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Coupling a bio-accumulator organism and MALDI-TOF MS: an early warning detection system for microcystins in water bodies

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Abstract Microcystins produced by freshwater cyanobacteria pose serious threats to human health and are a growing problem in drinking water supplies worldwide. Toxin detection and identification in water reservoirs when cyanobacterial density is still low is a key step to predict harmful algal blooms and to allow the safe use of the resource. For this purpose, developing sensitive and rapid methods of analysis is crucial. Adopting a strategy that couples the use of a filter-feeder organism (*Unio elongatulus,* Pfeiffer 1825 (Bivalvia: Unionidae)) with matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) technology, effective microcystin detection was achieved. The performances of MALDI-TOF MS, high-liquid-performance chromatography with diode array detector (HPLC-DAD) and enzyme-linked immunosorbent assays (ELISA) test were compared. The results obtained in this study suggest that the exploitation of an efficient bio-accumulator organism, coupled with a rapid and sensitive analytical method, can be a very useful strategy in monitoring programs for early and prompt risk management.

Keywords Microcystin detection · Sentinel organism · *Unio elongatulus* · MALDI-TOF MS · Early warning system

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Introduction

The increased frequency of toxic algal blooms (Paerl and Paul 2012; Taranu et al. 2015) requires the development of early warning strategies for prompt and efficient management of ecological and public health risks. In freshwaters, cyanobacteria are primarily responsible for the production of toxins, including the most common cyclic hepatotoxins microcystins (MCs) which are known to cause acute and chronic effects in both animals and humans (e.g., Chorus and Bartram 1999; Azevedo et al. 2002; Falconer 2005; IARC 2010; Peng et al. 2010; Giannuzzi et al. 2011; Lévesque et al. 2014). Among the more than 90 MC congeners identified, MC-leucine arginine (MC-LR, see Online Resource, Fig. S1) is one of the most commonly detected MC congeners in natural blooms (Chorus and Bartram 1999; Gupta et al. 2003; WHO 2011).

Since human exposure to MCs occurs most frequently through drinking or during recreational activities in which water is ingested or inhaled (IARC 2010), an efficient surveillance to detect the early stages of toxic cyanobacterial bloom

formation is mandatory. To this aim, the development of rapid, reliable, and sensitive monitoring methods is necessary.

The determination of MCs in the environment is a challenging issue and no unequivocal analytical strategy exists (Kaushik and Balasubramanian 2013; Moreira et al. 2014; Zhang and Zhang 2015). The current methods are immunoenzymatic (protein phosphatase inhibition assays, ELISAs) or chemical (liquid chromatography-mass spectrometry (LC-MS), HPLC, MALDI-TOF MS). ELISAs, now based on a diversity of kits using both monoclonal and polyclonal antibodies (An and Carmichael 1994; Ueno et al. 1996; Khreich et al. 2009), are effective and very sensitive (limit of detection >0.1 ppb) for rapid MCs screening, but lack specificity and may give false positive results in case of samples rich in organic matter (Botana 2014). Recently developed genetic methods are also effective and very sensitive (Singh et al. 2015), but they give indication only about the potential, not the real, ongoing toxicity of a cyanobacterial population or strain. Among the chemical methods, HPLC-DAD is still commonly used, although it does not always ensure optimum chromatographic separation, as more substances can be co-eluted. Detection is based on the retention time compared to a standard, but only few are commercially available. [UV-]DAD detection is susceptible to interferences from matrices and requires time-consuming sample purification and concentration (limit of detection >150 ppb) to achieve reliable quantification.

A more accurate determination can be achieved by mass spectrometry (MS) methods. Liquid chromatography in combination with multidimensional mass spectrometry (LC-MS^n), in particular, enables structural characterization and unambiguous identification of MCs, even if present in trace levels (limit of detection >0.01 ppb) (Allis et al. 2007; Draper et al. 2013; Ferranti et al. 2013; Greer et al. 2016). Although LC-MS/MS has become the preferred methodology for accurate determination of MCs, it is too time consuming to be the most suitable tool for early warning. In fact, only a MC per analysis run can be detected, while the application to commonly occurring congener mixtures requires extensive compound-dependent parameter optimization.

On the contrary, it is the easy and rapid analysis that has encouraged the use of an alternative mass spectrometry method—MALDI-TOF MS—for identification and rapid screening of MCs (Erhard et al. 1997; Welker et al. 2002; Ferranti et al. 2013; Flores and Caixach 2015). The method (limit of detection >10 ppb) presents many advantages for early warning: (1) tolerance of contaminants allows minimal sample handling for clean-up procedures; (2) intact cells/organisms can be analyzed; (3) small sample amount is needed; (4) simultaneous detection of various MC congeners in natural mixtures is possible; (5) analysis is very rapid (scan time < 30 s); (6) high resolution enables a reliable compound identification; and (7) confident-identification/structural-information is possible through post-source decay fragmentation of the molecular peak of the various MCs in the same sample. The main disadvantage of the method is the difficulty to achieve quantification (Howard and Boyer 2007a, b; Puddick et al. 2012), mainly when complex biological matrices are involved. However, quantification can have little relevance in an early warning system, which is simply intended to alert to the risk. A sustainable monitoring program can be accomplished in two steps: the first screening step, relying on a rapid, sensitive, and cheap method, is to decide whether to start the next procedure—longer and more expensive—for the quantification of the toxins.

This study aimed to develop a suitable strategy for a rapid and effective screening of MCs occurrence and identification, when the density of toxic cyanobacteria is far below the alert thresholds. To this purpose, an early warning system was tested, using MALDI-TOF MS as a rapid analytical method and a freshwater filter-feeder bivalve as bio-accumulator organism. Due to their high filtration rates mussels are ideal sentinel organisms widely used to improve the determination of trace contaminants when their concentration in water is lower than the detection limits (Farrington et al. 1983; Jeng et al. 2000; Gerhardt 2002; Dailianis 2011; De Solla et al. 2016). Acting as an integrator of toxic pollutant the mussel improves MC detection in spite of the high spatial-temporal variability of cyanobacteria populations, while phytoplankton sampling can fail to detect cyanobacteria at early stage of proliferation.

As bio-accumulator, we used *Unio elongatulus*, the most widely distributed and abundant native species in the study area. The performance of MALDI-TOF MS, HPLC-DAD, and ELISA test as suitable analytical methods for the first screening step for early warning were compared.

MCs can be accumulated by organisms in different forms (Williams et al. 1997; Takenaka 2001; Pires et al. 2004; Lance et al. 2010) that can be roughly divided into two groups: the covalently and irreversibly bound MC fraction, not extractable with methanol (or other) solvents, and the non-covalent, free fraction, extractable with methanol solvents. Here, only the presence of the free fraction was considered, assuming its presence as a proxy of in situ toxin production.

Materials and methods

Cyanobacterial cultures

Microcystis aeruginosa, Kützing, toxic strain PCC 7806, and *M. aeruginosa* non-toxic strain PCC 170 were kindly provided by Prof. Dr. Karl Gademann (Department of Chemistry, University of Basel). The PCC 7806 strain produces two MC variants: MC-LR and MC-[D-Asp_3]-LR (MC-DeLR) as major and minor component respectively. Both strains were cultured at 25 °C in three glass columns containing 0.5 L of BG11 medium (Rippka et al. 1979), mixed by bubbling a gas mixture consisting of air-CO_2 (*v*/*v* 97:3). Cultures were

illuminated with 70 μmol photons m^{-2} s^{-1} supplied by cold white lamps (Dulux L, 55 W/840, Osram, Italy) and with a 12-h light:12-h dark photoperiod. Incident light on the surface of the cultures was measured with a flat quantum radiophotometer (LICOR LI-250A, Biosciences).

Collection and maintenance of bivalves

Unio elongatulus specimens were collected in the oligotrophic Lake Maggiore (45° 50′ 19″ N, 8° 37′ 17″ E) and maintained in a 10 × 8 m artificial pond, 1.5 m deep. Water was renewed daily by pumping Lake Maggiore water from a depth of 20 m. MC absence in the pool water was checked periodically using ELISA test.

Bivalve acclimation

Prior to the experiments, mussels (6.4 ± 0.5 cm shell length) were acclimated in 80-L flow-through aerated glass aquaria at constant temperature (18 ± 1 °C), photoperiod 12-h light:12-h dark cycle and fed on the non-toxic *M. aeruginosa* strain for 1 week. Filtered (30 μm) lake water flow was regulated at 4 L h^{-1} to provide complete water renewal within the 24 h. Aquaria were provided with 10 cm clean artificial sand as bottom substrate. MC absence in the aquaria water was checked periodically using ELISA test.

Experimental setup

Filtered lake water at 18 ± 1 °C was used throughout the short-term and long-term feeding experiments, while dechlorinated tap water was used for the "short" (24 h) uptake experiment.

A mussel/water ratio of 1:6.5–7 L was adopted to avoid accumulation of excretion by-products, and water was completely renewed before each addition of food supply. Treated animals were fed on toxic *M. aeruginosa* strain PCC 7806, while control animals were fed on non-toxic *M. aeruginosa* strain PCC 170.

Both toxic and non-toxic *M. aeruginosa* culture cell densities were determined by a fluorometric probe (FluoroProbe, BBE-Moldaenke), using a calibration (Online Resource, Fig. S2) curve obtained by plotting in vivo cyanobacterial fluorescence versus cell density determined by cell counting. The samples were preserved in Lugol's solution for microscopic counting (Utermöhl 1958). MC content of the cyanobacteria supplied to the mussels at each feeding time was measured by HPLC-DAD.

Preliminary experiments

To verify *U. elongatulus* ability to accumulate MCs, identify the target organ, and test the extraction protocol, a preliminary experiment (Table 1, experiment 1) at high cyanobacterial density was performed. Mussels were placed in two 25-L glass tanks containing 20 L of filtered lake water and fed toxic *M. aeruginosa* strain PCC 7806 or non-toxic PCC 170 twice a day (morning and evening) making a density of 20,000 cells mL^{-1}, for 5 days.

Long-term experiments

Two long-term experiments were carried out at cell densities (Table 1, experiments 2 and 3) lower than the 20,000 cells mL^{-1} alert level proposed by WHO for recreational water (Chorus and Bartram 1999). Two 150-L glass tanks containing 100 L of filtered lake water and 15 mussels each were used for the first experiment. The experimental water was aerated to supply oxygen for the bivalves and mix *M. aeruginosa* cells and changed completely daily. Living toxic *M. aeruginosa* cells were administered to the animals every day, after water renewal, at mean concentrations of 12,200 (±800, SD) cells mL^{-1}, for 5 weeks. Concentration of MC in the aquarium was 2.8 (±0.6, SD) μg L^{-1} (ELISA test), corresponding to a daily dose of 1 (±0.2, SD) μg g^{-1} of mussels dry weight. The experiment was repeated with 9 mussels per tank (60 L filtered water) at lower cyanobacterial density (4500 (±300, SD) cells mL^{-1}) making a MC concentration of 1.1 (±0.2, SD) μg L^{-1} and a daily dose of 0.7 (±0.1 μg, SD) MC g^{-1} of mussels dry weight (Table 1, experiment 3) for 3 weeks. Three animals were randomly collected weekly for MCs analysis and were replaced by three other marked mussels in order to maintain the same animal density throughout the experiment. Only two specimens were collected at the end of the fifth week because of one casualty. The soft tissues were dissected, weighted, and frozen at −20 °C until extraction of the toxin. Three control animals were sacrificed at the start and three at the end of the experiment.

Short-term experiments

Short-term (24 h) exposure tests (Table 1, experiment 4) at decreasing toxic cyanobacteria densities were performed to evaluate the detection threshold of the proposed strategy (bio-accumulator + MALDI-TOF MS detection of MCs). Following 24 h acclimation, 36 mussels were randomly distributed in six glass tanks filled with 40 L of aerated dechlorinated tap water at 18 ± 1 °C. Cyanobacteria density in each treatment was checked every 20–30 min by a fluorometric probe (FluoroProbe, BBE-Moldaenke) and fresh *M. aeruginosa* stock aliquots added to keep the cell density constant over the exposure time. At the lower concentration tested, cell number was also estimated by direct counting, using an Olympus light microscope at 1000×, with 0.1-mm-deep counting hemocytomer (improved Neubauer chamber). After 24 h, the mussels were sacrificed and stored at −20 °C until posterior use.

Table 1 Set up features of feeding experiments

Feeding experiment		No. of animals	Aquarium water volume (L)	Uptake period	*M. aeruginosa* density (cells mL^{-1})
1	Cyanobacterial bloom Very high cell density	3	20	5 days	200,000 (±20,000, $n = 5$)[a]
2	Pre-cyanobacterial bloom High cell density	15	100	5 weeks	12,200 (±800, $n = 35$)[a]
3	Pre-cyanobacterial bloom Low cell density	9	60	3 weeks	4500 (±300, $n = 21$)[a]
4	Pre-cyanobacterial bloom Short-term uptake experiment	6	40	24 h	17,600 (±2600, $n = 24$)[a]
		6	40		12,300 (±2500, $n = 24$)[a]
		6	40		8800 (±2000, $n = 24$)[a]
		6	40		5400 (±800, $n = 30$)[a]
		6	40		2700 (±350, $n = 36$)[a]
		6	40		2000 (±450, $n = 44$)[a]

[a] Standard deviation (SD) of the analytical replicates

Three control animals were sacrificed at the beginning and three at the end of the experiment.

Extraction of MCs

Toxins present in cyanobacteria and in the mussels (extraction protocol was modified from Pires et al. (2004)) were extracted using a 75% (*v*/*v*) methanol/water solution prepared with 18 MΩ Milli-Q water, 0.1% formic acid (reagent grade, Sigma-Aldrich), and methanol (chromasolv for HPLC, Sigma-Aldrich).

Cyanobacterial cultures

MCs from *M. aeruginosa* strain PCC 7806 were extracted applying the following procedure: 5 mL of culture was sonicated for about 30–40 s (power 45%, pulse 0.5, sonotrode S2, Hielscher Ultrasonic Processor UP200S, 200 W, 24 kHz) and centrifuged at 12,000 rcf (at $T = 4$ °C) for 15 min. The supernatant was removed, and the pellets were extracted in 1.5 mL 75% MeOH (0.1% formic acid), homogenized (IKA Ultra-Turrax T25 homogenizer) at 9500 rpm for 5 min in a water/ice bath and centrifuged for 15 min (12,000 rcf, $t = -10$ °C). After supernatant removal, the pellet was re-extracted with the same procedure. The two methanol extracts were pooled, filtered with a disposable syringe filter (pore size 0.45 μm, PTFE), and analyzed (HPLC-DAD) for quantitative determination of MCs.

U. elongatulus

To verify the target organ for MCs detection in *U. elongatulus*, visceral sac and gills were separately extracted and analyzed. Extraction was performed by the following procedure: 1.0 g of homogenized tissue was added with 1 mL MeOH 75% (0.1% formic acid), sonicated five times for 40 s (power 75%, pulse 0.6, sonotrode S2, Hielscher Ultrasonic Processor UP200S, 200 W, 24 kHz) in water/ice bath, with 1 min pause in-between, and centrifuged for 15 min (12,000 rcf, $t = -10$ °C). The supernatant was collected, filtered with a disposable syringe filter (pore size 0.45 μm, PTFE), and analyzed by HPLC-DAD, ELISA test, and MALDI-TOF MS.

A simplified sample preparation procedure was also tested for MALDI-TOF MS analysis. Organs were simply homogenized, centrifuged, and the "crude" supernatant (no extraction solvent added) analyzed for MC presence. This procedure was not suitable for HPLC-DAD and ELISA test, which needed toxin extraction with solvent. Prior to ELISA test, a 200 fold dilution of methanol extracts was necessary to obtain reliable results.

Analytical methods

MALDI-TOF MS: sample preparation and spectra acquisition

U. elongatulus tissue extracts or crude supernatants were directly spotted in duplicates on a 48 spot steel target plate and air-dried at room temperature. Dried sample spots were overlaid with 1 μL of saturated alpha-cyano-4-hydroxycinnamic acid solution (Sigma-Aldrich, Switzerland) in 33% acetonitrile.

Spectra were acquired using an Axima confidence (Shimadzu-Biotech Corp., Japan) MALDI-TOF mass spectrometer in linear positive mode at a laser frequency of 50 Hz and within a mass range of m/z 500–3000 Da. Acceleration voltage was set at 20 kV, and the extraction delay time was 200 ns. A minimum of ten laser shots was used to generate each ion spectrum. For each sample, a total of 100 ion spectra per spot were averaged and processed using the Launchpad version 2.8 Software (Shimadzu-

Biotech Corp.). Each sample plate was externally calibrated by the use of the ProteoMass MALDI-MS Calibration Kit (MSCAL2, Sigma-Aldrich, Switzerland) with an accuracy of 50 ppm.

HPLC-DAD

Chromatographic determination was performed by a HPLC-DAD (UltiMate 3000 LC system, Thermo Scientific). The system consisted of a low-pressure quaternary pump, an autosampler, a thermostated column compartment and a UV-VIS diode-array detector. The separation of the analytes was achieved with a Luna 5 μm C18(2) column (250 mm × 4.6 mm id; particle diameter 5 μm, Phenomenex, USA); the column was protected by a pre-column (C18). The method used was modified from Guzzella et al. (2010). After sample injection (100 μL of the extract), a gradient program that ramped from 75% mobile-phase A (Milli-Q water with 0.1% formic acid) to 100% mobile-phase B (acetonitrile with 0.1% formic acid) in 30 min with a hold for 20 min provided sufficient resolution of all the compound of interest. The column was re-equilibrated between samples by linear ramping to 70% mobile-phase A for 5 min and maintenance for 10 min before sample injection. MC-LR and MC-[D-Asp_3]-LR for instrumental calibration were obtained by ALEXIS-Enzo Life Sciences. The chromatograms were recorded at 238 nm, and the spectra were recorded between 200 and 400 nm.

ELISA test

ELISA determination of microcystins was accomplished using the Quantiplate Kit for Microcystin (EnviroLogix Inc., ME, USA), with optimal detection range of 0.16–2.5 ppb (i.e., μg L^{-1}).

All samples were analyzed in duplicate. The resulting MC concentrations were calculated from the MC-LR standard calibration curve and were estimated as MC-LR equivalents. Samples which were under the range of the standard curve of each ELISA were considered to be below the limit of detection (<0.16 ppb).

Statistical analyses

Statistical analyses were performed on data presented in Table 4 (analyses of visceral sac methanol extracts). The different performance of the three methods (HPLC-DAD, MALDI-TOF MS, and ELISA test) was evaluated by chi-square test (R software).

Results

MC extraction from *U. elongatulus:* evaluation of analytical methods and critical organs

To evaluate the matrix effect (i.e., effects of mussel tissue) on the outcomes of the analytical methods, we performed blank determinations in non-exposed mussels and studied the response of the analytical methods on spiked samples (i.e., non-exposed mussel extract plus MC-LR). As expected, background microcystin values determined in non-exposed mussels were below detection limits of both HPLC-DAD and MALDI-TOF MS. On the contrary, the ELISA test gave positive responses, above the upper limit of the analytical range of the method, evidencing a severe matrix interference. Negative response of ELISA test on the extracting solvent excluded the possibility that false positives were due to the presence of MeOH. To check matrix interference, *U. elongatulus* extracts were progressively diluted and spiked with MC-LR (Online Resource, Table S1). Extracts had to be diluted 200 times to get reliable results from ELISA test.

Extraction efficiency was also checked, evaluating MC-LR recovery of spiked samples. Triplicate visceral sac samples (1.0 g) were respectively spiked with 2.0 and 10.0 μg g^{-1} MC-LR amounts (MC-LR limit of detection was about 0.15 μg mL^{-1} for HPLC-DAD method and 0.16 μg L^{-1} for the ELISA test kit used in this work). MALDI-TOF MS analysis was only qualitative, but MALDI-TOF MS spectra evidenced the presence of MC-LR in all the samples, as shown by the spectra of five successive extractions of an aliquot spiked with 2.0 μg of toxin (Fig. 1).

Accordingly, after proper dilution, ELISA test revealed a quantitative recovery (Online Resource, Table S2), with a moderate enhancement due to matrix effect (Pires et al. 2004).

Analysis of gills and visceral sac confirmed this latter as the target organ for MCs detection in *U. elongatulus* (Online Resource, Table S3).

Microcystin detection in pre-bloom conditions

During the long-term experiments, three bivalves died on the first and two on the fifth week at 12,200 (±800, SD) cells mL^{-1}, while only two animals died on the third week at 4500 (±300, SD) cells mL^{-1}. No adverse effects from the feeding behavior were found in the remainder of the bivalves. Clearance rate was determined for bivalves exposed to higher cell density. It ranged from 233 to 433 mL $ind.^{-1}$ h^{-1} as a consequence of active feeding (Online Resource, Table S4); the water always became clear, and aggregations of *Microcystis* cells, such as feces and pseudo-feces excreted by the bivalves, were laid on the sand 1 day after administration of the cells.

The number of specimens "positive" to MCs in the long-term experiment at the higher cyanobacterial density (Table 2)

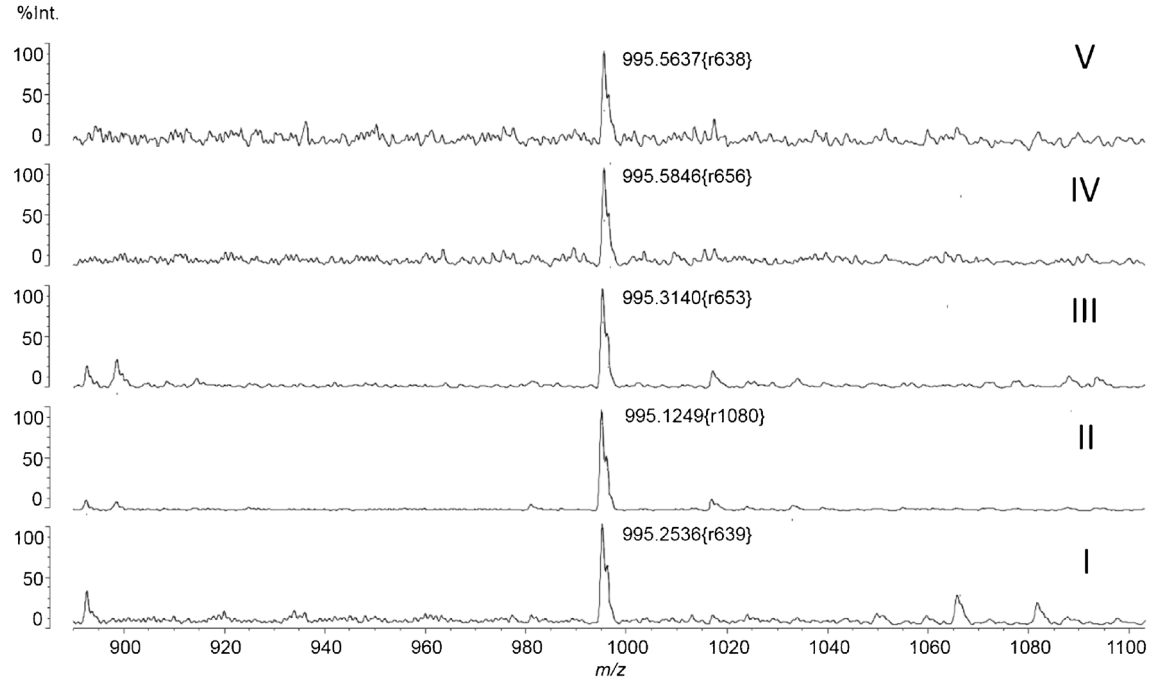

Fig. 1 MALDI-TOF MS spectra of five successive extraction cycles (curves I–V) of a 1.0 g aliquot of visceral sac sample spiked with 2.0 μg of MC-LR. MC-LR molecular peak (theoretic molecular peak = 995.20 *m/z*) is clearly visible in the spectrum of each extract. The resolution of the peak is reported in brackets

evidenced that all the three methods were able to detect MCs in almost all the samples after 1-week exposure. MALDI-TOF MS was efficient in 100% of the samples extracted, and in 80% of crude samples obtained as described in "*U. elongatulus*" section. ELISA test also evidenced the presence of MCs in all the sample extracted but was not suitable to analyze crude samples. Finally, HPLC failed in 14% of samples extracted and was unable to analyze crude samples.

Lowering the cyanobacterial supply at 4500 (±300, SD) cells mL^{-1} per day (0.7 (±0.1, SD) μg MC g^{-1} of mussels d.w.) enlightened the great accumulation ability of mussels and showed that MALDI-TOF MS could detect both MC-LR and MC-DeLR congeners in all the extracted and crude samples since the first week of exposure. MC presence in all the extracts of the exposed animals was also evidenced by ELISA test, while HPLC-DAD detection occurred from the second week of exposure (Table 3).

Short-term experiments ("Short-term experiments", Table 1, experiment 4) showed that at high cell density, after a single day's exposure, all the methods performed similarly (Table 4). HPLC-DAD was not able to measure the MC accumulation at cell concentrations equal to or lower than 5400 (±800, SD) cells mL^{-1}, while ELISA test and MALDI-TOF MS allowed MC detection in 66–100% of mussels down to the lowest cell density considered (2000 (±450, SD) cells mL^{-1}).

Bio-concentration factor estimated at the lowest cyanobacterial density ("Short-term experiments", Table 1, experiment 4) evidenced that mussels concentrated MCs 300–4000 times within 24 h (Table 5).

Discussion

Sample manipulation, matrix interference, and methods' analytical performance

To evaluate which is the organ where MCs are mainly accumulated in *U. elongatulus*, gill and visceral sac extracts were analyzed. MCs are hepato-toxins, and hapato-pancreas is expected to be the organ where MCs are mainly accumulated.

Table 2 Microcystin detection in the visceral sac of *U. elongatulus* exposed at a cyanobacterial density of 12,200 (±800, SD of analytical replicates, $n = 35$) cells mL^{-1}, corresponding to MC concentration of 2.8 (±0.6, SD of analytical replicates, $n = 35$) μg L^{-1}

Feeding period (week)	Visceral sac methanol extract		Visceral sac supernatant [a]
	HPLC-DAD no. of positive samples [no. of total samples]	MALDI-TOF MS and ELISA no. of positive samples [no. of total samples]	MALDI-TOF MS no. of positive samples [no. of total samples]
Control	0 [3]	0 [3]	0 [3]
1	2 [3]	3 [3]	3 [3]
2	2 [3]	3 [3]	3 [3]
3	3 [3]	3 [3]	2 [3]
4	3 [3]	3 [3]	2 [3]
5	2 [2]	2 [2]	2 [2]
Control	0 [3]	0 [3]	0 [3]

[a] That is, "crude" sample

Table 3 Microcystin detection in the visceral sac of *U. elongatulus* exposed at a cyanobacterial density of 4500 (±300, SD of analytical replicates, $n = 21$) cells mL^{-1}; corresponding to a MCs concentration of 1.1 (±0.2, SD of analytical replicates, $n = 21$) μg L^{-1}

Feeding period (week)	Visceral sac methanol extract HPLC-DAD no. of positive samples [no. of total samples]	Visceral sac supernatant [a]	
		MALDI-TOF MS and ELISA no. of positive samples [no. of total samples]	MALDI-TOF MS no. of positive samples [no. of total samples]
Control	0 [3]	0 [3]	0 [3]
1	0 [3]	3 [3]	3 [3]
2	2 [3]	3 [3]	3 [3]
3	3 [3]	3 [3]	3 [3]
Control	0 [3]	0 [3]	0 [3]

[a] That is, "crude" sample

However, the authors intended to exploit the MC accumulation ability of *U. elongatulus* very pragmatically, also considering the amount of cyanobacterial cells (and, consequently, of MCs) present in the gills or in the digestive gut. Cyanobacteria can be indeed concentrated in the gut or in the gills, even if temporary (MCs in the tissues are also depurated, but elimination occurs in much more time (Yokoyama and Park 2003; Pires et al. 2004; Smith and Haney 2006), and this can be advantageous for their detection. Analyses confirmed that the visceral sac (containing hapato-pancreas and gut) was the target organ for MCs detection in *U. elongatulus* (Online Resource, Table S3).

Since this study had the purpose to develop a strategy for an early but also rapid and effective screening of MC occurrence in water bodies, one of its goal was to minimize the complexity of sample purification steps, maintaining an acceptable MC detection limit. This requirement can be not straightforward and easy to achieve in a biological matrix.

Commercial available ELISA test kits (as the one used in this study, described in "ELISA test" section) are validated only for water samples. Indeed, a check of the response of the ELISA kit used evidenced that the biologic matrix interfered, invalidating the test outcome, which was always positive and exceeding the analytical range of the test. Only a previous 200-fold dilution of the extract before ELISA test application ensured a neutralization of the matrix effect (Online Resource, Table S1), which was mandatory to get reliable results.

The detection of MCs in *U. elongatulus* samples evidenced that HPLC-DAD was the least efficient method, while ELISA test and MALDI-TOF MS were best methods and showed similar performances.

Improved microcystin rapid detection through the combined use of a bio-accumulator organism and MALDI-TOF MS

The potentiality of the combination of a bio-accumulator organism and MALDI-TOF MS methodology for the detection of MCs at low concentrations was considered in relation to both the extraction procedure adopted and the particular organism used as bio-accumulator. MALDI-TOF MS was evaluated versus ELISA and HPLC-DAD, two common methods used for MC detection (Roegner et al. 2014; Moreira et al. 2014), taking into consideration the limits above discussed.

Bivalves were exposed to decreasing toxic cyanobacteria densities, and for gradually shorter periods. Cyanobacteria densities were chosen taking into account safeguard cell level and MC concentration suggested by WHO. WHO guidelines for safe recreational water environments (Chorus and Bartram

Table 4 Microcystin detection in *U. elongatulus* visceral sac by the compared analytical methods

Cyanobacterial density (cell mL^{-1}) [MCs_{tot} (μg L^{-1})]	Visceral sac methanol extract			χ^2 [p]	Visceral sac supernatant[a]
	HPLC-DAD no. of positive samples [no. of total samples]	ELISA test no. of positive samples [no. of total samples]	MALDI-TOF MS no. of positive samples [no. of total samples]		MALDI-TOF MS no. of positive samples [no. of total samples]
Control [0]	0 [6]	0 [6]	0 [6]	NA [NA]	0 [6]
17,600 ± 2600[b] [4.30]	4 [6]	6 [6]	5 [6]	2.4 [0.301]	4 [6]
12,300 ± 2500[b] [3.08]	3 [6]	6 [6]	6 [6]	7.2 [0.027]	5 [6]
8800 ± 2000[b] [2.31]	2 [6]	6 [6]	6 [6]	10.3 [0.006]	4 [6]
5400 ± 800[b] [1.27]	0 [6]	5 [6]	5 [6]	11.2 [0.004]	3 [6]
2700 ± 350[b] [0.45]	0 [6]	6 [6]	4 [6]	12.6 [0.002]	2 [6]
2000 ± 450[b] [0.25]	0 ([6]	5 [6]	5 [6]	11.2 [0.004]	0 [6]

Each row represents one short-term (24 h) uptake experiment

NA not applicable

[a] That is, "crude" sample

[b] Mean and standard deviation of analytical replicates are reported, see also Table 1

Table 5 Short-term uptake experiment at 2000 (±450, SD of analytical replicates, $n = 44$) cells mL^{-1} toxic *M. aeruginosa*

Bivalve specimen	MCs in visceral sac [ppb]	Bio-concentration factor (ppb MC_{tissue}/ppb MC_{water})
1	392	1568
2	<0.16	0
3	628	2512
4	484	1936
5	988	3952
6	88	352

ELISA determination of total microcystin content in *U. elongatulus* visceral sac samples and related bio-concentration factor

1999) suggest a first alert level of 20,000 cells mL^{-1} for cyanobacteria. At this cyanobacterial density, if the strain is toxic, 2–10 μg L^{-1} MCs should be expected (Falconer et al. 1999). These values imply that the WHO provisional drinking-water guideline value of 1 μg L^{-1} MCs (WHO 1998; WHO 2011) can be indicatively related to a cyanobacterial density of 2000–10,000 cells mL^{-1}. Consequently, bivalves (experiments 2, 3, and 4, Table 1) were exposed to cyanobacteria in densities lower than 20,000 cells mL^{-1}, down to 2000 cells mL^{-1}, which was the lowest cell density that our experimental apparatus permitted to check in real time.

The efficacy of *U. elongatulus* as fast bio-accumulator for early warning was evidenced by the average concentration factor (1720 ± (1450, SD of experimental replicates, $n = 6$) measured in the tissues of the specimens exposed for only 24 h to the lowest toxic cyanobacterial density (Table 5). Results (Table 4) also evidenced the poor detection ability of HPLC-DAD compared to ELISA and MALDI-TOF MS methods at decreasing cyanobacteria densities. MALDI-TOF MS and ELISA test were both able to detect MCs in methanol extracts, but only the mass method could identify MCs and was suitable to analyze crude extracts down to a MC concentration in water as low as 0.45 μg L^{-1}. The possibility to perform MALDI-TOF MS analyses directly on crude extracts provides a promising shortcut with the benefit of saving time and costs of analyses. The lack of false positive signals in any of the experiments performed in this study is encouraging as well; it suggests that even a single animal found to be positive to MC analysis can indicate the presence of toxins in the water body, permitting to direct and optimize analyses in water to confirm the actual presence of MCs and their concentration. However, deeper investigations are required in this direction. A mollusk may present some MCs in its tissues far longer after the presence of MCs in the water (Pires et al. 2004). Indeed a part of MCs can be covalently bound to tissues and therefore eliminated less rapidly compared to free MCs. This timing of eliminations and the proportions of free and bound MCs have already been demonstrated in mollusc gastropods (Lance et al. 2010). Therefore, the study of MC accumulation and depuration kinetics in *U. elongatulus* is necessary for an effective exploitation of this species as bio-accumulator organism.

Coupling MALDI-TOF MS method and a bio-accumulator (*U. elongatulus*, in this case) the presence of sub-μg L^{-1} quantities of MCs in water can be shown. In spite of the large individual variability, these animals are very effective and fast toxin concentrators even with as low as 0.25 μg L^{-1} concentrations of MC in water (Table 5). These characteristics make freshwater filter-feeder mussels promising candidates as bio-accumulator organisms for early and prompt risk assessment deriving from MCs, very useful in monitoring and screening programs, especially if coupled with rapid detection methods. Indeed, MALDI-TOF MS provides a rapid and straightforward tool for qualitative detection of MCs. This strategy allows a sensitivity comparable to ELISA test results to be achieved in water samples, but with the considerable advantage to establish the MC identity and to avoid false positive. Early and reliable MC detection is indispensable to ensure public health without causing economic loss deriving from risk under/overestimation and the adoption of late, and consequently more drastic, measures.

Conclusions

The results reported in this paper show that improved MCs detection can be achieved by coupling freshwater filter-feeder organisms, as bio-accumulators, and MALDI-TOF MS, as a rapid analytical method. MALDI-TOF MS is more sensitive than HPLC-DAD method and permits to overcome the lack of specificity that characterizes ELISA test.

The speed of analysis coupled to a simple and rapid sample preparation makes this technique well suitable for screening surveys and early microcystin detection and identification. On the other hand, filter-feeder species are optimal bio-accumulator organisms, able to store up many toxicants. In this work, the ability to bio-accumulate fast microcystins has been studied, but the same species could turn out to be suitable to monitor the presence of other toxicants than MCs. We showed that these animals are able to concentrate large amounts of toxin in a short period, but further studies are needed for the application of the proposed system in long-term monitoring programs. Finally, the authors believe that a more reliable detection of the MC presence in the water body can be achieved exploiting a sentinel species, because filter-feeders provide an integrated value of the density of toxic cells in the water body. Conversely, phytoplankton sampling can be biased by cell density variability due to spatial and temporal heterogeneity, hydrological conditions, and the presence or absence of surface accumulations of algae, and can even completely miss the cyanobacterial proliferation, especially in its early stage.

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