

# Cyanobacteria and cyanotoxins in estuarine water and sediment

M. Bormans, V. Savar, B. Legrand, E. Mineaud, E. Robert, E. Lance, Z.

Amzil

### ► To cite this version:

M. Bormans, V. Savar, B. Legrand, E. Mineaud, E. Robert, et al.. Cyanobacteria and cyanotoxins in estuarine water and sediment. Aquatic Ecology, 2020, 54 (2), pp.625-640. 10.1007/s10452-020-09764-y . hal-02862833

### HAL Id: hal-02862833 https://univ-rennes.hal.science/hal-02862833

Submitted on 25 Jun2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Cyanobacteria and cyanotoxins in estuarine water and sediment

- 4 Myriam Bormans<sup>\*1</sup>, Véronique Savar<sup>2</sup>, Benjamin Legrand<sup>3</sup>, Emilien Mineaud<sup>1</sup>, 5 Elise Robert<sup>2</sup>, Emilie Lance<sup>45</sup>, Zouher Amzil<sup>2</sup> 6 7 8 9 <sup>1</sup> Univ Rennes, CNRS, ECOBIO - UMR 6553, F-35000 Rennes, France 10 <sup>2</sup> IFREMER, Phycotoxins Laboratory, F-44311 Nantes, France 11 <sup>3</sup> ATHOS Environnement, 112 avenue du Brézet, 63100 Clermont Ferrand, France 12 <sup>4</sup> UMR-I 02 SEBIO, Bat 18, Campus du Moulin de la Housse, BP 1039, 51687 REIMS Cedex 2, 13 France 14 <sup>5</sup> UMR 7245 MNHN/CNRS MCAM, équipe CCE, Muséum National d'Histoire Naturelle, 12 rue Buffon, 15 F-75231, Paris. \*Corresponding author: mvriam.bormans@univ-rennes1.fr tel: +33 2 2323 5979 16 17 Key words: toxic cyanobacteria, microcystin, colonies, estuary, sediment 18 19 20
- 21 Abstract

1

2 3

22 While transfer of freshwater cyanobacteria to estuaries has been observed 23 worldwide, the associated transfer of cyanotoxins is less often reported, in particular the sediment contribution. During fall 2018, we monitored the co-occurrence of 24 25 cyanobacteria and microcystin (MC) in both the water column and in surface 26 sediments at 5 stations along a river continuum, from a freshwater reservoir to the 27 coastal area in Brittany, France. Cyanobacteria dominated the phytoplankton 28 community in the water column with high densities at the freshwater sites. 29 Microcystis cells and intracellular MC transfer to estuarine and marine sites were observed with decreasing concentrations in accordance with flow dilution. 30 Extracellular MC showed the opposite trend and increased from upstream to 31 32 downstream in accordance with the lysing of the cells at elevated salinities. Surface 33 sediment samples contained high densities of colonial *Microcystis* in freshwater and 34 with decreasing concentrations along the salinity gradient, similarly to cells 35 concentrations in the water column. Intracellular MC was detected in sediment at all sites except at the marine outlet suggesting the survival of intact cells. Extracellular 36 37 MC concentrations in sediment were up to 5 times higher than intracellular

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

43

#### 44 **1. Introduction**

45 46

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

61

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

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

74

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

85

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

98 99

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

101

103

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

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

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

116

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

123



124

125 **Fig. 1:** Map of the study site and sampling stations: F1 and F2 are located in the

126 freshwater section, E1 and E2 in the estuarine section and M at the marine outlet.

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

142 143

145

147

#### 144 2.2. Samples Analyses

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

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

### 164 2.2.1.1. Microcystin extraction

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

174

# 175176 2.2.2. Sediment samples

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

182

### 183 2.2.2.1. Extraction of cyanobacteria

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

191

192 2.2.2.2. Estimation of cyanobacterial abundance

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

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

208

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

210 Genomic DNA from sediment samples was extracted with the FastDNA® Spin kit for 211 soil (MP biomedicals, Santa Ana, USA) following the manufacturer's instructions. The 212 DNA concentration in each sample was estimated using a UV spectrophotometer 213 (Nanodrop® ND 2000). The cyanobacterial 16S rRNA gene was amplified in 214 sediment samples using the cya359F and cya781R set of primers from Nubel et al. 215 (1997). Then, two genes involved in the biosynthesis of microcystin, mcyA and mcyB 216 genes and one gene involved in the biosynthesis of anatoxin-a, anaC, were also 217 investigated in this study, given the cyanobacterial species found in sediment. anaC 218 and *mcyB* were amplified with nested PCRs whereas classic PCR was used to detect 219 mcvA. For mcvB nested PCR, MCY F1/R1 then MCY F1/R2 were used and 220 mcyA Cd1F and mcyA-Cd1R for mcyA. anaC was detected with the sets of primers 221 anxgen-F2/R and anaC 80F/706R for nested 1 and 2 PCR respectively. All PCR 222 mixtures and PCR programs were the same as described in Legrand et al. (2017). All 223 PCR products were revealed with 0.5% agarose gel with 0.3 mg L<sup>-1</sup> ethidium bromide 224 and migrated in a TAE buffer 0.5X at 100V for 30 minutes.

225

#### 226 2.2.2.4. Microcystin extraction

To analyse total MC (intracellular plus extracellular), the crude sediment (approximately 2 g) were extracted, according to the method of Umehara et al (2012), 4 times with 4 ml of MeOH/H<sub>2</sub>O (30%/70%), each time using sonication for 45 min and then centrifugation at 3500 g at 4 °C for 10 min. To analyse intracellular MC, the ludox supernatant containing cyanobacteria was filtered under pressure
(around 53kPA) using 25 µm nylon filtration tissues in order to eliminate akinetes and
extracellular MC. These tissues were cleaned with distilled water under pressure 3
times. Then, tissues containing *Microcystis* were cleaned with methanol 100%.
These solutions were kept in dark conditions at 4°C until further analysis.

- 236
- 237

# 238 2.2.3 Chemical analysis of microcystins by LC-MS / MS 239

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

248

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

- 257
- ~ -
- 258
- 259

Table S1: LC-MS/MS transitions for the 9 MC variants 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

#### **3. Results**

**3.1.** In the water column

### **3.1.1. Physico-chemical parameters**

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

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

_										
	Sitos	Temp	Cond	<b>O</b> <sub>2</sub>	Chl a	рН	P-PO <sub>4</sub>	N-NO₃	TSS	ОМ
	Siles	(°C)	(mS/cm)	(mg/L)	(µg/L)		(mg/L)	(mg/L)	(mg/L)	(%)
	F1	14.80	0.24	13.67	22.70	8.02	0.070	1.51	34	100.0
	F2	14.52	0.26	10.91	13.54	7.59	0.017	1.17	25	85.3
	E1	14.93	12.30	9.61	6.29	7.54	0.044	0.98	121	35.9
	E2	14.98	45.35	8.63	0.07	7.78	0.042	0.47	148	30.5
	М	14.96	40.21	7.84	1.06	7.77	0.055	0.48	135	24.7

### **3.1.2.** *Phytoplankton/Cyanobacteria community*

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



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

294 295 296

292 293

297 The biovolumes of the phytoplankton groups present together with the percentage of

298 cyanobacteria in the total biovolume of phytoplankton at each site are presented in

Table 2, demonstrating the strong dominance of cyanobacteria at the three mostupstream sites.

301

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

Biovolume (mm <sup>3</sup> L <sup>-1</sup> )	F1	F2	E1	E2	М
Cyanobacteria	21.655	4.721	2.434	0.000	0.000
Chlorophyceae	0.079	0.035	0.037	0.000	0.000
Bacillariophyceae	0.061	1.147	0.258	0.008	0.005

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

- 306
- 307

# 308 3.1.3. Intracellular and extracellular microcystins309

310 We observed a gradual decrease of intracellular MC along the continuum from 28  $\mu$ g/L in the freshwater reservoir, 3  $\mu$ g L<sup>-1</sup> in the estuary and down to 0.21  $\mu$ g L<sup>-1</sup> at the 311 312 marine station (Fig. 3A). This gradual decrease was mostly associated with the 313 decrease in cyanobacterial biovolume, in particular that of *M. aeruginosa*. Indeed, 314 based on *M. aeruginosa* densities, the cellular quotas corresponded to 88 fg cell<sup>-1</sup> at 315 F1, 69 fg cell<sup>-1</sup> at F2 and 80 fg cell<sup>-1</sup> at E1. Nine MC variants were recorded with the 316 dominance of MC-LR followed by MC-RR and MC-YR among all sites. No particular 317 selection of variants was observed with increasing salinity.



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

321 322

318 319

320

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



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

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

### **3.2.** In surface sediments

# 337338 3.2.1. Chemical characteristics

Overall, the surface sediment characteristics were not drastically different along the estuary (Table 3). Surface sediment showed a dominance of inorganic material at all 5 sites with a maximum of 17% of organic matter (OM) at the marine site. They contained a high proportion of silicate (SiO<sub>2</sub>) (50 to 73%), aluminum (Al<sub>2</sub>O<sub>3</sub>) (up to 15%), calcium (CaO) (up to 9.8%), iron (Fe<sub>2</sub>O<sub>3</sub>) (up to 6.7%), potassium (K<sub>2</sub>O) and sodium (Na<sub>2</sub>O) (up to 4%), magnesium (MgO) (up to 2.3%) and phosphorus ( $P_2O_5$ ) (up to 0.3%). The OM was maximal at F2 and M where sediment was fine and muddy while sediment at F1 and E2 was coarser and sandy. The water content of sediment varied between 30 and 71% being higher in muddy sediment. Coarser sandy sediment was also lower in Fe, Mn, Mg and OM.

Table 3 : Surface sediment characteristics along the continuum

Sites	SiO <sub>2</sub>	Al <sub>2</sub> O <sub>3</sub>	Fe <sub>2</sub> O <sub>3</sub>	MnO	MgO	CaO	Na₂O	K₂O	TiO <sub>2</sub>	$P_2O_5$	H₂O	ОМ
	%	%	%	%	%	%	%	%	%	%	%	%
F1	73.04	12.03	2.43	0.032	0.53	0.59	1.61	4.37	0.40	0.20	48	3.98
F2	53.54	15.17	6.72	0.22	1.76	1.97	0.75	2.63	0.75	0.34	71	15.45
E1	60.23	12.17	4.09	0.056	1.52	4.22	2.79	3.16	0.47	0.15	65	11.09
E2	73.71	2.76	0.92	0.018	0.44	9.82	0.74	0.78	0.12	< L.D.	30	9.92
Μ	51.32	12.25	4.95	0.11	2.30	5.67	3.74	2.40	0.61	0.20	68	16.49

#### 3.2.2. Cyanobacteria biomass

There was a dominance of Microcystis sp. (> 90%) with some akinetes of Dolichospermum sp, (Komàrek 2016) and few filaments of Planktothrix sp. at F2 in surface sediment. The presence of *Microcystis* cells in surface sediment was recorded at all 5 sites with concentrations varying between 3x10<sup>3</sup> and 3x10<sup>6</sup> cells g<sup>-1</sup> dry weight (DW) of sediment (Fig 4).



continuum.

the cells were not lysed (Fig. 5).

Photographs from epifluorescence microscopy together with counting suggested that 





377

378

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

379 380

# 381 3.2.3. Intracellular and extracellular microcystins382

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





Fig 6A: Intracellular MC in surface sediment along the continuum

Extracellular MC concentrations were inferred from subtracting the intracellular concentrations from the total MC. Extracellular MC in surface sediment varied also along the estuary between 733  $\mu$ g kg<sup>-1</sup> WW at F2 (1832  $\mu$ g kg<sup>-1</sup> DW), 70  $\mu$ g kg<sup>-1</sup> WW at E1 (175  $\mu$ g kg<sup>-1</sup> DW) and non detectable further downstream (Fig. 6B).





- 410
- 411
- 412 413

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

	Cyanobacterial 16S rRNA gene	тсуА	тсуВ	anaC
PCR	Classic	Classic	Nested	Nested
F1	+	+	+	
F2	+	+	+	ť
E1	+	+	+	
E2	+	+	+ 6	
Μ	+	-	+	<u> </u>

414

415 The PCR analyses using both classic and nested approaches confirmed the 416 presence of cyanobacterial species in sediment at all sites. Microcystin genotypes 417 were omnipresent. Indeed, microcystin biosynthesis genes were detected in the 418 sediment of all studied sites except mcyA at the most downstream station (Table 4). 419 In the freshwater section at F2, the anaC gene was also detected suggesting the 420 presence of species potentially producing anatoxin. This site exhibited the highest 421 cyanobacterial biomass including akinetes of *Dolichospermum sp.* and long filaments 422 of Planktothrix sp. (data not shown).

423 424

426

### 425 **4. Discussion**

This is the first study reporting on co-occurrence of both cyanobacteria and MC in estuarine/marine waters and sediments, resulting from a freshwater discharge of an upstream reservoir. Moreover we report separately the quantification of intracellular and extracellular forms of MC.

431

#### 432 4.1. In the water column

433 Cyanobacteria dominated the phytoplankton community in the water column with
434 high densities and biovolumes at the freshwater sites and decreasing concentrations
435 along the salinity gradient. Temperatures during sampling around 15°C at all 5 sites
436 were much lower than the growth optimal temperature of cyanobacteria in general

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

453

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

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

479

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

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

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

513

514 Quantifying cyanobacterial cells in sediments is difficult as reported by Pham and 515 Utsumi (2018). The presence of *Microcystis* was observed in surface sediments at all 516 5 sites along the freshwater marine continuum in relatively high densities even at the 517 marine outlet. Microcystis abundance was the highest at F2 where Microcystis 518 concentration reached 3 x10<sup>6</sup> cells g<sup>-1</sup> DW of sediment corresponding to bloom 519 concentrations. The higher number of cells at F2 compared to F1 is not surprising 520 given that the sediment sampling at F1 was done in the flowing river downstream of 521 the reservoir, while F2 corresponded to a much more stagnant water, associated with 522 muddy sediments rich in OM. Hence F2 acted as a sediment trap accumulating 523 *Microcystis* cells. The presence of *Microcystis* in sediments is consistent with 524 *Microcystis* being the dominant species being transferred in the water column from 525 the upstream reservoir freshwater discharge. Moreover, *Microcystis* concentration 526 gradient in surface sediments along the salinity gradient reflected *Microcystis* 527 concentration gradient in the water column over several months to years (Bormans et 528 al. 2019) rather than instantaneously (this study).

529

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

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

549

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

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

591

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

Moreover, at F2 all toxic genotypes and a high MC concentration were detected in the sediment. The same applies at E1 and E2 except with a smaller MC concentration. Only at the most downstream site (marine outlet) did we not detect 605 any MC in the sediment in accordance with the genes analysis that suggested the 606 presence of a very small number of toxic genotypes at the time of sampling. This 607 may confirm the previous hypothesis, about the importance of F2 station to transfer 608 *Microcystis* in the estuary. Our results are in line with the findings of Via-Ordorika et 609 al. (2004) who reported that most *M. aeruginosa* colonies from several freshwater 610 lakes contained the mcyA and mcyB genes and found a positive relationship 611 between genes frequency and colony size. Our results also support the measure of 612 MC production genes as a proxy of toxic genotypes as tested by Martinez et al. 613 (2017) and Otten et al. (2015) to evaluate the toxic potential of cyanobacteria along a 614 freshwater marine continuum.

615

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

- 623
- 624

#### 625 **5. Conclusions**

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

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

642

#### 643 Acknowledgments

The authors thank Maxime Georges des Aulnois for help with field work. Marion 644 645 Chorin for nutrient analyses and Gorenka Bojadzija Savic for finalisation of the 646 figures. Microscopy and biochemical analyses were performed at the Experimental 647 Ecology (ECOLEX) and the Analytical (PLAY) platforms of the UMR Ecobio. The 648 physicochemical caracteristics of the sediments were analysed by the SARM 649 laboratory of the CRPG-CNRS in Nancy, France. This project was financially 650 supported by the ANSES (Agence Nationale de Sécurité Sanitaire de l'Alimentation, 651 de l'Environnement et du Travail) under the Bieautox project EST-2015-191. The 652 helpful suggestions from the reviewers are also acknowledged.

- 653
- 654

# 655 References656

Anon (2005) Anonyme ISO 20179:2005(E). Water quality – Determination of microcystins – Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. International Standard ISO 20179, first edition 2005-10-01, 17 p

661

Bormans M, Amzil Z, Mineaud E, Brient L, Savar V, Robert E, Lance E (2019)
Demonstrated transfer of cyanobacteria and cyanotoxins along a freshwater-marine
continuum in France. Harmful Algae 87: 101639

- Bourrelly P (1985) Les algues d'eau douce: les algues bleues et rouges (Vol. 3).
  Lubrecht & Cramer Ltd.
- 668

Briand E, Escoffier N, Straub C, Sabart M, Quiblier C (2009) Spatiotemporal changes
in the genetic diversity of a bloom-forming *Microcystis aeruginosa* (cyanobacteria)
population. ISME J 3: 419–429

- 673 Briand E, Bormans M, Quiblier C, Salençon MJ, Humbert JF (2012) Evidence of the 674 cost of the production of microcystins by *Microcystis aeruginosa* under differing light 675 and nitrate environmental conditions. PLoS One 7 (1): e29981
- 676

Brient L, Lengronne M, Bertrand E, Rolland D, Sipel A, Steinmann D, Baudin I,
Legeas M, Le Rouzic N, Bormans M (2008) A phycocyanin probe as a tool for
monitoring cyanobacteria in freshwater bodies. J Environ Monit 10: 248–55

684 685 Bukaveckas PA, Lesutiene J, Gaisunaite ZR, Lozys L, Olenina I, Pilkaityte R, Putys 686 Z, Tassone S, Wood J (2017). Microcystin in aquatic food webs of the Baltic and 687 Chesapeake Bay regions. Estuarine, Coastal and Shelf Science 191 :50-59 688 689 Bukaveckas PA, Franklin R, Tassone S, Trache B, Egerton T (2018). Cyanobacteria 690 and cyanotoxins at the river-estuarine transition. Harmful Algae 76: 11-21 691 692 Carey CC, Ibelings BW, Hoffmann EP, Hamilton DP, Brookes JD (2012) Eco-693 physiological adaptations that favour freshwater cyanobacteria in a changing climate. 694 Water Res 46:1394–1407 695 696 Cires S, Wörmer L, Agha R, Quesada A (2013) Overwintering populations of 697 Anabaena, Aphanizomenon and Microcystis as potential inocula for summer blooms. 698 J Plankton Res 35(6): 1254–1266 699 700 Conley DJ (1997) Contribution of biogenic silica to the oceanic silica budget. Limnol 701 Oceanogr 42(4): 774–777 702 703 De Pace R, Vita V, Bucci MS, Gallo P, Bruno M (2014) Microcystin contamination in 704 sea mussel farms from the Italian southern Adriatic coast following cyanobacterial 705 blooms in the artificial reservoir. J. of Ecosystems ID 374027 706 http://dx.doi.org/10.1155/2014/374027 707 708 Devlin JP, Edwards OE, Gorham PR, Hunter NR, Pike RK, Stavric B (1977) 709 Anatoxin-a, a toxic alkaloid from Anabaena flos-aquae NRC-44h. Canadian Journal 710 of Chemistry 55(8): 1367-1371 711 712 Dörr FA, Pinto E, Soares RM, Azevedo SMFO (2010) Microcystins in South 713 American aquatic ecosystems: occurrence, toxicity and toxicological assays. Toxicon 714 56:1247-1256 715 716 Evans RD (1994) Empirical evidence of the importance of sediment resuspension in 717 lakes. Hydrobiologia 284:5–12 718 719 Folger D (1972) Characteristics of estuarine sediments of the United States USGS 720 report 2482 721 722 Fraisse S, Bormans M, Lagadeuc Y (2013) Morphofunctional traits reflect differences 723 in phytoplankton community between rivers of contrasted flow regime. Aquat Ecol 47: 724 315-327 doi:10.1007/s10452-013-9446-z 725 Georges des Aulnois M, Roux P, Caruana A, Réveillon D, Briand E, Hervé F, Savar 726 727 V, Bormans M, Amzil Z (2019) Physiological and metabolic responses of freshwater 728 and brackish waters strains of *Microcystis aeruginosa* acclimated to a salinity 729 aradient: tolerance. Applied insiaht into salt and Env Microbiology DOI: 10.1128/AEM.01614-19 730

Brient L, Lengronne M, Bormans M, Fastner J (2009) First occurrence of

cylindrospermopsin in freshwater in France. Environmental Toxicology 24(4): 415-

681

682

683

732 Gibble CM, Peacock MB, Kudela RM (2016) Evidence of freshwater algal toxins in 733 marine shellfish: implications for human and aquatic health. Harmful Algae 59: 59-66 734 735 Graham JL, Loftin KA, Meyer MT, Ziegler AC (2006) Co-occurrence of Toxins and 736 Taste-and-Odor Compounds in Cyanobacterial Blooms from the Midwestern United 737 States. Environ Sci Technol 44: 7361–7368 738 739 Grutzmacher G, Wessel G, Klitzke S, Chorus I (2010) Microcystin elimination during 740 sediment contact. Environ Sci Technol 44: 657–662 741 742 Harke MJ, Steffen MM, Gobler CJ, Otten TG, Wilhelm SW, Wood SA, Paerl HW 743 (2016) A review of the global ecology, genomics, and biogeography of the toxic 744 cyanobacterium Microcystis spp. Harmful Algae 54: 4-20 745 746 Henriksen A, Selmer-Olsen AR (1970) Automatic methods for determining nitrate and 747 nitrite in water and soil extracts. Analyst 95: 514-518 748 749 Komárek J, Anagnostidis K (2005) Süßwasserflora von Mitteleuropa, bd. 19/2: 750 Cyanoprokaryota: Oscillatoriales (Vol. 19). Spektrum Akademischer Verlag. 751 752 Komárek J, Anagnostidis K (2013) Süßwasserflora von Mitteleuropa, bd. 19/2: 753 Cyanoprokaryota: Heterocytous Genera (Vol. 19/3). Spektrum Akademischer Verlag. 754 755 Komárek J (2016) Review of the cyanobacterial genera implying planktic species after recent taxonomic revisions according to polyphasic methods: state as of 2014. 756 Hydrobiologia 764(1) : 259-270 757 758 759 Kravchuk ES, Ivanova EA, Gladyshev MI (2011) Spatial distribution of resting stages (akinetes) of the cyanobacteria Anabaena flos-aquae in sediments and its influence 760 761 on pelagic populations. Mar Fresh Res 62: 450-461 762 763 Kruk C, Segura AM, Nogueira L, Alcántara I, Calliaria D, Martínez de la Escalerad G, 764 Carballoa C, Cabreraa C, Sarthoua F, Scavone P, Piccini C (2017) A multilevel trait-765 based approach to the ecological performance of *Microcystis aeruginosa* complex 766 from headwaters to the ocean. Harmful Algae 70: 23-36 767 768 Labry C, Delmas D, Youenou A, Quere J, Leynaert A, Fraisse S, Raimonet M, 769 Raqueneau O (2016) High alkaline phosphatase activity in phosphate replete waters: 770 The case of two macrotidal estuaries. Limnol Oceanogr 61:1513–1529 771 772 Lance E, Brient L, Carpentier A, Acou A, Marion L, Bormans M, Gérard C (2010) 773 Impact of toxic cyanobacteria on gastropods and microcystin accumulation in a 774 eutrophic lake (Grand-Lieu, France) with special reference to Physa (= Physella) 775 acuta. Science of the Total Environment 408: 3560-3568 776 Legrand B, Lamarque A, Sabart M, Deltour D (2016) Characterization of akinetes 777 778 from cyanobacterial strains and lake sediment: A study of their resistance and toxic 779 potential. Harmful Algae 59: 42–50 780

781 Legrand B, Le Jeune AH, Colombet J, Thouvenot A, Latour D (2017) Akinetes May Be Representative of Past Nostocalean Blooms: a Case Study of Their Benthic 782 783 Spatiotemporal Distribution and Potential for Germination in a Eutrophic Lake. Appl 784 Environ Microbiol 83:e01571-17 785 786 Legrand B, Miras Y, Beauger A, Dussauze M, Latour D (2019)\_Akinetes and ancient 787 DNA reveal toxic cyanobacterial recurrences and their potential for resurrection in a 788 6700-year-old core from a eutrophic lake. Science of the Total Environment 687: 789 1369-1380 790 791 Lehman PW, Boyer G, Hall C, Waller S, Gehrts K (2005) Distribution and toxicity of a 792 new colonial *Microcystis aeruginosa* bloom in the San Francisco Bay Estuary, 793 California. Hydrobiologia 541: 87–99 794 795 Le Moal M, Gascuel-Odoux C, Menesquen A, Souchon Y, Etrillard C, Levain A, 796 Moatar F, Pannard A, Souchu P, Lefebvre A, Pinay G (2019) Eutrophication: A new 797 wine in old bottle? Science of the Total Environment an 798 DOI:10.1016/j.scitotenv.2018.09.139 799 800 Maavara T, Dürr HH, Van Cappellen P (2014) Worldwide retention of nutrient silicon 801 by river damming: From sparse data set to global estimate. Global Biogeochem 802 Cycles 28 : 842-855 doi:10.1002/2014GB004875 803 804 Martínez de la Escalera G, Kruk C, Segurad AM, Nogueira L, Alcántara I, Piccini C 805 (2017) Dynamics of toxic genotypes of Microcystis aeruginosa complex (MAC) 806 through a wide freshwater to marine environmental gradient. Harmful Algae 62:73-807 83 808 809 Merel S, Walker D, Chicana R, Snyder S, Baurés E, Thomas O (2013) State of 810 knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ Int 59: 811 303-327 812 813 Meriluoto J, Blaha L, Bojadzija G, Bormans M, Brient L, Codd GA, Drobac D, Faassen EJ, Fastner J, Hiskia A, Ibelings BW, Kaloudis T, Kokocinski M, Kurmayer 814 815 R, Pantelić D, Quesada A, Salmaso N, Tokodi N, Triantis TM, Visser PM, Svirčev Z 816 (2017) Toxic cyanobacteria and cyanotoxins in European waters - recent progress 817 achieved through the CYANOCOST Action and challenges for further research. 818 Advances in Oceanography and Limnology 8(1) DOI: 10.4081/aiol.2017.6429 819 820 Misson B, Sabart M, Amblard C, Latour D (2011) Involvement of microcystins and 821 colony size in the benthic recruitment of the cyanobacterium Microcystis 822 (Cyanophyceae). Journal of Phycology 47:42-51 823 824 Misson B, Sabart M, Amblard C, Latour D (2012) Benthic survival of Microcystis: 825 long-term viability and ability to transcribe microcystin genes. Harmful Algae 13: 20-826 25. 827 828 Ndlela LL, Oberholster PJ, Van Wyk JH, Cheng PH (2016) An overview of 829 cyanobacterial bloom occurrences and research in Africa over the last decade. 830 Harmful Algae 60 : 11–26

831 832 Nubel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16SrRNA genes 833 from cyanobacteria. Appl Environ Microbiol 63: 3327–3332 834 835 Omidi A, Esterhuizen-Londt M, Pflugmacher S (2017) Still challenging: the ecological 836 function of the cyanobacterial toxin microcystin - What we know so far. Toxin 837 Reviews 37: Issue 2. 838 839 O'Neil JM, Davis TW, Burford MA, Gobler CJ (2012) The rise of harmful 840 cyanobacteria blooms: The potential roles of eutrophication and climate change 841 Harmful Algae 14 : 313–334 842 843 Orr PT, Jones GJ (1998) Relationship between microcystin production and cell 844 division rates in nitrogen-limited Microcystis aeruginosa cultures. Limnol Oceanogr 845 43: 1604–1614 846 847 Orr PT, Jones GJ, Douglas GB (2004) Response of cultured *Microcystis aeruginosa* 848 from the Swan River, Australia, to elevated salt concentration and consequences for 849 bloom and toxin management in estuaries. Mar Freshw Res 55 (3): 277–283 850 851 Ortiz et al., (2017) A high throughput targeted and non-targeted method for the 852 analysis of microcystins and anatoxin-A using on-line solid phase extraction coupled 853 chromatography-guadrupole time-of-flight to liauid high resolution mass 854 spectrometry. Analytical and Bioanalytical Chemistry 409 (21): 4959 855 856 Otten TG, Crosswell JR, Mackey S, Dreher TW (2015) Application of molecular tools 857 for microbial source tracking and public health risk assessment of a Microcystis 858 bloom traversing 300 km of the Klamath River. Harmful Algae 46 : 71-81 859 860 Paerl HW (2018) Mitigating Toxic Planktonic Cyanobacterial Blooms in Aquatic 861 Ecosystems Facing Increasing Anthropogenic and Climatic Pressures. Toxins 10: 76 862 doi:10.3390/toxins10020076 863 864 Paerl HW, Otten TG, Kudela R (2018) Mitigating the Expansion of Harmful Algal 865 Blooms Across the Freshwater-to-Marine Continuum. Environ Sci Technol 52: 866 5519-5529 867 868 Paldavičiene A, Mazur-Marzec H, Razinokovas A (2009) Toxic cyanobacteria blooms 869 in the Lithuanian part of the Curonian Lagoon. Oceanologia 51 (2): 203-216 870 871 Paldavičiene A, Zaiko A, Mazur-Marzec H, Razinokovas-Baziukas A (2015) 872 Bioaccumulation of microcystins in invasive bivalves: a case study from the boreal 873 lagoon ecosystem. Oceanologia 57: 93-101 874 875 Pannard A, Guislain A, Chorin M, Mahé S, Bouger G, Crave A, Le Rouzic B, 876 Bormans M (2018) Phosphorus more than temperature controls the phytoplankton 877 community in a deep quarry lake: a combined field and laboratory approach. Inland 878 Waters, DOI:10.1080/20442041.2018.1424097 879

- Peacock MB, Gibble CM, Senn DB, Cloern JE, Kudela RM (2018) Blurred lines:
  Multiple freshwater and marine algal toxins at the land-sea interface of San Francisco
  Bay, California. Harmful Algae 73 : 138-147
- 883
  884 Pham TL, Utsumi M (2018) An overview of the accumulation of microcystins in
  885 aquatic ecosystems. Journal of Environmental Management 213: 520-529
  886
- Pitois F, Thoraval I, Baurès E, Thomas O (2014) Geographical Patterns in Cyanobacteria Distribution: Climate Influence at Regional Scale. Toxins 6: 509-522
- 889
  890 Pitois F, Fastner J, Pagotto C, Dechesne M (2018) Multi-Toxin Occurrences in Ten
  891 French Water Resource Reservoirs. Toxins 10: 283
- 892
- Preece EP, Moore BC, Hardy FJ (2015) Ecotoxicology and Environmental Safety Transfer of microcystin from freshwater lakes to Puget Sound, WA and toxin accumulation in marine mussels (*Mytilus trossulus*). Ecotoxicology and Environmental Safety 122: 98–105
- Preece EP, Hardy FJ, Moore BC, Bryan M (2017) A review of microcystin detections
  in Estuarine and Marine waters: Environmental implications and human health risk.
  Harmful Algae 61 : 31–45
- Puddick J, Prinsep MR, Wood SA, Kaufononga SAF, Cary SC, Hamilton DP (2014)
  High Levels of Structural Diversity Observed in Microcystins from *Microcystis*CAWBG11 and Characterization of Six New Microcystin Congeners. Mar Drugs 12:
  5372-5395; doi:10.3390/md12115372
- 905
  906 Reynolds CS (2006) Ecology of Phytoplankton. Cambridge University Press,
  907 Cambridge, UK
- 908
- Rigosi A, Carey CC, Ibelings BW, Brookes JD (2014) The interaction between
  climate warming and eutrophication to promote cyanobacteria is dependent on
  trophic state and varies among taxa. Limnol Oceanogr 59(1): 99–114
- 912
  913 Robson BJ, Hamilton DP (2003) Summer flow event induces a cyanobacterial bloom
  914 in a seasonal western Australia estuary. Mar Freshw Res 54: 139–151
- 915
- Sabart M, Crenn K, Perrière F, Abila A, Leremboure M, Colombet J, ... Latour D
  (2015) Co-occurrence of microcystin and anatoxin-a in the freshwater lake Aydat
  (France): Analytical and molecular approaches during a three-year survey. Harmful
  Algae 48: 12-20
- 920
- Taş S, Okus E, Aslan-Yilmaz A (2006) The blooms of a cyanobacterium, *Microcystis cf. aeruginosa* in a severely polluted estuary, the Golden Horn, Turkey. Estuar Coast.
  Shelf Sci 68, : 593–599
- 924
- Tatters AO, Howard MDA, Nagoda C, Busse L, Gellene AG, Caron DA (2017)
   Multiple stressors at the land-sea interface: cyanotoxins at the land-sea interface in
   the Southern California Bight Toxins doi:http://dx.doi.org/10.3390/
- 927 the Southern California Bight. Toxins doi:http://dx.doi.org/10.3390/
- 928 toxins9030095.
- 929

- Tillett D, Dittmann E, Erhard M, von Döhren H, Börner T, Neilan BA (2000) Structural
  organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an
  integrated peptide polyketide synthetase system. Chemistry & Biology 7:753-764
- 933
  934 Tonk L, Bosch K, Visser PM, Huisman J (2007) Salt tolerance of the harmful
  935 cyanobacterium *Microcystis aeruginosa*. Aquat Microb Ecol 46: 117–123
- 936
- Umehara A, Tsutsumi H, Takahashi T (2012) Blooming of *Microcystis aeruginosa* in
  the reservoir of the reclaimed land and discharge of microcystins to Isahaya Bay
  (Japan). Environ Sci Pollut Res 19: 3257–3267
- 940
- 941 Umehara A, Komorita T, Tai A, Takahashi T, Orita R, Tsutsumi H (2015) Short-term
  942 dynamics of cyanobacterial toxins (microcystins) following a discharge from a coastal
  943 reservoir in Isahaya Bay, Japan. Marine Pollution Bulletin 92: 73–79.
  944
- Umehara A, Takahashi T, Komorita T, Orita R, Choi JW, Takenaka R, Mabuchi R,
  Park HD, Tsutsumi H (2017) Widespread dispersal and bio-accumulation of toxic
  microcystins in benthic marine ecosystems. Chemosphere 167 : 492-500
- 948 949 Verspagen JMH, Snelder EOFM, Visser PM, Jöhnk KD, Ibelings BW, Mur LR,
- Huisman J (2005) Benthic-pelagic coupling in the population dynamics of the harmful cyanobacterium Microcystis. Freshw Biol 50: 854–867
- 952 <u>https://doi.org/10.1111/j.1365-2427.2005.01368.x</u>.
- 953
- Verspagen JMH, Passarge J, Jöhnk KD, Visser PM, Peperzak L, Boers P, Laanbroek
  HJ, Huisman J (2006) Water management strategies against toxic *Microcystis*blooms in the Dutch delta. Ecol Appl 16 : 313–327
- 957
  958 Vezie C, Brient L, Sivonen K, Bertru G, Lefeuvre JC, Salkinoja-Salonen M (1998)
  959 Variation of microcystin content of cyanobacterial blooms and isolated strains in Lake
  960 Grand-Lieu (France). Microb Ecol 35:126–35
- 961
  962 Viaggiu E, Melchiorre S, Volpi F, Di Corcia A, Mancini R, Garibaldi L, Bruno M (2004)
  963 Anatoxin-a toxin in the cyanobacterium Planktothrix rubescens from a fishing pond in
  964 northern Italy. Environmental Toxicology: An International Journal 19(3): 191-197
- Via-Ordorika L, Fastner J, Kurmayer R, Hisbergues M, Dittmann E, Komarek J,
  Erhard M, Chorus I (2004) Distribution of Microcystin-Producing and NonMicrocystin-Producing Microcystis sp. in European Freshwater Bodies: Detection of
  Microcystins and Microcystin Genes in Individual Colonies. Systematic and Applied
  Microbiology 27(5): 592-602
- 971
- Wood R (2016) Acute animal and human poisonings from cyanotoxin exposure A
   review of the literature. Environment International 91: 276-282
- 974
- Wood SA, Rueckert A, Hamilton DP, Cary SC, Dietrich DR (2011). Switching toxin
  production on and off : intermittent microcystin synthesis in a Microcystis bloom. Env.
  Microbiol. Report 3(1) : 118-124
- 978

Xiao M, Li M, Reynolds CS (2018) Colony formation in the cyanobacterium
Microcystis. Biol Rev 93: 1399-1420