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1 **Demonstrated transfer of cyanobacteria and cyanotoxins along a freshwater-**  
2 **marine continuum in France**

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13  
14 Key words: cyanobacteria, intracellular and extracellular microcystins, estuary,  
15 salinity  
16

17  
18 **Abstract**

19 The frequency of cyanobacterial proliferations in fresh waters is increasing worldwide  
20 and the presence of associated cyanotoxins represent a threat for ecosystems and  
21 human health. While the occurrence of microcystin (MC), the most widespread  
22 cyanotoxin, is well documented in freshwaters, only few studies have examined its  
23 occurrence in estuarine waters. In this study we evaluated the transfer of  
24 cyanobacteria and cyanotoxins along a river continuum from a freshwater reservoir  
25 through an interconnecting estuary to the coastal area in Brittany, France. We  
26 sampled regularly over 2 years at 5 stations along the river continuum and analysed  
27 for phytoplankton and cyanotoxins, together with physico-chemical parameters.  
28 Results show that cyanobacteria dominated the phytoplanktonic community with high  
29 densities (up to  $2 \times 10^6$  cells mL<sup>-1</sup>) at the freshwater sites during the summer and  
30 autumn periods of both years, with a cell transfer to estuarine (up to  $10^5$  cells mL<sup>-1</sup>)  
31 and marine ( $2 \times 10^3$  cells mL<sup>-1</sup>) sites. While the temporal variation in cyanobacterial  
32 densities was mainly associated with temperature, spatial variation was due to  
33 salinity while nutrients were non-limiting for cyanobacterial growth. Cyanobacterial  
34 biomass was dominated by several species of *Microcystis* that survived intermediate  
35 salinities. Intracellular MCs were detected in all the freshwater samples with  
36 concentrations up to  $60 \mu\text{g L}^{-1}$ , and more intermittently with concentrations up to  $1.15$   
37  $\mu\text{g L}^{-1}$ , at the most upstream estuarine site. Intracellular MC was only sporadically  
38 detected and in low concentration at the most downstream estuarine site and at the  
39 marine outlet (respectively  $< 0.14 \mu\text{g L}^{-1}$  and  $< 0.03 \mu\text{g L}^{-1}$ ). Different MC variants  
40 were detected with dominance of MC-LR, RR and YR and that dominance was  
41 conserved along the salinity gradient. Extracellular MC contribution to total MC was  
42 higher at the downstream sites in accordance with the lysing of the cells at elevated  
43 salinities. No nodularin (NOD) was detected in the particulate samples or in the  
44 filtrates.

45

## 46 **1. Introduction**

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). This tendency is also recorded in France and in particular in  
51 Brittany (AFSSA, 2006; Pitois et al., 2014; Le Moal et al., 2019) where most of the  
52 French agricultural lands are located. Freshwater cyanobacteria produce a variety of  
53 toxins (i.e. hepatotoxins, neurotoxins, dermatotoxins) which have strong negative  
54 impacts on animal and human health (Lance et al., 2010; Metcalf and Codd, 2012;  
55 Meriluoto et al., 2017). Reports of toxic cyanobacterial blooms in Brittany have been  
56 numerous in freshwater lakes and reservoirs (Vezie et al., 1998; Brient et al., 2009;  
57 Pitois et al., 2018). Among the diversity of cyanotoxins, the hepatotoxin microcystin  
58 (MC) is largely recognised as the most common and widespread in freshwater  
59 ecosystems (Harke et al., 2016). The general structure of that cyclic heptapeptide  
60 includes a specific beta amino acid- 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-  
61 4,6-decadienoic acid - Adda (Ortiz et al., 2017; Tillett et al., 2000) as well as two  
62 amino acids that can vary leading to the identification of more than 250 MC variants  
63 (Puddick et al., 2014). The regulation and synthesis of MC, as well as its ecological  
64 role, are complex and not yet fully understood (Neilan et al., 2013; Omidi et al.,  
65 2017). Nodularin (NOD) is also a potent cyanobacterial hepatotoxin occurring in  
66 brackish waters (Sivonen et al., 1989; Kaebernick and Neilan, 2001). It is a cyclic  
67 pentapeptide structurally similar to MC, consisting of Adda, D-glutamic acid (D-Glu),  
68 N-methyldehydrobutyrine (MeDhb), D-erythro--methylaspartic acid (D-MeAsp) and L-  
69 arginine (L-Arg) (Rinehart et al., 1988).

70

71 The transfer of cyanobacteria along the freshwater-marine continuum has been  
72 observed worldwide (Preece et al., 2017 for a review), in Africa (Ndlela et al., 2016),  
73 USA (Lehman et al., 2005; Peacock et al., 2018), South America (Dörr et al., 2010),  
74 Australia (Robson and Hamilton, 2003; Orr et al., 2004), Europe (Verspagen et al.,  
75 2006; Tonk et al., 2007; Paldavičiene et al., 2009), and Turkey (Taş et al., 2006). The  
76 majority of these studies reported on the cyanobacterial transfer being dominated by  
77 *Microcystis aeruginosa* demonstrating a certain salt tolerance of that species. The  
78 associated transfer of MC along the river continuum was less often reported (Preece

79 et al., 2017) and very few studies reported on MC levels high enough to affect  
80 recreational activities (Paldavičiene et al., 2009; Albay et al., 2005). In particular, MC  
81 transfer to the coastal environment resulting from freshwater discharge from an  
82 upstream reservoir has only been reported in Italy (De Pace et al., 2014) and Japan  
83 (Umehara et al., 2012). Both MC and NOD have been reported to accumulate in fish  
84 and bivalves resulting in a potential risk to humans from consumption of  
85 contaminated food (Lopes and Vasconcelos, 2011; Gobble et al., 2016; Karjalainen et  
86 al., 2007).

87

88 Here we report on the dynamics and transfer of cyanobacteria and cyanotoxins from  
89 a freshwater reservoir discharge to the marine environment during a 2 year field  
90 study, which to our knowledge is the first study in France reporting on such transfer.  
91 This study is part of a larger project aiming at the evaluation of the potential risk of  
92 contamination of aquatic organisms (i.e. bivalves) by cyanotoxins during transfer  
93 from a freshwater reservoir to an estuary mouth in Brittany, France. We present  
94 results on cyanobacterial biomass, species composition, as well as cyanotoxins  
95 concentrations of different variants of MC and NOD both in intracellular and  
96 extracellular forms. The quantification of those two forms of cyanotoxins are  
97 necessary as we anticipate gradual cells lysing along the salinity gradient.

98

## 99 **2. Materials and methods**

### 100 *2.1. Study site and sampling strategy*

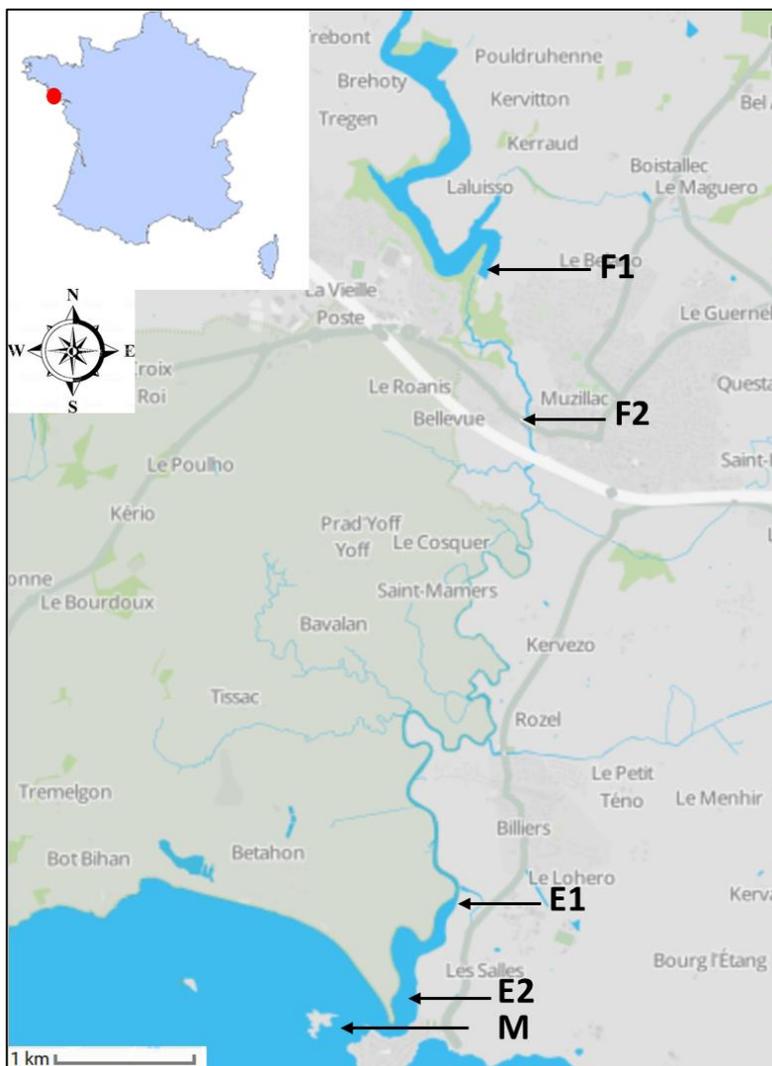
101 In Brittany, surface water dominated rivers have short residence times (Fraisie et al,  
102 2013) and reservoirs are generally close to marine outlets. The study site is located  
103 in the Morbihan (Brittany, France) along a continuum of moderate length (<10 km),  
104 from the Pen Mur freshwater reservoir upstream through the estuary and the marine  
105 outlet (Fig. 1). This study site was chosen as the Pen Mur reservoir, used for drinking  
106 water, is monitored by the Regional Health Agency (ARS) and undergoes recurrent  
107 intense cyanobacterial blooms dominated by the genus *Microcystis* (L. Brient, pers.  
108 comm.). Freshwater discharge from the reservoir to the estuary is frequently  
109 observed but not monitored and therefore not quantified.

110

111 From May 2016 to April 2018, we sampled at 5 stations along the freshwater-marine  
112 gradient (F1 in the Pen Mur reservoir and F2 in the river downstream of the reservoir

113 are both in the freshwater section, E1 and E2 are located in the estuarine section and  
114 the site M is located at the marine outlet). For each campaign, all 5 stations were  
115 sampled on the same day. We consistently sampled the 3 downstream stations  
116 within 1 hour of low tide to maximize the freshwater discharge and minimize the tidal  
117 contribution. At each station, fortnightly to monthly water sampling was carried out  
118 in the water column to: i) describe phytoplankton and cyanobacterial species  
119 (identification and enumeration), and (ii) quantify cyanotoxins (MC and NOD) in the  
120 cells and in the water. Physico-chemical parameters (temperature, conductivity,  
121 dissolved oxygen, phosphorus and nitrogen concentrations) using a YSI 6920 multi-  
122 parameter probe (YSI Environmental, Anhydre) and chemical analyses were also  
123 determined at a subset of the sampling dates.

124



125

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

128

## 129 *2.2. Samples analyses*

130 Samples were filtered upon arrival at the laboratory within a few hours of sampling.  
131 Dissolved nutrient concentrations were measured from filtered (GF/F) water using  
132 common colorimetric methods (Aminot and Chaussepied, 1983) with a Bran and  
133 Luebbe Autoanalyser 3 (Axflow, Norderstedt, Germany). Nitrate was measured after  
134 reduction to nitrite on a cadmium-copper column (Henriksen and Selmer-Olsen,  
135 1970). Phosphate was measured following the method of Murphy and Riley (1962).  
136 Phytoplankton and cyanobacteria identification and counts were conducted on fresh  
137 samples under an optical microscope (100x magnification) using a Nageotte  
138 chamber within 24 hours of sampling.

139

## 140 *2.3. Chemical analysis of cyanotoxins by LC-MS / MS*

141 Water samples containing cyanobacteria were filtered upon arrival at the laboratory  
142 through a 0.45 µm cellulose filter to separate the cell pellet for the intracellular  
143 cyanotoxin analysis and the filtrate for dissolved extracellular toxins and frozen at -  
144 20°C until chemical analysis. The filtrate was purified on a C<sub>18</sub> SPE cartridge (Solid  
145 Phase Extraction) according to the ISO 20179 standard method (Anon 2005). The  
146 fraction containing the toxins was frozen until LC-MS/MS analysis. The cell pellet was  
147 ground with 250 mg of glass beads (0.15-0.25 mm) and 1 ml of MeOH so that cells  
148 released their toxins. Both fractions (intracellular and extracellular) were filtered by a  
149 0.2 µm filter and analyzed by Ultra Fast Liquid Chromatography (Shimadzu, Marne  
150 La Vallee, France) coupled to 5500 QTrap tandem mass spectrometry (ABSciex,  
151 Villebon sur Yvette, France). Toxins were separated on a Kinetex XB C18 column  
152 (100 x 2.1 mm, 2.6 µm, Phenomenex), with water (A) and acetonitrile (B), both  
153 containing 0.1% formic acid at 0.3 mL min<sup>-1</sup> flow rate. The gradient was raised from  
154 30 to 80% B in 5 min and was held during 1 min before dropping down during 0.5 min  
155 to the initial conditions.

156

157 Mass spectrometry detection was carried out in multiple reactions monitoring (MRM)  
158 mode (positive ions). The electrospray ionization interface (ESI) was operated in  
159 positive mode using source setting: curtain gas set at 30 psi, ion spray at 5000 V, a  
160 turbogas temperature of 300°C, gas 1 and 2 set at 30 and 40 psi respectively and an  
161 entrance potential of 10 V. Each toxin was identified and quantified with two

162 transitions (Table S1): The toxin concentrations of all 10 lipophilic cyanotoxins were  
 163 determined using certified standards provided by CNRC (Halifax, NS, Canada). The  
 164 method was developed and validated internally in the IFREMER Phycotoxins  
 165 laboratory.

166

167

168

169 **Table S1** : LC-MS/MS transitions for the 9 MC variants and NOD tested with  
 170 standards

171

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
NOD	825.5	227.0	163.2

172

173

174 **2.4. Statistical analyses**

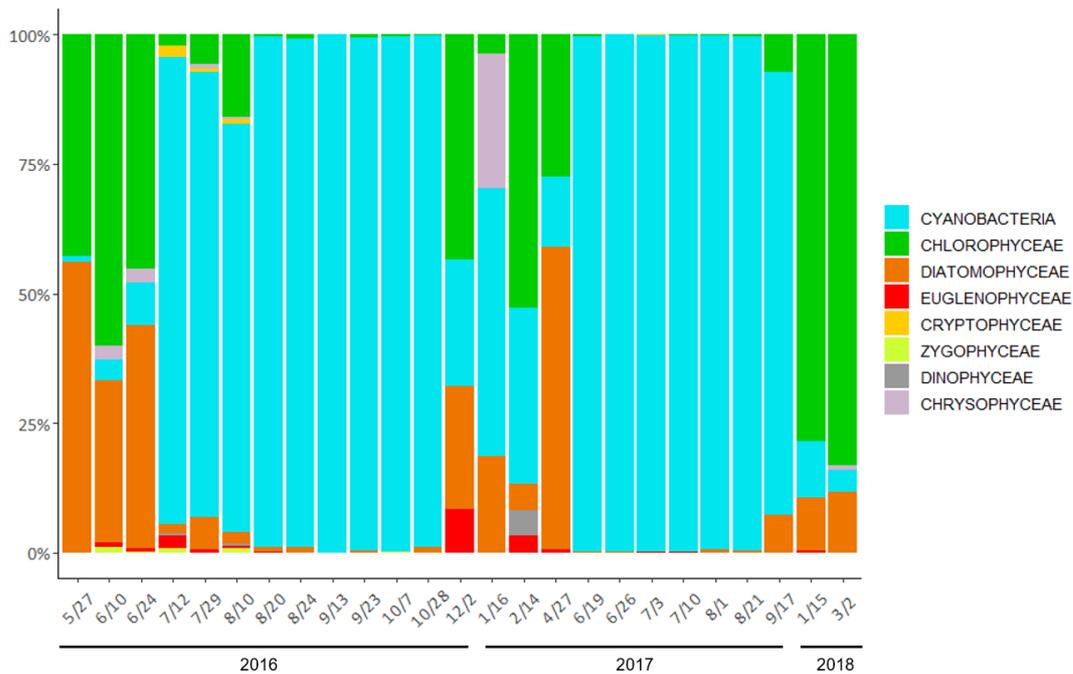
175 All statistical analyses were carried out using R studio software (R Development  
 176 Core Team, 2011). A Kruskal-Wallis analysis was used to test the temporal and  
 177 spatial effects on physico-chemical conditions and dissolved nutrient concentrations.  
 178 The significance threshold was set at  $p < 0.05$ . A Spearman correlation was applied  
 179 between cyanobacterial biomass and overall toxin concentrations. Changes in the  
 180 physico-chemical and biological conditions were characterized by a principal  
 181 component analyses (PCA). Analyses were performed on temperature, conductivity,  
 182 oxygen saturation, cyanobacterial biomass (cell/mL) and Shannon diversity index  
 183 data.

184

### 185 3. Results

#### 186 3.1. Dynamics and transfer of cyanobacteria along the freshwater marine continuum

187 Diatomophyceae and chlorophyceae occurred in winter and spring while  
188 cyanobacteria dominated the phytoplankton community in Pen Mur reservoir (F1 site)  
189 in summer and early fall during both years (Fig. 2). It is important to note that only  
190 few sampling campaigns (6) were performed in winter and spring, while the majority  
191 (18) occurred in summer and fall.

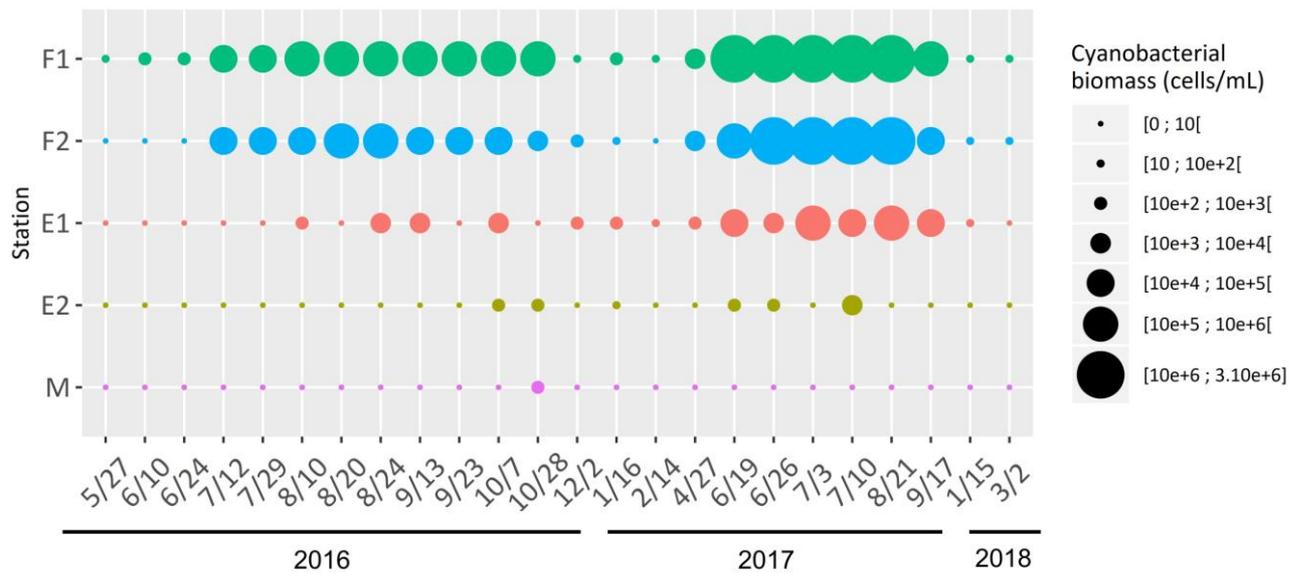


192

193 *Fig. 2: Dynamics of phytoplankton community structure at the freshwater site F1 over*  
194 *the 2 year field study. The x axis corresponds to sampling dates (and not time).*

195

196 The proliferation dynamics of cyanobacteria presented in Fig. 3 shows that the  
197 biomass reached very high concentrations in the freshwater reservoir (F1 site) ( $> 10^5$   
198  $\text{cells mL}^{-1}$ ) in summer and fall (from July to October) with a peak of  $10^6$   $\text{cells mL}^{-1}$  in  
199 2016 and of  $2 \times 10^6$   $\text{cells mL}^{-1}$  in 2017. These intense cyanobacterial blooms were  
200 also observed at F2 in the riverine section downstream of the reservoir with a slightly  
201 lower biomass.



202

203 *Fig. 3: Dynamics of the cyanobacterial biomass (cells/mL) along the freshwater-marine*  
 204 *continuum at the 5 sampling sites. The x axis corresponds to sampling dates (and not time).*

205

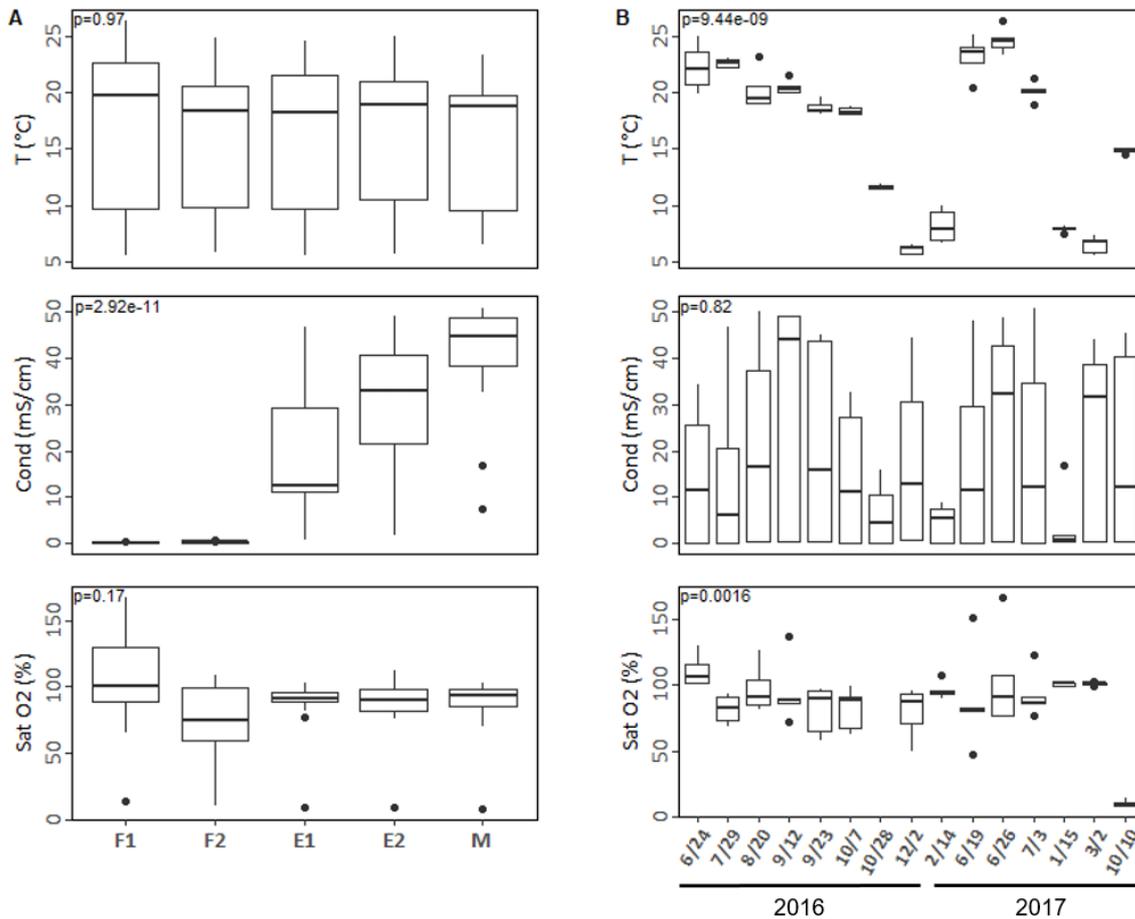
206 Transfer to the estuary was demonstrated with a progressively lower cyanobacterial  
 207 biomass along the estuary as the distance from the upstream reservoir increased.  
 208 Downstream cells concentrations were always related to upstream cells  
 209 concentrations suggesting that they were due to horizontal transfer and not to *in situ*  
 210 growth. A maximum of  $2 \times 10^5$  cells mL<sup>-1</sup> was recorded at E1 in June 2017 and a  
 211 maximum of  $1.2 \times 10^3$  cells mL<sup>-1</sup> was observed at E2 on the same day. At the most  
 212 downstream station M, coinciding with the marine outlet, a maximum of 240 cells/mL  
 213 was recorded (in October 2016).

214

### 215 3.2. Environmental factors associated with the cyanobacterial biomass

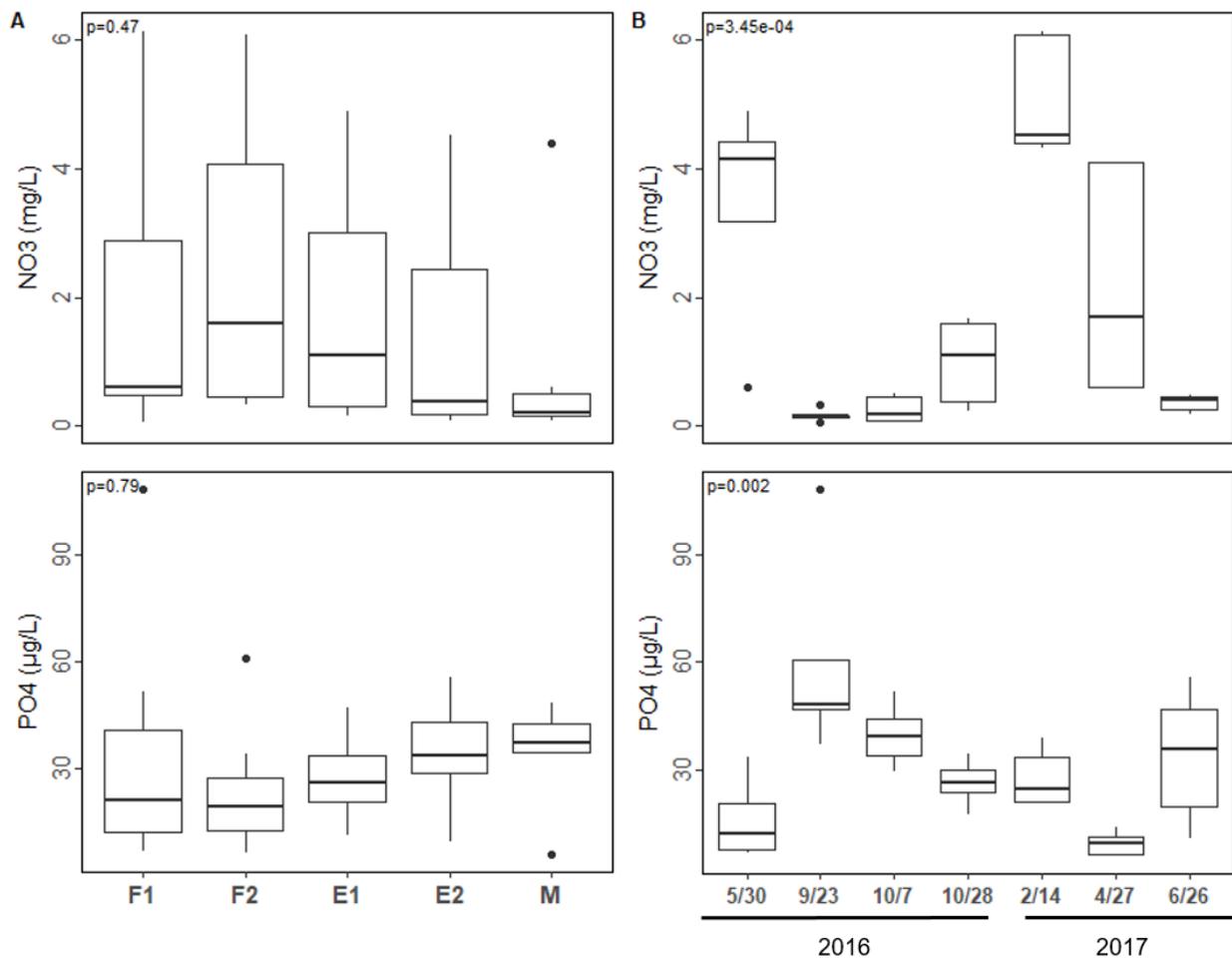
216 The spatial and temporal variations of cyanobacterial biomass were associated with  
 217 physico-chemical conditions presented in Fig. 4: a statistically significant seasonal  
 218 variation in water temperature was observed with maxima in June/August and  
 219 minima in December/February ( $p=10^{-8}$ ) but relatively little spatial variation along the  
 220 estuary ( $p=0.97$ ). On the contrary, conductivity exhibited a strong statistically  
 221 significant spatial variation ( $p=3 \times 10^{-11}$ ) and a relatively low temporal gradient  
 222 ( $p=0.82$ ). Hence temporal variation in cyanobacterial biomass was essentially  
 223 correlated with changes in temperature whereas spatial variation was mostly derived  
 224 from the longitudinal gradient of salinity/conductivity. A threshold of 18°C  
 225 corresponded to a biomass higher than  $10^5$  cells mL<sup>-1</sup> in the reservoir upstream. The

226 concentration variation in dissolved oxygen gave an indication of photosynthetic  
 227 activity, values greater than 100% saturation corresponding to photosynthetic  
 228 production conditions, values around 100% indicating a balance between the water  
 229 and the atmosphere and values below 100% indicating respiration or consumption by  
 230 bacteria.



231  
 232 *Fig. 4: Spatio-temporal variation of physicochemical conditions measured during the*  
 233 *2 year study along the freshwater to marine continuum.*

234  
 235 Dissolved oxygen displayed a statistically significant temporal variation ( $p=0.0016$ )  
 236 while no spatial variation ( $p=0.17$ ) was observed during the study period. In the  
 237 reservoir (F1 site) during high cyanobacterial biomass, the dissolved oxygen was  
 238 oversaturated, while in the shallow F2 site where low water level (< 1m) of near  
 239 stagnant waters coincided with high biological degradation and bacterial  
 240 consumption, the dissolved oxygen was strongly undersaturated. Further  
 241 downstream in the estuary (E1 and E2 sites) the dissolved oxygen was around 80 to  
 242 100 % saturation.



244

245 *Fig. 5: Spatio-temporal variation of dissolved nutrient concentrations measured*  
 246 *during the two year study along the freshwater to marine continuum.*

247

248 Both dissolved nutrients (NO3 and PO4) displayed a statistically significant temporal  
 249 variation while the spatial variation only showed tendencies (Fig. 5). Nitrate  
 250 concentrations presented an upstream to downstream decrease while phosphate  
 251 concentrations showed the opposing trend with higher values downstream.

252

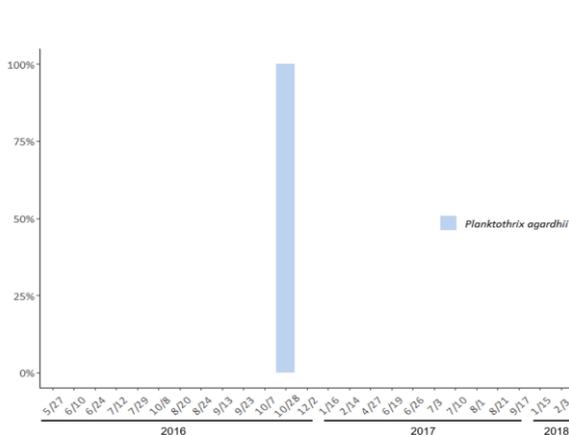
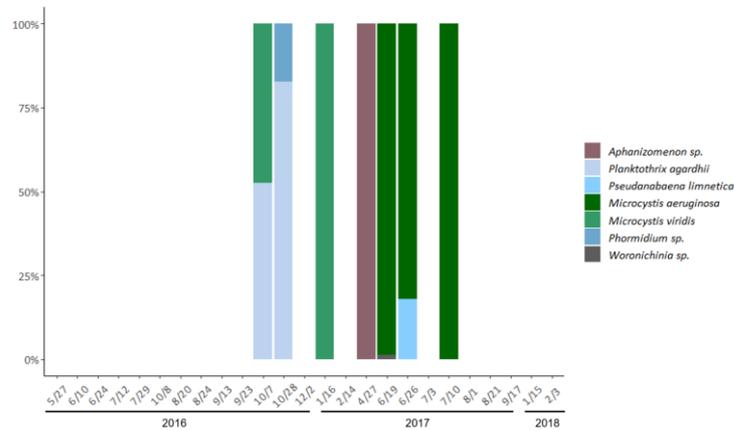
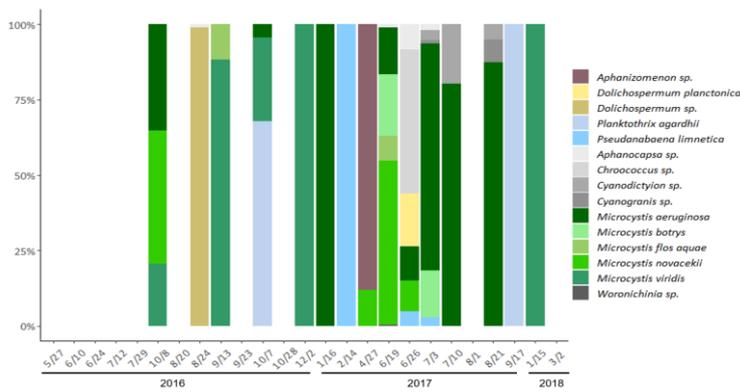
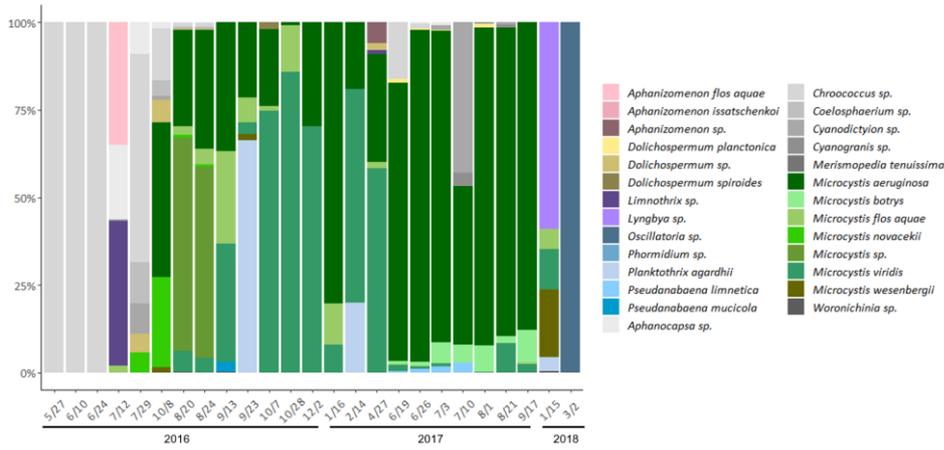
### 253 3.3. Cyanobacterial species composition along the salinity gradient

254 The composition of cyanobacterial populations was very diverse in the freshwater  
 255 reservoir with 27 species present during the study. As Fig. 6 shows, during the  
 256 strongest proliferations (from August to October both years), a dominance of the  
 257 genus *Microcystis* was observed at the F1 site with up to 7 different species of  
 258 *Microcystis* i.e. *M. aeruginosa*, *M. viridis*, *M. flos aquae*, *M. wesenbergii*, *M. novacekii*  
 259 and *Microcystis* sp. This same diversity and composition was observed at the F2 site

260 (not shown). There was a shift in the dominant *Microcystis* species between 2016  
261 and 2017 with a mix of *M. viridis*, *M. flos aquae*, *M. aeruginosa* and *Microcystis sp* in  
262 2016 and an almost monospecific bloom of *M. aeruginosa* in 2017.

263

264 The transfer to the estuary selected for certain species according to their adaptation  
265 to the new physicochemical conditions, mainly salinity (recorded as conductivity). Up  
266 to half of the cyanobacterial species were present in the estuarine section: 14  
267 species at E1, 7 at E2 and 1 at M. The maximum conductivity recorded during  
268 sampling in summer and fall was 12 mS cm<sup>-1</sup> at E1, 35 mS cm<sup>-1</sup> at E2 and 50 mS cm<sup>-1</sup>  
269 at M. The species that survived the transfer through the estuary were several  
270 species of *Microcystis*, *Aphanizomenon sp.*, *Pseudanabaena limnetica* and  
271 *Planktothrix agardhii*, all recorded at the estuarine site E2. *P. agardhii* was the only  
272 cyanobacterial species recorded at the marine outlet M. The transfer of *P. agardhii*  
273 was observed on 4 October 2016 between the freshwater site F2 (density *P. agardhii*  
274 7400 cells mL<sup>-1</sup>, *Microcystis sp* 22000 cells mL<sup>-1</sup>), through the estuarine E1 site  
275 (density *P. agardhii* 4480 cells mL<sup>-1</sup>, *Microcystis sp* 2200 cells mL<sup>-1</sup>), and the  
276 estuarine E2 site (*P. agardhii* density 533 cells mL<sup>-1</sup>, *Microcystis sp* 267 cells mL<sup>-1</sup>).  
277 Another transfer to the marine outlet was observed at the end of October 2016  
278 between E2 (432 cells mL<sup>-1</sup> of *P. agardhii*, 480 cells mL<sup>-1</sup> of total cyanobacteria) and  
279 M (240 cells mL<sup>-1</sup> of *P. agardhii*, 100% cyanobacteria), while *P. agardhii* was not the  
280 majority at F2 (160 cells mL<sup>-1</sup> *P. agardhii*, 4400 cells mL<sup>-1</sup> *Microcystis sp.*). Among  
281 the other filamentous cyanobacteria surviving the estuarine transfer, *Pseudanabaena*  
282 *limnetica* was observed on 26 June 2017 with a decreasing concentration from  
283 165120 cells/mL at F1 to 7680 cells mL<sup>-1</sup> at E1 down to 192 cells mL<sup>-1</sup> at E2 while  
284 *Aphanizomenon sp* was observed on 27 April 2017 from 220 cells mL<sup>-1</sup> at F2, down  
285 to 132 cells mL<sup>-1</sup> at E1 and 8 cells mL<sup>-1</sup> at E2. It is interesting to note that the relative  
286 transfer of filamentous cyanobacteria was higher than that of either unicellular  
287 (*Aphanocapsa*) or colonial (*Microcystis*). Of the transferred *Microcystis* species, *M.*  
288 *viridis* was observed further downstream than *M. aeruginosa* and *M. flos aquae* in  
289 2016 while the opposite was observed in 2017, in accordance with the relative  
290 biomass in the freshwater section.



295

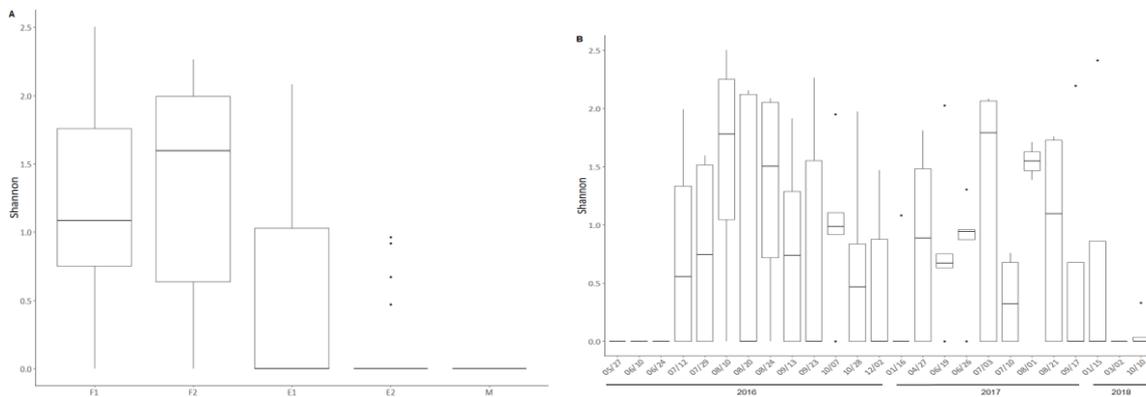
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Fig. 6: Dynamics of cyanobacterial species composition at stations F1, E1, E2 and M over the 2 year field study period.

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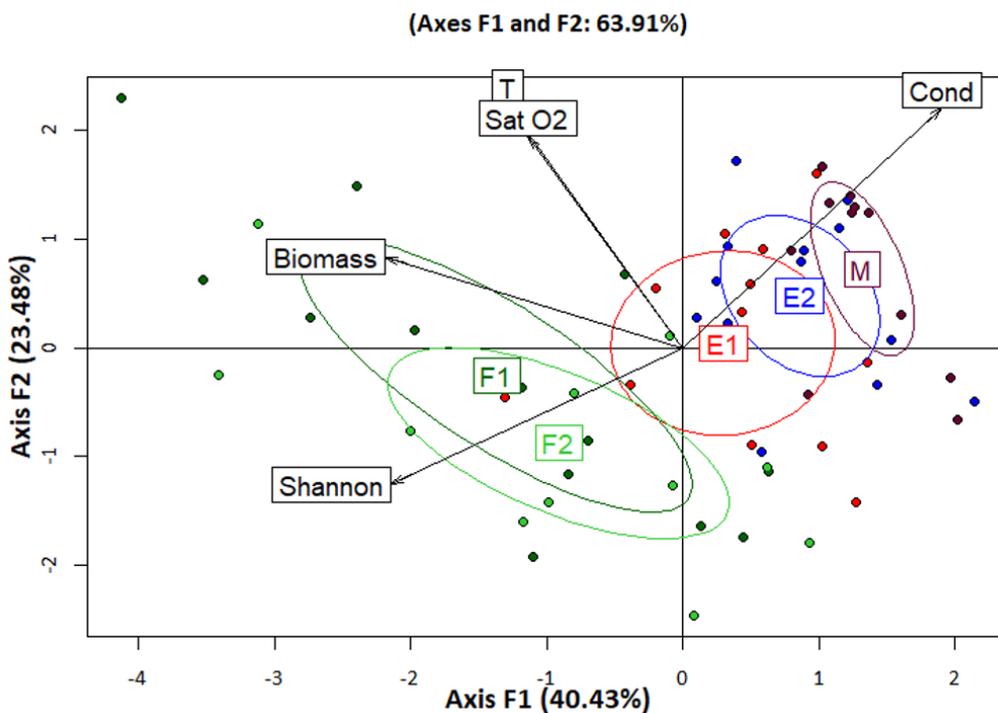
The cyanobacterial species diversity was also quantified through the cyanobacterial Shannon index which is presented in Fig. S1. A gradual decrease of the Shannon index along the salinity gradient is observed together with higher values in summer and fall.



304  
305  
306

*Fig. S1: Spatial and temporal variation of the cyanobacterial Shannon diversity index*

307 A PCA analysis performed on the complete dataset (Fig. S2) confirmed the stronger role of salinity (i.e. conductivity) in structuring the spatial distribution of the observations together with the Shannon diversity index, while the cyanobacterial biomass was most strongly associated with elevated temperature and dissolved oxygen concentration.



312

313 Fig. S2: PCA analysis highlighting the spatial variation in physico-chemical (T, O<sub>2</sub>,  
 314 Cond) and biological parameters (cyanobacterial biomass, Shannon index)

315

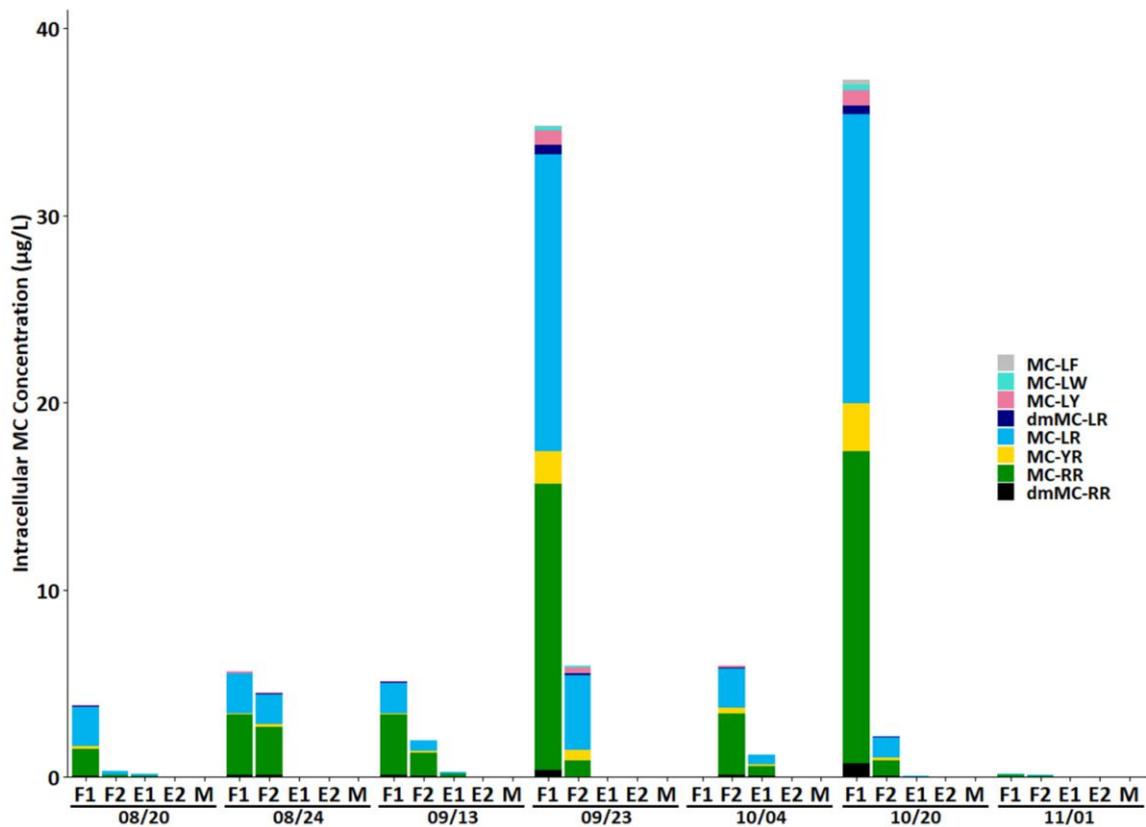
316 3.4 Transfer of cyanotoxins along the freshwater-marine continuum

317 This study presents the concentrations of non-protein bound lipophilic cyanotoxins,  
 318 MC and NOD in particulate samples and in the filtrates during the field campaigns of  
 319 2016 and 2017. In particular, the intracellular and extracellular forms of MC are  
 320 separated to consider the potential risk associated with the lysing of the cells at high  
 321 salinities.

322

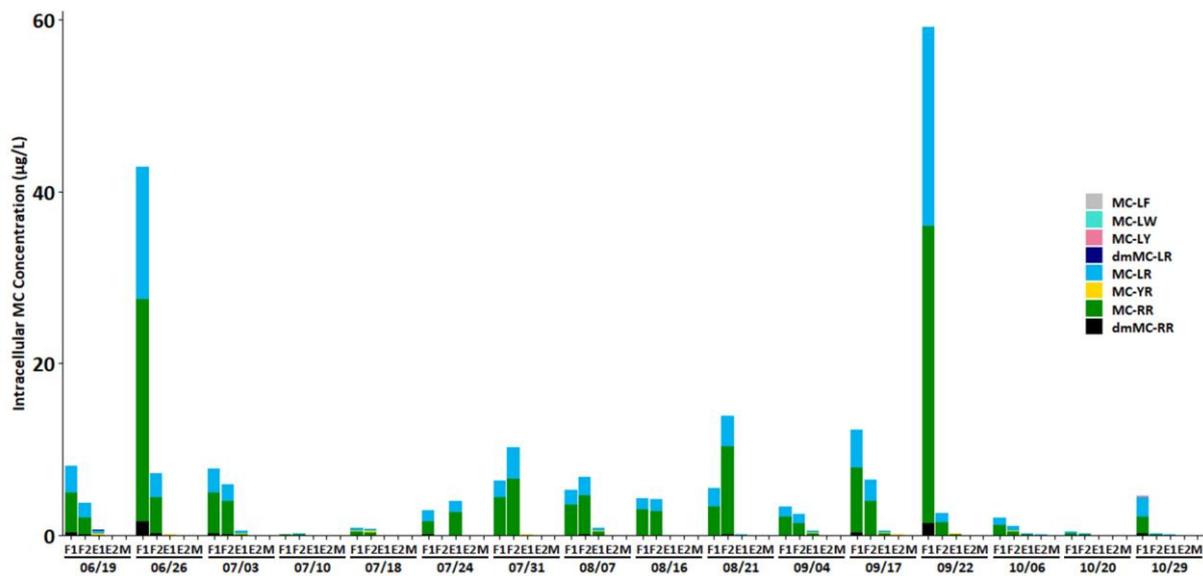
323 3.4.1. Intracellular toxins

324 Firstly, the toxins analysis of the particulate samples of the different stations didn't  
 325 reveal the presence of NOD, which corroborates the observation of cyanobacteria  
 326 species not potentially producing NOD. The time variation shows the annual  
 327 reproducibility with measured concentrations and transfer of MC occurring in both  
 328 2016 and 2017 with higher toxin concentrations reached in 2017 (Fig. 7).



329

330



331  
 332 *Fig. 7: Intracellular concentrations of MC variants in the cyanobacteria during the*  
 333 *field campaigns of A) 2016 and B) 2017. Note that the x axis corresponds to*  
 334 *sampling dates (and not time).*

335  
 336 While the presence of MC was recorded in all the freshwater samples (at F1 and F2),  
 337 a high temporal variation in concentration was observed with a maximum  
 338 concentration of total MC (sum of the MC analogs) of  $40 \mu\text{g L}^{-1}$  in September /  
 339 October 2016 and  $60 \mu\text{g L}^{-1}$  in September 2017. Among the MC variants observed,  
 340 some MC analogs (MC-RR, MC-LR, dmMC-RR and MC-YR) were detected in all  
 341 samples while others (dmMC-LR, MC-LY, MC-LW, MC-LF) were only recorded in  
 342 some samples. The three dominant variants at F1 were MC-LR, MC-RR and MC-YR.  
 343 The total MC values at F2 reached a maximum of  $14 \mu\text{g L}^{-1}$  with a dominance of MC-  
 344 RR, MC-LR, dmMC-RR and MC-YR. A demonstrated transfer of cyanotoxins through  
 345 the estuary was recorded on many occasions (Table 1) with progressively lower  
 346 intracellular toxins concentrations reaching  $1.15 \mu\text{g L}^{-1}$  at E1 (comprising of MC-RR,  
 347 MC-LR, MC-YR, dmMC-RR). In a less frequent manner, a maximum of  $0.14 \mu\text{g L}^{-1}$   
 348 was recorded at E2 (comprising of MC-RR, ML-LR) and a maximum of  $0.03 \mu\text{g L}^{-1}$  at  
 349 site M (MC-RR, MC-LR). In summary, we observed a gradual decrease in  
 350 occurrence and in concentrations of intracellular toxins from upstream to  
 351 downstream.

353

354

Sites	FO % intra MC (Jun – Nov)		Max intra MC µg/L
	2016	2017	
F1	100	100	60
F2	100	100	14
E1	67	87	1.15
E2	17	38	0.14
M	0	27	0.03

355

356 *Table 1: Frequency of occurrence (in %) and maximum concentrations of intracellular*  
357 *MC at the 5 stations during the period June to November of both sampling years*

358

359 The results of this study show relatively strong overall correlation between the  
360 amount of toxins in the phytoplankton samples and the total cells density of  
361 potentially toxic cyanobacteria (correlation coefficient of 0.85,  $p=10^{-11}$ ). However,  
362 when we consider relationships on specific dates, the correlation varies with the  
363 dominant cyanobacterial species. For example, in August 2016, a maximum of 5 µg  
364 MC L<sup>-1</sup> was observed at site F1, associated with a cyanobacterial density of 900,000  
365 cells/mL composed mainly of *Microcystis* sp, whereas up to 40 µg MC L<sup>-1</sup> were  
366 measured at the same site in October 2016, associated with a density of 360,000  
367 cells mL<sup>-1</sup> composed essentially of the species *P. agardhii*. Transfer of *P. agardhii*  
368 (4480 cells mL<sup>-1</sup>) to the estuarine site E1 in early October 2016 coincided with the  
369 presence of MC (1.2 µg MC L<sup>-1</sup>). MC was also measured at E1 in August, September  
370 and the end of October, at concentrations close to 1 µg MC L<sup>-1</sup>. MC in low  
371 concentration was measured in the particulate samples at E2 and at M despite the  
372 presence of low density cyanobacteria.

373

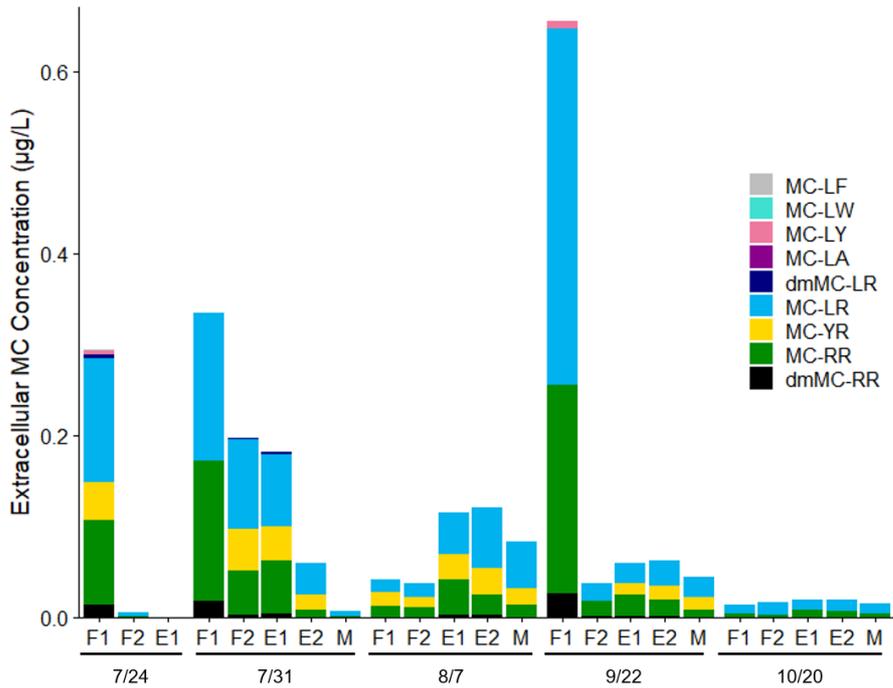
374

#### 375 3.4.2. Extracellular toxins

376

377 The extracellular toxins (measured in the filtered water) were analyzed only from July  
378 2017 to October 2017 (Fig. 8). Similarly to the intracellular fraction, no NOD was  
379 detected. The extracellular MC profiles revealed a dominance of MC-LR, MC- RR  
380 and MC-YR. Maximum MC concentrations of 0.65 µg L<sup>-1</sup> were found at F1 in the

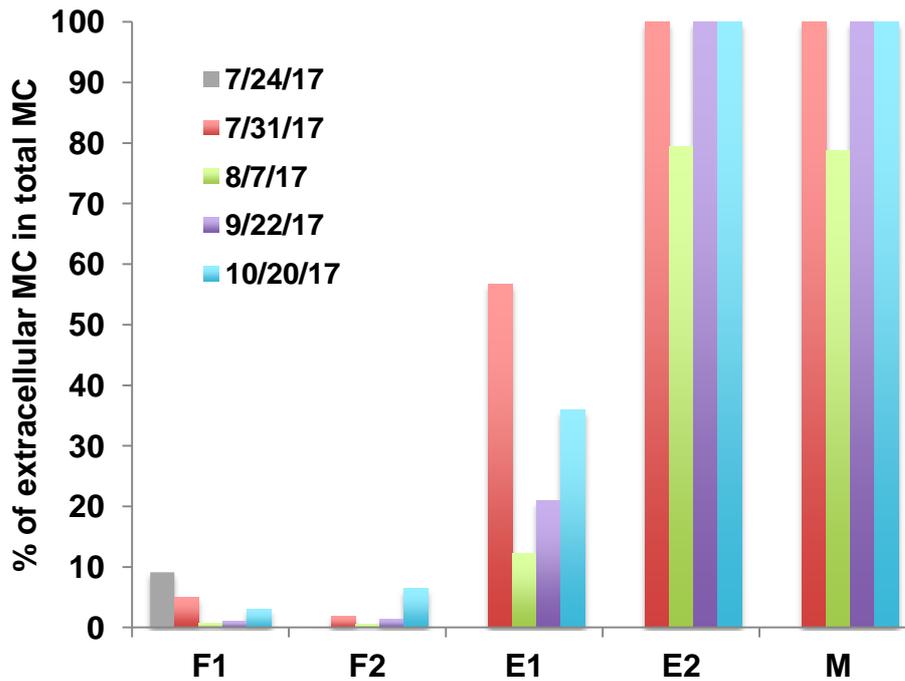
381 upstream freshwater reservoir. We observed higher MC concentrations downstream  
 382 of F2 during August and September 2017 in the estuarine section and marine site.



383  
 384 *Fig. 8: Extracellular concentrations of MC variants in the filtered water at the 5*  
 385 *sampling sites during summer and fall 2017*

386  
 387 When comparing ratios of extracellular MC to the total MC (intracellular +  
 388 extracellular fractions) along the salinity gradient, we observed a consistent trend  
 389 with minimal contribution of extracellular toxins in freshwater and a gradual increase  
 390 with increasing salinities (Fig. 9). At site E1 where the maximum conductivity  
 391 recorded during sampling during 2017 was 12 mS cm<sup>-1</sup>, the extracellular contribution  
 392 was up to 50%, while it increased to 100% at sites E2 and M where conductivities  
 393 were above 35 and 47 mS cm<sup>-1</sup> respectively.

394  
 395  
 396  
 397



398  
 399 *Fig. 9: Contribution of extracellular MC to the total MC concentrations at the 5*  
 400 *sampling sites during summer and fall 2017*

401  
 402

#### 403 **4. Discussion**

404  
 405 This study demonstrated over 2 successive years the recurrent transfer of  
 406 cyanobacteria and cyanotoxins along the freshwater – marine continuum in a Brittany  
 407 estuary, France. This result is in accordance with published studies worldwide. This  
 408 transfer was first reported in the late 1980's in Europe (Sivonen et al., 1989) and  
 409 USA (Paerl, 1988) but have gained an interest recently (Preece et al, 2017) and likely  
 410 to increase in frequency in the future (Paerl et al., 2018). Although the biomass was  
 411 lower than in the freshwater reservoir, cyanobacterial biomass of up to  $2 \times 10^5$  cells  
 412  $\text{mL}^{-1}$  was observed in the estuarine section. These cyanobacteria could therefore  
 413 contribute significantly to the phytoplankton community structure of the estuary,  
 414 which has typically a much lower biomass (usually reported in cells  $\text{L}^{-1}$ ) than in  
 415 freshwater, or serve as food source for invertebrates. While the temporal variation in  
 416 cyanobacterial densities was mainly associated with temperature, spatial variation  
 417 was due to salinity while nutrients were unlikely to limit cyanobacterial growth. Nitrate  
 418 presented an upstream to downstream decrease suggesting a freshwater dominant  
 419 source. Phosphate values showed the opposing trend with higher values

420 downstream and in particular at the outlet where we expect sediment resuspension  
421 to be the largest, based on the observed high concentrations of suspended particles.

422

423 Although the freshwater discharge was not monitored and therefore not quantified,  
424 the progressive increase in salinity near the surface was a good indicator of dilution.  
425 Possible light limitation could also be important but the short residence time (of the  
426 order of one to two days), inferred from observations of the surface velocities during  
427 sampling, would not permit *in situ* growth. Hence we suggest that the lower biomass  
428 in the estuary results from freshwater discharge dilution with estuarine waters.

429

430 The dominant blooming genus in the upstream freshwater reservoir was  
431 overwhelmingly *Microcystis*, consistent with the most widespread cyanobacterial  
432 occurrence of *Microcystis* in freshwaters worldwide (Harke et al., 2016) and as well  
433 as in Brittany (Pitois et al., 2014). While cyanobacterial transfer from freshwaters to  
434 estuaries has been reported for around 20 years, the majority of studies worldwide  
435 reported that *M. aeruginosa* was the dominant species transferred to coastal waters  
436 (Preece et al., 2017 for a review). *M. aeruginosa* is described as one of the  
437 freshwater cyanobacteria with the highest salinity tolerances (Verspagen et al., 2006)  
438 but variable thresholds have been observed from 4 ppt (Chen et al., 2015), 10 ppt  
439 (Lewitus et al., 2008; Tonk et al., 2007) to 35 ppt (Miller et al., 2010 ; Black et al.,  
440 2011) possibly showing strong intraspecific variability. In this study, different  
441 *Microcystis* species were transferred through the estuary in 2016 and 2017, in  
442 accordance with their relative biomass upstream. Therefore we cannot suggest a  
443 stronger resistance of either of the *Microcystis* species (*M. aeruginosa*, *M. viridis* and  
444 *M. flos aquae*). While the mucilage associated with the colonial form of *Microcystis* is  
445 likely to protect the cells from osmotic shock at high salinity, as suggested by Kruk et  
446 al., 2017 and Martínez de la Escalera et al., 2017, the relative resistance of the  
447 different *Microcystis* species is not known. Filamentous cyanobacteria i.e.  
448 *Planktothrix* and *Pseudanabaena* have been shown in this study to also survive the  
449 transfer through the estuary and therefore the salinity stress. The salinity tolerance of  
450 *P. agardhii* has been recently tested on brackish isolated species and a tolerance  
451 value of up to a salinity of 15 has been found (Vergalli et al., 2016). The transfer of  
452 *Planktothrix agardhii* through an estuary has been recently reported on one occasion  
453 although the bloom was non toxic (Churro et al., 2017). *Pseudanabaena sp.* has

454 been occasionally reported in the Baltic Sea (Lopes and Vasconcelos, 2011),  
455 however, the role of the mucilage/sheath around the filaments, as a potential  
456 protective morphological characteristic has not been suggested. On the other hand  
457 unicellular species present in relatively high numbers in the freshwater reservoir (i.e.  
458 *Aphanocapsa* sp., *Coelosphaerium* sp., *Cyanodiction* sp. or *Cyanogranis* sp.) were  
459 not found in the estuary. A possible explanation besides a sensitivity to salinity is that  
460 these species might be difficult to identify in samples with high suspended matter,  
461 and therefore not accounted for in the counting.

462

463 Intracellular MC was detected in all samples in the freshwater section with  
464 concentrations reaching 40  $\mu\text{g L}^{-1}$  in 2016 and 60  $\mu\text{g L}^{-1}$  in 2017. These levels are  
465 much higher than the alert 3 warning level of 10-20  $\mu\text{g L}^{-1}$  of the World Health  
466 Organization forbidding any recreational activities in waterbodies (Ibelings et al.,  
467 2014; Funari et al., 2017). In this study, the measured gradual decrease in  
468 cyanobacterial biomass was accompanied by a decrease in intracellular MC  
469 concentrations from upstream to downstream indicating a likely dilution effect as  
470 expected due to strong tidal influence, even though we minimized that influence by  
471 sampling within one hour of low tides. This decrease has also been reported in the  
472 San Francisco Estuary (Lehman et al., 2008) and in Monterey Bay (Gibble and  
473 Kudela, 2014). A concomitant physiological response to MC production could also be  
474 possible at high salinity. Indeed, the effect of salinity on the physiology of *M.*  
475 *aeruginosa* has demonstrated a reduction in the production of MCs under salt stress  
476 (Black et al., 2011; Martín-Luna et al., 2015).

477

478 A highly significant overall relationship between potentially toxic cyanobacterial  
479 biomass and intracellular MC concentrations was found. In 2017, there was an early  
480 summer dominance of the small unicellular non-toxic cyanobacteria *Cyanodiction* sp.  
481 and *Cyanogranis* sp. which did not occur in 2016 when the summer blooms were  
482 dominated by the potentially toxic *M. viridis*. The toxicity of *Microcystis* is also known  
483 to vary among species, *M. wesenbergii* being rarely found toxic, while the majority of  
484 *M. aeruginosa* and *M. viridis* is reported as toxic (Harke et al., 2016, Otten et al.,  
485 2017). The MC concentration in the reservoir and the estuary was most likely due to  
486 *Microcystis* sp., due to its overwhelming dominance and the high percentage of  
487 potentially toxic species within that genus in Brittany (Pitois et al., 2014). *P. agardhii*

488 is also known to produce MCs in relatively large quantities (Briand et al., 2009; Lance  
489 et al., 2007) and the most toxic bloom reported in Italy's estuarine waters was indeed  
490 one of *Planktothrix* (De Pace et al., 2014).

491

492 Few studies report on the different variants of MC during blooms in the natural  
493 environment although their identification is important as different variants display  
494 different toxicities and health risks (Lehman et al., 2008; Otten et al., 2017).  
495 Toxicological studies reported on similar LD<sub>50</sub> values for MC-LR, MC-LA, MC-YR and  
496 MC-YM while the value for MC-RR was 10 times higher (Sivonen and Jones, 1999),  
497 while Gupta et al., 2003 reported that MC-LR was twice as toxic as MC-LA. The  
498 dominant MCs variants (MC-LR and MC-RR in 2017 and (MC-LR, MC-RR and MC-  
499 YR in 2016) were transferred without specific selection along the salinity gradient.  
500 The difference between the two years of the dominant variants may be attributed to  
501 different dominant *Microcystis* species as it is known that each species and strain  
502 produced, in culture conditions or *in situ*, different variants in different proportions and  
503 cell quantities (Rios et al., 2014; Briand et al., 2016; Otten et al., 2017). The  
504 demonstrated concentrations of the three dominant variants of MC reported in this  
505 study might therefore induce potential impacts on cyanobacterial consumers. Our  
506 preliminary data (unpublished) show MC accumulation by filter feeding organisms  
507 supporting this hypothesis. While we quantified 9 variants of MC it is still possible that  
508 other variants were present in the samples.

509

510 In this study, a lysis of the cyanobacterial cells at high salinity was most likely as the  
511 ratio of extracellular MC to the total MC concentration increased downstream and in  
512 particular accounted for 100 % of the total MC concentrations at the two most  
513 downstream sites. In accordance with literature results the dominant form of MC in  
514 the freshwater section was intracellular indicating its constitutive nature (Orr and  
515 Jones, 1998; Briand et al., 2012), while the dominant form became extracellular as  
516 cells lysis increased at elevated salinity (Tonk et al., 2007).

517

## 518 **5. Conclusion**

519 This study reports on the transfer of both cyanobacteria and cyanotoxins from a  
520 freshwater reservoir to the marine outlet in France. Moreover, the transfer through  
521 the estuary occurred frequently over the 2 year study period. The higher the

522 concentrations upstream, the more intense the transfer. The very high correlation  
523 between the biomass of potentially toxic species and total MC concentrations  
524 suggests that the majority of the cyanobacterial present in the estuary were toxic.  
525 *Microcystis* which dominated the blooms in the freshwater reservoir was the most  
526 likely genus responsible for the measured MC concentrations in the estuary, followed  
527 by *P. agardhii* both being relatively resistant to the salinity gradient. The extracellular  
528 contribution to the total MCs increased from upstream to downstream in accordance  
529 with cells lysis at elevated salinity. Both intracellular and extracellular MC variants did  
530 not show specific selection along the salinity gradient and the dominance of two  
531 highly toxic variants (i.e. MC-LR and MC-YR) is worrisome, as it could impact  
532 cyanobacterial consumers.

533  
534

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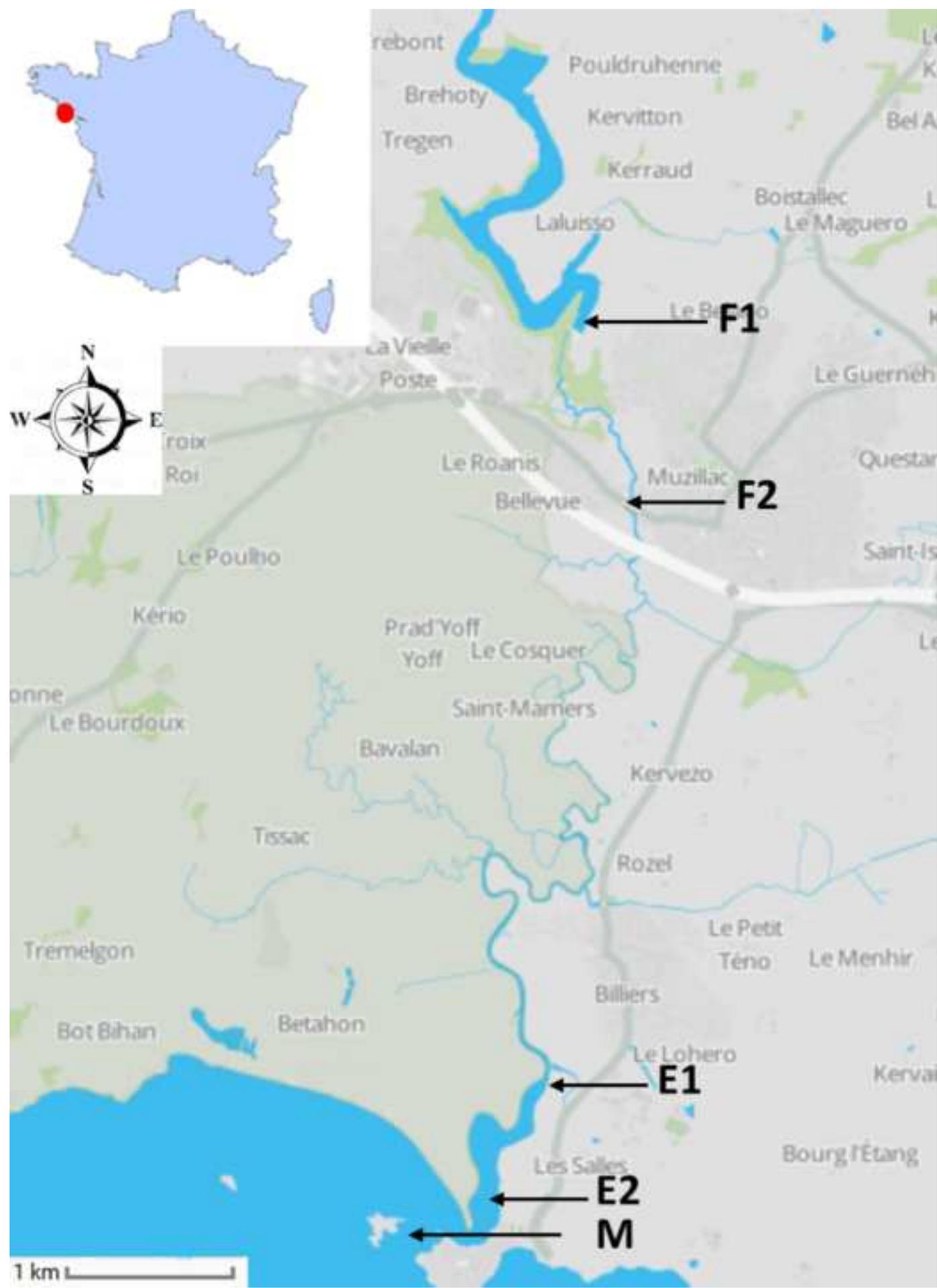


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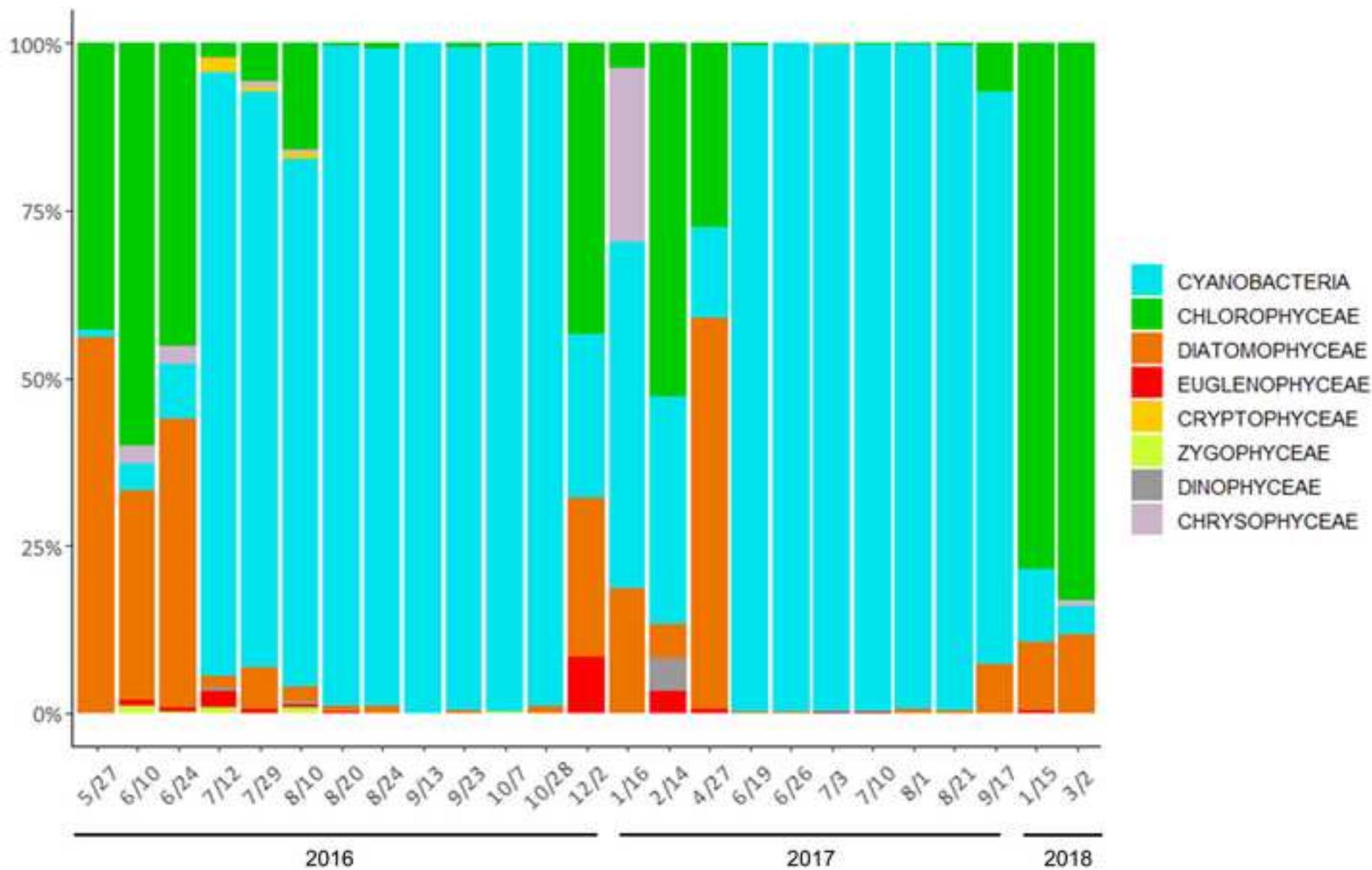
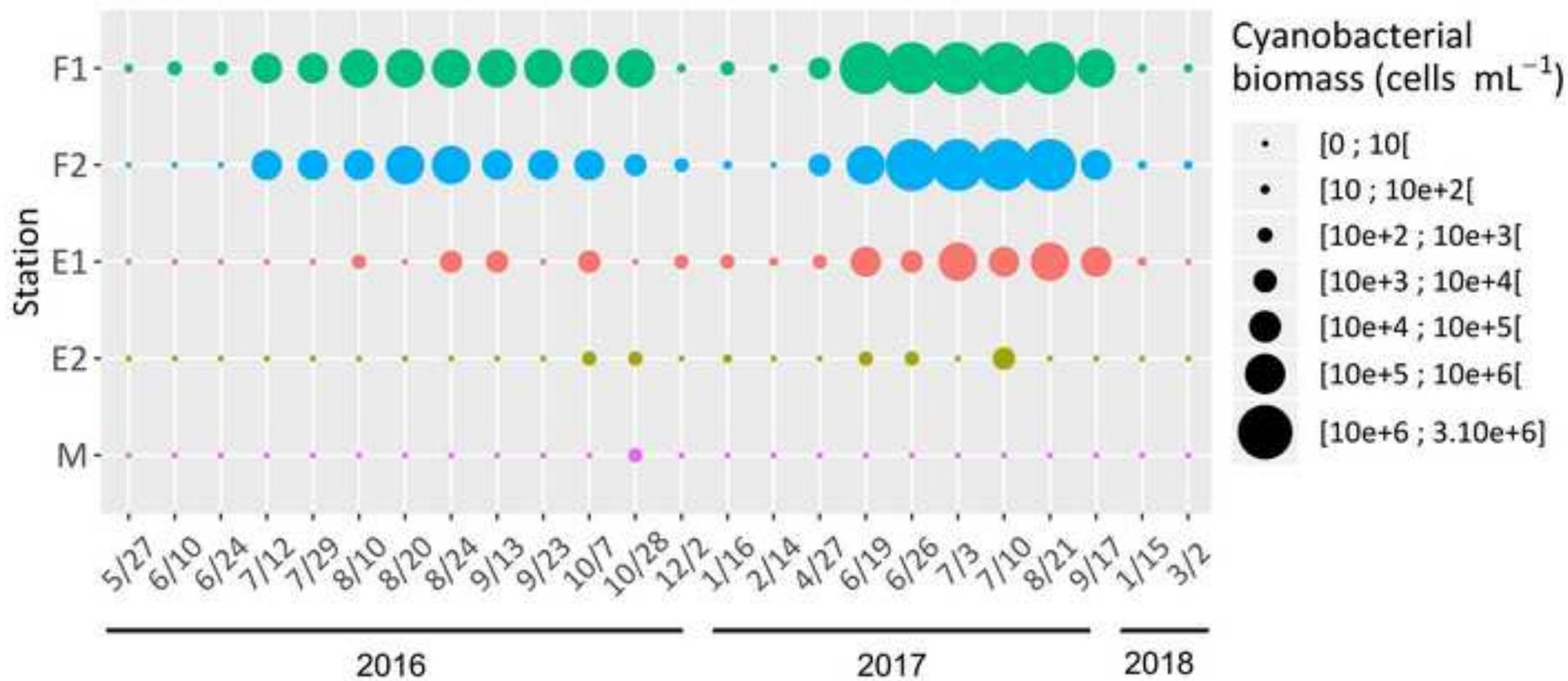


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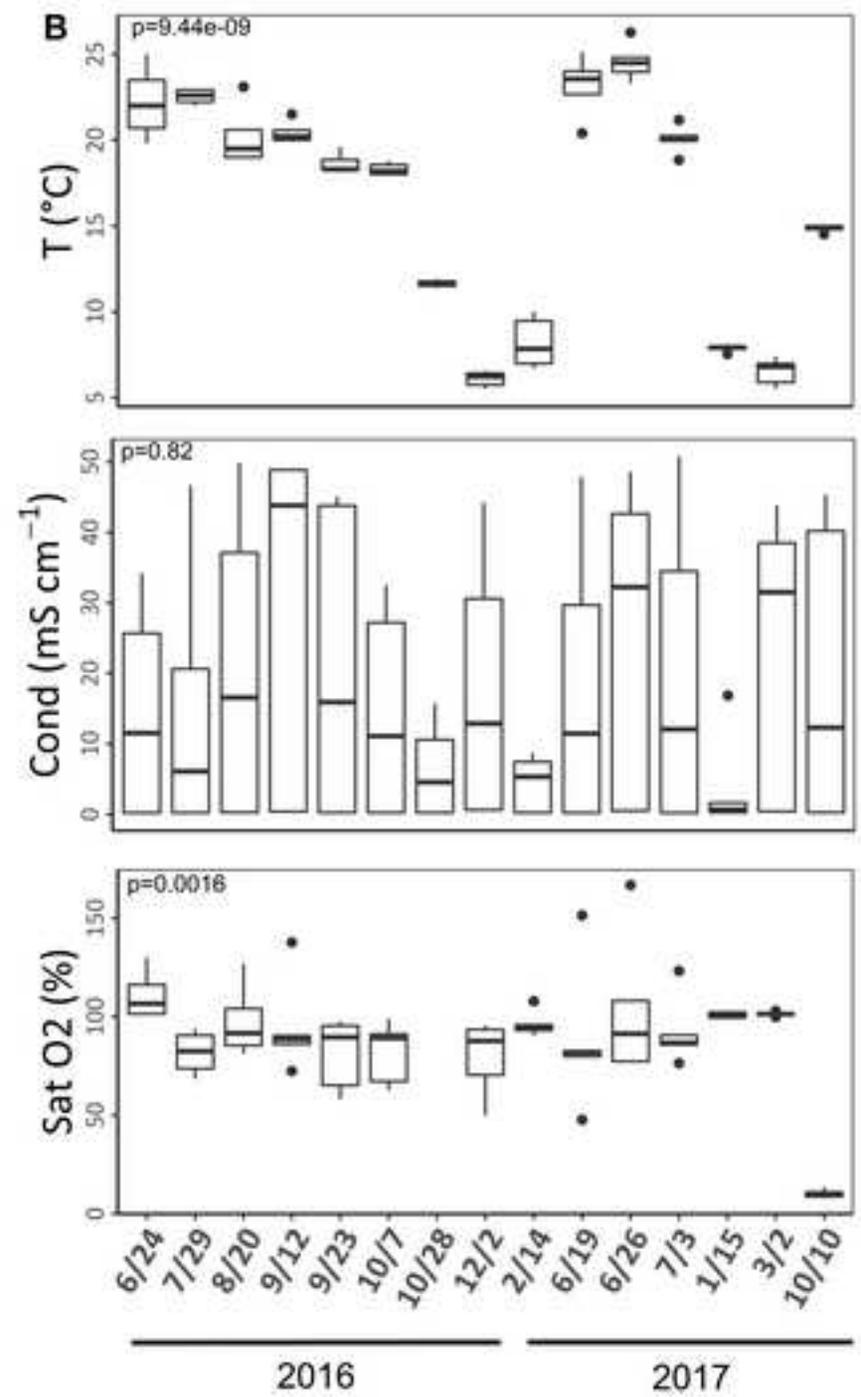
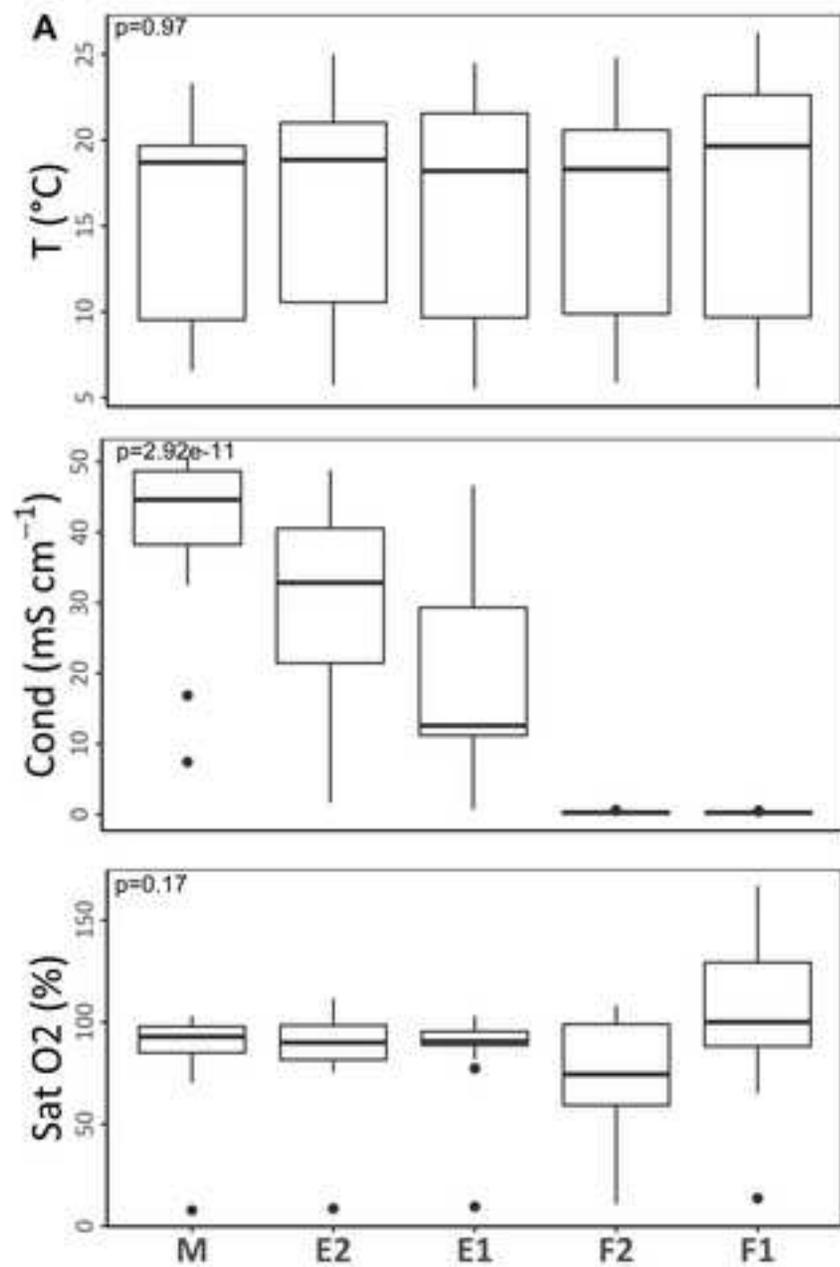


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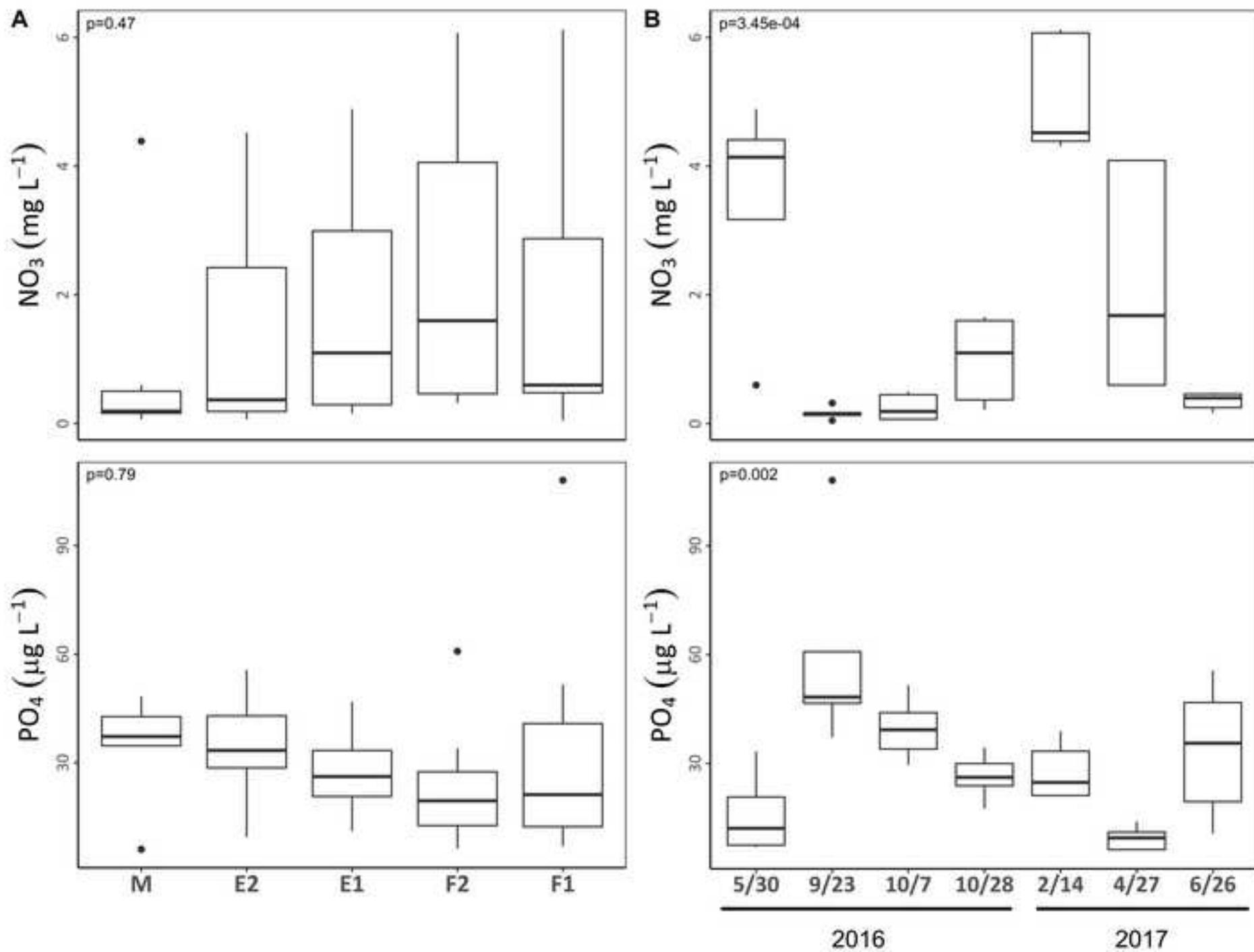






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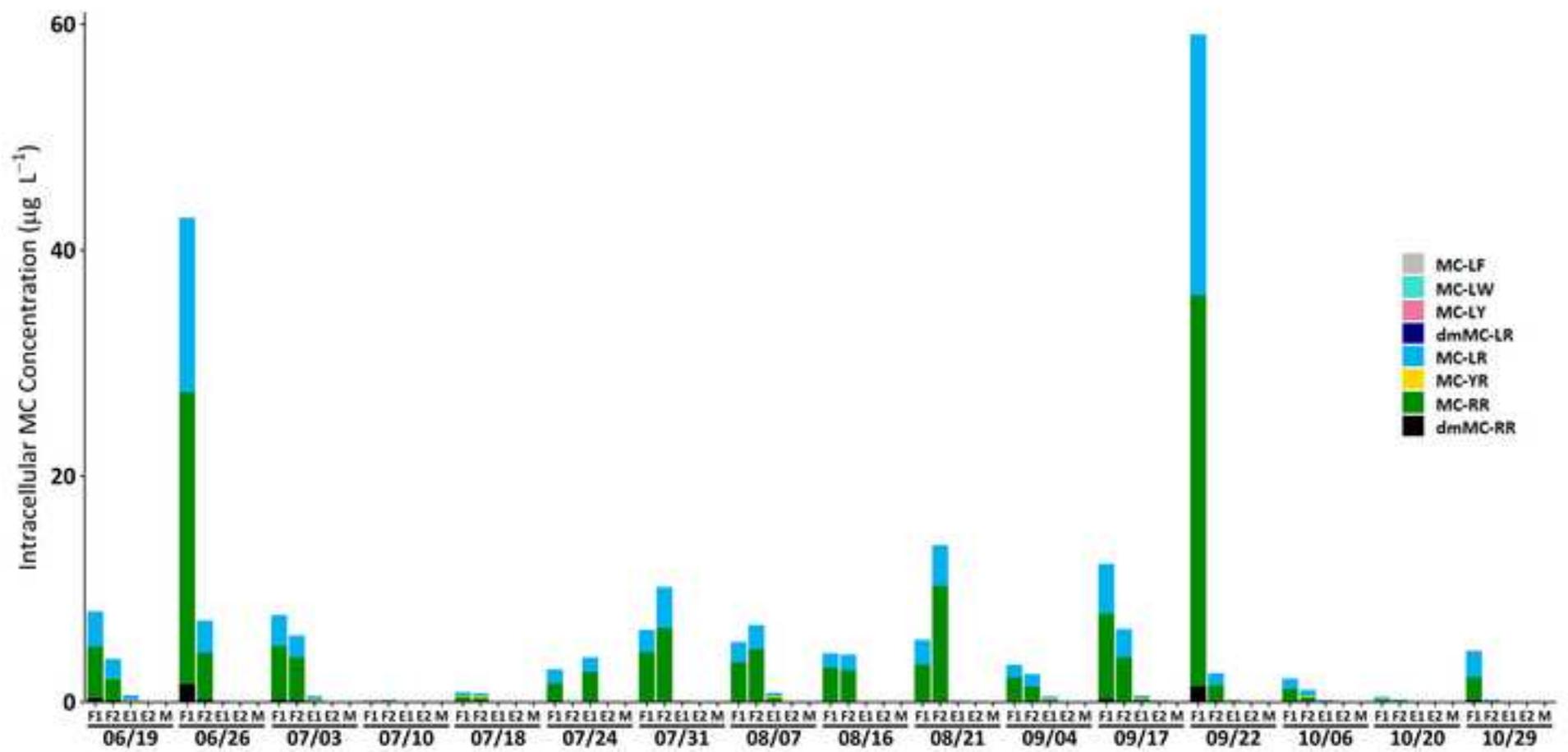
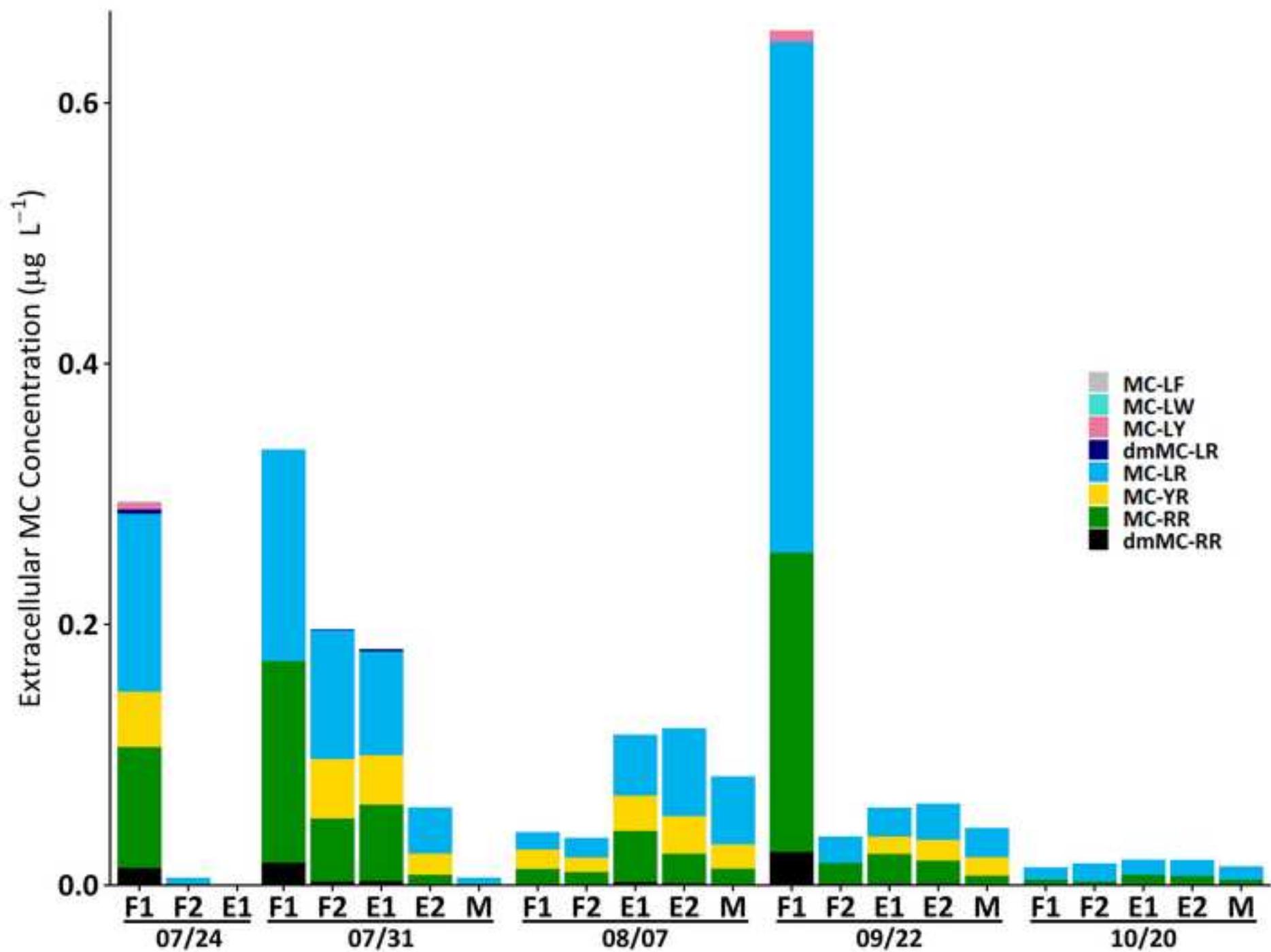
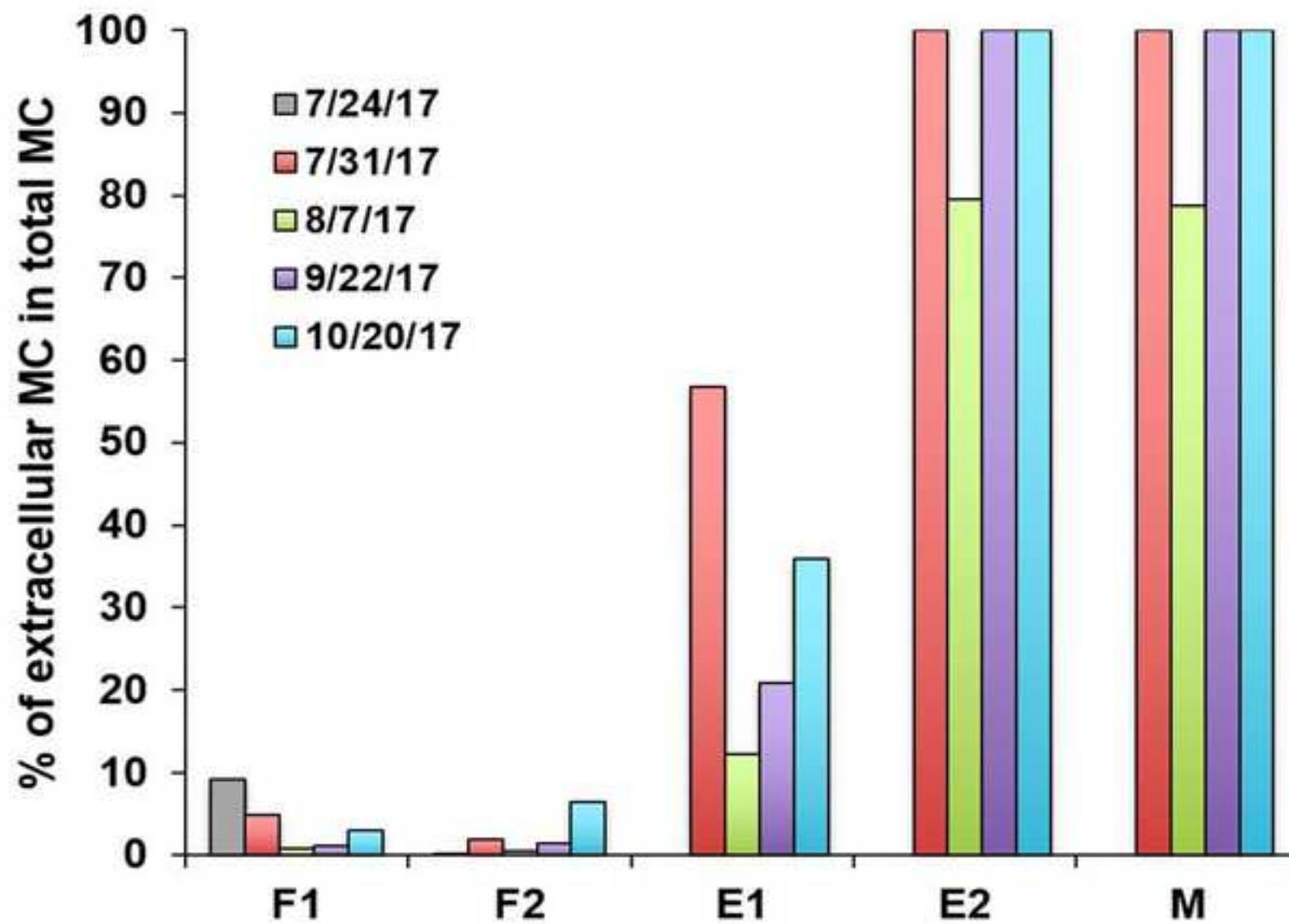


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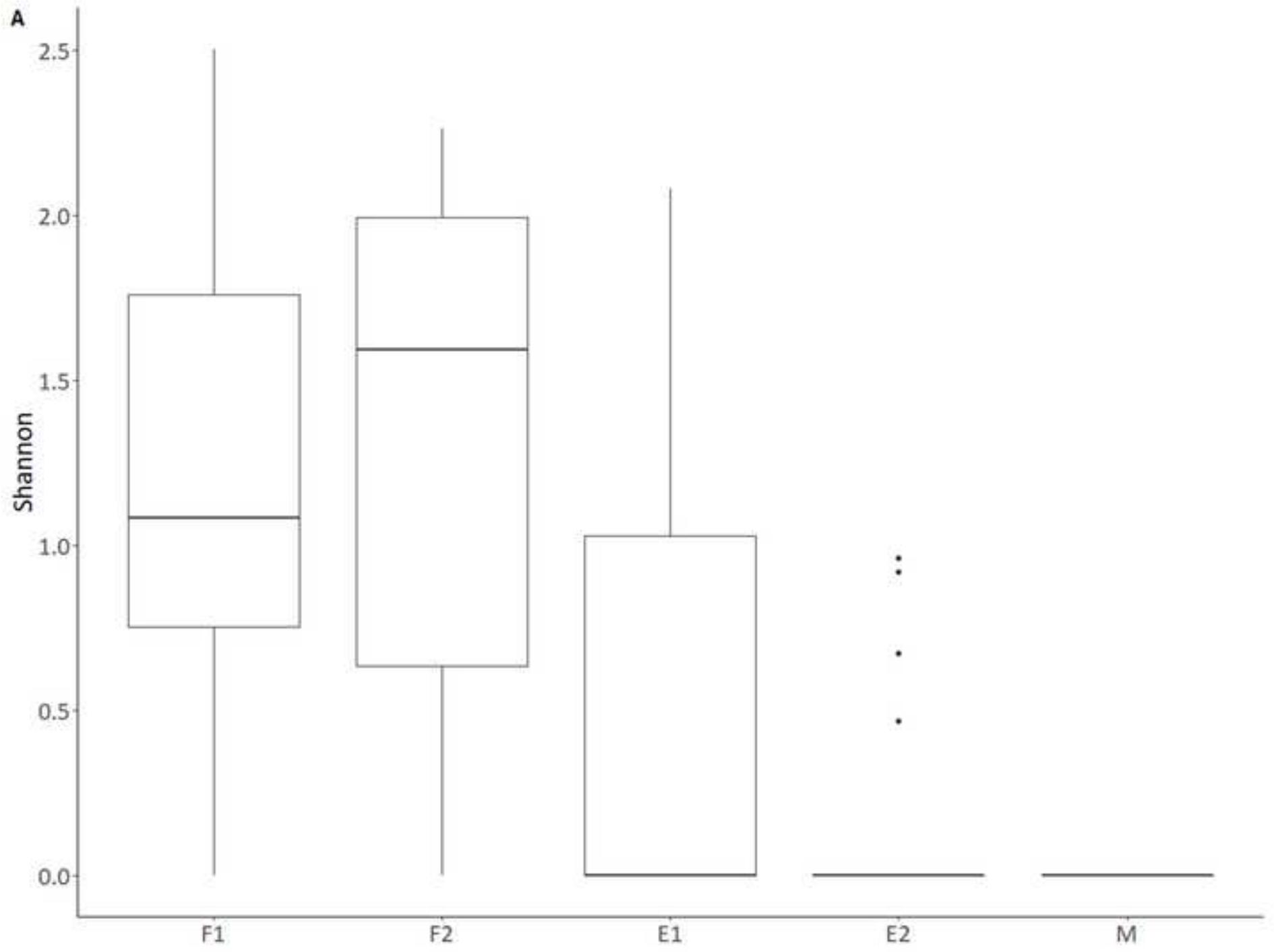


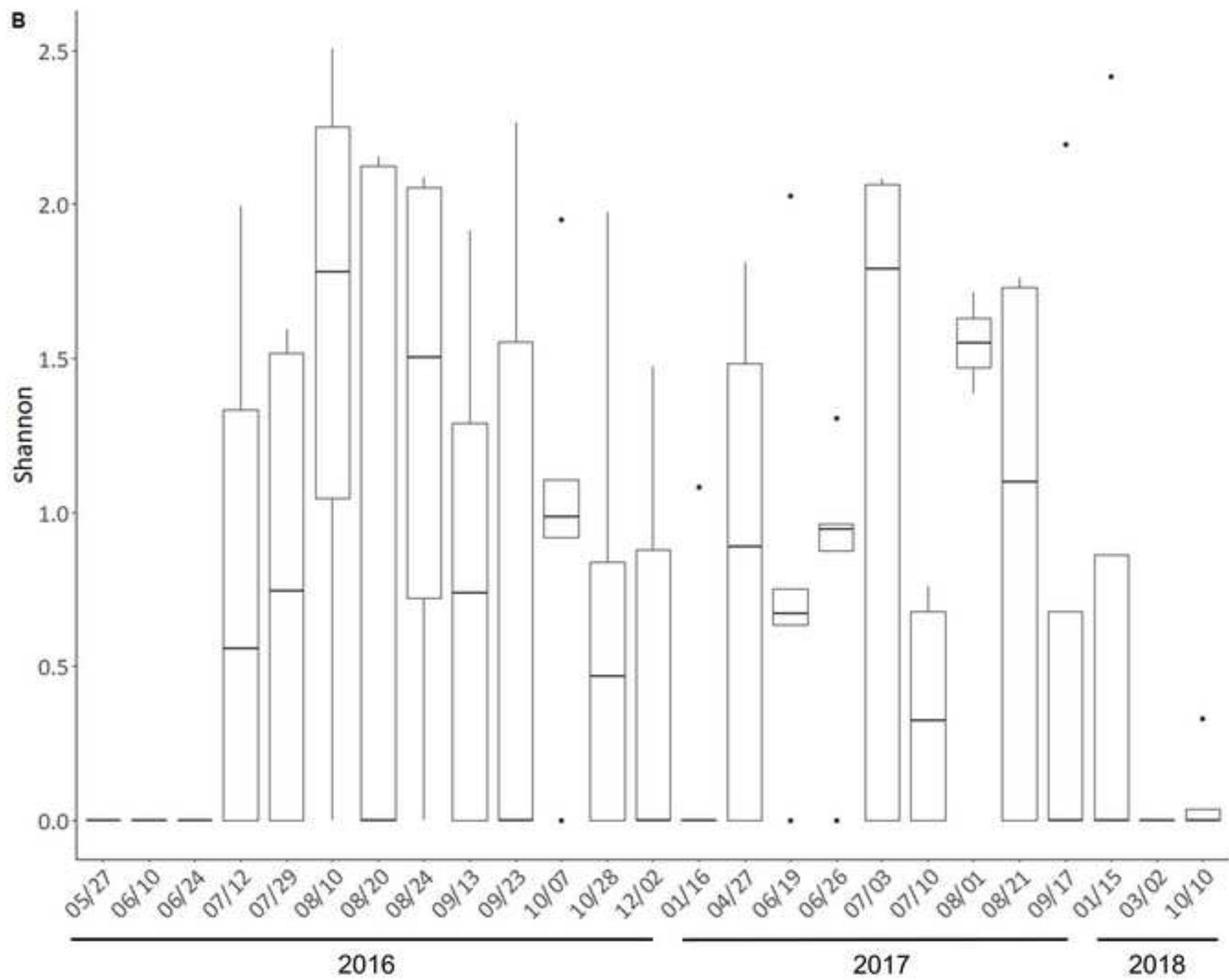
Sites	FO % intra MC (Jun – Nov)		Max intra MC µg/L
	2016	2017	
F1	100	100	60
F2	100	100	14
E1	67	87	1.15
E2	17	38	0.14
M	0	27	0.03

*Table 1: Frequency of occurrence (in %) and maximum concentrations of intracellular MC at the 5 stations during the period June to November of both sampling years*

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

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
NOD	825.5	227.0	163.2





(Axes F1 and F2: 63.91%)

