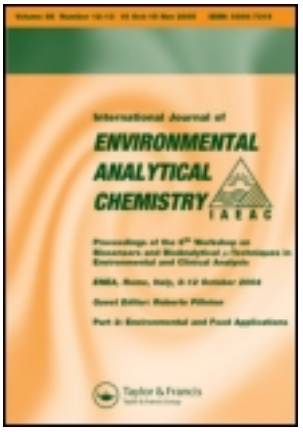

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/geac20

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Available online: 11 Apr 2011

To cite this article: Youness Ouahid, María C. Zaccaro, Gloria Zulpa, Mónica Storni, Ana M. Stella, Juan C. Bossio, Marian Tanuz & Francisca F. Del Campo (2011): A single microcystin in a toxic Microcystis bloom from the river Río de la Plata (Argentina), International Journal of Environmental Analytical Chemistry, 91:6, 525-536

To link to this article: http://dx.doi.org/10.1080/03067310903359492

Intern. J. Environ. Anal. Chem.
Vol. 91, No. 6, 15 May 2011, 525–536

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A single microcystin in a toxic *Microcystis* bloom from the river Río de la Plata (Argentina)

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(*Received 13 April 2009; final version received 25 August 2009*)

Microcystis is one of the most common bloom-forming cyanobacteria genera in diverse ecosystems. More than 80% of its strains are toxic, mainly due to their ability to produce metabolites known as microcystins (MC). Here we report on a *M. aeruginosa* bloom that appeared in the summer of 2001 at a site of the Río de la Plata, within the city of Buenos Aires. The symptoms in mice indicated that the bloom was hepatotoxic. LC-PDA analysis revealed a similar high concentration (0.9–1 mg g^{-1} d w) of just one MC in the bloom biomass during the 3-month study period. During this period most of the MC (ca. 98 %) was found intracellularly, and the content remained almost the same. The molecular mass of the bloom MC was 1036 Da, as deduced from LC-ESI-MS data. Fragment ion analysis by LC-IT-MS-MS allowed identifying 6 out of the 7 amino acids, as well as their position in the molecule. The molecular mass of the unidentified amino acid residue was 155 Da. According to the data obtained, the MC under study was MC-XR, X representing the unidentified amino acid. This is the first report both on the characterisation of MCs in an urban site of the Río de la Plata waters, and on an Argentinean bloom exhibiting only one MC variant.

Keywords: cyanobacteria; microcystin; *Microcystis aeruginosa*; cyanotoxicity; HPLC-PDA; LC-IT-MS/MS

1. Introduction

Microcystins (MCs), the most frequently found cyanobacterial toxins, are world-wide contaminants in diverse ecosystems, including water reservoirs for human use [1,2]. They affect organisms of different type, such as plants and animals [3,4]. In animals, some of the most apparent acute toxic effects are on the liver, haemorrhage and necrosis. MCs appear also to be liver-tumour promoters [5,6]. MC toxicity is based on the inhibition of type 1 and 2A protein phosphatases [7]. Due to the important health risk posed

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ISSN 0306–7319 print/ISSN 1029–0397 online

DOI: 10.1080/03067310903359492
http://www.informaworld.com

by MCs [8], drinking-water guidelines are being established in many countries, in which the maximum MC concentration is generally set to $1\,\mu g\,L^{-1}$ (as equivalent of MC-LR, the most common MC), following the recommendations of the World Health Organisation [9].

MCs are cyclic heptapeptides with the general structure, cyclo-(D.Ala-X-D.MeAsp-Z-Adda-D.Glu-Mdha), where X and Z are variable L-amino acids. MeAsp is erythro-ß-methyl aspartic acid, and Mdha N-methyldehydroalanine. Adda refers to (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid, an unusual amino acid restricted up to now to the cyanotoxins MCs and nodularins. It is essential, together with Glu and Mdha, for biological activity [10–12]. Mdha is needed for the covalent binding of MC to protein phosphatases [12]. To date, more than 80 structurally different MCs have been identified, the difference being mainly due to the variable amino acids in position 2 and 4 (X and Z, respectively), and to the methylation of amino acids in positions 3 (MeAsp) and/or 7 (Mdha). Other modifications include esterification of glutamic acid, replacement of Mdha by N-methyserine or serine, and modification of Adda to its 6 (Δ) Adda stereoisomer, the 9-hydroxy (DMAdda) or the 9-acetoxy (ADMAdda) derivatives [10,13,14].

Currently, liquid chromatography (HPLC) coupled to a photodiode array detector (HPLC-PDA) is a usual methodology for MC analysis, since it allows MC detection and quantification if MC standards are available. Detection is based both on retention time and UV-absorption spectrum, with different absorption maximum depending on the MC chemical structure: 239 nm in most MCs, but 232 or 222 when Tyr (Y) or Trp (W) respectively is present. However, HPLC suitability for MC identification is limited due to the few available commercial reference MCs. In the last decade, more versatile and accurate techniques are being successfully used, such as HPLC-mass spectrometry (MS), with different ion detection techniques.

Health risks of MC-contaminated waters increase when MC-producing cyanobacteria outgrow, forming blooms. *Microcystis* genus is responsible for many of the toxicity events caused by cyanobacteria blooms, since it is a usual component of these blooms, and more than 80% of its strains are able to produce MCs [1,9].

In Argentina, blooms of toxic cyanobacteria genera are quite common in rivers and water reservoirs, with the first report dating from more than twenty years ago [15]. It was stated that from a total of 170 cows, 72 died within 24 h after ingestion of a *M. aeruginosa* contaminated water in Goyena, Buenos Aires. Since then, other cyanobacteria toxic episodes have been reported, including those from the Roque Reservoir, Córdoba [16–18]. In 1999 a conspicuous *M. aeruginosa* bloom was observed at the Uruguayan side of the Río de la Plata river [19], with a high MC content. The Río de la Plata is the second largest river in South America, with its water being used by more than 125 million people. It is also the major source of water for human consumption in the city of Buenos Aires. Argentina and Uruguay share the last part of the river, before and at its estuary at the Atlantic Ocean, the Mar de Plata.

This paper deals with a bloom from an urban area in the Buenos Aires city, which was hepatotoxic and almost exclusively formed by *M. aeruginosa*. It exhibited only one major MC component, as deduced from analyses with HPLC-PDA and HPLC-ESI-IT-MS. As far as we know this is the first report on the chemical characterisation of MCs from toxic blooms in an urban site of the Río de la Plata, Argentina.

2. Experimental

2.1 *Biological material and MC extraction*

Most of the material used throughout the work was the biomass from a dense, pasty surface bloom that appeared in the summer of 2001 within Buenos Aires city, at Puerto Madero docks. At this urban site, water exchange and the influence of both wind and stream force are reduced. Bloom samples were collected monthly from October to December from the surface layer at a depth of 0.5–0.75 m, and passed through a 80 μm phytoplankton net, to separate biomass from water. The biomass was examined under a microscope to determine its phytoplankton composition, according to Komarek and Anagnostidis [20]. Both the biomass and water were lyophilised and stored at –20°C, till further use.

MCs were extracted from the lyophilised biomass with 70% aqueous methanol (100 mL g^{-1} dry weight, dw), and from the lyophylised water with methanol (25 mL L^{-1} water).The extracts were dried by rotary evaporation at 45°C.

2.2 *Toxicity bioassay*

Toxicity was assayed on male Swiss mice (36.85 g mean weight). Twenty mice were randomly separated into 4 groups, and one was utilised as control. For the assay, the dried MC extract was suspended in 0.9% NaCl, centrifuged and then passed through a 0.45 μm filter. The filtrate was diluted with 0.9% NaCl to obtain 3 different MC concentrations: 8, 4 and 2 μg mL^{-1}. One mL of each extract was administered to the test mice groups by intraperitoneal injection, with the control group receiving 1 mL of 0.9% NaCl.

2.3 *Microcystin detection and quantification by LC-PDA*

Detection of MCs by liquid chromatography was carried out with a HPLC quaternary gradient pump system (Alliance 2695 Waters), equipped with a PDA (Model 996). The column used was Kromasil C18 (5 μm, 250 mm × 4.6 mm ID). The mobile phase consisted of a non-linear gradient of 2 eluents: A, water with 0.05 % (v/v) trifluoroacetic acid (TFA); and B, acetonitrile with 0.05 % (v/v) TFA. The gradient profile was as follows (t in min): t_0, $A = 60\%$; t_2, $A = 60\%$; t_{12}, $A = 55\%$; t_{22}, $A = 55\%$; t_{32}, $A = 52\%$; t_{45}, $A = 35\%$; t_{50}, $A = 0\%$; t_{55}, $A = 0\%$; t_{57}, $A = 60\%$; t_{60}, $A = 60\%$. The flow rate was 1.0 mL min^{-1}. The sample volume injected was 50 μL of the dried methanolic extracts suspended in 75% methanol and passed through a GF/C glass filter.

MCs were identified by their UV-spectra (200–400 nm) and retention time. Reference material of MC-LR, -YR, -RR, -LF and -LW, of about 96% purity, were purchased from Calbiochem (Germany).

All chemicals were of chromatographic grade (Scharlau Chemie Barcelona, Spain).

Quantitative MC determination was carried out by comparing the peak area of the detected MC with that obtained with reference MC-LR. Content was expressed as equivalents of MC-LR per 1 L water sample or per 1 g of biomass dw.

2.4 *Microcystin characterisation by LC-ESI-IT-MS/MS*

Electrospray ionisation-mass spectrometry (ESI-MS) analysis was carried out in a LC-quadrupole ion trap (LCQIT) mass spectrometer (Deca XP Plus, Thermo Finnigan,

San Jose, CA). The column used was Zorbax C18 (5μm, 50 mm × 2.1 mm ID), and the mobile phase was as for LC-UV-DAD. The mobile phase gradient profile was as follows (t in min): t_0, $A = 70\%$; t_2, $A = 35\%$; t_4, $A = 10\%$; t_{20}, $A = 0\%$; t_{22}, $A = 0\%$; t_{23}, $A = 70\%$; t_{25}, $A = 70$. The flow rate was 0.2 mL min^{-1}. The sample injected volume was 5 μL.

Ions generated from the ESI source were introduced into the mass spectrometer through a heated capillary with the temperature and voltage maintained at 220°C and 54 V, respectively. Spray ionisation needle voltage was set at 4 KV. For all data dependent MS/MS experiments, the relative collision energy was adjusted to 35% relative to the base peak. An accurate mass calibration was obtained using a solution of phosphopeptide standard mixture (Agilent, Spain). Full scan mass spectra were acquired within the m/z 900–1200 range, using the X-Calibur software system for data processing.

3. Results

3.1 *Bloom cyanobacteria composition and toxicity*

Microscopic examination of fresh bloom samples revealed that in all cases the main species (>90%) was *M. aeruginosa* (Figure 1a). The cell size ranged between 6.5 and 7 μm (Figure 1b), within the largest described for this species [21].

To test bloom toxicity, mice behaviour was observed after the injection of the bloom extract. No apparent abnormal symptoms appeared within the first 2 h; after this time, dizziness and body tremors were observed, the tremors being more conspicuous in the mice that received more concentrated extracts. At 3 h, all of the mice from the group that received the most concentrated extract died. Thereafter, mice death continued, and by 4 h all mice were dead. Liver examination revealed that the bloom extract was hepatotoxic. Although a detailed histopathological study on the toxic effects was not conducted, clear structural lesions, as well as haemorrhage were observed.

3.2 *MC identification and quantification*

Considering the hepatotoxic nature of the *M. aeruginosa* bloom extracts, MCs appeared as the best toxic candidates; therefore, the concentration and the type of MCs were determined. HPLC chromatograms showed that the extracts contained only one component (Figure 2) with the typical microcystin UV-spectrum. The spectrum was of

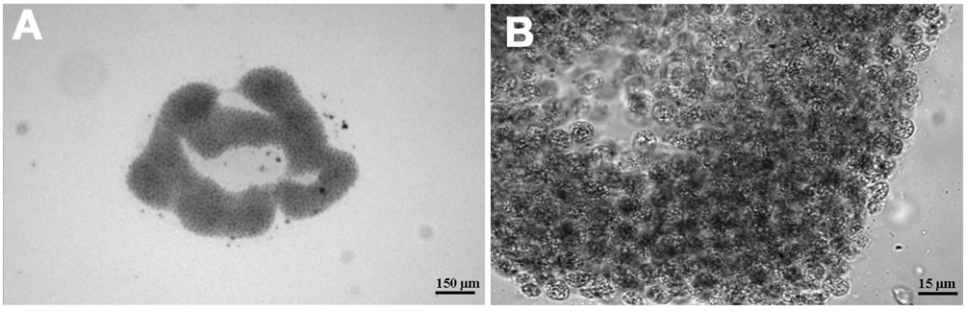

Figure 1. *M. aeruginosa* from the Río de la Plata bloom. (a) colony; (b) colony detail.

the MC-LR type, with a λ_{max} at about 239 nm, indicating the absence of tyrosine (Y) and/or tryptophane (W) residues in the molecule. The chromatogram obtained with the water samples was similar to that of the bloom extracts (not shown).

MC content in the bloom biomass (0.9–1 mg^{-1} g dw) remained almost the same along the study period. The concentration of intracellular MC (2.7 mg L^{-1}) was much larger than that solubilised in water (ca. 0.43 μg L^{-1}).

The retention time (19.6 min) of the MC in the extract differed from that of the reference MCs available (Figure 2): MC-RR (9.4 min), -YR (14.7 min), -LR (15.9 min), -LW (33.2 min) and -LF (33.8 min). Therefore, identification by HPLC-PDA of the detected MC component was not possible.

3.3 *MC characterisation*

ESI-MS analysis of the bloom MC variant (Figure 2a) generated a singly charged ion at *m/z* 1037.6 and a doubly charged ion at *m/z* 519.3 (Figure 3a). Thus, the molecular mass of the MC under study appeared to be 1036 Da.

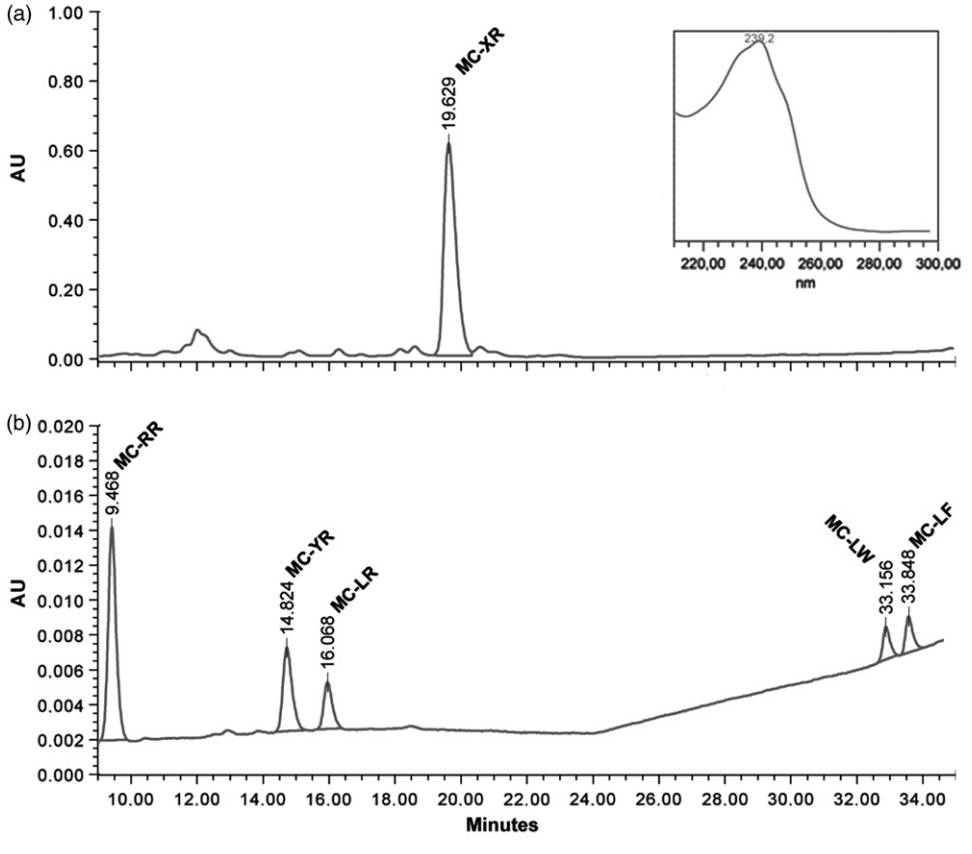

Figure 2. HPLC-UV chromatograms. (a) methanolic extract from bloom biomass; insert, UV spectrum of the single MC peak found. (b) MC reference material.

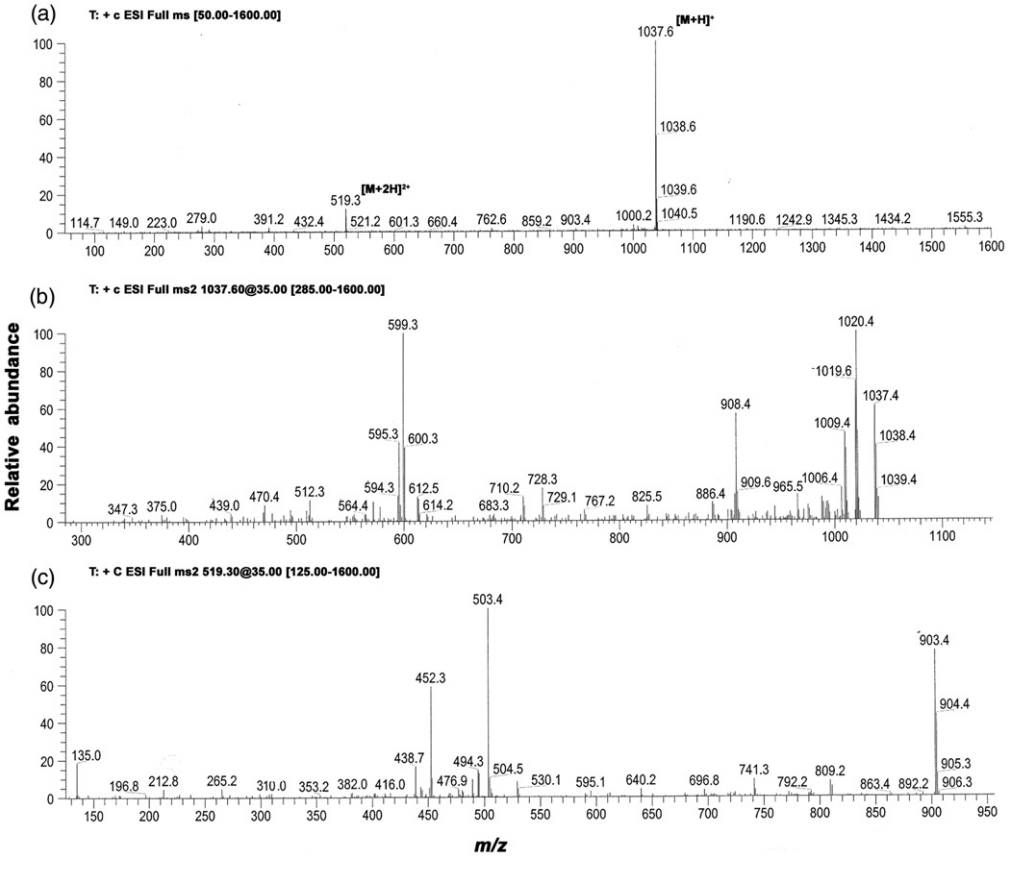

Figure 3. ESI mass spectra of the MC fraction from LC-UV. MS^1 (a) MS^2 of the *m*/*z* 1037.6 ion (b) and *m*/*z* ion 519.3 (c) from MS^1.

The 1037.6 ion may correspond to four previously reported MCs: two, of known structure, [ADMAdda5] MC-LHar, [D-Leu1] MC-LR; and the other two, MC-XR and [ADMAdda5] MC-XR, not completely characterised yet [22–25]. [ADMAdda5] MC-LHar was obtained from a *Nostoc* sp. culture [22], and [D-Leu1] MC-LR was first reported in 1996 by Matthiensen *et al*. [23] as the main MC from a *Microcystis* bloom at the Patos Lagoon estuary (southern Brazil), and from the *Microcystis* strain RST 9501 isolated from the bloom [23]**.** It was later identified by Park *et al*. [24] in a Canadian bloom [24]**.** MC-XR was one of the six MCs found in a *Microcystis* bloom from Patos Lagoon Brazil [25]**,** and [ADMAdda5] MC-XR was identified along with other four MCs in a *Nostoc* sp. culture [22].

Further characterisation of the MC variant was carried out by MS/MS analysis. Figures 3b and 3c show the MS^2 spectrum of the *m*/*z* 1037.6 and *m*/*z* 519.3 ions, respectively. Both ions fragmented well, the assignment of some of the product ions appearing in Table 1.

As a whole, the MS^2 product ions suggest the presence in the molecular structure of 5 known amino acid residues, Adda, Glu, Mdha, MeAsp and Arg. The presence of Glu

Table 1. MS^n product ions of $[M+H]^+$ at m/z 1037.6 and $[M+2H]^{2+}$ at m/z 519.3.

Ion assignment	m/z	MSn	Figure
Precursor ion	**1037.6**	1	3A
$[(M\text{-}NH_3)+H]^+$	1020.4	2	3B
$[(M\text{-}H_2O)+H]^+$	1019.6	2	3B
$[(M\text{-}CO)+H]^+$	1009.4	2	3B
$[(M\text{-Ala})+H]^+$	965.5	2	3B
$[C_{11}H_{14}O\text{-Glu-Mdha-Ala-(155)-MeAsp-Arg}+H]^+$	886.4	2	3B
$[C_{11}H_{14}O\text{-Glu-Mdha}+H]^+$	375.2	2	3B
$[\text{Adda-Arg-MeAsp-(155)-Ala-Mdha}+H]^+$	908.4	2	3B
$[\text{Adda-Arg-MeAsp}+H]^+$ or $[\text{Glu-Adda-Arg}+H]^+$	599.3	2	3B
$[\text{Arg-MeAsp-(155)-Ala-Mdha}+H]^+$	595.3	2	3B
$[\text{Arg-MeAsp-(155)-Ala}+H]^+$	512.2	2	3B
Precursor ion	**519.3**	1	3A
$[(M\text{-MeOH})+2H]^{2+}$	503.4	2	3C
$[(M\text{-}PhCH_2CH(OMe)+H]^+$	903.5	2	3C
$[PhCH_2CH\ (OMe)]^+$	135.0	2	3C
$[(155)\text{-Ala-Mdha}+H]^+$	310.0	2	3C
Precursor product ion	**908.4**	2	3B
$[\text{Arg-MeAsp-(155)-Ala-Mdha}+H]^+$	595.3	3	4A
$[\text{Adda-Arg-MeAsp-(155)-Ala-Mdha}+H]^+$	908.3	3	4A
$[\text{Adda-Arg-MeAsp-(155)}+H]^+$	754.3	3	4A
$[\text{Adda-Arg-MeAsp}+H]^+$	599.3	3	4A
$[\text{Adda-Arg}+H]^+$	470.2	3	4A
Precursor product ion	**599.3**	2	3B
$[\text{Glu-Adda-Arg}+H]^+$	599.3	3	4B
$[\text{Glu-(Adda-OMe)-Arg}+H]^+$	567.3	3	4B
$[\text{Glu-(Adda-OMe)}+H]^+$	411.0	3	4B
$[\text{(Glu-CO)-Adda-Arg}+H]^+$	571.2	3	4B
$[\text{(Glu-CO)-Adda}+H]^+$	415.4	3	4B
$[\text{Adda-Arg}+H]^+$	470.2	3	4B
$[C_{11}H_{14}O+H]^+$	163.1	3	4B
$[(Arg+NH_2)+2H]^+$	174.0	3	4B
$[Arg+H]^+$	157.2	3	4B
Precursor product ion	**503.4**	2	3C
$[C_{11}H_{14}O\text{-Glu-Mdha-Ala}+H]^+$	446.2	3	4C
$[(Arg+NH_2)\text{-MeAsp-(155)-Ala-Mdha-Glu}+2H]^+$	741.1	3	4C
$[(Arg+NH_2)\text{-MeAsp-(155)-Ala-Mdha}+2H]^+$	612.3	3	4C
$[(Arg+NH_2)\text{-MeAsp-(155)-Ala}+2H]^+$	529.3	3	4C
$[(Arg+NH_2)\text{-MeAsp}+2H]^+$	303.0	3	4C
$[\text{Arg-MeAsp-(155)-Ala-Mdha-Glu}+H]^+$	724.1	3	4C
$[\text{Arg-MeAsp}+H]^+$	286.1	3	4C
$[\text{Glu-(Adda-OMe)-Arg-MeAsp}]^+$	696.1	3	4C
$[\text{(Adda-OMe)-Arg-MeAsp}]^+$	567.4	3	4C
$[\text{(Adda-OMe)-Arg}]^+$	438.1	3	4C

and Mdha residues is indicated by the product ion at m/z 375.2 in the MS^2 of the m/z 1037.6 ion (Figure 3b), which could be assign to $[C_{11}H_{14}O\text{-Glu-Mdha}+H]^+$ (Table 1). The product ion at m/z 599.3 (Figure 3b) could correspond either to $[\text{Adda-Arg-MeAsp}+H]^+$ or to $[\text{Glu-Adda-Arg}+H]^+$ (Table 1) [26], since the residue of Glu and MeAsp exhibit the same molecular mass (129 Da).

Another feature in the MS^2 spectrum of the m/z 1037.6 ion was the presence of the m/z 965.5 product (Figure 3a), assigned to [M+H – Ala]+ (Table 1). This assignment suggests

Ala to be the amino acid in position 1, and discards the molecular structure of [D-Leu1] MC-LR.

The MC nature of the molecule under study was confirmed by the MS^2 product ions of the doubly charged *m/z* 519.3 ion (Figure 3c and Table 1): *m/z* 503.4, 903.5, and 135. The product ion $[(M\text{-}MeOH)+2H]^{2+}$ at *m/z* 503.4 (Figure 3c, Table 1), would correspond to the neutral loss of MeOH [27]. The *m/z* 903.5 ion could be assigned to $[(M\text{-}135)+2H]^{+}$, due to the loss of O-methyl-phenyl-acetaldehyde $[PhCH_2CH(OMe)]^{+}$ ion, *m/z* 135.0 [26]. The production of the *m/z* 135.0 ion, resulting from α-cleavage of the methoxy group of the Adda chain side (Table 1), denotes an unmodified Adda side chain [22,27], and allows to discard the MC under study to be [ADMAdda[5]] MC-LHar or [ADMAdda[5]] MC-XR. The absence of the fragment ions at *m/z* 121 $[PhCH_2CH(OMe)]^{+}$ and *m/z* 265 $[(ADMAdda\text{–}CH_3COOH\text{–}NH_3)+H]^{+}$, resulting from hydroxy Adda (DMAdda) and acetoxy Adda (ADMAdda respectively [22,27], also supports the presence of an unmodified Adda residue. Besides, in the case of [ADMAdda[5]] MC-LHar, the loss of acetic acid from the amino acid ADMAdda expected at *m/z* 977 from its published fragmentation pattern was not observed [25].

To confirm the location of the amino acid residues and to decipher the remaining amino acids, MS^3 analysis was carried out (Figure 4), using as parent ions major products obtained in MS^2 analysis. Three product ions were subjected to MS^3 analysis: two, at *m/z* 908.4 and 599.3, derived from the singly charged ion (1037.6) (Figure 3b and Table 1), and the third, at *m/z* 503.4, derived from the doubly charged ion (519.3) obtained in MS^1 (Figure 3a). Assignation of the MS^3 ion products is listed in Table 1.

MS^3 spectrum of the product ion at *m/z* 599.3 strongly suggested that this ion was $[Glu\text{-}Adda\text{-}Arg+H]^{+}$, due to the product ions at *m/z* 567.3 $[Glu\text{-}(Adda\text{-}OMe)\text{-}Arg+H]^{+}$, 411.0 $[Glu\text{-}(Adda\text{-}OMe)+H]^{+}$, 571.2 $[(Glu\text{-}CO)\text{-}Adda\text{-}Arg+H]^{+,}$ and 415.4 $[(Glu\text{-}CO)\text{-}Adda+H]^{+}$ (Figure 4b and Table 1). All these fragment ions derived from *m/z* 599.3 clearly indicate that Glu and Arg are at positions 6 and 4, respectively. The $C_{11}H_{14}O$ unit (162 Da), derived from the Adda residue by the loss of the $PhCH_2CH(OMe)$ unit (135 Da) and C–N bond cleavage [27], confirms the presence of an unmodified Adda.

The ion at *m/z* 446.2 $[C_{11}H_{14}O\text{-}Glu\text{-}Mdha\text{-}Ala+H]^{+}$ (Figure 4c, Table 1) resulting from the fragmentation of the product ion at *m/z* 503.4, along with the MS^2 product *m/z* 375.2 mentioned before (Figure 3b), support the presence of the amino acid residues Glu at position 6, Mdha at position 7, and Ala at position 1 [28]. These data also allow discarding the MC under study to be [D-Leu[1]] MC-LR.

The position of MeAsp at 3 was also confirmed, first, by the characteristic ion C-type series which retained the amide at *C*-terminus of the Arg residue at *m/z* 303.0 $[(Arg+NH_2)\text{-}MeAsp+2H]^{+}$, obtained from the *m/z* 503.4; and second, by the B-type ion series starting with $[Arg+H]^{+}$ residue at *m/z* 268.1 $[Arg\text{-}MeAsp+H]^{+}$ [27] obtained respectively from the *m/z* 908.3 ion (Table 1, Figure 4a and 4c).

Some of the ions produced by MS^3 analysis strongly indicate that 155 is the molecular mass of the amino acid residue in position 2 of the under study MC (Table 1): *m/z* 908.3 $[Adda\text{-}Arg\text{-}MeAsp\text{-}(155)\text{-}Ala\text{-}Mdha+H]^{+}$ and *m/z* 754.3 $[Adda\text{-}Arg\text{-}MeAsp\text{-}(155)+H]^{+}$, originated by fragmentation of the MS^2 product ion *m/z* 908.3 (Figure 4a); and *m/z* 741.1 $[(Arg+NH_2)\text{-}MeAsp\text{-}(155)\text{-}Ala\text{-}Mdha\text{-}Glu+2H]^{+}$ and 612.3 $[(Arg+NH_2)\text{-}MeAsp\text{-}(155)\text{-}Ala\text{-}Mdha+2H]^{+}$, originated by fragmentation of the MS^2 product ion *m/z* 503.4 (Figure 4c).

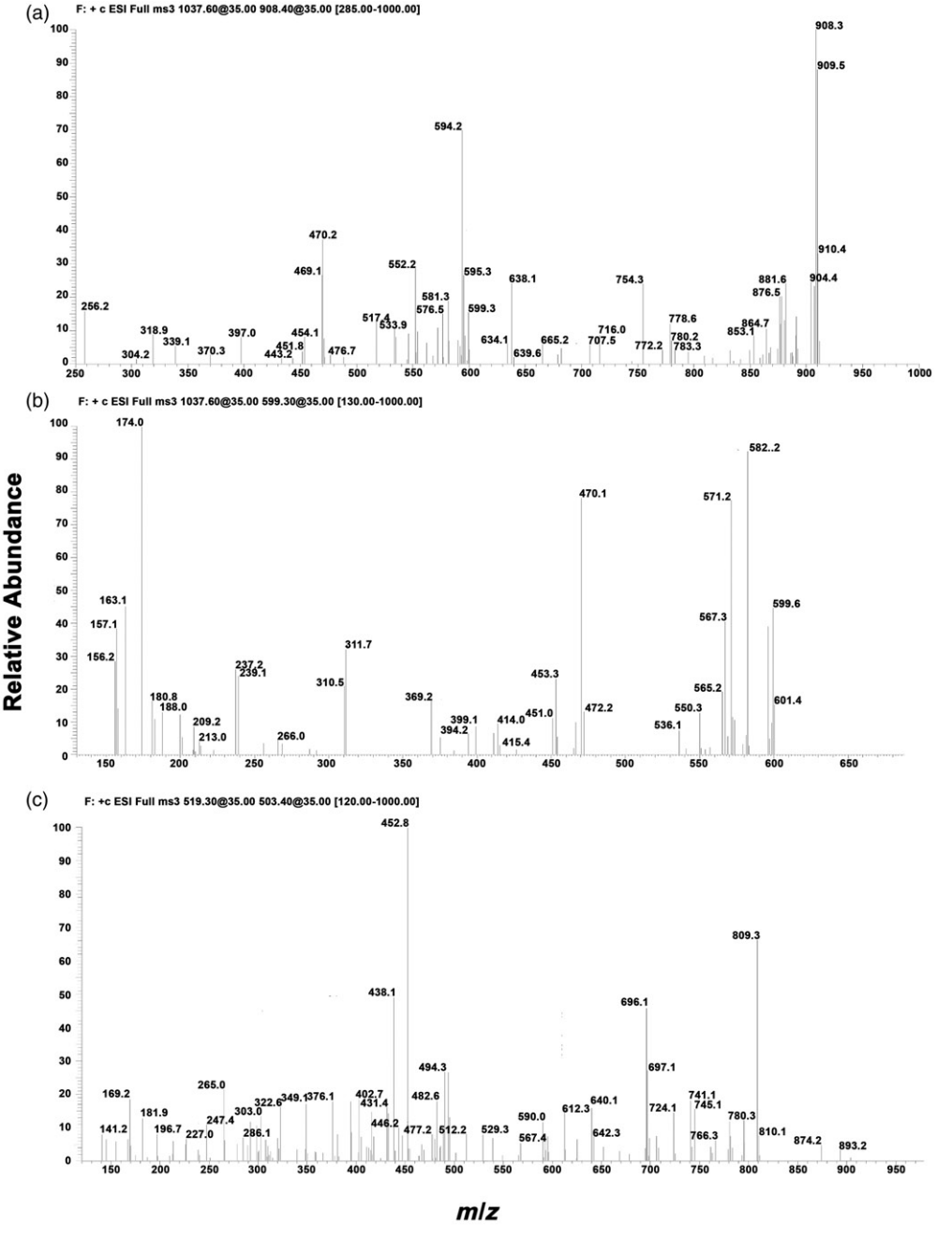

Figure 4. ESI MS^3 of product ions. Product ion at m/z 908.3, (a); at 599.3, (b); and at 503.4, (c).

4. Discussion

Most of the experimental work has been focused in elucidating the structure of an MC variant, which by HPLC appeared to be the only MC component of a toxic bloom appearing in the urban area of the Buenos Aires city during the summer of 2001, and

formed almost exclusively by a *M. aeruginosa* strain of large size. The methodology used, HPLC-PDA and HPLC coupled to ESI-IT-MS/MS, allowed to determine the molecular mass of the MC, 1036 Da, and to decipher 6 out of the 7 amino acids as well as their position within the molecule (Ala-X-MeAsp-R-Adda-Glu-Mdha).

From all MCs with a molecular mass of 1036 Da, the fragment ion pattern of the MC variant studied here by LC-ESI-IT-MS/MS agrees with that of the MC-XR reported by Zweigenbaum *et al*. [25]. The data available do not permit the elucidation of the nature of the amino acid X in position 2. To accomplish the final structure other methodology, such as NMR, would be needed. Unfortunately, sufficient bloom material to obtain the amount of the purified MC needed for NMR is not available. Nevertheless, significant clues can be drawn from our observations. For example, the UV spectrum (Figure 2) shows that the unidentified amino acid is not Y or W. To the best of our knowledge, the molecular mass of this amino acid differs from that of known MC amino acids. Considering the MC-XR molecular mass and the $-NH_2$ contribution of the assigned amino acids from tandem MS (Figures 3 and 4, Table 1), the unidentified amino acid should have none or a pair number of $-NH_2$ residues [29].

As previously stated, the MC-XR appeared as the sole MC variant of the bloom, a striking fact since MC-producing cyanobacteria usually exhibit several MC variants. An exception to this is the report by Park *et al*. [24] in which only one MC, [D-Leu^1] MC-LR, was described.

Other aspects to be considered are the almost even MC content and composition of the bloom, as well as the intracellular localisation of the MC throughout the 3 month-study period. This latter feature indicates a steady metabolic status and MC-producing capacity of the bloom. Although no attempt was made to study in detail the toxicity of the bloom, the bloom appeared to be hepatotoxic on the basis of the symptoms observed in the mice used for the toxicity bioassay.

We would like to emphasise that as far as we know this is the first report on a detailed characterisation of MCs in urban waters of the Río de la Plata. The first report on MC-containing blooms in Río de la Plata was in 2001 [19]. It referred to samples from the Uruguayan part of the river, and MC analysis was just performed by ELISA. Later, several authors identified MC variants in various Argentinean water systems [16–18], but in no case MC-XR was reported.

In 2008 Andrinolo *et al*. [30] studied a toxic bloom at the Argentinean coast of the River de la Plata, that showed 2 MCs, MC-LR and another one, with the retention time in HPLC of MC-YR. MS analysis allowed to discard the unidentified MC being MC-YR. The UV spectrum would have also discarded an MC-YR identity. The unknown MC could well be the same variant, since it gave rise in MS to a molecular mass ion of 1037.8. Confirmation of both MCs identity would require at least to compare MS fragmentation data, but this is not possible, since no tandem MS appeared in the Andrinolo *et al*. report [30]. It will be interesting to observe if in the future other blooms from the Río de la Plata, other Argentinean or South America water ecosystems exhibit the MC variant here studied.

Acknowledgements

We acknowledge the financial support of the Comunidad Autónoma de Madrid (Project 07M/0058/2000), Universidad de Buenos Aires (UBACYT X042) and PREFECTURA NAVAL ARGENTINA.

We wish to thank Dr. Lee Robertson for language help. Y. Ouahid was a recipient of a fellowship from the Ministry of Higher Education, Morocco.

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