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The characterisation of the biotoxins produced by a cyanobacteria bloom in Lake Averno by different techniques based on LC-MS

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Key words: cyanobacteria; microcystins; anabaenopeptins; ion trap LC-MS; MALDI-TOF/MS

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Abstract

The cyanobacteria (blue-green algae), widely known worldwide for causing blooms in eutrophic lakes and drinking water reservoirs, produce many groups of biotoxins; among these, microcystins (MCs) are highly toxic cyclic heptapeptides, that have caused several animal and human poisoning outbreaks. In this paper we present a method for the analysis of four MCs by ion trap LC-MS and MALDI-TOF/MS. The data were compared to evaluate the performance and reliability of the different MS detection systems. The method was applied to the analysis of water and algae samples from Lake Averno, near Naples, as a consequence of a cyanobacteria bloom. The analysis of algae cell extracts showed that no contamination by known microcystins was present, but three main substances were detected. MALDI-TOF/MS was successful in wide range screening of the biotoxins in the samples, leading to the identification of Anabaenopeptin B and Anabaenopeptin F as the major contaminants, on the basis of the literature mass spectrometry data. The structure of the third compound was not identified, and is under further investigation. This way, we characterised the biotoxins produced in Lake Averno, for an evaluation of the risk related to their presence.

Introduction

Cyanobacteria, known also as blue-green algae (BGA), are prokaryotic organisms widely distributed in surface waters, producing blooms and scums. These phenomena in lakes and freshwaters are seasonal, caused by several species belonging to the genera *Microcystis*, *Planktothrix*, *Anabaena*, *Nostoc*, *Oscillatoria*, the most frequently observed. Some BGA genera produce biotoxins, that can be toxic to wildlife, domestic livestock and even to humans; not all cyanobacteria strains are toxic within one species.

Microcystins (MCs) are the most abundant and dangerous toxins produced by cyanobacteria; MCs are cyclic heptapeptides of about 1000 g mol⁻¹, showing the general structure cyclo[D-Ala⁽¹⁾–L-X⁽²⁾–D-MeAsp⁽³⁾–L-Z⁽⁴⁾–Adda⁽⁵⁾–D-Glu⁽⁶⁾–Mdha⁽⁷⁾] (Figure 1). L-X and L-Z in position 2 and 4 of the ring are variable L-amino acids, D-MeAsp is D-erythro-β-methylaspartic acid, Mdha is N-methyldehydroalanine. The most unusual structural feature of these biotoxins is the presence of ADDA moiety, that is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid, which is essential for the toxicity of MCs. Each microcystin is named depending on the identity of X and Z amino acids. MCs are hydrophobic compounds, exhibiting high structural variability because of:

- 1) the identity of the amino acid residues in position 2 and 4 of the ring;
- 2) several side chain modifications occurring, such as de-methylation in position 3 and 7 (desMe-derivatives);
- 3) and variation of the ADDA moiety (Table 1).

After MC-LR (microcystin-Leucine-Arginine), the first one identified [Carmichael, 1994], more than 75 MCs have been isolated and characterised worldwide. MCs are genotoxic [Bouaicha, et al. 2005], inhibit protein phosphatase 1 (PP-1) and 2 (PP-2A) activities, thereby causing both acute and chronic diseases. Liver is the main target of MCs, followed by lungs and kidney [Chorus, 1999]. Moreover, MCs are nephrotoxic and can act as tumour promoters. MCs can be lethal for eutrophic aquatic organisms [Zimba, et al. 2001], but they can also accumulate in tissues and transfer to other animals and to humans through food chain [de Magalhaes et al. 2001]; apart large scale death of aquatic animals [Zimba, et al. 2001] sub-lethal exposure to MCs caused several intoxication outbreaks to consumers [Carmichael, 1994; Carmichael, 1992; Carmichael, 1997; Falconer et al. 1983; Falconer, 1998; Rodger et al. 1994; Harada, and Teuji 1998; Teixeira et al 1993; Pouria et al.1998; Azevedo, and Carmouze, 1994]. The World Health Organization (WHO) set for MC-LR a Tolerable Daily Intake (TDI) of $0.04 \mu\text{g kg}^{-1}$ body weight/per day for humans [WHO, 1998]; a provisional limit at $1 \mu\text{g L}^{-1}$ in drinking water has been suggested, until further studies regarding the carcinogenesis potency of MCs will be completed [Chorus, 1999]. The Italian law set $0.84 \mu\text{g L}^{-1}$ total MCs as the tolerable limit in freshwaters [Italian Ministry of Health, 1998], to prevent possible intoxication due to swimming and recreation activities. Apart MCs, many other biotoxins produced in freshwaters are well known, such as cylindrospermopsin, anatoxin-a and analogues, anabaenopeptins, nodularins, cyanopeptolins, microviridins, oscillapeptins, saxitoxin-analogues [Welker, et al. 2002].

As the presence of cyanobacteria in Italian freshwaters is common in the last years, it is urgent to monitor the risks for consumer's health deriving from possible intake of

biotoxins from contaminated foods. In this work, the study of algae biotoxin contamination from a bloom of *Planktothrix rubescens* in the Lake Averno, near Naples, is described. *P. rubescens* is a cyanobacterium already known for producing several biotoxins, that is MCs, anabaenopeptins, aeruginosins, microginins, cyanopeptolins and microviridins [Zweigenbaum et al. 2000; Welker et al 2004; Ernst, et al. 2006; Erhard et al. 1999]. The red bloom from *P. rubescens* in the Lake Averno was studied between March and June 2007, and water and algae samples were analysed by different confirmatory methods based on ion trap (IT) and matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry. The performances of these methods and MS techniques used were reported, in terms of repeatability, trueness, limit of quantification, to describe their relative potency as analytical tools for rapid, sensitive and reliable detection of early contamination of water bodies.

Experimental

Chemicals and reagents

Microcystin-LR (MC-LR) and Microcystin-RR (MC-RR) were supplied by DHI Water and Environment (Danmark); Microcystin-LF (MC-LF) and Microcystin-LW (MC-LW) were supplied by Vinci Biochem (Vinci, Italy). All the reference materials were of analytical grade purity.

Trifluoroacetic acid (TFA), HPLC grade acetonitrile, methanol and glacial acetic acid (99%) were from Carlo Erba (Milan, Italy). HPLC grade water was produced using a

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4 MilliQ system (Millipore, Bedford, MA, USA). α -cyano-4-hydroxycinnamic acid was
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6 supplied by Fluka (Switzerland).
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11 C18 end-capped (EC) solid-phase extraction (SPE) cartridges with 1 g sorbent bed and
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13 6 mL reservoir volume were purchased from Isolute (Step-Bio, Bologna, Italy).
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18 19 *Standard solutions of MCs*

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21 MC-RR and MC-LR reference materials were purchased in standard stock solutions at
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23 $10 \mu\text{g mL}^{-1}$ in methanol. To prepare the standard stock solutions of MC-LF and MC-
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25 LW at $10 \mu\text{g mL}^{-1}$, 2.5 ± 0.1 mg of each compound were weighed and dissolved in 2.5
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27 mL of methanol. A MCs mix standard solution at $2.5 \mu\text{g mL}^{-1}$, containing the four
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29 compounds, was prepared by mixing equal volumes of each standard stock solution at
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31 $10 \mu\text{g mL}^{-1}$. All the standard stock solutions were stable for at least six months if stored
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33 at -20°C . MCs mix working standard solutions at 125, 250, 500, 1000 ng mL^{-1} were
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35 prepared daily from the standard mix solutions at $2.5 \mu\text{g mL}^{-1}$ by diluting with
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37 methanol.
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45 46 *Water and algae samples*

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48 A red bloom of *Planktothrix rubescens* was observed in the brackish Lake Averno, near
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50 Naples, between March and June 2007. The samples of water and algae from Lake
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52 Averno were drawn by the local health authority, in different points at 1 m deep. The
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54 samples were immediately stored at 4°C , and analysed within 48 h.
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Water and algae sample clean up

Water sample clean up was carried on SPE C18 end-capped cartridges, rinsed with 5 mL methanol and 5 mL MilliQ water; then, 20 mL of sample were loaded by gravity, the cartridge was washed with 5 mL of MilliQ water, dried under vacuum for 30 min. The SPE column was eluted with 5 mL TFA 1% in methanol v/v; the sample was dried under a nitrogen stream at 40°C, then re-dissolved in 1 mL methanol and analysed by ion trap LC/ESI-MS/MS and MALDI-TOF/MS.

The algae samples (50 mL in volume) were centrifuged into a falcon tube at 1086 g for 10 min, then the pellet was separated and suspended in 500 µL MilliQ water, transferred into a 2 mL eppendorf tube and sonicated for 10 min. The sample was centrifuged at 18790 g, the supernatant separated and analysed by ion trap LC/ESI-MS/MS and MALDI-TOF/MS.

Ion trap LC/ESI-MS/MS analysis

Analyses were performed using a LC/ESI-MS system, equipped with P2000 pump, SCM 1000 degasser, AS1000 autosampler and LCQ Advantage ion trap mass spectrometer with an electrospray ion source (ESI) (ThermoElectron, Milan, Italy). Chromatographic separation was performed injecting 50 µL sample volume on a 4 µm particle 250 × 3.0 mm Max RP 80 Å Synergi stainless steel column (Phenomenex, Torrance, CA, USA), at 0.3 mL min⁻¹ flow rate, using 0,05% TFA in water as mobile phase A, and 0,05% TFA in acetonitrile (ACN) as mobile phase B. The chromatography was carried on by linear gradient at room temperature, according to the following

programme: 2 min at 30% B, then from 30% B at time 2 to 100% B in 16 min, holding on for 8 min, finally to 30% B in 3 min; the equilibrium time between runs was 12 min. The mass spectrometer was periodically calibrated with standard solutions of Ultramark, caffeine and Met-Arg-Phe-Ala peptide provided by the manufacturer. During the LC/ESI-MS/MS experiments, mass spectra were acquired in the positive ionisation mode; the spectrometer parameters were optimised by tuning on the $[M+H]^+$ ions of MC-RR, at m/z 1038.5, and of MC-LF, at m/z 986.3. Tuning was performed at 0.3 mL min⁻¹ LC flow rate. The following experimental LC/ESI-MS/MS parameters were set: capillary temperature 300 °C, spray voltage 4.5 kV, microscan number 3, maximum inject time 200 ms. Further experimental conditions (collision energy, isolation width), and the diagnostic ions for MS/MS qualitative analysis, are also reported (Table 2). The LC/ESI-MS/MS analysis was performed by monitoring the signals of the precursor ion → product ion transitions (selected reaction monitoring, SRM mode) from the LC/ESI-MS/MS dataset of each MC; to improve method specificity, a SIM event was selected for both MC-LW and MC-LF, apart SRM events for MS/MS analysis (Table 2). The data were acquired and processed using the XcaliburTM software, version 1.3, from ThermoElectron.

Both blank fresh water samples and water samples spiked at 10 ng mL⁻¹ of each MC were analysed. The chromatograms obtained extracting the precursor ion→main product ion transition (SRM) signals from the LC/ESI-MS/MS dataset were integrated to calculate the calibration curves. Calibration curves for testing method linearity in solvent were calculated by linear regression, using standard solutions in methanol 125 – 250 – 500 – 750 - 1000 ng mL⁻¹. Method linearity was tested also by standard

calibration curves in matrix. To this aim, uncontaminated water samples were purified, then spiked with the standards prior to the chromatographic analysis; the data were managed by linear regression of peak area versus the concentrations of each MC added at 10 - 20 -50 - 125 ng mL⁻¹. During each working session, we analysed a blank reagent, blank and spiked samples.

MALDI-TOF/MS analysis

Mass spectrometry experiments were carried out on a PerSeptive Biosystems (Framingham, MA) Voyager DE-PRO instrument equipped with a N₂ laser (337 nm, 3 ns pulse width). α -cyano-4-hydroxycinnamic acid was used as matrix; the sample (1 μ L from a solution in water) was loaded on the target and dried. Afterwards, 1 μ L of a mixture composed by 0.1% TFA in H₂O/acetonitrile (1/1, v/v), and 10 mg mL⁻¹ of matrix were added. For each sample, mass spectrum acquisition was performed in the positive linear or reflector mode accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with low-mass peptide standards (PerSeptive Biosystems, Framingham, MA).

Specificity, recovery and precision data

Method specificity was assessed by testing 20 uncontaminated water samples from different origin (lakes, ponds), showing no matrix interference in the LC-MS chromatograms. All samples were analysed by ion trap LC-MS/MS and MALDI-TOF/MS. Method repeatability was calculated analysing six different water samples spiked at 10 ng mL⁻¹, over two different days. The mean recoveries were also calculated. The limit of quantification (LOQ) for each MC was evaluated analysing by

both ion trap LC-MS/MS and MALDI-TOF/MS some water samples spiked at 0.2, 0.5, 1, 2 and 5 $\mu\text{g L}^{-1}$ of each MC.

Results and Discussion

Ion trap LC/ESI-MS/MS analysis

The four MCs were completely resolved by the LC chromatography. A comparison between the SRM and SIM chromatograms of a mix standard at 500 ng mL^{-1} and a water sample spiked at 10 ng mL^{-1} shows that MC-LR MC-LW and MC-LF were all identified on the basis of the precursor ion and two product ions (Figure 2); only MC-RR was identified on the basis of two product ions. No matrix interference was observed. The response of the ion trap MS detector was linear both in solvent and in matrix, all over the concentration ranges studied ($0.9772 < R^2 < 0.9915$ for MC-RR; $0.9655 < R^2 < 0.9915$ for MC-LR; $0.9604 < R^2 < 0.9994$ for MC-LW; $0.9812 < R^2 < 0.9947$ for MC-LF).

MALDI-TOF/MS analysis

MALDI-TOF/MS was able to detect all the four MCs studied; the spectrum of a standard mix at 20 ng mL^{-1} showing the molecular ions of MC-RR, MC-LR, MC-LW and MC-LF is reported (Figure 3). The results show the response of the detector was much better for MC-RR and MC-LR than for MC-LW and MC-LF, similarly to the ion trap MS analysis. The response of the TOF MS detector was linear in the range 10 – 250 ng mL^{-1} ; the $R^2 = 0.9981$ for MC-RR is reported as an example. The run-to-run stability of the detector was tested over a period of several hours, analysing a mix standard at 20 ng mL^{-1} .

Specificity, recovery and precision data

No matrix interference was observed during both ion trap LC/ESI-MS/MS and MALDI-TOF/MS, proving method specificity. The trueness and precision of the method were assessed on the basis of repeatability and recovery data (Table 3). The mean recoveries were higher than 72.6%, apart for MC-LW in MALDI-TOF/MS, repeatability data, showing CV% lower than 18.2% accounted for satisfactory method reliability. Moreover, the limit of detection (LOD) was calculated for each microcystin, on the basis of the signal-to-noise (S/N) ratio = 3; the LOQ for each compound was experimentally determined by repeating tests at different decreasing concentrations. The results (Table 3) show the ion trap MS detector is slightly more sensitive than TOF MS, even if both allow determine the four MCs down to 0.5 – 2 ng mL⁻¹.

Water and algae samples from Lake Averno

No significant signal was observed analysing water samples drawn from the Lake Averno, neither by ion trap LC/ESI-MS/MS nor by MALDI-TOF/MS. The samples were drawn during the last phase of the bloom, therefore the absence of MCs could be due to a lower production by the cyanobacteria. Similarly, none of the four MCs studied was detected in algae samples by ion trap LC/ESI-MS/MS. Unlike, the analysis of algae extracts by MALDI-TOF/MS detected the presence of three main substances (Figure 4); surprisingly, no signal was detected in the mass range area of known microcystins, thus excluding this kind of contamination. The post source decay (PSD) mode allowed to detect the product ions from the selected molecular ions at 837 and 851 *m/z*, respectively; the case of the most abundant compound is reported (Figure 5). A

comparison with the data in the literature [Erhard et., 1999] allowed us to identify these biotoxins as Anabaenopeptin B and Anabaenopeptin F, respectively. Anabaenopeptins are a group of hexapeptide biotoxins from cyanobacteria, less toxic than MCs, showing the general structure $\text{cyclo}[\text{Phe}^{(1)}\text{-MeAla}^{(2)}\text{-HTyr}^{(3)}\text{-Val}^{(4)}\text{-Lys}^{(5)}]\text{-CO-L-X}^{(6)}$; HTyr is homotyrosine, MeAla is *N*-methylalanine, CO is a carbonyl bridging group, and L-X in position 6, linked to the peptide ring, is a variable L-amino acid (usually Tyr, Arg, Lys or Phe). The anabaenopeptin B structure reported in Figure 5 shows an arginine residue in position 6; anabaenopeptin F is characterised by the presence of isoleucine in position 4 and arginine in position 6. It was not possible to identify unambiguously the third compound, on the basis of the molecular mass and the PSD spectrum; no data available in the literature for MCs, anabaenopeptins, cyanopeptolins, nodularins were comparable. Therefore, further studies are in progress to define completely its molecular structure, and, accordingly, to evaluate the toxicity of the bloom observed in the Lake Averno.

A monitoring of many Italian lakes to detect the contamination by MCs and other biotoxins was carried on during the last three years by the Italian Istituto Superiore di Sanità, using both ELISA and triple quadrupole LC-MS/MS (Milena Bruno, personal communication); the survey allowed to characterise biotoxin contamination in many lakes. The results herein described give further evidences that cyanobacteria contamination is present also in Southern Italy, as well as in other areas of our country, and improve the knowledge about the effects of BGA blooms (Figure 6). It is noteworthy that MALDI-TOF/MS analysis allowed to detect for the first time anabaenopeptins in Italian lakes.

Conclusions

The method we developed for MCs determination in water and algae samples was simple, rapid, reliable and showed satisfactory mean recoveries. Both ion trap MS and MALDI-TOF/MS were successfully used to determine MC-RR, MC-LR, MC-LW and MC-LF down to the 0.5 – 2 ng mL⁻¹ contamination level in water. The performances of the two MS equipments were comparable, but their use for studying a *P. rubescens* bloom in Lake Averno demonstrated that ion trap detector allowed only to exclude the presence of the four MCs, whereas MALDI-TOF/MS detected three main compounds. Moreover, on the basis of their fragmentation patterns, two of them were identified as Anabaenopeptin B and Anabaenopeptin F, that are less toxic than microcystins. As a consequence, ion trap MS can be applied to MCs screening and confirmatory analysis, while MALDI-TOF/MS allows rapid wide range screening and identification of cyanobacteria biotoxins. The results of our work give new information about algae biotoxins detectable in Italian lakes, and improve the knowledge regarding the contamination by cyanobacteria in our country.

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Figure captions.**Figure 1**

The general molecular structure of a microcystin.

Figure 2

The ion trap LC/ESI-MS/MS SRM chromatograms and the MS/MS spectra of a standard mix of MCs at 500 ng mL⁻¹ (a) and lake water sample spiked at 10 ng mL⁻¹ of each MC (b); for MC-LW and MC-LF the SIM chromatograms are also reported.

Figure 3.

The MALDI-TOF/MS spectrum of a mix standard of MCs at 20 ng mL⁻¹. The inset shows the linearity of the TOF MS detector response for MC-RR in solvent.

Figure 4.

The MALDI-TOF/MS spectrum of an extract from *P. rubescens* algae from Lake Averno.

Figure 5.

The MALDI-TOF/MS post source decay (PSD) spectrum of the ion at 837 *m/z* and its product ions; in the inset the molecular structure of Anabaenopeptin B is reported.

Figure 6.

The distribution of cyanobacteria contamination and the biotoxins characterised in the Italian Lakes.

Table 1.

The R₁ and R₂ amino acids in position 2 and 4 characterising some microcystins.

Microcystin	Amino acids		Molecular weight, amu
	R ₁	R ₂	
MC-LR	Leu	Arg	994
MC-RR	Arg	Arg	1037
MC-YR	Tyr	Arg	1044
MC-LA	Leu	Ala	909
MC-LW	Leu	Trp	1024
MC-LF	Leu	Phe	986
MC-LL	Leu	Leu	950
MC-YM	Tyr	Met	1018
DesMe-MC-LR	Leu	Arg	980
DesMe-MC-RR	Arg	Arg	1023
DesMe-MC-YR	Tyr	Arg	1030

Table 2.

The experimental conditions for ion trap LC/ESI-MS/MS analysis of MCs in the positive ionisation mode. For MC-LW and MC-LF both the precursor molecular ions and the fragments arising from ADDA loss are reported.

MS/MS conditions				
	MC-RR	MC-LR	MC-LW	MC-LF
Isolation width (m/z)	2	2	2	2
Collision energy (%)	48	40	28	28
Selected precursor ion (m/z)	1038.5 $[M+H]^+$	995.5 $[M+H]^+$	1025.2 $[M+H]^+$ 891.1 ($[M+H]^+ - 134$)	986.3 $[M+H]^+$ 852.2 ($[M+H]^+ - 134$)
Selected transitions (m/z)	SRM 1038.5→1020.4 SRM 1038.5→909	SRM 995.5→977.4 SRM 995.5→967.3	SRM 891.1→873.2 SRM 891.1→790.1 SIM 1025.2	SRM 852.2→834.2 SRM 852.2→751.1 SIM 986.3
Selected product ions (m/z)	1020.4 (base peak) 909.3	977.4 967.3 (base peak)	873.2 (base peak) 790.1	834.2 (base peak) 751.1

Table 3.

The repeatability (CV%, n = 6) and mean recovery data (n = 6) of the method, measured by both ion trap and TOF MS detector. The LOD and LOQ value calculated for each MC and the linearity ranges in solvent and matrix are also reported.

	Ion trap MS				MALDI-TOF/MS			
	MC-RR	MC-LR	MC-LW	MC-LF	MC-RR	MC-LR	MC-LW	MC-LF
Mean recovery % (n = 6)	88.6	72.6	83.6	83.9	90.1	91.4	69.5	79.8
CV% (n = 6)	8.4	6.9	18.2	3.8	5.5	6.2	14.2	13.0
LOD	0.1	0.1	1	1	0.1	0.2	0.5	0.5
LOQ	0.5	0.5	1	1	1	2	2	2
Linearity range in solvent, ng mL ⁻¹	125 - 1000				10 - 250			
Linearity range in matrix, ng mL ⁻¹	10 - 125				10 - 250			

Figure 1.

The general molecular structure of a microcystin.

209x296mm (144 x 144 DPI)

Figure 2.

The ion trap LC/ESI-MS/MS SRM chromatograms and the MS/MS spectra of a standard mix of MCs at 500 ng mL⁻¹ (a) and lake water sample spiked at 10 ng mL⁻¹ of each MC (b); for MC-LW and MC-LF the SIM chromatograms are also reported.

209x296mm (200 x 200 DPI)

Figure 3.

The MALDI-TOF/MS spectrum of a mix standard of MCs at 20 ng mL⁻¹. The inset shows the linearity of the TOF MS detector response for MC-RR in solvent.

210x297mm (211 x 211 DPI)

Figure 4.
The MALDI-TOF/MS spectrum of an extract from *P. rubescens* algae from Lake Averno.

209x296mm (225 x 225 DPI)

The MALDI-TOF/MS post source decay (PSD) spectrum of the ion at 837 m/z and its product ions; in the inset the molecular structure of Anabaenopeptin B is reported.

209x296mm (197 x 197 DPI)

Italy Distribution Map:

- Sicily:**
 - Palermo: *P. rubescens* MC-RR, desMC-RR
- Calabria:**
 - Crotone: *P. rubescens* Anatoxin-a, MC-RR, desMC-RR, MC-YR, MC-LR
- Campania:**
 - Naples area: *M. aeruginosa* MC-YR, MC-LR
 - Avellino: *M. aeruginosa* MC-RR, desMC-RR
 - Salerno: *P. rubescens* MC-LR, MC-RR, desMC-RR
 - Avellino: *P. rubescens* MC-LR, MC-RR, desMC-RR
 - Avellino: *M. aeruginosa* MC-LR
 - Avellino: *M. aeruginosa* MC-YR
 - Avellino: *M. aeruginosa* MC-RR, desMC-RR, MC-LR
 - Avellino: *P. rubescens* MC-RR, desMC-RR, MC-YR
 - Avellino: *P. rubescens* MC-YR
 - Avellino: *M. aeruginosa* MC-LR
- Lazio:**
 - Rome area: *P. rubescens* Anatoxin-a, MC-RR, desMC-RR, MC-YR, MC-LR, MC-LA
 - Rome area: *Microcystis* spp., *O. tenuis*, *P. isothrix*, *P. rubescens*, *Anabaena spiroides*, *Aphanocapsa* spp, Anatoxin-a MC-RR, desMC-RR, MC-YR, MC-LR, MC-LA
 - Rome area: *P. rubescens* Anabaenopeptin B, Anabaenopeptin F
- Umbria:**
 - Perugia area: *P. rubescens* Anatoxin-a, MC-RR, desMC-RR, MC-YR, MC-LR, MC-LA

Averno Lake: *P. rubescens* MC-YR

210x297mm (150 x 150 DPI)