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1 ***In situ* use of bivalves and passive samplers to reveal water contamination by microcystins**
2 **along a freshwater-marine continuum in France**

3

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12

13 **Key words:** cyanotoxins, biosurvey, molluscs, solid phase adsorption toxin tracking (SPATT)

14

15

16 **Abstract**

17 Cyanobacteria are a potential threat to aquatic ecosystems and human health because of their
18 ability to produce cyanotoxins, such as microcystins (MCs). MCs are regularly monitored in
19 fresh waters, but rarely in estuarine and marine waters despite the possibility of their
20 downstream export. Over a period of two years, we monthly analysed intracellular (in
21 phytoplankton) and extracellular (dissolved in water) MCs at five stations along a river
22 continuum from a freshwater reservoir with ongoing cyanobacterial blooms to the coast of
23 Brittany, France. MCs were quantified using two integrative samplers placed at each site: solid
24 phase adsorption toxin tracking (SPATT) samplers for collecting extracellular MCs and caged
25 mussels (*Anodonta anatina* and *Mytilus edulis*) filter-feeding on MC-producing cyanobacteria.
26 The MC transfer was demonstrated each year during five months at estuarine sites and
27 sporadically at the marine outlet. SPATT samplers integrated extracellular MCs, notably at low
28 environmental concentrations (0.2 µg/L) and with the same variant profile as in water. The
29 mussel *A. anatina* highlighted the presence of MCs including at intracellular concentrations
30 below 1 µg/L. *M. edulis* more efficiently revealed the MC transfer at estuarine sites than
31 punctual water samplings. Bivalves showed the same MC variant profile as phytoplankton
32 samples, but with differential accumulation capacities between the variants and the two species.
33 Using SPATT or bivalves can give a more accurate assessment of the contamination level of a
34 freshwater-marine continuum, in which the MC transfer can be episodic. MC content in *M.*
35 *edulis* represents a potent threat to human health if considering updated French guideline values,
36 and particularly the total (free and protein-bound) MC content, highlighting the necessity to
37 include cyanotoxins in the monitoring of seafood originating from estuarine areas.

38

39

40

41 **Introduction**

42 Proliferations of freshwater cyanobacteria are a source of growing concern because of the
43 ecological and economical disturbances they create, as well as the significant impact of their
44 toxins on animal and human health (for reviews: Wood et al., 2016; Svirčev et al., 2017). The
45 most frequent cyanotoxins found in freshwater ecosystems are the MCs, among which 246
46 variants have been described (Spoof and Arnaud, 2017). MCs are mostly within cyanobacteria
47 cells (intracellular) during the cyanobacterial bloom but are released into the water as free or
48 adsorbed on particles (extracellular) when blooms collapse. MCs are mainly hepatotoxic, but
49 were also recently recognised as neurotoxic and reprotoxic (Buratti et al., 2017). Provisional
50 MC thresholds (guideline values) of 1 and 12 µg/L for lifetime and short-term drinking-water
51 respectively have been established by the World Health Organization (WHO) for the
52 widespread and most often studied MC-LR variant (WHO, 2020). The WHO also proposed a
53 provisional upper limit for recreational water of 24 µg/L and a lifetime tolerable daily intake
54 (TDI) of 40 ng/day/kg of body weight for MC-LR.

55 Most studies on cyanobacteria and cyanotoxin occurrence were conducted in freshwater
56 ecosystems, but intermittent cyanobacteria and MC transfer during a freshwater discharge into
57 the coastal environment were recently reported in California (Gibble and Kudela, 2014), Italy
58 (De Pace et al, 2014), Japan (Umehara et al, 2015), Washington state (Preece et al., 2015; 2017),
59 Korea (Kim et al., 2019), France (Bormans et al., 2019), Lithuania (Overlinge et al., 2020) and
60 Uruguay (Kruk et al., 2021). The resulting MC contamination of marine organisms such as
61 molluscs, crustaceans, fish and even sea otters, has been reported in several countries (Lehman
62 et al, 2010; Miller et al, 2010; Gibble et al, 2016; Tatters et al. 2017; Peacock et al., 2018; Kim
63 et al., 2019), and could have a large health and economic impact. However, the contamination
64 level in organisms consumed by humans may have been underestimated because only the free
65 (not protein-bound) fraction of MC accumulation is routinely quantified in tissues. After

66 ingestion by organisms and distribution in target cells, MCs either remain free or covalently
67 bind to protein phosphatases (PPases) and proteins containing cystein group (Campos and
68 Vasconcelos, 2010). The protein-bound fraction of MCs has been demonstrated to be
69 predominant in animal tissues and is slowly eliminated (Lance et al., 2010; Lepoutre et al.,
70 2020). MC-cysteine conjugates are still toxic but less so than free MCs (Kondo et al., 1992);
71 the toxicity of MCs bound to PPases has not been determined. The bioavailability and
72 bioaccessibility of protein-bound MCs, that could become free during the digestion process in
73 consumers is also unknown (Mohamed et al., 2018). In the context of increasing average global
74 temperature and periodic droughts and floods, the occurrence of cyanobacterial blooms may
75 become more frequent and intense in the future (Paerl and Paul, 2020), as may cyanotoxin
76 transfer in estuarine zones and marine coastal areas. It may therefore be useful to evaluate the
77 free and total (free plus protein-bound) MC content in seafood from estuarine zones.

78 The present study represents the second part of a project on cyanobacteria and cyanotoxin
79 transfer along a freshwater-marine continuum in Brittany, France (Bormans et al., 2019, 2020),
80 during which only MCs were found in phytoplankton and water samples, while
81 cylindrospermopsin, anatoxins, and nodularin were absent. The occurrence of MC-producing
82 cyanobacteria in water samples can be difficult to assess because of the spatial (vertical and
83 horizontal migrations) and temporal (rapid proliferation and collapse) dynamics of
84 cyanobacteria populations (Deng et al., 2016; Salmaso et al., 2017). The episodic occurrence
85 of cyanotoxins in marine waters and the effects of tidal water dilution may both influence the
86 reliability and relevance of intermittent water samplings. The use of monitoring devices that
87 integrate cyanotoxins over time may overcome this issue. Passive integrators like SPATT have
88 been developed, but their use is limited because the samplers only adsorb extracellular
89 cyanotoxins and they are subject to rapid clogging and saturation, which shortens their useful
90 life (Kudela, 2011). Bivalves, filter-feeders with high filtration rates, have been proposed as
91 tools for detecting the presence of MC-producing cyanobacteria in water (Preece et al., 2015;

92 Lauceri et al., 2017; Lepoutre et al., 2020). Bivalves accumulate MCs primarily in the digestive
93 gland, mainly after ingestion of MC-producing cyanobacteria and to a lesser extent through the
94 filtration of dissolved toxins (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Gkelis et al., 2006;
95 Camacho et al., 2021).

96 In this study, five stations along a freshwater-marine continuum were sampled monthly over
97 two years, and weekly during proliferation periods. The freshwater mussel *Anodonta anatina*
98 was caged at two upstream freshwater sites exposed to intense annual cyanobacterial blooms.
99 The marine mussel *Mytilus edulis* was caged at two estuarine sites and also sampled at a most
100 marine site. Nine MC variants (MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-YR, MC-LA,
101 MC-LY, MC-LW, MC-LF) were measured in phytoplankton biomass (intracellular), in filtered
102 water (extracellular), in SPATT samplers (extracellular adsorbed on membranes) and in tissues
103 of mussels (free accumulation fraction). Total (free plus protein-bound) MCs were also
104 determined in some mussel tissues. This study aimed at increasing knowledge about: i) the
105 effectiveness of SPATT samplers as an integrative tools of extracellular MCs, notably at low
106 concentrations and in salt water, ii) the value of freshwater and marine bivalves as bioindicators
107 of the presence of MC-producing cyanobacteria in aquatic systems compared to phytoplankton
108 samplings, iii) the potential concern for human food safety by comparing the levels of MC
109 contamination in marine bivalves with the guidelines from the WHO and French policy makers.

110

111 **2. Materials and methods**

112 2.1. Study sites

113 The study site was located in the Morbihan (Brittany, France) along a continuum of moderate
114 length (about 8 km), from a freshwater reservoir (Pen Mur) to the estuary and the marine outlet
115 (Pen Lan harbour), through the St Eloi river. The reservoir undergoes annual intense
116 cyanobacterial blooms dominated by the genus *Microcystis*, and freshwater discharges into the

117 estuary have been highlighted (Bormans et al. 2019). Field investigations were performed at
118 five stations: (1) F1 reservoir (freshwater), (2) F2 downstream river (freshwater), (3) E1
119 brackish downstream river (estuarine), (4) E2 harbour near the mouth of the river (estuarine),
120 and (5) M, the foreshore zone (marine) close to a shellfish farming area (**Fig.1**).

121

122 2.2. Caging of bivalves

123 *A. anatina* were collected at site F2 four months before starting caging, sized and placed by
124 groups of 20 at $14 \pm 2^\circ\text{C}$ in aerated tanks containing 25 L of Cristalline® water (Saint Yorre,
125 France). *M. edulis* were collected during winter 2016 at the M site, and placed by groups of 50
126 at $8 \pm 2^\circ\text{C}$ in aerated tanks containing 20 L of sea water. During the depuration period, both
127 bivalve species were fed twice a week with 3.7×10^7 cells of *Chlorella vulgaris* (Greensea,
128 Mèze, France) / individual / day. The caging of bivalves was done from May 2016 to November
129 2017. *A. anatina* were distributed per groups of 60 in 3 mm mesh polyethylene cages (40 x 40
130 x 60 cm) immersed at 1-meter depth at the F1 and F2 sites (two cages per site). *M. edulis* were
131 distributed per groups of 100 in 3 mm mesh polyethylene cages (15 x 30 x 5 cm) immersed at
132 E1 and E2 sites (three cages per site) at a depth ensuring an immersion in the freshwater flow
133 at low tide. Due to the technical impossibility of placing cages at the marine site M, *M. edulis*
134 were sampled in a wild population attached to a buoy.

135

136 2.3. Passive sampler devices (SPATT): design and deployment

137 SPATT samplers were prepared using a 68 mm embroidery frame (Singer, Nantes, France).
138 Two resins were tested, HP-20 and Strata-X, in 500 mL of fresh and salt waters with MC-YR
139 (100 ng/L), MC-RR (412 ng/L) and MC-LR (808 ng/L) under agitation at 30 rpm. Absorption
140 yield were higher with HP-20 resins compared to Strata-X resins in fresh waters and equivalent
141 in marine waters. HP-20 resin was then chosen and 3 g of resin were placed between two layers

142 of a 30 µm nylon mesh (Mougel, France), and clamped in an embroidery frame. Before
143 deployment, the SPATT samplers were soaked for 3 h in methanol to activate the resin and
144 rinsed twice with deionized water to remove methanol residues (Zendong et al., 2014). Three
145 SPATT samplers were deployed at each of the five sites from July to October 2017, with
146 sampling and renewal of samplers every ten days.

147

148 2.4. Water, bivalve and SPATT sampling and processing

149 Water and bivalve samples were obtained once a month from May 2016 to November 2017,
150 twice per month during the proliferation period, and weekly from mid-June to early July 2017
151 due to a heavy cyanobacterial bloom. At estuarine and marine sites, samplings were performed
152 within one hour of low tide to maximize the freshwater discharge and minimize tidal dilution.
153 Water samples (500 mL) were filtered using Cyclopore track-etched membranes (1 µm pore
154 size, Whatman, Maidstone, UK). The phytoplankton biomass and the filtrate were respectively
155 used for analysis of intracellular and extracellular MCs. The SPATT samplers were retrieved
156 and rinsed with seawater. Water samples for November 2016 to April 2017 were not analysed.
157 MC concentrations were measured in five pools of six digestive glands of *M. edulis*, and six
158 digestive glands of *A. anatina*. On specific dates, MC concentrations were measured in whole
159 *M. edulis* to evaluate the MC ratio in the digestive gland versus the body. After dissection, the
160 fresh tissues were washed with Milli-Q water. All samples were stored at -80°C until chemical
161 analysis.

162

163 2.5. Chemical analysis of MCs by liquid chromatography coupled to tandem mass 164 spectrometry (LC-MS/MS)

165

166 2.5.1. Analysis of MCs in water, SPATT samplers, phytoplankton and bivalves
167 (free accumulation fraction)

168 The nine MC variants (MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-YR, MC-LA, MC-LY,
169 MC-LW, MC-LF, Novakits, France) were measured in phytoplankton, water, mussels and
170 SPATT samplers. Extracellular MCs were purified by solid-phase extraction on 200 mg C18
171 cartridges (Bond Elut, Agilent). After conditioning with 10 ml of MeOH and 10 ml of Milli-Q
172 water, 500 ml of filtrate was loaded, rinsed with 4 ml of MeOH/Milli-Q water (5:95, v/v), and
173 eluted with 2 ml of MeOH. The eluate was dried under a nitrogen stream, and dissolved in 0.5
174 mL of MeOH. The phytoplankton biomass was ground in a bead mill with 250 mg of glass
175 beads (0.15-0.25 mm) in 1 mL of MeOH at 30 Hz for 30 min, and centrifuged at 15,000 g for
176 5 min at 4°C. The supernatant was kept at 4°C and another millilitre of MeOH was added to
177 the pellet to repeat the extraction. Then both supernatants were pooled together, and 400 µL
178 was filtered through a membrane filter (0.2 µm) and centrifuged 1 min at 14,000 g before
179 analysis. The concentrations of extracellular and of intracellular MCs were expressed as µg/L
180 of water sampled *in situ*. For analysis of free MCs accumulated in bivalves, 200 mg tissue
181 samples were homogenized using an Ultra-Turrax homogenizer for 30 sec at 15,000 tr/min.
182 Extraction was performed with 750 µL of 100% MeOH. Samples were ground with 250 mg of
183 glass beads (0.15-0.25 mm) at 30 Hz for 3 min, and centrifuged at 15,000 g for 5 min at 4°C.
184 The supernatant was kept at 4°C and 750 µL of 100% MeOH was added to the pellet to repeat
185 the extraction. Both supernatants were pooled together. All samples were spin-filtered through
186 0.2 µm membranes by centrifugation for 10 min at 14,000 g before LC-MS/MS analysis. Free
187 MC contents in mussel tissues were expressed as ng/g of fresh weight (FW). The SPATT
188 samplers were extracted according to Zendong et al. (2014). Briefly, HP-20 resins were rinsed
189 twice in 500 mL deionized water, transferred into empty polypropylene columns and eluted
190 drop-wise with 10 ml of MeOH (2 x 5 ml). The extracts were evaporated at 40°C under nitrogen
191 stream. The dry residue was reconstituted in 500 µL of MeOH, filtered through Nanosep MF

192 centrifugal filters 0.2 μm and transferred into LC vials. The MC concentrations in SPATT were
193 expressed as ng/g of resin/day of deployment. The instrument settings for LC-MS/MS analysis
194 of MCs in water, SPATT, cyanobacteria and bivalves, their specific transitions, as well as their
195 LOD and LOQ, are presented in supplementary material (**Table S1**). The quantification of MCs
196 was done using an external calibration curve with six concentrations of standards dissolved in
197 MeOH. Chromatograms of MC variants are shown in figure S1.

198

199 2.5.2. Analysis of total (free plus protein-bound) MCs in *A. anatina* and *M. edulis*

200 The total MC content in bivalve digestive glands was determined by quantification of the
201 erythro-3-methoxy-2-methyl-4-phenylbutyric acid (MMPB, Tokyo Chemical Industry, Japan),
202 a product of MC oxidation. The method was adapted from Neffling et al. (2010), with
203 modification of the extraction step: two oxidant concentrations, 0.1 M and 0.025 M
204 $\text{KMnO}_4/\text{INAO}_4^-$ were tested, and extractions were done using SPE cartridges (HLB 3 cc, 60
205 mg, Waters, MA, USA) versus liquid-liquid extraction with AcOEt. Based on the results, the
206 0.025 M oxidant solution and liquid-liquid extraction were chosen as most efficient. Fresh 100
207 mg tissue samples were homogenized using an Ultra-Turrax instrument and digested in
208 Sorensen's buffer (0.1 M, pH 7.5) with 20 μL of trypsin (25 g/L) at 37°C with stirring at 650
209 rpm for protein hydrolysis. The oxidation was performed with 5 mL of 0.025 M
210 $\text{KMnO}_4/\text{INAO}_4^-$ (0.1 M Sorensen's, pH 8.5) for 3 h with mild stirring at 37°C. The reaction
211 was quenched with sodium bisulfite (NaHSO_3) until the liquid became transparent, and pH was
212 adjusted to 3 with 10% sulfuric acid. The liquid-liquid extraction was performed with 5 mL of
213 AcOEt, and samples were centrifuged 5 min at 2500 g and 4° C. The procedure was repeated
214 and both supernatants were pooled, dried under nitrogen at 35°C, resuspended in 1 mL of 40%
215 MeOH and filtered (0.2 μm). The quantification of MMPB was performed by spiking blank
216 samples with 1, 5, 10, 25, 50, 75, 100 ng of MMPB to generate an external calibration curve.
217 The percent recovery of total MCs in mussel tissues was determined by spiking blank tissues

218 of each species with MC-LR (10.1 µg/mL) before oxidation, with MMPB (1 µg/mL) after
219 oxidation and before liquid-liquid extraction, and with MMPB (1 µg/mL) after extraction and
220 drying. The recoveries were 26% from oxidation, 81% from extraction, and 89% from
221 ionisation, with a global percent recovery of 19%. Total MC concentrations in the whole
222 mussels (ng/g FW) were calculated based on the global percent recovery of the method and on
223 the proportions of MCs in the digestive glands versus in the whole mussels established on *M.*
224 *edulis* during this study (93%, data not shown) and on *A. anatina* (89%, Lepoutre et al., 2020).
225 Instrumental conditions for LC-MS/MS analysis of the MMPB and its specific transition are
226 presented in supplementary material (**Table S1**).

227

228 2.6. Statistics

229 Statistical analyses were performed with Statistica (Version 8.0.360.0, Statsoft, Tulsa, USA,
230 2007). The normality was verified by Shapiro-Wilk test, and the homogeneity of variances was
231 studied with a Levene test. The comparison of two independent samples was done with Mann-
232 Whitney tests and the comparison of multiple independent samples was done with Kruskal-
233 Wallis tests. A chi-squared test was used to compare percentages of each MC variant to the
234 total MC concentration in water samples, SPATT samplers, phytoplankton, and mussel tissues.
235 The significance threshold was set at $p < 0.05$.

236

237 3. Results and Discussion

238

239 3.1. Efficiency of SPATT samplers for adsorbing extracellular MCs from water

240 Extracellular MCs were found at low concentrations in water from July to October 2017, with
241 a maximum of 0.65 µg/L in the freshwater reservoir (**Fig. 2**). The MC concentrations extracted

242 from SPATT membranes (expressed as $\mu\text{g MCs/g}$ of resin/day) show that SPATT samplers
243 continuously revealed the presence of extracellular MCs, including for low environmental
244 concentrations ($0.12 \mu\text{g/L}$) and in saltwater. The use of passive SPATT samplers allowed
245 integration of extracellular MCs and to follow their transfer to the foreshore. The qualitative
246 extracellular MC profile at freshwater sites (**Fig. 3A**) showed a dominance of MC-LR (mean
247 percentage of MC concentration, %var, of 51-62% at F1 and F2 in water, and 47-49% on
248 SPATT samplers), followed by MC-RR (%var of 26-35% at F1 and F2 in water, and 36-42%
249 on SPATT samplers), and MC-YR (%var of 10% at F1 and F2 in water, and 7-11% on SPATT
250 samplers). The MC variant profiles were similar between filtered waters and SPATT samplers,
251 showing that SPATT membranes adsorbed all MC variants equally. This was also true at
252 estuarine and marine sites, where the proportions of MC variants were of the same rank-order
253 on SPATT samplers as with filtered water: MC-LR, MC-RR and MC-YR by order. The %var
254 for extracellular MCs was stable along the salinity gradient.

255 SPATT can represent an efficient warning tool highlighting low water concentrations of
256 extracellular MCs that may occur at early phytoplanktonic bloom development, in case of
257 benthic cyanobacteria, or after bloom collapse. However, these passive samplers only adsorb
258 and cumulate extracellular MCs without release after an episodic and low contamination event,
259 and do not provide a quantitative representation of environmental concentrations. Moreover,
260 SPATT are not usable for intracellular MCs (in cyanobacteria) that can be predominant during
261 the cyanobacterial blooms. We therefore used two filter-feeding mussel species as an
262 integrating tool of intracellular MCs and of their variation in time through their ingestion of
263 cyanobacteria and dynamic of MC accumulation and elimination.

264

265 3.2. Ability of *A. anatina* to reveal the presence of MC producers at freshwater sites

266 3.2.1. Dynamics of MCs in freshwater phytoplankton and bivalves

267 At freshwater sites, cyanobacteria dominated the phytoplankton communities in summer and
268 autumn of 2016 and 2017 (density >10,000 cells/mL with a maximum of 2 million cells/mL),
269 with 27 species, among which species of the *Microcystis* genus were most abundant (Bormans
270 et al. 2019). The concentrations of MCs in phytoplankton (intracellular) and in digestive gland
271 tissues (free MCs) from caged *A. anatina* are presented in **Fig. 4**, with a selection of pertinent
272 dates between August 2016 and October 2017. As intracellular MCs were not measured
273 between November 2016 and May 2017, we considered two different periods to evaluate the
274 frequency of occurrence (FO) of MCs in phytoplankton and in bivalves (**Table 1**). At the F1
275 site, intracellular MCs were measured from August to October 2016, with a maximum of 37
276 µg/L, and from June to October 2017, with a maximum of 57 µg/L. During these two periods,
277 the bivalves accumulated free MCs, with a FO of 100% and a maximum concentration of 3764
278 ng/g FW in June 2017 (**Fig. 4, Table 1**). At the F2 site, intracellular MCs were quantified from
279 August to October 2016, with a maximum of 5.9 µg/L, and from June to October 2017, with a
280 maximum of 14 µg/L. During these two periods, the FO of MCs in bivalves was 100%, with a
281 maximum of 3782 ng/g FW in September 2016 (**Fig. 4, Table 1**). These results are in line with
282 previous findings demonstrating that *Anodonta sp.* ingest either colonial (i.e., *Microcystis*) or
283 filamentous (i.e., *Planktothrix*) cyanobacteria, and subsequently accumulate MCs (Gkelis et al.,
284 2006; Dionisio Pires et al., 2007; Barda et al., 2015; Bontes et al., 2016; Lauceri et al., 2017;
285 Lepoutre et al., 2020). In this study, MC accumulation levels varied depending on which genus
286 dominated the phytoplankton community. The MC levels in mussels were lower when
287 *Planktothrix agardhii* dominated in September 2016 (with 35 µg/L intracellular MCs), and
288 higher when *M. viridis* and *M. aeruginosa* dominated in October 2016 and June 2017 (with 37
289 and 42 µg/L intracellular MCs, respectively), as also reported by Barda et al. (2015).
290 Nevertheless, *A. anatina* accumulated large amounts of MCs when the environmental
291 contamination was high. A study in the Gulf of Riga, Latvia, showed that *A. anatina*
292 accumulated the greatest amount of MCs (from 0.5 to 88 µg/g DW) among various organisms

293 including fish, gastropods and other bivalves (Barda et al., 2015). Our previous laboratory
294 investigations with *A. anatina* and the freshwater mussel *Dreissena polymorpha* also
295 demonstrated this result, as well as the ability of *A. anatina* to ingest cyanobacteria, accumulate
296 MCs and reveal an environmental contamination as low as 1 µg/L (Lepoutre et al., 2020). This
297 ability of *A. anatina* to indicate a low level of MC contamination was demonstrated in the
298 present *in situ* study in early 2017. Bivalves continuously showed MCs in their digestive glands
299 during the winter period, except in December 2016, suggesting an elimination of MCs
300 previously accumulated, and a *de novo* accumulation in the first month of 2017 when a low
301 cyanobacteria density was observed (1000 cells/mL) (Bormans et al., 2019). *A. anatina* then
302 represents a sensitive bioindicator for environmental monitoring, which integrates intracellular
303 MCs over time between sampling dates of phytoplankton, including low and high
304 contamination levels.

305

306 3.2.2. MC variant profiles in phytoplankton and bivalves of freshwater sites

307 Among the nine MC variants, only MC-LA was never detected in the freshwater sites. The five
308 lowest occurring variants, dmMC-RR, dmMC-LR, MC-LY, MC-LW, MC-LF, represented 1%
309 or less of the MC concentration in phytoplankton and less than 5% in mussels (**Fig. 3B**). At
310 freshwater sites, the qualitative MC profiles in phytoplankton and mussels differed slightly
311 from those of filtered water and SPATT samplers. The three dominant variants in
312 cyanobacterial samples were MC-RR (%_{var} from 51 to 55%), MC-LR (%_{var} from 39 to 43%),
313 and MC-YR (%_{var} 4%), as already reported (Vasas et al. 2010; Kim et al., 2021). The same MC
314 profile was observed in mussels at both sites (%_{var} from 64 to 80% for MC-RR, from 10 to
315 19% for MC-LR and from 5 to 7% for MC-YR), but with a significant (chi-squared, $p < 0.05$)
316 increase for MC-RR or decrease for MC-LR in mussels compared to phytoplankton (**Fig. 3B**).
317 This suggests that *A. anatina* accumulated a higher proportion of MC-RR and a lower

318 proportion of MC-LR in their tissues than the amount available in their food, probably due to
319 differential absorption/distribution or metabolization. In contrast, Gupta et al. (2003) reported
320 that MC-LR, MC-YR and MC-RR were highly toxic to mice but that MC-LR had the most
321 severe effects. Detoxification of MC-RR is more efficient than MC-LR in rodents, but the
322 differences are much smaller in human samples (Buratti and Testai 2015). The different
323 hydrophilicities of MC variants may influence their respective bioavailability and propensity
324 for bioaccumulation (McCord et al., 2018). To date, the regulatory guidelines are based on data
325 on MC-LR toxicity because of a lack of toxicological data concerning other variants for which
326 no standards were available. As the amount of purified MC standards has increased recently,
327 differences in toxicokinetics among the most common MC variants have to be investigated.

328

329 3.3. Ability of *M. edulis* to reflect MC transfer to estuarine sites

330 3.3.1. Dynamics of MCs in phytoplankton and bivalves at estuarine sites

331 The cyanobacteria transfer to the estuary was observed with a progressive decrease in biomass,
332 frequency of occurrence, and species diversity (Bormans et al., 2019). At the first estuarine site,
333 intracellular MCs were quantified from August to October 2016 and from June to October 2017,
334 with a maximum concentration of 5.9 µg/L and a FO of 86%. During these two periods, the
335 bivalves accumulated MCs with a higher FO (100%) than in phytoplankton samples. Maximum
336 concentrations in *M. edulis* at E1 were 1482 ng/g FW in September 2016 and 447 ng/g FW in
337 September 2017 (**Fig. 4, Table 1**). At the harbour site E2, intracellular MCs were quantified
338 only occasionally in phytoplankton with FO from 31 to 42%, and a maximum concentration of
339 0.07 µg/L. The FOs of MCs in bivalves were higher than in phytoplankton samples, 100% and
340 50% in 2016 and 2017, respectively, with maximum MC content of 544 ng/g FW in October
341 2016 and 166 ng/g FW in June 2017 (**Fig. 4, Table 1**). Finally, at the marine site M, MCs were
342 not detected between August and October 2016, and in only 14% of phytoplankton samples

343 between June and October 2017, with a maximum concentration of 0.03 µg/L. During these
344 two periods, the FOs of free MCs in mussel tissues were 0 and 15% (respectively for 2016 and
345 2017 periods) with a maximum MC concentration of 22.6 ng/g FW.

346 We observed a gradual decrease in MC occurrence and accumulation levels from upstream to
347 downstream, probably resulting from the sequential filtration of cyanobacteria flooding from
348 upstream by *M. edulis*, according to the daily rhythm of the tides. Nevertheless, the FO of MCs
349 in *M. edulis* was higher than in phytoplankton samples, demonstrating their ability to integrate
350 environmental MCs between two water samplings, and to mark their transfer from freshwater
351 to estuarine and marine sites. *M. edulis* and *M. trossulus* have already demonstrated their ability
352 to reveal an environmental MC contamination more efficiently than intermittent water
353 samplings (Pobel et al., 2011; Preece et al., 2015).

354 No quantification of intracellular MCs was performed from November 2016 to April 2017 on
355 phytoplankton samples, but blue mussels still showed MC content (mean of 37 ± 15 ng/g, $47 \pm$
356 29 ng/g, and 19 ng/g at the E1, E2 and M sites respectively) in their tissues during the winter
357 period. At E1, low densities (around 100 cell/mL) of cyanobacteria were routinely detected,
358 with a dominance of *M. viridis* (November), *M. aeruginosa* (January), *P. agardhii* (February)
359 and *Aphanizomenon sp* (April) (Bormans et al., 2019). Similarly, at E2, a cyanobacterial density
360 of 80 cell/mL was observed in January. At the marine site, no cyanobacteria were detected in
361 water samplings during winter, but *M. edulis* sporadically accumulated MCs (after a period
362 without MCs in its tissues) demonstrating its sensitivity as a bioindicator of low environmental
363 contamination levels.

364

365 3.3.2. MC variant profiles in phytoplankton and bivalves of estuarine sites

366 MC-LF and MC-LA were not detected at the estuarine sites, in contrast with what was reported
367 in the Curonian Lagoon, Southern Baltic Sea (Overlingè et al., 2020) and in the freshwater-

368 marine continuum of Puget Sound, Washington (Preece et al., 2015). The variants dmMC-LR,
369 MC-LY, and MC-LW, represented < 1% of the MC concentration in phytoplankton and mussel
370 samples at E1 and completely disappeared at E2 and M (**Fig. 3B**). The rank-order of the three
371 dominant MC variants in phytoplankton samples was slightly changed along the salinity
372 gradient: in order MC-LR (%_{var} from 53 to 56%), MC-RR (%_{var} from 29 to 38%), and MC-YR
373 (%_{var} from 5 to 16%) at E1 and E2 sites, and MC-RR (%_{var} 67%), MC-LR (%_{var} 32%) and
374 dmMC-RR (%_{var} 1.62%) at site M. The mean %_{var} of MC-RR in phytoplankton increased along
375 the salinity gradient from E1 to M, whereas the mean %_{var} of MC-YR decreased up to total
376 disappearance from sample. These changes in variant profiles can be attributed to the selection
377 of cyanobacteria species and strains along the salinity gradient (Bormans et al., 2019). The %_{var}
378 of MC-YR was similar between phytoplankton and mussel tissues, and decreased in mussel
379 tissues along the salinity gradient from 13% at E1 to 0% at M. The MC-LR variant showed a
380 similar and stable %_{var} between mussel and phytoplankton samples at the three estuarine sites,
381 suggesting that *M. edulis* accumulated MC-LR and MC-YR in proportions similar to what they
382 ingest. This observation is in contradiction to the lower MC-LR ratio observed in mussel tissues
383 compared to cyanobacteria at freshwater sites of this study and in the laboratory exposure of *M.*
384 *edulis* to *M. aeruginosa* (Camacho et al., 2021). While the MC-RR variant occurred more
385 frequently in tissues of *A. anatina* than in phytoplankton at freshwater sites, the contrary was
386 observed at estuarine sites where the %_{var} of MC-RR decreased in mussels along the salinity
387 gradients (from 42% at E1 to 6% at M) despite a concomitant increase in phytoplankton (from
388 28% at E1 to 66% at M). This suggests either that *M. edulis* accumulates less MC-RR as the
389 salinity increases, as a result of changes in metabolic processes, or also filter MCs dissolved in
390 water (extracellular) or adsorbed on organic or inorganic particles. However, we did not observe
391 a change in the proportion of extracellular MC-RR along the salinity gradient. Moreover, the
392 proportion of MC-YR was similar between phytoplankton and mussel tissues, both decreasing
393 along the salinity gradient, and was stable in water and on SPATT samplers (extracellular)

394 along the freshwater-marine continuum. This reinforces the hypothesis that contamination of
395 mussels with MCs occurs mainly through the filtration of cyanobacteria flowing downstream
396 with differential accumulation kinetics between variants, probably influenced by environmental
397 parameters such as the salinity; a hypothesis that should be further assessed. Similarly, Kim et
398 al. (2019; 2021) showed that the concentration of MC-RR was lower than MC-LR and MC-YR
399 in clams, worms, crabs, and fish from the Geum river estuary in Korea. Interestingly, in the
400 present study, marine mussels accumulated less MC-RR but more dmMC-RR than was
401 available in food. The proportion of dmMC-RR significantly increased in mussels (from 3 to
402 50%) when the salinity increased, despite little change (1 to 2%) in phytoplankton. One could
403 hypothesize metabolism of MC-RR, abundant in phytoplankton, to dmMC-RR, in mussel
404 tissues, but this is unlikely. Lastly, *M. edulis* may also accumulate MCs from sediment, which
405 could have a different MC variant profile than the one in cyanobacteria flowing from upstream.
406 This could explain the presence of extracellular MC-YR at estuarine and marine sites, with
407 lower levels of intracellular MC-YR. A recent study reported that sediments from a river delta
408 contained MCs, even during periods that were not typical proliferation months (Bolotaolo et
409 al., 2020). These MCs may originate from planktonic cyanobacteria sinking onto the sediments
410 or from benthic MC-producing cyanobacteria. In a joint survey, we reported the presence of
411 intact colonies of *Microcystis* with *mcyB* genes and of intracellular MCs in the sediment at the
412 E2 site (harbour) (Bormans et al., 2020). MCs in sediment may also originate from extracellular
413 MCs contained in or adsorbed onto particles that are periodically released into the water during
414 a rising tide (Henaio et al., 2020). The periodic adsorption and release of different MC variants
415 from sediment probably varies according to their respective partitioning coefficient, which is
416 influenced by water pH (McCord et al., 2018).

417

418 3.4. Free and total MC accumulation in *M. edulis* and risk for consumers

419 The potent risk for human was evaluated using data on MC accumulation in *M. edulis* sampled
420 at the harbour and marine sites where mussels collection for human consumption is realistic.
421 Mean free MC contents in whole *M. edulis* (15.7 ± 6.8 ng/g FW at E2, 1.9 ± 0.8 ng/g FW at M)
422 were in agreement with values (6.5 ng/g) reported for *M. trossulus* by Preece et al. (2015) but
423 lower than the ones reported for *M. galloprovincialis* from the Adriatic Sea, Italy (1.7-256 ng/g)
424 and from the Amvrakikos Gulf, Greece (45-142 ng/g) (De Pace et al., 2014 ; Vareli et al., 2012).
425 We calculated the safe MC threshold concentration in mussels based on the WHO TDI of 40
426 ng MCs/day/kg of body weight, a standard body weight of 70 kg, and a mean daily intake of
427 fresh shellfish for the population of 27 and 77 g/day for average and high consumers,
428 respectively (ANSES, 2017; Rodríguez-Hernández et al., 2019). The threshold MC
429 concentrations in mussels were 103 and 36 ng/g FW for average and high consumers,
430 respectively. At the harbour site E2, concentrations of free MCs in *M. edulis* only exceeded the
431 threshold for high consumers on two occasions in October and December 2016 (**Fig. 5**), and
432 were therefore not sufficient to provoke adverse human health effects with respect to the
433 provisional WHO TDI. However, the French Agency for Food, Environmental and
434 Occupational Health & Safety (ANSES) recently revised the sub-chronic exposure criteria
435 value based on a study of the reprotoxic effects of MC-LR on rodents (Chen et al., 2011), and
436 proposed a reference value of 1 ng MCs/day/kg BW (ANSES, 2020). Based on this updated
437 value, the threshold MC concentration in mussels for average and high consumers (2.6 and 0.9
438 ng/g FW) would have been exceeded during 6 and 8 months, respectively, in mussels from the
439 harbour site from September 2016 to September 2017 (**Fig. 5**). Mussels from the marine site
440 close to farming areas exceeded the threshold MC concentration for high consumers on two
441 dates. The consumption of mussels from the harbour over a period of several weeks would be
442 sufficient to expose consumers to potent reprotoxic effects described in rodents (decreased
443 sperm count and mobility, increase in sperm anomalies). However, when consumption exceeds
444 the recommendations, the potent sub-chronic effects are unlikely to be diagnosed because of a

445 lack of specific diagnostic criteria and of knowledge of the risk by health practitioners. These
446 poisonings are likely to occur during summer and autumn from mussels farmed or collected
447 close to the tideline at sites receiving flows of fresh waters that experience cyanobacterial
448 blooms. Mussel consumers might also be co-exposed to other freshwater cyanotoxins and to
449 marine phycotoxins such as domoic acid and brevetoxins (Peacock et al., 2018; Metcalf et al.,
450 2020).

451 Previous studies demonstrated that from 60 to 90% of total (free plus protein-bound) MCs exist
452 in protein-bound form in mollusc tissues, and that protein-bound MCs remained longer in
453 tissues than the free MCs (Lance et al., 2010; Pham et al., 2015; Lepoutre et al., 2020). At the
454 freshwater sites, total MC content in *A. anatina* was significantly higher than free MC (Mann-
455 Whitney Test, $p < 0.01$), but the FO of positive samples was similar (**Fig. 6**). At estuarine and
456 marine sites, both the total MC contents in mussels and the FO of positive samples were higher
457 than for free MCs (Mann-Whitney Test, $p < 0.01$). Measuring the total MCs allowed us to
458 highlight an accumulation at the marine site for the three investigation dates at which no free
459 MCs were quantified. This suggests that the frequency of MCs in mussels at marine sites could
460 have been far higher than 14% if we had quantified total MCs at every sampling date. If
461 considering total MC content, the threshold MC concentration in mussels for average and high
462 consumers based on WHO and French provisional guideline values would have been exceeded
463 at each sampling date. However, the toxicity of protein-bound MCs for consumers has not been
464 investigated so far. If MCs bound to proteins remain toxic, become free through release by
465 proteolytic enzymes, or are metabolized as a toxic compound during the digestive process, then
466 measuring only free MCs in seafood may underestimate the risk for consumers. While awaiting
467 data on the bioavailability, bioaccessibility, and toxicity of total MCs, their analysis in seafood
468 may improve available data for future mussel consumption advisories.

469

470 **4. Conclusions**

471 Our study suggests that the bivalves *A. anatina* and *M. edulis* represent useful integrative tools
472 for monitoring the contamination of fresh, estuarine and marine waters by MCs. While SPATT
473 only collect extracellular MCs and are passive cumulative samplers, they remain a
474 complementary tool to bivalve especially to highlight low MC concentrations. Globally, the
475 qualitative profiles of MC variants were similar between phytoplankton and mussels, and
476 between filtered waters and SPATT samplers, with a dominance of MC-LR, -RR and -YR, and
477 with some differences in variant accumulation capacities by mussels. Marine mussels were
478 better indicators of MC transfer from fresh to estuarine waters than were monthly water
479 samplings. Compared to intermittent water samplings, using SPATT or bivalves could give a
480 fairer view of the contamination level of a freshwater-marine continuum, in which the
481 downstream transfer of cyanobacteria and cyanotoxins can be episodic. This study is the first
482 to report the contamination of marine mussels *M. edulis* by cyanotoxins at a French estuarine
483 site. Based on the updated French sanitary guideline values, the level of free MCs in *M. edulis*
484 tissues may present a potent risk for people who regularly consume mussels. The level of total
485 (free and protein-bound) MCs in mussel tissues may be of greater concern, but the use of total
486 MC content in health risk assessment is still being debated as no data on their toxicity are
487 available. Our results demonstrate the importance of including both free and total MCs in
488 national monitoring programs for marine food originating from estuarine zones, and for health
489 practitioners to improve reporting of cases of cyanotoxin poisoning and related symptoms.

490

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496

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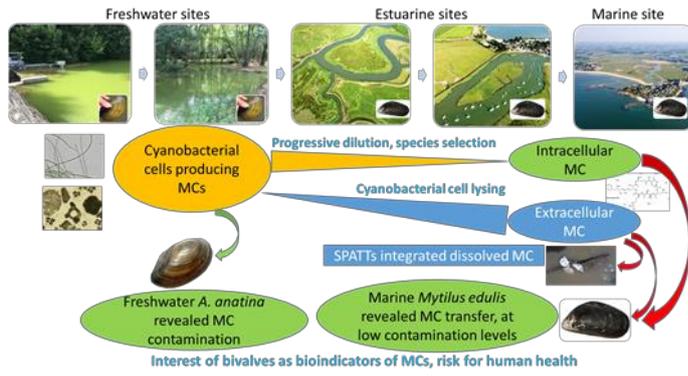


Table 1: Frequency of occurrence (FO in %) and mean + SD positive concentrations of MCs in phytoplankton biomass (intracellular) and in tissues of freshwater and marine bivalves (free MCs = are not protein-bound), from August to October 2016 and from June to October 2017. The FO and mean + SD concentrations of intracellular MCs (in phytoplankton) are indicated in brackets.

| Sites | FO (%) MCs in bivalves (FO MCs in phytoplankton) | | Mean+SD positive free MCs concentration (ng/g FW) in bivalves | |
|-----------------------------|---|------------------|--|----------------|
| | 2016 | 2017 | 2016 | 2017 |
| | F1 <i>A. anatina</i> | 100 (100) | 100 (100) | 4.1 ± 1.8 |
| F2 <i>A. anatina</i> | 100 (100) | 100 (100) | 946.9 ± 644.9 | 1002.7 ± 331.6 |
| E1 <i>M. edulis</i> | 100 (86) | 100 (87) | 996,0 ± 336.9 | 181.1 ± 60.6 |
| E2 <i>M. edulis</i> | 100 (42) | 50 (31) | 223.1 ± 139.5 | 93.1 ± 33.9 |
| M <i>M. edulis</i> | 0 (0) | 15 (14) | 0 | 22.6 |

Figure 1. Map of the freshwater (F1, F2), estuarine (E1, E2) and marine (M) sites, with the two caged bivalves species (*A. anatina* in F1 and F2, and *M. edulis* in E1, E2 and M), during the two-year investigation period.

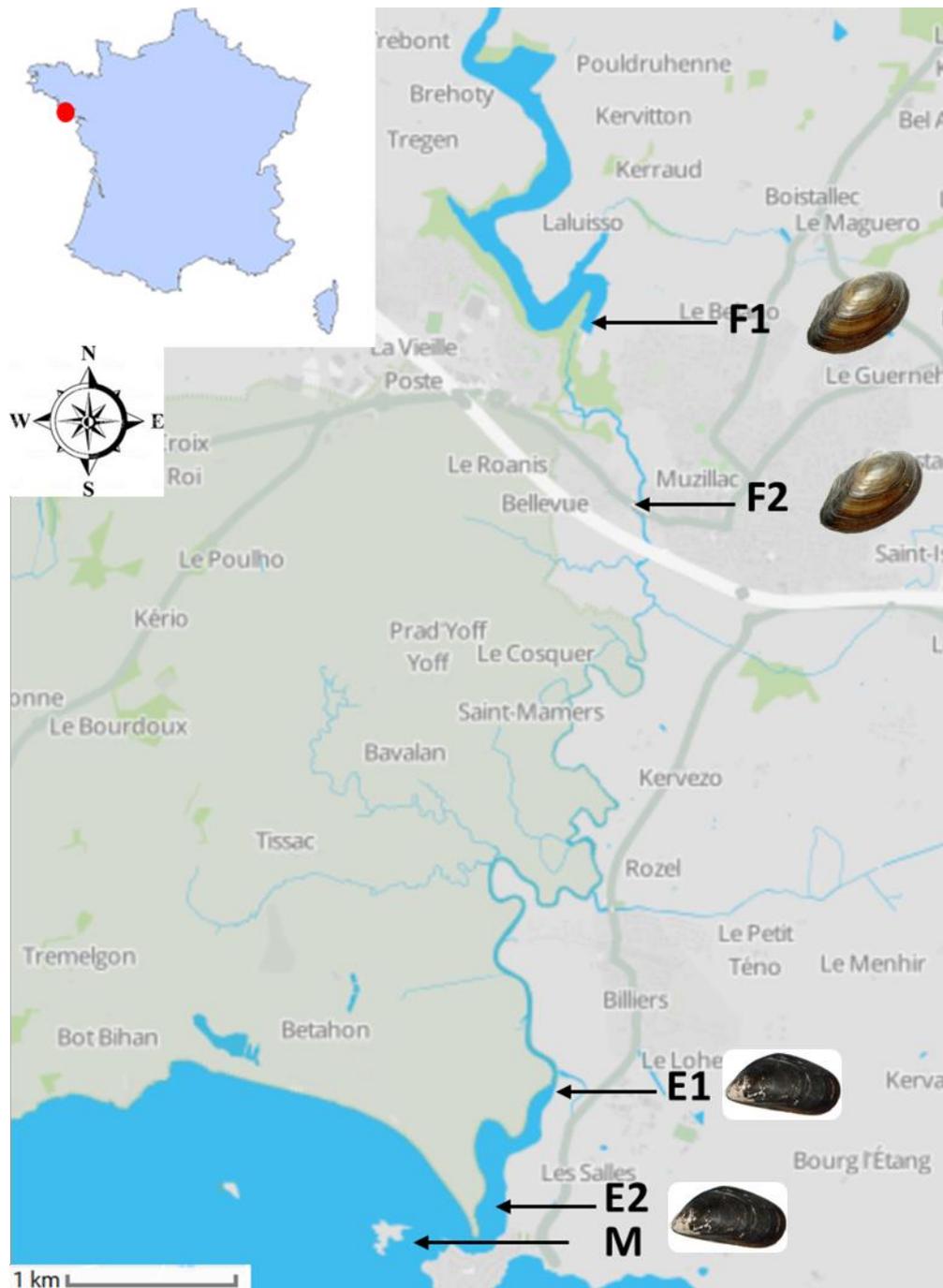


Figure 2. Concentration of MCs (sum of the nine MC variants dmMC-RR, MC-RR, MC-YR, MC-LR, dmMC-LR, MC-LA, MC-LY, MC-LW, MC-LF) measured in filtered water (extracellular MCs in $\mu\text{g/L}$, bar chart) and in SPATT samplers (amount of MCs adsorbed in the membranes during the deployment expressed in ng/g of resin/day, areas), from July to October 2017 at the freshwater (F1, F2), estuarine (E1, E2) and marine (M) sites. ND: Not Determined.

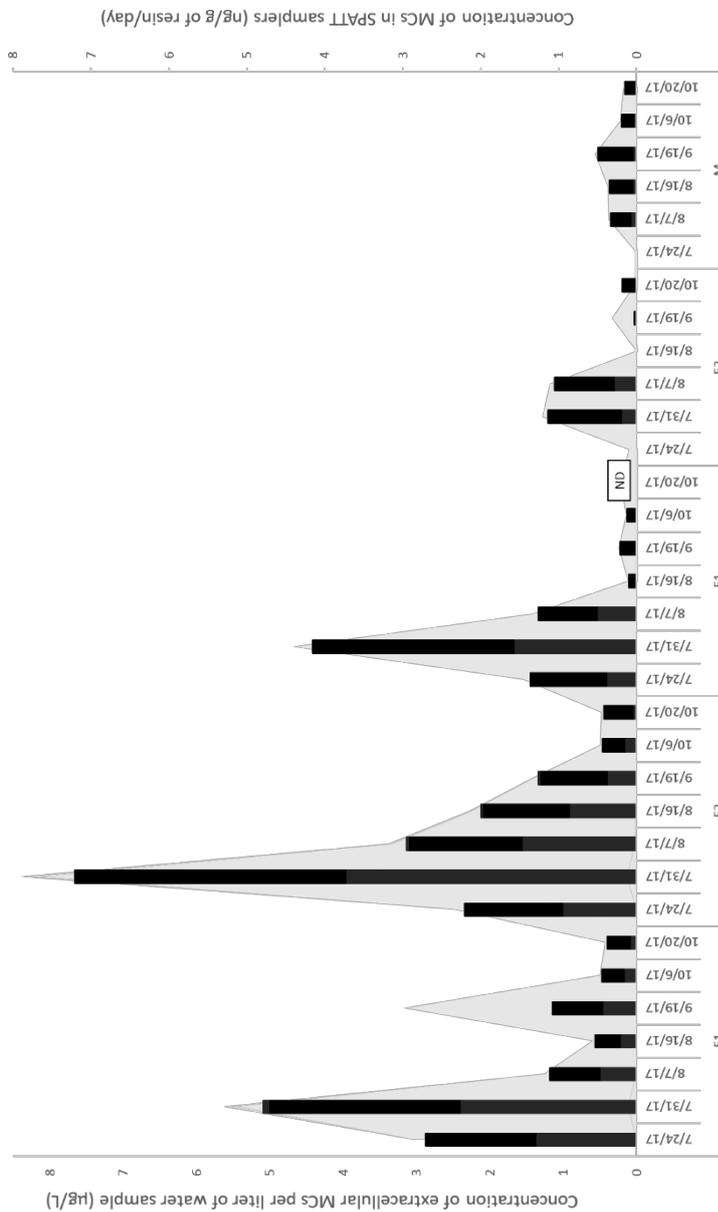


Figure 3: Mean percentages of the nine MC variants (dmMC-RR, MC-RR, MC-YR, MC-LR, dmMC-LR, MC-LA, MC-LY, MC-LW, MC-LF) in A) filtered water (extracellular MCs) and SPATT samplers (extracellular MCs) from July to October 2017, and B) phytoplankton samples (intracellular MCs) and tissues of caged mussels *A. anatina* and *M. edulis* (free accumulated MCs) from August 2016 to October 2017.

(color should be used for this figure)

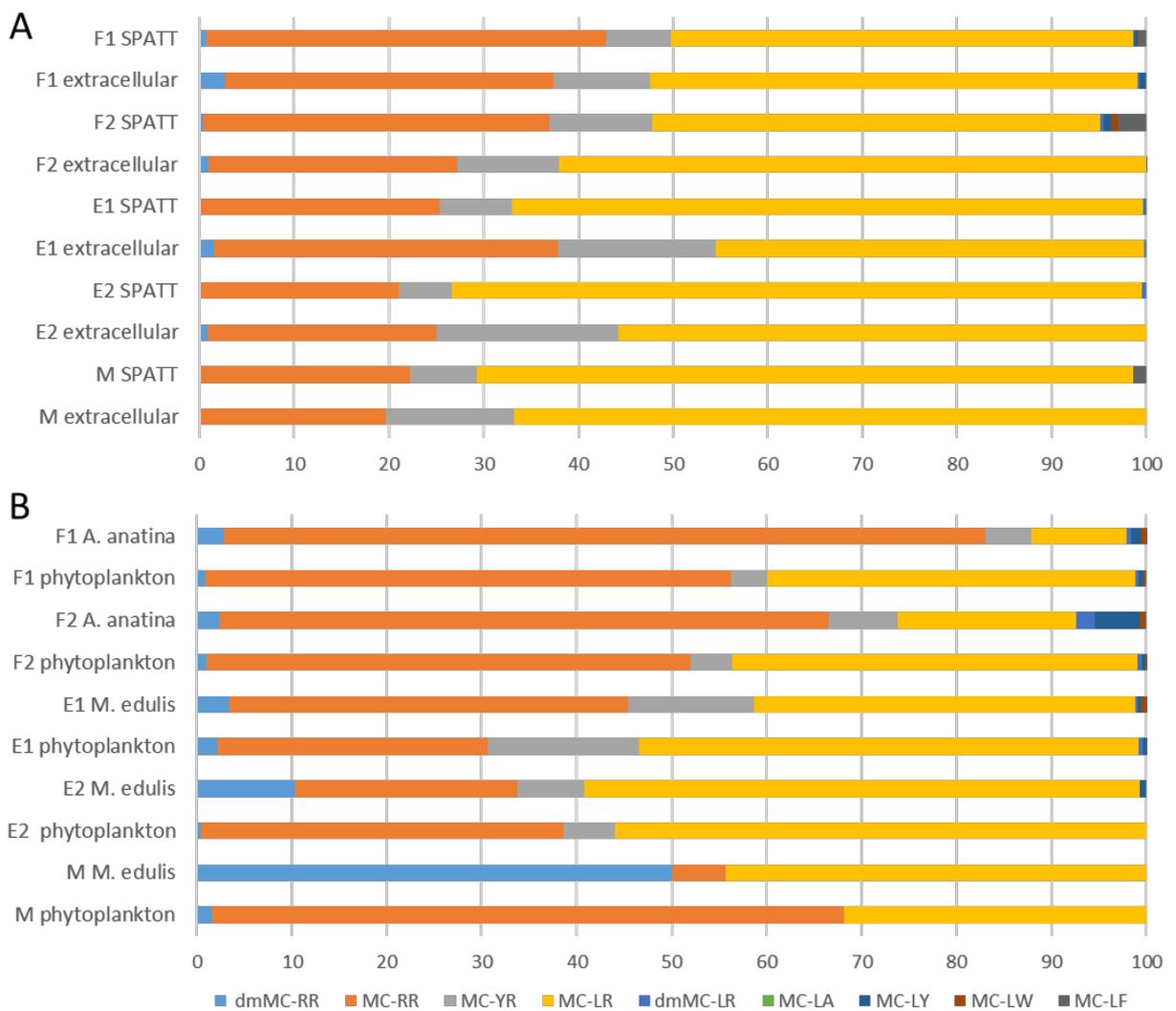


Figure 5: Free MC concentrations (in ng/g FW) in whole *M. edulis* from September 2016 to September 2017 at the harbour site E2 (grey bar chart) and the marine site M (white bar chart). The safe MC threshold concentrations in mussels for human were evaluated for average (black full dash) or high (black dotted dash) consumers based on the new French guideline value of ANSES, and for average (not visible, value of 103 ng/g FW which extends beyond the scale of the y-axis) or high (grey dotted dash) consumers based on the WHO TDI.

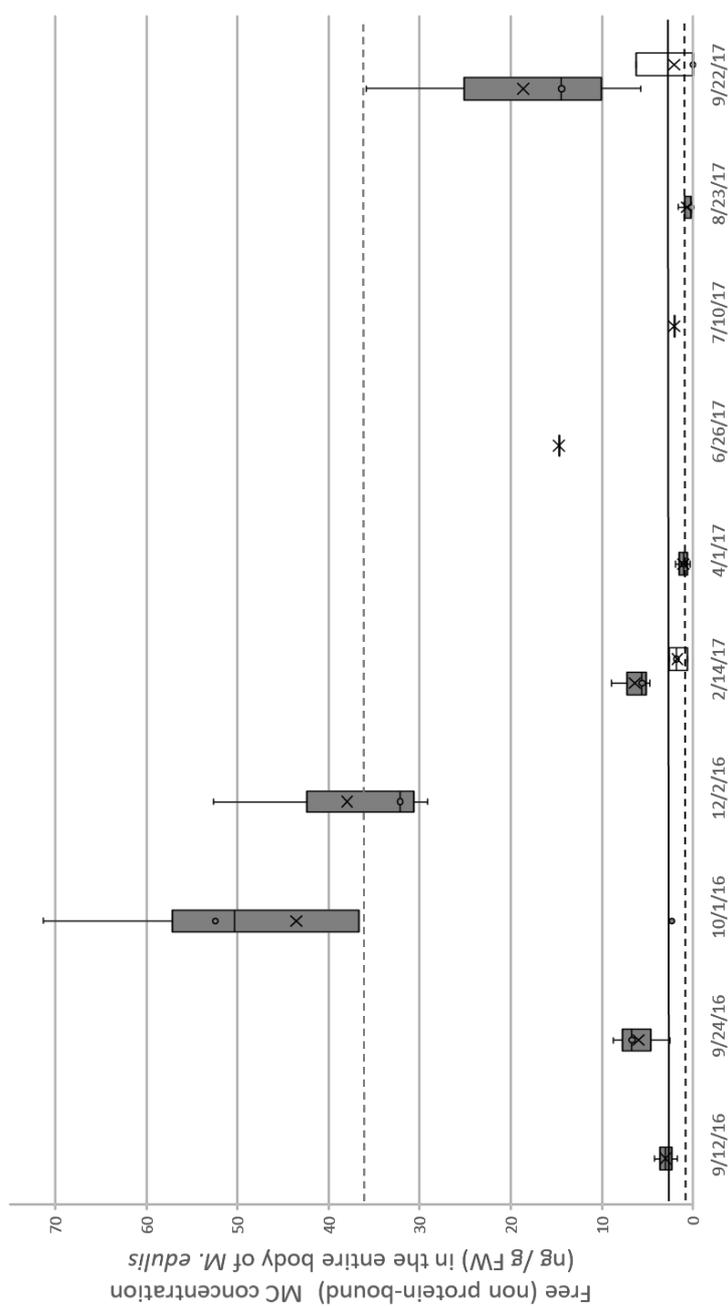
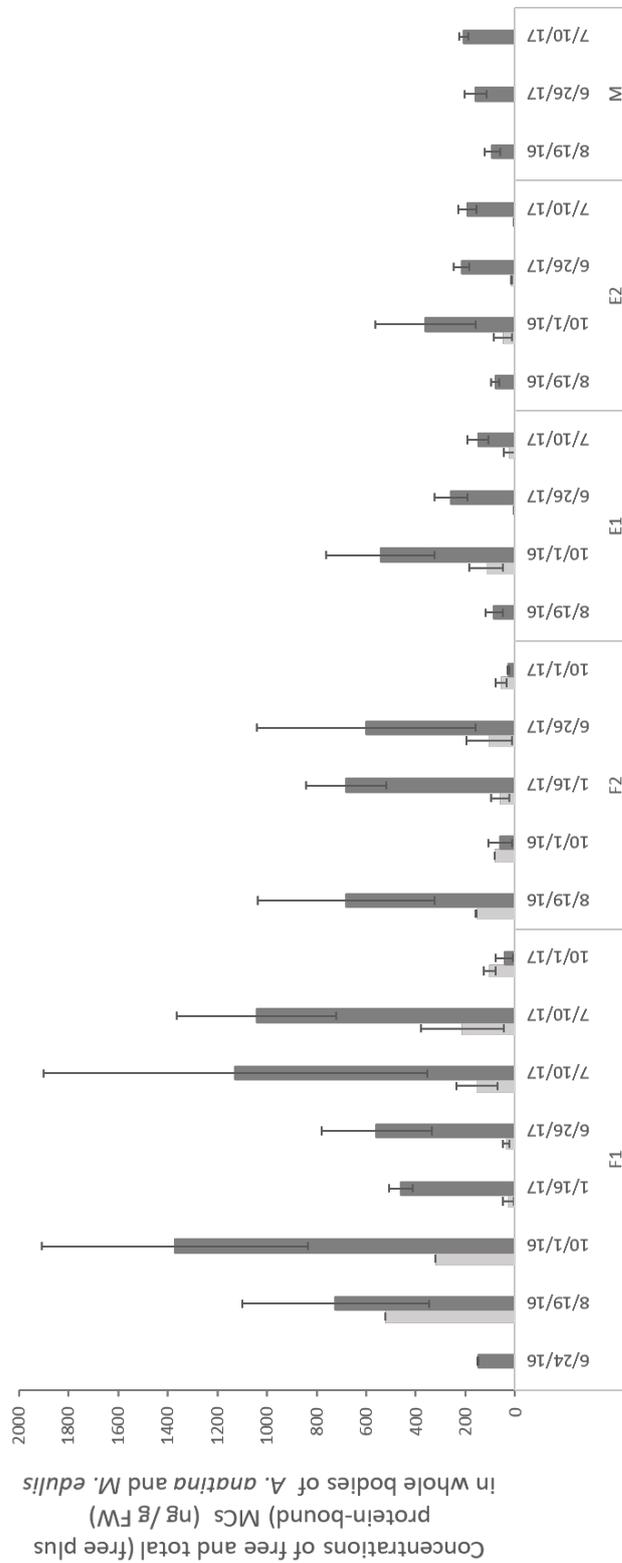
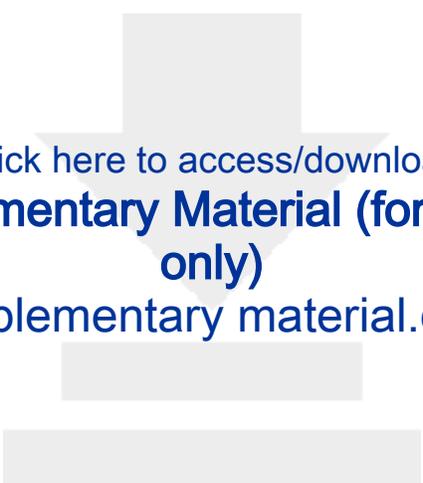


Figure 6: Free (light grey color) and total (including free plus protein-bound, dark grey color) MC concentrations (in ng/g FW) in whole bodies of some individuals of *A. anatina* (F1 and F2 sites) and *M. edulis* (E1, E2 and M sites) from June 2016 to October 2017.





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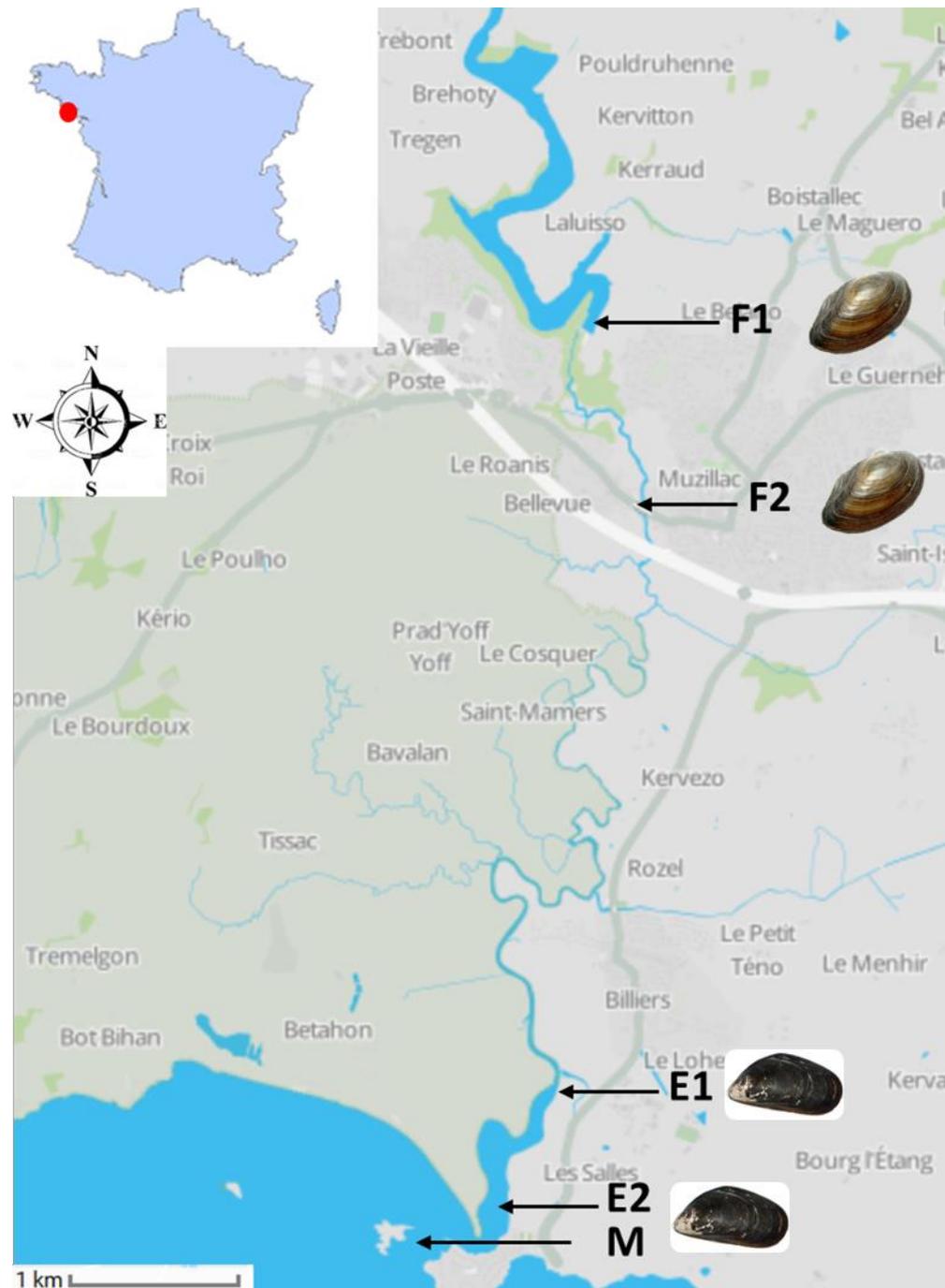


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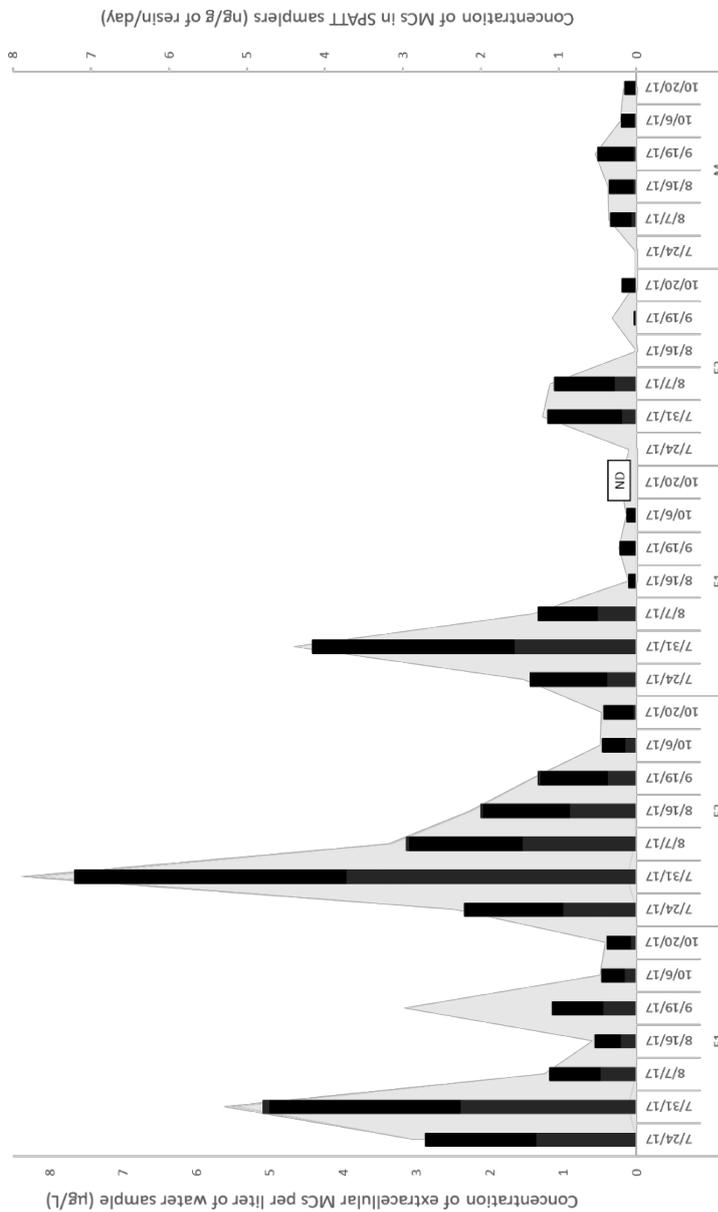


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(color should be used for this figure)

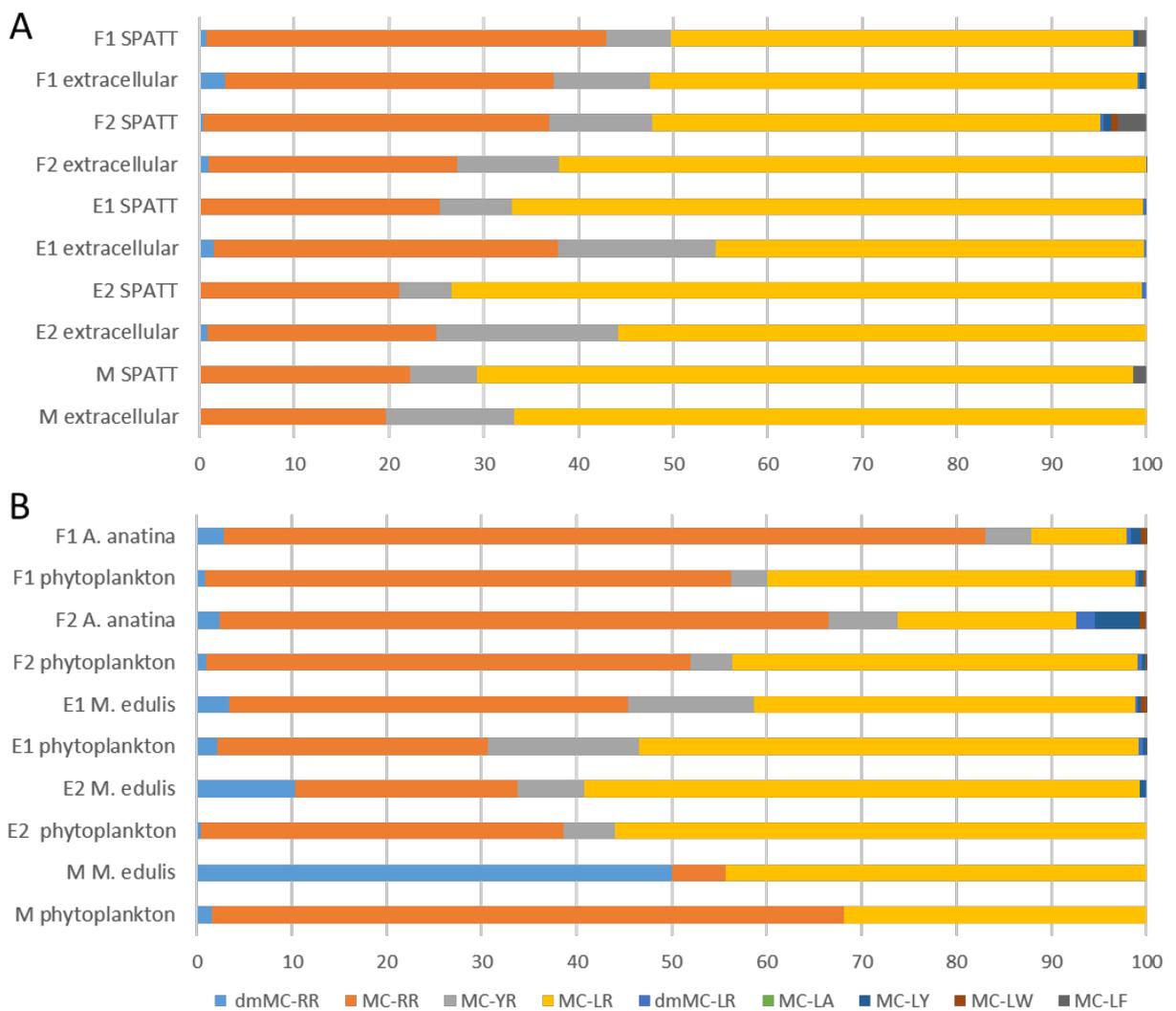


Figure 4: Concentration of MCs (sum of the nine MC variants) measured in phytoplankton (intracellular in $\mu\text{g/L}$, bar chart) and in caged mussels (free/non-protein bound accumulation fraction in *A. anatina* and *M. edulis* in ng/g FW , areas) sampled from August 2016 to October 2017 (selected dates are presented here) at the freshwater (F1, F2), estuarine (E1, E2) and marine (M) sites. Value scales in Y axis changes among sites. ND: Not Determined.

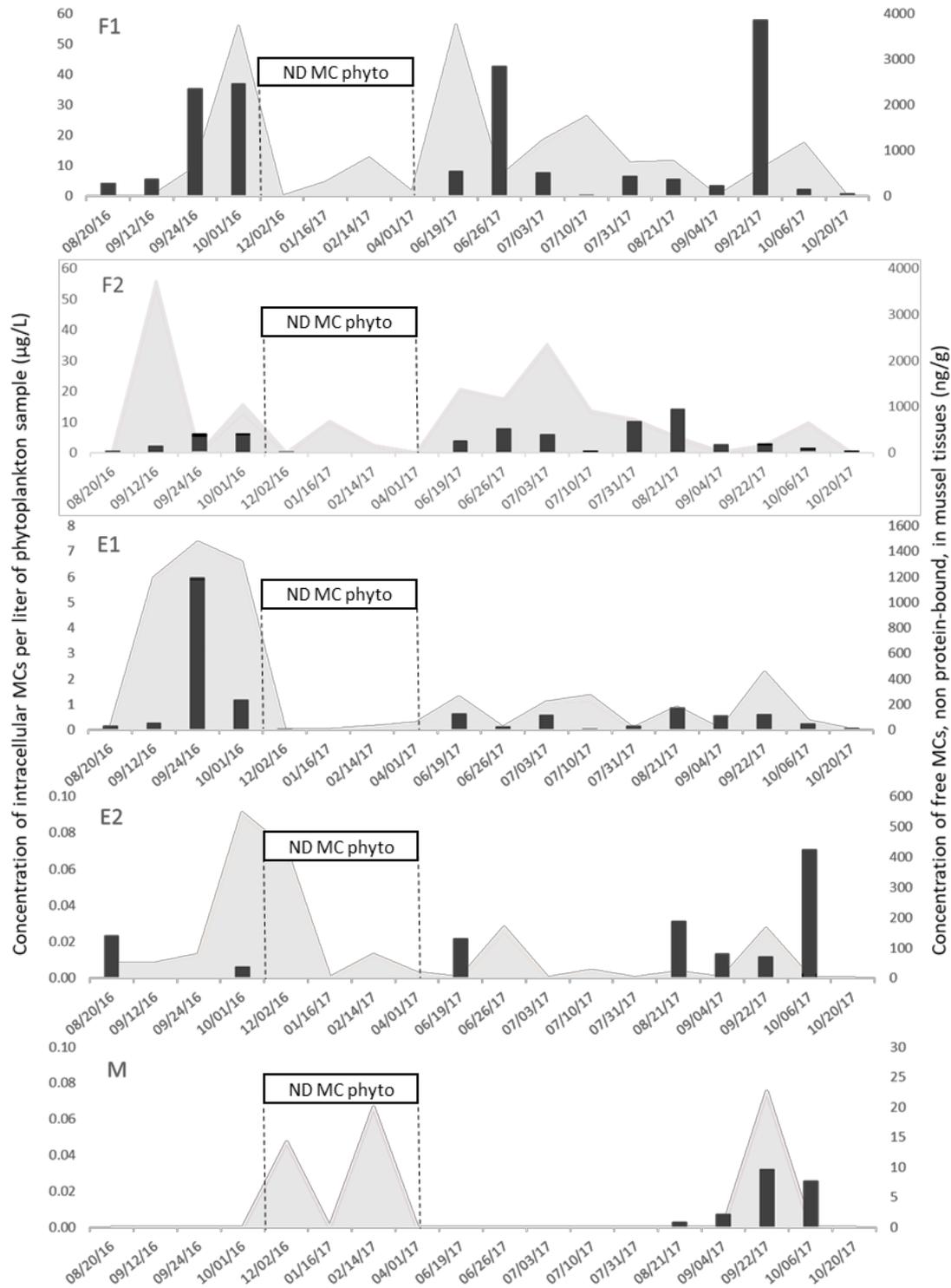


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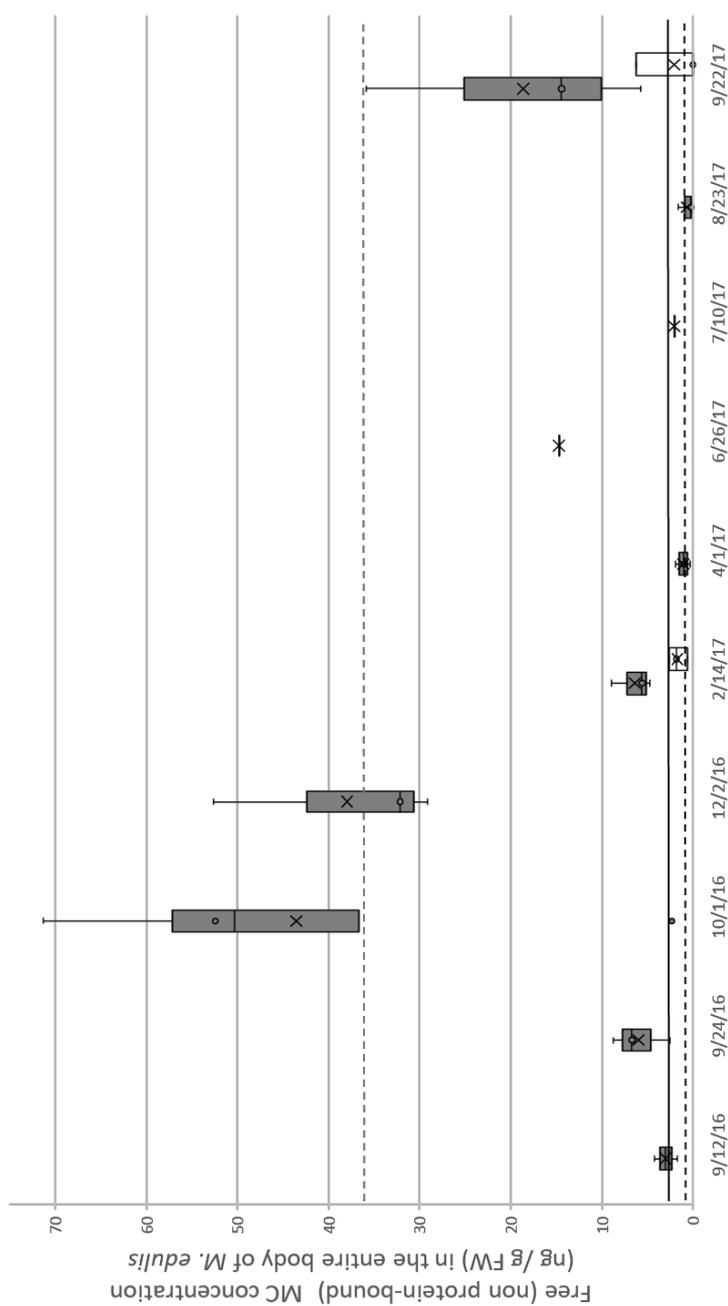


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