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# Cyanobacteria and cyanotoxins in estuarine water and sediment

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Key words: toxic cyanobacteria, microcystin, colonies, estuary, sediment

## Abstract

While transfer of freshwater cyanobacteria to estuaries has been observed worldwide, the associated transfer of cyanotoxins is less often reported, in particular the sediment contribution. During fall 2018, we monitored the co-occurrence of cyanobacteria and microcystin (MC) in both the water column and in surface sediments at 5 stations along a river continuum, from a freshwater reservoir to the coastal area in Brittany, France. Cyanobacteria dominated the phytoplankton community in the water column with high densities at the freshwater sites. *Microcystis* cells and intracellular MC transfer to estuarine and marine sites were observed with decreasing concentrations in accordance with flow dilution. Extracellular MC showed the opposite trend and increased from upstream to downstream in accordance with the lysing of the cells at elevated salinities. Surface sediment samples contained high densities of colonial *Microcystis* in freshwater and with decreasing concentrations along the salinity gradient, similarly to cells concentrations in the water column. Intracellular MC was detected in sediment at all sites except at the marine outlet suggesting the survival of intact cells. Extracellular MC concentrations in sediment were up to 5 times higher than intracellular

38 concentrations suggesting incomplete MC degradation. *mcyB* genes were present at  
39 all sites while *mcyA* genes were absent at the marine outlet suggesting the presence  
40 of toxic strains along the estuary. The high densities of intact colonies of potentially  
41 toxic *Microcystis* in the estuarine sediment strongly suggest that sediments can act  
42 as an inoculum of cyanobacteria and cyanotoxins in estuaries.

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## 44 **1. Introduction**

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46

47 Cyanobacterial blooms have been reported worldwide (Merel et al. 2013) and their  
48 proliferations have been increasing in recent years as a result of anthropogenic  
49 activities including eutrophication and climate warming (O'Neil et al. 2012; Rigosi et  
50 al. 2014; Paerl 2018). Freshwater cyanobacteria produce a large variety of toxins  
51 (including hepatotoxins, neurotoxins, dermatotoxins) which have strong negative  
52 impact on animal and human health (Lance et al. 2010; Metcalf and Codd 2012;  
53 Wood 2016 for a review, Meriluoto et al. 2017). Among the diversity of cyanotoxins,  
54 microcystin (MC) is largely recognized as the most common and widespread in  
55 freshwater ecosystems (Harke et al. 2016). The general structure of those cyclic  
56 heptapeptides includes a specific beta amino acid-3-amino-9-methoxy-2,6,8-  
57 trimethyl-10-phenyl-4,6-decadienoic acid-Adda (Ortiz et al. 2017; Tillett et al. 2000)  
58 as well as two amino acids that can vary leading to the identification of more than  
59 250 MC variants (Puddick et al. 2014). The regulation and synthesis of MC, as well  
60 as its ecological role, are complex and not yet fully understood (Omidi et al. 2017).

61

62 The transfer of freshwater cyanobacteria to estuaries has been observed worldwide  
63 (Preece et al. 2017 for a review), in Africa (Ndelela et al. 2016), USA (e.g. Lehman et  
64 al. 2005; Peacock et al. 2018), South America (e.g. Dörr et al. 2010), Australia  
65 (Robson and Hamilton 2003; Orr et al. 2004), Europe (e.g. Verspagen et al. 2006;  
66 Tonk et al. 2007; Paldavičiene et al. 2009; Bormans et al. 2019), and Turkey (Taş et  
67 al. 2006). The majority of these studies reported on the cyanobacterial transfer being  
68 dominated by *Microcystis aeruginosa* demonstrating a certain salt tolerance of that  
69 species. The associated transfer of intracellular or extracellular MC along the river  
70 continuum was less often reported (Preece et al. 2017). In particular MC transfer to  
71 the coastal environment resulting from freshwater discharge from an upstream

72 reservoir has only been reported in Italy (De Pace et al. 2014), Japan (Umehara et al.  
73 2012; 2015) and recently in France (Bormans et al. 2019).

74  
75 To our knowledge, MC occurrence in estuarine sediment has only been reported in  
76 Isahaya Bay, Japan (Umehara et al. 2012, 2015), and in sub-estuaries of the Baltic  
77 Sea (Paldaviciene et al. 2015) and Chesapeake Bay, USA (Bukaveckas et al. 2017)  
78 while cyanobacterial (e.g. *Microcystis*) occurrence in estuarine sediment has never  
79 been demonstrated. Hence, given the demonstrated contamination of estuarine and  
80 marine benthic and pelagic organisms (Preece et al. 2015; Gible et al. 2016;  
81 Bukaveckas et al. 2017, 2018), as well as the high potential for tidal derived sediment  
82 resuspension in estuaries (Labry et al. 2016) we wanted to investigate the sediment's  
83 contribution of a French estuary as a potential source/sink of both cyanobacteria and  
84 MC.

85  
86 Here we report on the co-occurrence of both cyanobacteria and microcystin in the  
87 water column and in surface sediment along a river continuum from a freshwater  
88 reservoir discharge to the marine environment during fall 2018. This study is part of a  
89 larger project aiming at the evaluation of the potential risk of contamination of aquatic  
90 organisms (i.e. bivalves) by cyanotoxins during transfer from a freshwater reservoir to  
91 an estuary mouth in Brittany, France. We present results on  
92 phytoplankton/cyanobacterial species composition, and concentrations of different  
93 variants of MC both in intracellular and extracellular forms. The quantification of  
94 those two forms of toxins are necessary as we anticipate gradual cells lysing along  
95 the salinity gradient. We also report on the presence of microcystin producing genes  
96 *mcyA* and *mcyB* together with the *anaC* gene in surface sediment to assess the  
97 potential toxicity of estuarine sediment.

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## 100 **2. Materials and methods**

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### 102 **2.1. Study site and sampling protocols**

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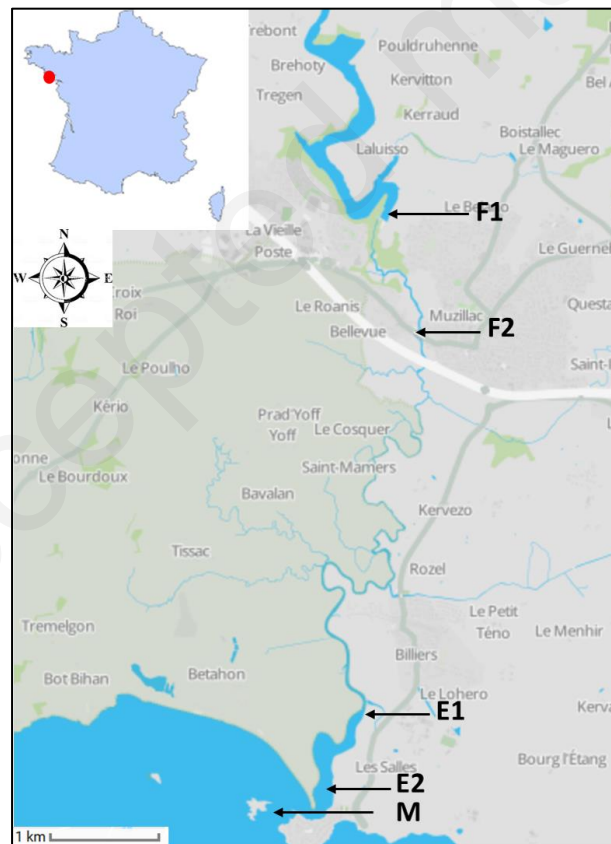
104 We chose to investigate a site in Brittany where most of the French agricultural lands  
105 are located and where reports of toxic blooms have been numerous in freshwater  
106 lakes and reservoirs (Vezie et al. 1998; Brient et al. 2009; Pitois et al. 2018).  
107 Moreover, in Brittany, most rivers have short residence times (Fraisie et al, 2013)

108 and as a result reservoirs are generally close to marine outlets. The study site is  
109 located in the Morbihan (Brittany, France) along a continuum of moderate length (<10  
110 km), from the Pen Mur freshwater reservoir upstream through the Saint Eloi River,  
111 the Pen Lann estuary and the marine outlet (Fig. 1). Pen Mur reservoir is used for  
112 drinking water and it is monitored by the Regional Health Agency (ARS). The  
113 reservoir experiences recurrent intense cyanobacterial blooms dominated by the  
114 genus *Microcystis* (Bormans et al. 2019). Freshwater discharge from the reservoir to  
115 the estuary is frequently observed but not monitored and therefore not quantified.

116

117 During fall 2018, we sampled on October 10, at 5 stations along a freshwater-marine  
118 gradient, where F1 in the Pen Mur reservoir and F2 in the river downstream of the  
119 reservoir are both in the freshwater section, E1 and E2 are located in the estuarine  
120 section, and the site M is located at the entrance of the marine section (Fig. 1). In  
121 particular, we sampled the 3 downstream stations within 1 hour of low tide to  
122 maximize the freshwater discharge and minimize the tidal contribution.

123



124

125 **Fig. 1:** Map of the study site and sampling stations: F1 and F2 are located in the  
126 freshwater section, E1 and E2 in the estuarine section and M at the marine outlet.

127

128 At each station, subsurface grab water sampling was carried out in the water column  
129 to: i) determine physico-chemical parameters (temperature, conductivity, dissolved  
130 oxygen, Phosphorus and Nitrogen) using a YSI 6920 multi-parameter probe (YSI  
131 Environmental, Anhydre) and physico-chemical analyses, ii) describe phytoplankton  
132 and cyanobacterial species (identification and enumeration), and (iii) quantify  
133 microcystins in the phytoplanktonic biomass and in the filtered water. At each station,  
134 surface sediment sampling (top 1 to 2 cm) was carried out to (i) determine sediment  
135 characteristics including percentage of organic matter (OM) and major elements, ii)  
136 identify and count phytoplankton species including cyanobacteria, (iii) quantify  
137 cyanotoxins and (iv) determine the presence of MC and anatoxin production genes. It  
138 is important to note that for the sampling of surface sediment, the site F1 was chosen  
139 in the river directly downstream of the reservoir, as no sediment was found in the  
140 reservoir itself at the site where the water column sampling was performed, the  
141 bottom there being dominated by larger rocks.

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## 144 **2.2. Samples Analyses**

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### 146 **2.2.1. Water analyses**

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148 Water column samples were transported in dark and cold conditions and filtered  
149 upon arrival at the laboratory within few hours of sampling. Dissolved inorganic  
150 nutrient concentrations were measured from filtered (GF/F 0.7  $\mu\text{m}$ ) water using  
151 common colorimetric methods with a Bran and Luebbe Autoanalyser 3 (Axflow,  
152 Norderstedt, Germany). Nitrate was measured after reduction to nitrite on a  
153 cadmium-copper column (Henriksen and Selmer-Olsen 1970). Phosphate was  
154 measured following the method of Murphy and Riley (1962). Filtration was also done  
155 to determine total suspended solids (TSS). Whatman glass fiber filters (GF/F 0.7  $\mu\text{m}$ )  
156 were dried at 105°C and weighed. Ashing to 550°C was performed to determine the  
157 organic contribution. Phytoplankton and cyanobacteria identification and counts were  
158 conducted on both fresh and lugolised samples under an optical microscope (100x  
159 magnification) using a Nageotte chamber within 48 hours of sampling as reported in  
160 Lance et al. (2010) and Pannard et al. (2018). This was preferred over a  
161 haemocytometer chamber for the larger volume sampled to limit counting errors as  
162 described in Brient et al. (2008).

163

#### 164 *2.2.1.1. Microcystin extraction*

165 Water samples containing cyanobacteria were filtered upon arrival at the laboratory  
166 through a 0.1 µm GF filter to separate the cell pellet for the intracellular cyanotoxin  
167 analysis and the filtrate which may contain dissolved extracellular toxins. Both filters  
168 and filtrates were frozen at -20°C until chemical analysis. The filtrate was purified on  
169 a C<sub>18</sub> SPE cartridge (Solid Phase Extraction) according to the ISO 20179 standard  
170 method (Anon, 2005). The fraction containing the toxins was frozen until LC-MS/MS  
171 analysis. The GF filter was sonicated with 10 mL of MeOH for 15 minutes. The  
172 MeOH extract was evaporated under nitrogen and then the residue was diluted in 1.5  
173 mL of methanol.

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#### 176 **2.2.2. Sediment samples**

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178 Fresh surface sediment was kept in the dark and at 4°C until analyses. Sediment  
179 characteristics including major elements and % organic matter (OM) were analysed  
180 by the SARM (Service d'Analyse des Roches et des Minéraux) laboratory (Nancy,  
181 France) using an ICP-MS iCapQ instrument.

182

#### 183 *2.2.2.1. Extraction of cyanobacteria*

184 Cyanobacteria were extracted from sediment using a density gradient with Ludox  
185 TM-50 (Sigma-Aldrich, Saint Louis, USA), as described in Legrand et al. (2016). 0.5g  
186 of fresh sediment was diluted with 9.5 ml of distilled water and 4 ml of Ludox. These  
187 solutions were sonicated for 30 seconds with a frequency of 50% and a power of 80  
188 W (Sonoplus, Bandelin, Berlin, Germany). Finally, solutions were centrifuged at  
189 10,000 x g for 30 minutes and after the first 6 ml was pipetted and kept in the dark at  
190 4°C for further analysis.

191

#### 192 *2.2.2.2. Estimation of cyanobacterial abundance*

193 Genus determination for vegetative cells and akinetes (Nostocale resting stage cells)  
194 was based on morphological criteria using reference books (Bourelly 1985; Komàrek  
195 and Anagnostidis 2005, 2013). 2 ml of Ludox solution was filtered through an 8-µm  
196 mesh (TETP filter; Merck Millipore, Tullagreen, Ireland). Counts were performed in  
197 triplicate for each sample in this study. Cyanobacteria were enumerated at a

198 magnification of x200 with an epifluorescence microscope (Axiovert 200 M; Zeiss,  
199 Oberkochen, Germany) and a minimum of 40 fields was counted for each filter. One  
200 sample (F2) was tested for cell viability with SYTOX green following Legrand et al.  
201 (2016). Briefly, 1 µL of SYTOX green (50 mM) was added for 1 mL of isolated  
202 cyanobacterial solution. Resulting solution was incubated in the dark at least for 30  
203 min. 1 mL of this solution was filtered using an 8 µm filter (TETP, Merck Millipore,  
204 Tullagreen, Ireland). The sample was enumerated with the same epifluorescence  
205 microscope than previously. Two separate counts were performed: the first at 546  
206 nm to determine intact cells still containing chlorophyll pigment; and the second at  
207 448 nm to discriminate damaged cells with SYTOX green staining.

208

#### 209 2.2.2.3. DNA extraction and target genes amplification

210 Genomic DNA from sediment samples was extracted with the FastDNA® Spin kit for  
211 soil (MP biomedical, Santa Ana, USA) following the manufacturer's instructions. The  
212 DNA concentration in each sample was estimated using a UV spectrophotometer  
213 (Nanodrop® ND 2000). The cyanobacterial 16S rRNA gene was amplified in  
214 sediment samples using the *cya359F* and *cya781R* set of primers from Nubel et al.  
215 (1997). Then, two genes involved in the biosynthesis of microcystin, *mcyA* and *mcyB*  
216 genes and one gene involved in the biosynthesis of anatoxin-a, *anaC*, were also  
217 investigated in this study, given the cyanobacterial species found in sediment. *anaC*  
218 and *mcyB* were amplified with nested PCRs whereas classic PCR was used to detect  
219 *mcyA*. For *mcyB* nested PCR, MCY F1/R1 then MCY F1/R2 were used and  
220 *mcyA\_Cd1F* and *mcyA-Cd1R* for *mcyA*. *anaC* was detected with the sets of primers  
221 *anxgen-F2/R* and *anaC 80F/706R* for nested 1 and 2 PCR respectively. All PCR  
222 mixtures and PCR programs were the same as described in Legrand et al. (2017). All  
223 PCR products were revealed with 0.5% agarose gel with 0.3 mg L<sup>-1</sup> ethidium bromide  
224 and migrated in a TAE buffer 0.5X at 100V for 30 minutes.

225

#### 226 2.2.2.4. Microcystin extraction

227 To analyse total MC (intracellular plus extracellular), the crude sediment  
228 (approximately 2 g) were extracted, according to the method of Umehara et al  
229 (2012), 4 times with 4 ml of MeOH/H<sub>2</sub>O (30%/70%), each time using sonication for 45  
230 min and then centrifugation at 3500 g at 4 °C for 10 min. To analyse intracellular



231 MC, the ludox supernatant containing cyanobacteria was filtered under pressure  
232 (around 53kPA) using 25 µm nylon filtration tissues in order to eliminate akinetes and  
233 extracellular MC. These tissues were cleaned with distilled water under pressure 3  
234 times. Then, tissues containing *Microcystis* were cleaned with methanol 100%.  
235 These solutions were kept in dark conditions at 4°C until further analysis.

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237

### 238 **2.2.3 Chemical analysis of microcystins by LC-MS / MS**

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240 All extracts obtained from the water column and surface sediment were filtered  
241 through a 0.2 µm filter and analyzed by Ultra Fast Liquid Chromatography  
242 (Shimadzu, Marne la Vallée, France) coupled to API-5500 QTrap tandem mass  
243 spectrometry (ABSciex, Villebon sur Yvette, France). Toxins were separated on a  
244 Kinetex XB C18 column (100 x 2.1 mm, 2.6 µm, Phenomenex) at 25°C, with water  
245 (A) and acetonitrile (B), both containing 0.1% formic acid at 0.3 mL min<sup>-1</sup> flow rate.  
246 The gradient was raised from 30 to 80% B in 5 min and was held during 1 min before  
247 dropping down during 0.5 min to the initial conditions.

248

249 Mass spectrometry detection was carried out in multiple reactions monitoring (MRM)  
250 mode. The electrospray ionization interface (ESI) was operated in positive mode  
251 using source setting: curtain gas set at 30 psi, ion spray at 5000 V, a turbogas  
252 temperature of 300°C, gas 1 and 2 set at 30 and 40 psi respectively and an entrance  
253 potential of 10 V. Each toxin was identified and quantified with two transitions as  
254 follow (Table S1): The toxin concentrations were determined using certified  
255 standards provided by CNRC (Halifax, NS, Canada). The method was developed  
256 and validated internally in the IFREMER Phycotoxins laboratory.

257

258 **Table S1:** LC-MS/MS transitions for the 9 MC variants tested with standards

259

Toxin	Precursor ion ( <i>m/z</i> )	Transition ( <i>m/z</i> ) - Quantification	Transition ( <i>m/z</i> ) - identification
MC-LR	995.6	213.2	374.5
MC-LW	1025.6	375.2	135.2
MC-LF	986.6	375.2	135.2

MC-LY	1002.6	375.2	135.2
dmMC-LR	981.4	103.0	135.2
MC-RR	520.1	135.2	213.2
dmMC-RR	512.8	135.0	103.0
MC-LA	910.7	375.2	135.2
MC-YR	1045.6	213.2	375.2

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### 3. Results

#### 3.1. In the water column

##### 3.1.1. Physico-chemical parameters

The physico-chemical characteristics of the water sampled at each site along the continuum are presented in Table 1. While temperature was constant along the continuum, conductivity (as a proxy of salinity) increased from upstream to downstream. Dissolved oxygen exhibited a decreasing concentration associated with a simultaneous decrease in Chl a concentration. Nitrate presented an upstream to downstream decrease while phosphate values showed the opposing trend with higher values downstream. The total suspended solids in the water column exhibited higher concentrations downstream in the estuary and revealed a decreasing gradient in percentage of organic matter (OM) varying from 100% in freshwater to 24% at the marine site.

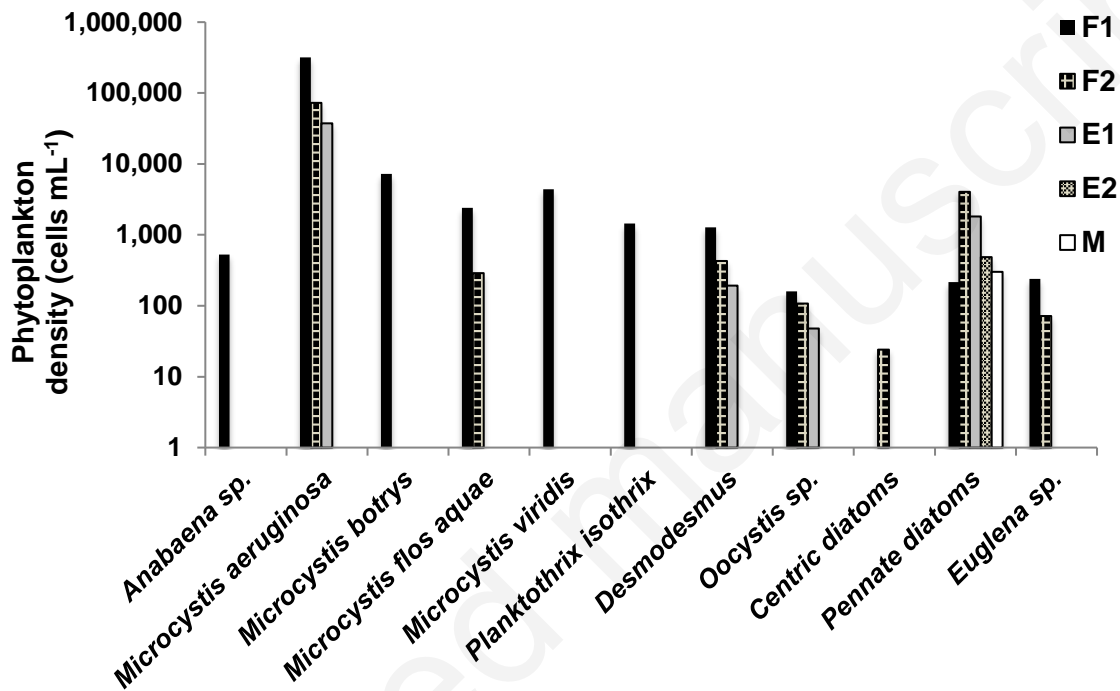
**Table 1** : Physico-chemical parameters measured in the water at the 5 sampling stations

Sites	Temp (°C)	Cond (mS/cm)	O <sub>2</sub> (mg/L)	Chl a (µg/L)	pH	P-PO <sub>4</sub> (mg/L)	N-NO <sub>3</sub> (mg/L)	TSS (mg/L)	OM (%)
F1	14.80	0.24	13.67	22.70	8.02	0.070	1.51	34	100.0
F2	14.52	0.26	10.91	13.54	7.59	0.017	1.17	25	85.3
E1	14.93	12.30	9.61	6.29	7.54	0.044	0.98	121	35.9
E2	14.98	45.35	8.63	0.07	7.78	0.042	0.47	148	30.5
M	14.96	40.21	7.84	1.06	7.77	0.055	0.48	135	24.7

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##### 3.1.2. Phytoplankton/Cyanobacteria community

285 In the water column, the phytoplankton community was largely dominated by  
 286 cyanobacteria in cells density (Fig. 2). In particular several species of the *Microcystis*  
 287 genus (*M. aeruginosa*, *M. botrys*, *M. viridis* and *M. flos-aquae*) accounted for most of  
 288 the cells density at the freshwater sites. *M. aeruginosa* was the only cyanobacterial  
 289 species observed in the estuary with a concentration of  $3.7 \times 10^4$  cells  $\text{mL}^{-1}$  at E1.  
 290 Several Chlorophyceae and pennate diatoms were also recorded in the estuary in  
 291 low numbers ( $< 10^3$  cells  $\text{mL}^{-1}$ ).



292  
 293 **Fig 2:** Species composition and phytoplankton density in the water column at the 5  
 294 stations along the freshwater-marine continuum on October 10, 2018

297 The biovolumes of the phytoplankton groups present together with the percentage of  
 298 cyanobacteria in the total biovolume of phytoplankton at each site are presented in  
 299 Table 2, demonstrating the strong dominance of cyanobacteria at the three most  
 300 upstream sites.

301  
 302 **Table 2 :** Biovolume of phytoplankton groups at each sampling site

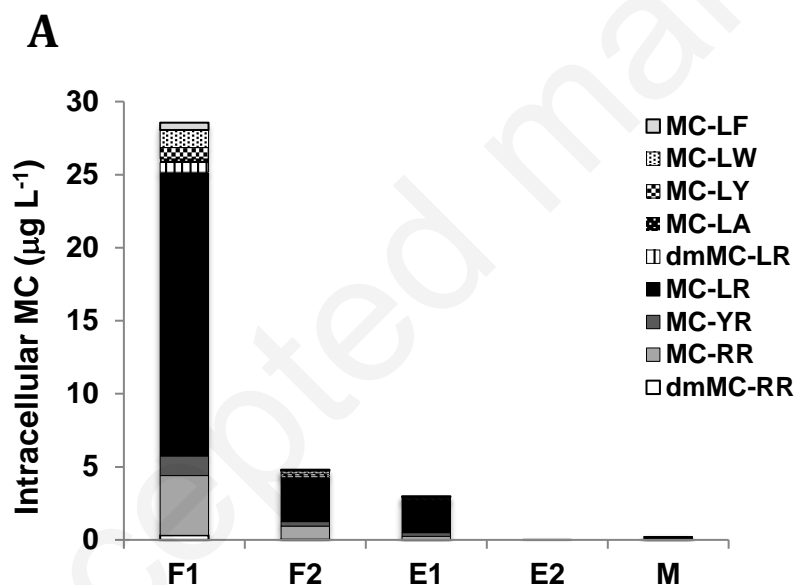
Biovolume ( $\text{mm}^3 \text{L}^{-1}$ )	F1	F2	E1	E2	M
Cyanobacteria	21.655	4.721	2.434	0.000	0.000
Chlorophyceae	0.079	0.035	0.037	0.000	0.000
Bacillariophyceae	0.061	1.147	0.258	0.008	0.005

Euglenophyceae	0.201	0.060	0.000	0.000	0.000
% Cyanobacteria	0.984	0.792	0.892	0.000	0.000

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### 3.1.3. Intracellular and extracellular microcystins

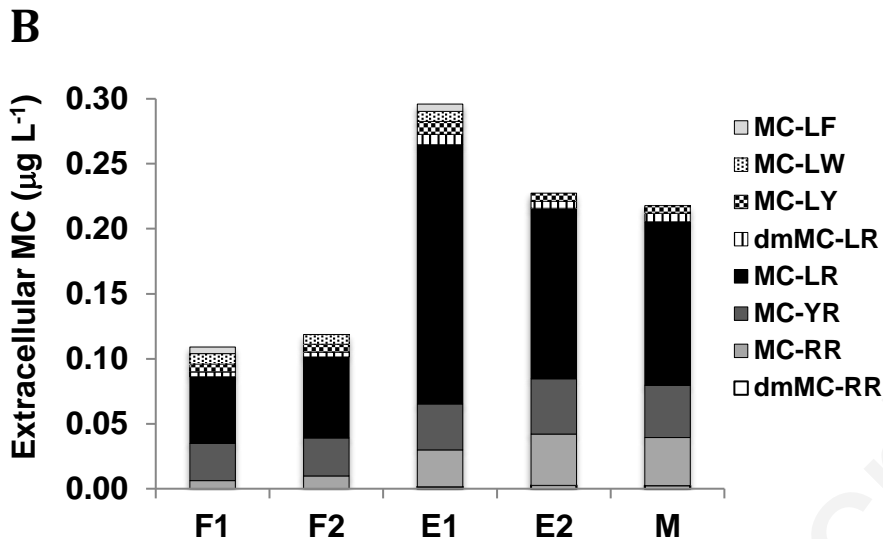
We observed a gradual decrease of intracellular MC along the continuum from 28  $\mu\text{g/L}$  in the freshwater reservoir, 3  $\mu\text{g L}^{-1}$  in the estuary and down to 0.21  $\mu\text{g L}^{-1}$  at the marine station (Fig. 3A). This gradual decrease was mostly associated with the decrease in cyanobacterial biovolume, in particular that of *M. aeruginosa*. Indeed, based on *M. aeruginosa* densities, the cellular quotas corresponded to 88 fg cell<sup>-1</sup> at F1, 69 fg cell<sup>-1</sup> at F2 and 80 fg cell<sup>-1</sup> at E1. Nine MC variants were recorded with the dominance of MC-LR followed by MC-RR and MC-YR among all sites. No particular selection of variants was observed with increasing salinity.



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**Fig 3A)** Intracellular MC present in the cyanobacterial biomass in the water at the investigated sites

323 The extracellular MC measured in the filtrate was much smaller (100 to 400 times)  
324 than the intracellular fraction, varying between 0.1 and 0.3  $\mu\text{g L}^{-1}$ , but increased from  
325 upstream to downstream (Fig. 3B). In particular, at the two most downstream sites,  
326 the proportion of extracellular/total MC reached 90% and 55% respectively.



**Fig. 3B) Extracellular MC present in dissolved form in the water**

Similarly to the intracellular fraction, 9 MC variants were recorded in the extracellular fraction and the dominant MC variant was MC-LR. It was followed by MC-YR and MC-RR at low salinities while MC-RR became more abundant at higher salinities.

### 3.2. In surface sediments

#### 3.2.1. Chemical characteristics

Overall, the surface sediment characteristics were not drastically different along the estuary (Table 3). Surface sediment showed a dominance of inorganic material at all 5 sites with a maximum of 17% of organic matter (OM) at the marine site. They contained a high proportion of silicate ( $\text{SiO}_2$ ) (50 to 73%), aluminum ( $\text{Al}_2\text{O}_3$ ) (up to 15%), calcium ( $\text{CaO}$ ) (up to 9.8%), iron ( $\text{Fe}_2\text{O}_3$ ) (up to 6.7%), potassium ( $\text{K}_2\text{O}$ ) and sodium ( $\text{Na}_2\text{O}$ ) (up to 4%), magnesium ( $\text{MgO}$ ) (up to 2.3%) and phosphorus ( $\text{P}_2\text{O}_5$ ) (up to 0.3%). The OM was maximal at F2 and M where sediment was fine and muddy while sediment at F1 and E2 was coarser and sandy. The water content of sediment varied between 30 and 71% being higher in muddy sediment. Coarser sandy sediment was also lower in Fe, Mn, Mg and OM.

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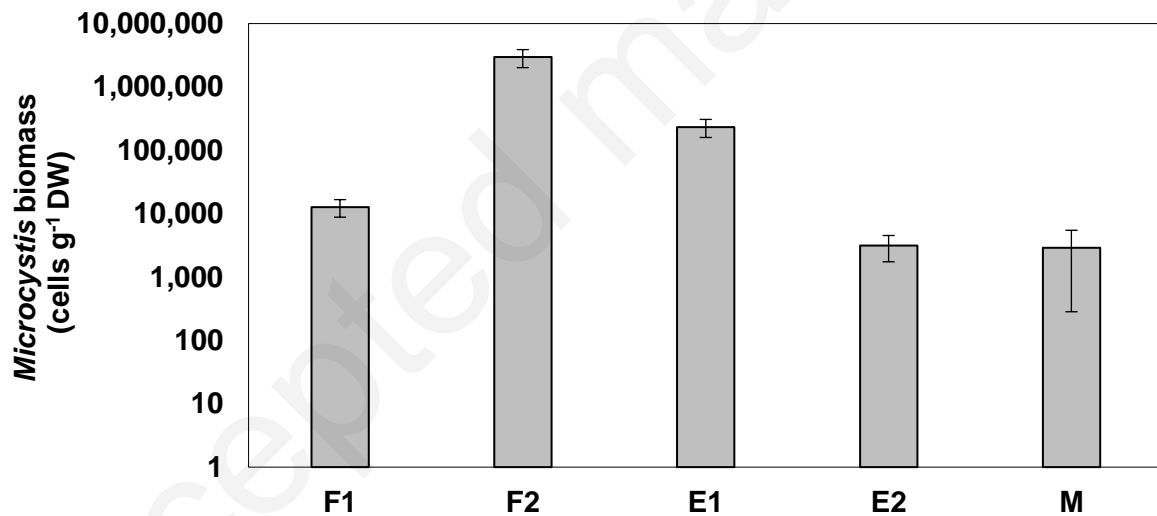
**Table 3 :** Surface sediment characteristics along the continuum

Sites	SiO <sub>2</sub>	Al <sub>2</sub> O <sub>3</sub>	Fe <sub>2</sub> O <sub>3</sub>	MnO	MgO	CaO	Na <sub>2</sub> O	K <sub>2</sub> O	TiO <sub>2</sub>	P <sub>2</sub> O <sub>5</sub>	H <sub>2</sub> O	OM
	%	%	%	%	%	%	%	%	%	%	%	%
F1	73.04	12.03	2.43	0.032	0.53	0.59	1.61	4.37	0.40	0.20	48	3.98
F2	53.54	15.17	6.72	0.22	1.76	1.97	0.75	2.63	0.75	0.34	71	15.45
E1	60.23	12.17	4.09	0.056	1.52	4.22	2.79	3.16	0.47	0.15	65	11.09
E2	73.71	2.76	0.92	0.018	0.44	9.82	0.74	0.78	0.12	< L.D.	30	9.92
M	51.32	12.25	4.95	0.11	2.30	5.67	3.74	2.40	0.61	0.20	68	16.49

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### 3.2.2. Cyanobacteria biomass

362 There was a dominance of *Microcystis sp.* (> 90%) with some akinetes of  
363 *Dolichospermum sp.* (Komàrek 2016) and few filaments of *Planktothrix sp.* at F2 in  
364 surface sediment. The presence of *Microcystis* cells in surface sediment was  
365 recorded at all 5 sites with concentrations varying between  $3 \times 10^3$  and  $3 \times 10^6$  cells g<sup>-1</sup>  
366 dry weight (DW) of sediment (Fig 4).

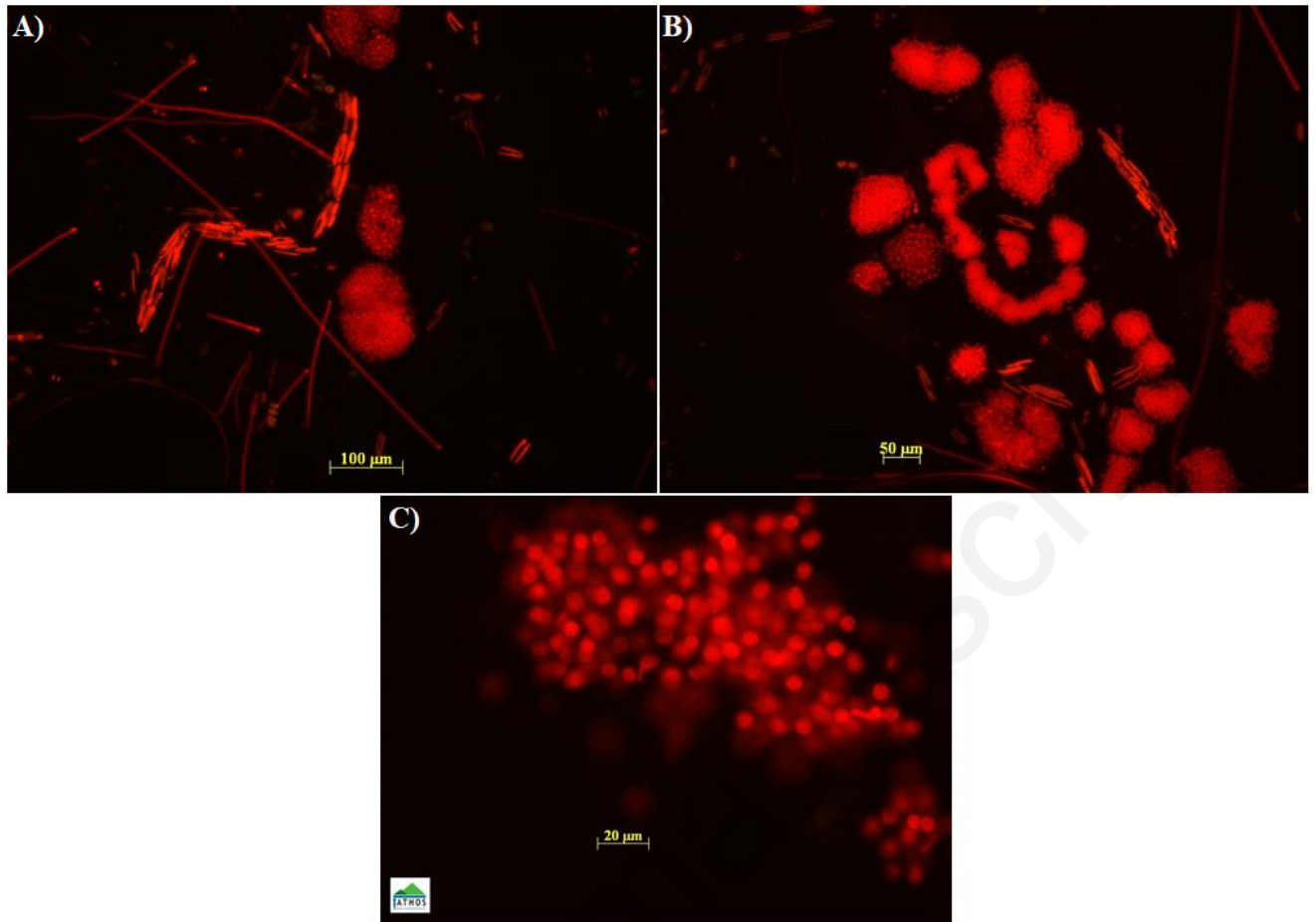


367  
368

**Fig 4:** *Microcystis* biomass in surface sediment at the 5 sampling sites along the continuum.

369  
370  
371

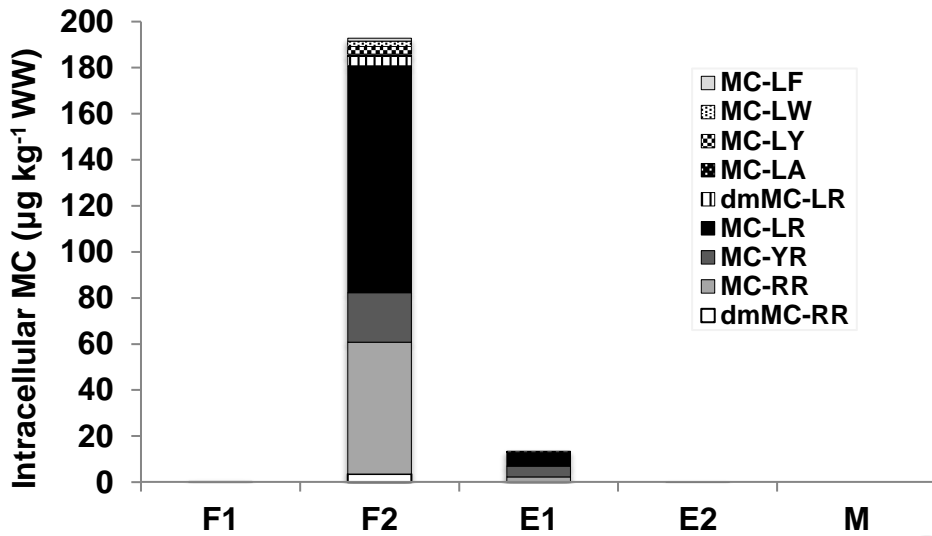
372 Photographs from epifluorescence microscopy together with counting suggested that  
373 the cells were not lysed (Fig. 5).



374  
 375  
 376 **Fig 5:** Photomicrographs of extracted *Microcystis* from studied sediment  
 377 (Epifluorescence light): colonies with filaments and diatoms (x100), focus on colony  
 378 (x200), focus on *Microcystis* intact cells (x400)  
 379  
 380

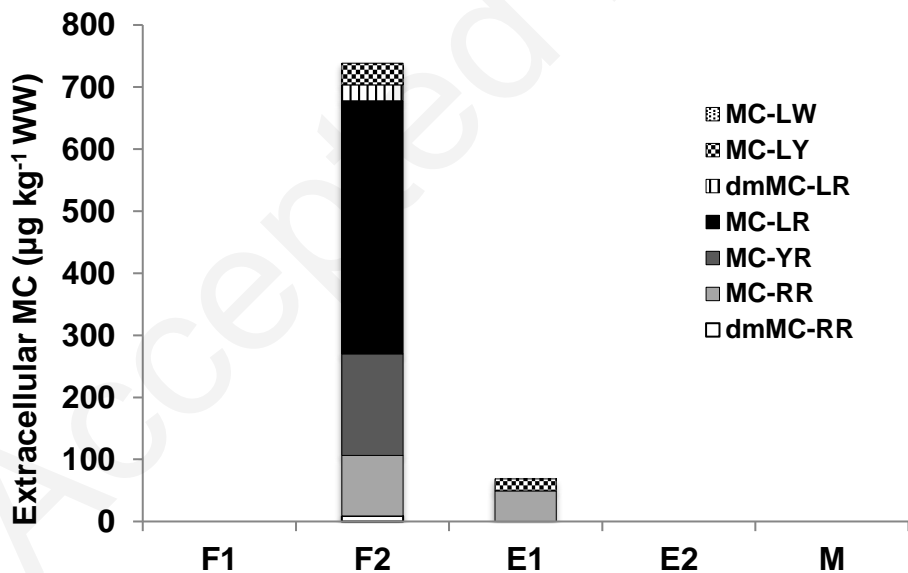
### 381 **3.2.3. Intracellular and extracellular microcystins**

382  
 383 Intracellular MC varied between 193  $\mu\text{g kg}^{-1}$  wet weight (WW) at F2, 14.4  $\mu\text{g kg}^{-1}$  WW  
 384 of sediment at E1, 0.2  $\mu\text{g kg}^{-1}$  WW at E2 and non detectable at site M (Fig. 6A).  
 385 These intracellular concentrations corresponded respectively to 482  $\mu\text{g kg}^{-1}$  DW at  
 386 F2, 36  $\mu\text{g kg}^{-1}$  DW at E1 and 0.5  $\mu\text{g kg}^{-1}$  DW at E2. The quantification of intracellular  
 387 MC in the estuarine sediment indicates that cells of *M. aeruginosa* were not lysed  
 388 and that sediment preserved at least some cells intact. Different MC variants were  
 389 recorded in the sediments with the dominance of MC-LR, RR and YR similarly to the  
 390 proportions observed in the water column.



391  
392  
393 **Fig 6A:** Intracellular MC in surface sediment along the continuum  
394

395 Extracellular MC concentrations were inferred from subtracting the intracellular  
396 concentrations from the total MC. Extracellular MC in surface sediment varied also  
397 along the estuary between 733 µg kg⁻¹ WW at F2 (1832 µg kg⁻¹ DW), 70 µg kg⁻¹ WW  
398 at E1 (175 µg kg⁻¹ DW) and non detectable further downstream (Fig. 6B).



401  
402  
403 **Fig 6B:** Extracellular MC in surface sediment along the continuum  
404  
405  
406  
407  
408



409 **3.2.4. Genes presence**

410

411

412 **Table 4:** Detection of the targeted genes in studied surface sediments

413

	Cyanobacterial 16S rRNA gene	<i>mcyA</i>	<i>mcyB</i>	<i>anaC</i>
PCR	Classic	Classic	Nested	Nested
F1	+	+	+	-
F2	+	+	+	+
E1	+	+	+	-
E2	+	+	+	-
M	+	-	+	-

414

415 The PCR analyses using both classic and nested approaches confirmed the

416 presence of cyanobacterial species in sediment at all sites. Microcystin genotypes

417 were omnipresent. Indeed, microcystin biosynthesis genes were detected in the

418 sediment of all studied sites except *mcyA* at the most downstream station (Table 4).

419 In the freshwater section at F2, the *anaC* gene was also detected suggesting the

420 presence of species potentially producing anatoxin. This site exhibited the highest

421 cyanobacterial biomass including akinetes of *Dolichospermum sp.* and long filaments

422 of *Planktothrix sp.* (data not shown).

423

424

425 **4. Discussion**

426

427 This is the first study reporting on co-occurrence of both cyanobacteria and MC in

428 estuarine/marine waters and sediments, resulting from a freshwater discharge of an

429 upstream reservoir. Moreover we report separately the quantification of intracellular

430 and extracellular forms of MC.

431

432 **4.1. In the water column**

433 Cyanobacteria dominated the phytoplankton community in the water column with

434 high densities and biovolumes at the freshwater sites and decreasing concentrations

435 along the salinity gradient. Temperatures during sampling around 15°C at all 5 sites

436 were much lower than the growth optimal temperature of cyanobacteria in general

437 and of *Microcystis* in particular (Carey et al. 2012) but still within growth potential  
438 observed in freshwater lakes and reservoirs (Kruk et al. 2017). Spatial variation of  
439 cyanobacterial biomass was most likely due to salinity as nutrients were unlikely to  
440 limit cyanobacterial growth (Reynolds 2006). Nitrate showed an upstream to  
441 downstream decrease suggesting a freshwater dominant source consistent with high  
442 nitrate from agricultural lands in the upstream catchment (Le Moal et al. 2019).  
443 Phosphate values showed the opposing trend with higher values downstream in the  
444 estuary where we expect sediment resuspension to be the largest, based on the  
445 observed high concentrations of total suspended solids and high tidal energy flows.  
446 Although the freshwater discharge was not monitored and therefore not quantified,  
447 the progressive increase in salinity near the surface was a good indicator of dilution.  
448 The short residence time (of the order of one day), inferred from observations of the  
449 surface velocities during sampling, would not permit *in situ* growth. Hence we  
450 suggest that the lower biomass in the estuary results from freshwater discharge  
451 dilution with estuarine water even though we minimized that influence by sampling  
452 within one hour of low tides.

453

454 The dominant blooming genus in the upstream freshwater reservoir was  
455 overwhelmingly *Microcystis*, consistent with the most widespread cyanobacterial  
456 occurrence of *Microcystis* in freshwaters worldwide (Harke et al. 2016) and indeed in  
457 Brittany (Pitois et al. 2014). While several species of *Microcystis* co-occurred at the  
458 two freshwater sites, *M. aeruginosa* was the only species found in the estuary. This  
459 observation is in accordance with several other studies worldwide which report on *M.*  
460 *aeruginosa* as the dominant species in estuaries (Preece et al. 2017) in relation with  
461 its highest salinity tolerance among cyanobacteria (Verspagen et al. 2006).  
462 Conductivities in the estuary and at the marine outlet ranged during sampling from 12  
463 to 45 mS cm<sup>-1</sup> corresponding to salinities from 7 to 27, the maximum value beyond  
464 reported values for *M. aeruginosa* salinity tolerance (Verspagen et al. 2006) and  
465 justifying that no *M. aeruginosa* cells were found in the water column at the two most  
466 downstream stations. The mechanisms of salinity tolerance in *M. aeruginosa* are still  
467 unclear and current hypotheses include the presence of sucrose genes acting as  
468 osmolytes (Tanabe et al. 2018) or the role of mucilage protection against osmotic  
469 shock (Kruk et al. 2017).

470

471 *M. aeruginosa* density was high in the estuarine section (at E1) at intermediate  
472 salinity reaching  $3.7 \times 10^4$  cells mL<sup>-1</sup> and associated with intracellular MC of 3 µg L<sup>-1</sup>,  
473 resulting in a MC quota of 80 fg cell<sup>-1</sup>, if we assume that MC was only due to *M.*  
474 *aeruginosa* cells and that all *M. aeruginosa* cells were toxic. This quota is similar to  
475 that measured in freshwater at F1 and F2, suggesting no particular change with  
476 salinity. Very few studies report on MC quotas in natural environments especially in  
477 estuaries. Moreover according to Wood et al 2011, MC quotas *in situ* can vary by 20  
478 fold in 5 hours, and potentially are the response to a potential stress.

479

480 The toxins profiles in the cyanobacterial biomass (intracellular form) and dissolved in  
481 the water (extracellular form) were dominated by MC-LR, RR and YR similarly to  
482 those of a previous study when *Microcystis* was the dominant genus at the same  
483 sites (Bormans et al. 2019). Both intracellular and extracellular MC exhibited here  
484 higher concentrations than previously reported in the literature. The downstream  
485 gradient of intracellular MC was consistent with the downstream gradient in  
486 *Microcystis* biovolume in the water column while extracellular MC showed a reverse  
487 trend and increased at higher salinities suggesting some cells lysing. In accordance  
488 with literature results the dominant form of MC in the freshwater part was intracellular  
489 (Orr and Jones 1998; Briand et al. 2012), while the dominant form became  
490 extracellular as cells lysis increased at elevated salinity (Tonk et al. 2007; Bormans  
491 et al. 2019). Both intracellular and extracellular toxins were measured in the water (at  
492 E2 and M) while cyanobacterial cells were not counted at those sites. This result  
493 confirms previous reports that toxins can persist as extracellular form in the water  
494 after the presence of cyanobacteria (Briand et al. 2009). Concerning the presence of  
495 intracellular MCs in the water column, cyanobacterial cells may have been too scarce  
496 at the marine site to be visualised. Bukaveckas et al. 2018 also reported MC  
497 concentrations in the water column at the river estuarine interface of the James River  
498 while no cyanobacterial cells were recorded. However their values of total MC were  
499 very low (< 0.3 µg L<sup>-1</sup>) and only measurable in the freshwater section (salinity < 0.5).  
500 While Umehara et al. (2015) reported in Isahaya Bay total MC values of 0.06 µg L<sup>-1</sup>  
501 at salinities approaching 30, we found a much higher concentration with total MC =  
502 0.4 µg L<sup>-1</sup>, most likely due to the high toxin concentration in the upstream reservoir.

503

#### 504 **4.2. In surface sediments**

505 Most bottom sediments that accumulate in the estuarine zone consist of terrestrial  
506 detritus and biogenic debris. The surface sediments were dominated by inorganic  
507 material with a high proportion of silicate, which can have both a riverine and marine  
508 origin, if associated with biogenic production by diatoms (Conley 1997; Maavara et  
509 al. 2014). The organic matter percentage was maximum at F2 and M where the  
510 sediments were fine and muddy while sediments at F1 and E2 were coarse and  
511 sandy. Coarser sandy sediments were also lower in Fe, Mn, Mg and OM as is often  
512 found in estuaries (Folger 1972).

513

514 Quantifying cyanobacterial cells in sediments is difficult as reported by Pham and  
515 Utsumi (2018). The presence of *Microcystis* was observed in surface sediments at all  
516 5 sites along the freshwater marine continuum in relatively high densities even at the  
517 marine outlet. *Microcystis* abundance was the highest at F2 where *Microcystis*  
518 concentration reached  $3 \times 10^6$  cells  $g^{-1}$  DW of sediment corresponding to bloom  
519 concentrations. The higher number of cells at F2 compared to F1 is not surprising  
520 given that the sediment sampling at F1 was done in the flowing river downstream of  
521 the reservoir, while F2 corresponded to a much more stagnant water, associated with  
522 muddy sediments rich in OM. Hence F2 acted as a sediment trap accumulating  
523 *Microcystis* cells. The presence of *Microcystis* in sediments is consistent with  
524 *Microcystis* being the dominant species being transferred in the water column from  
525 the upstream reservoir freshwater discharge. Moreover, *Microcystis* concentration  
526 gradient in surface sediments along the salinity gradient reflected *Microcystis*  
527 concentration gradient in the water column over several months to years (Bormans et  
528 al. 2019) rather than instantaneously (this study).

529

530 *Microcystis* cells in the sediments were found intact and in high numbers suggesting  
531 a preservation role of sediments even at high salinities. The exact quantification of  
532 abundance of colonial cells is always a challenge. The presence of colonies in the  
533 sediments indicated that the mucilage was probably preserving cells from lysing  
534 (Misson et al 2012; Xiao et al. 2018) and that these cells had been deposited in the  
535 sediments during freshwater discharge (Kruk et al. 2017). At the two most  
536 downstream sites where no *Microcystis* cells were found in the water column due to  
537 high salinities, some *Microcystis* colonies were still intact in the sediments. While the

538 filtration at 8  $\mu\text{m}$  did not allow the observation of individual *Microcystis* cells, it is very  
539 unlikely that cells without the protection of the mucilage would survive degradation  
540 and salinity osmotic shock as demonstrated for unicellular *M. aeruginosa* (Georges  
541 des Aulnois et al. 2019). Cyanobacterial cells can survive in sediments depending on  
542 numerous factors such as river flow, depth, bathymetry, sediment composition or  
543 redox conditions (Kravchuk et al. 2011; Legrand et al. 2017; Grutzmacher et al.  
544 2010). Hence, some *Microcystis* cells can be punctually released in the estuary  
545 explaining the decreasing gradient from the beginning of the estuary to the marine  
546 section. These phenomena of primary and secondary sedimentation have already  
547 highlighted in lacustrine ecosystems (Evans 1994; Verspagen et al. 2005; Cirés et al.  
548 2013; Legrand et al. 2017).

549  
550 Concentrations of both extracellular and intracellular toxins measured in the  
551 sediments were relatively high compared to literature values. We measured total MC  
552 concentrations of 926  $\mu\text{g}/\text{kg}$  WW (or 2315  $\mu\text{g kg}^{-1}$  DW) at the freshwater site F2 and  
553 70  $\mu\text{g kg}^{-1}$  WW (or 175  $\mu\text{g kg}^{-1}$  DW) in the estuary at E1. These values were higher  
554 than the value of 3  $\mu\text{g kg}^{-1}$  WW reported by Umehara et al. (2012) in Isahaya Bay,  
555 Japan associated with MC concentration of 100  $\mu\text{g kg}^{-1}$  WW in the freshwater  
556 reservoir upstream. High values of MC in surface silty sediments, reaching 40  $\mu\text{g kg}^{-1}$   
557 DW, were also reported in Curonian lagoon after a large bloom of *Microcystis*  
558 (Paldaviciene et al 2015; Bukaveckas et al 2017). Intracellular MC measured in the  
559 sediments were most likely due to *Microcystis* species (*M. aeruginosa* in particular),  
560 given its dominance in the sediments. Assuming that *Microcystis* was the only MC  
561 producer, these intracellular concentrations corresponded at both F2 and E1 to  
562 cellular quotas of 160 fg cell<sup>-1</sup>. Higher MC quotas in sediments compared with the  
563 overlying water column has already been observed in lakes where the authors have  
564 suggested that MC may intervene in cells preservation reducing grazing by benthic  
565 invertebrate or reducing microbial activities (Misson et al 2012). In surface  
566 sediments, extracellular MC concentrations were almost 5 times higher than  
567 intracellular concentrations, in contrast with the water column. This result suggests  
568 either some cells lysis from individual cells or small colonies and subsequent release  
569 in the porous sediments, or active transport of MC from intracellular to extracellular  
570 form possibly associated with the signaling of stress conditions. Relatively high

571 values of extracellular MC also indicated that MC degradation by bacteria or other  
572 abiotic factors was not completely occurring. Hence our results indicate that  
573 estuarine sediments are preserving both colonial cyanobacteria from cells lysing and  
574 probably also MC from degradation. Nevertheless, these results are still preliminary  
575 and there is still a need to investigate the duration of the toxins presence in  
576 sediments after the bloom period as well as their presence deeper in the sediments.  
577 Our results are in line with reports from Curonian lagoon where relatively high  
578 sediment MC concentrations have been measured many years after a large  
579 *Microcystis* bloom (Paldaviciene et al 2015). These results have important  
580 consequences for contamination of aquatic food webs as reported for zebra mussels  
581 by Paldaviciene et al (2015) and planktivorous fish by Bukaveckas et al (2017). In  
582 lacustrine systems, MC can persist in sediments several years (Misson et al. 2011)  
583 probably due to of the preservation conditions found in these environments (no light,  
584 low temperature, anoxic condition). Biodegradation in sediments of MC-LR was  
585 indeed found more rapid in aerobic than in anaerobic conditions (Grutzmacher et al  
586 2010). Interestingly, Misson et al. (2012) have highlighted that benthic *Microcystis*  
587 from 6-years sediments were able to initiate MC production implying that MC could  
588 be involved in the benthic survival mechanisms of *Microcystis*. Here, it is evident that  
589 salinity may affect significantly intra or extracellular MC in terms of degradation or  
590 inducing *Microcystis* metabolism stimuli but no studies has examined these factors.

591  
592 Generally, we observed a decrease of MC concentrations along the salinity gradient.  
593 A gradient of MC concentration in surface sediments was also observed in Isahaya  
594 Bay, Japan (Umehara et al. 2012; 2017) related to concentrations in the water  
595 column with maximum values during bloom periods. Temporal variability in MC  
596 concentrations in sediment is expected as demonstrated by Umehara et al. (2012;  
597 2015) where the dynamics is governed by variations in the discharge from the  
598 reservoir, as well as sedimentation and resuspension processes associated with tidal  
599 flows. However, while we did not investigate the temporal variability, we  
600 demonstrated unambiguously the presence of *Microcystis* colonies along the entire  
601 freshwater-marine continuum, at the time of a toxic bloom in the upstream reservoir.  
602 Moreover, at F2 all toxic genotypes and a high MC concentration were detected in  
603 the sediment. The same applies at E1 and E2 except with a smaller MC  
604 concentration. Only at the most downstream site (marine outlet) did we not detect

605 any MC in the sediment in accordance with the genes analysis that suggested the  
606 presence of a very small number of toxic genotypes at the time of sampling. This  
607 may confirm the previous hypothesis, about the importance of F2 station to transfer  
608 *Microcystis* in the estuary. Our results are in line with the findings of Via-Ordorika et  
609 al. (2004) who reported that most *M. aeruginosa* colonies from several freshwater  
610 lakes contained the *mcyA* and *mcyB* genes and found a positive relationship  
611 between genes frequency and colony size. Our results also support the measure of  
612 MC production genes as a proxy of toxic genotypes as tested by Martinez et al.  
613 (2017) and Otten et al. (2015) to evaluate the toxic potential of cyanobacteria along a  
614 freshwater marine continuum.

615

616 Interestingly, *anaC* was also detected at F2 revealing the presence of potentially  
617 anatoxin-a producer such as *Planktothrix* sp. (Viaggiu et al. 2004) or  
618 *Dolichospermum* sp. (Delvin et al. 1977). These results suggest a potential co-  
619 occurrence between MC and anatoxin-a which has already observed in several lakes  
620 (Graham et al. (2006); Sabart et al. (2015) and the co-occurrence of several  
621 cyanotoxins in coastal areas (Tatters et al. 2017). This potential co-occurrence may  
622 link to a high number of toxigenic species present here.

623

624

## 625 **5. Conclusions**

626 The results of this study confirm the transfer of cyanobacteria and MC from a  
627 freshwater reservoir discharge, through an estuary and up to the marine outlet  
628 together with the co-occurrence of both cyanobacterial cells and MC in the estuary's  
629 sediments. Similarly to studies in freshwater lakes both *Microcystis* cells and MC  
630 were found in sediments even when no cells or MC was recorded in the water  
631 column at the same site, indicating that sediments integrate over time as well as  
632 preserve cells from dilution and degradation. Hence the sediments act as a reservoir  
633 of cyanobacteria and MC. The majority of cyanobacterial cells in sediments were  
634 found in colonial form suggesting that the mucilage plays a crucial role in maintaining  
635 colonies in sediments. This study highlights the survival of *Microcystis* cells in the  
636 water column at salinities in the range 5 to 10, and in surface sediments up to a  
637 salinity of 27. MC concentrations measured here in estuarine sediments were much  
638 higher than reported elsewhere in the literature. This is the first study reporting on the

639 presence of *mcy* genes in estuarine sediments, indicating the toxicity potential of  
640 estuarine sediments for benthic organisms and also as potential source to be  
641 resuspended in the water column.

642

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653

654

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