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1 Relative effect of hydraulics, physico-chemistry and other biofilm algae on
2 benthic cyanobacteria assemblages in a regulated river

3

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13

14

15 **Highlights**

- 16 • We investigated the response of benthic cyanobacteria assemblages to
17 hydraulics, physico-chemical and biotic variables considering three sampling
18 zones differing in terms of velocity variability according to discharge.
- 19 • In the studied regulated river, benthic cyanobacteria communities were
20 dominated by potentially toxic genera (>50% of the biovolume).
- 21 • The development of cyanobacteria assemblages was limited spatially and
22 temporally by the ecological preferences of genera and high-flow events.
- 23 • Warmer water temperatures in mid-summer and higher nutrient
24 concentrations in the end of summer favoured the development of
25 cyanobacteria assemblages.
- 26 • The development of Chlorophyta coincides with the decline of cyanobacteria
27 suggesting potential competition between the two phyla.
- 28 • Discharges over $100 \text{ m}^3 \cdot \text{s}^{-1}$ only had a negative influence on cyanobacteria
29 biovolumes when the flow variability was high for at least two weeks before
30 sampling, and did not influence BC in late summer.
- 31 • Biofilm management through flushing flows of $100 \text{ m}^3 \cdot \text{s}^{-1}$ is not a viable
32 solution to reduce biofilm development if it lasts less than one day.

33

34 **Abstract**

35 The development of benthic cyanobacteria currently raises concern worldwide
36 because of their potential to produce toxins. As a result, understanding which
37 measures of biotic and abiotic parameters influence the development of
38 cyanobacterial assemblages is of great importance to guide management actions. In
39 this study, we investigate the relative contributions of abiotic and biotic parameters
40 that may drive the development of cyanobacterial assemblages in river biofilms.
41 First, a 2D hydrodynamic model allowed us to retrace changes in depths and
42 velocities according to discharge at a 4 m² resolution. From this model, we set up
43 three hydraulic zones in each of the 4 reaches investigated along a 50-km-long river
44 stretch. We further used univariate, multivariate and variance partitioning analyses to
45 assess the contribution of past and present hydraulics, present physical and
46 chemical parameters and algae to the temporal variability of cyanobacterial
47 assemblage composition. The cyanobacterial assemblages were generally
48 dominated by *Phormidium* sp., *Lyngbya* sp., *Planktolyngbya* sp. and *Oscillatoria* sp.,
49 four genera known to contain potentially toxic species. The highest biovolumes of
50 cyanobacteria were present in low velocity zones in early summer and shifted to high
51 velocity zones in late summer, highlighting the major influence of hydraulic
52 parameters on benthic cyanobacteria settlement and development in rivers.
53 Considering the identified genera, biofilms present a potentially high risk of toxin
54 production. Relations between cyanobacterial development, toxin production and
55 environmental parameters need to be further assessed to better estimate this risk.

56

57 **Keywords:**

58 benthic cyanobacteria; biofilm development; hydraulics; regulated river

59 1. Introduction

60 Cyanobacteria blooms have been rising worldwide for the last two decades.
61 Benthic cyanobacteria (BC) are found in rivers and lakes (Mez et al., 1998;
62 Vadeboncoeur et al., 2021). They have raised concern for the last two decades due
63 to the ability of some species to produce cyanotoxins. These toxins can be harmful
64 to aquatic and terrestrial organisms and may cause mammalian neurotoxicosis and
65 death (Quiblier et al., 2013). For example, dog deaths have been reported to be
66 caused by benthic cyanobacteria in New Zealand (Hamill, 2001; Wood et al., 2007),
67 France (Cadel-Six et al., 2007; Gugger et al., 2005), Germany (Bauer et al., 2020),
68 North America (Bouma-Gregson and Higgins, 2015; Puschner et al., 2008), the UK
69 (Edwards et al., 1992) and the Netherlands (Faassen et al., 2012). The rise of
70 intoxications due to BC has translated into an increasing bulk of studies in the last 5
71 years (Wood et al., 2020), especially focusing on the autecology of BC. While it is
72 widely accepted that BC can have a large ecological niche (Quiblier et al., 2013), the
73 environmental factors allowing their development in rivers are not fully understood
74 and often limited to specific taxa or species. For example, *Phormidium* sp. and
75 *Microcoleus* sp. have been extensively studied, but they may not represent the full
76 diversity of existing development strategies in BC (Wood et al., 2020).

77

78 Although studies have proven that temperature and nutrient inputs are major
79 factors acting on planktonic cyanobacteria (Paerl, 1988), the influence of these
80 parameters seems more contrasted for river BC. In rivers, BC also usually develop
81 under warmer water temperatures than the yearly average of the streams, i.e.,
82 exceeding 14 °C or 16 °C (in NZ and France, respectively; Echenique-Subiabre et
83 al., 2018; Heath et al., 2015), but the link between their development and nutrient

84 concentrations is less clear. Studies conducted in eutrophic rivers have shown a
85 preference of certain BC species for nutrient-rich environments (Loza et al., 2013),
86 whereas in oligotrophic rivers, BC species still developed because they were able to
87 use alternative sources of nutrients (McAllister, 2018; McAllister et al., 2018a). For
88 example, Wood et al. (2015) showed that entrapped fine sediments could provide
89 biofilm communities with dissolved reactive phosphorus concentrations on average
90 320 times higher than the concentration in the water column. In addition, the
91 development of nitrogen-fixing cyanobacteria within biofilms is not inhibited in
92 nutrient-poor environments (Douterelo et al., 2004; Perona et al., 1998). Considering
93 the complex structure and exchanges occurring within biofilms, these nitrogen-fixing
94 cyanobacteria could provide nutrients for other biofilm organisms (cyanobacteria and
95 microalgae) and thus enable the development of complex biofilm communities even
96 in oligotrophic waters (Stolz, 2000).

97

98 Hydrological conditions are also known to influence BC settlement and growth
99 in rivers (Hart et al., 2013; Stevenson, 2009). On one side, high-flow events may
100 remove BC (Heath et al., 2011). Wood et al. (2017) additionally stated that our ability
101 to predict the magnitude of peak flows necessary to scour off *Phormidium* cover
102 could vary among sampling sites. On the other side flow velocities represent one
103 niche axis for BC species (McAllister et al., 2019, 2018b). However, varying
104 responses to hydraulic constraints occur in the literature. Recent studies indicated
105 that BC could develop at flow velocities ranging from 0.3 to 1.1 m.s⁻¹ (Echenique-
106 Subiabre et al., 2018; Heath et al., 2015). In contrast, other studies from low
107 discharge rivers have shown that BC could develop with velocities <0.1 m.s⁻¹
108 (Sabater et al., 2003). Finally, Thomson-Laing et al. (2021) found no relationship

109 between near-bed velocity and accrual rates of *Microcoleus autumnalis* mats.
110 Substrate is also an important component, with large substrate sized (diameter
111 between 5-26 cm) and stable substrate being more favourable to BC development
112 (Echenique-Subiabre et al., 2018; Heath et al., 2015). Other physical parameters,
113 such as light intensity, may also influence BC development. Using water depth as a
114 proxy for received light intensity, Echenique-Subiabre et al. (2018) demonstrated that
115 biofilms expanded widely under low light (when water depth increased), whereas BC
116 thickness increased under high light to protect cells against ultraviolet radiation.

117 Finally, Wood et al. (2020) suggested potential competition between
118 cyanobacteria and other benthic algae for the colonisation of the substrate. Indeed,
119 cyanobacteria and filamentous green algae (Chlorophyta) can develop during biofilm
120 maturation (see also, Barranguet et al., 2005; Battin et al., 2003; Besemer et al.,
121 2007; Brasell et al., 2015; Majdi et al., 2012; Roeselers et al., 2007; Villanueva et al.,
122 2011), making them potential competitors for space and nutrients necessary for their
123 growth.

124

125 As a result, the above-listed BC ecological preferences make them more likely
126 to grow during spring and summer. European climate scenarios predict an increase
127 in water temperature and more variable rainfall patterns that will involve more
128 frequent and intense low-water periods (IPCC, 2018), which might favour BC
129 development (Codd et al., 2017). Such facilitation in development can be
130 exacerbated in regulated rivers. This is the case for the Ain River, a 190 km-long
131 tributary to the Rhône regulated by five dams (built on its main course between 1928
132 and 1968). During the summer season, this regulation involves long periods of low
133 flow and occasional high-flow events to flush out algal biofilm. This regulated river

134 represents an appropriate experimental situation for conducting experiments
135 assessing the relative contribution of biotic and abiotic factors to the development of
136 cyanobacterial communities in river biofilms.

137 In this study, based on our literature review, we hypothesise that the relative
138 contribution of hydraulics, physico-chemistry and the presence of other algae at
139 explaining the composition of benthic cyanobacteria assemblages should vary
140 according to the river regulation regime across summer. In particular, the
141 development of cyanobacteria assemblages should be (i) limited spatially and
142 temporally by hydraulic constraints both in terms of ecological preferences (high
143 velocity, small and unstable substrate), and in terms of high-flow events; (ii) favoured
144 by warmer water temperatures; (iii) weakly impacted by nutrient concentrations; and
145 (iv) limited by intrabiofilm competition with other phyla, such as Chlorophyta.

146

147 **2. Materials and methods**

148

149 *2.1. Sampling design*

150

151 Four locations along a 50-km stretch of river, each comprising 270- to 500-m-
152 long reaches, were selected using preliminary surveys of past occurrences of
153 benthic cyanobacteria (SR3A Ain River Authorities; Table 1). Five sampling
154 campaigns were performed during summer 2020 in four locations: 2-3 June (named
155 early June), 29-30 June (late June), 15-16 July (mid-July), 27-28 July (late July) and
156 24-25 August (late August). At each location, three hydraulic zones (HZ) were
157 delimited from maps resulting from a 2-D hydrodynamic model (INRAE/EDF). This 2-
158 D hydrodynamic model was first used by Judes et al. (2022) for relating the

159 abundance of fish and invertebrates species to variations of water depth and velocity
160 according to discharge variation. The model predicts mean water depth and velocity
161 in each grid cell (resolution of 4m^2) at 40% of the water depth measured from the
162 bottom in the cell for various discharges. This enables the assessment of past local
163 hydraulic variations of water depth and velocity. To extract values from the 2D-
164 hydrodynamic model, we considered discharges ranging from 15 to $40\text{ m}^3\cdot\text{s}^{-1}$, the
165 most frequent discharge values during spring and summer on the Lower Ain River
166 (i.e., this range had a frequency $\geq 50\%$ calculated from May to October in the last 5
167 years according to two gauging stations). In addition, the threshold of $0.8\text{ m}\cdot\text{s}^{-1}$ was
168 used in agreement with literature records to determine zones of velocities exceeding
169 BC preferences (Wood et al., 2020). Finally, we delimited (i) HZ#1 experiencing
170 velocities $\leq 0.8\text{ m}\cdot\text{s}^{-1}$ for flow between $20\text{ m}^3\cdot\text{s}^{-1}$ and $40\text{ m}^3\cdot\text{s}^{-1}$ and a low magnitude of
171 variation of velocities with increasing discharges ($<0.1\text{ m}\cdot\text{s}^{-1}$ for flow $<20\text{ m}^3\cdot\text{s}^{-1}$
172 remaining $<0.2\text{ m}\cdot\text{s}^{-1}$ for flow between $20\text{ m}^3\cdot\text{s}^{-1}$ and $40\text{ m}^3\cdot\text{s}^{-1}$); (ii) HZ#2
173 experiencing velocity $\leq 0.8\text{ m}\cdot\text{s}^{-1}$ for a low flow of $15\text{ m}^3\cdot\text{s}^{-1}$ and a high magnitude of
174 variation of velocities with increasing discharges ($<0.1\text{ m}\cdot\text{s}^{-1}$ for flow $<20\text{ m}^3\cdot\text{s}^{-1}$ going
175 $>0.2\text{ m}\cdot\text{s}^{-1}$ for flow between $20\text{ m}^3\cdot\text{s}^{-1}$ and $40\text{ m}^3\cdot\text{s}^{-1}$); and (iii) HZ#3 with velocities
176 $>0.8\text{ m}\cdot\text{s}^{-1}$ even for flows of $15\text{ m}^3\cdot\text{s}^{-1}$ (Fig. 1). It should be noted that depth variations
177 were similar for the three hydraulic zones for flow changes between $15\text{ m}^3\cdot\text{s}^{-1}$ and 40
178 $\text{m}^3\cdot\text{s}^{-1}$. From the literature review (see introduction), HZ#1 was considered
179 favourable to BC development for all discharge values, HZ#2 was considered
180 favourable at low discharge values ($<20\text{ m}^3\cdot\text{s}^{-1}$), and HZ#3 was considered
181 unfavourable at all discharge values. Stratified random sampling was further
182 performed according to the surface of each hydraulic zone in the four locations.
183 HZ#1 represented 38% of the surface of the sampled reaches, HZ#2 represented

184 50% and HZ#3 represented 12%. In coherence with the 2D-model design, units of 4
185 m² were used as sampling units. In total, 172 units were sampled over summer 2020
186 (65, 81, and 26 in HZ#1, HZ#2 and HZ#3, respectively, according to the surface of
187 each hydraulic zone).

188

189 *2.2. Field measurements*

190

191 *2.2.1. Benthic algae*

192

193 Biofilm samples were collected from three cobbles randomly selected within
194 each 4 m²-unit yielding a total of 516 biofilm samples. Both thin periphyton biofilms
195 and thick macroscopic biofilms were sampled (Fig.2). Each sample was scrubbed,
196 homogenised in river water, fixed with Lugol's iodine solution in the field and stored
197 in the dark until microscopic identification in the laboratory. We delimited the area
198 scrubbed on a given pebble using a piece of translucent paper. We further digitised
199 each piece of translucent paper with a scanner and determined the scrubbed area
200 with ImageJ software (Abràmoff et al., 2004).

201

202 *2.2.2. Environmental variables*

203

204 At each pebble, we measured the water depth (D in cm) with a meter stick
205 and the average flow velocity of the water column (U in m.s⁻¹, measured at 40% of
206 water depth from the pebble) with an electromagnetic current metre (FLO-MATE
207 2000, Cometec). At each 4 m²-unit, water temperature (WT), pH and conductivity
208 (EC) were measured with a multiparameter probe (AQUAREAD AP-5000). Water

209 samples were collected to determine ammonium (NH₄), nitrate (NO₃) and
210 orthophosphate (PO₄) concentrations, following standard colorimetric methods
211 (automatic analyser Smartchem 200, AMS Alliance, Roma, Italy). We estimated the
212 percentage cover of substrate types (sand, gravel, cobble and boulder) and derived
213 the mean grain size (GR). To take into account the recent hydrological changes in
214 the river associated with hydropeaking events, we considered the past 15-day
215 maximal discharge (Q15 in m³.s⁻¹) measured from two gauging stations maintained
216 by the RMC Water Agency: Pont d'Ain (Station V2712010) for site PA and Chazey-
217 sur-Ain (Station V2942010) for sites GI, BU and SJ (see Table 1 for acronyms).

218

219 *2.2.3. Taxa identification and counting*

220

221 Biofilm samples were homogenised before identification and enumeration.
222 Subsampling allowed the identification and counting of all algal cells using a light
223 microscope and a Malassez chamber. Photosynthetic communities were identified at
224 the genus level following several guidelines (Bourrelly, 1985, 1970, 1968, 1966;
225 Huber-Pestalozzi, 1968, 1961, 1955, 1941; John et al., 2002; Komárek and
226 Anagnostidis, 2005). Counts were further grouped into phyla for diatoms
227 (Bacillariophyta), Cyanobacteria and Chlorophyta, and less abundant taxa not
228 included in these phyla were grouped as others. The biovolume (μm³.cm⁻²) of each
229 phylum was calculated using PHYTOBS (2021) and ImageJ software (Rasband,
230 1997), which allowed scaling biovolume values to the pebble surface.

231

232 *2.3. Data analysis*

233

234 To compare each hydraulic variable between HZ and each physical and
235 chemical variable between campaigns, we used a Kruskal–Wallis test followed by a
236 Dunn’s test. The combined effect of campaigns and HZ on cyanobacteria
237 biovolumes was investigated with two-way ANOVA and post-hoc Tukey test.
238 Furthermore, to select drivers best explaining the composition of biofilm and
239 cyanobacteria assemblages, we performed a redundancy analysis (RDA; van den
240 Wollenberg, 1977) at each sampling campaign. RDA is a common multivariate
241 technique in ecology that allows to explain variation in a set of response variables
242 (e.g. cyanobacteria taxa) as a function of combinations of explanatory variables (e.g.
243 physical and chemical variables) (ter Braak and Prentice, 1988). Prior to RDA, the
244 variance inflation factor was used to detect multicollinearity between explanatory
245 variables (Dodge, 2008). Only variables with a $VIF < 4$ were included in the RDA
246 models. Variables in RDA models were selected by permutation tests using a
247 forward selection on the adjusted R^2 . In addition, the hydraulic zone (HZ) was used
248 as a conditioning variable in the model to assess its independent effect on
249 cyanobacteria assemblages. The significance of global RDA, of the first-two RDA
250 axes, and of each physical and chemical variable was further assessed by a
251 permutation test. In addition, variance partitioning (VP) allowed us to quantify the
252 amount of variance (by means of adjusted R^2 in RDA; Borcard et al., 1992) in the
253 cyanobacteria biovolumes explained by each group of explanatory variables, i.e. the
254 other biofilm algae (chlorophyta, diatoms, and other algae), hydraulic variables (Q15,
255 U, D, GR) and physical and chemical variables (WT, pH, EC, NH_4 , NO_3 , PO_4)
256 without removing collinear variables as recommended by Oksanen et al. (2020).
257 Biovolumes were log-transformed prior to statistical analysis to reduce numerical

258 discrepancies. All analyses were performed with R version 4.1.2 (R Core Team,
259 2021) using the package *vegan* (Oksanen et al., 2020).

260

261 **3. Results**

262

263 *3.1. Environmental context*

264

265 The first two campaigns occurred after a long period of high discharges >100
266 $\text{m}^3\cdot\text{s}^{-1}$ (maximum discharge = 224 and $117 \text{ m}^3\cdot\text{s}^{-1}$ in early and late June, respectively;
267 Fig. 3). The early June campaign occurred 15 days after the last dam release event,
268 while the late June campaign occurred only 5 days after the last dam release event.
269 The two July campaigns were performed during low and stable flows (12 to $17 \text{ m}^3\cdot\text{s}^{-1}$
270 1). From late July to August, dam releases led to three flushing flow events
271 (maximum discharge = $101 \text{ m}^3\cdot\text{s}^{-1}$, $99.3 \text{ m}^3\cdot\text{s}^{-1}$ and $97.9 \text{ m}^3\cdot\text{s}^{-1}$, respectively) in order
272 to scour off algae and cyanobacteria. The latter flushing event occurred 3 days
273 before the last sampling campaign (late August). As we could not measure flow
274 velocities in the field for these high discharges, they were predicted using the 2D-
275 hydrodynamic model (Fig.1). At $100 \text{ m}^3\cdot\text{s}^{-1}$, velocities ranged from 1.3 to $1.8 \text{ m}\cdot\text{s}^{-1}$ in
276 most of the river section, while at $200 \text{ m}^3\cdot\text{s}^{-1}$ velocity could reach $2.5 \text{ m}\cdot\text{s}^{-1}$ in the
277 middle of the river section.

278 The water velocity increased from HZ#1 to HZ#2 and HZ#3 as expected (Fig.
279 4; Kruskal–Wallis test, $P<0.0001$, $n = 172$). The depth was significantly lower in
280 HZ#1 (Kruskal–Wallis test, $P<0.0001$), while the mean grain size was coarser in
281 HZ#2 and HZ#3 than in HZ#1 (Kruskal–Wallis test, $P<0.0001$). NH_4 concentrations
282 were significantly lower in early June than in the other sampling campaigns and

283 significantly lower in late August than in mid-July (Table 2; Kruskal–Wallis test,
284 $P<0.0001$). The NO₃ concentrations were the highest in early June and the lowest in
285 mid-July (4.0 mg. L⁻¹ and 1.4 mg. L⁻¹, respectively; Kruskal–Wallis test, $P<0.0001$),
286 while concentrations in late June, July and August covered a similar range (i.e.,
287 between 2.3 and 2.8 mg.L⁻¹). PO₄ concentrations decreased significantly between
288 early and late June and reached the lowest values in mid-July (Kruskal–Wallis test,
289 $P<0.0001$). Finally, the water temperature was significantly higher in late July and
290 lower in mid-July (Kruskal–Wallis test, $P<0.0001$).

291

292 3.2. Biofilm composition

293

294 The mean cyanobacteria biovolume significantly differed over time and
295 hydraulic zones ($F_{8,157}= 5.36$, $p<0.0001$). It tended to decrease during the summer
296 period, ranging from 4.33E+07 to 1.18E+07 $\mu\text{m}^3.\text{cm}^{-2}$. Overall, cyanobacteria
297 biovolume was the highest in June (maximum in late June with 55. E+07 $\mu\text{m}^3.\text{cm}^{-2}$)
298 and the lowest in July (Tukey test, $P<0.05$). All campaigns combined, biovolumes
299 were significantly lower in HZ#1 than in HZ#2 and HZ#3 (Fig. 5; Tukey test, $P<0.05$).
300 Investigation of the combined effects of HZ and campaign revealed a high temporal
301 variability of cyanobacteria biovolumes in HZ#1, with mean biovolumes being higher
302 in the June and August campaigns than in the July campaigns (Tukey test, $P<0.01$),
303 while no significant differences appeared for HZ#2 and HZ#3. These low biovolumes
304 in HZ#1 occurred during campaigns characterised by stable low flow (mid-July and
305 late July).

306 A total of 23 genera of cyanobacteria were identified in the biofilm samples,
307 10 of which are known to comprise potentially toxic species such as *Anabaena* sp.,

308 *Leptolyngbya* sp., *Limnothrix* sp., *Lyngbya* sp., *Nodularia* sp., *Oscillatoria* sp.,
309 *Phormidium* sp., *Planktolyngbya* sp., *Planktothrix* sp. and *Pseudanabaena* sp.
310 (supplementary information; Table S1).

311 The 4 most abundant genera of cyanobacteria (mean relative contribution to
312 total cyanobacteria biovolume >10%) included *Lyngbya* sp., *Phormidium* sp.,
313 *Planktolyngbya* sp., *Oscillatoria* sp. (17.2%, 29.4%, 20.2% and 17.2%, respectively).
314 These genera were also the most frequently found in the samples (occurrence
315 >10%). They were present at each campaign, with the exception of *Planktolyngbya*
316 sp. being absent in mid-July only. Indeed, *Phormidium* sp. was the major component
317 of cyanobacteria biovolume (Fig. 6A). Its biovolume reached a maximum in mid-July
318 and late July during the low flow period and was significantly at its lowest in June
319 and August during periods of high flow variability. *Phormidium* sp. biovolumes were
320 significantly lower in HZ#1 than in HZ#2 (Tukey test, $P<0.05$). *Planktolyngbya* sp.
321 was the second major component of cyanobacteria biovolume (Fig. 6B);
322 *Planktolyngbya* sp. contribution significantly decreased from June to July (from 3-5%
323 to <0.4%) and was highest in late August (22.1%) after hydropeaking events.
324 *Planktolyngbya* sp. biovolumes were significantly lower in HZ#1 than in HZ#2 and
325 HZ#3 (Tukey test, $P<0.01$). *Lyngbya* sp. was more prominent in early June than in
326 July (Fig. 6C). During the low-flow period in mid-July, it was more abundant in HZ#3
327 than in HZ#1 and HZ#2 (Tukey test, $P<0.05$). Finally, *Oscillatoria* sp. did not exhibit
328 any spatiotemporal trends in its biovolumes across the campaigns or HZ (Fig. 6D).

329 Diatoms dominated the total biofilm composition in June, while Chlorophyta
330 increased greatly from mid-July (supplementary information, Table S2 and Fig. S1).

331

332 **3.3. Drivers of benthic cyanobacteria biovolume**

333

334 Abiotic and biotic variables used to investigate their influence for explaining
335 cyanobacteria assemblages at each campaign are available in the supplementary
336 information (Table S3). The overall variance of cyanobacteria biovolume explained
337 by the RDA models ranged between 17.3 and 41.7%, (i.e. constrained models using
338 the selection of significant variables), with the first canonical axis explaining between
339 16.0 and 25.7% of the total variance (Table 3). Past 15-day maximum discharge
340 (Q15) and local hydraulic variables (D, depth; U, flow velocity; and GR, mean grain
341 size) had a prominent influence throughout summer (R^2 ranging from 0.06 to 0.165).
342 In addition, chlorophyta biovolume explained 11.5% of the cyanobacteria biovolume
343 variance in mid-July. Finally, physical and chemical variables were mostly influential
344 in late August, with NH_4 and NO_3 being responsible for 11.2 and 10.3% of
345 cyanobacteria biovolume variance, respectively.

346 The amount of variance explained by each group of explanatory variables
347 ranged between 20.9 and 32.9% (Figure 7). Hydraulic variables were the main
348 source of variation in each campaign (variance explained ranged from 14.5% to
349 19.4%). Physico-chemistry and the biovolume of non-cyanobacteria algae
350 represented a secondary source of variation. The biovolume of other algae was
351 influential in late June and mid-July (14.0% and 10.5%). Physico-chemistry was a
352 significant source of variation only in early June (13.4%).

353

354 **4. Discussion**

355

356 Our study showed that past flow and local hydraulic patterns mainly
357 influenced the development of BC, as expected from our first hypothesis. However,

358 contrary to our first hypothesis, BC development was not limited by high water
359 velocities, which facilitated the development of some genera. Only periods with high
360 flow magnitudes and variations altered their development. However, large-sized and
361 stable substrates facilitated cyanobacterial development, as expected from our first
362 hypothesis. In agreement with our second hypothesis, warmer temperatures
363 favoured BC development when water temperature was the highest. In contrast,
364 nutrient concentrations had only a minor effect on BC, validating our third
365 hypothesis. Finally, in agreement with our fourth hypothesis, a negative correlation
366 between BC and other biofilm algae developments suggested intrabiofilm
367 competition in at least two campaigns (mid-July and late July).

368

369 4.1. Hydraulic conditions

370

371 The total cyanobacterial biovolume was overall smaller in HZ#1, and most
372 genera were positively related to high current velocity variation across discharge
373 (HZ#2 and HZ#3). Among the four most abundant cyanobacteria genera, some
374 occurred during stable flow periods (*Phormidium* sp. and *Oscillatoria* sp.), while
375 some others occurred during periods of high flow variations (*Lyngbya* sp. and
376 *Planktolyngbya* sp.). However, our study does not allow us to discuss in detail the
377 ecological preferences of each genus despite scrutinising this pattern, which needs
378 further investigation in the future. Our result showing that the most abundant
379 cyanobacteria genera were associated with high velocities (HZ#2 and HZ#3) is
380 somewhat in contradiction with the results of Sabater et al. (2003), who worked on a
381 low discharge river but in agreement with studies conducted in active dynamic rivers
382 (Bouma-Gregson et al., 2019; Echenique-Subiabre et al., 2018; Heath et al., 2015;

383 McAllister et al., 2019). This suggests that BC might be adapted to specific river
384 habitats, with different communities from one river to another depending on their
385 flowing regimes. In addition, as suggested by Wood et al. (2020), macroinvertebrate
386 colonisation might be reduced in hydraulic zones with high velocities, which may limit
387 grazers (see Hart et al., 2013 and McAllister et al., 2019 in Wood et al., 2020). This
388 could enable biofilms and BC to develop at higher biovolumes at high velocities.

389

390 RDA and variance partitioning showed that hydraulic variables were the major
391 drivers of BC biovolumes, and past flow and local hydraulic variables were influential
392 at every campaign. High discharge events ($>100 \text{ m}^3 \cdot \text{s}^{-1}$) decreased BC biovolume as
393 expected (e.g. Heath et al., 2011; Wood et al., 2017) in early summer (early June
394 and late June), whereas in late August, they did not induce any decrease in BC
395 biovolume. According to Heath et al. (2011), BC development increases when the
396 river discharge is below half the yearly average and water temperature increases. In
397 the Ain River, all the sampling campaigns occurred for discharge below half the
398 yearly average of $51.5 \text{ m}^3 \cdot \text{s}^{-1}$, but hydraulic conditions preceding sampling
399 campaigns differed greatly between campaigns. Even though Q15 in late August
400 was of similar magnitude to that in the June campaigns, it is possible that its short
401 duration (less than a few hours) was not sufficient to induce a decrease in BC
402 biovolumes. The high flow events of June presented a higher flow variation and
403 duration, which might be a key to reduce BC development, along with a high
404 discharge magnitude. Moreover, this suggests that during periods of favourable
405 environmental conditions on already developed BC, high discharge events may not
406 be as effective in decreasing BC biovolumes compared to early colonisation stages.
407 This may be seen as counterintuitive since as benthic algal biomass increased, drag

408 also increases, and high-flows can more easily remove biomass (e.g. Biggs et al.,
409 1998). For example, in New Zealand benthic cyanobacteria decreased at 2-3x
410 median/mean flows (Heath et al., 2011; Wood et al., 2017). Translated to the Ain
411 River that has a yearly average discharge of $103 \text{ m}^3 \cdot \text{s}^{-1}$, this would mean that
412 flushing flows with discharges from 206 to $309 \text{ m}^3 \cdot \text{s}^{-1}$ would be necessary to reduce
413 BC development. In our study, the highest discharge event in late August was of ~
414 $100 \text{ m}^3 \cdot \text{s}^{-1}$ (close to the yearly average discharge). At this discharge value, flow
415 velocities range from 1.3 to $1.8 \text{ m} \cdot \text{s}^{-1}$ in most of the river section, which is slightly
416 superior to previously recorded velocities for BC development (e.g. $1.1 \text{ m} \cdot \text{s}^{-1}$; Heath
417 et al., 2015) but might still be fitting their ecological niche. This discharge value may
418 not be high enough to scour off benthic cyanobacteria, and higher discharges should
419 be considered (for example, $200 \text{ m}^3 \cdot \text{s}^{-1}$).

420 Finally, a larger grain size facilitated the settlement of cyanobacteria, as
421 expected. This result is in agreement with previous results that showed a larger BC
422 coverage on stable substrates (cobbles and boulders), and BC coverage increased
423 with grain size (Echenique-Subiabre et al., 2018; Heath et al., 2015).

424

425 *4.2. Warming temperatures and nutrient concentrations*

426

427 BC development occurred despite the oligo-mesotrophic water quality of the
428 Ain River (according to nutrients thresholds provided by Loza et al., 2013),
429 suggesting non-limiting nutrient concentrations. Our results showed evidence for
430 nutrient concentrations influencing BC biovolume only in late August. According to
431 the reviews of Quiblier et al. (2013) and Wood et al. (2020) on drivers of BC
432 development, nutrients and other physical and chemical variables have been

433 reported to have an effect on BC in only a few studies. Moreover, their effect is
434 reported to be more important during the implantation phase of BC, which seems
435 consistent with our findings. Indeed, in our study, physical and chemical variables
436 had an influence in late summer after flushing flows that could have greatly modified
437 the ecosystem and led to a new colonization phase.

438

439 Nutrient concentrations only influenced BC biovolumes in late August:
440 cyanobacteria biovolumes were influenced positively by NH₄ and negatively by NO₃.
441 NH₄ availability probably favoured BC, as NH₄ is the most easily assimilated
442 nitrogen source (Bryant, 1994). The fact that nutrient concentrations did not influence
443 cyanobacteria biovolumes at other campaigns suggests that their development was
444 not nutrient-limited in the Ain river. Indeed, important BC proliferations have been
445 observed in very clear and oligotrophic rivers with lower NH₄, NO₃ and PO₄
446 concentrations than those measured in the Ain river (McAllister, 2018; McAllister et
447 al., 2018a). As nutrient uptake is faster in taxa with small cell sizes, such as
448 cyanobacteria, it can explain their dominance in low-nutrient conditions (Reynolds,
449 1989). Moreover, the increase in BC development and biofilm thickness during
450 summer might have allowed the uptake of phosphorus in sediments (Wood et al.,
451 2015), independently from dissolved phosphate concentrations.

452

453 Water temperature positively influenced BC development in late July. At this
454 campaign, the water temperature was the highest (20.9 ± 0.1 °C). This result is
455 coherent with findings showing that BC development is associated with temperatures
456 warmer than the seasonal average (>14 °C in New Zealand rivers, Heath et al.,
457 2015; >16 °C in the Tarn River, France, Echenique-Subiabre et al., 2018).

458

459 **4.3. Intrabiofilm competition**

460

461 In mid and late July, the cyanobacterial assemblages were negatively
462 correlated with those of other biofilm algae, suggesting a trend of intrabiofilm
463 competition between BC and other benthic algae (diatoms, chlorophyta and other
464 less abundant algae). This pattern could result from real spatial competition or from
465 different environmental preferences. Indeed, low and stable flow associated to flow
466 regulation might have allowed the fast development of other biofilm algae (mean
467 discharge = $14.9 \text{ m}^3 \cdot \text{s}^{-1}$ and $Q15 = 66.2 \text{ m}^3 \cdot \text{s}^{-1}$ in mid-July), along with an increase in
468 NH_4 concentrations, temperature and light availability. Under these conditions, we
469 hypothesise that the development of Chlorophyta was faster than that of
470 cyanobacteria and limited the growth of cyanobacteria. Indeed, under stable
471 conditions, the Chlorophyta growth rate is higher than that of cyanobacteria under
472 the same temperature conditions (Lüring et al., 2013). The growth of macroalgae
473 might have caused a light limitation for cyanobacterial cells in the biofilm, with the
474 filaments leading to shading (Dodds et al., 1999). However, cyanobacteria
475 development continued in high velocity zones and deep water zones, suggesting that
476 the competition with other algae for space and resources was more intense in zones
477 with low and stable velocity (e.g. HZ#1). Indeed, Chlorophyta were the most
478 prominent in HZ#1. Their development might have been limited by hydraulic
479 constraints in June and late August, leaving space for cyanobacteria to grow. This
480 would be coherent with findings showing that filamentous green algae have a low
481 tolerance to shear stress (Biggs and Thomsen, 1995). This pattern could also result
482 from the successional stages of biofilm development in river ecosystems. Biofilm

483 development generally starts with bacteria and diatoms (Azim and Asaeda, 2005;
484 Stal, 2012, 1995), followed by filamentous algae, mostly Chlorophyta, as a late
485 successional stage (Burns and Walker, 2000; Vadeboncoeur et al., 2021), which is
486 consistent with our findings showing the growth of Chlorophyta and other algae
487 overgrowing cyanobacteria in biofilms.

488

489 5. *Outcome*

490

491 This study allowed us to show the different environmental preferences of
492 cyanobacteria genera. Even if our RDA models had a low explanatory power when
493 constrained with the selection of significant variables, constrained models explained
494 between 35.7 to 59.2% of the variance of BC biovolumes. This low explanatory
495 power might be the result of the temporal variability in explanatory variables.
496 Different variables influenced BC biovolumes over time, and the same variable could
497 influence BC biovolumes differently at different campaigns, depending on the current
498 combination of environmental conditions. This unexplained variance might also come
499 from the number of genera implied in the model (23 genera), whose responses to
500 environmental variables could differ, inducing more variability. Overall, a dominant
501 pattern of environmental preferences was observed. Further analysis considering
502 each genus could help to determine their specific preferences.

503 Currently, we were able to show that hydraulic variables prominently shaped
504 BC assemblages in rivers, with varying responses over time. Discharges over 100
505 $\text{m}^3 \cdot \text{s}^{-1}$ only had a negative influence on cyanobacteria biovolumes when the flow
506 variability was high for at least two weeks before sampling, and did not influence BC
507 in late summer. Therefore, biofilm management through flushing flows of $100 \text{ m}^3 \cdot \text{s}^{-1}$

508 is not a viable solution to reduce biofilm development if it lasts less than one day.
509 Higher discharge variability, or longer flushing flows, should be considered to truly
510 affect BC development. Flushing flows of $200 \text{ m}^3 \cdot \text{s}^{-1}$ might be more effective for
511 scouring off benthic cyanobacteria by exceeding their ecological preferences in
512 terms of flow velocity. More importantly, discharge is a parameter that depends on
513 the studied river, therefore more precise hydraulic indicators, such as flow velocity,
514 should be used to make management recommendations. In light of our study, it
515 appears that flushing flows should induce flow velocities reaching 1.3 to $2.5 \text{ m} \cdot \text{s}^{-1}$ in
516 order to scour off benthic cyanobacteria. Further investigations could enable the
517 depiction of more precise hydraulic limitations to their development. Indeed, the
518 results from the 2-D hydrodynamic model could be used more extensively by
519 deriving fine hydraulic parameters from hourly discharge chronicles on the river
520 during the sampling period. For example, the number of hours above or under a
521 certain velocity value could be taken into account in a predictive model, as Judes et
522 al. (2022) performed to describe past hydraulic variables in their study. This opens
523 new perspectives in terms of biofilm management in regulated rivers in the future.

524

525 Similar to the recent study of Bauer et al. (2022) on benthic cyanobacteria,
526 our biofilm samples contained a variety of cyanobacteria genera, with only a few
527 dominant ones containing potentially toxic species. Among all identified
528 cyanobacteria genera, 10 out of 23 included species known to produce cyanotoxins,
529 and the four most abundant genera found in our samples are potentially toxic
530 (*Phormidium* sp., *Planktolyngbya* sp., *Lyngbya* sp., *Oscillatoria* sp.). Cyanotoxins
531 produced by these genera are mostly neurotoxic (Cadel-Six et al., 2007; Gugger et
532 al., 2005; Lajeunesse et al., 2012; Puschner et al., 2008; Seifert et al., 2007; Wood

533 et al., 2010, 2006). Therefore, BC development in the Ain River is associated with a
534 high toxicity potential, even though genetic analyses or toxin extraction would be
535 needed to better assess this toxic risk. Further analysis assessing toxin production
536 by these biofilms would determine the risk associated with their development and,
537 more importantly, determine the environmental parameters favouring cyanotoxin
538 production.

539

540 **Declaration of competing interest**

541 The authors declare that they have no known competing financial interests or
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544

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556

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801

802 **Table 1.** Sampling site characteristics (PA=Pont d'Ain, BU=Buchin, GI= Giron, SJ=St Jean de Niost)

Site	GPS coordinates (Lambert 93)	Distance to Allement dam (km)	Mean T°C ¹	Median discharge (m ³ .s ⁻¹) ¹	Geomorphology ²
PA	880109.5 ; 6552094.0	13.6	18.2 ± 3.0	14.5	Low lateral mobility, incised, paved
BU	873932.5 ; 6535675.0	35	18.9 ± 3.0	19.8	Low lateral mobility, stable channel
GI	872498.5 ; 6533919.5	38.6	18.9 ± 3.0	19.8	Low lateral mobility, stable channel
SJ	873516.0 ; 6529243.3	43.6	18.9 ± 3.0	19.8	Lateral mobility, dynamic channel

803
804 ¹ Calculated for the summer periods of the past 5 years (2014-2019, early June to late August)

805 ² From Rollet et al. (2014)

806

807 **Table 2.** Average values (n = 29; 30; 43; 40; 30; respectively) of physical and chemical variables measured at the five sampling
 808 campaigns in 2020 on the Lower Ain River.

	Discharge (m ³ .s ⁻¹)	Q15 (m ³ .s ⁻¹)	T°C	pH	NH4 (µg.L ⁻¹)	NO3 (mg.L ⁻¹)	PO4 (µg.L ⁻¹)
Early June	17.1	69.8	19.2 ± 1.8	8.1 ± 0.6	10.7 ± 4.3	4.0 ± 0.9	5.8 ± 2.8
Late June	20.1	124.4	19.5 ± 1.7	8.2 ± 0.1	28.2 ± 14.8	2.5 ± 0.9	3.5 ± 2.6
Mid-July	14.9	66.2	18.6 ± 0.8	8.1 ± 0.4	32.9 ± 14.6	1.4 ± 0.5	2.1 ± 2.0
Late July	13.5	45.9	20.9 ± 0.1	8.1 ± 0.3	29.7 ± 8.8	2.3 ± 0.7	4.1 ± 2.9
Late August	10.9	99.0	19.9 ± 1.2	8.3 ± 0.2	21.8 ± 10.5	2.8 ± 1.0	4.6 ± 3.0

809

810 **Table 3.** Evolution of the influence of biofilm algae (green), local hydraulics (light blue), past
811 hydraulics (dark blue) and physico-chemical variables (pink) on the biovolume of 23 genera of
812 cyanobacteria at 5 sampling campaigns in 2020 on the Ain River (RDA models) (GR, mean grain size;
813 Q15, maximum discharge 15 days before sampling; D, water depth; U, flow velocity; Chloro,
814 biovolume of Chlorophyta; Others, biovolume of other biofilm algae; NH₄, ammonium concentration;
815 NO₃, nitrate concentration; EC, conductivity). Dot size is proportional to the score of each genus or
816 each variable along the first RDA axis. Closed dots represent positive scores, open dots represent
817 negative scores, and the absence of points shows the absence of a genus in samples in this
818 campaign. For a given genus and a given variable, a similar dot filling indicates a similar positive
819 effect of the variable on the genus (e.g., Q15 and *Planktolyngbya* in early June or GR and
820 *Phormidium* in late June), whereas different dot filling (open vs. closed) indicates a negative effect of
821 the variable on the genus (e.g., Q15 and *Phormidium* in late June). In this table, genera are separated
822 into two groups according to their potential toxin production and are ordered according to their relative
823 contribution to the total cyanobacteria biovolume, from the most to the least contributing genus. The
824 percentage of variance explained by the conditional model, the constrained model, and the
825 constrained model including only the selection of significant variables and the two RDA axes are
826 indicated in the last five lines.

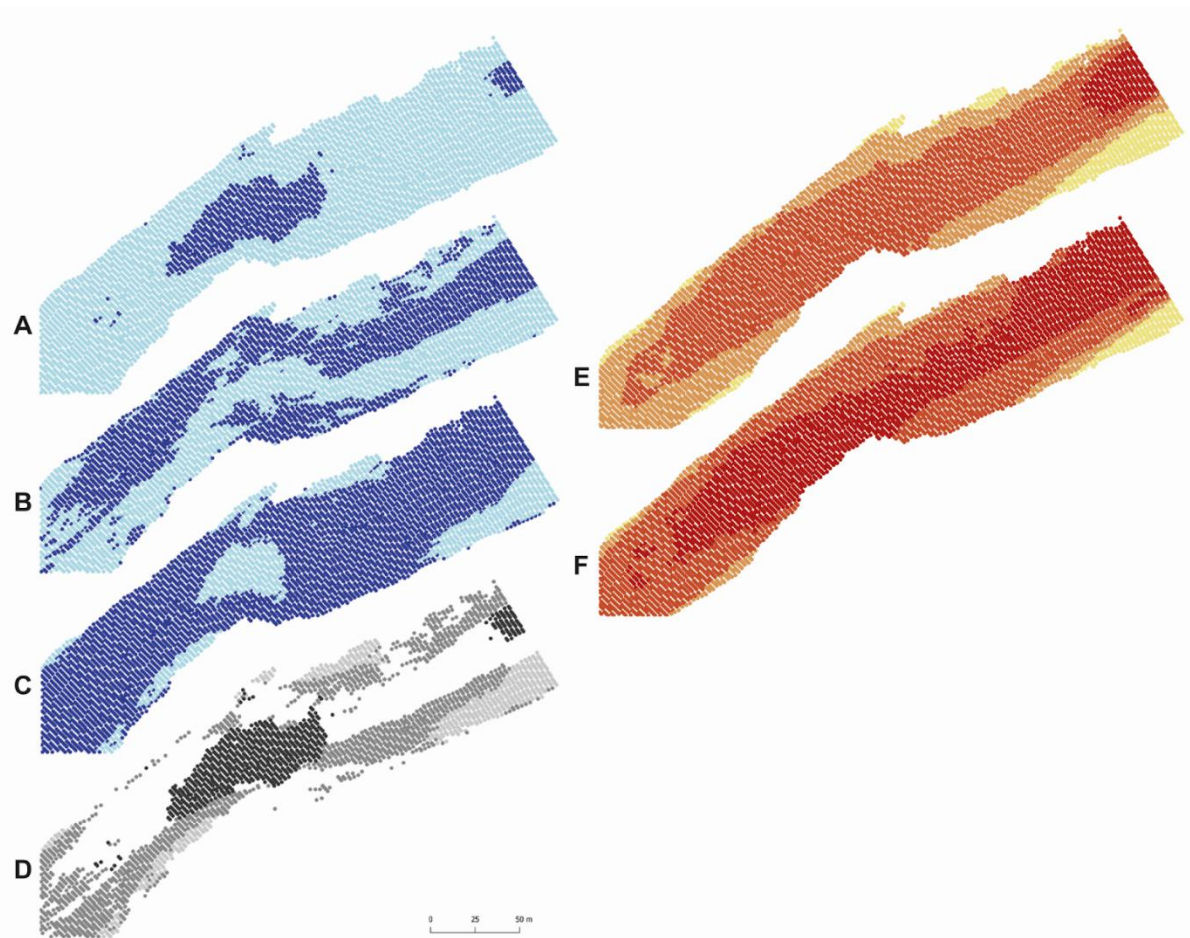
827

		Early June		Late June			Mid July				Late July			Late August		
Variable		Q15	EC	Q15	GR	pH	GR	Chloro	U	D	GR	Others	WT	NH4	NO3	D
Potential toxin-producing genera	<i>Phormidium</i>	●	●	○	○	○	○	○	○	○	●	○	●	○	○	○
	<i>Planktolyngbya</i>	○	○	●	●	●	○	○	○	○	●	●	●	○	○	○
	<i>Lyngbya</i>	●	●	○	○	○	○	○	○	○	●	●	●	○	○	○
	<i>Oscillatoria</i>	●	●	○	○	○	○	○	○	○	●	●	●	○	○	○
	<i>Pseudanabaena</i>	○	○	○	○	○	●	●	●	●	●	●	●	●	●	●
	<i>Anabaena</i>	○	○	○	○	○	○	○	○	○	●	●	●	○	○	○
	<i>Leptolyngbya</i>	●	●	●	●	●						●	●	●	○	○
	<i>Nodularia-like</i>															
	<i>Planktothrix</i>	●	●	○	○	○	○	○	○	○				○	○	○
	<i>Limnothrix</i>			○	○	○	○	○	○	○	●	●	●	○	○	○
	Non toxin-producing genera	<i>Merismopedia</i>			○	○	○	●	●	●	●				○	○
<i>Schizothrix</i>				○	○	○	○	○	○	○	●	●	●	○	○	○
<i>Pleurocapsa</i>		●	●								○	○	○			
<i>Myxosarcina</i>				○	○	○					●	●	●			
<i>Dermocarpa</i>														●	●	●
<i>Chroococcus</i>											○	○	○			
<i>Aphanothece</i>							○	○	○	○	●	●	●			
<i>Coelosphaerium</i>							○	○	○	○	○	○	○			
<i>Hyella</i>											●	●	●			
<i>Aphanocapsa</i>							○	○	○	○	●	●	●			
<i>Snowella</i>							●	●	●	●	●	●	●			
<i>Coelomoron</i>							○	○	○	○						
<i>Synechocystis</i>							○	○	○	○						
Variance explained (%)		Conditional	8.5		3.2			14.7				11.6			1.3	
	Constrained	40.5		59.2			35.9				35.7			53.5		
	Constrained*	27.0		41.7			18.9				17.3			21.4		
	RDA axis 1*	25.7**		20.8**			16.3**				16.0**			17.4**		
	RDA axis 2*	3.2		12.7**			4.2				2.0			4.3		

828

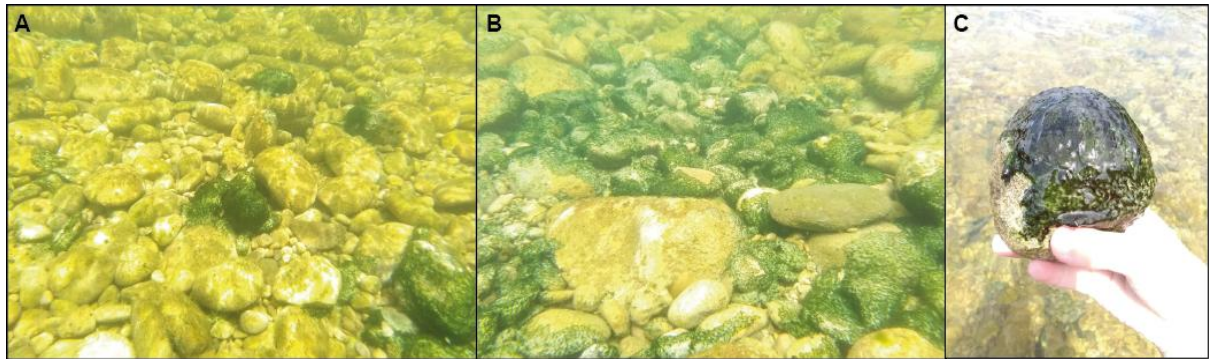
829 * model using the selection of significant variables

830 ** statistically significant from a permutation test



832

833 **Figure 1.** Example of delimitation of the three hydraulic zones from a 2-D hydraulic model in the
 834 upstream site (PA). (A) Distribution of water velocity for a discharge of $15 \text{ m}^3 \cdot \text{s}^{-1}$ (light blue $\leq 0.8 \text{ m} \cdot \text{s}^{-1}$,
 835 dark blue $> 0.8 \text{ m} \cdot \text{s}^{-1}$), allowing us to identify HZ#3 in dark blue; (B) distribution of velocity variation for
 836 discharges between 15 and $20 \text{ m}^3 \cdot \text{s}^{-1}$ (light blue $< 0.1 \text{ m} \cdot \text{s}^{-1}$, dark blue $> 0.1 \text{ m} \cdot \text{s}^{-1}$), allowing us to
 837 identify HZ#1 and HZ#2 in light blue; (C) distribution of velocity variation for discharges between 20
 838 and $40 \text{ m}^3 \cdot \text{s}^{-1}$ (light blue $< 0.2 \text{ m} \cdot \text{s}^{-1}$, dark blue $> 0.2 \text{ m} \cdot \text{s}^{-1}$), allowing us to discriminate HZ#1 from
 839 HZ#2; (D) selection of the final hydraulic zones with HZ#1 (light grey), HZ#2 (medium grey), and
 840 HZ#3 (dark grey). Water velocity predictions for discharges of $100 \text{ m}^3 \cdot \text{s}^{-1}$ (E) and $200 \text{ m}^3 \cdot \text{s}^{-1}$ (F)
 841 (yellow $\leq 0.8 \text{ m} \cdot \text{s}^{-1}$, orange $]0.8-1.3] \text{ m} \cdot \text{s}^{-1}$, light red $]1.3-1.8] \text{ m} \cdot \text{s}^{-1}$, and dark red $]1.8-2.5] \text{ m} \cdot \text{s}^{-1}$).

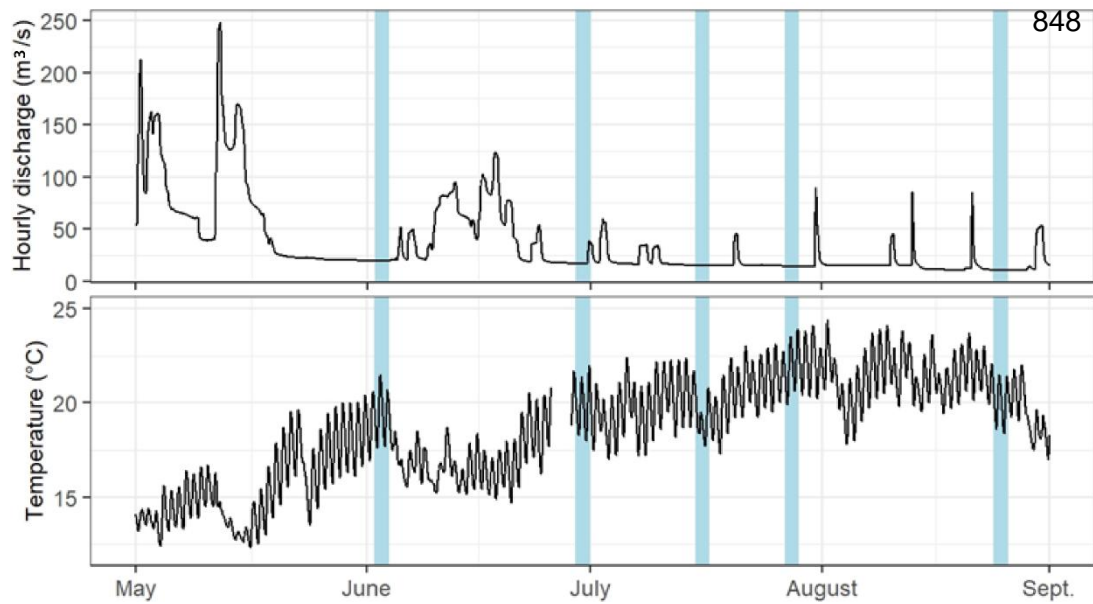


842

843 **Figure 2.** Examples of sampling plots presenting mostly thin periphyton biofilms (A)
844 and mostly thick macroscopic biofilms (B). Macroscopic biofilms were identified by
845 their dark-green colour (C).

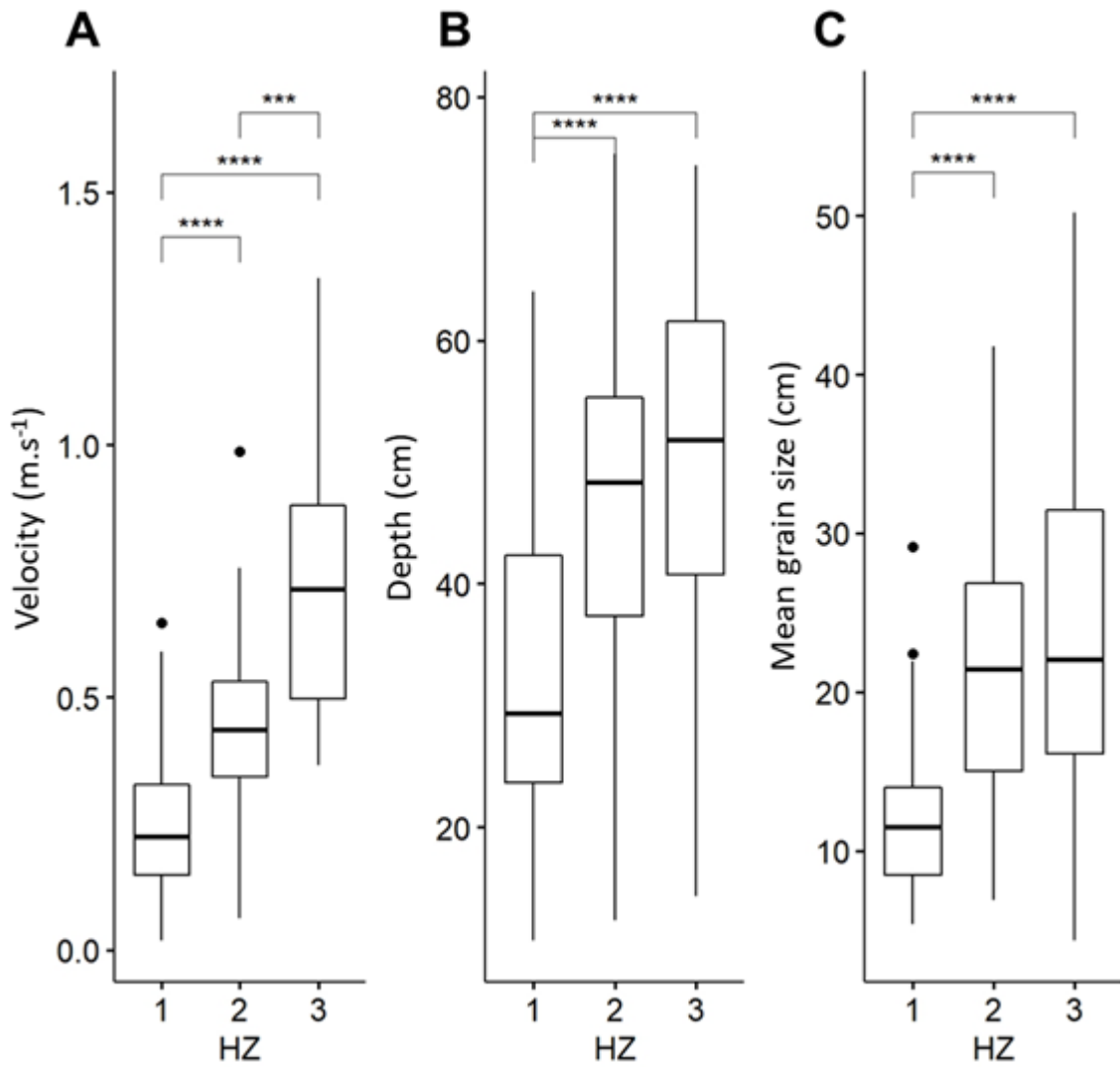
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847



849 **Figure 3.** Hourly discharge and water temperature at the gauging station and
850 multiparameter probe of Chazey-sur-Ain (RMC Water Agency, Station V2942010)
851 during summer 2020. Vertical blue bars correspond to the 5 sampling campaigns.
852 The absence of temperature data in late June was due to a probe malfunction.

853



854

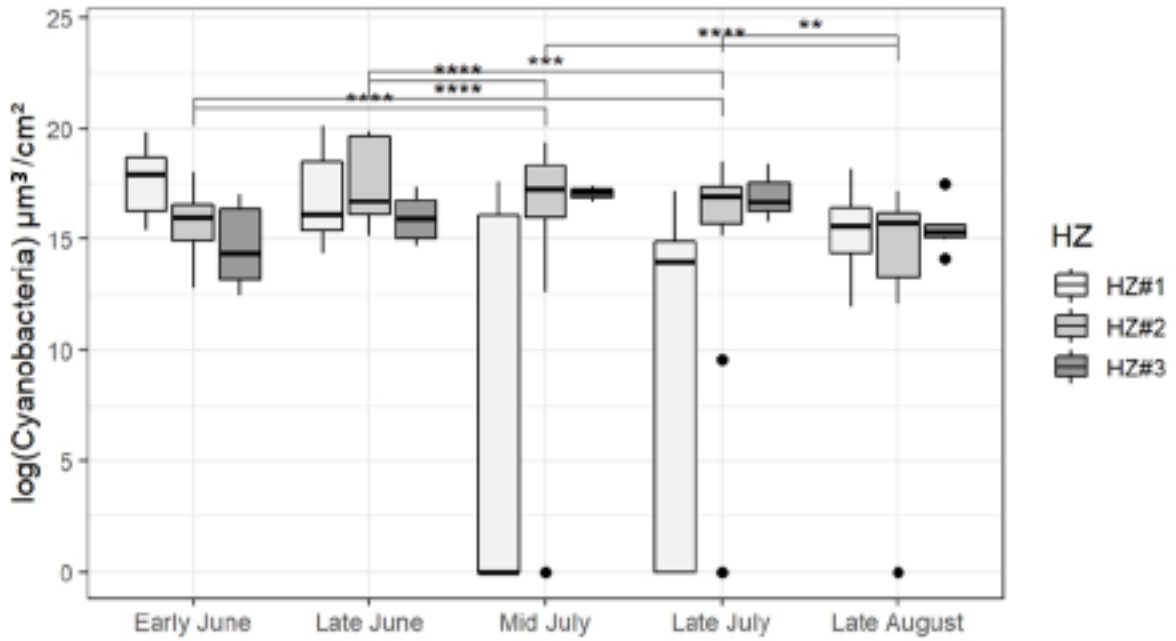
855

856 **Figure 4.** Variation in mean current velocity (A), depth (B) and mean grain size (C)

857 across the three HZs (Significant Kruskal–Wallis tests and Dunn’s tests of multiple

858 comparisons are shown with an asterisk).

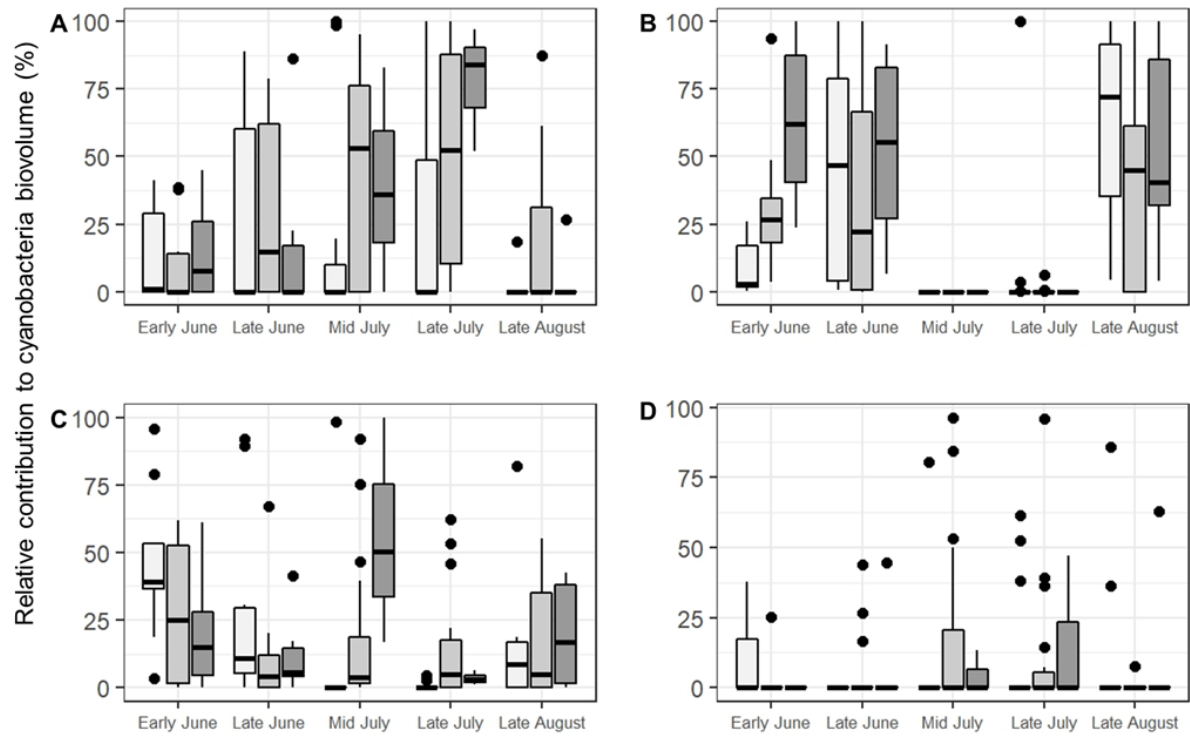
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860

861 **Figure 5.** Average biovolume of cyanobacteria collected at the 5 sampling
 862 campaigns separated by hydraulic zone (Significant Tukey tests of multiple
 863 comparisons are shown with an asterisk).

864

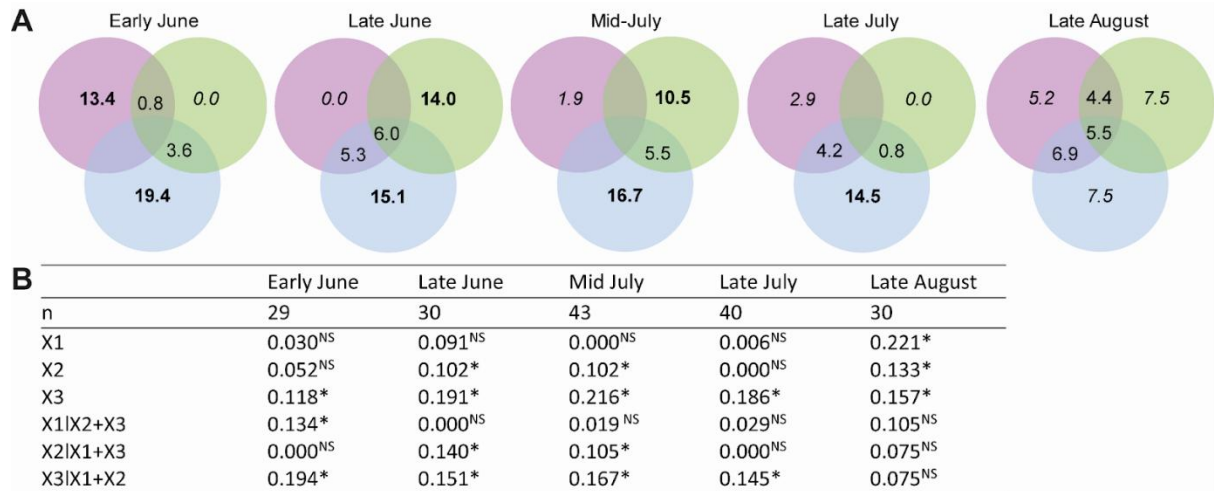


865

866

867 **Figure 6.** Mean relative contribution of the four most abundant genera (mean
 868 contribution > 15%) to the total biovolume in cyanobacteria at the 5 sampling
 869 campaigns and in each hydraulic zone: HZ#1 in light grey, HZ#2 in grey, HZ#3 in
 870 dark grey. (A) *Phormidium* sp., (B) *Planktolyngbya* sp., (C) *Lyngbya* sp., (D)
 871 *Oscillatoria* sp.

872



873

874 * statistically significant from a permutation test ($P < 0.05$)

875

876 **Figure 7.** (A) Percentage of variance of the cyanobacteria biovolumes explained by
 877 the three groups of explanatory variables: other biofilm algae in green (chlorophyta,
 878 diatoms, and other algae), hydraulic variables in blue (Q15, U, D, GR) and physical
 879 and chemical variables in pink (WT, pH, EC, NH₄, NO₃, PO₄). The residual variance
 880 at each campaign equals 75.1%, 69.1%, 68.9%, 79.1% and 67.1%, respectively. (B)
 881 Tests of significance of the amount of variance explained by the three explanatory
 882 tables alone or in combination (X1 = Physico-chemistry, X2 = Other algae, X3 =
 883 Hydraulics, n = number of plots).