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Extraction and analysis by liquid chromatography – Tandem mass spectrometry of intra- and extracellular microcystins and nodularin to study the fate of cyanobacteria and cyanotoxins across the freshwater-marine continuum

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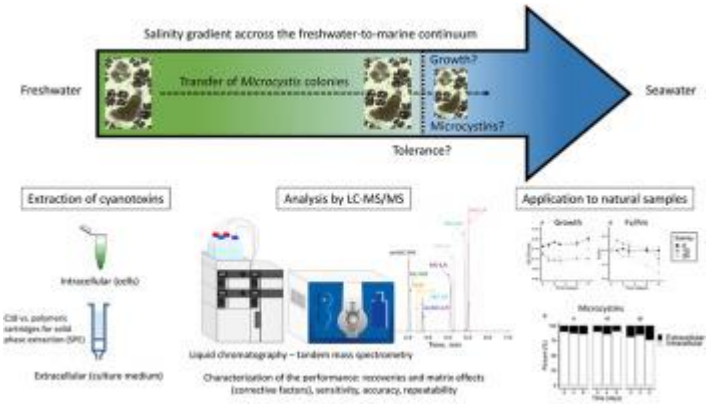
Abstract :

The presence of microcystins (MCs) is increasingly being reported in coastal areas worldwide. To provide reliable data regarding this emerging concern, reproducible and accurate methods are required to quantify MCs in salt-containing samples. Herein, we characterized methods of extraction and analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for nine MCs and one nodularin (NOD) variants in both cyanobacteria (intracellular) and dissolved forms (extracellular). Different approaches have been used to cope with salinity for the extraction of dissolved MCs but none assessed solid phase extraction (SPE) so far. It was found that salt had negligible effect on the SPE recovery of dissolved MCs using the C18 cartridge while an overestimation up to 67% was noted for some variants with a polymeric sorbent. The limits of detection (LOD) and quantification (LOQ) were 1.0–22 and 5.5–124 pg on column for the intracellular toxins, while 0.05–0.81 and 0.13–2.4 ng/mL were obtained for dissolved toxins. Extraction recoveries were excellent for intracellular (89–121%) and good to excellent for extracellular cyanotoxins (73–102%) while matrix effects were considered neglectable (<12% for 16/20 toxin-matrix combinations), except for the two MC-RR variants. The strategy based on the application of a corrective factor to compensate for losses proved useful as the accuracy was satisfactory (73–117% for intra- and 81–139% for extracellular cyanotoxins, bias <10% for 46/60 conditions, with a few exceptions), with acceptable precisions (intra- and inter-days variabilities <11%). We then applied this method on natural colonies of Microcystis spp. subjected to a salt shock, mimicking their estuarine transfer, in order to assess their survival and to quantify their toxins. The colonies of Microcystis spp. had both their growth and photosynthetic activity impaired at salinities from 10, while toxins remained mainly intracellular (>76%) even at salinity 20, suggesting a potential health risk and contamination of estuarine organisms.

Graphical abstract



Highlights

► Successful implementation of Solid Phase Extraction for extracellular microcystins and nodularin in (salt)water samples. ► Accurate and repeatable methods for analysis of intracellular and extracellular cyanotoxins using a corrective factor. ► Application of the analytical methods on natural *Microcystis* spp. colonies subjected to a salinity shock. ► Colonies survived and toxins remained mainly intracellular, highlighting a potential health risk for estuarine organisms.

Keywords : Coastal water, Microcystins, LC-MS/MS, Salinity, Solid Phase Extraction, colonial Microcystis

1 Introduction

Since the first report of microcystins (MCs) in American lakes in the 1930s (Bishop et al., 1959), their presence has been documented on every continent, in aquatic ecosystems from lakes to oceans as well as in terrestrial and extreme ecosystems (Buratti et al., 2017; Cirés et al., 2017). Nearly 50 MC-producing cyanobacterial species have been reported (Catherine et al., 2017), belonging mainly to common freshwater blooming genera such as *Microcystis, Planktothrix, Aphanizomenon, Dolichospermum* and *Oscillatoria* (Buratti et al., 2017). These non-ribosomally synthesized heptapeptides share a common structure: cyclo-(D-Ala^{1}-X^{2}-D-Masp^{3}-MeD^{3}-Z^{4}-Adda^{5}-D-γ-Glu^{6}-Mdha^{7}) (Dittmann et al., 2013). Due to the numerous combinations of amino acids, mainly at positions 2 and 4, and the occurrence of demethylations, there is a huge diversity of MCs with more than 321 analogs reported

in CyanoMetDB (Jones et al., 2021), while new congeners are regularly being discovered (e.g. Baliu-Rodriguez et al., 2022). Another family of at least 10 cyclic pentapeptides (Jones et al., 2021) shares some similarities but was first detected in the genus *Nodularia* and named nodularins accordingly (NODs).

Both MCs and NODs are hepatotoxins acting as specific inhibitors of eukaryotic protein phosphatases (Bouaïcha et al., 2019; Mackintosh et al., 1990; Yoshizawa et al., 1990) but they are also immunotoxic and show tumor-promoting, endocrine-disrupting and oxidative DNA damage activities (Catherine et al., 2017). Consequently, MCs and NODs are a threat for both humans and animals because of the global occurrence of toxic cyanobacterial blooms (Huisman et al., 2018). The review by Svircev et al. (2019) showed that almost 700 studies reported the detection of MCs in the environment which accounted for 63% of the cyanotoxins reports published in peer-reviewed journals until 2018. Besides, MCs were involved in most of the cyanotoxin-derived poisoning events worldwide with 79 over the 189 cases reported in the literature up to 2018 (Svircev et al., 2019). Therefore, aquatic ecosystems and users are particularly exposed while these cyanobacterial blooms are likely to increase and spread as a consequence of human activities and climate changes (Paerl and Paul, 2012; Paerl et al., 2018; Visser et al., 2016).

Proliferations of toxin-producing cyanobacteria also impact non-freshwater aquatic ecosystems. In Europe, Baltic Sea is historically the main water ecosystem (i.e. brackish) affected by MCs and NODs due to dense blooms of species of the genera *Dolichospermum*, *Microcystis* and *Nodularia* (Brutemark et al., 2015; Mazur-Marzec et al., 2010; Paldaviciene et al., 2015; Teikari et al., 2018). However, several studies pointed out the occurrence of MCs in brackish and marine coastal waters as a consequence of the physical transfer of cyanobacterial blooms and MCs along the freshwater-to-marine continuum (Bormans et al., 2019; Gibble et al., 2014; Lehman et al., 2005; Robson and Hamilton, 2003). This phenomenon frequently recorded in North America (Lehman et al., 2005; 2010; 2013; 2017; Moisander et al., 2009; Peacock et al., 2018) has also been more recently observed in western European coastal waters, both in Portugal (Churro et al., 2017; Lopes and Vasconcelos., 2011;) and France (Bormans et al., 2019; Lance et al. 2021) where the presence of *Microcystis* spp. and MCs in sediments was also reported (Bormans et al., 2020). This transfer results in a salinity increase in the surrounding medium of cells with subsequent cell lysis when

salt tolerance is overpassed (Ross et al., 2019) and can ultimately lead to the release of MCs in the water and to the accumulation of MCs in aquatic organisms e.g., shellfish, fish and mammals (Bukaveckas et al., 2017; Lance et al., 2021; Lehman et al 2017; Miller et al., 2010). In addition, some research groups consider that the contamination of mussels may have originated from marine cyanobacteria in Greece (Kalaitzidou et al., 2021; Vareli et al., 2013), although the MC-producing organisms remain to be unequivocally discovered. Altogether, these observations call for the need to better monitor the land-sea interface, where multiple sampling strategies are necessary (Tatters et al., 2021).

Accordingly, the development of reliable and sensitive analytical methods is required to accurately assess the presence of MCs in different matrices (Balest et al., 2016; Massey et al., 2020; Turner et al., 2018), especially in the marine environment where they are considered as emerging toxins (Howard et al., 2023; Paerl et al., 2018; Tatters et al., 2017; 2021). Hitherto, most of the published methods focused on freshwater ecosystems (e.g. lakes, rivers, reservoirs) for the extraction and quantification of MCs and NODs in matrices such as natural and drinking waters, cyanobacteria, as well as vegetal and animal tissues (Massey et al., 2020). However, seawater and more generally salts can be responsible for “matrix effect” which can significantly affect method performances (Bienvenu et al., 2017) from the sample preparation (extraction recovery) up to the analysis (retention time (Bruns et al., 2022) or signal intensity (Bruns et al., 2022; Munoz et al., 2017)) for LC-MS/MS methods, with potential strong implications on the result accuracy if not properly assessed and controlled. This matrix effect may also apply for other quantification methods such as enzyme linked immunoassay (ELISA) or protein phosphatase inhibition assay (PPIA) and should be further characterized as they have been used in the context of elevated salinity studies (e.g. Lehman et al., 2017, Ross et al., 2019).

Nowadays, the most accurate and reliable methods to detect and quantify cyanotoxins are based on liquid chromatography coupled to mass spectrometry (Beltran et al., 2012; Benke et al., 2015; Haddad et al., 2019; Oehrle et al., 2010; Ortiz et al., 2017; Romera-García et al., 2021; Turner et al., 2018). Therefore, we characterized a LC-MS/MS method to quantify simultaneously nine MCs and one NOD analogs in cyanobacteria and in water containing salts. Among the different sample preparation available for environmental contaminants (Ali et al., 2019), a C18-SPE step for small

volume was implemented for the extraction of dissolved MCs (e.g. for laboratory study purpose). Once characterized, this method was tested on natural bloom samples of colonial *Microcystis* spp. exposed to two salinities (10 and 20), to mimic the transfer of MC-producing freshwater cyanobacteria into estuarine waters and to assess the health risk on estuarine organisms. Most previous experiments of salt-shock on *Microcystis* species involved unicellular strains with no mucilage (Allakhverdiev et al., 2008; Georges des Aulnois et al., 2020; Ross et al., 2019; Tonk et al., 2007) while colony formation is suspected to protect the cells against environmental stress (including salinity) (Bormans et al., 2023; Kehr and Dittmann, 2015). Therefore, we also monitored the growth, cell lysis and MC intra- and extracellular concentrations of the colonial *Microcystis* spp. in response to this salt increase.

2 Material and methods

2.1 Chemicals and reagents

Ultra-pure water was provided by a Milli-Q integral 3 system (Millipore). Methanol (MeOH) and acetonitrile were of LC-MS grade (Honeywell) while formic acid >98% purity (Fluka). Reference toxin standards (purity > 95%, MC-LR, MC-RR, MC-LY, MC-LF, MC-LA, MC-YR, MC-LW, dmMC-RR, dmMC-LR and NOD) were purchased from Novakits. Cyanobacterial growth media BG_{11} and [BG_{11} + 36 g/L NaCl] were prepared according to Rippka et al. (1979) using chemicals from Merck.

2.2 Extraction of dissolved MCs: effect of salt on the recovery of two SPE cartridges

The effect of salt on SPE was assessed by comparing (n=3) recoveries in media with and without salts (i.e. [BG_{11} + 36 g/L NaCl] versus BG_{11}) spiked with toxin standards at concentrations corresponding to 18 times the LOQs. Two types of SPE sorbents were compared: a C18 (Bond Elut C18, 200 mg, 10 mL, Agilent) and a polymeric (Oasis HLB, 200 mg, 6 mL, Waters). The same SPE procedure was applied for both: conditioning with 3 mL of MeOH followed by 3 mL of water, then 15 mL of the spiked matrix were loaded onto the cartridge before washing with 2.4 mL of MeOH/ water (5:95, v/v) and elution with 4 mL of MeOH.

2.3 Characterization of the performance of the analytical method

All conditions were tested in triplicate, by spiking the blank matrices with the 10 reference toxin standards. All extracts were stored at -20 °C until LC-MS/MS analysis.

159 2.3.1 Extraction of intra- and extracellular toxins

160 The method was characterized for both intra- and extracellular toxins.
161 For the intracellular blank matrix, *M. aeruginosa* PCC 7806 *mcyB*$^{-}$ (Dittman et al., 1997)
162 was used. This non-MC-producing mutant strain is available from the Pasteur Culture
163 collection of Cyanobacteria (PCC, http://cyanobacteria.web.pasteur.fr/). Cell pellets
164 (corresponding to 5 billion cells), obtained after centrifugation (4000 g, 15 min at room
165 temperature), were mechanically grinded in 1 mL of MeOH with 250 mg of glass beads
166 (0.15-0.25 mm; VWR) using a mixer mill (MM400; Retsch) for 30 min at 30 Hz
167 (Réveillon et al., 2014). Tubes were centrifuged at 13000 g and extracts were
168 ultrafiltered (Nanosep MF Bio-inert, 0.2 µm, Pall).
169 For the extracellular blank matrix, as recoveries were considered non-affected by salts
170 with the C18 sorbent, 15 mL of BG_{11} medium were used and extracted with the Bond
171 Elut C18 cartridge, as aforementioned.

172 2.3.2 Liquid Chromatography-tandem Mass Spectrometry analysis (LC-MS/MS) of
173 MCs and NOD

174 Ultrafast liquid chromatography (Nexera; Shimadzu) coupled to a hybrid triple
175 quadrupole mass spectrometer (QTRAP 5500, Sciex) equipped with a TurboV
176 ionization source were used for LC-MS/MS analyses.

177 The chromatography was conducted using a Kinetex XB C18 column (100 x 2.1 mm;
178 2.6 µm; Phenomenex) equipped with a suited guard column and maintained at 25 °C.
179 The injection volume was 5 µL and the samples were kept at 4°C. The flow rate was
180 0.3 mL/min and the elution gradient with water (A) and acetonitrile (B) both containing
181 0.1% formic acid was 30-80% B (0 to 5 min), 80% B (5 to 6 min), and 30% B (6.5 to 11
182 min). The total runtime per sample was 11 min.

183 The MS/MS detection was performed using multiple reaction monitoring (MRM). The
184 electrospray ionization interface (ESI) was operated in positive mode using the
185 following settings: curtain gas 30 psi, ion spray 5000 V, temperature 300 °C, gas 1 and
186 2 set at 30 and 40 psi respectively, and entrance potential 10 V. Two transitions per
187 toxin were monitored (Table 1) with a dwell time of 40 ms for each. Quantification was
188 carried out using a 6-point external calibration curve (from 0.6 to 145 ng/mL) prepared
189 with the 10 standards (see Supplementary Fig. S1 for an example of chromatogram).
190 Data acquisition and processing were performed using Analyst 1.6.3 (Sciex).
191

Table 1 : Optimized transitions and mass spectrometer parameters for the MRM detection of MCs and NOD.

Analytes	MRM transitions (*m/z)*	DP (V)	CE (eV)	CXP (V)
dmMC-RR	512.8 > 135.0*; 103.0	121	35; 89	6; 16
MC-RR	520.1 > 135.2*; 200.1	76	45; 49	6; 12
MC-YR	1045.6 > 213.2*; 375.2	221	87; 71	12; 22
MC-LR	995.6 > 213.2*; 375.2	186	85; 70	14; 22
dmMC-LR	981.4 > 103.0*; 135.0	101	129; 117	12; 18
MC-LA	910.7 > 375.2*; 135.2	81	45; 91	8; 6
MC-LY	1002.6 > 135.2*; 375.3	106	93; 49	6; 8
MC-LW	1025.6 > 135.2*; 375.2	106	103; 53	12; 22
MC-LF	986.6 > 375.2*; 135.2	96	47; 93	8; 6
NOD	825.5 > 227.0*; 163.2	151	69; 67	12; 14

DP = declustering potential; CE = collision energy; CXP = cell exit potential

* quantitative MRM transition

2.3.3 Matrix effect, extraction recovery and corrective factor

Matrix effects (i.e. signal suppression or enhancement) on electrospray ionization of MCs and NOD were estimated by spiking the intra- and extracellular extracted matrices just before LC-MS/MS analysis at concentrations corresponding to 18 and 22 LOQs (i.e. intermediate level of concentration used to assess the performance of the method, see section 2.3.4). Results from the spiked extracts were compared to pure cyanotoxin standards in 100% MeOH to calculate the matrix effect as in Gosetti et al. (2010):

$$\text{Matrix effect}_{(\%)} = \frac{\text{Peak area}_{\text{in matrix}}}{\text{Peak area}_{\text{in solvant}}} \times 100$$

Extraction recoveries then corresponded to the deviation between the final concentration (i.e. with a matrix spiked before extraction) and the expected concentration:

$$\text{Extraction recovery}_{(\%)} = \frac{\text{Concentration}_{\text{matrix spiked before extraction}}}{\text{Concentration}_{\text{solvant spiked before analysis}}}$$

A corrective factor was finally calculated and applied to compensate the biases resulting from the sample preparation and the matrix effect:

$$\text{Corrective factor (CF)} = \text{extraction recovery} \times \text{matrix effect}$$

212 2.3.4 Determination of limits of detection and quantification (LOD, LOQ)

213 The limits of detection (LOD) and quantification (LOQ) were determined for intracellular
214 and extracellular toxins. LODs were defined as the lowest concentrations giving a
215 mean (three consecutive injections) signal-to-noise (S/N) ratio of 3 for the qualifier
216 transition. LOQs were defined as the lowest concentrations giving a mean (for three
217 consecutive injections) signal-to-noise (S/N) ratio of 10 for the quantifier transition. For
218 both, S/N were calculated considering three times the standard deviation of the noise.

219 2.3.5 Performance of the method: accuracy, intra- and inter-day variabilities

220 The accuracy and intra- and inter-day variabilities of both analytical methods
221 (intracellular and extracellular) were then characterized at 3 levels of concentration
222 prepared in triplicate (4, 18, 80 LOQs and 4, 22, 125 LOQs respectively for intracellular
223 and extracellular fractions). The two blank matrices (i.e. toxin-free cell pellets of the
224 *mcyB*$^{-}$ mutant strain of *M. aeruginosa* PCC 7806 and 15 mL of BG_{11} medium extracted
225 with the Bond Elut C18 cartridge) were spiked before extraction and LC-MS/MS
226 analysis.

227 The accuracy was obtained after dividing by a corrective factor (see section 2.4.2).
228 Intra-day variability of the method (relative standard deviation, RSD) was evaluated
229 with three consecutive injections the same day while inter-day variability was evaluated
230 by comparing three injections every two days over one week (inter-batch, n=9).

231 2.4 Application to natural cyanobacterial bloom samples

232 Cyanobacterial bloom samples were collected in the freshwater reservoir of Pen Mur
233 in Brittany, France (47°33'44.4"N, 2°29'31.7"W). This reservoir is used for drinking
234 water and undergoes recurrent and intense cyanobacterial blooms dominated by MC-
235 producing *Microcystis* spp. (Bormans et al., 2019). This site is close to the ocean (< 10
236 km) and the water level in the reservoir is regulated by a flood barrier. In August 2018,
237 a surface scum sample (2 L) was collected and immediately brought back to the
238 laboratory to perform a salt shock experiment. Cyanobacterial identification was
239 performed under optical microscope, following the morphological descriptions of
240 Komárek and Komárková (2002).

241 A water sample (5 L) from the reservoir was also retrieved one meter below the surface
242 and used to prepare the media. After filtration on 1 µm glass microfiber filter (Whatman)
243 to remove cyanobacterial cells, it was mixed with natural filtered seawater (0.45 µm

cellulose filter; Whatman) in order to constitute two salt conditions corresponding to a salinity of 10 and 20 as checked with a conductivity meter (Cond 3110 Set 1; WTW).

Triplicate 500 mL culture media at different salinities (0: control, 10 and 20) were inoculated with the same volume of the surface scum sample and maintained for 8 days at 22±0.6°C under a 12:12 h light:dark cycle using cool-white fluorescent tubes (Philips) with 35 µmol photons /m²/s illumination.

The optical density (OD, 750 nm) was used as a proxy of cell concentration and monitored every day (Infinite® M200 plate reader; Tecan). The maximum quantum efficiency of the photosystem II (F_V/F_M) was used as a proxy of the physiological status of the cyanobacterial cells and monitored daily on 15 min dark-adapted samples (630 nm, no DCMU addition and the basal fluorescence was not subtracted) (Ogawa et al., 2017) using an Aquapen-C fluorimeter (Photon Systems Instruments).

The cultures were sampled every two days by centrifugation (15 mL, 4000 g 15 min at room temperature) and both pellets and supernatants were stored at -20°C before extraction and LC-MS/MS analysis. The quantification of intracellular and extracellular MCs was obtained by applying the appropriate corrective factor.

3 Results

3.1 Effect of salt on SPE recovery

In order to assess the effect of salt, the SPE recoveries obtained with the salt-containing medium (i.e., BG_{11} + 36 g/L NaCl) were compared with those obtained with the salt-free medium (i.e., BG_{11} as reference) and expressed as a relative recovery (Fig. 1). The C18 cartridge gave similar and more repeatable relative recoveries with and without salt for all toxins (i.e. 0.87-1.13) while only 6 toxins were in that range for the HLB cartridge. In addition, the relative recovery of 3 MCs was markedly affected with the HLB cartridge (1.22, 1.30 and 1.74 for dmMC-RR, MC-YR and MC-RR) for which an increase in recovery due to the salt effect was stronger.

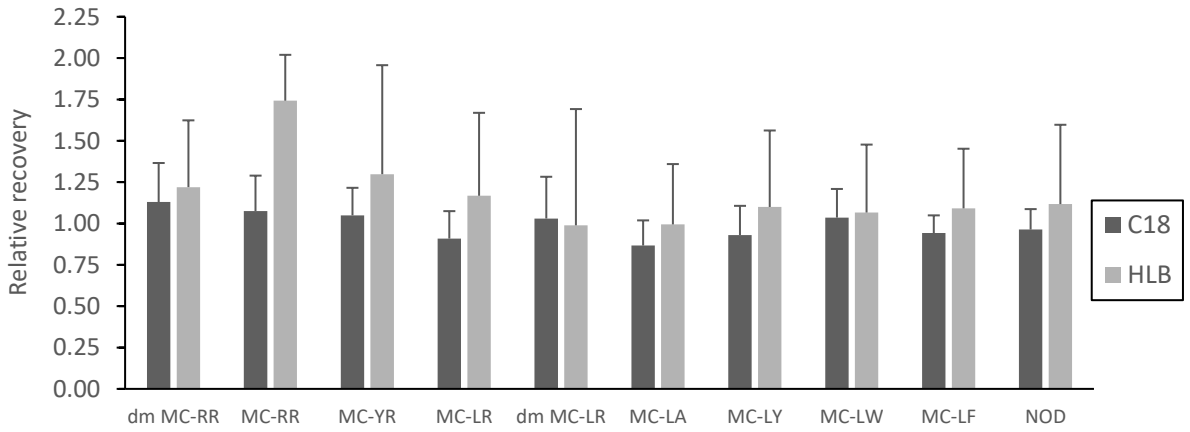


Figure 1: Relative recoveries between [BG_{11} + 36 g/L NaCl] and BG_{11} media for each MC analog and the two SPE cartridges tested (C18 and HLB) (n = 3). Error bars denote the standard deviation calculated based on the standard approach of error propagation in calculated ratios (Holmes and Buhr, 2007).

As a result, the C18 cartridge was selected as it showed less salt-derived matrix effects compared to HLB, under the conditions tested.

3.2 Characterization of the methods for intra- and extracellular MCs and NOD

3.2.1 LODs and LOQs

Limits of detection and quantification for intracellular and extracellular matrices are provided in Table S1. The LODs and LOQs ranged from 1.0 to 22 pg and from 5.5 to 124 pg (on column), in the cyanobacterial cell matrix and from 0.59 to 11 pg and from 1.8 to 34 pg (on column) in the extracellular matrix, respectively. For the latter, the method detection limit (MDL) and method quantification limit (MQL) ranged from 0.04 to 0.81 ng/mL and from 0.13 to 2.4 ng/mL, respectively (i.e. for a 15 mL sample concentrated by a factor 3.75 by SPE, after application of the corrective factors defined in Table 2). There was a ca. 20-fold difference between the lowest (for dmMC-RR) and the highest (for MC-LR or MC-YR) LOD and LOQ, for both the intracellular and extracellular matrices.

3.2.2 Extraction recovery, matrix effect and corrective factor

Extraction recovery, matrix effect on ionization and the resulting corrective factor (CF) were evaluated for the intermediate spiking level for both intracellular and extracellular toxins (Table 2).

Table 2: Extraction recovery, matrix effect and corrective factor (CF) for each toxin in the intracellular and extracellular matrices (mean ± SD, n = 3).

Variant	Intracellular			Extracellular		
	Extraction recovery ± SD (%)	Matrix effect ± SD (%)	CF	Extraction recovery ± SD (%)	Matrix effect ± SD (%)	CF
dmMC-RR	89 ± 4	132 ± 4	1.2	30 ± 2	228 ± 4.6	0.68
MC-RR	90 ± 3	111 ± 3	0.99	43 ± 8	152 ± 11	0.65
MC-YR	116 ± 5	101 ± 1	1.2	73 ± 1	103 ± 1.0	0.75
MC-LR	121 ± 4	100 ± 1	1.2	77 ± 2	102 ± 1.0	0.79
dmMC-LR	108 ± 6	96 ± 2	1.0	83 ± 3	102 ± 1.0	0.85
MC-LA	96 ± 2	98 ± 4	0.94	102 ± 5	98 ± 2.9	1.0
MC-LY	93 ± 4	104 ± 4	0.97	99 ± 4	100 ± 1.0	0.99
MC-LW	95 ± 3	112 ± 3	1.1	81 ± 3	103 ± 2.1	0.83
MC-LF	100 ± 3	120 ± 4	1.2	77 ± 3	100 ± 1.0	0.77
NOD	96 ± 4	98 ± 3	0.94	95 ± 1	101 ± 2.0	0.96

Overall, extraction recoveries ranged between 89 ± 4% and 121 ± 4% for the intracellular toxins and between 73 ± 1% and 102 ± 5% for the extracellular toxins. Only, dmMC-RR and MC-RR showed poor SPE extraction recoveries of 30 ± 2% and 43 ± 8% respectively.

Generally, low matrix effects were observed for the great majority of MC variants, except for MC-LF (120 ± 3.6%) and dmMC-RR (132 ± 4.0%) for the intracellular matrix and for dmMC-RR (152 ± 11%) and MC-RR (228 ± 4.6%) for the extracellular matrix. As a result, the corrective factors were weak for the intracellular toxins (0.94-1.2) while they were higher for the extracellular toxins (0.65-1.0), reflecting the balance between lower extraction recoveries and strong matrix effect, especially for MC-RR and dmMC-RR.

3.2.3 Accuracy for intra- and extracellular toxins

The accuracy was calculated for each MC variant and NOD at each of the three levels of the spiked, extracted and analyzed matrices (Fig. 2, Table S2). For the intracellular matrix, accuracies ranged between 73 ± 3.1% and 117 ± 8.1%. An excellent performance close to 100% (i.e. 95-102%) for the three spiking levels was noted for 7

variants (dmMC-RR, MC-RR, -LA, -LY, -LW, -LF and NOD). For MC-YR, MC-LR and dmMC-LR, the accuracy range was wider, between 77 – 116%, 73 – 98% and 83 – 117% depending on the spiking level considered (Fig. 2 A).

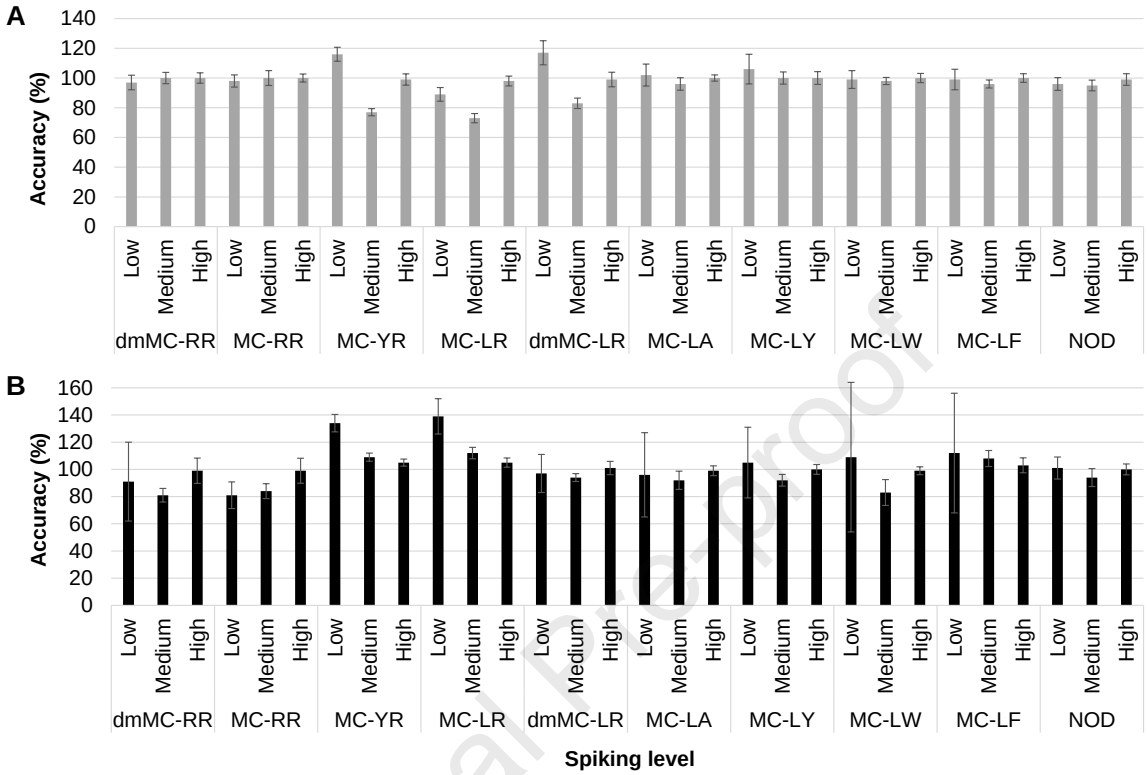


Figure 2: Accuracies for intracellular (A) and extracellular toxins (B) for each spiking level (mean ± SD, n=3). Low, Medium and High spiking levels corresponded to 4, 18 and 80 LOQs for the intracellular matrix and 4, 22 and 125 LOQs for the extracellular matrix.

For the extracellular matrix, accuracies ranged between 81 ± 9.8% and 139 ± 13% (Fig. 2 B). The performance was considered excellent (i.e. accuracy of 90-110%) for 22/30 conditions (combination of 10 variants and 3 spiking levels) while for 3 variants at the lowest level of spiking, an overestimation was noted (i.e. accuracy of 112-139% for MC-YR, MC-LR and MC-LF), and some underestimation were also observed for the other conditions (e.g. for MC-RR at the low and medium levels of spiking).

3.2.4 Intra- and inter-day variabilities

Intra- and inter-day variabilities were good to excellent for both intracellular and extracellular toxins for all variants at medium and high spiking levels (RSD of 0.3-11%, Table S3). For the low level of spiking, a wider inter-day variability was obtained for dmMC-RR, MC-LA, -LY and -LW (RSD of 25-51%).

3.3 Salt shock experiment on natural colonies of *Microcystis* spp.

In the natural bloom sample, the genus *Microcystis* dominated the phytoplankton community (>95%) and the morpho-species *M. aeruginosa* was dominant while some colonies of *M. botrys* and *M. viridis* were also noted. Over the 8-day experiment, OD increased only in the control condition from 0.16 ± 0.01 to 0.20 ± 0.01 (Fig. 3A). At a salinity of 10, after an initial decline from 0.12 ± 0.01 on day 1, OD remained stable around 0.10 ± 0.01. The same trend was obtained at a salinity of 20 except between days 6 and 8 where the OD increased from 0.10 ± 0.02 to 0.15 ± 0.01 (Fig. 3A). The maximum quantum yield of photosystem II (Fv/Fm) remained stable in the control condition between 0.39 ± 0.03 and 0.42 ± 0.01 (Fig. 3B). At a salinity of 10, Fv/Fm values increased from 0.40 ± 0.04 to 0.54 ± 0.01 on day 1, before decreasing steadily to 0.28 ± 0.11 at the end of the experiment. At a salinity of 20, a linear decrease from 0.40 ± 0.04 to 0.08 ± 0.03 was observed (Fig. 3B).

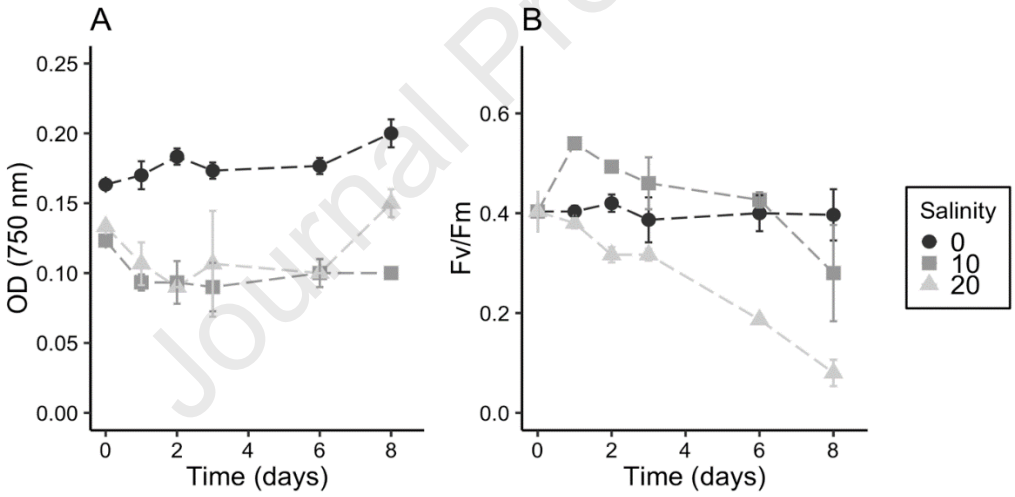


Figure 3: Effect of different salinity conditions on optical density (A) and on the maximum quantum yield of photosystem II (Fv/Fm; B) of natural bloom samples (mean ± SD, n = 3).

In the cyanobacterial scum, 5 MCs were detected (MC-LR, MC-YR, MC-RR, dmMC-LR and dmMC-RR) (Fig. 4). Irrespective of salinity and time, the proportion of the different variants was 45/31/21/2/1% (MC-LR/-YR/-RR/dmMC-LR/dmMC-RR).

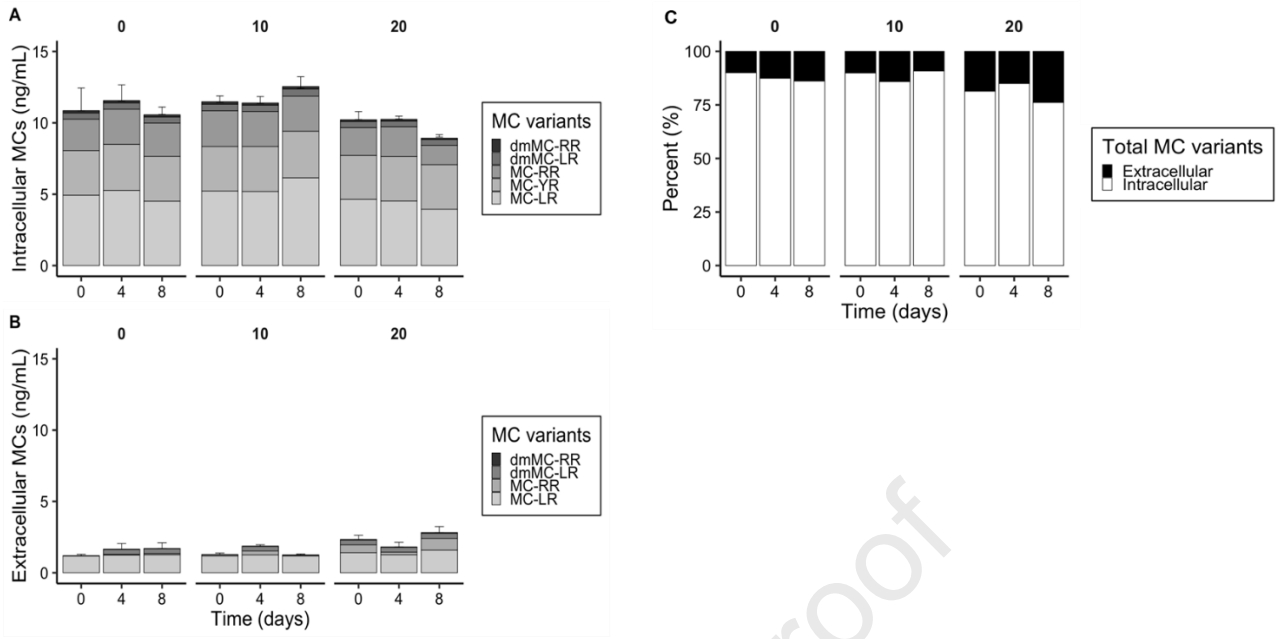


Figure 4: (A) Intracellular and (B) extracellular MC profiles (mean ± SD, n=3; SD was calculated on the total of each MC variant quantified) and (C) proportion (%) of intra- and extracellular toxins of natural bloom samples submitted to three salt treatments (0, 10 and 20) and over time (4, 8 days).

The total amounts of intracellular MCs were in a close range and remained stable over time, irrespective of salinity (from 9.0 ± 0.18 to 12 ± 0.81 ng/mL; Fig. 4A). In the extracellular fraction, only four MCs were quantified as MC-YR was detected but below the LOQ (Fig. 4B). At salinities 0 and 10, the extracellular fraction represented 10 - 14% and 9 - 14% of total MCs over time (Fig. 4C) while at a salinity of 20, this proportion was slightly higher (15 - 24%). Overall, the proportion of extracellular MCs tended to increase with salinity and time (Fig. 4C).

4 Discussion

This study aimed to characterize a method for the simultaneous extraction and quantification of nine MCs and one NOD in cyanobacterial cells and as dissolved in both fresh- and salt-containing waters, corresponding to intra and extracellular MCs. First, an SPE procedure designed for laboratory studies on relatively small volume samples was implemented for the dissolved toxins. Afterwards, the LC-MS/MS method was characterized for the detection and quantification of these ten cyanotoxins. Finally, the characterized methods (extraction and analysis) were applied to test the effect of a salinity shock on *Microcystis* spp. colonies and on intra- and extracellular MC

388 contents by exposing a natural bloom sample to a sudden salt increase, to mimic a
389 transfer across the freshwater-marine continuum.

390 Extractions by SPE are routinely used for the analysis of dissolved MCs in freshwater,
391 allowing for concentration of MCs before LC-MS/MS analysis (Mashile and
392 Nomngongo, 2016) while direct injections are also possible when sensitivity is not an
393 issue (e.g. Mekebri et al., 2009; Tran et al., 2020; Turner et al., 2018). By contrast, a
394 direct injection of seawater samples in mass spectrometers is not recommended due
395 to the high concentration of salts possibly resulting in interferences during the
396 electrospray ionization process (Hemmati et al., 2019). Such a phenomenon has been
397 reported for the analysis of total MCs in surface waters but due to the oxidation
398 process, with a severe decrease as high as 75% of the signal (Munoz et al., 2017). As
399 matrix effects can significantly affect method performance, they have to be evaluated
400 first, and then reduced as much as possible, either by appropriate sample preparation
401 or correction or a combination of both. While SPATT (Solid Phase Adsorption Toxin
402 Tracking) using HP20 resin and SALLE (salting-out assisted liquid-liquid extraction)
403 were successfully implemented for the extraction of dissolved MCs from saltwaters
404 (Hemmati et al., 2019; Kudela et al., 2011), we chose to assess the effectiveness of
405 SPE, by comparing the effect of salt on the recovery of the two types of cartridges
406 mainly used for MCs, namely C18 and HLB (Mashile and Nomngongo, 2016). Unlike
407 for HLB cartridge, recoveries with the C18 cartridge were not affected by salt addition
408 (i.e. relative recovery with and without salt between 0.87 and 1.13 for the 10 toxins
409 considered). Therefore, the C18-SPE appeared suitable to both remove salts and
410 concentrate extracellular MCs and NOD. Concerning intracellular MCs, we considered
411 that salinity would not affect the extraction, based on previous data (Georges des
412 Aulnois et al., 2019). Finally, the methods' performances to extract and analyze
413 intracellular and extracellular MCs were assessed, using these two extraction
414 protocols.

415 While aqueous methanol gave better recoveries for intracellular MCs extracted from
416 cyanobacteria in previous studies (Fastner et al., 1998; Romera- García et al., 2021;
417 Turner et al., 2018), here recoveries obtained with pure methanol using a mixer mill
418 and glass beads were excellent in the spiked *Microcystis* matrix (89-121%). This
419 suggests that methanol associated with an efficient cell lysis (Réveillon et al., 2014) is

appropriate to extract the 9 MCs and NOD from wet cyanobacteria, despite their different polarities.

Recoveries of dissolved cyanotoxins extracted by C18-SPE from BG_{11} medium were good to excellent (73-102%) except for the two more hydrophilic and doubly-arginated variants (30 and 43% for dmMC-RR and MC-RR). Despite differences in protocols, these recoveries are in agreement with previous studies that used SPE (e.g. 48-157% with C18-SPE in Mekebri et al. (2009) or 70-107% with HLB-SPE in Kaloudis et al. (2013)) or on-line C18-SPE (94-102% for Fayad et al. (2015)) or other techniques such as SALLE (53-100%, Hemmati et al., 2019) to extract dissolved cyanotoxins from water samples, although some studies reported better recoveries for MC-RR (e.g. 100% in Fayad et al. (2015) or total recovery of 76-143% in Mekebri et al. (2009)). In our case, this low recovery was counterbalanced by the unusual strong matrix-derived signal enhancement observed for the two -RR variants, leading to an acceptable total recovery (i.e. extraction recovery and matrix effect) of 65-68%. Ion suppressions are generally reported for MCs, but strong signal enhancements have already been observed for some phycotoxins, such as DTX1 (>300% in mussel matrix, Zendong et al., 2015). Except for these two conditions, matrix effects observed here were considered negligible (i.e. lower than 12% for 16 out of the 20 toxin-matrix combinations). Assessing matrix effects is essential, as it is analyte-, matrix-, sample preparation- and instrument-dependent (Zendong et al., 2015) and can have a significant impact on the signal intensity. For examples, Turner et al. (2018) observed different matrix effects between water, cyanobacteria, shellfish and algal supplement tablet, while Hemmati et al. (2017) or Tran et al. (2020) noted strong signal suppression of 80% for MC-RR and NOD extracted by SALLE from hypersaline water or ca. 55% vs. 18% for MC-RR and dmMC-RR extracted by HLB-SPE from freshwater vs. after direct injection.

While different strategies have been successfully implemented to provide accurate results, such as matrix-matched calibration curves (Munoz et al., 2017) or the use of internal standards (Haddad et al., 2019; Tran et al., 2020), we tried to compensate for losses (i.e. due to extraction recoveries and residual matrix effects) by applying a predefined corrective factor for each analyte in each matrix while relying on external calibration curves in solvent for the quantification. This strategy provided an excellent accuracy for most variants and spiking levels for the intracellular and extracellular

toxins (<10% bias for 26/30 and 22/30 combinations, respectively), with only two conditions for intracellular and two for extracellular toxins showing a bias >20% (MC-YR and MC-LR at low or intermediate levels of spiking). Similar level of performance were reported when using a matrix-matched calibration curve for water samples (Munoz et al., 2017), making this approach a suitable alternative, but not as accurate as methods using isotopically labelled MCs as internal standards (Haddad et al., 2019; Tran et al., 2020). Still, we obtained highly repeatable recoveries and matrix effects (standard deviations <8 and 11%, respectively), which is as good as or even better than Haddad et al. (2019) and Tran et al. (2020) despite their use of internal standards. Both intra- and inter-day variabilities were excellent for intracellular cyanotoxins (RSD <10%) suggesting a high precision within and between batches. The variabilities were slightly higher for extracellular cyanotoxins, especially for the inter-day comparison and for the lowest spiking level, but still acceptable for most conditions (RSD <17% for 26/30 combinations). Overall, these levels of precision are in line with previous studies for cyanobacterial cells (Turner et al., 2018) and waters (Kaloudis et al., 2013; Romera-García et al., 2021; Turner et al., 2018) or when considering similar levels of spiking (Ortiz et al., 2017).

The LC-MS/MS methods characterized here showed LOD and LOQ in the range of 1.0-22 pg and 5.5-124 pg (on column) in the intracellular matrix (*M. aeruginosa* cells) and 0.59-11 pg and 1.8-34 pg (on column) in the extracellular matrix (BG11 medium). These information are not systematically reported unfortunately, while they would ease the comparison between methods and mass spectrometer sensitivity, but when comparing to the available or inferred data, our sensitivities seem similar (for four variants) or higher than Turner et al. (2018) by a factor 9-22 in cyanobacterial cells (i.e. when comparing the ng spiked), but ca. 7 to 40 times better than Haddad et al. (2019) (20-80 and 70-280 pg) for dissolved toxins (i.e. in a spiked blank water sample). Although not directly comparable, Tran et al. (2020) reported excellent instrumental LOD and LOQ (i.e. based on standards) of 0.06-2.5 and 0.20-8.0 pg. When considering the factor of SPE concentration (i.e. 3.75) and after application of the corrective factors, method detection and quantification limits (MDL and MQL) were 0.04-0.81 ng/mL and 0.13-2.4 ng/mL. Except for the two least sensitive MC variants (MC-YR and -LR), these values are in the higher range of what is generally reported in the literature when using pre-concentration by SPE (Kaloudis et al., 2013; Haddad et al., 2019; Munoz et al.,

2017; Tran et al., 2020; and see the table ESI1 in Romera et al. (2021) for other examples) or other technique (Hemmati et al., 2019), as low to hundreds ng/L sensitivities are common. However, the most sensitive methods used very different concentration factors (3.75 here vs. 27-1000 in the above-mentioned studies) so a direct comparison should be avoided. The MDL and MQL obtained here are in good compliance with existing guidelines or sanitary threshold values for drinking and recreational waters (Chorus and Welker, 2021), although the emerging risk caused by MCs across the freshwater-marine continuum and in coastal waters has not been properly addressed yet.

Still, our analytical methods could be improved. It should be noted that the optimization of the SPE protocols was beyond the scope of the current study, however, a better reduction of matrix effect may be pursued, for example by modifying the cleaning step, as the wash volume was found to be a critical parameter for the appropriate desalting in Munoz et al. (2017). Similarly, extraction recovery could be increased, especially for the more polar variants (i.e. MC-RR, MC-YR and dmMC-RR) and future considerations should concern the pH of the samples (Haddad et al., 2019; Tran et al., 2020), the solvent(s) for elution, the type of filter (Fayad et al., 2015; Ortiz et al., 2017) or the losses associated with adsorptions as depicted by Altaner et al. (2017). Also, more MC variants could be included (e.g. 12 MCs in Tran et al. (2020) and 17 MCs in Pekar et al. (2016) vs. 9 variants here) to better quantify the chemodiversity. In addition, we could increase the factor of concentration for dissolved cyanotoxins, to improve the method sensitivity but this can significantly increase the matrix effects by concentrating both toxins and interferences (Tran et al., 2020). Ultimately, the accuracy would be further increased by using internal standards. To this end, many studies have been using NOD, a strategy questioned by Munoz et al. (2017) as affecting negatively the accuracy compared to matrix-matched calibration curves. On the opposite, the use of isotopically labelled MCs as internal standards gave excellent performance (Haddad et al., 2019; Tran et al., 2020).

Overall, the corrective factors satisfactorily improved the accuracies of the methods while the degree of variability (i.e. extractions for both intra and extracellular toxins, matrix effects), precision (intra/inter-day) and the sensitivity obtained here were fit for purpose.

518 The sample preparations and LC-MS/MS method were then applied on a natural
519 *Microcystis* spp. bloom sample mainly composed of *M. aeruginosa* colonies and
520 subjected to two salt treatments in the laboratory. Our objective was to assess the
521 potential growth and induced cell lysis of the colonies, together with detecting and
522 quantifying the MC analogs. A sudden increase in salinity inhibited the growth of
523 *Microcystis* spp., associated with a decrease in the maximum quantum efficiency of
524 photosystem II, and an increase in the proportion of extracellular toxins, in a
525 concentration dependent manner. The MC profile (5 congeners: MC-LR, MC-YR, MC-
526 RR, dmMC-LR and dmMC-RR) of the natural bloom remained similar between salinity
527 conditions, as previously observed with isolated strains (Georges des Aulnois et al.,
528 2019). Similar growth inhibition and/or photosynthetic activity responses to salt shocks
529 have been previously reported on unicellular strains (Georges des Aulnois et al., 2020;
530 Ross et al., 2019; Tonk et al., 2007) and colonies (Orr et al., 2004; Bormans et al.,
531 2023) of *Microcystis* spp. The range of salt tolerance of *Microcystis* spp. corresponds
532 to the mesohaline condition (e.g. 0-18) (Georges des Aulnois et al., 2019) with higher
533 thresholds for colonial forms, as observed here. Indeed, even at salinity 20, the optical
534 density remained high and toxins mainly intracellular, most likely due to the colonial
535 form of the natural bloom and the role of mucilage (Bormans et al., 2023; Reigner et
536 al., 2023; Xiao et al., 2019). Taken together, these results suggested that *Microcystis*
537 spp. produced no particular MC analogs in response to salt shock, that natural blooms
538 could survive in high salinity estuaries while releasing more toxins into the surrounding
539 environment, the latter indicating the beginning of cell lysis though (Ross et al., 2019).

540 The present results suggest that natural blooms of *Microcystis* spp. could persist during
541 their transfer to the estuarine zones of the Pen Mur outflow even at salinities higher
542 than mesohaline values, while extracellular MCs could increase. These hypotheses
543 are corroborated by both laboratory experiment and field monitoring that reported the
544 presence of intact *Microcystis* spp. colonies and detection of similar MC profile in
545 coastal water and sediment in this area (Bormans et al., 2019; 2020).

546 This study pointed out the necessity to characterize analytical methods for several MC
547 analogs based on seawater matrices to better assess the actual risk for animal and
548 human health. In addition to their application in the research field, the development
549 and implementation of these analytical methods for monitoring MCs and NOD in fresh
550 and coastal waters meet a need for assistance tools to decision intended for health

551 and environmental risk managers. This is of particular importance as the transfer of
552 freshwater cyanotoxins across the freshwater-marine continuum (Paerl et al., 2018) is
553 increasing, as attested by numerous reports of MCs in coastal areas and diverse
554 matrices (e.g., dissolved in water, bloom samples and animal and vegetal tissues)
555 (Peacock et al., 2018; Preece et al., 2017).

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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