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## Biofilm community composition is changing in remote mountain lakes with a relative increase in potentially toxigenic algae

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12 **Title page**

13 a) Manuscript title: **Biofilm community composition is changing in remote**  
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30

## 31 **Abstract**

32 Mountain lakes provide clear drinking water to humankind but are strongly impacted by global  
33 change. Productive benthic biofilms are crucial for maintaining water quality in these  
34 oligotrophic lakes, yet little is known about the effects of global change on biofilm  
35 communities. By combining analyses of metabarcoding data on 16S and 18S rRNA genes with  
36 climatic and environmental data, we were able to investigate global change effects on the  
37 composition of biofilm prokaryotic and micro-eukaryotic assemblages in a five-year  
38 monitoring program of 26 Pyrenean lakes (2016-2020). Data derived from meteorological  
39 observations indicate that climate has drastically changed at our study sites with an average  
40 increase of 1.5–2°C since 1950. Our results show that the composition of both prokaryotic and  
41 micro-eukaryotic assemblages of biofilm communities significantly shifted and their  
42 biodiversity declined over the study period. In particular, we observed an increase in the  
43 richness and relative abundance of cyanobacteria, including potentially toxigenic  
44 cyanobacteria, and a concomitant decrease in diatom richness and relative abundance.  
45 Variations in water pH and hardness were the main drivers of dissimilarity for both prokaryotic  
46 and micro-eukaryotic assemblages. Water pH and hardness increased in our lakes over the study  
47 period, and are known to increase in Pyrenean lakes due to the intensification of rock  
48 weathering as a result of climate change. Given predicted climate trends, the changes in benthic  
49 biofilms observed here might be further exacerbated in the future. Such biofilm compositional  
50 shifts may induce cascading effects in mountain food webs, threatening the resilience of the  
51 entire lake ecosystem. The rise in potentially toxigenic cyanobacteria also increases intoxication  
52 risks for humans, pets, wild animals, and livestock that use mountain lakes. Therefore, our study  
53 has implications for water quality, ecosystem health, public health, as well as local economies  
54 (farming, tourism), and highlights the possible impacts of global change on mountain lakes.

## 55 **Keywords**

56 climate change, cyanotoxins, metabarcoding, microbial diversity, time-decay relationships,

57 water quality

58

## 59 **1. Introduction**

60 Biodiversity is dramatically declining due to human activities (Díaz *et al.* 2019).  
61 Anthropogenic impacts have mainly been analysed for animals and plants, but much less  
62 attention has been paid to microbial communities (Cavicchioli *et al.* 2019). This is in stark  
63 contrast to their quantitative and qualitative importance for ecosystem functioning, as microbes  
64 are very abundant and diverse, play essential roles in many biogeochemical processes, and  
65 support the health of multicellular organisms and ecosystems (Cavicchioli *et al.* 2019; Sentenac  
66 *et al.* 2022). Therefore, microbes play a crucial role in the response of ecosystems to global  
67 environmental changes and, consequently, in their ability to keep providing goods and services  
68 to human populations (Cavicchioli *et al.* 2019; Sentenac *et al.* 2022). One of the most important  
69 goods provided through microbial processes is drinking water. Mountains have been named  
70 ‘the water towers of the world’, as more than half of the human population currently depends  
71 on water from mountains (Immerzeel *et al.* 2020; Viviroli *et al.* 2020). However, mountains  
72 and their freshwater ecosystems are threatened by global change, including climate change,  
73 nutrient and synthetic chemical pollution, land-use change, and the introduction of invasive  
74 species (Schmeller *et al.* 2022). This vulnerability directly threatens the quantity of water that  
75 mountains can deliver (Immerzeel *et al.* 2020; Schmeller *et al.* 2022), but we still know little  
76 about how global change affects the quality of mountain water through its impacts on aquatic  
77 microbiomes, in particular microbial communities in benthic biofilms.

78 Benthic biofilms are very diverse and highly organized matrix-enclosed communities  
79 which form the basis of food webs and a very abundant life-form in mountain freshwater  
80 ecosystems (Sentenac *et al.* 2022). As such, biofilms exert an important control on water quality  
81 by ensuring organic matter decomposition, primary production, nutrient cycling and by acting  
82 as a buffer against both synthetic and nutrient pollutants (Sentenac *et al.* 2022). Diatoms are  
83 normally abundant and important biofilm autotrophs because they produce nutrients such as  
84 polyunsaturated fatty acids, which are vital for a wide range of organisms to meet physiological  
85 requirements (Guo *et al.* 2015). Few organisms can synthesize polyunsaturated acids and most  
86 terrestrial vertebrates, for example, are consequently directly or indirectly dependent on aquatic  
87 ecosystems to obtain them (Hixson *et al.* 2015). However, diatoms are very sensitive to  
88 environmental changes and easily replaced by other more resistant photoautotrophs of lower  
89 dietary quality such as green algae and cyanobacteria (Guo *et al.* 2015; Leflaive *et al.* 2015;  
90 Crenier *et al.* 2019). In addition, cyanobacteria can significantly deteriorate water quality and  
91 ecosystem health, either by proliferating and outcompeting other lifeforms in regard to oxygen,  
92 light or nutrients, or by producing cyanotoxins that can have acute or chronic toxic effects for  
93 many organisms living in or dependent on aquatic ecosystems, including humans (Quiblier *et*  
94 *al.* 2013; Wood *et al.* 2020). The impacts of cyanobacteria on the health of domestic and wild  
95 animals is considerable, even causing mass mortalities (Roegner *et al.* 2014; Huisman *et al.*  
96 2018). In humans, acute exposure induces various digestive, respiratory and/or neurological  
97 disorders that can lead to death, while chronic exposure is linked with an increased incidence  
98 of liver cancer (Roegner *et al.* 2014). In contrast to planktonic cyanobacteria, little is known on  
99 the ecology of toxigenic (i.e. toxin-producing) benthic cyanobacteria (Wood *et al.* 2020).  
100 However, deaths of cattle and dogs due to ingestion of water containing cyanotoxins produced  
101 by biofilm cyanobacteria, or cyanobacteria-laden biofilm flocs (i.e. loose pieces of floating  
102 biofilms) have already been observed (Mez *et al.* 1997, 1998; Gugger *et al.* 2005). Therefore,

103 toxigenic benthic cyanobacteria represent a threat to human, animal and ecosystem health, as  
104 well as to local economies.

105         Yet, the composition of benthic biofilm communities in mountain ecosystems, and their  
106 trends in the context of global change, are poorly studied compared to other microbiomes  
107 (Wang *et al.* 2022). To date, biofilm micro-eukaryotic assemblages have rarely been  
108 investigated as a whole, but only for particular taxa (e.g. fungi or diatoms), and most studies  
109 have remained purely descriptive or highly restricted in their temporal and spatial scales,  
110 attempting to predict climate change effects through space-for-time substitution (Bartrons *et al.*  
111 2012; Teittinen *et al.* 2017; Yeh *et al.* 2019). These shortcomings restrict our understanding on  
112 how microbial communities and ecosystems respond to global environmental change. In this  
113 regard, benthic biofilms of mountain lakes offer an excellent opportunity to fill this knowledge  
114 gap (Catalan *et al.* 2006; Schmeller *et al.* 2018; Moser *et al.* 2019), as climate change impacts  
115 are particularly pronounced in mountains and represent one of the most important threats to  
116 mountain lake biodiversity (Pepin *et al.* 2015; Schmeller *et al.* 2022) . Climate change leads to  
117 increased water temperatures and evaporation, glacier retreat, alterations of aquatic mixing  
118 regimes, as well as changes in water chemistry mainly related to increased rock weathering and  
119 glacier runoff (Koinig *et al.* 1998; Pepin *et al.* 2015; Woolway *et al.* 2020). Depending on the  
120 lithology of the catchment and the initial levels of alkalinity and base cations, water pH can  
121 decrease (acidification: Skjelkvåle and Wright 1998) or increase (basification; Curtis *et al.*  
122 2009). All of these environmental changes are likely to have considerable consequences for the  
123 biodiversity and structure of benthic biofilm microbial assemblages.

124         Here, we used metabarcoding data on the 16S and 18S rRNA genes to study the  
125 successions of prokaryotic and micro-eukaryotic assemblages in biofilms over a five-year  
126 period in 26 mountain lakes spread across six elevational gradients of the French Pyrenees. We  
127 analysed climatic and water chemistry variables to assess their effects on the composition of

128 the benthic biofilm microbial assemblages and to identify temporal trends. We hypothesized  
129 that, due to observed environmental modifications, the composition of biofilm assemblages  
130 would change over time, with a potential loss of biodiversity and relative increase of  
131 cyanobacteria as well as a decrease in diatoms. Our study is the first to simultaneously assess  
132 the temporal trends and environmental drivers of prokaryotic and micro-eukaryotic  
133 assemblages of benthic biofilm communities in mountain lakes, with the aim to provide in-  
134 depth insights on aquatic biodiversity and water quality in these sensitive ecosystems under  
135 ongoing global change.

## 136 **2. Materials and methods**

### 137 **2.1. Biofilm taxonomic and community data**

138 We sampled biofilms of 26 lakes from six altitudinal gradients located from West to  
139 East in the French Pyrenees (**Figure 1, Table S1**). Lakes were visited in summer: twice in 2016;  
140 thrice in 2017 and 2018, and once both in 2019 and 2020 (for only a subset of the lakes in 2020).  
141 At each visit, epilithic (i.e. growing-on-rock) biofilms were sampled (n=235) at the same  
142 location in each lake, with a metal spatula (disinfected with chlorhexidine and rinsed with sterile  
143 water) by scraping four or five rocks located at a depth of 15-30 cm. Samples were directly  
144 frozen on dry ice. Biofilm DNA was extracted and purified from 400 mg of thawed sample  
145 using the NucleoSpin Soil kit<sup>TM</sup> (Macherey-Nagel<sup>TM</sup>, Düren, Germany) according to the  
146 manufacturer protocol. We amplified the V3-V4 region of the 16S rRNA gene (F:5'-  
147 CCTACGGGNGGCWGCAG and R:5'-GACTACHVGGGTATCTAATCC; Klindworth *et al.*  
148 (2013)) and the V8-V9 region of the 18S rRNA gene (F:5'-ATAACAGGTCTGTGATGCCCT  
149 and R: 5'-CCTTCYGCAGGTTACCTAC; Bradley *et al.* (2016)), respectively, by  
150 Polymerase Chain Reaction (PCR; for more details see **Appendix A**). Samples were sequenced  
151 on a MiSeq Illumina platform (2x250bp V3).



152 Demultiplexing and the removal of primer and adapter sequences was performed using  
153 Cutadapt v3.4 (Martin 2011). Additional trimming, formation of contiguous sequences,  
154 identification of unique amplicon sequence variants (ASVs), and chimera removal were  
155 performed in R v4.2.0 (R Core Team 2022) using the DADA2 v1.20.0 pipeline (Callahan *et al.*  
156 2016). Taxonomy of ASVs of both 16S and 18S rRNA genes was assigned using SINA v1.7.2  
157 and the SILVA 138.1 reference database (Pruesse *et al.* 2012; Quast *et al.* 2013). For the 16S  
158 rRNA gene library, ASVs unclassified at the class level, or (mis)classified as eukaryotes,  
159 chloroplasts, or mitochondria were removed in R. For the 18S rRNA library, unclassified ASVs  
160 at the second and third taxonomic ranks (in general corresponding to the superkingdom or  
161 superphylum levels, respectively, but that depends on the ASV) as well as Metazoan taxa  
162 belonging to Vertebrata, Arthropoda, Platyhelminthes, Annelida, and Mollusca were removed.  
163 We excluded four and six samples due to low total number of reads (<1,000) in the prokaryotic  
164 and micro-eukaryotic libraries, respectively.

165 We used the Chao1 index for  $\alpha$ -diversity, which estimates richness while accounting for  
166 differences in library sizes (Chao and Chiu 2016), calculated on the raw dataset with the  
167 function `estimateR` of package *vegan* (Oksanen *et al.* 2022). We also used the Chao 1 index to  
168 estimate the richness of indicator taxa (cyanobacteria, toxigenic cyanobacteria and diatoms) in  
169 each sample. Potentially toxigenic cyanobacteria were identified as such when we detected  
170 cyanobacterial genera known to produce cyanotoxins while living in biofilms (Quiblier *et al.*  
171 2013). For all other analyses, we used the compositional approach of Gloor *et al.* (2017). First,  
172 datasets were filtered out in R to only keep ASVs having at least five reads in at least five  
173 samples, to eliminate rare ASVs resulting from sequencing errors. Then, a centered-log-ratio  
174 (clr) transformation of the relative abundance data was performed (hereafter termed  
175 “transformed abundance”), with the function `transform` (method = “clr”) of package  
176 *microbiome* (Lahti and Shetty 2012). The Aitchison distance (Aitchison 1982) was then

177 obtained with the *vegan* function `vegdist` (method = “euclidean”) and used as an index for  
178 pairwise  $\beta$ -diversity (Gloor *et al.* 2017). To ensure the robustness of our results, we repeated all  
179 analyses using i) Total Sum Scaled (TSS) data and ii) rarefaction (**Appendix A**), which are  
180 other approaches to account for differences in sample library sizes, but they produced similar  
181 results (**Figures S11-S16, Table S5**).

## 182 **2.2. Environmental metadata**

183 We retained hardness, pH, chloride (denoted Cl), sodium (Na), potassium (K), total  
184 copper (TCu), total organic carbon (TC), total nitrogen (TN), and biogenic silica (SiO<sub>2</sub>)  
185 amongst other measured variables (**Appendix A**). Even if pH and SiO<sub>2</sub> had many missing  
186 values, we retained them given their known importance in shaping microbial communities  
187 (Curtis *et al.* 2009). Missing values were filled with Multivariate Imputation by Chained  
188 Equation (R package *MICE*; Buuren and Groothuis-Oudshoorn 2011) i) using the method  
189 *2L.lmer* for hardness, chloride and potassium, with time as fixed effect and lake as random  
190 effect, because a temporal trend in each lake was detected for these variables; ii) using again  
191 the same method for pH, but with hardness and time as fixed effects, due to the existing  
192 correlation between hardness and pH ( $r_{48} = 0.62$ ,  $p < 0.001$ ); iii) using the default method  
193 *predictive mean matching* for other variables for which no trend was initially detected. Because  
194 pH and hardness were not independent, we clustered them together with the R package  
195 *ClustOfVar* (Chavent *et al.* 2011), which clusters correlated variables and create one synthetic  
196 variable per cluster. We obtained a synthetic variable, termed pH-hardness ( $r = 0.96$  with pH  
197 and hardness). Using public E-OBS climate datasets on daily precipitation and air temperature  
198 as well as our own water temperature data, we described climatic changes in the French  
199 Pyrenees along the different altitudinal gradients (**Appendix A**). In addition, we computed for  
200 each sample i) the mean air temperature from the 15<sup>th</sup> of November to the 1<sup>st</sup> of June to capture  
201 winter temperatures prior to sampling (denoted LT\_T for long-term temperatures), ii) the sum

202 of precipitations over this winter period (denoted LT\_PRCP), and iii) the mean air and water  
203 temperatures over three weeks prior to sampling. As both were highly correlated ( $r = 0.73$ ,  $p <$   
204  $0.001$ ), they were clustered using *ClustOfVar* in a synthetic variable denoted recentT ( $r = 0.93$   
205 with mean air and water temperatures). We also computed iv) the sum of precipitations over  
206 three weeks prior to sampling (denoted recentPRCP), and v) the average daily temperature  
207 range over three weeks prior to sampling (being right-skewed, it was log-transformed and  
208 denoted as ln.wDTR). In total, we retained 14 environmental predictors (all centered and  
209 scaled): recentPRCP, recentT, LT\_T, LT\_PRCP, ln.wDTR, pH-hardness, SiO<sub>2</sub>, TC, TN, Cl,  
210 Na, K, TCu, and lake area.

### 211 **2.3. Temporal trends in biofilm biodiversity.**

212 Using linear mixed models (LMMs) fitted with package *lmerTest* (Kuznetsova *et al.*  
213 2017), we tested the effect of time (fixed effect) on eight different response variables: the Chao1  
214 indices of i) the whole prokaryotic and ii) micro-eukaryotic assemblages, of iii) cyanobacteria,  
215 iv) potentially toxigenic cyanobacteria, v) and diatoms and the transformed abundances of vi)  
216 cyanobacteria, vii) potentially toxigenic cyanobacteria, and viii) diatoms. We included time as  
217 random slope and lake as random intercept. We also tested whether the temporal trends for  
218 these eight response variables differed between Eastern and Western gradients which exhibited  
219 different climatic conditions.

220 To explore within-lake temporal dissimilarity, we used two approaches. First, we  
221 measured time-decay relationships (TDRs) of the prokaryotic and micro-eukaryotic  
222 assemblages, using a linear regression between Aitchison distance (pairwise dissimilarity) and  
223 logarithmic temporal distance (between any two samples) with time as random slope and lake  
224 as random intercept. As in Guo *et al.* (2018), we partitioned our time series data into subset  
225 windows of one year (four windows: 2016-17, 2017-18, etc), two years (2016-18, 2017-2019,  
226 etc.), three years, and four years and restricted pairwise comparisons to samples taken in a same

227 lake at the same period of the year (to remove seasonal variation). For each year, we defined  
228 three periods which correspond to our three sampling campaigns: i) before mid-July (spring in  
229 mountains), ii) from mid-July to end August (summer), and iii) September onward (fall). The  
230 TDR model estimates a general slope  $\nu$  (the TDR value), which reflects the temporal turnover  
231 rate of microbial assemblages (Guo *et al.* 2018). We also looked whether these temporal  
232 turnover rates differed between the western and eastern groups. Second, we used a Global  
233 Dissimilarity Model (GDM, Ferrier *et al.* 2007) with the temporal approach of Blois *et al.*  
234 (2013) and comparisons restricted to samples belonging to the same lake and period (as above).  
235 To determine the environmental drivers associated with the temporal dissimilarity, we included  
236 in the model the 14 retained environmental predictors. We used the `gdm.varImp` function of  
237 package *gdm* to assess the respective importance and significance of each predictor, as well as  
238 the significance explained by the full model (Fitzpatrick *et al.* 2022).

#### 239 **2.4. Environmental drivers of biofilm biodiversity**

240 We used the 14 retained environmental variables as fixed effects in eight LMMs with  
241 the same eight response variables detailed in the previous section, but only with lake random  
242 intercept models. After fitting, collinearity was assessed with the package *performance*  
243 (Lüdecke *et al.* 2021) to ensure that the Variance Inflation Factor was low ( $VIF < 5$ ) for each  
244 predictor. We then fitted eight other models with only location (East or West) as categorical  
245 predictor and lake as random effect. We used GDMs to determine the environmental factors  
246 explaining variation in the prokaryotic and micro-eukaryotic assemblages. GDMs relate  
247 pairwise dissimilarity (here, the scaled Aitchison distance, as the dissimilarity must be between  
248 0 and 1) to environmental and spatial ('geographical') distances between samples. The  
249 significance of the GDMs and the predictors, as well as predictor importance (deviance  
250 explained by that predictor), were determined using the `gdm.varImp` function with 1,000  
251 permutations. Pairwise comparisons were restricted to samples taken at the same period of the

252 same year, to obtain the drivers of between-lake dissimilarity and eliminate the influence of  
253 season and time. We drew Principal Component Analysis (PCA) ordination plots and  
254 performed permutational analysis of variance (PERMANOVA) with 1,000 permutations, after  
255 testing for heterogeneity in multivariate dispersion (Anderson 2006, 2017) to respectively  
256 visualize and test potential compositional differences between western and eastern gradients  
257 with the package *vegan*.

## 258 **3. Results**

### 259 **3.1. Climatic and environmental changes in Pyrenean lakes under study**

260 Since 1950, the annual mean temperature has increased between 1.63 and 1.92°C in our  
261 gradients, but other aspects of climate change differ markedly between eastern (gradients Arbu-  
262 lers-Estagnon, Bassies, Bethmale) and western sites (Neouvielle, Fache, Lescun; **Figures 1 &**  
263 **2, Table S2**). The annual maximum temperature (TXx) and the number of extremely hot days  
264 (WSDI) increased significantly in the east, with total precipitations (PRCPTOT) being stable  
265 and their intensity (SDII) decreasing. In contrast, PRCPTOT and minimum temperatures  
266 considerably increased in the west, the latter resulting in a reduced daily temperature range  
267 (**Figure 2**). Based on air temperature and in-situ logged water temperature data, our model  
268 showed that average annual water temperatures in the ice-free period significantly increased in  
269 almost all our lakes from 2007 to 2021, especially in eastern sites (**Figure S1, Table S3**).

270 While no trend was detectable in any of the lakes when taken alone, some water  
271 chemical variables showed significant trends when the whole dataset was analysed with lake as  
272 random effect (**Figures S2-S4, Table S3**). Hardness and potassium had a significant overall  
273 positive temporal trend, whereas chloride had a significantly negative temporal trend. These  
274 trends did not differ between western and eastern sites (**Table S3**). There was no significant  
275 overall temporal trend for sodium.

## 276 **3.2. Taxonomic overview**

277 We obtained 231 prokaryotic and 229 micro-eukaryotic libraries from 26 Pyrenean lakes  
278 during the sampling period 2016-2020, for which we identified a total of 44,165 and 19,320  
279 ASVs, respectively. Cyanobacteria (in particular the class Cyanobacteriia) and Proteobacteria  
280 (now called Pseudomonadota, mainly represented by  $\alpha$ - and  $\gamma$ -Proteobacteria) were the two  
281 main phyla in benthic biofilm prokaryotic assemblages (**Figure S5-S6**). The micro-eukaryotic  
282 communities were dominated by three supergroups: the Archaeplastida (i.e. plants, in particular  
283 the green algae belonging to the class of Chlorophyceae), the Stramenopiles-Alveolata-Rhizaria  
284 (SAR, with notably the Diatomea class), and the Amorphea, a vast supergroup gathering the  
285 animal and fungal kingdoms (**Figure S7** and **S8**). The PERMANOVA showed that  
286 compositions of both biofilm prokaryotic and micro-eukaryotic assemblages were statistically  
287 different between eastern and western sites ( $p = 0.001$  for both). However, there was a  
288 significant heterogeneity in multivariate dispersion between these two groups for micro-  
289 eukaryotes, but not prokaryotes ( $p = 0.032$  and  $p = 0.715$ , respectively, see also **Figure S10**).

## 290 **3.3. Temporal trends in biofilm diversity and indicator taxa**

291 We found a significant decline in prokaryotic, micro-eukaryotic, and diatom richness,  
292 and a significant increase in richness and transformed abundance of both cyanobacteria and  
293 toxigenic cyanobacteria (**Table 1, Figure 3**). Time-decay relationship analyses revealed that  
294 the Aitchison distance between biofilm assemblages (comparing samples taken in a same lake  
295 and the same period over different years) significantly increased with temporal distance, both  
296 for prokaryotes and micro-eukaryotes (**Table 1, Figure 4**). The GDMs supported these results:  
297 temporal distance was a significant predictor of prokaryotic within-lake dissimilarity  
298 (importance = 49.29,  $p = 0.010$ : deviance explained by the full model = 12.23%, model  $p =$   
299 0.001) and a marginally significant one for micro-eukaryotic within-lake dissimilarity  
300 (importance = 14.33,  $p = 0.090$ , 15.94%, model  $p < 0.001$ , **Figure 4**). The latter was also driven

301 by variations in pH-hardness (importance = 25.33,  $p = 0.035$ ). For none of the studied response  
302 variables were there significant differences between the temporal trends of the western and  
303 eastern sites (**Table 1**).

### 304 **3.4. Environmental drivers of biofilm diversity and indicator taxa**

305 Drivers of the estimated richness (Chao1 index, proxy for  $\alpha$ -diversity) of both the  
306 prokaryotic and micro-eukaryotic assemblages, and of the estimated richness and transformed  
307 abundance of indicator taxa, are shown in **Figure 5** and **Table S4**. The GDM of between-lake  
308 prokaryotic dissimilarity (deviance explained = 16.60%, model  $p < 0.001$ ) showed that  
309 Aitchison distance was greater with increasing differences in pH-hardness (importance = 44.34,  
310  $p < 0.001$ ) and biogenic silica (15.12,  $p = 0.012$ ). Similar results were found for the micro-  
311 eukaryotic assemblage (deviance explained = 15.58%, model  $p < 0.001$ ), with pH-hardness  
312 (importance = 22.6,  $p = 0.018$ ), biogenic silica (12.32,  $p = 0.055$ ) and geographic distance  
313 (importance = 2.76,  $p = 0.017$ ) being the predictors best explaining between-lake dissimilarity  
314 (**Figure 4, Table S4**).

## 315 **4. Discussion**

316 We studied the composition of prokaryotic and micro-eukaryotic assemblages of  
317 benthic biofilm communities in 26 mountain lakes of the French Pyrenees from 2016 to 2020  
318 and linked it to climate and environmental changes. Our study clearly shows that the  
319 compositions of both prokaryotic and micro-eukaryotic assemblages have significantly shifted  
320 and their diversity has significantly declined during the study period. These changes were more  
321 pronounced for prokaryotes than microeukaryotes and translated into a significant increase in  
322 cyanobacteria (including toxigenic ones) richness and relative abundance, and a significant  
323 decrease in diatom richness and relative abundance. Our findings suggest that ongoing global  
324 change in the Pyrenees is leading to important alterations in benthic microbial communities in

325 mountain lakes, mainly through changes in water acidity and hardness, with potentially  
326 important consequences for water quality and ecosystem functioning.

327         The relative increase in potentially toxigenic benthic cyanobacteria observed in our  
328 study is of particular concern for water quality as cyanotoxin poisoning presents a non-  
329 negligible risk for humans and animals (Roegner *et al.* 2014; Huisman *et al.* 2018). While we  
330 acknowledge that toxin production is not systematic but context dependent, we observed a high  
331 proportion of *Phormidium* and, to a lesser extent *Oscillatoria*, two genera responsible for  
332 numerous animal deaths following biofilm and/or cyanotoxin ingestion in French mountain  
333 ranges (Mez *et al.* 1997; Gugger *et al.* 2005, **Figure S9**). Mountain lakes are popular touristic  
334 areas (hiking, fishing) and often the sole source of water for livestock (Mayer *et al.* 2022;  
335 Schmeller *et al.* 2022). Humans as well as domestic and wild animals may be exposed to  
336 cyanotoxin by ingesting contaminated water or by eating intoxicated fish (Buratti *et al.* 2017;  
337 Schmeller *et al.* 2022). The potential presence of cyanotoxins would also ultimately raise the  
338 costs inherent in drinking water production (Roegner *et al.* 2014). Furthermore, when mountain  
339 lakes experience substantial benthic cyanobacteria blooms, oxygen levels at the sediment-water  
340 interface and even in the water column can drop dramatically, leading to hypoxia or even anoxia  
341 (Vadeboncoeur *et al.* 2020). Oxygen deprivation has profound consequences for aquatic food  
342 webs, particularly in naturally highly oxygenated mountain water bodies (Brune *et al.* 2000;  
343 Schuster *et al.* 2021). Additionally, dense phytoplankton or benthic filamentous blooms may  
344 decrease light penetration to the bottom and further impact benthic flora and fauna with  
345 cascading effects to the entire lake ecosystem (Brothers *et al.* 2013). Changes in mountain lake  
346 biofilms may also have nutritional consequences for food webs due to the relative decline in  
347 highly nutritious diatoms. Combined with the increase in cyanobacteria, this relative decline  
348 could mean that the availability of polyunsaturated fatty acids is reduced, with deleterious  
349 health consequences on consumers (Crenier *et al.* 2019), and difficult-to-predict cascading



350 effects on all organisms in aquatic food webs and beyond. These nutritional consequences are  
351 especially concerning in the context of mountain ecosystems, which are usually oligotrophic  
352 (Schmeller *et al.* 2018).

353 Our study confirms that climate change has been pronounced in the Pyrenees over the  
354 last decades (Pepin *et al.* 2015; Schmeller *et al.* 2022) and we argue that this might have various  
355 direct and indirect impacts on mountain lake biofilms. Regarding direct effects, variations in  
356 winter precipitations, both between years (for a same lake) and between lake (for a same year),  
357 were associated with changes in biofilm prokaryotic and micro-eukaryotic compositions when  
358 other methods than clr-transformation were used to process the data. Warmer and drier winters  
359 significantly and negatively impacted the richness and transformed abundance of  
360 cyanobacteria, toxigenic cyanobacteria and diatoms. With temperatures generally rising and  
361 precipitations increasing only in some places, our results suggest that cyanobacteria might  
362 especially increase in the Western Pyrenees. In addition to these direct impacts, our study  
363 indicates that climate change could also indirectly impact biofilms through modifications of  
364 lake water chemistry. First, we found that hardness generally increased in our study sites,  
365 suggesting that pH also increased. This is supported by other studies showing that pH and  
366 hardness increased in mountain lakes presumably as a consequence of intensified climate-  
367 induced rock weathering (in the Pyrenees: Curtis *et al.* 2009; in the Alps: Rogora *et al.* 2020).  
368 Second, our study identifies pH and water hardness as one of, if not, the main environmental  
369 determinants of biofilm microbial composition: hardness and pH had the most important and  
370 significant impact on between-lake (spatial) dissimilarity for both prokaryotes and micro-  
371 eukaryotes, as well as on within-lake (temporal) dissimilarity especially for micro-eukaryotes  
372 (with TSS data only for prokaryotes). Hardness and pH also had significant effects on micro-  
373 eukaryotic  $\alpha$ -diversity, notably on diatom richness and relative abundance. Taken together, our  
374 results suggest that, if climate-change-induced basification and changes in water hardness

375 continue to occur in Pyrenean lakes, the prokaryotic and micro-eukaryotic assemblages of  
376 microbial biofilm communities will further change. This is not to mention other pressures on  
377 mountain freshwater ecosystems, such as chemical pollution (Machate *et al.* 2022, 2023),  
378 eutrophication through livestock and atmospheric deposition (Oleksy *et al.* 2020a; Schmeller  
379 *et al.* 2022), invasive species, as well as fish introductions (Moser *et al.* 2019; Schmeller *et al.*  
380 2022). Considering this, we can expect biofilms to enter unprecedented ecological states  
381 dominated by benthic cyanobacteria, including potentially toxigenic ones, and providing  
382 ecosystem disservices instead of services (Oleksy *et al.* 2020b).

## 383 **5. Conclusion**

384 Our study indicates that the composition of biofilms in French Pyrenean mountain lakes  
385 has rapidly shifted and their quality deteriorated. Such changes in biofilms may have profound  
386 negative consequences for water quality as well as ecosystem, animal, and human health and  
387 shed light on the possible impacts of ongoing global change in lakes of mountain ranges around  
388 the world. The implications extend to public health and also local economies as toxigenic  
389 cyanobacteria threaten tourism and farming, and might cause the costs inherent to drinking  
390 water production to increase. Temporal and spatial patterns of cyanotoxin concentrations, as  
391 well as of both planktonic and biofilm cyanobacteria abundance, are therefore urgently needed  
392 to better predict the risks cyanobacteria may pose. Future studies should confirm these trends  
393 in biofilm composition, while also assessing the consequences on water quality and the impacts  
394 of other anthropogenic drivers than climate change, such as chemical pollution or fish stocking  
395 on biofilms. Mitigating the effects of global change on mountain aquatic ecosystems, in  
396 particular on their benthic microbial communities, must be considered a top priority in order to  
397 avoid serious health risks for billions of people worldwide and to protect mountain  
398 environments as an important component of our global life-support system.

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## 406 **Author contributions**

407 A.L., and D.S.S. conceived the study. A.L. and D.S.S. performed most of the fieldwork, with  
408 help of H.S. in 2020. H.S. performed laboratory analyses. L.Z. performed the bioinformatics  
409 processing. H.S. analysed the data, with help of A.L., D.S.S. and V.E.J.J. H.S. wrote the first  
410 draft of the manuscript, with contributions from A.L., D.S.S. and V.E.J.J. All authors  
411 contributed to the final version of the manuscript.

## 412 **Conflict of interest statement**

413 The authors declare no competing interests.

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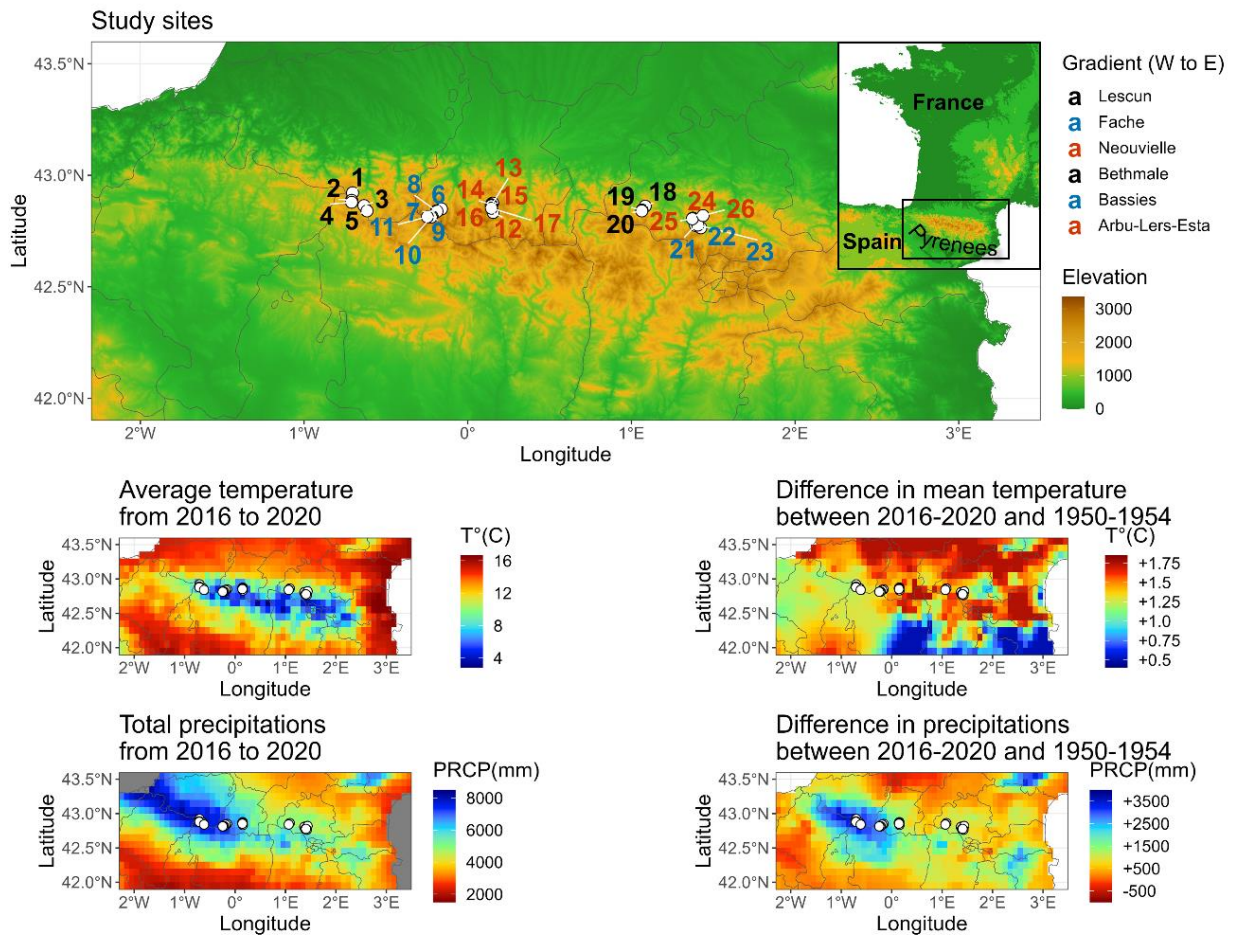
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660 **Figure 1**



661

662 **Figure 1: Location and climatic conditions of study sites.** For further details on lakes, see

663 **Table S1:** 1: Lhurs, 2: Ansabere, 3: Puits d'Arrious, 4: Acherito, 5: Arlet, 6: Paradis, 7:

664 Embarrat, 8: Vallon, 9: Petite-Fache, 10: Grande-Fache, 11: Fache-Espagne, 12: Laquettes, 13:

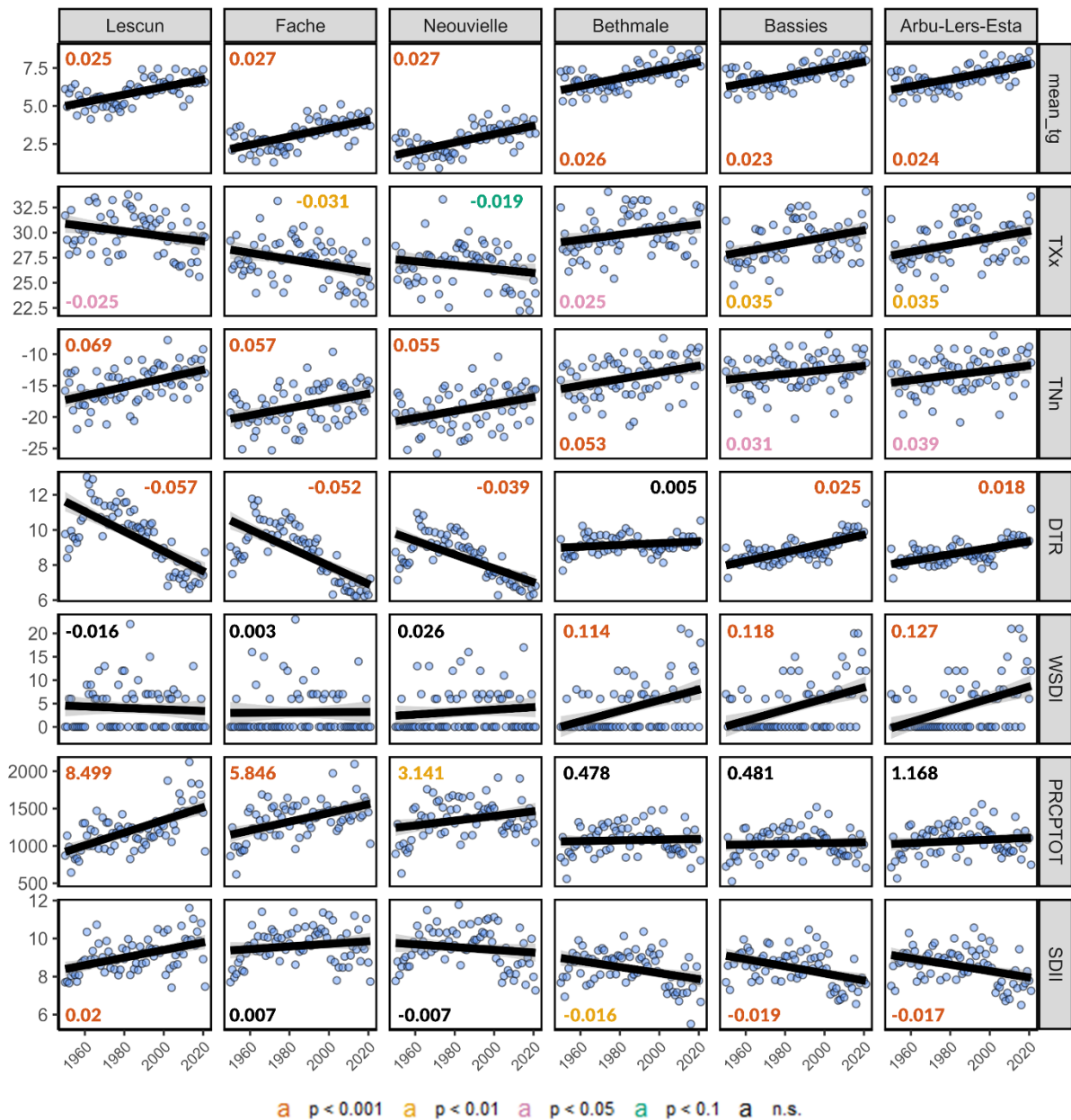
665 Coueyla-Gran, 14: Madamete-Bas, 15: Pecheur, 16: Madamete-Haut, 17: Gourg-de-Rabas, 18:

666 Bethmale, 19: Ayes, 20: Bellonguere, 21: Labant, 22: Mort, 23: Alate, 24: Lers, 25: Estagnon,

667 26: Arbu.

668

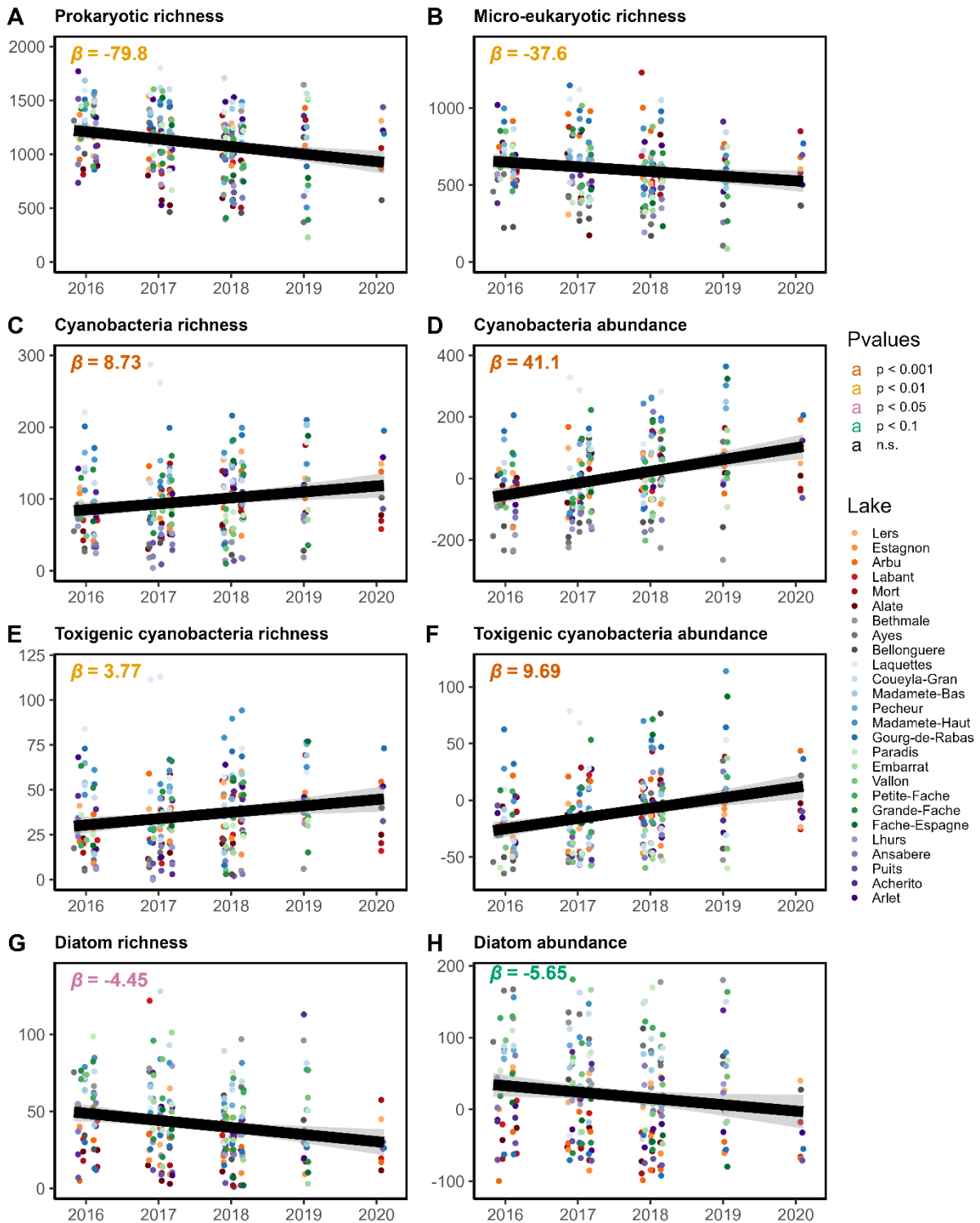
Figure 2



670

671 **Figure 2: Temporal trends in climatic variables from 1950 to 2021.** Slopes of linear  
 672 regression are displayed in each facet. Abbreviations: Mean\_tg: average annual temperature  
 673 (°C); TXx: maximum annual temperature; TNn: minimum annual temperature; DTR: average  
 674 annual daily temperature range; WSDI: number of extremely hot days; PRCPTOT: annual total  
 675 precipitations (mm); SDII: intensity of precipitations (see [Table S2](#) for more details).

## Figure 3



677

678 **Figure 3:** Temporal trends in biofilm  $\alpha$ -diversity (A-B, Chao1 index) and in indicator taxa

679 richness and abundance (C-H) in the period from 2016-2020. Abundance were clr-

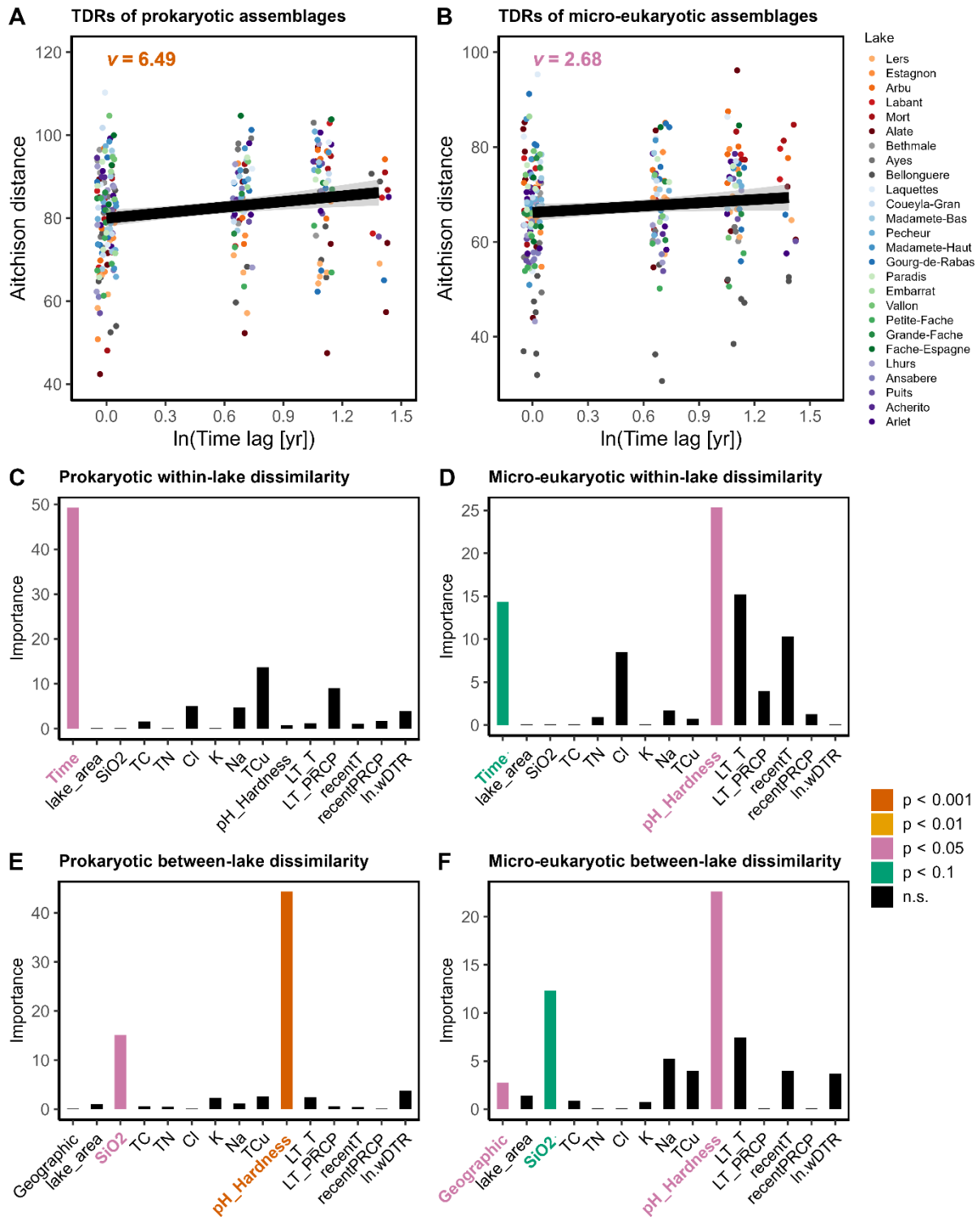
680 transformed prior to fitting. The general trend is shown in black for each panel, with 95%

681 confidence interval in grey shade;  $\beta$  indicates the estimated slope coefficient, coloured  
682 according to its significance.

683

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Figure 4



685

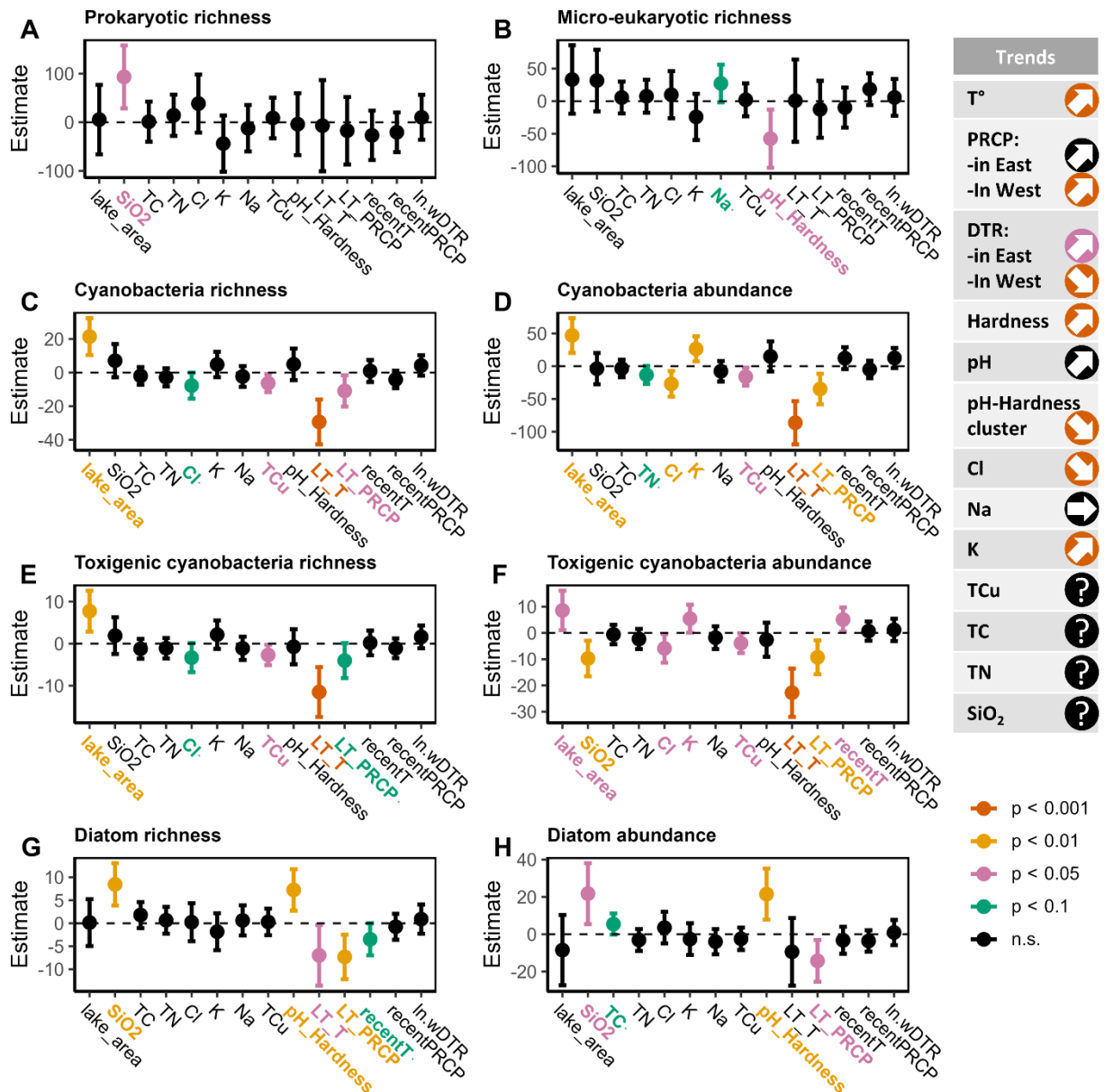
686 **Figure 4: Drivers of microbial biofilm  $\beta$ -diversity for prokaryotic (left) and micro-**687 **eukaryotic assemblages (right). A-B: Time-Decay Relationships (TDRs, or temporal**688 **microbial turnover, indicated by  $v$ , coloured by significance). C-F: drivers of within- and**

689 between-lake dissimilarity determined by GDM. Abbreviations: lake\_area: Lake size; SiO<sub>2</sub>:  
690 Biogenic silica; TC: Total Organic Carbon; TN: Total Nitrogen; Cl: Chloride; K: Potassium;  
691 Na: Sodium; Cu: Copper; LT\_T: temperatures during winter prior to sampling; LT\_PRCP:  
692 precipitations during winter prior to sampling; pH\_Hardness: synthetic variable reflecting pH  
693 and hardness; recentT: same with mean air and water temperatures over three weeks prior to  
694 sampling, recentPRCP: same with precipitation variables, ln.wDTR: average water daily  
695 temperature range over three weeks prior to sampling (log-transformed), Geographic:  
696 geographical distance between samples.

697

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Figure 5



699

700 **Figure 5: Environmental drivers of the  $\alpha$ -diversity (Chao1, A-B) and the richness and clr-**701 **transformed abundance of indicator taxa (C-H) in biofilm prokaryotic and micro-**702 **eukaryotic assemblages.** Points and segments representing the mean and 95% confidence

703 intervals, respectively, estimated by linear mixed models. Details are given in Table Temporal

704 trends over the last decades or years for each of the 14 environmental predictors are displayed

705 on the right-hand side. Abbreviations are given in [Fig. 4](#).

706 **Tables**

707 **Table 1: Summary statistics of the temporal trends in biofilm diversity.**

708 For each response variable, the first line displays the overall temporal trend and the second line  
 709 displays the contrast between the temporal trend of eastern sites minus that in western sites.  
 710 Significant p-values are in boldface. Richness indicates the estimated number of ASVs per  
 711 sample for the given variable (Chao1 index) and relative abundance were clr-transformed prior  
 712 to fitting.

Response variable	Overall temporal trend ( $\beta$ or $v$ )	95% CI	Conditional $R^2$	Marginal $R^2$	statistics	p
	East vs. West					
Prokaryotic richness	-79.8	[-125.9, -33.7]	0.33	0.06	$t_{225} = -3.41$	<b>0.003</b>
	71.2	[-28.2, 171]	0.34	0.09	$t_{21} = 1.49$	0.151
Micro-eukaryotic richness	-37.6	[-61.9, -13.3]	0.44	0.03	$t_{223} = -3.05$	<b>0.006</b>
	40.9	[-10.2, 91.9]	0.45	0.05	$t_{20,1} = 1.67$	0.111
Cyanobacteria richness	8.73	[4.20, 13.25]	0.55	0.03	$t_{204} = 3.81$	<b>&lt;0.001</b>
	-0.56	[-9.72, 8.6]	0.55	0.09	$t_{204} = -0.12$	0.904
Cyanobacteria abundance	41.1	[26.8, 55.3]	0.53	0.11	$t_{19,8} = 5.68$	<b>&lt;0.001</b>
	-13.9	[-44.7, 16.9]	0.54	0.15	$t_{20,6} = -0.94$	0.357
Toxigenic cyanobacteria richness	3.77	[1.59, 5.95]	0.47	0.03	$t_{12,5} = 3.41$	<b>0.005</b>
	0.87	[-3.25, 5.0]	0.47	0.08	$t_{204} = 0.42$	0.677
Toxigenic cyanobacteria abundance	9.69	[5.38, 14]	0.55	0.08	$t_{20,75} = 4.43$	<b>&lt;0.001</b>
	-2.75	[-12.3, 6.8]	0.56	0.08	$t_{21,4} = -0.59$	0.556
Diatom richness	-4.45	[-4.44, -0.45]	0.50	0.03	$t_{18,8} = -2.86$	<b>0.010</b>
	3.63	[-3.15, 10.4]	0.51	0.08	$t_{20,9} = 1.11$	0.278
Diatom abundance	-5.65	[-2.03, 43.52]	0.72	0.01	$t_{15,2} = -2.03$	0.061
	1.37	[-11, 13.8]	0.73	0.02	$t_{20,2} = 0.23$	0.820
Prokaryotic turnover (TDR)	6.49	[3.27, 9.70]	0.36	0.07	$t_{24,3} = 3.97$	<b>&lt;0.001</b>
	2.24	[-4.79, 9.27]	0.36	0.12	$t_{19,6} = 0.66$	0.513
Micro-eukaryotic turnover (TDR)	2.68	[-0.55, -0.03]	0.49	0.02	$t_{23,9} = 2.21$	<b>0.036</b>
	2.8	[-2.3, 7.89]	0.50	0.03	$t_{19,4} = 1.15$	0.265

713



714 **Supporting information**

715 Additional information includes Supplementary methods in Appendix A, and Supplementary  
716 figures and tables in Appendix B.

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