
IR30[b]		<LOQ	0.0796
IR31[b]		0.0205	0.0855
IR32[b]		<LOQ	0.0586
IR33[b]		<LOQ	0.1013
IR34[b]		<LOQ	0.0810
IR35[b]		<LOQ	0.0813
IR36[b]		<LOQ	0.0890
IR37[b]		<LOQ	0.0570
IR38	1.387	0.0447	0.0881
IR39	0.139		
IR43	0.022		
IR44	0.189		
IR45	0.751		
IR47	0.061	<LOQ	0.0887
IR48[b]		0.0249	0.0873
IR49[b]		0.0262	0.0876
IR50[b]		0.0181	0.0912
IR51[b]		0.0031	0.0749
IR52[b]		<LOQ	0.0550
IR53[b]			0.0848
IR54[b]			0.0931
IR55[b]		<LOQ	0.0837

[a]Positive results (μg toxin/L water collected) of samples collected in Ireland in 2012 and 2014 analyzed by microsphere-based assay and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method.
[b]Analyzed only by UPLC-MS/MS.
ATX-a = anatoxin-a; H_2-homoATX-a and H_2-ATX-a = dihydro derivatives of anatoxin-a; <LOQ = traces below the limit of quantification.

Table 7. Samples for Italy[a]

Code	Microsphere Assay		UPLC-MS/MS	
	ATX-a (μg/L)	MCs (μg/L)	H_2-homoATX-a (μg/L)	H_2-ATX-a (μg/L)
I1	3.815		0.0345	0.0174
I2	0.603		<LOQ	0.0746
I3	0.560			0.0751
I4	1.750	1.295	<LOQ	0.0712
I5	0.294			0.0687
I6	0.920		0.0167	0.0697

[a]Positive results (μg toxin/L water collected) of samples collected in Italy in 2012 and 2013 analyzed by microsphere-based assay and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method.
ATX-a = anatoxin-a; MCs = microcystins; H_2-homoATX-a and H_2-ATX-a = dihydro derivatives of anatoxin-a; <LOQ = traces below the limit of quantification.

described for the first time from the present study. Whereas ATX-a was detected in only 1 sample from Germany (G9) and homoATX-a was detected in 3 samples (G1, G9, and G15), the dihydro derivatives of these toxins were detected in almost all samples collected in the 5 countries involved. In this sense, H_2-ATX-a was detected in most samples and in higher amounts. The toxins H_2-ATX-a and H_2-homoATX-a were considered as degradation products of ATX-a and homoATX-a, respectively [47]. However, enzymatic steps to produce this degradation are still largely unknown, and the dihydro derivatives sometimes represent a higher percentage of the total toxin content in field samples [26,55]. In addition, the cluster of genes responsible of the natural production of dihydro toxins was recently identified in several cyanobacteria [18]. Therefore, the wide distribution of dihydro derivatives with respect to the ATX-a and homoATX-a observed in our results points to their natural occurrence. These toxins are neurotoxic compounds responsible for many animal poisonings worldwide [23,56]. Anatoxin-a is transformed into a non-toxic compound after sunlight exposure, and the dihydro derivatives show an approximately 10-fold reduction of affinity for nicotinic receptors and lower toxicity [2,55]. Because of the lack of standards, however, no studies about toxicity and transformations of pure dihydro compounds are available; therefore, the toxicological risk of these compounds is unknown. Although there are no official guidelines for maximum levels of ATX-a in drinking waters in Europe, several US states have implemented threshold levels for this toxin. In addition, the US EPA has recently published a health effects support document for ATX-a [57], but there is not adequate information to support a health advisory for these toxins, ATX-a, and derivatives, as that which exists for MCs and CYN [7,57]. Therefore, considering the present study, ATX-a toxins and especially the dihydro derivatives should be taken under consideration in future regulations and monitoring protocols for water quality, because all of them are naturally present everywhere in Europe and their potential health risk should be assessed.

CONCLUSIONS

The toxins MCs, ATX-a, homoATX-a, and mainly H_2-ATX-a and H_2-homoATX-a are present in aquatic habitats throughout Europe. The wide natural distribution of dihydro derivatives of ATX-a toxins is an important issue that should be taken under consideration in monitoring programs to avoid intoxications. The combination of 2 multidetection assays—the microsphere-based method, as a screening assay, and the analytical method UPLC-MS/MS, which requires highly trained personnel—might be a useful approach to monitoring freshwater toxins in a sampling program. Nevertheless, the microsphere-based method as a semi-quantitative approximation to detect the toxins seems to require further refining of antibodies cross reactivity. This initial screening indicates presence or absence of toxins and reduces the number of samples to be analyzed by

Table 8. Samples from Turkey[a]

	Microsphere assay		UPLC-MS/MS		
Code	ATX-a (μg/L)	MCs (μg/L)	H_2-homoATX-a (μg/L)	H_2-ATX-a (μg/L)	MC-RR (μg/L)
T1	0.557	0.101		<LOQ	
T2	2.553		<LOQ	<LOQ	
T3	0.094	0.418			
T4	0.081	0.900	<LOQ	0.079	
T5	0.168	1.487	<LOQ	0.075	
T6	0.207	2.068			
T7	0.080	1.384	<LOQ	<LOQ	0.191
T8	0.123	0.725	<LOQ	0.055	
T9	0.318		0.021	0.075	
T10	0.096		<LOQ	0.088	
T11	0.083	0.326	<LOQ	0.066	
T12	0.127	0.132	<LOQ	0.066	
T13	1.474		<LOQ	0.089	
T14[b]			<LOQ	0.079	
T15[b]			<LOQ	0.059	
T16[b]			<LOQ	0.055	
T17[b]			<LOQ	0.035	
T18[b]			0.038	0.035	
T19[b]			<LOQ	0.037	
T20[b]			<LOQ	0.041	

[a]Positive results (μg toxin/L water collected) of samples collected in Turkey in 2012 and 2014 analyzed by microsphere-based assay and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method.
[b]Analyzed only by UPLC-MS/MS.
ATX-a = anatoxin-a; MCs = microcystins; H_2-homoATX-a and H_2-ATX-a = dihydro derivatives of anatoxin-a; <LOQ = traces below the limit of quantification.

confirmatory analytical methods, which typically are more laborious. Therefore, the combination of methodologies described in the present study should reduce the number of samples and the time of analysis, especially since both methodologies are designed as multidetection assays.

Acknowledgment—The research leading to these results has received funding from the following FEDER cofunded-grants: from CDTI and Technological Funds, supported by Ministerio de Economía y Competitividad, AGL2012-40185-CO2-01, AGL2014-58210-R, and Consellería de Cultura, Educación e Ordenación Universitaria, GRC2013-016; and from the European Union's Seventh Framework Program managed by the Research Executive Agency (FP7/2007-2013), 265409 MicroAqua. I. Rodríguez is supported by a fellowship from Subprograma de Formación de Personal Investigador (AGL2012-40185-CO2-01), Spain. Authors participated in the present study as follows: I. Rodriguez: sample processing, UPLC-MS/MS analysis, table elaboration, and manuscript preparation; M. Fraga: sample processing, microsphere-assay analysis, and manuscript preparation; A. Alfonso and L.M. Botana: analysis design, data supervision, and manuscript elaboration. Collection of 50 L of water samples and concentration to 1 L was performed as follows: D. Guillebault, L. Medlin, and J. Baudart: samples from France (Canet Lagoon and Mondony River); P. Jacob and K. Helmi: samples from France (tap water, Sine River, Oise River, and Marne River); T. Meyer and U. Breitenbach: samples from Germany; N.M. Holden and B. Boots: samples from Ireland; R. Spurio, L. Cimarelli, L. Mancini, and S. Marcheggiani: samples from Italy; M. Albay, R. Akcaalan, and L. Köker: samples from Turkey.

Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (amparo.alfonso@usc.es; Luis.Botana@usc.es).

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