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1 **Occurrence and seasonal dynamics of ALNs in**
2 **freshwater lakes are influenced by their biological**
3 **environment**

4
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9
10 **Abstract**

11 Aster Like Nanoparticles (ALNs) are femtoentities, recently discovered in different aquatic
12 environments, whose intrinsic nature and ecological features remain to be determined. In this
13 study, we investigate the *in-situ* temporal dynamics of ALNs during one year in 3 different
14 lakes, in relation with the physico-chemical and biological environment. ALN abundances in
15 investigated lakes showed a marked seasonal dynamic (from no detectable to $4.28 \pm 0.75 \times 10^6$
16 ALNs.mL⁻¹), with characteristic peaks in spring. We recorded correlation between ALNs and
17 some prokaryotic phyla suggesting a broad and non-specific relationship. From their seasonal
18 dynamics and potential link with prokaryotes, we conclude that ALNs represent an important
19 ecological actor in the functioning of aquatic ecosystems.

20
21 **Keywords:** Aster Like Nanoparticles (ALNs) – Femtoplankton – Seasonal variability –
22 Ecological significance

23 1/ INTRODUCTION

24 The ecological importance of planktonic communities closely depends on their diversity and
25 the associated spatio-temporal dynamics, in response to environmental changes. In the past, the
26 ecological importance of picoplankton (0.2-2 μm) [1, 2] and nanoplankton (2-20 μm) [3, 4]
27 making up the bulk of the so-called microbial loop has been studied extensively. Their roles in
28 the flow of energy and matter on a global scale has become evident [5]. Highlighting the
29 importance of viruses resulted in the inclusion of femtoplankton ($< 0.2 \mu\text{m}$) in ecological
30 models [6–8]. Viruses have long been considered as the main, if not the only, compartment of
31 femtoplankton, mainly because of their interactions within the microbial loop where they shunt
32 microbial particles into dissolved matter, *i.e.* the ‘viral shunt’ [9, 10]. However, in the recent
33 years, the discovery of an unsuspected diversity of femtoplankton entities as Candidate Phyla
34 Radiation (CPR), Diapherotrites-Parvarchaeota-Aenigmarchaeota-Nanoarchaeota-
35 Nanohaloarchaeota (DPANN), Bio-Mineral Organic Particles (BMOPs), Extracellular
36 Vesicles (EVs), and Aster Like Nanoparticles (ALNs), leads to a necessary reconsideration of
37 the ecological role of femtoplankton compartment, at least through the study of the diversity
38 and dynamics of their representatives (Colombet *et al.*, [11]). Attempts to evaluate the
39 quantitative and qualitative importance of CPR / DPANN [12, 13], BMOPs [14], EVs [15]
40 underscores this necessity. Likewise, pioneering reports on ALNs also showed that these
41 entities might have important ecological functions [16, 17]. ALNs are original aster-like
42 entities composed of C, O, Ca, N, K with distinct morphotypes depending on the number of
43 arms (4, 11 and 20 arms) present and their budding (**Fig 1**). Their size (on average from 110 to
44 430 nm) and volume (less than $1.4 \times 10^{-3} \mu\text{m}^3$) lower than the minimum required for life
45 expression (Theoretical Minimal Cell Volume, *i.e.* $0.008 \mu\text{m}^3$) [18], together with their organic
46 nature, their sensitivity to different biocidal treatments, and their capacity to change their
47 numbers in the absence of potential hosts [16], question their exact nature. The presence of

48 nucleic acids remains to be proven [11, 16]. However, (i) their ability to colonize different
49 continental and coastal aquatic environments according to their trophic status [17], (ii) their
50 capabilities to produce bloom situations, with abundance peaks reaching up to $9.0 \pm 0.5 \times 10^7$
51 ALNs.mL⁻¹ [16], and (iii) their putative functional interactions with prokaryotes [16, 17]
52 strongly suggest that they are potentially significant actors in the flow of energy and matter
53 transiting in aquatic systems. To understand the quantitative and functional importance of
54 ALNs in the environment and their exact nature, it seems to be crucial to realize ecological
55 studies integrating temporal variations of ALNs in various ecosystems considering possible
56 environmental forcing parameters.

57 For this purpose, we monitored the *in-situ* dynamic of ALNs as well as the physico-chemical
58 and biological parameters in three lakes of the Massif Central, France, during a one-year period.
59 Our results indicate that ALNs are new dynamic players in the ecology of aquatic systems,
60 with potential links with microbial phyla.

61

62 **2/ MATERIALS & METHODS**

63 **2.1/ Study sites and sample collections**

64 Samples were collected every month during one year (10-2018 to 09-2019) in three artificial
65 freshwater lakes: Fargette (45°44'39''N ; 3°27'21''E ; 465 m altitude ; surface area 1.2 ha ;
66 maximum depth 2.5 m), Lapeyrouse (46°13'20''N ; 2°53'15''E ; 490 m altitude ; surface area
67 12 ha ; maximum depth 6 m) and Saint-Gervais-d'Auvergne (St. Gervais) (46°02'15''N ;
68 2°48'43''E ; 680 m altitude ; surface area 10.5 ha ; maximum depth 4.5 m). These lakes are in
69 the French Massif Central (within a 120 km area). Fargette is a hyper eutrophic lake while St.
70 Gervais and Lapeyrouse are eutrophic lakes with a significant human presence. Five liters of
71 water was collected at the surface (0-40cm) of the deepest point of the lake, using a sampling

72 bottle (5L-Niskin-type, Bionef, Fr). St. Gervais has the particularity of having been emptied
73 during November, December 2018 and January 2019 and returned to its maximum depth in
74 December 2019. During the empty period, samples were taken from a permanent puddle at the
75 center of the lake supplied by the upstream source flowing into the lake. Monitoring of the
76 number of Aster Like Nanoparticles (ALNs) in this upstream source was performed during all
77 the survey, in addition to the lake. Samples of virus-like particles (VLPs), prokaryotes and
78 Aster Like Nanoparticles (ALNs) were fixed with 1% (v/v) formaldehyde and stored at 4°C
79 until counts within 4 hours following sampling. Samples of heterotrophic eukaryotes (HE) were
80 fixed with 1% (v/v) formaldehyde and stored at -20°C until counts.

81 Unfixed samples for analyses of phytoplanktonic communities, microbial diversity, nutrients
82 concentrations and pH measurements were transported at 4°C and treated in the 4 hours
83 following sampling.

84

85 **2.2/ Abiotic parameters measurements**

86 Dissolved oxygen content (mg.L⁻¹), temperature (°Celsius) and conductivity (µSiemens.cm⁻¹)
87 were measured *in-situ* with a submersible probe (ProDSS YSI, Yellow Springs, Ohio, USA),
88 while pH was measured in the laboratory (probe Thermo Scientific, Orion Star A111, Waltham,
89 MA). Organic phosphorus, chlorides and total organic nitrogen were analyzed by UV-VIS
90 spectrophotometry (NF ISO 15923-1). Total organic carbon was determined by IR
91 spectrophotometry after persulfate oxidation (NF EN 1484) and total organic calcium,
92 potassium, magnesium and sodium were determined by ion chromatography (NF EN ISO
93 14911). These standardized and normed measurements were carried out by an accredited
94 institute (Eurofins, Saint-Etienne, France NF EN ISO/IEC 17025:2005 COFRAC 1-2091).

95

96

2.3/ Biotic parameters measurements

97

2.3.1/ Phytoplanktonic communities

98 The biomasses of different groups of the Nanophytoplankton (*i.e.* *Chlorophyceae*,
99 cyanobacteria, *Cryptophyceae* and diatoms) were inferred from *in situ* measurements of
100 chlorophyll *a*, phycoerythrin and phycocyanin ($\mu\text{g.L}^{-1}$) using a submersible
101 spectrofluorometric probe (BBE FluoroProbe, Moldaenke GmbH, DE). Counts of
102 picophytoplankton populations were determined by flow cytometry as described elsewhere
103 [19] using a BD FACSCalibur (BD Sciences, San Jose, CA) equipped with an air-cooled laser,
104 delivering 15 mW at 488 nm with the standard filter set-up.

105

106

2.3.2/ VLP, Prokaryote, Heterotrophic eukaryote and ALN counts

107 Counts of VLPs, prokaryotes and heterotrophic eukaryotes (HE) were performed in triplicates
108 by flow cytometry according to Brussaard [20] and Christaki *et al.*, [21] respectively using a
109 BD FACSAria Fusion SORP (BD Sciences, San Jose, CA) equipped with an air-cooled laser,
110 delivering 50 mW at 488 nm with 502 longpass, and 530/30 bandpass filter set-up. ALN (and
111 their different forms) detection and quantification were realized by transmission electron
112 microscopy (TEM) using a Jeol JEM 2100-Plus microscope (JEOL, Akishima, Tokyo, Japan)
113 operating at 80 kV and x50,000 magnifications as described in Colombet *et al.*, [16].
114 ALN/prokaryote ratios were determined in triplicate following uranyl salt contrasting of
115 particles centrifuged at 18,000xg; 20 min; 14°C directly onto 400-mesh electron microscopy
116 copper grids covered with carbon-coated Formvar film (Pelanne Instruments, Toulouse,
117 France). The number of ALN results from the multiplication of this ratio-fold by prokaryote
118 concentration obtained by cytometry.

119

120 2.3.3/ Diversity of microbial communities

121 Nucleic acid extractions and amplifications

122 For each unfixed sample, microbial communities were collected on a 0.2 µm (Millipore)
123 polycarbonate filter (until saturation, pressure < 25 kPa) and stored at -20°C until DNA
124 extraction. The filters were covered with a lysing buffer (lysozyme 2 mg.mL⁻¹, SDS 0.5%,
125 Proteinase K 100 µg.mL⁻¹ and RNase A 8.33 µg.mL⁻¹ in TE buffer pH 8) at 37°C for 90
126 minutes. A CTAB 10% / NaCl 5M solution was added, and the samples were incubated at 65°C
127 for 30 minutes. The nucleic acids were extracted with phenol-chloroform-isoamyl alcohol
128 (25:24:1) ; the aqueous phase containing the nucleic acids was recovered and purified by
129 adding chloroform-isoamyl alcohol (24:1). The nucleic acids were then precipitated with a
130 mixture of glycogen 5mg.mL⁻¹, sodium acetate 3M and ethanol 100% overnight at -20°C. The
131 DNA pellet was rinsed with ethanol (70%), dried and dissolved in the TE buffer. DNA was
132 then purified using a commercial kit (NucleoSpin® gDNA Clean-up, Macherey-Nagel).
133 For archae, amplification of the V4-V6 region of the small subunit rDNA was amplified using
134 the universal archae 519F and 1017R primers [22] (**Tab s1**). For bacteria, the V4-V5 region of
135 the bacterial small subunit rDNA was amplified using the universal bacteria 515F and 928R
136 primers [23] (**Tab s1**). In addition, the ITS region of fungi was amplified using common fungi
137 primers ITS7f and ITS4r [24] (**Tab s1**). Each PCR was performed in a total volume of 50µL
138 containing 1x final reaction buffer, 2 mM MgCl₂, 0.2mM dNTP, 100 µg.mL⁻¹ BSA, 0.2 µM of
139 each primer, 0.025 U.µL⁻¹ PROMEGA GoTaq HotsStart G2 and 5 µL of DNA. The different
140 PCR programs are detailed in the supplemental (**Tab s1**).

141

142 Amplicon analysis and taxonomic affiliation

143 Bacterial sequencing data were processed with the PANAM2 pipeline [25]. Briefly, sequence
144 reads were assembled with Vsearch [26], excluding reads <200pb, with ambiguous calls (N)

145 or having mismatch in the forward/reverse primer. Demultiplexed amplicons were clustered
146 into OTUs using Vsearch at a 95% id threshold. Representative sequence of OTUs were
147 compared against the SILVA SSURef 115 database restricted to sequences with length > 1,200
148 pb, quality score > 75% and a pintail value > 50. Archaea and Fungi sequencing data were
149 analysed through the FROGs pipeline [27]. Sequence reads were assembled with Vsearch [26],
150 excluding reads < 200pb, with ambiguous calls (N) or having mismatch in the forward/reverse
151 primer. Demultiplexed amplicons were clustered into OTUs using Swarm [28] with distance
152 (d) = 1. OTUs sequences were compared against the SILVA_132_16s database for archaea and
153 Unite_Fungi_8,0 database for fungi.

154

155 **2.4/ Data analysis**

156 Differences in physico-chemical and biological variables between seasons (Spring: March-
157 May, Summer: June-August, Autumn: September-November, Winter: December-February)
158 and lakes were tested by Kruskal-Wallis and Dunn tests. To investigate the potential proximity
159 between our sampling points, function of lakes or seasons, principal component analysis (PCA)
160 was used with the FactoMineR package. Potential relationships among all variables were tested
161 by linear pair-wise correlations (Pearson correlation analyses). Potential relationships between
162 the presence/absence of ALNs and OTUs were tested by pairwise correlations (Spearman
163 correlation analyses).

164 Link between the log-transformed abundance of ALNs and prokaryotes was tested by linear
165 regression using data obtained in the 3 lakes and those obtained from a previous study in
166 Colombet *et al.*, [16]. Cross effects of measured parameters were tested by the omega-square
167 method ω^2 [29].

168

169

170 **3/ RESULTS**

171 **3.1/ Environmental parameters**

172 *3.1.1/ Physico-chemical environment of the studied sites*

173 Absolute values and seasonal variations in oxygen content, temperature and pH were
174 characteristics of freshwater lakes in temperate regions. The three lakes showed similar average
175 values ($p > 0.05$, **Table 1-A**) with marked statistical differences ($p < 0.01$) between seasons
176 (**Table 1-B**). As expected, temperature was inversely correlated to oxygen ($r^2 = -0.77$, $p < 0.01$).
177 The summer and autumn warming periods of the lakes corresponded to oxygen depletion while
178 the cold spring and winter periods corresponded to a rise in oxygenation of lake waters (**Table**
179 **1-B**). pH was stable during spring, summer and autumn and decreased significantly in winter
180 ($p < 0.01$) (**Table 1-B**).

181 The availability of mineral elements (K, Mg, Na, Ca) showed different distributions between
182 lakes (**Table 1-A**). K and Mg had similar distributions with significant differences ($p < 0.01$)
183 between the three lakes, with highest and lowest values recorded respectively in Fargette and
184 St. Gervais (**Table 1-A**). Na concentration was significantly lower in Fargette compared to St.
185 Gervais and Lapeyrouse ($p < 0.01$). Ca was significantly lower in St. Gervais compared to
186 Fargette and Lapeyrouse ($p < 0.01$) (**Table 1-A**).

187 The main chemical factors that discriminated the three lakes appeared to be productivity
188 indicators such as Phosphorus (P), Total Organic Carbon (TOC) or Total Nitrogen (TN). P,
189 TOC and TN were significantly higher in Fargette compared to Lapeyrouse while St. Gervais
190 displayed intermediate values ($p < 0.01$) (**Table 1-A**). At the seasonal scale, no significant
191 variation ($p > 0.05$) was recorded for the three lakes in the availability of mineral elements, P,
192 TOC and TN (**Table 1-B**).

193

194 3.1.2/ Standing stocks of Viruses like particles, prokaryotes,
195 phytoplankton and heterotrophic eukaryotes

196 The variations of biological environment (Viruses Like Particles (VLPs), prokaryotes,
197 phytoplankton and heterotrophic eukaryotes (HE)) between lakes and seasons are listed in
198 **Table 1** and **Figs s1-s2**. The average numbers of VLPs were similar for the three lakes ($p >$
199 0.05) (**Table 1-A**). Prokaryote and HE numbers followed the spatio-temporal dynamic of the
200 productivity indicators (P, TOC and TN) with highest average recorded in Lake Fargette
201 compared to Lapeyrouse ($p < 0.01$) and no significant difference with lake St. Gervais ($p >$
202 0.05). Lapeyrouse had higher content in nanophytoplankton and lower content in
203 picophytoplankton compared to Fargette and St. Gervais ($p < 0.01$).

204 At the seasonal scale, no significant variations ($p > 0.05$) were recorded for the three lakes
205 (**Table 1-B**), excepted for HE. HE dynamic was significantly affected by seasons ($p < 0.01$)
206 with a maximum number in autumns and minimal number in winter. Detailed lake-specific
207 monthly variations for each biological parameter are presented in **Figs s1-s2**.

208 3.1.3/ Composition of communities

209 Metabarcoding of bacterial, archaeal, and fungal communities revealed the predominance of
210 the same phyla, whatever the lake and the season (**Fig s3**). Bacterial communities in the three
211 lakes were dominated by Proteobacteria (30%), *Bacteroidetes* (15%) and unclassified bacteria
212 (13%) (**Fig s3**). Euryarchaeota (43%), Nanoarchaeota (40%) and Crenarchaeota (11%) were
213 the major phyla for Archaea (**Fig s3**). While the fungus community is dominated by
214 unclassified fungi (40%), major known phyla correspond to Chytridiomycota (27%) and
215 Basidiomycota (17%) (**Fig s3**). The mean percentage of the relative abundance of these major
216 phyla presents important temporal variations specific to each lake. For Lake Fargette, we can
217 notice a peak of Proteobacteria in March and April (More than 60% of total abundance),
218 Bacteroidetes in May (50%) and the dominance of unclassified fungi in April (98%) (**Fig s3**).

219 In St. Gervais, Bacteroidetes (40%) increase in March, Euryarchaeota in February and April
220 (50%), and Basidiomycota in December (90%) (**Fig s3**). For Lapeyrouse lake, Bacteroidetes
221 increase in October and May (35 and 40 % respectively) (**Fig s3**).

222 **3.2/ Aster-Like Nanoparticles distribution**

223 As for productivity indicators (P, TOC, TN) and prokaryotes concentrations, significant
224 differences between ALN abundances ($p < 0.01$) were recorded between lakes (**Table 1-A**).

225 As the above-mentioned parameters, the highest and lowest annual mean abundance of Aster
226 Like Nanoparticles (ALNs) were recorded in lake Fargette ($6.12 \pm 11.7 \times 10^5$ ALNs.mL⁻¹) and
227 Lapeyrouse ($0.83 \pm 1.99 \times 10^5$ ALNs.mL⁻¹). St. Gervais had an intermediate average value
228 ($2.32 \pm 2.60 \times 10^5$ ALNs.mL⁻¹) (**Table 1-A**). ALN dynamic was significantly affected by
229 seasons ($p < 0.01$) with remarkable spring peaks in the three lakes, recorded in March 2019
230 (**Table 1-B**). In these lakes, ALN abundances fluctuated from undetectable to a low range from
231 October 2018 to February 2019 before a strong increase towards the main peaks recorded in
232 March 2019 (from no detectable to 10^6 for Fargette, 2.4×10^5 to 7.0×10^5 for St. Gervais and
233 from no detectable to 10^5 ALNs.mL⁻¹ for Lapeyrouse) (**Fig 2-A**), immediately followed by a
234 decline in April in the three lakes. A second increasing phase was recorded from May until
235 July, especially in St. Gervais.

236 The mean distribution combined from the three lakes of different forms (**Fig 1**) varied with
237 time (**Fig 2-B**). The 4-10 arms forms were recorded preferentially from October (15%) to
238 November (27%) and showed highest values from March (45%) to July (35%) during and
239 following the spring peaks of ALNs (**Fig 2-B**). The 11 arms forms dominated the ALNs
240 communities in October (71%), December (100%), January (87%), April (43%) and May
241 (61%) mainly when the lowest concentrations of ALNs were recorded, whereas the 11 budding
242 arms form dominated from June (53%) to September (63%) (**Fig 2-B**). The 20 arms were

243 detected from October (13%) to November (13%) and had highest values from February (35%)
244 to April (15%) concomitantly with the spring peaks of ALNs (**Fig 2-B**). During the ALN peaks
245 of (March 2019), the contributions of the 4 different forms (4-10, 11, 11 budding and 20 arms)
246 were at 45, 25, 21 and 9%, respectively (**Fig 2-B**). When the abundances of ALNs were
247 particularly low, *i.e.*, in December and January (**Fig 2-A**), only the 11-arm form was
248 represented, with absence or very low proportion of the budding form.

249 All parameters and ALNs were integrated in a principal component analysis (**Fig 3**). 54.1% of
250 the variance is explained by the first 2 axes. This analysis showed a pronounced inter-lake
251 variability, mainly due to the biological variables. Like the patterns of ALN abundance,
252 Fargette Lake was clearly distinguished from Lake Lapeyrouse with St. Gervais in intermediary
253 (**Fig 3-A**). The summer/winter seasonal discrimination with spring and autumn in between is
254 classic for temperate zone lake systems (**Fig 3-B**). The remarkable point of Fargette in March
255 stands out clearly from this analysis which coincides with the bloom of ALNs.

256

257 **3.3/ Interaction between Aster-like Nanoparticles and their** 258 **environment**

259 There was no significant correlation between ALNs and any of the measured physico-chemical
260 parameters (**Table 2**). In contrast, significant correlations ($p \leq 0.05$) were recorded between
261 ALNs and their biotic environment, primarily with VLPs ($r^2 = 0.47$) and prokaryotes ($r^2 = 0.32$)
262 (**Table 2**).

263 Linear regression of data including data obtained from a former study in lake Fargette
264 (Colombet et al.,[16]), revealed a significant relation between ALNs and prokaryotes (**Fig 4**).
265 The regression line linking number of ALNs as a function of number of prokaryotes appears
266 highly significant (ALN concentration = $-3 + 1.2 \times$ prokaryotes concentration, $R^2 = 0.43$, $\omega^2 =$

267 0.43, one way ANOVA ($F(1,62) = 49.14, p < 0.001$) (**Fig 4**). As revealed by $\omega^2 = 0.43$, one
268 of the two parameters can explain 43 % of the distribution of the other (**Fig 4**).

269 No significant correlations were observed between ALNs and archaeal OTUs during the
270 sampling period in the three lakes (**Table 3**). On the other hand, ALNs were significantly
271 correlated ($p < 0.01$) with 5 bacterial phyla (Acidobacteria, Cyanobacteria, Firmicutes,
272 Planctomycetes, Proteobacteria) and 2 fungal phyla (Ascomycota and Chytridiomycota)
273 (**Table 3**).

274

275 **4/ DISCUSSION**

276 Here we report the seasonal fluctuations of Aster Like Nanoparticles (ALNs) concentrations in
277 3 different lakes. These entities were first described in 2019 [16] and colonize different
278 continental and coastal environments, reaching important concentrations in the order of 10^7
279 ALNs.mL⁻¹ [17]. We recently demonstrated that their distribution could be influenced by the
280 trophic state of the environment [17]. These pioneering works result in a partial understanding
281 of the ecology of ALNs. Information on their spatial and seasonal dynamics in relation to their
282 environment are crucial and could allow us to (i) reveal their environmental forcing factors,
283 (ii) understand their contribution to the flow of matter and energy and (iii) perhaps open a
284 window on their exact nature.

285 The seasonal variations of ALN abundances were similar in the three lakes which were in a
286 restricted geographical area (French Massif Central) and were apparently driven more by
287 differences in biological parameters. This is in agreement with our previous study [17] where
288 ALNs increased in environments with high microbial density and nutrients content, such as
289 Lake Fargette. The study of the temporal dynamics of ALNs revealed a marked seasonality.
290 The seasonal patterns of ALNs in the three lakes was relatively similar, with characteristic

291 peaks in spring. The ALNs communities exhibited a rapid growth capacity with a high
292 amplitude of abundance variations (from undetectable to $4.28 \pm 0.74 \times 10^6$ ALNs.mL⁻¹ between
293 two consecutive time point in lake Fargette for example). ALNs are therefore a rapid
294 mobilization and sequestration factor of their constituent elements. Colombet and colleagues
295 [16] showed that Ca is a preponderant constitutive element of ALNs. It is therefore very likely
296 that ALNs have a key role in the aquatic Ca cycle. Considering the biomass dynamics of ALNs
297 can therefore be fundamental in understanding the flow of elements through aquatic
298 ecosystems, as has been demonstrated for viruses for example [8].

299 Seasonal variations in the form of bloom are a recurring pattern in aquatic microbial and viral
300 communities [8, 30–32]. All the models showing this kind of dynamics (free or symbiotic),
301 have a prominent role in the functioning of the ecosystem. It is therefore undoubtedly that
302 ALNs are intrinsic actors in the ecology of aquatic systems. However, the understanding of
303 their ecology is intimately linked to the understanding of their control factors.

304 Different factors can explain temporal variations of microbial entities, including geographic
305 and environmental factors [33], abiotic and biotic factors, bottom-up and top-down factors [34],
306 or the presence/absence of hosts for the life cycle such as for viruses [35]. The case of lake
307 Saint Gervais, which was emptied for several weeks, makes it possible to specify here that the
308 ALNs are aboriginal to the lake. Indeed, during the drying phase and until the peak of ALNs,
309 we carried out regular sampling in the tributaries feeding the lake without ever detecting the
310 presence of ALNs. The controlling factors of ALNs are thus intrinsic to the lake. No abiotic
311 factors seem to have an impact on the variations of ALNs abundance in lake ecosystems.

312 It's interesting to note that with equal temperature between spring and fall, we recorded a
313 significant development of ALNs in spring. The development of ALNs could therefore be more
314 associated with the seasonal succession of biological communities forced by the interaction of
315 physico-chemical environmental variables rather than with a single physico-chemical variable.
316 Among biotic factors measured, correlations with prokaryotes and VLPs were identified. We

317 further explored these putative links to the microbial environment by integrating data
318 previously acquired in Fargette Lake [16]. The linear regression model used allows us to
319 highlight a strong link between ALNs and prokaryotes. The ω^2 allowed us to estimate that 47
320 % of the variation in number of ALNs could be explained by the prokaryotes. These results
321 support the observations described in Colombet *et al.*, [16] where one can clearly distinguish a
322 close physical interaction between ALNs and prokaryotes. These data raise the hypothesis that
323 ALNs could have strong interactions with prokaryotes. One of the particularities of ALNs is
324 their pleomorphism. Each form (see description in Colombet *et al.*, [16]) has a particular
325 seasonal dynamic (**Fig 2-B**). In the hypothesis that they are dependent on each other within a
326 developmental cycle, it is likely that a partner, possibly prokaryotic, may intervene at certain
327 stages and regulate the dynamics of the different forms. 11-arms forms could be a resistant
328 form.

329 To identify more precisely which microbial communities may be involved in these interactions
330 with ALNs, we analyzed the covariation of the presence of ALNs with those of the different
331 bacterial, archaeal, and fungal phyla identified in the three lakes. ALNs appeared strongly
332 positively correlated ($r^2 > 0,7$, $p < 0.05$) with some fungal (Ascomycota and Chytridiomycota)
333 and bacterial (Acidobacteria, Cyanobacteria, Firmicutes, Planctomycetes, Proteobacteria)
334 OTUs in lakes. Ascomycota is the most common fungal phylum in aquatic environments [36]
335 and Chytridiomycota is a phylum with a huge implication in the regulation of aquatic
336 organisms, especially influencing directly or indirectly the microbial loop [37, 38]. Although
337 we cannot rule out a direct relationship, it is more likely that fungi interact indirectly with
338 ALNs via a third-party microbe. The positive correlations between ALNs and prokaryotes seem
339 to target a large heterogeneity of bacterial phyla. Acidobacteria, Cyanobacteria, Firmicutes,
340 Planctomycetes, and Proteobacteria represent most lake bacteria [39]. Thus, although we
341 cannot formally infer a direct relationship, these data suggest that the spectrum of possible
342 prokaryotic partners for ALNs may be broad and non-specific.

343 The data acquired here allow a better understanding of the ecology of ALNs, namely, a
344 pronounced seasonal dynamic in the form of spring “bloom” that can be controlled by the
345 microbial and probably prokaryotic environment. The hypothesis that ALNs have strong
346 interactions with prokaryotes is emerging but will have to be verified in the future as well as
347 the possibility of a development cycle linking the different morphotypes. The functionality of
348 ALNs is certain for the transfer of their constitutive elements within the microbial network but
349 their role on other microbial components (neutrality, mutualism, parasitism...) remains to be
350 determined. Through their dynamics, ALNs are undoubtedly new actors to consider in the
351 functioning of aquatic ecosystems. More generally, our data highlight the seasonal importance
352 of overlooked femtoplankton in aquatic ecosystems and their potential roles in the ecosystem
353 functioning.

354 **Author’s contribution** MF, HB and JC designed and performed the experiments. MF and GB
355 analyze genomic datas. MF, HB, GB, TSN and JC wrote the manuscript. All authors have read,
356 commented, and approved the final version of the manuscript.

357

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365

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371

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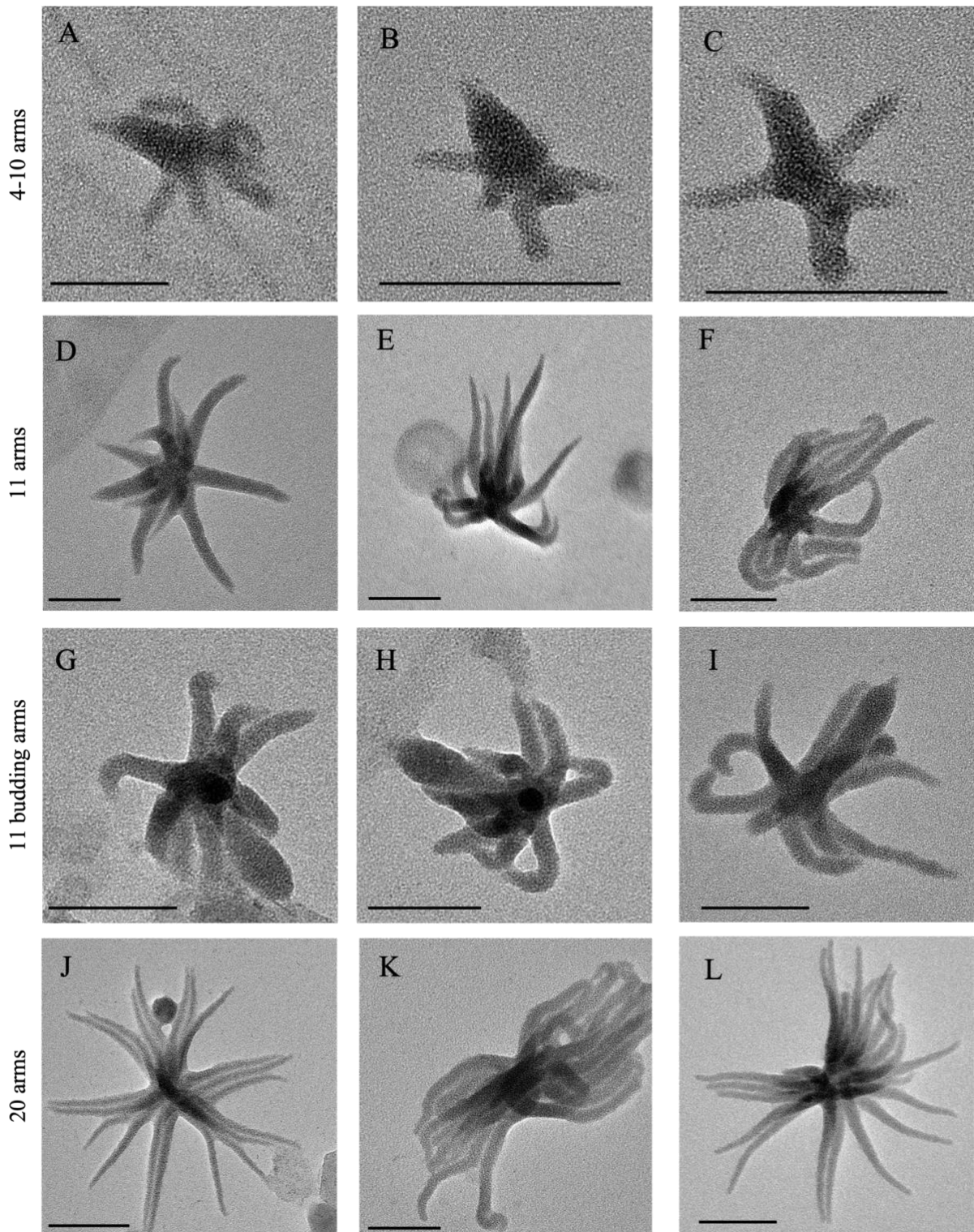
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Figure 1. Transmission electron microscopy (TEM) micrographs of different morphotypes of aster-like nanoparticles (ALNs).
 A-C, 4-10 arms forms. D-F, 11 arms forms and their budding variants G-I. J-L 20 arms forms.
 Scale Bars = 100 nm

524 **Table 1. Mean \pm SD of physico-chemical and biological characteristics according to the lakes (A) or the**
 525 **seasons (B)*.**

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(A)	Fargette	St. Gervais	Lapeyrouse
O ₂ ^{ns} (mg.L ⁻¹)	8.46 \pm 3.45	9.36 \pm 3.02	9.86 \pm 2.07
Temperature ^{ns} (°C)	12.63 \pm 7.13	12.79 \pm 8.04	13.32 \pm 7.72
pH ^{ns}	7.54 \pm 0.67	7.88 \pm 0.83	7.53 \pm 0.56
K (mg.L ⁻¹)	31.15 \pm 5.53 ^a	2.78 \pm 0.51 ^b	5.11 \pm 0.49 ^c
Mg (mg.L ⁻¹)	4.84 \pm 0.52 ^a	3.22 \pm 1.13 ^b	4.23 \pm 0.48 ^c
Na (mg.L ⁻¹)	8.98 \pm 2.27 ^a	12.52 \pm 1.29 ^b	13.04 \pm 1.41 ^b
Ca (mg.L ⁻¹)	22.94 \pm 2.44 ^a	17.61 \pm 6.08 ^b	24.06 \pm 2.75 ^a
P (mg.L ⁻¹)	0.23 \pm 0.06 ^a	0.14 \pm 0.12 ^{ab}	0.06 \pm 0.02 ^b
T.O.C. (mg.L ⁻¹)	23.92 \pm 4.34 ^a	10.78 \pm 3.70 ^b	10.67 \pm 1.05 ^b
T.N. (mg.L ⁻¹)	4.73 \pm 2.65 ^a	3.13 \pm 1.89 ^{ab}	1.75 \pm 0.67 ^b
VLP ^{ns} (VLPs.mL ⁻¹)	1.31 \pm 0.70 x10 ⁸	1.08 \pm 0.88 x 10 ⁸	0.78 \pm 0.66 x 10 ⁸
Prokaryotes (cells.mL ⁻¹)	2.73 \pm 1.77 x10 ⁷ ^a	1.59 \pm 0.92 x 10 ⁷ ^a	0.39 \pm 0.36 x 10 ⁷ ^b
Nanophytoplankton (µg.L ⁻¹)	178.20 \pm 98.80 ^a	106.27 \pm 70.84 ^a	222.44 \pm 13.13 ^b
Picophytoplankton (cells.mL ⁻¹)	4.62 \pm 2.01 x 10 ⁵ ^a	4.97 \pm 6.43 x 10 ⁵ ^a	0.76 \pm 0.93 x 10 ⁵ ^b
Heterotrophic eukaryotes (cells.mL ⁻¹)	2.34 \pm 1.46 x10 ⁴ ^a	2.08 \pm 1.56 x10 ⁴ ^{ab}	1.23 \pm 0.77 x10 ⁴ ^b
ALN (ALNs.mL ⁻¹)	6.12 \pm 11.7 x10 ⁵ ^a	2.32 \pm 2.60 x10 ⁵ ^{ab}	0.83 \pm 1.99 x10 ⁵ ^b

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(B)	Spring	Summer	Autumn	Winter
O ₂ (mg.L ⁻¹)	10.22 \pm 2.80 ^{bc}	6.29 \pm 1.31 ^a	8.46 \pm 1.82 ^{ac}	11.94 \pm 1.93 ^b
Temperature (°C)	12.70 \pm 3.18 ^a	22.39 \pm 2.54 ^b	11.87 \pm 6.68 ^a	4.70 \pm 1.36 ^c
pH	8.31 \pm 0.44 ^a	7.69 \pm 0.44 ^a	7.60 \pm 0.83 ^{ab}	7.01 \pm 0.29 ^b
K ^{ns} (mg.L ⁻¹)	11.75 \pm 12.18	14.94 \pm 16.23	13.97 \pm 15.02	11.40 \pm 11.84
Mg ^{ns} (mg.L ⁻¹)	3.97 \pm 0.95	4.45 \pm 0.87	4.37 \pm 1.15	3.59 \pm 0.96

Na ^{ns} (mg.L ⁻¹)	11.95 ± 2.75	12.54 ± 2.15	11.40 ± 2.43	10.16 ± 2.27
Ca ^{ns} (mg.L ⁻¹)	21.33 ± 4.08	23.78 ± 3.64	21.86 ± 5.42	19.18 ± 5.80
P ^{ns} (mg.L ⁻¹)	0.14 ± 0.12	0.16 ± 0.08	0.17 ± 0.13	0.11 ± 0.09
T.O.C. ^{ns} (mg.L ⁻¹)	14.34 ± 7.78	16.10 ± 6.56	17.44 ± 7.95	12.60 ± 6.21
T.N. ^{ns} (mg.L ⁻¹)	3.46 ± 3.28	2.61 ± 1.34	3.14 ± 1.95	3.59 ± 2.20
VLP ^{ns} (VLPs.mL ⁻¹)	0.88 ± 0.51 x 10 ⁸	1.32 ± 0.87 x 10 ⁸	1.07 ± 1.09 x 10 ⁸	0.95 ± 0.44 x 10 ⁸
Prokaryotes ^{ns} (cells.mL ⁻¹)	2.09 ± 2.33 x 10 ⁷	1.46 ± 1.10 x 10 ⁷	1.33 ± 1.25 x 10 ⁷	1.39 ± 1.05 x 10 ⁷
Nanophytoplankton ^{ns} (µg.L ⁻¹)	137.84 ± 138.11	103.03 ± 88.35	111.31 ± 78.03	56.79 ± 45.02
Picophytoplankton ^{ns} (cells.mL ⁻¹)	2.69 ± 2.77 x 10 ⁵	5.32 ± 6.32 x 10 ⁵	4.27 ± 4.58 x 10 ⁵	1.54 ± 1.38 x 10 ⁵
Heterotrophic eukaryotes (cells.mL ⁻¹)	1.80 ± 1.75 x 10 ⁴ ab	2.35 ± 0.91 x 10 ⁴ ac	2.69 ± 1.20 x 10 ⁴ c	0.69 ± 0.39 x 10 ⁴ b
ALN (ALNs.mL ⁻¹)	7.14 ± 13.30 x 10 ⁵ a	2.11 ± 2.16 x 10 ⁵ ab	1.72 ± 2.50 x 10 ⁵ ab	1.39 ± 2.55 x 10 ⁵ b

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531 O₂ : Oxygen; K: Potassium; Mg: Magnesium; Na: Sodium; Ca: Calcium; P: Phosphorus; T.O.C.: Total

532 Organic Carbon; T.N.: Total Nitrogen; VLP: Viral Like Particles; ALN: Aster Like Nanoparticles

533 ^{a,b,c} indicate significant differences between lakes or seasons (*Dunn test, p < 0.01*)

534 ^{ns} indicate no effect of lakes or seasons on this parameter.

535 * = data are obtained from mean of the 3 lakes

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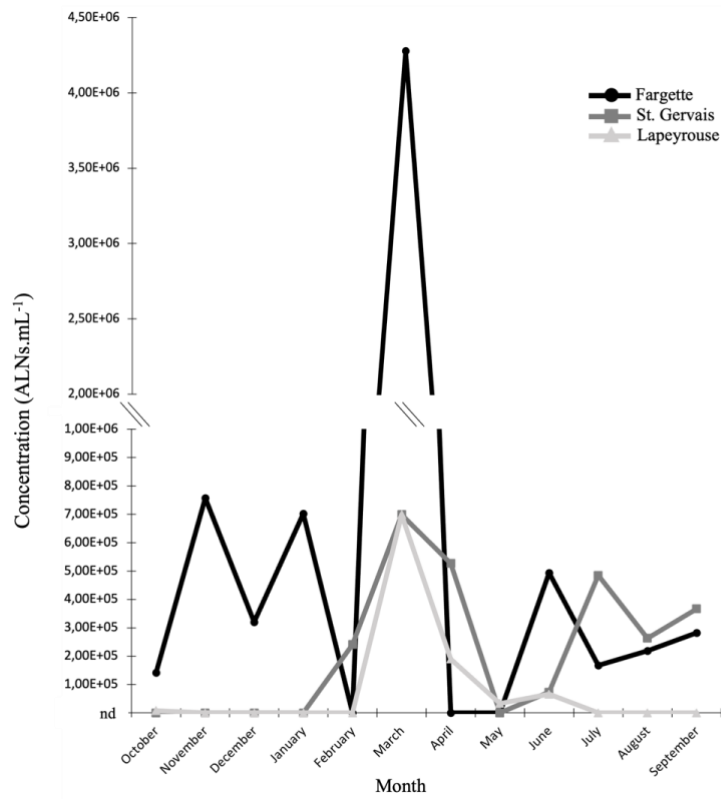
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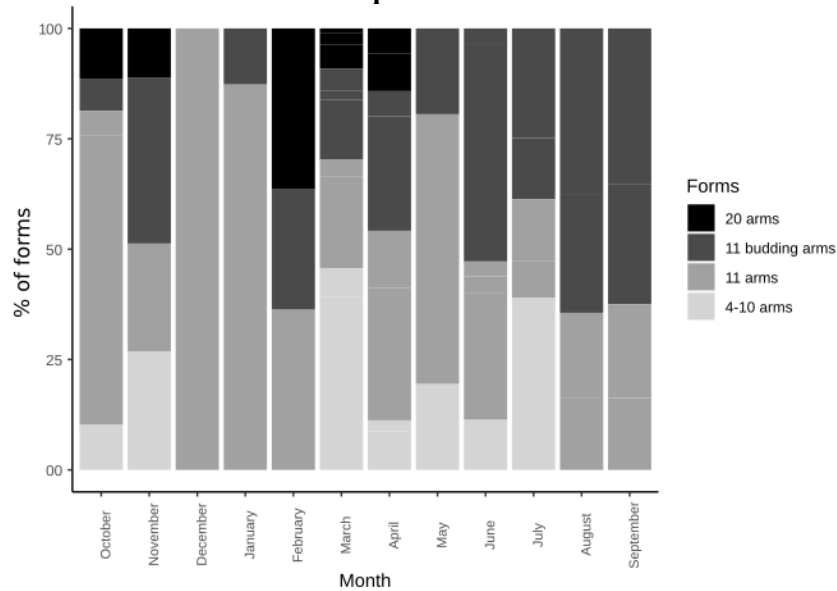
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(A) 1-year variation of Aster Like Nanoparticles concentrations



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(B) 1-year variation of Aster Like Nanoparticles different forms



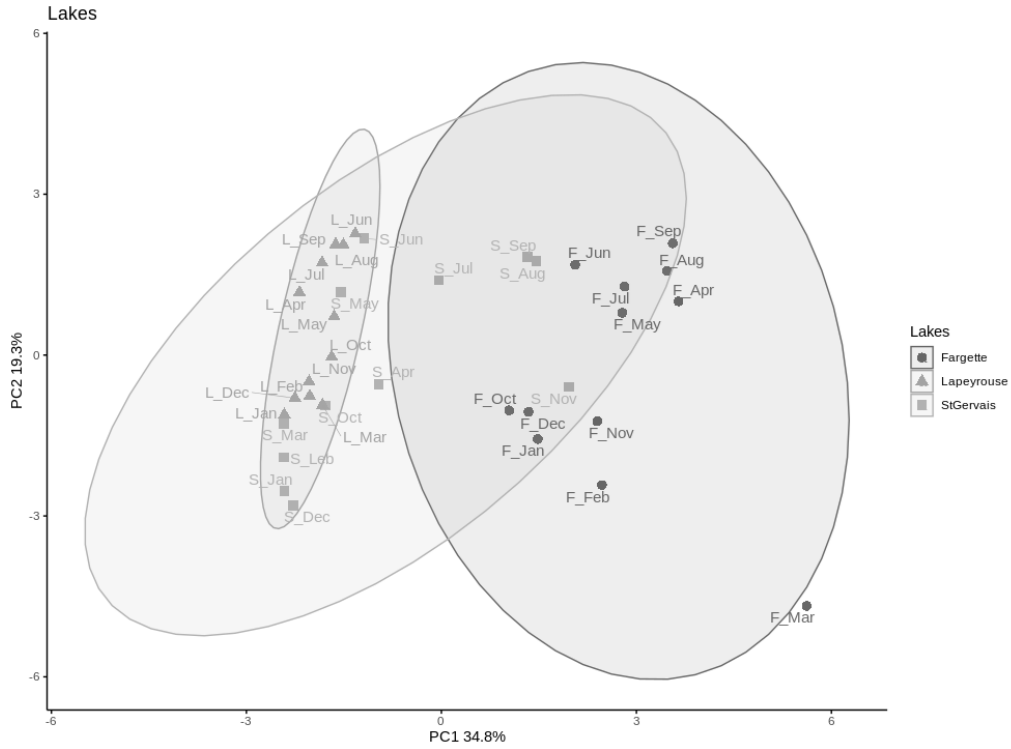
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Figure 2. Dynamics of Aster Like Nanoparticles (ALNs) concentrations (A) and their different forms (B).

Total ALNs concentration in the 3 lakes during the 12 months of sampling (A). Dynamics of the different forms during this period (B)*. ND = No Detectable. * = data are obtained from mean of the 3 lakes

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(A) Individuals according to the lakes

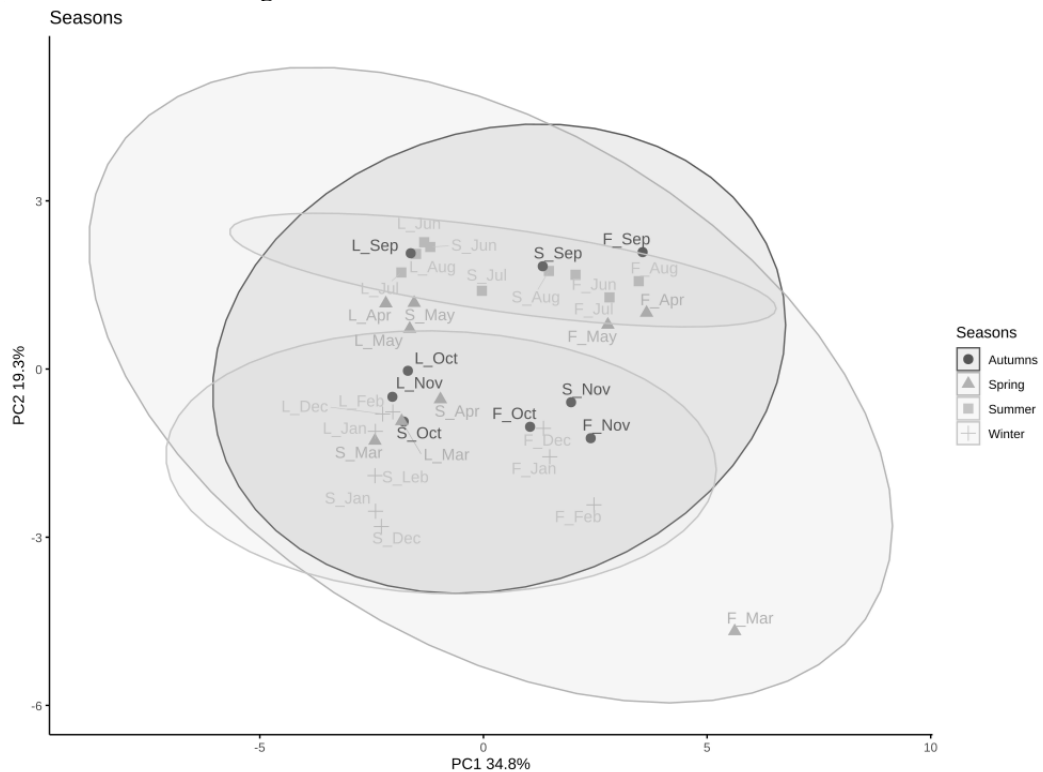


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(B) Individuals according to the seasons



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Figure 3. Principal Component Analysis of individuals (sampling points) in function of lakes (A) or seasons (B).

F: Fargette ; S: St. Gervais ; L: Lapeyrouse ; Oct: October ; Nov: November ; Dec: December ; Jan: January ; Feb: February ; Mar: March ; Apr: April ; May: May ; Jun: June ; Jul: July ; Aug: August ; sep: September

577 **Table 2. Pearson correlation between the Aster Like Nanoparticles (ALNs) and the**
 578 **environmental variables in the 3 lakes.**

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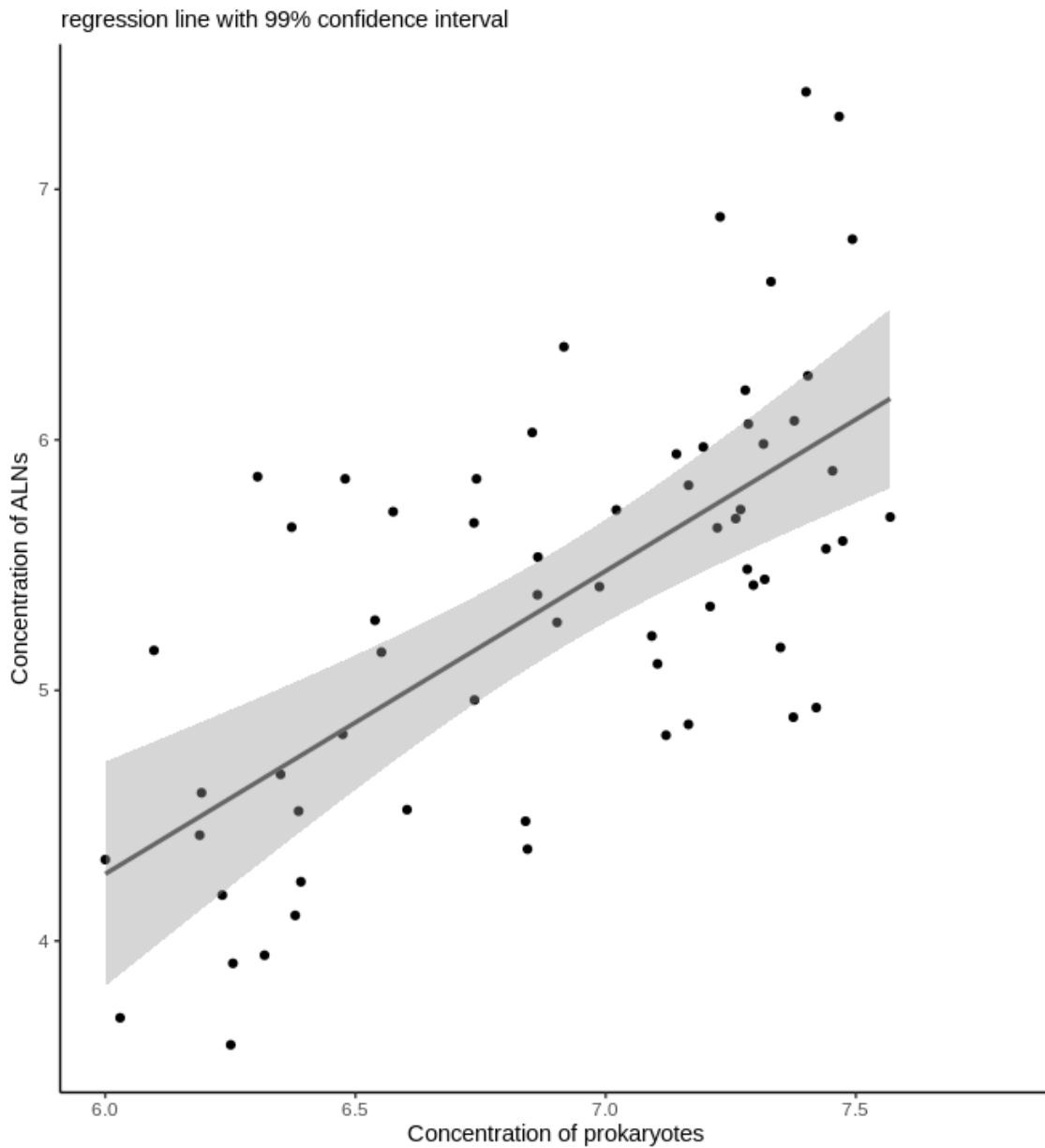
	O ₂	T [°] c	pH	K	Mg	Na	Ca	P	TOC	TN	VLP	Prok	NPP	PPP	HE
ALN	-0.26	0.28	0.24	0.22	-0.01	-	0	0.23	0.25	0.17	0.47	0.32	0.31	0.26	-0.01
						0.21					*	*			

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Prok: prokaryotes; VLP: Viral Like Particles; N.P.P:Nanophytoplankton; P.P.P: Picophytoplankton;
 HE:Heterotrophic Eukaryotes; T[°]c: Temperature; O₂ : Oxygen; T.O.C: Total organic Carbon; T.N:
 Total Nitrogen; Mg: Magnesium; K: Potassium; Ca: Calcium; Na: Sodium; P: Phosphorus
*Pearson test, *p ≤ 0.05*

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623 **Figure 4: Linear regression between ALNs concentration and concentration of prokaryotes.**

624 Linear regression ($y = -3 + 1,2 \cdot x$) was performed with data from the 3 lakes during the 12 months of
625 sampling and data from Colombet *et al.*, [13].

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636 **Table 3. Significant Spearman correlation coefficients between the presence of ALNs and the**
 637 **presence of OTUs.**

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Kingdom	Phylum	Lake	R ²
	Acidobacteria	St. Gervais	0.85
	Cyanobacteria	St. Gervais	0.71
		Lapeyrouse	0.71
Bacteria	Firmicutes	Fargettes	0.82
	Planctomycetes	Fargettes	0.77
		Fargettes_1	0.77
	Proteobacteria	Fargettes_2	1
		St. Gervais	0.84
		Fargettes_1	0.77
	Ascomycota	Fargettes_2	0.77
		St. Gervais_1	0.85
Fungi		St. Gervais_2	0.85
	Chytridiomycota	Lapeyrouse	0.84

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Positive correlation analyses (Spearman's test, $p < 0.01$) between the presence of different OTUs (and their affiliations) and the presence of ALNs.

_(x) = different OTU

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650 **Table S1: Primers used for the amplification and their PCR programs.**

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	Bacteria	Archae	Fungi
Forward primer	515f-GTGYCAGCMGCCGCGGTA	519f- CAGCMGCCGCGGTAA	its7f- GTGARTCATCGAATCTTTG
Reverse Primer	928r- CCCCgycaattcmtttragt	1017r- GGCCATGCACCWCCTCTC	its4r- TCCTCCGCTTATTGATATGC
Program	95°c 3mn -----x32----- 98°c 20s 65°c 40s 72°c 30s ----- 72°c. 10mn	95°c 3mn -----x32----- 98°c 20s 68°c 30s 72°c 30s ----- 72°c. 5mn	95°c 3mn -----x32----- 98°c 20s 57°c 30s 72°c 30s ----- 72°c. 5mn

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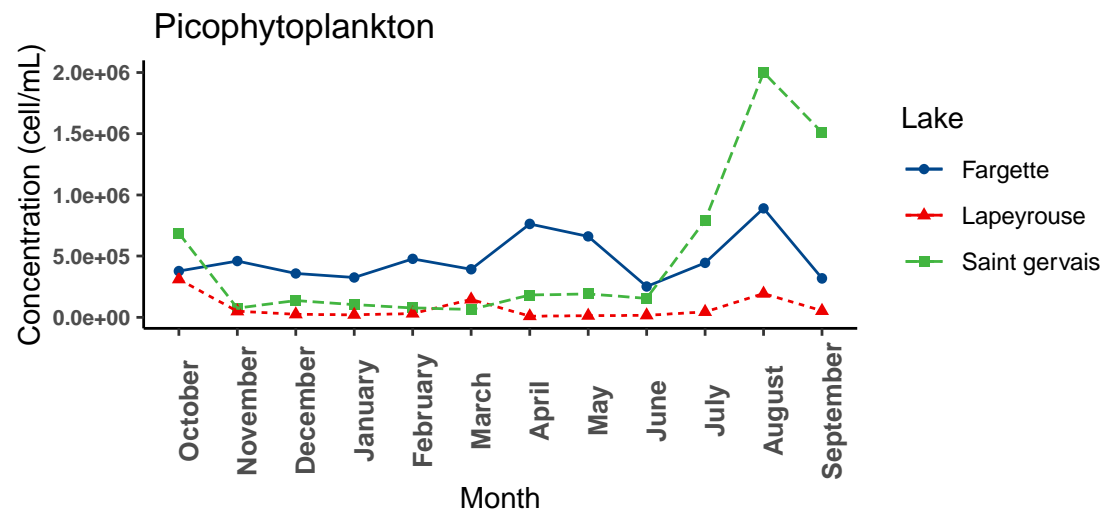
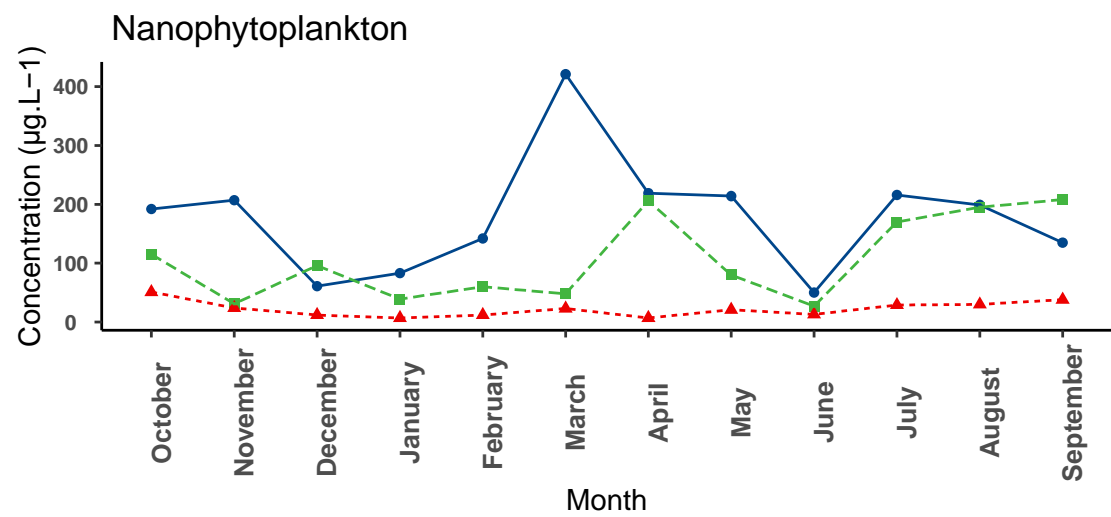
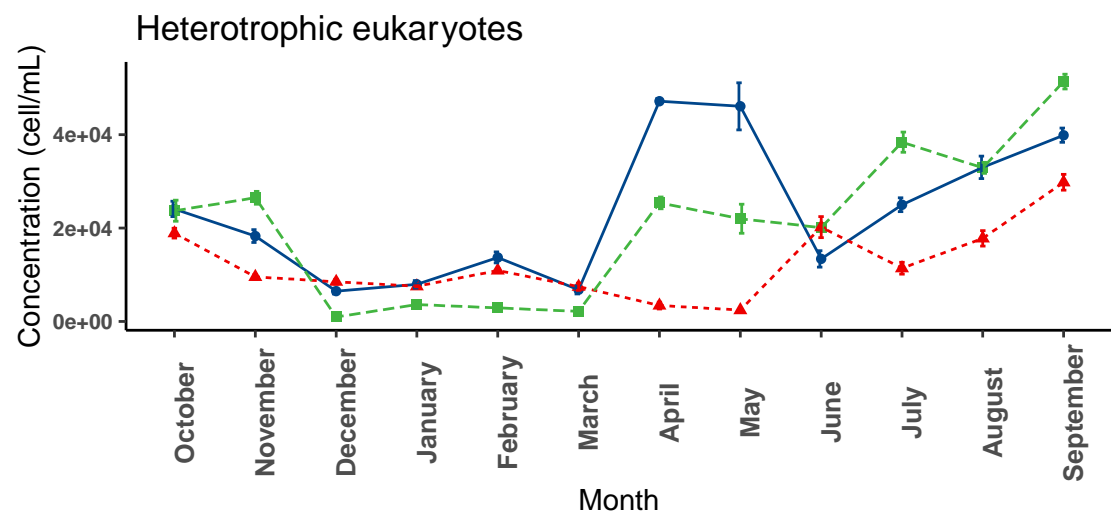
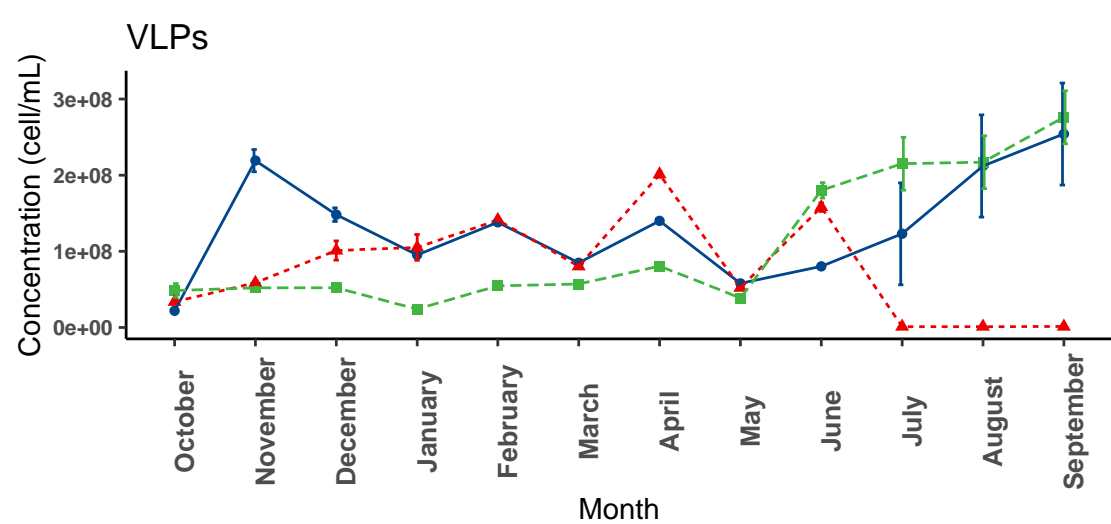
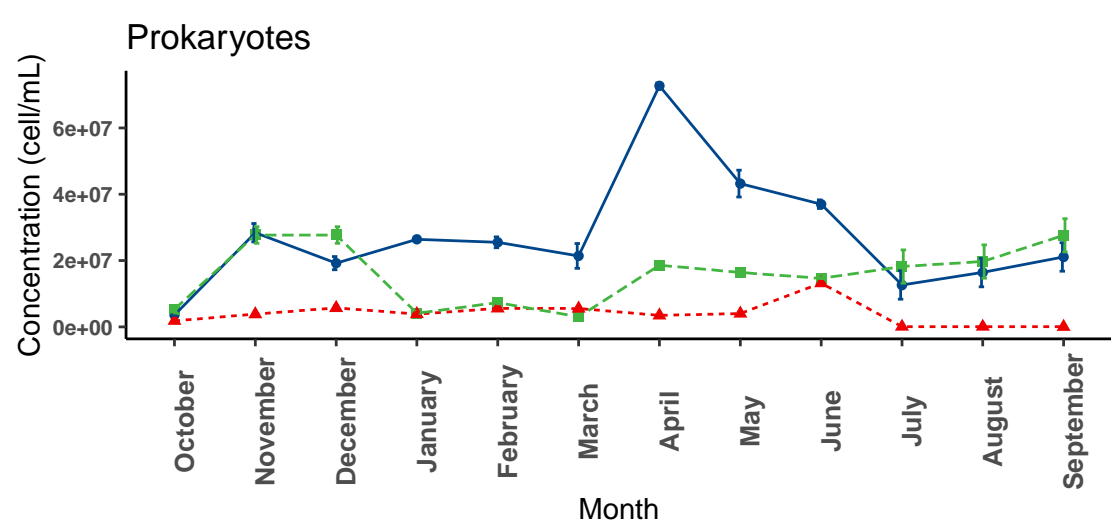


Figure S1. Variation of prokaryotic (A), Viral Like Particles (B), heterotrophic eukaryotes (C), nanophytoplanktonic (D) and picophytoplanktonic (E) concentrations over the 12-months sampling period.

Lake

- Fargette
- ▲ Lapeyrouse
- Saint gervais



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Figure S2. Variation of different groups of nanophytoplanktonic communities (Chlorophyceae, Cryptophyceae, Cyanobacteria and Diatoms) in our 3 lakes over the 12-months sampling period.

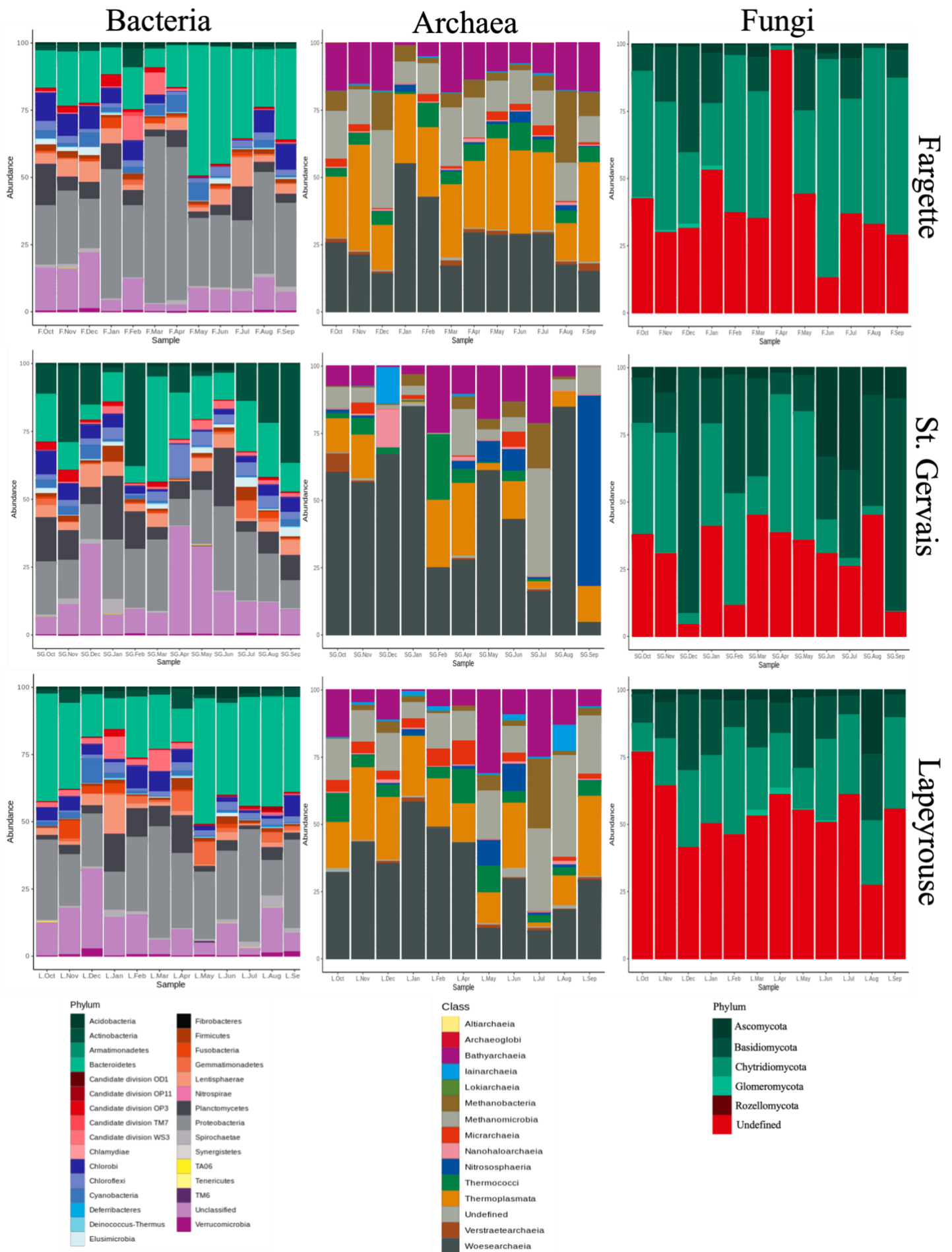


Figure S3. Variation of the different bacterial, archaeal and fungal phyla in the 3 lakes during the 12-months of sampling period.