1 AN INTEGRATED STRATEGY FOR RAPID AND ACCURATE DETERMINATION
2 OF FREE AND CELL-BOUND MICROCYSTINS AND RELATED PEPTIDES IN
3 NATURAL BLOOMS BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY-HIGH
4 RESOLUTION MASS SPECTROMETRY AND MATRIX-ASSISTED LASER
5 DESORPTION/IONIZATION TIME-OF-FLIGHT/TIME-OF-FLIGHT MASS
6 SPECTROMETRY USING BOTH POSITIVE AND NEGATIVE IONIZATION MODES

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10 **ABSTRACT**

11 An integrated high resolution mass spectrometry (HRMS) strategy has been developed
12 for rapid and accurate determination of free and cell-bound microcystins (MCs) and
13 related peptides in water blooms. The natural samples (water and algae) were filtered
14 for independent analysis of aqueous and sestonic fractions. These fractions were
15 analyzed by MALDI-TOF/TOF-MS and ESI-Orbitrap-HCD-MS. MALDI, ESI and the
16 study of fragmentation sequences have been provided crucial structural information.
17 The potential of combined positive and negative ionization modes, full scan and
18 fragmentation acquisition modes (TOF/TOF and HCD) by HRMS and high resolution
19 and accurate mass was investigated in order to allow unequivocal determination of
20 MCs. Besides, a reliable quantitation has been possible by HRMS. This composition
21 helped to decrease the probability of false positives and negatives, as alternative to
22 commonly used LC-ESI-MS/MS methods. The analysis was non-target, therefore
23 covered the possibility to analyze all MC analogues concurrently without any pre-
24 selection of target MC. Furthermore, archived data was subjected to retrospective “post-
25 targeted” analysis and a screening of other potential toxins and related peptides as
26 anabaenopeptins in the samples was done. Finally, the MS protocol and identification
27 tools suggested were applied to the analysis of characteristic water blooms from
28 Spanish reservoirs.

29 **Keywords:** High resolution mass spectrometry; MALDI-TOF/TOF-MS; ESI-Orbitrap-
30 HCD-MS; Whole natural samples; Microcystins; Anabaenopeptins

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1. Introduction

Blue-green algae tend to proliferate massively (blooms) in eutrophic waters, under certain conditions of growth and environment factors. Microcystins (MCs) are the most commonly reported cyanobacterial hepatotoxins produced by several genera including *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* [1, 2]. MCs are usually cell-bound in healthy cyanobacterial cells. However, in senescent blooms, cell lysis occurs leading to release of toxins. These cyclic heptapeptide toxins show potent hepatotoxicity and tumor-promoting activity through inhibition of protein phosphatases 1 and 2A and have been responsible for the death of domestic, farm and wild animals and some outbreaks in human beings [1, 3]. Excellent work guides and reviews [1-4] on the cyanobacterial toxins description and their behavior in the aquatic environment have been reported in the literature.

Over the last few decades, cyanotoxins creates a significant water quality problem and have become a serious ecological, health and economical issue due to the massive occurrence in eutrophied waters worldwide. The World Health Organization recommended in 1998 a maximum guideline level for total MC-LR (free plus cell-bound) in water of $1 \mu\text{g L}^{-1}$ (WHO, 1998) [5]. Several worldwide countries have adopted this maximum residue level in their national guidelines [6]. Particularly, the Spanish authorities established a maximum level for MC in drinking water of $1 \mu\text{g L}^{-1}$ (RD 140/2003) [7] and include three levels of probability for cyanobacterial proliferation in water-bodies used for recreation (RD 1341/2007) [8]. Also, cyanobacteria and their toxins are included on the current Drinking Water Contaminant Candidate List of US Environmental Protection Agency (EPA, CCL3) [9].

The general structure of MCs is cyclo(D-Ala¹-L-X²-D-MeAsp³-L-Z⁴-Adda⁵-D-Glu⁶-Mdha⁷), in which MeAsp is D-erythro- β -methylaspartic acid, Mdha is N-methyldehydroalanine, Adda is 2S, 3S, 8S, 9S-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid (Fig. S1 of supplementary data). The nomenclature of MCs uses the single-letter abbreviations for residues at positions 2 and 4 (e.g., MC-LR, has a leucine (L) residue on position 2 and an arginine (R) residue on position 4) [2]. The most common structural substitutions occur at positions 2 and 4, and demethylation at positions 3 and 7. In other cases, substitutions of Ala at position 1 for Leu, Ser, and methyl-esterification at Glu at position 6 have also been observed [6, 10]. To date, more than 80 MCs have been tentatively identified in the literature but only about twelve commercial standards are available. MC-LR, -RR and -YR are the most common variants detected, but many studies describe the presence of other MCs. As an example, blooms of *Planktothrix* sp. in which the congeners dominant were [Asp³]MC-RR, [Asp³]MC-LR and [Asp³]MC-HtyR demethylated variants were detected in Europe [11-14].

Mass spectrometry (MS) techniques are a powerful tool for the analysis of biotoxins. Specifically, liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is a technique with excellent selectivity, specificity, sensitivity, quantitation abilities owing to their wide linear dynamic range and using commercially affordable instruments (triple quadrupoles, QqQ). Consequently, in the last years, LC-ESI-MS/MS is the most commonly used MS-based technique in the determination of MC in environmental samples [15-18]. On the other hand, direct Matrix-assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) analysis has been widely used because allows accurate identification of toxin congeners simultaneously without preceding separation. Typically, the positive ionization mode is

the most usually ionization mode employed. Additionally, electrospray (ESI) recorded in negative ion mode has recently been applied to the analysis of MCs [19-21]. In order to have faster methods for toxin analysis: ultra-high performance liquid chromatography (UHPLC) [22-24], on-line solid phase extraction (SPE) [25-28] and direct analysis without prior extraction [29, 30] and chromatographic separation [31, 32] have been implemented in several works.

The use of full scan MS approaches employing high-resolution mass spectrometers and accurate mass measurements (AMM) has also strongly emerged as attractive alternative for the analysis of biotoxins [31-38]. Specifically, the application of Orbitrap higher resolution mass analyzer was capable of supporting fairly rapid and reliable identifications of known toxins even when standards were not available [33-36, 38]. The use of high resolution mass spectrometry (HRMS) instead of MS/MS offers several advantages [39]. False positive and negative findings in LC-MS/MS using a QqQ analyzer were prevented by LC-HRMS. HRMS allows resolving the corresponding peaks of interfering compounds that are co-eluting with the monitored analyte [39-41]. On the other hand, the possibility of reporting a false negative by LC-MS/MS when the interference causes ion-ratio errors between the two transitions registered by selected reaction monitored (SRM) acquisition mode higher than the legislated tolerance [42] can be prevented with LC-HRMS [43]. These ion-ratio errors make infeasible the confirmation of this compound and a possible harmful analyte would be ignored.

Combined MS techniques (accurate mass measurements, MS/MS and MSⁿ experiments) and analytical techniques complementary to LC-MS such as thiol derivatization, chiral amino-acid analyses, nuclear magnetic resonance and ultraviolet-

visible spectroscopy, or deuterium exchange experiments are useful for unambiguous structural identification and confirmation of cyanotoxins. [44-48].

This work presents an integrated strategy for rapid analysis of free and cell-bound cyanotoxins and related peptides in natural blooms based on HRMS by TOF/TOF and Orbitrap-Exactive HCD analyzers using MALDI and ESI ionization sources. The main aims of this study were:

- (1) Rapid analysis of cyanotoxins with minimum treatment of sample, and
- (2) Reliable identification and quantitation of toxins present in cyanobacteria and water samples by HRMS and fragmentation techniques.

In this regard, we have developed and combined different MS methodologies to quantify and identify MC variants and related peptides in water blooms: direct injection of water samples and analysis of intracellular extracts by LC/ESI-HRMS onto Orbitrap mass spectrometer; and, intracellular toxin analysis by MALDI-TOF/TOF-MS. We report on the application of the Orbitrap-Exactive HCD and TOF/TOF analyzers in terms of AMM and fragmentation studies. To the best of our knowledge, this is the first report on TOF/TOF mass analyzer has been applied to MC analysis. Positive and negative ionization modes by ESI and MALDI have been extensively applied together in order to combine and use cumulative information provided for unambiguous identification and confirmation of cyanotoxins. The identification points (IPs) implemented according to 2002/657/EC European Commission Decision [42] for MC analysis by the integrated HRMS strategy have been discussed. The accurate mass techniques proposed showed more selectivity versus the alternative MS/MS approaches.

Finally, an analytical protocol combining all techniques, diagnostic signals and restrictive criteria for reliable identification and quantitation of MCs have been proposed and successfully applied to the analysis of natural blooms.

2. Materials and methods

2.1. Standards and reagents

All reagents used were of analytical or high-performance liquid chromatographic (HPLC) grade. Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid was from Panreac (Montcada i Reixac, Barcelona, Spain) and trifluoroacetic acid (TFA) was from Fisons (Loughborough, UK). High purity water produced with a Milli-Q Synergy UV system (Millipore, Bedford, MA, USA) was used. α -Cyano-4-hydroxycinnamic acid (α -CHCA) used as matrix in MALDI-TOF/TOF-MS analysis was obtained from Sigma (Steinheim, Germany).

MC-LA, -LR, -RR, -WR, -YR, and nodularin standards were purchased from Alexis Biochemicals (San Diego, CA, USA). Stock standard solutions of each analyte (100 or 500 $\mu\text{g mL}^{-1}$) were individually prepared by weight in methanol and stored at -20°C. Intermediate solutions were prepared weekly from the stock standard solution by appropriate dilution in methanol. MC-LF, -LW and -LY 5-10 $\mu\text{g mL}^{-1}$ methanolic solutions were supplied from Sigma (Sigma-Aldrich, St Louis, MO, USA). MC-dmRR 10 $\mu\text{g mL}^{-1}$ in methanol was acquired from Cyano Biotech GmbH (Berlin, Germany). Finally, MC-dmLR 10 $\mu\text{g mL}^{-1}$ methanolic solution was purchased from DHI (Hørsholm, Denmark). Mixed calibration standard solutions of all MCs were prepared daily with nodularin as internal standard (IS).

Nitrogen (purity > 99.999%) supplied by Air Liquide (Madrid, Spain) was used for the ESI source and as a collision-induced dissociation (CID) gas in the Orbitrap mass spectrometer.

2.2. Cyanobacterial bloom samples and MC extraction

Two samples of natural MCs-producing microalgae bloom were collected in the Alcántara reservoir (Tajo River, Cáceres, central Spain) in July 2009 and Vilasouto reservoir (Mao River, Lugo, northwest Spain) in April 2011. *Microcystis aeruginosa* and *Planktothrix rubescens* were the most dominant cyanobacteria of both blooms, respectively. These two events correspond to the most representative water bloom episodes of common cyanobacteria genera in Spanish waters [11, 12].

The water plus algae samples were collected in wide-mouthed polypropylene (Alcántara) and brown glass bottles (Vilasouto) and immediately placed in darkness at 4°C until analysis were performed (typically within 48 h of collection). 50 and 420 mL of Alcántara and Vilasouto bloom samples (water and algae), respectively, were filtered through Whatman GF/F filters (0.7 µm) for independent analysis of aqueous (extracellular) and sestonic (intracellular, defined as the particles retained on 0.7 µm filters) fractions. The filter was frozen-thawed and lyophilized. The sestonic fraction was analyzed directly by MALDI-TOF/TOF-MS for qualitative purposes and after extraction with methanol acidified by LC/ESI-HRMS for quantitative analysis. The aqueous extracellular fraction was analyzed by LC/ESI-HRMS via direct injection into Orbitrap mass spectrometer.

For MALDI-TOF/TOF-MS qualitative analysis of sestonic fraction, a small amount of lyophilized sample was dissolved in 0.1% of TFA in H₂O/acetonitrile (1/1, v/v), blended with the matrix and placed on the MALDI sample plate. For quantitative analysis, the lyophilized sestonic fraction was extracted three times with methanol acidified according to Barco et al. (2005) [49]. Nodularin was added as IS to the

lyophilized fraction to obtain a final concentration of $1 \text{ ng } \mu\text{L}^{-1}$. Alcántara and Vilasouto extracts were concentrated to dryness and reconstituted in 8 and 4 mL of methanol, respectively. Toxins present in the extract were identified and quantified by LC/ESI-HRMS.

Finally, direct analysis of 10 or 100 μL of aqueous fraction (extracellular) for high or low level concentrated samples, respectively, was performed by LC/ESI-HRMS. Before analysis, a 10 mL subsample (filtered water) was taken and a standard solution containing nodularin was added as IS to obtain a final concentration of $1 \text{ } \mu\text{g L}^{-1}$ (10 μL at $1 \text{ ng } \mu\text{L}^{-1}$).

2.3. MALDI-TOF/TOF-MS analysis

MALDI-TOF/TOF-MS and high-energy collision dissociation CID-MS/MS spectra were acquired with a 4800 Plus MALDI TOF/TOF-MS spectrometer (ABSciex - 2010, Foster City, CA) equipped with a Nd:YAG laser (355 nm, 200 Hz frequency, 3-7 ns pulse). The MS and MS/MS measurements were performed in α -CHCA matrix, by mixing 24 μL of matrix solution with 1 μL of sample on the sample target and allowing it to dry at room temperature. α -CHCA matrix solution consisted in 10 mg mL^{-1} α -CHCA in 0.1% TFA in H_2O /acetonitrile (1/1, v/v). To obtain the individual mass spectrum, the instrument was operated in reflector mode, and the experiments were carried out in positive and negative ion modes in the mass range m/z 400-2,000. At 400 nanoseconds after laser firing, an accelerating voltage of 20 kV was applied. Ions were refocused in the two-stage reflector and detected in the second detector. The Mirror 2 to Source 1 voltage was kept constant at a ratio value of 1.105. For MS/MS acquisitions, the Source 1 voltage was held at 8 kV, and the laser value was set 1,000 units higher

than in the MS experiments. Precursor ions were selected for fragmentation by means of a timed ion selector at a resolution value of 250 (full width at half maximum, FWHM). Selected ions were decelerated before their arrival to the collision chamber at the deceleration stack, and were dissociated upon collisions with air at a kinetic energy of 1 keV (high-energy collision-induced dissociation). Fragment ions were reaccelerated in the second source at 15 kV after a short delay. The Metastable Suppressor was activated in all the MS/MS experiments to avoid the detection of remaining precursor ions and unwanted metastable decay fragments. MS/MS spectra in the mass range m/z 9-1,200 were acquired in positive and negative ion modes. External mass calibration was performed with low-mass peptide standards (PerSeptiveBiosystems, Monza, Italy). Data Explorer software was used for spectral treatment (including smoothing and noise filtering).

2.4. LC/ESI-HRMS analysis

A Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer equipped with heated electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS autosampler kept at 15 °C (Thermo Fisher Scientific, San Jose, California) were used for the analysis by LC/ESI-HRMS.

The chromatographic separation was performed on a reversed-phase Phenomenex Luna C18(2) column (150×2.0 mm, 5 µm). The mobile phase was composed of Milli Q water as solvent A and acetonitrile as solvent B, both containing 0.08% (v/v) formic acid at a flow rate of 200 µL min⁻¹. The linear gradient elution program for the analysis was: 10-30% B 10 min, 30-35% B 20 min, 35-55% B 15 min, 55% B 5 min, 55-90% B 2 min, 90% B 3 min and return to initial conditions for re-

equilibrate (10% B 10 min). The total duration of the method was 65 min. The injection volume was 10 μL for direct water analysis of high level concentration extracellular fractions and quantitative analysis of intracellular extracts or 100 μL for direct water analysis of low level concentration extracellular fractions. The retention time in standards and samples must agree: the ratio of the chromatographic retention time of the analyte to that of the IS (nodularin), shall correspond to that of the calibration solution at a tolerance of $\pm 2.5\%$.

The analyses were carried out in positive and negative ion ESI and the instrument was daily calibrated in both ionization modes. To optimize the source, transmission and HRMS conditions, a 1 mg L^{-1} stock solution in methanol of each MC was infused at a flow rate of 1-5 $\mu\text{L min}^{-1}$ by the syringe pump and mixed with the mobile phase (200 $\mu\text{L min}^{-1}$, $\text{H}_2\text{O}:\text{ACN}:\text{HCOOH}$ (50:50:0.08, v/v) using a Valco zero dead volume tee piece (Supelco, USA). Nitrogen was used as sheath gas, auxiliary gas and collision gas. In both modes the final parameters were: capillary temperature of 250 $^{\circ}\text{C}$, heater temperature of 30 $^{\circ}\text{C}$, sheath gas flow rate of 42 psi, auxiliary gas flow rate of 10 (arbitrary units) and sweep gas flow rate of 0 (arbitrary units). In ESI using the positive ion mode, spray voltage of 4.25 kV, capillary voltage of 35 V, tube lens voltage of 186 V and skimmer voltage of 35 V were used. In ESI using the negative ion mode, spray voltage of -2.75 kV, capillary voltage of -35 V, tube lens voltage of -186 V and skimmer voltage of -18 V were used. Data were acquired simultaneously in full scan and all ion fragmentation (AIF) modes. The ESI-HCD-MS fragmentation [38] by Exactive mass spectrometer occurs in a special collision chamber essentially comparable with those used in QqQ without precursor mass selection (AIF mode) similar to in-source fragmentation [50]. But, a comprehensive chromatographic separation allowed a reliable HCD fragmentation without isolating the precursor. Three

time segments were set in each ionization mode. In ESI using positive ion mode: two scan events in the first segment (0–12 min) without and with AIF (HCD 30 eV); three scan events in the second segment (12–30 min) without and with AIF (HCD 50 and 70 eV); and three scan events in the third segment (30–65 min) without and with AIF (HCD 32 and 60 eV) were adopted. In ESI using negative ion mode: two scan events in the first segment (0–12 min) without and with AIF (HCD 32 eV); three scan events in the second segment (12–30 min) without and with AIF (HCD 51 and 57 eV); and three scan events in the third segment (30–65 min) without and with AIF (HCD 50 and 60 eV) were applied. In some segments two different energies are used because according to their structure, each MC needs more or less energy to fragment. The Orbitrap-Exactive HCD mass spectrometer is fast enough (2Hz at high resolution 50,000) to perform three experiments simultaneously and keep suitable points per peak (at least 10) for quantitation purposes. The mass range was m/z 400–1,200 in full scan and m/z 60–1,200 in AIF mode. The automatic gain control (AGC) was set as “balanced” with a maximum injection time of 250 ms. The data was processed with Xcalibur 2.1 software (Thermo Fisher Scientific, Bremen, Germany).

2.5. High resolution and mass accuracy

Table 1 shows the instrumental specifications concerning resolution and accuracy for these instruments. The experimental resolution (FWHM) was better than 18,000 by TOF/TOF instrument and 40,000 by Orbitrap analyzer, in all the mass range of interest. It should be noted that Orbitrap worked in same HRMS by full scan and AIF modes. In contrast, TOF/TOF functioned at lower resolution (9,000) for CID-MS/MS fragmentation mode with respect to the full scan mode (resolution: 18,000).

External calibration and mass accuracy expressed as parts per million (ppm) were used. Mass accuracy in all cases was <3 ppm for molecular and fragment ions. So a maximum of ± 3 ppm extraction window was allowed for peak identification.

2.6. Restrictive criteria for propose elemental formulae

For structural elucidation and identification of toxins is crucial to know the exact mass and correct elemental composition of analytes. To calculate the theoretical accurate mass (m/z calculated) of analytes, the mass of the electron has been taken into account as 0.00055 Da [51]. In addition of convenient HRMS and accuracy, in the present study, to be confident of identification and elemental formulae proposed, the following criteria were used: elements considered were restricted in accordance with MC molecular formulae (C:30-60, H:50-90, O:0-20, N:0-20, S:0-1); the isotopic pattern considering $M+1$ and $M+2$ was matched ($\Delta < 20\%$); and, the charge, the ring plus double bond equivalents (RDBE) and nitrogen rule were taken into account. The combination of high resolution, AMM and restrictive criteria was crucial for unequivocal identification of target and unknown compounds as well as precise quantitation of analytes.

2.7. LC-MS quantitation, quality control and quality assurance

Quantitation of analytes by LC/ESI-HRMS was performed using the internal calibration method. As there are no commercial isotopic-labeled MCs, nodularin was used as IS. Nodularin was chosen as IS because it is a pentacyclic peptide that contains

the MC conserved Adda moiety and so far is only produced by the cyanobacterium, *Nodularia spumigena*, frequently found in brackish waters rather than freshwaters. Toxins different from the available standards were quantified related to MC-LR. Calibration standard solutions of ten commercially available MCs at different concentration levels (n=5) were prepared daily from 0.06 to 110 $\mu\text{g L}^{-1}$ for direct analysis of water (extracellular) samples and from 0.05 to 20 $\text{ng } \mu\text{L}^{-1}$ for extracts of sestonic fraction (intracellular samples) with 1.0 $\mu\text{g L}^{-1}$ and 1.0 $\text{ng } \mu\text{L}^{-1}$ final concentration of nodularin, respectively.

The matrix effect, linearity, limit of detection and quantitation, trueness and precision were studied for direct water analysis by LC/ESI-HRMS in positive and negative ionization modes. Each calibration curves were tested in different matrices: drinking, surface and bottled water; and equivalent curves were obtained for the three matrices. Therefore, the validation study was carried out on bottled water. In order to evaluate linearity, r^2 coefficient and residuals were studied. The limit of detection (LOD) was estimated for a signal-to-noise (S/N) ratio equal to 3 from the chromatograms of the samples spiked at the lowest validated level and the lowest level of linearity range was considered the limit of quantitation (LOQ). Trueness and precision were performed by spiking experiments using fortified bottled water at three levels (0.2, 0.6 and 6 $\mu\text{g L}^{-1}$, respectively). These samples were extracted following the same procedure used for real samples. Trueness was expressed as the recovery of fortified water samples (n=5). Recovery was calculated as the amount of toxin detected in front of the amount fortified. To check the trueness in all samples, the recovery of nodularin, added before extraction, was calculated. The interday and intraday precisions were expressed as the relative standard deviation (RSD) of 5 consecutive measurements at 0.6 times the legislated limit.

Blank spikes were used as quality controls. A blank sample (bottled water and blank filter for extracellular and intracellular analysis, respectively) was always extracted with every batch of samples showing good repeatability for the method. Instrumental blanks (water and methanol for extracellular and intracellular analysis, respectively) were injected between samples and three additional blanks were injected after the most concentrated internal calibration standard in order to avoid cross talking between injections. External quality assurance was performed by participation in intercomparison exercises.

3. Results and discussion

3.1. Determination of MCs. MS analytical protocol

Following the WHO recommended guideline [5], the total MC concentration and whole water sample (free plus cell-bound) should be considered. Therefore, a HRMS integrated approach utilizing the MS methodologies developed for intracellular (cell bound) and extracellular (free) MCs and related peptides identification and determination was presented. The Fig. 1 shows the scheme of analytical methodology.

The natural samples (water and algae) were filtered for independent analysis of aqueous and sestonic fractions. Initially, rapid qualitative analysis of toxins including identification tools described in the following *Identification and confirmation criteria* section was performed. Lyophilized sestonic and aqueous fractions were analyzed directly (without prior extraction) by positive/negative ion MALDI-TOF/TOF-MS and LC/ESI-HRMS (Orbitrap-Exactive HCD) techniques, respectively. If these analyses do not reveal the presence of any toxin our routine/process ends here. Otherwise,

quantitative analysis of both extracellular toxins by direct injection and intracellular toxins in the extract of lyophilized algae using LC/ESI-HRMS method was performed.

3.2. Throughput analysis (direct MALDI and ESI analysis)

In order to have faster methods for toxin analysis, MALDI-TOF/TOF-MS and direct injection by LC/ESI-HRMS were implemented for direct analysis of lyophilized sestonic and filtered water fractions, respectively.

Initially, MALDI-TOF/TOF-MS method was employed for qualitative analysis and preliminary identification of cyanobacteria biotoxins [31], as a simple and rapid screening of lyophilized sestonic fraction samples. It is a high-resolution and sensitive technique that required little sample handling, had low sample consumption, and allowed accurate identification of toxin congeners simultaneously without preceding separation. Additionally, the sample preparation method was very easy. MALDI ionization technique allowed direct analysis of solid biomass without prior extraction and chromatographic separation. As a result, all the MCs were simultaneously ionized and a fingerprint of MC presence was provided quickly and easily by the full scan mass spectra. However, the poor spot-to-spot and shot-to-shot reproducibility of analyte signal intensities makes it necessary to use an IS for quantitation [32] but with solid samples was difficult to handle. Therefore, for quantitative analysis, methanolic extracts of lyophilized sestonic fraction were analyzed using LC/ESI-HRMS as chosen method.

On the other hand, by direct injection and LC/ESI-HRMS analysis of aqueous fraction, the pre-treatment was restricted to filtration and IS addition, avoiding sample manipulation and contamination during the pre-treatment steps. Although different large volume injection approaches have been proposed over the last years, in this work 10 μ L

of filtered water were enough for direct injection and analysis of high level concentration water samples. However, low level concentration water samples required a large volume injection of 100 μL in few specific cases. Large volume injection technique is easier to set up, it was needed only small hardware modifications to used autosampler.

Quality parameters for direct analysis of commercially available MCs (-dmLR, -dmRR, -LA, -LF, -LR, -LW, -LY, -RR -WR and -YR) in filtered water by LC/ESI-HRMS are shown in Table S1 of supplementary data. Linearity was tested between 0.06 and 110 $\mu\text{g L}^{-1}$ ($n=5$) and Table S1 lists the linearity range for each MC. All MCs showed satisfactory r^2 values higher than 0.98 and residuals lower than 32%. LODs ranged from 0.01 (MC-dmRR, -LA, -LR and -RR) to 0.05 $\mu\text{g L}^{-1}$ (MC-dmLR, -LF and -LW) and from 0.01 (MC-dmLR, -LA, -LF, -LR, -LY and -YR) to 0.07 $\mu\text{g L}^{-1}$ (MC-RR) for positive and negative ionization modes, respectively. The lowest level of linearity range (0.06 or 0.20 $\mu\text{g L}^{-1}$, based on MC and ionization mode) was considered the LOQ for each MC analyzed. Negative ionization mode showed lowest LODs, except for MCs containing two Arg residues (MC-dmRR and -RR), but this low concentration level (0.06 $\mu\text{g L}^{-1}$) did not retain linearity, except for MC containing no Arg residue (MC-LA, -LF and -LY) and the LOQs were higher by ESI(-), 0.2 $\mu\text{g L}^{-1}$ for MC-LR, -LW, -RR, -WR and -YR. In contrast, this ionization mode was linear until higher concentrations, 110 $\mu\text{g L}^{-1}$ for MC-LY and 70 $\mu\text{g L}^{-1}$ for MC-LR, -LW, -WR and -YR versus 6 $\mu\text{g L}^{-1}$ by ESI(+). The recoveries for all MCs were in the range of 67-133%, for MC-LF and MC-WR at the lowest and highest validated levels, respectively. The interday and intraday precisions of common MCs (-LR, -RR and -YR) at 0.6 times the legislated limit were satisfactory with RSDs lower than 12 and 27%, respectively.

3.3. MS and fragmentation characterization

To perform suitable mass spectral characterizations of the molecular and fragment ions obtained we have used the full scan MALDI-TOF/TOF-MS and ESI-Orbitrap-Exactive-HCD-MS instruments and MS/MS and AIF fragmentation modes, respectively.

Initially, the ten commercially available MC standards were tested alone and in combination. Subsequently, the ionization techniques were applied to real samples. The presence of different amino acids within the cyclic MC structure allows multiple possibilities for the localization of charge, generated by both protonation (or cationization) or deprotonation. Accordingly, MALDI and ESI sources were implemented in positive and negative ionization modes in order to compare, combine and use cumulative information provided for unambiguous identification and confirmation of cyanotoxins.

MALDI and ESI-MS spectra in positive and negative ionization modes yield the singly protonated or deprotonated ion, $[M+H]^+$ and $[M-H]^-$, respectively, except for two arginine-containing MCs (MC-dmRR and MC-RR) which were detected as doubly charged species $[M+2H]^{2+}$ by ESI using the positive ion mode [52, 53]. MC with only one arginine (MC-dmLR, -LR, -WR and -YR) residue presented both signals by ESI, $[M+H]^+$ and $[M+2H]^{2+}$, but the singly protonated ion was usually the most intense signal. The characteristic doubly charged ion (Fig. S2 of supplementary data) was used for the differentiation of MCs from other types of peptides and, as discussed below, was adopted as diagnostic ion of arginine-containing MC detection. Additionally, the acidic conditions of proposed analysis (0.1% of TFA or 0.08% of formic acid by MALDI and ESI techniques, respectively) were able to minimize the presence of other adduct ions

such as $[M+Na]^+$ and/or $[M+K]^+$, enhanced the $[M+H]^+$ ion signal and improved the sensitivity of analysis. Yuan et al. [53] described that some MC, especially those having no arginine (Arg) residue as MC-LA, may show these cationized ions derived from trace alkali metal salts in environmental samples and cultured strains or glass tube.

Although ESI- recorded in the positive ion mode is the most commonly used in the analysis of MCs, ESI- and MALDI-MS in the negative ion mode were also applied by sources in order to compare the sensitivity, to improve the selectivity and to achieve complementary information to the positive ion mode results. These changes in the selectivity of the ion mode aided the characterization of unknown toxins and to confirm structure proposals. Negative ionization mode by MALDI and ESI presented low absolute intensities but also low background and therefore provided suitable S/N ratios. According to other authors [19-21, 44], as shown Table S1 of supplementary data, the analysis of MCs containing no Arg residue such as MC-LA, -LF, -LW and -LY presented high sensitivity in negative ionization mode.

In the present work, two different techniques of fragmentation in both positive and negative ionization modes were applied to cyanotoxin analysis: high energy CID fragmentation by TOF/TOF and high energy collision dissociation (HCD) by Orbitrap-Exactive. As shown in Fig. 2 for anabaenopeptin F, found in both algal bloom events, equivalent fragmentation spectra were obtained using the two different mass spectrometers for all MCs and related peptides studied and detected in the samples. The fragmentation energy applied was typically 1 keV in TOF/TOF analysis and 30-70 eV in HCD experiments, depending if the signal was doubly or singly charged. Normally, less energy was needed to fragment $[M+2H]^{2+}$ MC signals [54]. In our MS method doubly charged signals (MC-dmRR and MC-RR) were fragmented at 30 eV and single charged signals of other variants needed more energy to fragment, typically 50-70 eV.

As an example, Fig. S2c of supplementary data shows the ESI-HRMS AIF spectra of [MC-dmRR+2H]²⁺ by Orbitrap instrument.

3.4. MALDI-TOF/TOF-MS, ESI-Orbitrap-Exactive-HCD-MS approaches and high-energy CID-MS/MS

Table 1 compares the HRMS methods used in this work with the most common MS-based technique applied for the analysis of MCs in the last years (LC-ESI-MS/MS by QqQ analyzer). As shown in this table, the acquisition modes operated by QqQ are SRM and MS/MS pre-target experiments for quantitative and qualitative purposes, respectively. In contrast, TOF and Orbitrap instruments result in non-target analysis [55-57] due to full scan acquisition mode. Non-target analysis covered the possibility to analyze all MC analogues concurrently without any pre-selection of target MC. Additionally, any compound present in the sample (unexpected analytes, transformation products and/or impurities, or even untargeted compounds that can be toxic) could be detected and multi-class of toxins could be included in the method. Furthermore, archived data can be subjected to retrospective “post-targeted” analysis to determine non-a priori selected analytes as new toxins or toxin analogues once discovered. Accordingly, a screening of other potential toxins and related peptides as anabaenopeptins was performed in the analyzed samples.

Moreover, although low resolution QqQ instruments by SRM acquisition mode could be considered as highly selective, the occurrence of false positives and negatives arises in the analysis of dirty samples. The HRMS integrated proposed in the present work combined capabilities of high resolution and AMM with information provided by fragmentation studies. Consequently, this approach offered high selectivity and many

IPs, as discussed later. TOF/TOF and Orbitrap-Exactive HCD instruments allowed achieve the measurements of the full scan MS and product ion spectra with high mass accuracy. This combination helped to overcome confirmatory problems experienced when using low resolution MS (LRMS) [43, 58]. It is noteworthy that the MS experiment set up by Orbitrap analyzer operated simultaneously in AIF and full scan HRMS modes (see Fig. 3). From this MS experiment, the full scan and fragmentation mass spectra were registered alternatively and AMM (<3ppm with external calibration) for all molecular and product ions of MCs were obtained by HRMS in a single run. As an example, mass accuracy of main signals obtained from ESI-Orbitrap fragmentation spectra of MC-LR by positive and negative ionization modes are listed in Table 2. In this case, experimental mass accuracy ranged from 0.10 to 1.67 ppm (m/z 60-1200) absolute values.

3.5. Identification and confirmation criteria

MALDI-TOF/TOF-MS and LC/ESI-HRMS allowed a comprehensive monitoring of the following tools for identification purposes: i) proof exact mass (mass accuracy <3 ppm) of molecular and fragment signals by positive and negative ionization modes; ii) propose correct elemental formulae of these ions taking into account the restrictive criteria described in the *Restrictive criteria for propose elemental formulae* section; iii) detection of fragment diagnostic ions also in both ionization modes, m/z 135.0804 and 128.0353, respectively. The fragmentation mass spectra of analytes were recorded to obtain his fingerprint fragmentation pattern, detailed structural information and supplementary characteristic fragments of the compounds under study, and to increase the number of IPs and selectivity of the proposed methodologies. Additionally,

LC/ESI-HRMS also must satisfy the following criteria: i) the distinctive retention time of each toxin was used as primary identification tool due to chromatography separation; ii) presence of double charged species, such as $[M+2H]^{2+}$, characteristics of ESI ionization mode and MCs with some Arg-residues in its structure. In fact, the LC/ESI-HRMS methodology provided more identification tools.

3.5.1. Fragmentation pattern

The fragmentation mass spectra of analytes were obtained for detailed qualitative analysis. Every MC has a specific fingerprint pattern in its fragmentation spectrum. This typical fingerprint spectrum was used for structural identification of the different MC variants and related peptides, especially when there were no commercial standards. The different fragment ions provided relevant information about amino acid structure of MCs. Combining positive and negative elucidation exhibited complementary information for structural assignments.

As an example, Fig. S3 of supplementary data includes the fragmentation spectra of MC-LR from Alcántara bloom by ESI-Orbitrap in positive and negative ionization modes. The main fragment ions of MC-LR have been included in Table 2. These fragment ions have been already described in the literature [19, 21, 52] but in the present manuscript all the fragment ions have been evaluated for confirmatory purposes. As shown in Fig. S3a and Table 2, MC important generic characteristic ions [6] in positive ionization mode are: m/z 135.0804, which is a product ion of the Adda side chain, unusual amino acid present in all MCs, formed by α -cleavage of the methoxyl group ($\text{PhCH}_2\text{CH}(\text{OMe})$); m/z 213.0870, which corresponds to a product ion of Glu+Mdha; and m/z 375.1915 from Adda+Glu+Mdha. Furthermore, Fig. S3a includes a typical dissociation product at m/z 70.0651 representative of MCs containing Arg (MC-

LR in this spectrum). Also, by negative ionization mode a product as a result of nonspecific loss of water and a generic characteristic fragment common to all MCs of m/z 128.0353 (see Fig. S3b) were obtained. Dörr et al. 2011 identified this fragment ion as a product of a charge-transfer reaction at the leaving cyclic acid unit [19].

3.5.2. Diagnostic ions

All MCs in their fragmentation spectra showed some common fragments. The fragmentation spectra of $[\text{MC-dmRR}+2\text{H}]^{2+}$ signal and MC-LR ($[\text{M}+\text{H}]^+$ and $[\text{M}-\text{H}]^-$ signals) from the studied blooms by ESI(+) and (-) Orbitrap are displayed as examples in Fig. S2c and S3 of supplementary data, respectively. The two most selective and intense fragment ions are m/z 135.0804 ($\text{C}_9\text{H}_{11}\text{O}$) and 128.0353 ($\text{C}_5\text{H}_6\text{O}_3\text{N}$) arising from the characteristic Adda side chain and the cyclic glutamic and β -methylasspartic acids in positive and negative ionization modes, respectively. Both were used as diagnostic ions in their reconstructed ion signal of MALDI-TOF/TOF and ESI-Orbitrap methods. The reconstructed chromatograms of diagnostic fragment ions in the extracellular fraction from Vilasouto bloom by LC/ESI-HRMS analysis in both ionization modes are shown in Fig. 3. As a result of working with the reconstructed chromatograms of diagnostic fragment ions, observed signals are simplified and the chromatogram is more specific and selective, easily identifying potential MC that may be in the sample. As is known, the retrospective analysis of data working by HRMS and full scan acquisition mode is done by extracting the accurate target m/z signals. In the reconstructed chromatograms of diagnostic fragment ions, only the peaks having the characteristic fragments of MCs are observed. Noteworthy that the extraction window used was ± 3 ppm. The proper setting of this extraction window increases the selectivity and specificity of the method. Additionally, the doubly charged specie was used as diagnostic ion for the identification

of MCs containing Arg. A compound with a doubly charged signal could potentially be identified as a toxin with one (weak $[M+2H]^{2+}$ signal) or two (intense $[M+2H]^{2+}$ signal) arginine residues. As is well known, a doubly charged signal shows a characteristic isotopic profile with a M+1 peak at a distance of half unit mass, see Fig. S2b of supplementary data.

3.5.3. *IPs*

The concept of IPs described in the 2002/657/EC Decision [42] has been applied for guarantee an effective and reliable MC identification and quantitation. This EC guideline assigns the number of IPs that each of the basic MS techniques can confer. The main advantage of using IPs is that the verification of identity can be done in a well-described and internationally accepted way.

The European guideline assumes that a molecular or fragment ion measured with a HRMS would give one IP more than LRMS and sets high resolution typically as greater than 10,000 at 10% valley. The difficulties possibly appear when one tries to classify mass analyzers as low or high resolution mass spectrometers. By TOF and Orbitrap instruments the resolution are usually measured at 50% height (FWHM). As has been previously described in the literature, FWHM resolutions must divide by two or three times to make them equivalent to 10% valley resolutions. Accordingly, we considered the fragment ions derive from TOF/TOF analysis as LRMS product ions. Should be mentioned that the use of exact mass as a confirmatory tool is not explicitly specified in the legislation [42], where the IPs are assigned based on the resolution of traditional high resolution mass spectrometers (magnetic sector field, FT-ICR). According to other authors [59], in addition to resolution, the use of mass accuracy

provided by TOF and Orbitrap (<3 ppm) has been taken into account for compute the number of IPs.

The accurate mass techniques proposed in our integrated HRMS strategy showed more selectivity versus the alternative MS/MS approaches as evidenced by the highest IPs proved and included in Table 1. The EC Decision proposes 1.0, 2.0, 1.5 and 2.5 IPs for each LRMS and HRMS precursor ion, LRMS and HRMS product ion, respectively. To decrease the probability of false positives and negatives, a minimum of four exact masses were always considered for each analyte. This includes $[M+H]^+$ and $[M-H]^-$ molecular ions and the fragment diagnostic ions of m/z 135.0804 and 128.0353 in positive and negative ionization modes, respectively. Taking into account these molecular and diagnostic fragments, MALDI-TOF/TOF-MS and LC/ESI-HRMS methods provided seven and nine IPs for MC confirmation, respectively (Table 1), compared to five of the LC/ESI-QqQ technique. Additionally, $[M+2H]^{2+}$ by ESI and other fragment ions by TOF/TOF and AIF were considered. As a result selectivity and IPs increases. Namely, for arginine-containing MCs, the doubly charged molecular ion contributed adding one and two IPs to LC/ESI-QqQ and LC/ESI-HRMS methods (total six and eleven IPs, respectively). Finally, each supplementary product ion from fragmentation spectra added 1.5 and 2.5 IPs to total MALDI-TOF/TOF-MS and LC/ESI-HRMS IPs, respectively, balancing that no ion ratio was calculated. All fragmentation spectra of this manuscript include the number of IPs that provides each analysis. Therefore, the results obtained have a significant rate of MC presence confirmation in the samples. In contrast, MALDI(+)-TOF/TOF-MS method only could provide 3.5 IPs (1.5 IPs for the LRMS product ion + 2 IPs for the HRMS parent ion) and no ion ratio was measured. The 2002/657/EC Decision establishes the need to obtain at least three (group B: compounds with an established maximum residue level)

or four (group A: banned compounds) IPs in order to confirm organic residues and contaminants in live animals and animal products. This evidences the advantage of working with both ionization (positive and negative) and acquisition (full scan and fragmentation) modes. Studying the complementary information obtained from MS and MS/MS in both ionization modes was very useful for confirmation purposes, significantly increasing the selectivity and number of IPs. As the number of IPs increases, to use the integrated methodology proposed minimizes the probability of false positive and negatives.

3.6. Sample Results

The MS protocol and identification tools proposed were applied to the analysis of two representatives bloom episodes of common cyanobacteria genera in Spanish waters: *Microcystis aeruginosa* and *Planktothrix rubescens* from Alcántara and Vilasouto reservoirs, respectively.

Table 3 shows the predominant signals identified as potential MCs and related peptides in water (extracellular) and sestonic (intracellular) fractions of bloom samples and their concentration levels expressed in $\mu\text{g L}^{-1}$. As the cyanobacteria produce toxins intracellularly, a greater variety of toxins and higher concentration levels were present in sestonic fraction. Taking into account that negative ionization mode usually presents worse LOQ, except for MCs containing no Arg residue, the detection of less concentrated MCs were not confirmed in negative ionization mode. Screening of other potential toxins and related peptides was performed as full scan methods were used. Overall, eleven MCs and two anabaenopeptins (B and F) were detected in the blooms. According to other authors, the blue-green algae *Microcystis aeruginosa* and

Planktothrix rubescens have shown to be a rich source of unique cyclic peptides such as anabaenopeptins [11, 60, 61]. Fig. 2 includes the TOF/TOF and HCD fragmentation spectra of anabaenopeptin F in positive ionization mode. The main species in the fragmentation spectra of anabaenopeptin F were the side chain (CO+Arg, m/z 201.0982) and Arg from the side chain (Arg+2H, m/z 175.1190). Several fragments at m/z 84.0808, 263.1390 and 651.3865 were also detected. The related ion (m/z 84.0808) from cleavage of lysine is characteristic of all anabaenopeptins (Lys-immonium ion), whereas m/z 651.3865 corresponded to the complete ring (Lys+Phe+Mala+Htyr+Ile+2H) and the fragment m/z 263.1390 was identified as [Mala+HTyr+H]⁺ in a previous study [60].

Alcántara bloom showed in their intracellular fraction a large number of MC variants: two MC-dmLR, -LR, -dmRR, two -dmYR, -RR, -YR and -WR; which were confirmed by AMM (<3ppm), fragmentation spectra and presence of diagnostic ions. In contrast, the identification of four signals (m/z 945.4589, 959.4799, 965.4123, 973.5586) tentatively proposed as MCs by its mass spectrum (see Table 3) has been rejected by AMM exceeding 3 ppm and/or not detected the presence of diagnostic ions discussed in this work. Fig. 4 displays MALDI-TOF-MS spectra of Alcántara bloom in positive and negative ionization modes. As no chromatographic separation was carried out, all the MCs were simultaneously ionized and a fingerprint of compounds detected in the sample was obtained. On the other hand, the analysis of the methanolic extract by LC/ESI-HRMS revealed the presence of two different signals at m/z 1045.5353 with a different retention time (Fig. 5). The comparative pattern isotopic in positive ionization mode of a simulated MC-YR spectrum and m/z 1045.5353 signals from Alcántara bloom spectra by MALDI-TOF-MS and LC/ESI-HRMS are included in the Fig. 5. In MALDI-TOF-MS, any isobaric interference including congeners with same or similar molecular weight can interfere and overlap the isotopic patterns. In contrast, the

LC/ESI-HRMS analysis allowed separate and identify two MC variants of m/z 1045.5353 present in the sample through their different retention times. The M+2 signal in MALDI-TOF-MS spectrum (Fig. 5) corresponds to an intensity of 30% of the base signal due to all MC variants with theoretical m/z 1045.5353 overlap, whereas in the ESI-HRMS spectra this signal corresponds to 17% and the isotopic pattern matches with the theoretical simulated (intensity of 17% for M+2 signal) . The first specie was confirmed as MC-YR and the other signal should be a demethylated variant of MC-HtyR or MC-RY in agreement with the variants with this base peak reported in the literature. The other signal was not included in the final results table (Table 3). Intracellular MC concentrations observed in Alcántara bloom ranged from 20 to 2271 $\mu\text{g L}^{-1}$ equivalents, taking into account filtered water volume. MC-RR and -LR were found as the major variant represented the 43 and 26% of total concentration, respectively. Fig. S2a of supplementary data displays the mass spectrum of MC-RR by LC/ESI-HRMS, this variant showed the typical two Arg-residues doubly charged ion $[\text{M}+2\text{H}]^{2+}$ as the base peak.

Vilasouto sample only presented two MC variants, MC-dmLR and -dmRR, both demethylated. This was consistent with other water blooms of *Planktothrix rubescens* reported in the literature [11, 13]. Fig. 3 shows the total and reconstructed chromatograms of Vilasouto extracellular fraction by LC/ESI-HRMS analysis in full scan and AIF acquisition modes and both ionization modes, positive and negative as example. An aliquot of the extracts of these samples were also analyzed by thiol derivatization and LC-MS methodology [48] in order to obtain evidence for the presence of Mdha or Dha and to differentiate between the isobaric amino acids Mdha and Dhb in candidate cyanotoxin peaks. Noteworthy, the demethylated variants detected in Vilasouto sample contained isobaric amino acid Dhb in position 7 instead of Dha.

Intracellular MC concentrations observed in Vilasouto bloom were MC-dmRR (41 $\mu\text{g L}^{-1}$) and MC-dmLR (2.9 $\mu\text{g L}^{-1}$), which corresponds to 93% and 7% of total MC concentration, respectively.

As examples, Fig. S2c and S3 of supplementary data show HCD fragmentation spectra by LC/ESI-HRMS of two major variants, MC-dmRR from Vilasouto sample and MC-LR from Alcántara sample, respectively. Diagnostic ($[\text{MC-RR}+2\text{H}]^{2+}$, m/z 135.0805 and 128.0350) and characteristic fragments of MCs are observed in these fragmentation spectra.

The aqueous fraction usually contains the most hydrophilic MCs. MC-LR and MC-RR were detected in Alcántara extracellular water sample at 4.5 and 1.8 $\mu\text{g L}^{-1}$, 71 and 29% of extracellular MC content, respectively. MC-dmRR and -dmLR, were present in the Vilasouto extracellular fraction at 19 and 3.1 $\mu\text{g L}^{-1}$, respectively. Equivalent concentration ratios MC-dmRR:-dmLR were observed in extracellular and intracellular fractions of Vilasouto sample.

Both samples presented extracellular total MC concentrations exceeding the Spanish legislated and WHO guidance levels (1 $\mu\text{g L}^{-1}$). In addition, the very high intracellular concentrations detected could represent a potential danger by contact or ingestion of the algae and because these cyanobacterial strains could release their internal toxins to the water. It indicates the necessity of management protocols to the presence of a bloom and monitoring programs to prevent the humans and animals health risk.

4. Conclusions

The developed methodologies composed an integrated strategy for determination of MCs in whole natural samples that combines quickly capabilities of high resolution and AMM with information provided by fragmentation studies. In order to have faster methods, direct analysis of lyophilized biomass and direct injection of filtered water by MALDI-TOF/TOF-MS and LC/ESI-HRMS, respectively, were implemented. The integration of ESI and MALDI sources, positive and negative ionization modes and HRMS and fragmentation techniques has resulted in a powerful and helpful MS-based approach for the detection and the confirmation of free and cell-bound MCs and related peptides. Characteristic MC signals in the MS and fragmentation spectra were suggested as diagnostic ions. Additionally, some confirmatory criteria for reliable HRMS identification and quantitation were used. As a result, the HRMS strategy proposed in this work allowed a comprehensive monitoring of several tools for identification and confirmation purposes. The IP concept according to 2002/657/EC Decision has been applied for confirmation of MC identification. The typical fingerprint of fragmentation mass spectra of analytes were obtained for structural identification of the different MC variants and related peptides, especially when there were no commercial standards. Each supplementary ion from fragmentation spectra and both positive and ionization modes added supplementary IPs.

The use of the present HRMS procedure versus the alternative QqQ methodologies, offers several advantages: more selectivity, higher IPs, less probability of false positives and negatives, non-target and retrospective “post-targeted” analysis. TOF/TOF and Orbitrap are full scan MS approaches that perform non-target analysis

and covered the possibility to detect all MC analogues concurrently and any compound present in the sample. Therefore, it is possible to make retrospective analysis of samples without re-injecting for analysis of emerging toxins. It is worth mentioning that the Orbitrap-Exactive HCD instrument allows AMM of molecular ions and fragments simultaneously.

Finally, the MS protocol and identifications tools proposed were successfully applied to analysis of two characteristic water blooms from Spain.

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Figure captions

Fig. 1. Scheme of analytical methodology.

Fig. 2. Fragmentation spectra of $[\text{Anabaenopeptin F} + \text{H}]^+$ signal (exact mass m/z 851.47740) from Vilasouto bloom by: **a** ESI-Orbitrap acquired at an HCD of 50 eV; **b** MALDI-TOF/TOF acquired at 1 keV (b).

Fig. 3. Chromatogram of LC/ESI-HRMS analysis of extracellular fraction from Vilasouto bloom in positive and negative ionization modes in: **a** and **c** full scan mode acquisitions; **b** and **d** HCD fragmentation mode monitoring the diagnostic fragment ions. Reconstructed chromatogram of analytes identified in negative ionization mode: **e** anabaenopeptin B (m/z 835.4472); **f** anabaenopeptin F (m/z 849.4629); **g** MC-dmLR (m/z 979.5281); **h** MC-dmRR (m/z 1022.5429).

Fig. 4. MALDI-TOF-MS spectra from Alcántara bloom by: **a** positive; **b** negative ionization modes.

Fig. 5. Comparative pattern isotopic of m/z 1045.5353 ($\text{C}_{52}\text{H}_{73}\text{N}_{10}\text{O}_{13}$) positive signals from: **a** simulated mass spectrum; **b** MALDI-TOF-MS; and **c** LC/ESI-HRMS spectra of Alcántara bloom. **d** Reconstructed chromatogram of m/z 1045.5353 signals of Alcántara bloom analyzed by LC/ESI-HRMS.

Fig 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

C52H72N10O13 +H: C52 H73 N10 O13 pa Chrg 1

RT: 0.54 - 34.48

Table 1
Comparison of mass spectrometry techniques

Analyzer	QqQ	TOF/TOF	Orbitrap
Ionization	ESI (+/-)	MALDI (+/-)	ESI (+/-)
HPLC	Chromatography Separation	Direct analysis (without prior extraction and separation chromatography)	Chromatography Separation
Analysis	Quantitative/qualitative Target	Qualitative Non target	Quantitative/qualitative Non target
	SRM	Full Scan HRMS	Full Scan Ultra-HRMS
Acquisition mode	MS/MS (structural information)	MS/MS (structural information)	All Ion Fragmentation (AIF) (structural information)
Resolution	Unitary Δm : 0.7	20,000	100,000 (m/z 200; 1 Hz) ^a 50,000 (m/z 200; 2 Hz) ^b
Accuracy (instrument specifications)	-	<3ppm internal calibration	<1ppm internal calibration
Minimum IPs^c	5 or 6 (if the $[M+2H]^{2+}$ signal is also characteristic)	7	9 or 11 (if the $[M+2H]^{2+}$ signal is also characteristic)

^a Best Exactive specifications

^b Resolution specified in our MS experiment

^c Considering only diagnostic ions, not all fragment ions of spectrum

Table 2

Ion composition, theoretical accurate and measured m/z and mass accuracy of main signals obtained from ESI-Orbitrap fragmentation spectra of MC-LR by positive and negative ionization modes in Alcántara bloom sample

Ion composition	Theoretical accurate m/z	Measured m/z	Error (ppm)	Ion composition	Theoretical accurate m/z	Measured m/z	Error (ppm)
$[M+H]^+$	995.5560	995.5577	1.67	$[M-H]^-$	993.5415	993.5420	0.51
$[H+MeAsp-Arg-Adda]^+$	599.3552	599.3555	0.57	$[M-H-H_2O]^-$	975.5309	975.5315	0.58
$[H+Mdha-Ala-Arg-MeAsp-Leu]^+$	553.3093	553.3097	0.77	$[C_5H_4O_3-Arg-Adda]^-$	580.3141	580.3140	-0.10
$[C_{11}H_{14}O-Glu-Mdha+H]^+$	375.1914	375.1916	0.41	$[C_3H_4O-Glu-Mdha-Ala-Leu-NH_2]^-$	468.2464	468.2456	-1.67
$[H+Glu-Mdha]^+$	213.0870	213.0872	1.03	$[Glu-Mdha-Ala-Leu-NH_2]^-$	412.2202	412.2199	-0.63
$[PhCH_2CH(OCH_3)]^+$ $([C_9H_{11}O]^+)$	135.0804	135.0805	0.74	$[CO-Mdha-Ala-Leu-NH_2]^-$	311.1725	311.1721	-1.22
Arg side chain $([C_4H_8N]^+)$	70.0651	70.0651	-0.43	$[Mdha-Ala-Leu-NH_2]^-$	283.1776	283.1773	-0.95
				$[C_3H_6O_3N]^-$	128.0353	128.0352	-0.94

Fragments used as diagnostic ions are shown in bold.

Table 3

Signals observed in bloom samples that can be identified as likely microcystins and related peptides. Concentration levels ($\mu\text{g L}^{-1}$) of predominant microcystins detected in extracellular and intracellular fractions from bloom samples

Extracellular and intracellular fractions from bloom samples							
<i>m/z</i>	Analyte identification	Ionization Mode	Confirmation ^a	Alcántara		Vilasouto	
				<i>Microcystis aeruginosa</i>		<i>Planktothrix rubescens</i>	
				$\mu\text{g L}^{-1}$			
				Extracellular	Intracellular ^b	Extracellular	Intracellular ^b
837.4631/ 835.4442	Anabaenopeptin B	+/-	YES	n.q.	n.q.	n.q.	n.q.
851.4751/ 849.4629	Anabaenopeptin F	+/-	YES	n.q.	n.q.	n.q.	n.q.
945.4589	X	-	NO	X	X	n.d.	n.d.
959.4799	X	-	NO	X	X	n.d.	n.d.
965.4123	X	+	NO	X	X	n.d.	n.d.
973.5586	X	+	NO	X	X	n.d.	n.d.
981.5409 ^c	[Asp ³]MC-LR (-dmLR)	+	YES	n.d.	68	n.d.	n.d.
981.5389 ^c	[Asp ³ , Dhb ⁷]MC-LR ^e (-dmLR)	+	YES	n.d.	n.d.	3.1	2.9
981.5392/ 979.5248 ^c	[Dha ⁷]MC-LR (-dmLR)	+/-	YES	n.d.	312	n.d.	n.d.
995.5559/ 993.5389	MC-LR	+/-	YES	1.8	1353	n.d.	n.d.
512.7813 (1024.5571)/ 1022.5440 ^c	[Asp ³]MC-RR (-dmRR)	+/-	YES	n.d.	406	n.d.	n.d.
512.7846 (1024.5512)/ 1022.5421 ^c	[Asp ³ , Dhb ⁷]MC-RR ^e (-dmRR)	+/-	YES	n.d.	n.d.	19	41
1031.5239 ^c	[Asp ³]MC-YR (-dmYR)	+	YES	n.d.	20 ^d	n.d.	n.d.
1031.5189 ^c	[Dha ⁷]MC-YR (-dmYR)	+/-	YES	n.d.	176 ^d	n.d.	n.d.
519.7892 (1038.5710)/ 1036.5571	MC-RR	+/-	YES	4.5	2271	n.d.	n.d.
1045.5330/ 1043.5251	MC-YR	+/-	YES	n.d.	522	n.d.	n.d.
1068.5503	MC-WR	+	YES	n.d.	94	n.d.	n.d.
TOTAL MCs				6.3	5222	22.1	43.9

n.d.: not detected (<LOD, S/N<3).

n.q.: quantitation not performed.

X: not confirmed. It does not fulfill any or all of the criteria for confirmation.

^a Confirmation by HRMS (accuracy <3ppm) and TOF/TOF and HCD fragmentation experiments (detection of diagnostic ions).

^b $\mu\text{g L}^{-1}$ equivalents taking into account filtered water volume in each case.

^c Different retention time by LC/ESI-HRMS analysis.

^d $\mu\text{g L}^{-1}$ equivalents of MC-LR. Toxins different from the available standards were quantified related to MC-LR.

^e Identification confirmed by thiol derivatization prior LC-MS analysis.

SUPPLEMENTARY DATA

AN INTEGRATED STRATEGY FOR RAPID AND ACCURATE DETERMINATION OF FREE AND CELL-BOUND MICROCYSTINS AND RELATED PEPTIDES IN NATURAL BLOOMS BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-HIGH RESOLUTION MASS SPECTROMETRY AND MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT/TIME-OF-FLIGHT MASS SPECTROMETRY USING BOTH POSITIVE AND NEGATIVE IONIZATION MODES.

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Contents of Supplementary Data

Table S1 Analytical parameters validated for MC-dmLR, -dmRR, -LA, -LF, -LR, -LW, -LY, -RR, -WR-and -YR by direct inject LC/ESI-HRMS methodology

Fig. S1. General chemical structure of microcystins.

Fig. S2. **a** ESI(+)-MS spectrum of MC-RR from Alcántara bloom; **b** characteristic isotopic pattern of their signal $[\text{MC-RR}+2\text{H}]^{2+}$ (zoom m/z 519.6-521.4); **c** HCD fragmentation spectra of $[\text{MC-dmRR}+2\text{H}]^{2+}$ (m/z 512.7824) from Vilasouto bloom by ESI(+)-Orbitrap.

Fig. S3. Fragmentation spectra of MC-LR from Alcántara bloom by ESI-Orbitrap acquired at an HCD of 50 eV: **a** $[\text{MC-LR}+\text{H}]^+$ (m/z 995.5577) in positive ionization mode; **b** $[\text{M-LR-H}]^-$ (m/z 993.5415) in negative ionization mode.

Table S1

Analytical parameters validated for MC-dmLR, -dmRR, -LA, -LF, -LR, -LW, -LY, -RR, -WR and -YR by direct inject LC/ESI-HRMS methodology

Compound	Linearity		LOD ($\mu\text{g L}^{-1}$)	LOQ ($\mu\text{g L}^{-1}$)	%Recovery		
	$\mu\text{g L}^{-1}$	Residuals (%)			0.2 $\mu\text{g L}^{-1}$	0.6 $\mu\text{g L}^{-1}$	6 $\mu\text{g L}^{-1}$
ESI positive							
MC-dmLR	LOQ-110	<30	0.05	0.20	109	72	85
MC-dmRR	LOQ-70	<26	0.01	0.20	108	74	95
MC-LA	LOQ-70	<24	0.01	0.06	78	88	101
MC-LF	LOQ-6	<30	0.05	0.06	67	101	120
MC-LR	LOQ-6	<29	0.01	0.06	76	85	129
MC-LW	LOQ-6	<22	0.05	0.06	78	93	121
MC-LY	LOQ-6	<28	0.03	0.06	72	101	118
MC-RR	LOQ-70	<26	0.01	0.06	107	74	103
MC-WR	LOQ-6	<30	0.04	0.06	85	75	133
MC-YR	LOQ-6	<30	0.04	0.06	73	87	130
ESI negative							
MC-dmLR	LOQ-110	<17	0.01	0.20	105	82	99
MC-dmRR	LOQ-70	<32	0.05	0.20	109	72	86
MC-LA	LOQ-70	<25	0.01	0.06	80	100	110
MC-LF	LOQ-6	<25	0.01	0.06	75	103	121
MC-LR	LOQ-70	<17	0.01	0.20	117	83	99
MC-LW	LOQ-70	<19	0.04	0.20	105	82	94
MC-LY	LOQ-110	<29	0.01	0.06	71	93	110
MC-RR	LOQ-70	<24	0.07	0.20	93	77	91
MC-WR	LOQ-70	<30	0.04	0.20	110	66	101
MC-YR	LOQ-70	<25	0.01	0.20	107	77	91

Fig. S1.

11052302 #1239-1246 RT: 10.97-11.02 AV: 4 NL: 7.00E6
T: FTMS {1,1} + p ESI Full ms [400.00-1200.00]

13112126 #366-369 RT: 3.81-3.84 AV: 2 NL: 4.47E5
F: FTMS {1,3} + p ESI Full ms2 1000.00@hcd33.00 [60.00-1200.00]

Fig. S2.

Fig. S3.