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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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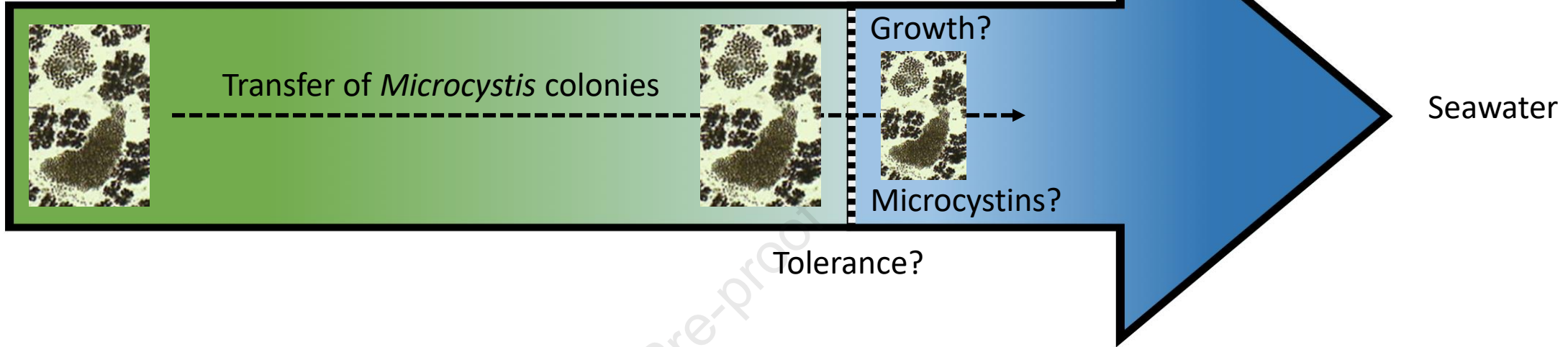
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## Salinity gradient across the freshwater-to-marine continuum

Freshwater

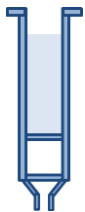


Seawater

## Extraction of cyanotoxins

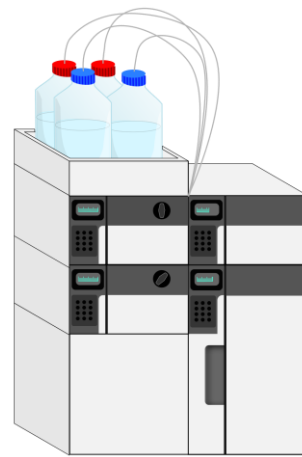


Intracellular (cells)

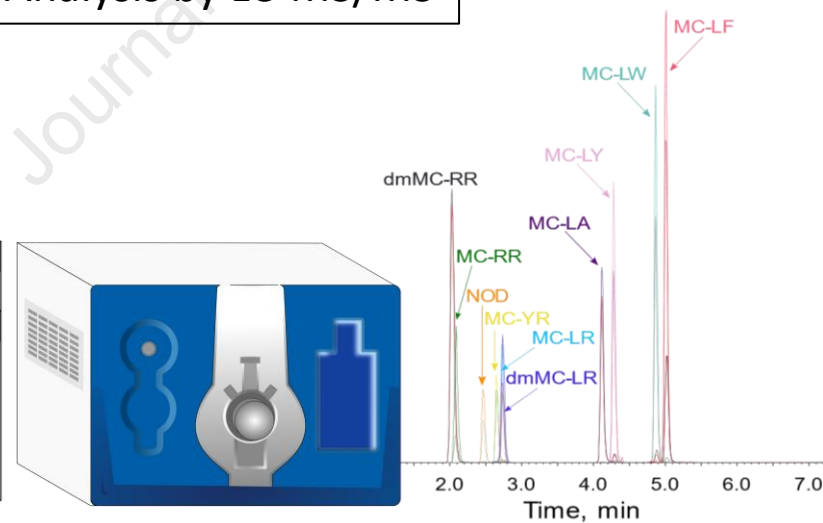
C18 vs. polymeric  
cartridges for solid  
phase extraction (SPE)

Extracellular (culture medium)

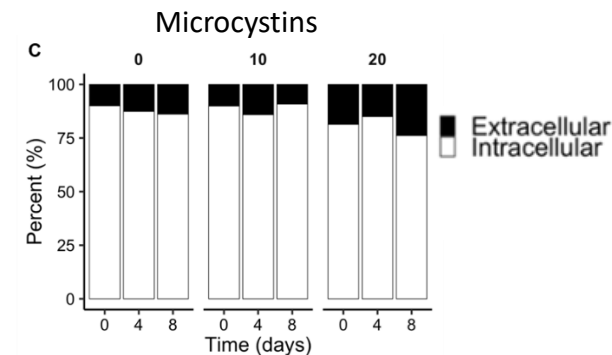
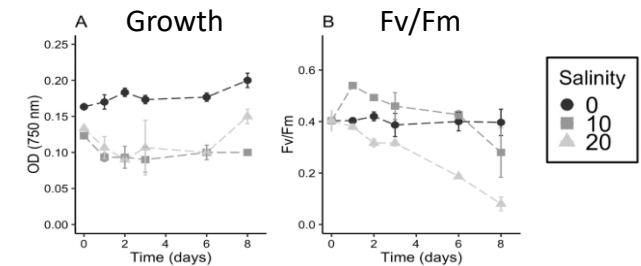
## Analysis by LC-MS/MS



Liquid chromatography – tandem mass spectrometry

Characterization of the performance: recoveries and matrix effects  
(corrective factors), sensitivity, accuracy, repeatability

## Application to natural samples



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

4

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10

11 **Highlights**

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

20

21 **Abstract:**

22 The presence of microcystins (MCs) is increasingly being reported in coastal areas  
23 worldwide. To provide reliable data regarding this emerging concern, reproducible and  
24 accurate methods are required to quantify MCs in salt-containing samples. Herein, we  
25 characterized methods of extraction and analysis by liquid chromatography coupled to  
26 tandem mass spectrometry (LC-MS/MS) for nine MCs and one nodularin (NOD)  
27 variants in both cyanobacteria (intracellular) and dissolved forms (extracellular).  
28 Different approaches have been used to cope with salinity for the extraction of  
29 dissolved MCs but none assessed solid phase extraction (SPE) so far. It was found

30 that salt had negligible effect on the SPE recovery of dissolved MCs using the C18  
31 cartridge while an overestimation up to 67% was noted for some variants with a  
32 polymeric sorbent. The limits of detection (LOD) and quantification (LOQ) were 1.0-22  
33 and 5.5-124 pg on column for the intracellular toxins, while 0.05-0.81 and 0.13-2.4  
34 ng/mL were obtained for dissolved toxins. Extraction recoveries were excellent for  
35 intracellular (89-121%) and good to excellent for extracellular cyanotoxins (73-102%)  
36 while matrix effects were considered neglectable (<12% for 16/20 toxin-matrix  
37 combinations), except for the two MC-RR variants. The strategy based on the  
38 application of a corrective factor to compensate for losses proved useful as the  
39 accuracy was satisfactory (73-117% for intra- and 81-139% for extracellular  
40 cyanotoxins, bias <10% for 46/60 conditions, with a few exceptions), with acceptable  
41 precisions (intra- and inter-days variabilities <11%). We then applied this method on  
42 natural colonies of *Microcystis* spp. subjected to a salt shock, mimicking their estuarine  
43 transfer, in order to assess their survival and to quantify their toxins. The colonies of  
44 *Microcystis* spp. had both their growth and photosynthetic activity impaired at salinities  
45 from 10, while toxins remained mainly intracellular (>76%) even at salinity 20,  
46 suggesting a potential health risk and contamination of estuarine organisms.

47 Key words: Coastal water, Microcystins, LC-MS/MS, Salinity, Solid Phase Extraction,  
48 colonial *Microcystis*.

49

## 50 1 Introduction

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

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

66 Both MCs and NODs are hepatotoxins acting as specific inhibitors of eukaryotic protein  
67 phosphatases (Bouaïcha et al., 2019; Mackintosh et al., 1990; Yoshizawa et al., 1990)  
68 but they are also immunotoxic and show tumor-promoting, endocrine-disrupting and  
69 oxidative DNA damage activities (Catherine et al., 2017). Consequently, MCs and  
70 NODs are a threat for both humans and animals because of the global occurrence of  
71 toxic cyanobacterial blooms (Huisman et al., 2018). The review by Svircev et al. (2019)  
72 showed that almost 700 studies reported the detection of MCs in the environment  
73 which accounted for 63% of the cyanotoxins reports published in peer-reviewed  
74 journals until 2018. Besides, MCs were involved in most of the cyanotoxin-derived  
75 poisoning events worldwide with 79 over the 189 cases reported in the literature up to  
76 2018 (Svircev et al., 2019). Therefore, aquatic ecosystems and users are particularly  
77 exposed while these cyanobacterial blooms are likely to increase and spread as a  
78 consequence of human activities and climate changes (Paerl and Paul, 2012; Paerl et  
79 al., 2018; Visser et al., 2016).

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

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

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

121 Nowadays, the most accurate and reliable methods to detect and quantify cyanotoxins  
122 are based on liquid chromatography coupled to mass spectrometry (Beltran et al.,  
123 2012; Benke et al., 2015; Haddad et al., 2019; Oehrle et al., 2010; Ortiz et al., 2017;  
124 Romera-García et al., 2021; Turner et al., 2018). Therefore, we characterized a LC-  
125 MS/MS method to quantify simultaneously nine MCs and one NOD analogs in  
126 cyanobacteria and in water containing salts. Among the different sample preparation  
127 available for environmental contaminants (Ali et al., 2019), a C18-SPE step for small



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

## 139 **2 Material and methods**

### 140 2.1 Chemicals and reagents

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

### 147 2.2 Extraction of dissolved MCs: effect of salt on the recovery of two SPE cartridges

148 The effect of salt on SPE was assessed by comparing (n=3) recoveries in media with  
149 and without salts (i.e. [BG<sub>11</sub> + 36 g/L NaCl] versus BG<sub>11</sub>) spiked with toxin standards at  
150 concentrations corresponding to 18 times the LOQs. Two types of SPE sorbents were  
151 compared: a C18 (Bond Elut C18, 200 mg, 10 mL, Agilent) and a polymeric (Oasis  
152 HLB, 200 mg, 6 mL, Waters). The same SPE procedure was applied for both:  
153 conditioning with 3 mL of MeOH followed by 3 mL of water, then 15 mL of the spiked  
154 matrix were loaded onto the cartridge before washing with 2.4 mL of MeOH/ water  
155 (5:95, v/v) and elution with 4 mL of MeOH.

### 156 2.3 Characterization of the performance of the analytical method

157 All conditions were tested in triplicate, by spiking the blank matrices with the 10  
158 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 *mcvB*<sup>-</sup> (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<sub>11</sub> 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

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

Analytes	MRM transitions ( <i>m/z</i> )	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

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

195 \* quantitative MRM transition

196

### 197 2.3.3 Matrix effect, extraction recovery and corrective factor

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

$$204 \text{ Matrix effect (\%)} = \frac{\text{Peak area in matrix}}{\text{Peak area in solvent}} \times 100$$

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

$$208 \text{ Extraction recovery (\%)} = \frac{\text{Concentration}_{\text{matrix spiked before extraction}}}{\text{Concentration}_{\text{solvent spiked before analysis}}}$$

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

$$211 \text{ Corrective factor (CF)} = \text{extraction recovery} \times \text{matrix effect}$$

#### 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 *mcvB* mutant strain of *M. aeruginosa* PCC 7806 and 15 mL of BG<sub>11</sub> 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

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

246 Triplicate 500 mL culture media at different salinities (0: control, 10 and 20) were  
247 inoculated with the same volume of the surface scum sample and maintained for 8  
248 days at  $22 \pm 0.6^\circ\text{C}$  under a 12:12 h light:dark cycle using cool-white fluorescent tubes  
249 (Philips) with  $35 \mu\text{mol photons /m}^2/\text{s}$  illumination.

250 The optical density (OD, 750 nm) was used as a proxy of cell concentration and  
251 monitored every day (Infinite® M200 plate reader; Tecan). The maximum quantum  
252 efficiency of the photosystem II ( $F_v/F_m$ ) was used as a proxy of the physiological status  
253 of the cyanobacterial cells and monitored daily on 15 min dark-adapted samples (630  
254 nm, no DCMU addition and the basal fluorescence was not subtracted) (Ogawa et al.,  
255 2017) using an Aquapen-C fluorimeter (Photon Systems Instruments).

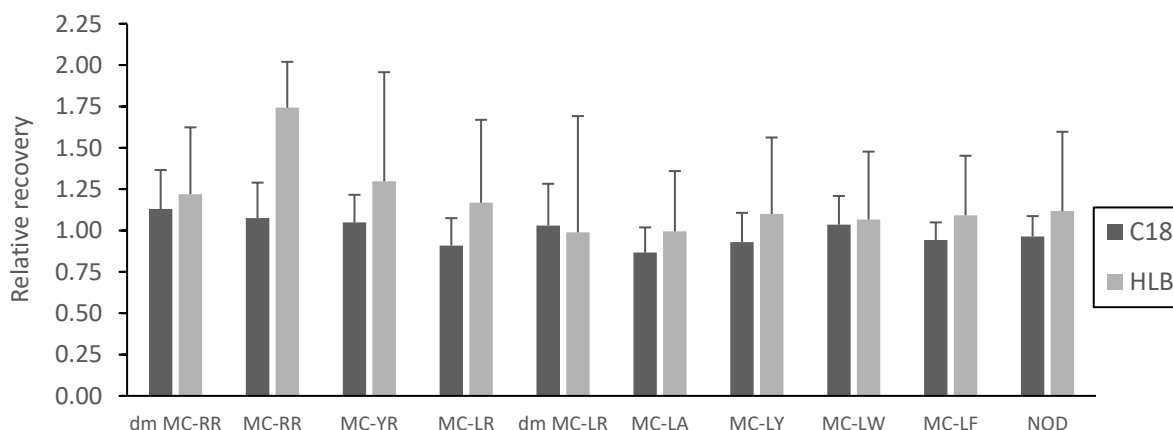
256 The cultures were sampled every two days by centrifugation (15 mL, 4000 g 15 min at  
257 room temperature) and both pellets and supernatants were stored at  $-20^\circ\text{C}$  before  
258 extraction and LC-MS/MS analysis. The quantification of intracellular and extracellular  
259 MCs was obtained by applying the appropriate corrective factor.

260

### 261 **3 Results**

#### 262 3.1 Effect of salt on SPE recovery

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



271  
 272 Figure 1: Relative recoveries between [BG<sub>11</sub> + 36 g/L NaCl] and BG<sub>11</sub> media for each MC  
 273 analog and the two SPE cartridges tested (C18 and HLB) (n = 3). Error bars denote the  
 274 standard deviation calculated based on the standard approach of error propagation in  
 275 calculated ratios (Holmes and Buhr, 2007).

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

## 278 3.2 Characterization of the methods for intra- and extracellular MCs and NOD

### 279 3.2.1 LODs and LOQs

280 Limits of detection and quantification for intracellular and extracellular matrices are  
 281 provided in Table S1. The LODs and LOQs ranged from 1.0 to 22 pg and from 5.5 to  
 282 124 pg (on column), in the cyanobacterial cell matrix and from 0.59 to 11 pg and from  
 283 1.8 to 34 pg (on column) in the extracellular matrix, respectively. For the latter, the  
 284 method detection limit (MDL) and method quantification limit (MQL) ranged from 0.04  
 285 to 0.81 ng/mL and from 0.13 to 2.4 ng/mL, respectively (i.e. for a 15 mL sample  
 286 concentrated by a factor 3.75 by SPE, after application of the corrective factors defined  
 287 in Table 2). There was a ca. 20-fold difference between the lowest (for dmMC-RR) and  
 288 the highest (for MC-LR or MC-YR) LOD and LOQ, for both the intracellular and  
 289 extracellular matrices.

### 290 3.2.2 Extraction recovery, matrix effect and corrective factor

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

294

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

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

310  
 311 Overall, extraction recoveries ranged between 89  $\pm$  4% and 121  $\pm$  4% for the  
 312 intracellular toxins and between 73  $\pm$  1% and 102  $\pm$  5% for the extracellular toxins.  
 313 Only, dmMC-RR and MC-RR showed poor SPE extraction recoveries of 30  $\pm$  2% and  
 314 43  $\pm$  8% respectively.

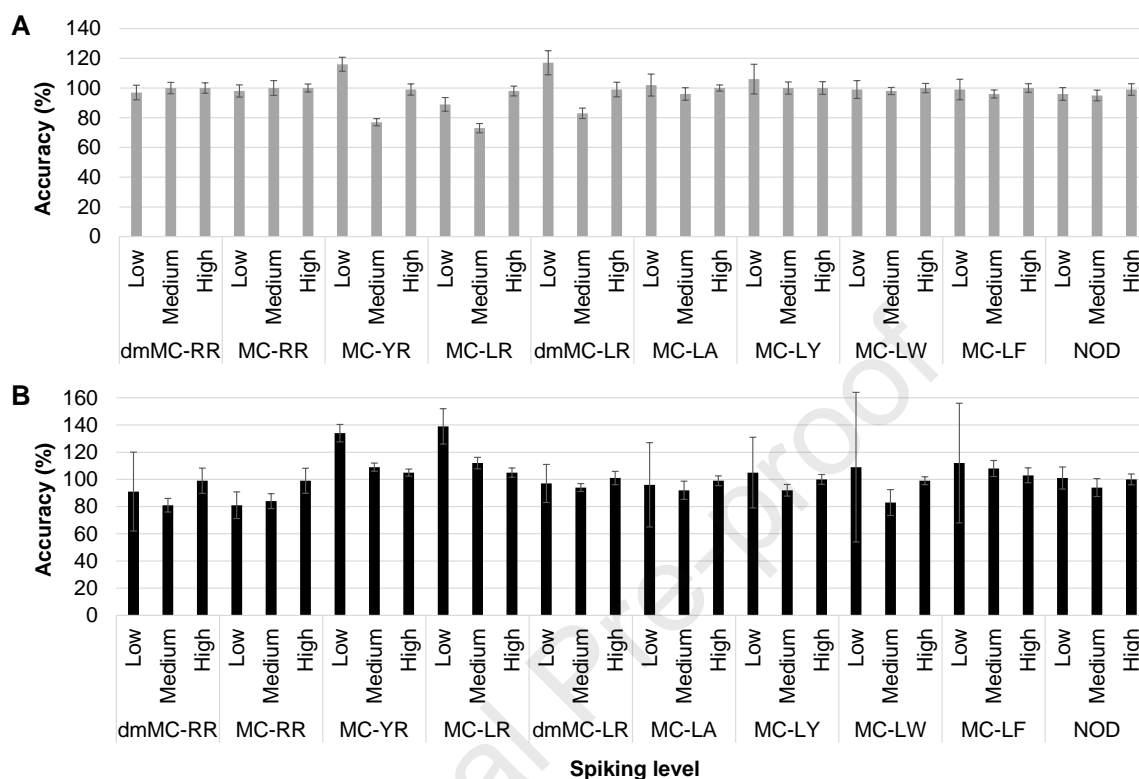
315 Generally, low matrix effects were observed for the great majority of MC variants,  
 316 except for MC-LF (120  $\pm$  3.6%) and dmMC-RR (132  $\pm$  4.0%) for the intracellular matrix  
 317 and for dmMC-RR (152  $\pm$  11%) and MC-RR (228  $\pm$  4.6%) for the extracellular matrix.

318 As a result, the corrective factors were weak for the intracellular toxins (0.94-1.2) while  
 319 they were higher for the extracellular toxins (0.65-1.0), reflecting the balance between  
 320 lower extraction recoveries and strong matrix effect, especially for MC-RR and dmMC-  
 321 RR.

### 323 3.2.3 Accuracy for intra- and extracellular toxins

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

328 variants (dmMC-RR, MC-RR, -LA, -LY, -LW, -LF and NOD). For MC-YR, MC-LR and  
 329 dmMC-LR, the accuracy range was wider, between 77 – 116%, 73 – 98% and 83 –  
 330 117% depending on the spiking level considered (Fig. 2 A).



331  
 332 Figure 2: Accuracies for intracellular (A) and extracellular toxins (B) for each spiking level  
 333 (mean  $\pm$  SD, n=3). Low, Medium and High spiking levels corresponded to 4, 18 and 80 LOQs  
 334 for the intracellular matrix and 4, 22 and 125 LOQs for the extracellular matrix.

335 For the extracellular matrix, accuracies ranged between  $81 \pm 9.8\%$  and  $139 \pm 13\%$   
 336 (Fig. 2 B). The performance was considered excellent (i.e. accuracy of 90-110%) for  
 337 22/30 conditions (combination of 10 variants and 3 spiking levels) while for 3 variants  
 338 at the lowest level of spiking, an overestimation was noted (i.e. accuracy of 112-139%  
 339 for MC-YR, MC-LR and MC-LF), and some underestimation were also observed for  
 340 the other conditions (e.g. for MC-RR at the low and medium levels of spiking).

#### 341 3.2.4 Intra- and inter-day variabilities

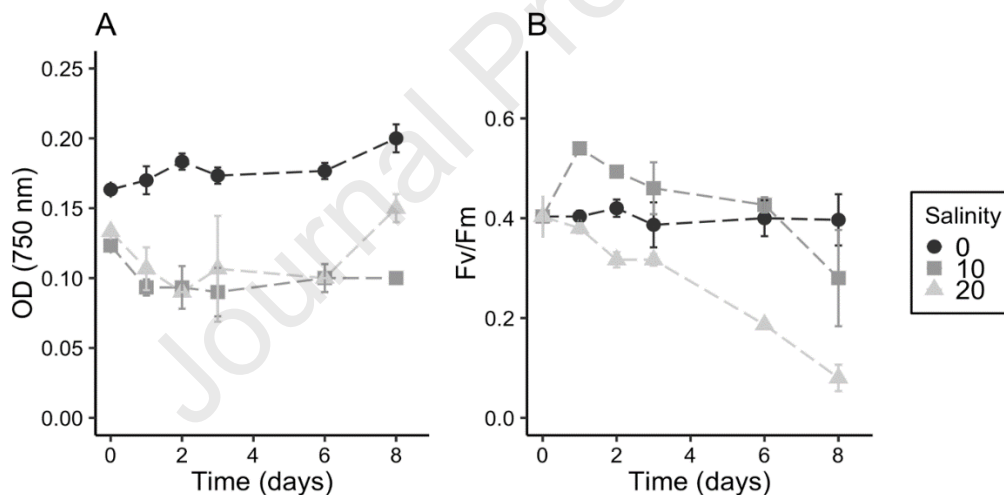
342 Intra- and inter-day variabilities were good to excellent for both intracellular and  
 343 extracellular toxins for all variants at medium and high spiking levels (RSD of 0.3-11%,  
 344 Table S3). For the low level of spiking, a wider inter-day variability was obtained for  
 345 dmMC-RR, MC-LA,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

#### 346 3.3 Salt shock experiment on natural colonies of *Microcystis* spp.



347 In the natural bloom sample, the genus *Microcystis* dominated the phytoplankton  
 348 community (>95%) and the morpho-species *M. aeruginosa* was dominant while some  
 349 colonies of *M. botrys* and *M. viridis* were also noted. Over the 8-day experiment, OD  
 350 increased only in the control condition from  $0.16 \pm 0.01$  to  $0.20 \pm 0.01$  (Fig. 3A). At a  
 351 salinity of 10, after an initial decline from  $0.12 \pm 0.01$  on day 1, OD remained stable  
 352 around  $0.10 \pm 0.01$ . The same trend was obtained at a salinity of 20 except between  
 353 days 6 and 8 where the OD increased from  $0.10 \pm 0.02$  to  $0.15 \pm 0.01$  (Fig. 3A). The  
 354 maximum quantum yield of photosystem II (Fv/Fm) remained stable in the control  
 355 condition between  $0.39 \pm 0.03$  and  $0.42 \pm 0.01$  (Fig. 3B). At a salinity of 10, Fv/Fm  
 356 values increased from  $0.40 \pm 0.04$  to  $0.54 \pm 0.01$  on day 1, before decreasing steadily  
 357 to  $0.28 \pm 0.11$  at the end of the experiment. At a salinity of 20, a linear decrease from  
 358  $0.40 \pm 0.04$  to  $0.08 \pm 0.03$  was observed (Fig. 3B).

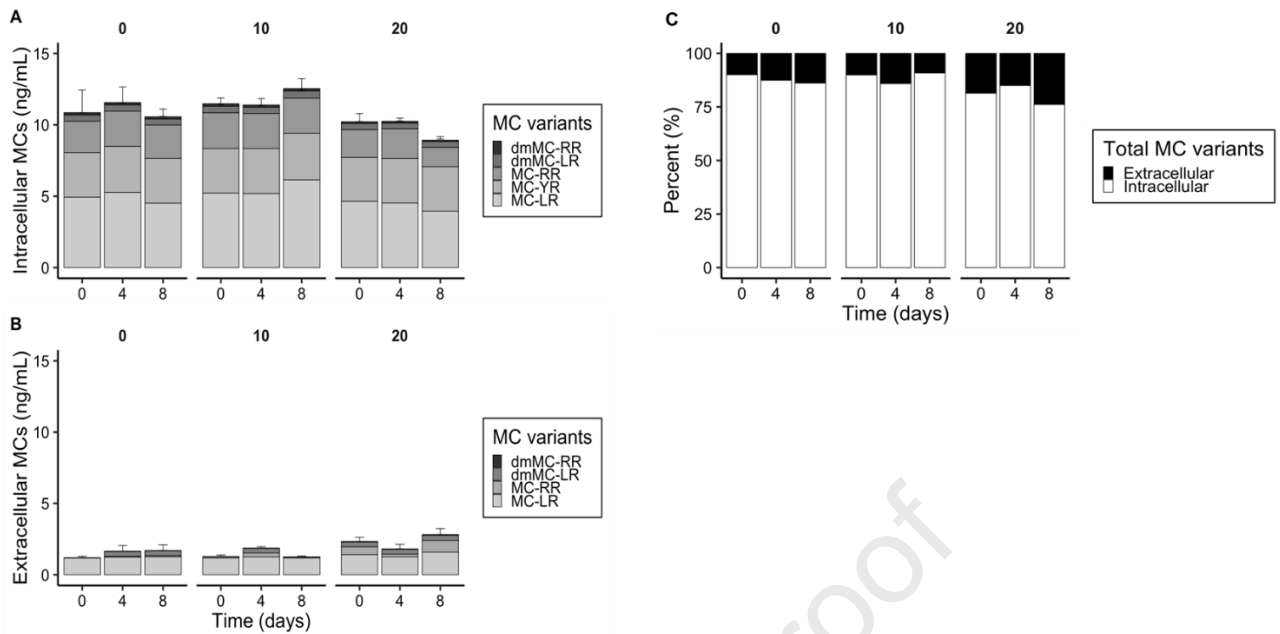
359



360

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

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



366

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

371 The total amounts of intracellular MCs were in a close range and remained stable over  
 372 time, irrespective of salinity (from  $9.0 \pm 0.18$  to  $12 \pm 0.81$  ng/mL; Fig. 4A). In the  
 373 extracellular fraction, only four MCs were quantified as MC-YR was detected but below  
 374 the LOQ (Fig. 4B). At salinities 0 and 10, the extracellular fraction represented 10 -  
 375 14% and 9 - 14% of total MCs over time (Fig. 4C) while at a salinity of 20, this proportion  
 376 was slightly higher (15 - 24%). Overall, the proportion of extracellular MCs tended to  
 377 increase with salinity and time (Fig. 4C).

378

## 379 4 Discussion

380 This study aimed to characterize a method for the simultaneous extraction and  
 381 quantification of nine MCs and one NOD in cyanobacterial cells and as dissolved in  
 382 both fresh- and salt-containing waters, corresponding to intra and extracellular MCs.  
 383 First, an SPE procedure designed for laboratory studies on relatively small volume  
 384 samples was implemented for the dissolved toxins. Afterwards, the LC-MS/MS method  
 385 was characterized for the detection and quantification of these ten cyanotoxins. Finally,  
 386 the characterized methods (extraction and analysis) were applied to test the effect of  
 387 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

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

422 Recoveries of dissolved cyanotoxins extracted by C18-SPE from BG<sub>11</sub> medium were  
423 good to excellent (73-102%) except for the two more hydrophilic and doubly-arginated  
424 variants (30 and 43% for dmMC-RR and MC-RR). Despite differences in protocols,  
425 these recoveries are in agreement with previous studies that used SPE (e.g. 48-157%  
426 with C18-SPE in Mekebri et al. (2009) or 70-107% with HLB-SPE in Kaloudis et al.  
427 (2013)) or on-line C18-SPE (94-102% for Fayad et al. (2015)) or other techniques such  
428 as SALLE (53-100%, Hemmati et al., 2019) to extract dissolved cyanotoxins from water  
429 samples, although some studies reported better recoveries for MC-RR (e.g. 100% in  
430 Fayad et al. (2015) or total recovery of 76-143% in Mekebri et al. (2009)). In our case,  
431 this low recovery was counterbalanced by the unusual strong matrix-derived signal  
432 enhancement observed for the two -RR variants, leading to an acceptable total  
433 recovery (i.e. extraction recovery and matrix effect) of 65-68%. Ion suppressions are  
434 generally reported for MCs, but strong signal enhancements have already been  
435 observed for some phycotoxins, such as DTX1 (>300% in mussel matrix, Zendong et  
436 al., 2015). Except for these two conditions, matrix effects observed here were  
437 considered negligible (i.e. lower than 12% for 16 out of the 20 toxin-matrix  
438 combinations). Assessing matrix effects is essential, as it is analyte-, matrix-, sample  
439 preparation- and instrument-dependent (Zendong et al., 2015) and can have a  
440 significant impact on the signal intensity. For examples, Turner et al. (2018) observed  
441 different matrix effects between water, cyanobacteria, shellfish and algal supplement  
442 tablet, while Hemmati et al. (2017) or Tran et al. (2020) noted strong signal suppression  
443 of 80% for MC-RR and NOD extracted by SALLE from hypersaline water or ca. 55%  
444 vs. 18% for MC-RR and dmMC-RR extracted by HLB-SPE from freshwater vs. after  
445 direct injection.

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

453 toxins (<10% bias for 26/30 and 22/30 combinations, respectively), with only two  
454 conditions for intracellular and two for extracellular toxins showing a bias >20% (MC-  
455 YR and MC-LR at low or intermediate levels of spiking). Similar level of performance  
456 were reported when using a matrix-matched calibration curve for water samples  
457 (Munoz et al., 2017), making this approach a suitable alternative, but not as accurate  
458 as methods using isotopically labelled MCs as internal standards (Haddad et al., 2019;  
459 Tran et al., 2020). Still, we obtained highly repeatable recoveries and matrix effects  
460 (standard deviations <8 and 11%, respectively), which is as good as or even better  
461 than Haddad et al. (2019) and Tran et al. (2020) despite their use of internal standards.  
462 Both intra- and inter-day variabilities were excellent for intracellular cyanotoxins (RSD  
463 <10%) suggesting a high precision within and between batches. The variabilities were  
464 slightly higher for extracellular cyanotoxins, especially for the inter-day comparison and  
465 for the lowest spiking level, but still acceptable for most conditions (RSD <17% for  
466 26/30 combinations). Overall, these levels of precision are in line with previous studies  
467 for cyanobacterial cells (Turner et al., 2018) and waters (Kaloudis et al., 2013; Romera-  
468 García et al., 2021; Turner et al., 2018) or when considering similar levels of spiking  
469 (Ortiz et al., 2017).

470 The LC-MS/MS methods characterized here showed LOD and LOQ in the range of  
471 1.0-22 pg and 5.5-124 pg (on column) in the intracellular matrix (*M. aeruginosa* cells)  
472 and 0.59-11 pg and 1.8-34 pg (on column) in the extracellular matrix (BG11 medium).  
473 These information are not systematically reported unfortunately, while they would ease  
474 the comparison between methods and mass spectrometer sensitivity, but when  
475 comparing to the available or inferred data, our sensitivities seem similar (for four  
476 variants) or higher than Turner et al. (2018) by a factor 9-22 in cyanobacterial cells (i.e.  
477 when comparing the ng spiked), but ca. 7 to 40 times better than Haddad et al. (2019)  
478 (20-80 and 70-280 pg) for dissolved toxins (i.e. in a spiked blank water sample).  
479 Although not directly comparable, Tran et al. (2020) reported excellent instrumental  
480 LOD and LOQ (i.e. based on standards) of 0.06-2.5 and 0.20-8.0 pg. When considering  
481 the factor of SPE concentration (i.e. 3.75) and after application of the corrective factors,  
482 method detection and quantification limits (MDL and MQL) were 0.04-0.81 ng/mL and  
483 0.13-2.4 ng/mL. Except for the two least sensitive MC variants (MC-YR and -LR), these  
484 values are in the higher range of what is generally reported in the literature when using  
485 pre-concentration by SPE (Kaloudis et al., 2013; Haddad et al., 2019; Munoz et al.,

486 2017; Tran et al., 2020; and see the table ESI1 in Romera et al. (2021) for other  
487 examples) or other technique (Hemmati et al., 2019), as low to hundreds ng/L  
488 sensitivities are common. However, the most sensitive methods used very different  
489 concentration factors (3.75 here vs. 27-1000 in the above-mentioned studies) so a  
490 direct comparison should be avoided. The MDL and MQL obtained here are in good  
491 compliance with existing guidelines or sanitary threshold values for drinking and  
492 recreational waters (Chorus and Welker, 2021), although the emerging risk caused by  
493 MCs across the freshwater-marine continuum and in coastal waters has not been  
494 properly addressed yet.

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

514 Overall, the corrective factors satisfactorily improved the accuracies of the methods  
515 while the degree of variability (i.e. extractions for both intra and extracellular toxins,  
516 matrix effects), precision (intra/inter-day) and the sensitivity obtained here were fit for  
517 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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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