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## Phytoplanktonic species in the haloalkaline Lake Dziani Dzaha select their archaeal microbiome

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25 **Running head:** Archaeal phycospheric microbiome

26 **Keywords:** Phycosphere, microscale, microbial diversity, archaea, thalassohaline  
27 ecosystem

## 28 **Abstract**

29 Microorganisms are key contributors of aquatic biogeochemical cycles, but their microscale  
30 ecology remains largely unexplored, especially interactions occurring between  
31 phytoplankton and microorganisms in the phycosphere, *i.e.*, the region immediately  
32 surrounding phytoplankton cells. The current study aimed to provide evidence of the  
33 phycosphere taking advantage of a unique hypersaline, hyperalkaline ecosystem, Lake  
34 Dziani Dzaha (Mayotte), where two phytoplanktonic species permanently co-dominate: a  
35 cyanobacterium, *Arthrospira fusiformis*, and a green microalga, *Picocystis salinarum*. To  
36 assay phycospheric microbial diversity from *in situ* sampling, we set up a flow cytometry  
37 cell-sorting methodology for both phytoplanktonic populations, coupled with  
38 metabarcoding and comparative microbiome diversity. We focused on archaeal  
39 communities as they represent a non-negligible part of the phycospheric diversity, however  
40 their role is poorly understood. This work is the first which successfully explores *in situ*  
41 archaeal diversity distribution showing contrasted phycospheric compositions, with *P.*  
42 *salinarum* phycosphere notably enriched in Woesearchaeales OTUs while *A. fusiformis*  
43 phycosphere was enriched in methanogenic lineages affiliated OTUs such as  
44 Methanomicrobiales or Methanofastidiosales. Most archaeal OTUs, including  
45 Woesearchaeales considered in literature as symbionts, were either ubiquitous or specific of  
46 the free-living microbiome (*i.e.*, present in the 3-0.2 $\mu$ m fraction). Seminally, several

47 archaeal OTUs were enriched from the free-living microbiome to the phytoplankton  
48 phycospheres, suggesting (i) either the inhibition or decrease of other OTUs, or (ii) the  
49 selection of specific OTUs resulting from the physical influence of phytoplanktonic species  
50 on surrounding Archaea.

51

## 52 **Introduction**

53 Microorganisms, including bacteria, microeukaryotes and archaea drive and supply many  
54 ecological processes especially through their interactions (Allison & Martiny, 2008; Liu et  
55 al., 2019). Interactions conditioning the structure and dynamics of microbial communities  
56 (Cordero & Datta, 2016; Orland et al., 2020) take place within micrometre-scale (*i.e.*  
57 microscale) cell aggregates and only in a short-range of a few cell diameters (Dal Co et al.,  
58 2020). However, the microbial interactions occurring at this scale and their consequences  
59 for ecosystem functioning, stability and resilience remain largely unexplored.

60         In aquatic ecosystems, the production and maintenance of high phytoplanktonic  
61 biomass, as observed during blooms, does not depend solely on the functional capabilities  
62 of the dominant species but also on the interactions they are engaged in (Woodhouse et al.,  
63 2016; Yang et al., 2017). These interactions mainly take place in the intimate zone of  
64 molecule exchanges between bacterial and archaeal communities and phytoplanktonic cells  
65 (Bell & Mitchell, 1972), *i.e.* the phycosphere. This microscale region, analogous to the  
66 plant root rhizosphere, serves as the interface for phytoplankton and their microbiome  
67 interactions.

68 Most of these interactions are based on the release by the host cell of exudates that fuel  
69 their heterotrophic microbiome, which in turn can either release growth hormones and

70 vitamins (Amin et al., 2015; Croft et al., 2005) to stimulate phytoplanktonic species, or  
71 protecting them from pathogens (Seyedsayamdost et al., 2014) or facilitate their iron uptake  
72 (Amin et al., 2009). It has been shown that this nutrient dependence relies upon the  
73 functional complementarity of the species involved (Halary et al., 2022; Pascault et al.,  
74 2021), which has been engineered over evolutionary times by the host cells. Moreover, the  
75 class of metabolites exuded by phytoplanktonic species can influence the phycosphere  
76 microbiome composition (Fu et al., 2020; Shibl et al., 2020). While recent works suggested  
77 that the phycosphere contains many new and undiscovered microbial lineages and genetic  
78 resources (Ashraf et al., 2023; Zhang et al., 2019), few investigated the phycospheric  
79 microbiome considering bacteria, microeukaryotes and archaea simultaneously. Moreover,  
80 classical studies target the phycosphere, considered as a particle-attached-fraction, through  
81 cultivation of large algae (from 8 to 200 $\mu\text{m}$ ), mesocosms or gradual filtration (Halary et al.,  
82 2022; Sapp et al., 2007; Zhou et al., 2019) showing that particle-attached and free-living  
83 microbiomes exhibit different taxonomic composition, physiology and metabolism  
84 corresponding to their lifestyle and ecological behavior (Li et al., 2021, Suzuki et al., 2017,  
85 Yang et al., 2017).

86 Lake Dziani Dzaha (Mayotte, Indian Ocean) is an extreme thalassohaline,  
87 hypersaline, hyperalkaline lake characterized by many unique geochemical and biological  
88 features (Leboulanger et al., 2017; Sarazin et al., 2021). One of these features is the high  
89 and stable chlorophyll *a* concentration over time (mean concentrations ranging from 566 to  
90 702  $\mu\text{g.L}^{-1}$ , reported over the 2010-2015 period (Leboulanger et al., 2017), 587  $\mu\text{g.L}^{-1}$  in  
91 2018), which is of great interest in the study of phytoplanktonic phycosphere compared  
92 with other ecosystems where phytoplanktonic blooms occurs only periodically. In  
93 temperate areas where cyanobacterial blooms occur in lakes, they only appear during the

94 summer period (Louati et al., 2023). Indeed, whatever the season, the cyanobacterium  
95 *Arthrospira fusiformis* and the picoeukaryotic algae *Picocystis salinarum* dominate the  
96 phototrophic microbial assemblage (Bernard et al., 2019) in the two main contrasted  
97 ecological niches of the water column: the photic upper layer (i.e. above 2 m), where  
98 oxygen and light were detected and the aphotic, anoxic deeper layer (i.e. below 2 m)  
99 (Bernard et al., 2019; Hugoni et al., 2018; Leboulanger et al., 2017), presenting high  
100 concentrations in  $\text{H}_2\text{S}/\text{HS}^-$ , pH values higher than 9 and salinities exceeding 60 psu  
101 (Leboulanger et al., 2017; Sarazin et al., 2021). This co-dominance contrasts with the  
102 dominance of a single successful species as observed in other hypersaline lakes (Dadheech  
103 et al., 2013; Krienitz et al., 2013; Sorokin et al., 2014) and is likely due to differences in  
104 their pigment composition (Bernard et al., 2019) and the ability of *P. salinarum* to adapt to  
105 low light intensity (Pálmai et al., 2020; Roesler, 2002). Although a sharp decrease of *A.*  
106 *fusiformis* and *P. salinarum* abundances along the water column depth has been reported  
107 (Bernard et al., 2019) associated to an unknown physiological status, it does not result from  
108 grazing by aquatic metazoans as these are absent from the lake (Hugoni et al., 2018).

109         While the phycospheric diversity is suspected to be important for phytoplankton life  
110 cycle, allowing niche protection, nutrient and/or metabolite exchanges with the  
111 phototrophic cells (through exudates consumption, chemotaxis, etc.), the microbiome  
112 associated with the dominant phytoplanktonic species, *A. fusiformis* and *P. salinarum* has  
113 not been identified yet. Despite much larger size, *A. fusiformis* (filaments average length  
114 335  $\mu\text{m}$  [min 170  $\mu\text{m}$  - max 2,390  $\mu\text{m}$ ] (Cellamare et al., 2018)) appears to co-occur with a  
115 very limited number of archaeal and bacterial taxa (OTUs) compared to *P. salinarum*  
116 (diameters from 2.5 to 4.5  $\mu\text{m}$  (Cellamare et al., 2018)), according to co-occurrence  
117 microbial networks (CMN) (Escalas et al., 2021). This counter-intuitive observation

118 advocates for a high selectivity in *A. fusiformis* phycosphere and the possible lack of a  
119 specific *P. salinarum* phycosphere for which the detected microbiome might only reflect  
120 the free-living communities in the vicinity of the cells. The hypothesis of an absence of a *P.*  
121 *salinarum* phycosphere by Escalas *et al.* (2021) was congruent with predictions suggesting  
122 that chemotactic associations could not be detected for phytoplanktonic cells smaller than 4  
123  $\mu\text{m}$  (J. R. Seymour *et al.*, 2017). However, associations between taxa identified using CMN  
124 correspond to statistical relationships that are observed at a relatively large observational  
125 scale compared with the scale at which microbial interactions do actually occur, being  
126 relevant for the phycosphere study.

127         Here, we used a cell-level observational scale, based on flow cytometry cell-sorting,  
128 to test the existence of specific phycospheric microbiomes (*i.e.* particle-attached),  
129 hypothesized previously at larger scale (Escalas *et al.*, 2021), for the two-codominant  
130 phytoplanktonic species, *A. fusiformis* and *P. salinarum*, retrieved in the same ecosystem,  
131 Lake Dziani Dzaha. We investigated the diversity of bacterial, microeukaryotic and  
132 archaeal communities retrieved within the phycosphere of the two primary producers to test  
133 whether these two phytoplanktonic species harbor specific phycospheric microbiomes. By  
134 comparing the phycosphere microbiomes with the surrounding water free-living  
135 microbiome (3-0.2 $\mu\text{m}$  fraction), we evidenced (1) distinct microbiomes composition in the  
136 phycospheres (particle-attached) *versus* the free-living fraction (non-phycospheric), (2) the  
137 presence of unexpected archaeal phycosphere communities for *P. salinarum* which were  
138 different in OTUs and sequence abundance from the free-living fractions. (3) We evidenced  
139 the selection of specific archaeal OTUs by the phytoplanktonic cells to thrive in their  
140 intimacy.

141

## 142 **Materials and Methods**

### 143 *Study site, sampling, and environmental parameters*

144 Lake Dziani Dzaha is a volcanic crater lake located in the Comoros archipelago (Western  
145 Indian Ocean) on the Petite Terre Island of Mayotte (12°46' 15.6" S; 45° 17' 19.2" E;  
146 Latitude: -12.771, Longitude: 45.2887). The lake surface is approximately 0.24 km<sup>2</sup> with a  
147 mean depth of ~3.5 m except for the eastern part of the lake where a narrow pit reaching a  
148 maximum depth of 18 m is present. The elevation of the lake surface is close to the average  
149 sea level. This lake is characterized by a higher-than-seawater salinity (ranging from 34 to  
150 71 psu), a strong alkalinity (0.23 mol L<sup>-1</sup>) and a permanent green color due to a high  
151 primary production (Leboulanger et al., 2017). Eight water samples (1.2 L) were collected  
152 on November 14<sup>th</sup>, 2018, along a depth profile (0.25 m, 1 m, 1.5 m, 2.5 m, 5 m, 11 m, 14 m  
153 and 16 m) located above the deepest point of the lake, using a horizontal Niskin bottle, and  
154 were processed within 2 hours following collection. Because of the clogging effect, to  
155 collect the free-living microbiome, a 45 mL subsample was filtered successively through 3-  
156 µm and 0.2-µm (30 mL) pore-size polycarbonate filters. The filters were stored at -20°C  
157 until nucleic acid extraction was performed. Another 250 mL water subsample was stored  
158 at room temperature in the dark in Nalgene bottles until cell-sorting by flow cytometry  
159 performed from November 20<sup>th</sup> to 23<sup>rd</sup>, 2018 (+ 4-7 days). Vertical profiles for pH,  
160 dissolved O<sub>2</sub>, temperature and conductivity were measured using a WTW 3630 probe. The  
161 salinity was calculated based on conductivity and temperature. Chl *a* was analyzed after  
162 extraction using 96% ethanol by ultrasonication in an ice bath for 30 sec, and further  
163 extraction was allowed overnight at 4 °C in the dark. The extract was filtered, and the



164 filtrate was analyzed spectrophotometrically at 400–750 nm. The concentration of Chl *a*  
165 was calculated according to Ritchie (Ritchie, 2006).

166

### 167 ***Flow cytometry cell-sorting***

168 The workflow for flow cytometry cell-sorting was determined as a compromise of different  
169 requirements (i) the amount of DNA needed to prepare libraries, (ii) the efficiency of *A.*  
170 *fusiformis* and *P. salinarum* cell-sorting, and (iii) the time necessary to perform the  
171 experimentation, which should not affect microbial diversity.

172 Autotrophic populations consisting of *Picocystis salinarum* and *Arthrospira*  
173 *fusiformis* were sorted using a FACS Aria Flow cytometer (Becton Dickinson, San Jose,  
174 CA, USA), equipped with a blue laser (488nm), following the procedure described in  
175 Figure S1. Our working hypothesis is that the microbial members present in the  
176 phycosphere of *P. salinarum* and *A. fusiformis* were sorted concomitantly with the  
177 phytoplanktonic populations as they are closely associated with them.

178 Samples were pre-filtered on a 20µm sieve and sorting parameters were: nozzle of  
179 70µm (70PSI) with Facs flow (stream) and BD FACSDiva software: amplitude: 88 Mhz  
180 voltage sorting 5v, the Drop Delay: 38.4 µs and GAP = 6 pixels. The sorting quality (fine  
181 tube) using accudrop beads (BD) for 1600 to 2000 events/s, was obtained with a sorting  
182 efficiency percentage > to 95%. The procedure allowed to sort about 10<sup>6</sup> events for *P.*  
183 *salinarum*, and 5000 events for *A. fusiformis*, Figure S2 and Table S1) in a limited time  
184 (2h) to prevent any alteration of the sorted cells. *P. salinarum* and *A. fusiformis* cells were  
185 excited at 488 nm and sorted on parameters depending on their FALS and RALS properties  
186 and red fluorescence (>650 nm) from chlorophyll pigments. The sorted cells were collected

187 in a polypropylene tube containing 500 µl of Dziani Dzaha lake water, which was filtered  
188 at 0.2 µm and heated to 60°C to denature viral particles beforehand.

189

### 190 ***DNA extraction, Illumina sequencing and sequence processing***

191 DNA extractions were performed on the free-living fraction (*i.e.* 0.2µm filters) using the  
192 ZymoBIOMICS DNA Miniprep kit (Zymo Research) following the manufacturer's  
193 recommendations but amended with a phenol/chloroform/isoamyl alcohol (25:24:1) step to  
194 further purify the DNA. DNA quality was checked by 1% (w/v) agarose gel electrophoresis  
195 and quantified using NanoDrop (Table S1). The same extraction procedure was used for  
196 flow cytometry cell-sorted fractions (Table S1).

197 The V3-V5 region of the 16S rRNA genes was amplified in triplicate for *Bacteria*  
198 and *Archaea* using the universal primers 357F (Schuurman et al., 2004) and 926R (Walters  
199 et al., 2016) and 519F and 915R (Hugoni et al., 2015), respectively (Table S2). For  
200 Eukaryota, the V4 region of the 18S rRNA genes was amplified in triplicate using the  
201 universal primer 515F (Caporaso et al. 2011) and the eukaryotic primer 951R (Lepère et al.,  
202 2016) (Table S2). Additionally, a DNA extraction was carried out in absence of biological  
203 matrix to obtain a negative control of lab-induced contamination. High-throughput  
204 sequencing was achieved after pooling PCR triplicates and performing a multiplexing step  
205 using MiSeq 300 bp PE technology (Biofidal, Vaulx-en-Velin, France -  
206 <http://www.biofidal-lab.com>).

207 Bacterial and archaeal 16S rRNA and eukaryotic 18S rRNA paired-end reads were  
208 merged with a maximum of 10% mismatches in the overlap region using VSEARCH  
209 (Rognes et al., 2016). Denoising procedures consisted of discarding reads containing  
210 ambiguous bases (N) or reads outside the range of expected length (*i.e.*, 450 to 580 bp for

211 bacterial 16S rRNA genes, 370 to 580 bp for archaeal 16S rRNA genes, and 250 to 420 bp  
212 for eukaryotic 18S rRNA genes). After dereplication, sequences were clustered using  
213 SWARM (Mahé et al., 2014) with a local clustering threshold. Chimeric OTUs were  
214 removed with VSEARCH (Rognes et al., 2016) and along with OTUs representing less  
215 than 0.005% of the total number of sequences (Bokulich et al., 2013) and singletons. The  
216 eukaryotic dataset was cured of eukaryotic contaminations (higher metazoan sequences) to  
217 create the microeukaryotic dataset. Taxonomic assignment was performed with both RDP  
218 Classifier (Wang et al., 2007) and BLASTN+ (Camacho et al., 2009) against the 138.1  
219 SILVA database (Pruesse et al., 2007). This procedure was automated in the FROGS  
220 pipeline (Escudié et al., 2017). Contaminant Operational Taxonomic Units (OTUs)  
221 identified from the control samples were removed. Last, samples were randomly resampled  
222 to allow comparisons between samples, Table S3).

223

#### 224 ***Statistical analyses***

225 For each sample, richness (Chao-1) and diversity (Shannon) indices were computed using  
226 the VEGAN package (<http://cran.r-project.org/web/packages/vegan/index.html>) in R.  
227 Distribution of OTUs among the different conditions was illustrated with Venn diagrams  
228 (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). An ordination of the samples was  
229 realized using NMDS approach based on Jaccard index computed from presence-absence  
230 matrices and on Bray-Curtis dissimilarity computed from sequence abundance data  
231 (function *metaMDS* in R). A stress value was calculated to measure the difference between  
232 the ranks on the ordination configuration and the ranks in the original similarity matrix for  
233 each repetition (Ramette, 2007). An acceptable stress value should be below 0.2. An  
234 analysis of similarity (ANOSIM) was calculated to test the differences between each

235 fraction and to further confirm the results observed in the NMDS plot. These analyses were  
236 performed with the VEGAN package ([http://cran.r-](http://cran.r-project.org/web/packages/vegan/index.html)  
237 [project.org/web/packages/vegan/index.html](http://cran.r-project.org/web/packages/vegan/index.html)) in R.

238

### 239 *Archaeal phylogeny*

240 Archaeal OTUs sequences retrieved in the *A. fusiformis* and *P. salinarum* phycospheres and  
241 in the free-living microbiome were extracted for the three datasets and aligned using  
242 MAFFT (Kuraku et al., 2013). Gaps and poorly conserved regions were eliminated using  
243 Gblocks (Castresana, 2000; Talavera & Castresana, 2007). The phylogeny was  
244 reconstructed using fastme 2.1.5 (Lefort et al., 2015), with the F84 nucleotide model and  
245 NNI+SPR topological optimization under the balanced minimum evolution principle. Node  
246 support was estimated using the bootstrap method (1000 replicates). The tree was  
247 visualized together with the presence/absence matrix of OTUs in the *A. fusiformis* and *P.*  
248 *salinarum* phycospheres and in the free-living microbiome, using iTOL  
249 (<https://itol.embl.de>).

250

## 251 **Results**

### 252 **Physico-chemical properties of the water column**

253 Physico-chemical parameters recorded during November 2018 indicated that the water  
254 column was not stratified in terms of salinity, pH and temperature (Figure 1). It was  
255 however stratified in terms of O<sub>2</sub> saturation, with an oxycline between 0.25 and 1m, where  
256 O<sub>2</sub> saturation decrease from 89.9% to 21.7%, and anoxic waters at depth, with 0% O<sub>2</sub>  
257 saturation at 11m depth.

258

### 259 **Flow-cytometry cell-sorting methodological validations**

260 The microscale resolution of the present work implies that DNA extracted from cell sorted  
261 fractions was at least ~ten-fold magnitude lower as compared to DNA retrieved from the  
262 filtered water samples that correspond to upstream and downstream cell sorting controls  
263 (Table S1). Hence, an average of 2.4 ng. $\mu\text{L}^{-1}$  of DNA in the *A. fusiformis* panel and 3.6  
264 ng. $\mu\text{L}^{-1}$  in the *P. salinarum* panel were obtained, which contrasted with the average 46.8  
265 and 49.1 ng. $\mu\text{L}^{-1}$  of DNA extracted from the unsorted 20-0.2 $\mu\text{m}$  fractions.

266         Nonetheless, in the bacterial dataset, a clear enrichment in *P. salinarum*  
267 mitochondrion and chloroplast sequences (i.e. 16S rRNA genes) in the *P. salinarum*  
268 phycosphere associated with an increase in *A. fusiformis* sequences in the *A. fusiformis*  
269 phycosphere fraction demonstrated the cell sorting efficiency (Table S3). In the same way,  
270 *P. salinarum* affiliated sequences represented close to all sequences in the *P. salinarum*  
271 phycosphere eucaryotic dataset (Table S3). However, because of the small amounts of  
272 DNA extracted and the sequencing depth in *P. salinarum* and *A. fusiformis* phycospheres  
273 fractions, we needed to adapt the normalization procedures for the bacterial and  
274 microeukaryotic datasets by investigating 2,360 and 101 sequences per sample for Bacteria  
275 and 2,097 and 196 for microeukaryotes, respectively, which allowed the collection of  
276 almost all diversity (Figure S3). When retaining 2,360 sequences per sample in the bacterial  
277 dataset, we excluded all *P. salinarum* phycosphere samples that contained mostly *P.*  
278 *salinarum* mitochondrion sequences, thus we decided to apply a complementary  
279 normalization threshold corresponding to 101 sequences per sample (Table S2). The same  
280 protocol was applied for the microeukaryotic dataset as the 2,097 sequences threshold

281 excluded all the *A. fusiformis* phycosphere samples, we decided to investigate the  
282 microeukaryotic members of *A. fusiformis* phycosphere using a 196 sequences threshold.  
283 Normalization thresholds retained for *Archaea* was 4,000 sequences per sample allowing to  
284 capture most of the diversity present in the samples (Figure S3).

285 Last, as time necessary to process enough cell events by flow cytometry could affect  
286 phycosphere microbial composition, we first compared microbial communities issued from  
287 upstream and downstream controls. By computing Jaccard indices at the OTUs presence-  
288 absence level, we showed that bacterial, microeukaryotic and archaeal communities are  
289 highly similar in upstream and downstream control with stresses being respectively: 0.16,  
290 0.21, 0.11, and supported by the ANOSIM (p-value  $1.10^{-6}$ , Figure 2). The same patterns  
291 were recorded at the OTUs abundance, using Bray-Curtis indexes, showing the proximity  
292 of communities in upstream and downstream controls (Figure S4). The microbial  
293 community composition evaluated at the phylum\_class level was also highly similar  
294 between upstream and downstream controls (Figure S5). Thus, the three days necessary to  
295 sort *A. fusiformis* and *P. salinarum* with their respective phycosphere did not alter  
296 microbial communities' composition, validating our method to explore the microbiomes  
297 from the *A. fusiformis* and *P. salinarum* phycospheres.

298

### 299 **Community structure retrieved in *A. fusiformis* and *P. salinarum* phycosphere**

300 Cell-sorting based on the fluorescence and size properties of cell populations from the  
301 different samples allowed to collect *A. fusiformis* individuals and their associated  
302 phycosphere (27,172 events on average) and *P. salinarum* individuals and their  
303 phycosphere (1,025,034 events on average) (Table S1, Figure S1 and S2). Since we could  
304 not sample biological replicates at the different sampled depth because of field constraints,

305 we chose to analyze the phycosphere diversity by pooling all depths together in the  
306 abundance table. As expected, a dominance of ribosomal sequences affiliated to the  
307 targeted phytoplanktonic species either in the 16S or 18S rRNA genes datasets was  
308 observed (*i.e.*, the dominance of *A. fusiformis* 16S sequences in the bacterial 16S rRNA  
309 genes dataset of the *A. fusiformis* phycosphere and that of *P. salinarum* in the eukaryotic  
310 18S rRNA genes dataset sequences of the *P. salinarum* phycosphere, Figure S6 and Table  
311 S3), confirming the successful enrichments of the target groups. The direct consequence of  
312 the presence of the target host sequences in the bacterial and eucaryotic datasets is the  
313 reduction of the apparent sequencing depth for these particular pools. Nevertheless, the *A.*  
314 *fusiformis* and *P. salinarum* OTUs were conserved, and the datasets were analyzed focusing  
315 on OTUs presence-absence patterns rather than classically the number of sequences. This  
316 allows to obtain a representative view of the diversity which is less biased by the over  
317 representation of the dominant taxon, and which can be compared between samples to give  
318 trends of microbial structure divergences. Since the archaeal dataset was free of host  
319 sequences and not impacted by the targeted photosynthetic microorganism, we could  
320 investigate archaeal OTUs presence-absence, abundance, and phylogeny in detail.

321         Based on flow cytometry cell-sorting, we showed that microbial OTUs  
322 composition from *A. fusiformis* and *P. salinarum* phycospheres were different, as illustrated  
323 on NMDS plots for bacterial, microeukaryotic and archaeal communities (based on OTUs  
324 presence-absence, Figure 2 and OTUs abundance, Figure S4) and by the presence of  
325 specific OTUs in each phycosphere (Figure S7). Moreover, phycospheric microbial  
326 composition is slightly different from the microbiome retrieved as free-living in the water  
327 column of Lake Dziani Dzaha as suggested by the NMDS plots (Figure 2 and Figure S4)  
328 and the composition in OTUs (Figure 3). This was further supported by the number of

329 specific free-living OTUs that represented 31.9, 36.8 and 26.6% of bacterial,  
330 microeukaryotic and archaeal communities (Figure 4).

331 In more details, in the *A. fusiformis* phycosphere, bacterial communities were  
332 mostly composed of cyanobacterial OTUs (all being affiliated to *A. fusiformis*) accounting  
333 for 52.9 % of the 274 observed OTUs. Other OTUs were affiliated with Bacteroidia (mostly  
334 Bacteroidales and Chitinophagales), Alphaproteobacteria (mainly Rhodobacterales) and  
335 Dethiobacteria, accounting for 10.6, 8.8 and 7.3% of bacterial OTUs, respectively (Figure  
336 3A). This pattern contrasted with the *P. salinarum* phycosphere (Figure 3A) in which  
337 bacterial OTUs communities consisted mainly of Alphaproteobacteria (mainly  
338 Rhodobacterales) accounting for 20.4% of OTUs, and of several phyla each representing  
339 about 10% of bacterial OTUs (among 206 OTUs) including Cyanobacteria (all being  
340 affiliated to the *Arthrospira* PCC-7345 genus), Actinobacteria (mainly Nitriliruptorales),  
341 Bacteroidia (mostly Bacteroidales) and Gammaproteobacteria (mainly  
342 Ectothiorhodospirales). In contrast, the bacterial free-living microbiome was dominated  
343 by OTUs affiliated with Actinobacteria, Alphaproteobacteria, Bacteroidia and  
344 Dethiobacteria, each representing over 10% of the identified OTUs (Figure 3A).

345 The microeukaryotic composition retrieved in both phycospheres was mainly  
346 represented by OTUs affiliated to the Picocystophyceae class, representing 44.4 and 62.5%  
347 of OTUs in *A. fusiformis* and *P. salinarum* phycospheres, respectively (all being *P.*  
348 *salinarum* OTUs) and by a Chlorophyceae class (Figure 3B). This latter Chlorophyceae  
349 class contained 3 OTUs affiliated with the rare volvocalean green algal genus *Lobomonas*  
350 and represented 31.1 and 27.5% of the microeukaryotic OTUs in *A. fusiformis* and *P.*  
351 *salinarum* phycospheres, respectively. *A. fusiformis* was also associated with Bikosea and  
352 Placididae classes: 11.1 and 6.7% of OTUs, respectively, that were less frequent in the *P.*



353 *salinarum* phycosphere, 2.5 and 2.5%, respectively (Figure 3B). On the other hand, the  
354 free-living microbiome was mainly composed of the Picocystophyceae class (*i.e.*  
355 *Picocystis*), Jakobea and Bikosea classes, representing 56.4, 16.4 and 14.5% of  
356 microeukaryotic OTUs, respectively (Figure 3B).

357 Analyses of archaeal communities also showed contrasted patterns in  
358 phycospheric communities between *A. fusiformis* and *P. salinarum*. This was particularly  
359 true for Woesearchaeales (Woesearchaeia), enriched in the *P. salinarum* phycosphere (*i.e.*,  
360 70.1% of archaeal OTUs in the *P. salinarum* phycosphere versus 51% in the *A. fusiformis*  
361 phycosphere, Figure 3). Opposite composition patterns were reported for  
362 Methanomicrobiales (Halobacteriota), Methanofastidiosales (Euryarchaeota) or  
363 Thermoplasmata MBGD and DHVEG-1 (Thermoplasmatota) that were lower in the *P.*  
364 *salinarum* phycosphere (8.4, 4.8 and 5.3% of OTUs, respectively) compared to the *A.*  
365 *fusiformis* phycosphere (13.5, 5.75 and 12.5% of OTUs, respectively). The archaeal free-  
366 living microbiome is mainly composed of Woesearchaeales, Thermoplasmata MBGD and  
367 DHVEG-1, Methanomicrobiales and Methanofastidiosales (73.2, 6.9, 5.4 and 5.1% of  
368 OTUs, respectively). Despite an apparently similar composition to that found in the *A.*  
369 *fusiformis* and even more *P. salinarum* phycosphere microbiomes, the Jaccard matrices,  
370 established on an OTUs presence-absence matrix, suggested that OTUs from the three  
371 fractions were different (Figure 2). This was congruent with the occurrence of OTUs  
372 reported on the archaeal phylogeny (Figure 5), based on 421bp positions, showing that  
373 whatever their taxonomic affiliation, most archaeal OTUs were retrieved in the free-living  
374 microbiome (185 over 196 archaeal OTUs) suggesting the ubiquity of archaea in the water  
375 column. A total of 116 archaeal OTUs were retrieved in *A. fusiformis* phycosphere and 116  
376 OTUs retrieved in the *P. salinarum* phycosphere. A focus on Woesearchaeales (129 OTUs

377 over 196 archaeal ones) highlighted that 125 OTUs were retrieved in the free-living  
378 microbiome, 60 in the *A. fusiformis* phycosphere and 76 in the *P. salinarum* phycosphere,  
379 and interestingly 47 woesearchaeal OTUs were retrieved in both phycospheres and free-  
380 living microbiomes (Figure 5). Noticeably, 43 woesearchaeal OTUs were specific to the  
381 free-living microbiome, many of which are phylogenetically close, while only 4 OTUs  
382 were specific to the phycospheres (*i.e.* particule-attached). The abundance of archaeal  
383 OTUs allowed the characterization of three contrasted profiles: First, OTUs for which the  
384 abundance in phycospheres was in the same range as that in the free-living microbiome, for  
385 example OTUs 2, 4, 13, 36, 148, 261, 138. Second, some OTUs that were less abundant in  
386 the free-living microbiome compared to the phycospheres, such as OTUs 558, 235 or 441.  
387 Finally, some OTUs that were more abundant in the free-living microbiome compared to  
388 the phycospheres, for example OTUs 10, 34, 47, 49, 57, 75, 91, 222, 202, 390 (Figure 5).

389

## 390 **Discussion**

391

392 We used flow cytometry cell-sorting to collect both *A. fusiformis* and *P. salinarum* cells in  
393 association with their surrounding associated phycospheres, offering the possibility to work  
394 at the microbial scale compared to previous studies which were performed at a larger scale.  
395 In fact, inherently to its functioning, flow cytometry isolates the objects to analyze, *e.g.*  
396 here the *Picocystis* or *Arthrospira* cells, in micro-droplets. By setting the sorting to favor  
397 unique events (one cell per droplet), we can isolate the cell with its closely associated  
398 surrounding water layer, *e.g.* its phycosphere. The size of the droplets used here, 60µm, is  
399 in adequation to theoretical ranges of phycosphere radius calculated by Seymour and  
400 colleagues (2017).

401

402 **Different composition of *A. fusiformis* and *P. salinarum* phycospheres**

403 The present work showed different microbial compositions, at the OTUs level, between the  
404 *A. fusiformis* and *P. salinarum* phycospheres, with contrasted dominant OTUs being  
405 reported. Previous works, conducted on microorganisms associated with photosynthetic  
406 cells in marine ecosystems, reported mostly Rhodobacterales (*i.e.* Alphaproteobacteria),  
407 such as in the *P. salinarum* phycosphere, and Bacteroidia, such as in both *P. salinarum* and  
408 *A. fusiformis* phycospheres (Buchan et al., 2014; Teeling et al., 2012). The specificity of  
409 some associated bacterial members recovered here in the *A. fusiformis* and *P. salinarum*  
410 phycosphere microenvironments, such as Nitriliruptorales or Ectothiorhodospirillales is in  
411 agreement with the uniqueness of Lake Dziani Dzaha and the adapted communities  
412 retrieved in its water column (Hugoni et al., 2018). Moreover, the retrieval of eukaryotic  
413 members affiliated with Bikosea and Placididae, mainly from the *A. fusiformis*  
414 phycosphere, is consistent with their size, Bikosea being on average 3–4 µm wide and 5–6  
415 µm length, (Jirsová et al., 2019) and Placididae, 3-10µm length and 2-7µm wide, (Rybarski  
416 et al., 2021), which are essentially larger than Picocystis. While being previously retrieved  
417 in hypersaline environments, the Bikosea ecology remains unclear (Jirsová et al., 2019;  
418 Park & Simpson, 2015). The presence of several Chlorophyceae OTUs affiliated with the  
419 *Lobomonas* genus in both phycospheres is highly interesting as we did not detect those  
420 OTUs in the large ecosystemic studies we previously performed (Bernard et al., 2019;  
421 Hugoni et al., 2018), suggesting that we missed those OTUs because of the large scale  
422 employed, proving further value to the microscaled studies as performed here.

423

424 **Contrasted free-living microbiome compared to phycospheric microbiomes**

425 Noticeably, our work highlighted that the free-living microbiome was different from the  
426 one retrieved from the phycospheres and did not reflect a possible leakage of the *A.*  
427 *fusiformis* or *P. salinarum* phycosphere microbiomes in the filtrate or a possible capture of  
428 microbial cells by the *A. fusiformis* or *P. salinarum* cells at the filtration step. This  
429 demonstrates our postulate that the microalgae *Picocystis* indeed harbors a phycosphere.  
430 We show here that this phycosphere microbiome is different from that of the *A. fusiformis*  
431 one. The proximity of the *P. salinarum* phycosphere microbiome and the free-living  
432 microbiome may have been enhanced in our study due to technical issues and may only  
433 partially represent the true *P. salinarum* phycosphere microbiome. Indeed, it is widely  
434 considered that the free-living microbiome is accessible through the classical differential  
435 filtering considering the 3 to 0.2  $\mu\text{m}$  fraction. In consequence, we could not exclude that  
436 some *P. salinarum* small cells pass the 3 $\mu\text{m}$  filters and their phycosphere could be retrieved  
437 in the "free living" microbiome (3 to 0.2  $\mu\text{m}$  fraction). The presence of small cells  
438 phycosphere is congruent with previous studies focusing on *Prochlorococcus* and  
439 *Synechococcus* (from 0.7 to 1.5  $\mu\text{m}$ ) reporting their physical associations with heterotrophic  
440 bacteria (Malfatti & Azam, 2009) and chemotaxis through their exudates (J. Seymour et al.,  
441 2010).

442

#### 443 **New insights into archaeal microbiomes in the phycosphere**

444 The archaeal assemblage retrieved both at a large ecosystemic scale in the water column of  
445 Lake Dziani Dzaha and at a microscale in both phycospheres is largely dominated by OTUs  
446 affiliated with the Woesearchaeales. Woesearchaeales are members of the DPANN  
447 superphyla, which includes fast-evolving archaeal species (Adam et al., 2017; Dombrowski  
448 et al., 2020), that are diverse and broadly distributed in the biosphere. Few culturable

449 representatives of DPANN are available. Nevertheless, studies revealed that they presented  
450 an extremely small cell size (0.1-0.8  $\mu\text{m}$ ; (Dombrowski et al., 2020)) and a reduced genome  
451 ( $\approx$ 0.5 to 3 Mb, (Castelle et al., 2018)) associated with limited metabolic capabilities,  
452 suggesting they may depend on symbiotic interactions with other organisms or may even  
453 comprise novel parasitic species (Castelle et al., 2015; Dombrowski et al., 2019). While  
454 numerous studies hypothesized an obligate symbiotic and/or parasitic lifestyle for DPANN  
455 archaea (Golyshina et al., 2017; Hamm et al., 2019; Huber et al., 2002; Schwank et al.,  
456 2019), a growing number of recent works suggested wider metabolic capabilities allowing a  
457 free-living lifestyle in some DPANN members, such as Micrarchaeota (Kadnikov et al.,  
458 2020) or “*Ca. Iainarchaeum andersonii*” affiliated with Diapherotrites (Youssef et al.,  
459 2015). Here, we can hypothesize that, in Lake Dziani Dzaha, there are two kinds of  
460 Woesearchaeales, (i) the ones that lives as particle-attached OTUs to a phytoplanktonic  
461 species and (ii) the ones that lives as free-living (*i.e.* not as attached to phytoplankton:  
462 fraction < 3 $\mu\text{m}$ ) for which we could not exclude interactions with other small microbial  
463 cells. While the Woesearchaeales metabolism remains unclear, a recent study of Lake A  
464 (Canadian High Arctic lake), which presents extreme physico-chemical gradients and  
465 sulfidic waters as observed in Lake Dziani Dzaha, detected sulfur/thiosulfate oxidation and  
466 sulfite and sulfur reduction potentials in Woesearchaeota reconstructed genomes (Vigneron  
467 et al., 2022). We suppose that in the water column conditions of Lake Dziani Dzaha, that  
468 are organic-rich and where oxygen saturation decreases sharply from the surface to 5m  
469 depth (from 180 to 2.7%) and becomes anoxic below, that free-living Woesearchaeales  
470 might possess key metabolic pathways for heterotrophic autonomous growth such as  
471 glycolysis, ATP generation or nucleotide biosynthesis. Thus, we hypothesize that, in this  
472 anoxic zone, the ecological role of free-living Woesearchaeales might be the fermentation

473 of organic compounds that come from photosynthetic biomass degradation and which  
474 fermentation by-products will be further consumed by methanogenic Archaea, performing  
475 the final step of organic matter mineralization (Huang et al., 2021). In contrast, in the  
476 phycosphere, attached-Woeseearchaeales might take direct advantage from low molecular  
477 compounds from photosyntheticates exudated by their host cell, from transparent  
478 exopolymeric particles (TEP) and exopolymeric substances (EPS) (Christian & Anderson,  
479 2002), and in turn ferment some of these compounds making their secondary metabolites  
480 accessible to the cells. Over time, Woeseearchaeota that have benefited of key nutrients and  
481 cellular building blocks from their hosts might have adapted to these conditions by losing  
482 key pathways involved in their synthesis in central metabolism, as reported for DPANN  
483 archaea.

484         Among other archaeal classes retrieved in Lake Dziani Dzaha, most are  
485 methanogens and thus, strictly anaerobic. Their presence in the water column, even in  
486 layers where oxygen is still present suggested either a tolerance to oxygen exposure (Angel  
487 et al., 2011) and/or the presence of anoxic micro-niches in the *A. fusiformis* cells periphery  
488 and to a lesser extent in *P. salinarum* cells periphery, because of its lower size. High  
489 methane fluxes were observed in Lake Dziani Dzaha (Cadeau et al., 2020), that could not  
490 only depend on abiotic lithogenic inputs, and which could be the consequence of the  
491 “methane paradox” (unexplained accumulation of methane in oxic layers of aquatic  
492 ecosystems) (Günthel et al., 2019). Different mechanisms could explain this methane fluxes  
493 in Lake Dziani Dzaha. The first is based on the ability of different microorganisms to  
494 demethylate organic matter occurring when bacteria, including cyanobacteria, scavenge  
495 phosphorus, sulfur or nitrogen sources, cleave a methyl group to convert it in methane  
496 (Bizic, 2021). Second, regardless and independently of the presence of methylated

497 compounds in the water column, it has been shown that cyanobacteria could produce  
498 methane from CO<sub>2</sub> concomitantly with oxygen emission (Bižić et al., 2020). It has also  
499 been shown that the eukaryotic phytoplankton could also produce methane through  
500 different ways (Klitzsch et al., 2019). In classical aquatic ecosystems, in which  
501 cyanobacteria and eukaryotic green algae do not represent the vast majority of the biomass,  
502 photosynthesis-associated methane emissions might not be sufficient to account for a  
503 significant fraction of methanogenesis in comparison to methanogenic archaea (Bižić,  
504 2021). However, in Lake Dziani Dzaha were *A. fusiformis* and *P. salinarum* account for  
505 almost 566 to 702 µg.L<sup>-1</sup> of chlorophyll *a* (Leboulanger et al., 2017), the photosynthesis-  
506 associated methane emissions might become significant to apprehend lake functioning.

507

#### 508 **Analogies with the terrestrial plant-root interface, *i.e.* the rhizosphere**

509 While being a fundamental ecological interface, the phycosphere micro-environment has  
510 been paid much less attention compared to its terrestrial analogue, *i.e.* the rhizosphere.  
511 Here, we showed for the archaeal compartment, that most of the OTUs are widespread in  
512 the water column as free-living microorganisms and that several of them, being  
513 phylogenetically close, could also be retrieved in the phycosphere environment. This result  
514 suggested that phytoplanktonic species select specific archaeal OTUs, which sounded very  
515 similar to what is known as the rhizosphere effect, *i.e.*, the stimulation and selection of  
516 microbial communities by organic material exudated by plants in their rhizosphere, the  
517 recruitment of a root-adapted microbiome (Haichar et al., 2008; Uroz et al., 2019). In brief,  
518 our results confirm that the phycosphere is a dynamic microenvironment promoting  
519 microbial interactions, to which the ecological concepts described in the rhizosphere might  
520 be transposed.

## 521 **Conclusions**

522 Our work is the first to explore the microbial communities thriving in the phycosphere of  
523 bacterial (*A. fusiformis*), and eukaryotic (*P. salinarum*) phytoplanktonic populations by  
524 coupling flow-cytometry cell-sorting and metabarcoding of the three-domains-of-life. We  
525 took advantage of the comparison of microorganisms associated to the phycosphere *versus*  
526 free-living ones to identify contrasts in the microbiomes of the phycospheres and the water  
527 column. By investigating archaeal patterns, we showed that most of the Woesearchaeales  
528 from Lake Dziani Dzaha were preferentially free-living members and that the  
529 phytoplanktonic species promoted the selection of specific archaeal OTUs.

530 Further work will be needed to confirm this selection for bacteria and  
531 microeukaryotes, and a deeper investigation of the mechanisms involved in OTU selection  
532 will be necessary, in order to prove that a phycosphere effect exist (selection of specific  
533 OTUs through the interaction with phytoplanktonic species) and to identify the key  
534 metabolites involved in these interactions. However, the current study sets the stage for a  
535 better understanding of the relationships between microbials cells and how each and  
536 everyone, regardless of their size, can influence their (very) local environment.

537

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544

545 **Conflict of interest statement**

546 The authors declare that there is no conflict of interest.

547

548 **Benefit-Sharing statement**

549 The field permit was granted by the Conservatoire du Littoral et des Rivages Lacustres,  
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551 body with free public access but restricted activities, under the control of the French agency  
552 for littoral ecosystems conservation (<http://www.conservatoire-du-littoral.fr/>).

553

554 **Authors contribution**

555 C.B, M.T, M.H designed research.

556 P.G, L.G, M.H, G.S, D.J performed research.

557 M.B, P.M.O, D.M, S.D, C.B, M.T, M.A, M.H, C.B, H.A, A.E analyze data.

558 L.A.C, M.B, M.H, prepared the figures.

559 M.B, P.M.O, D.M, S.D, C.B, M.T, M.A, M.H wrote the manuscript.

560

561

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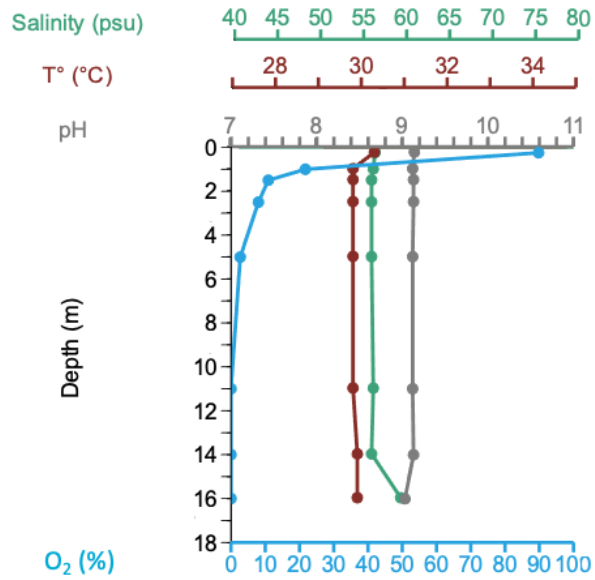
886 **Data accessibility statement**

887 The sequence data generated in this study were deposited in the EBI-ENA public database  
888 under the following accession number PRJEB60179, PRJEB60179, PRJEB42914,  
889 PRJEB42913 for microeukaryotes, bacteria and archaea, respectively.

890

891 **Figures and tables**

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895 **Figure 1. Vertical profiles of environmental parameters recorded along the water**

896 **column.** Salinity (psu), temperature (°C), pH, and O<sub>2</sub> saturation (%) profiles were recorded

897 during November 2018.

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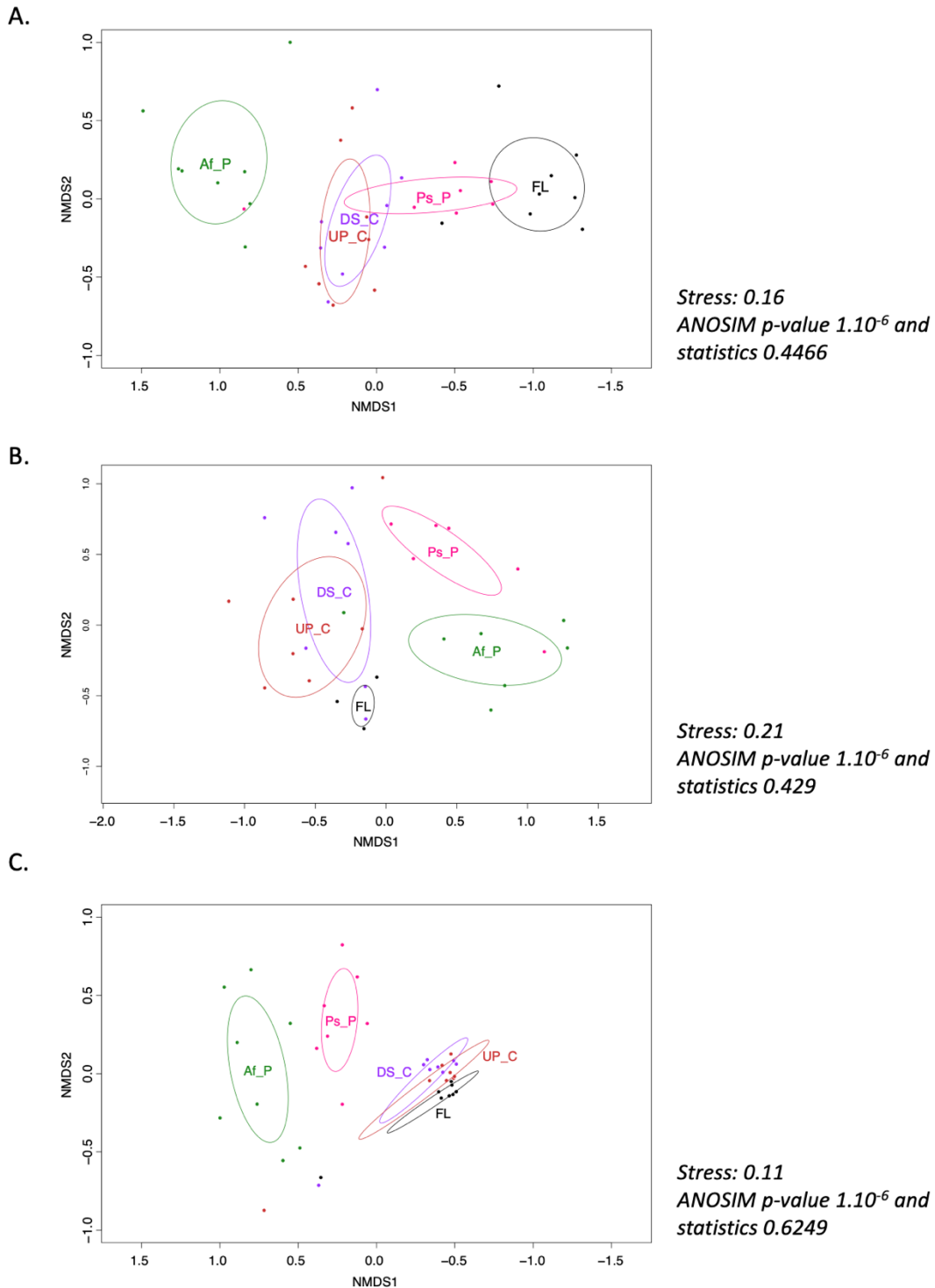
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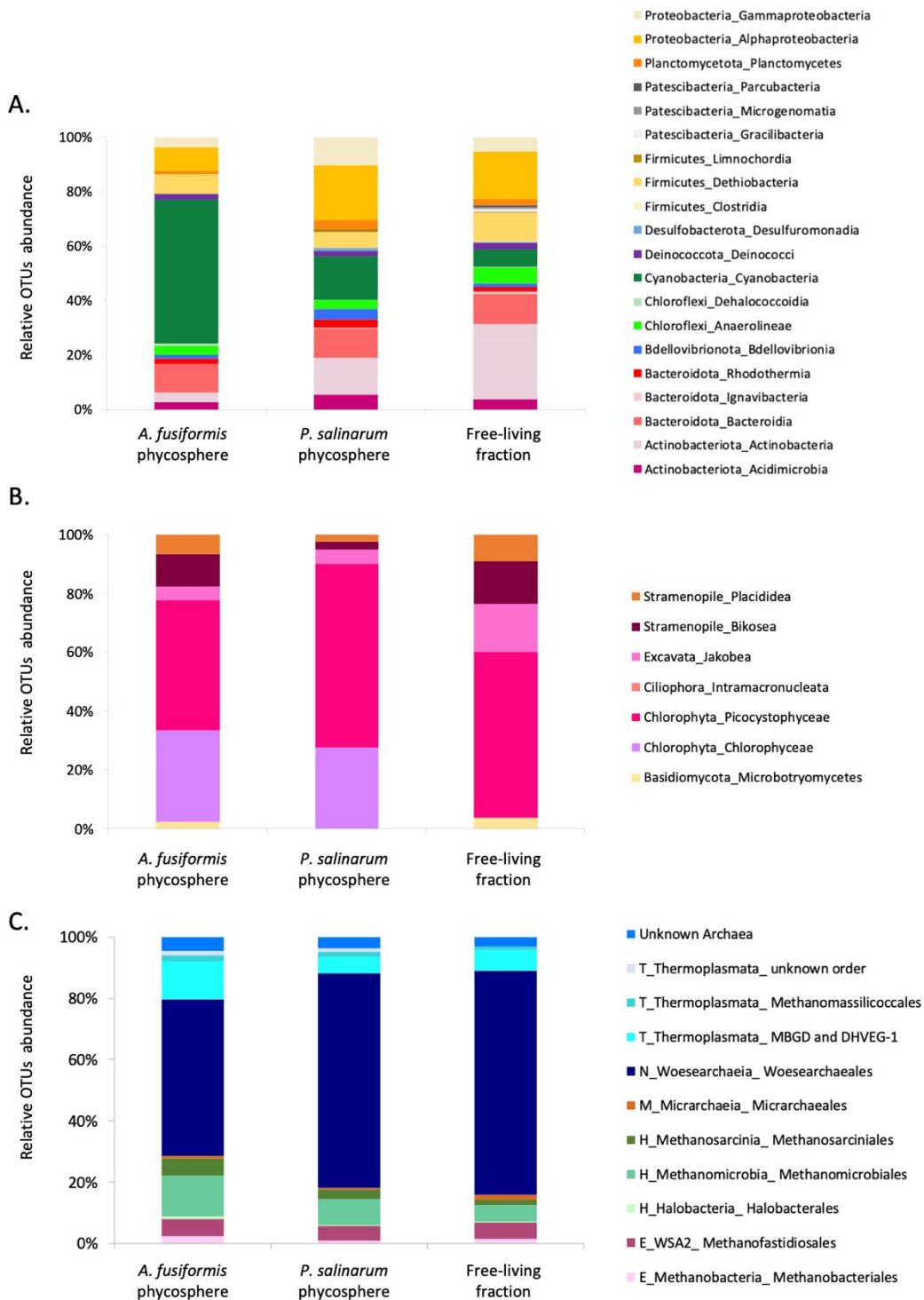
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908 **Figure 2. Differences in (A) bacterial, (B) microeukaryotic and (C) archaeal**

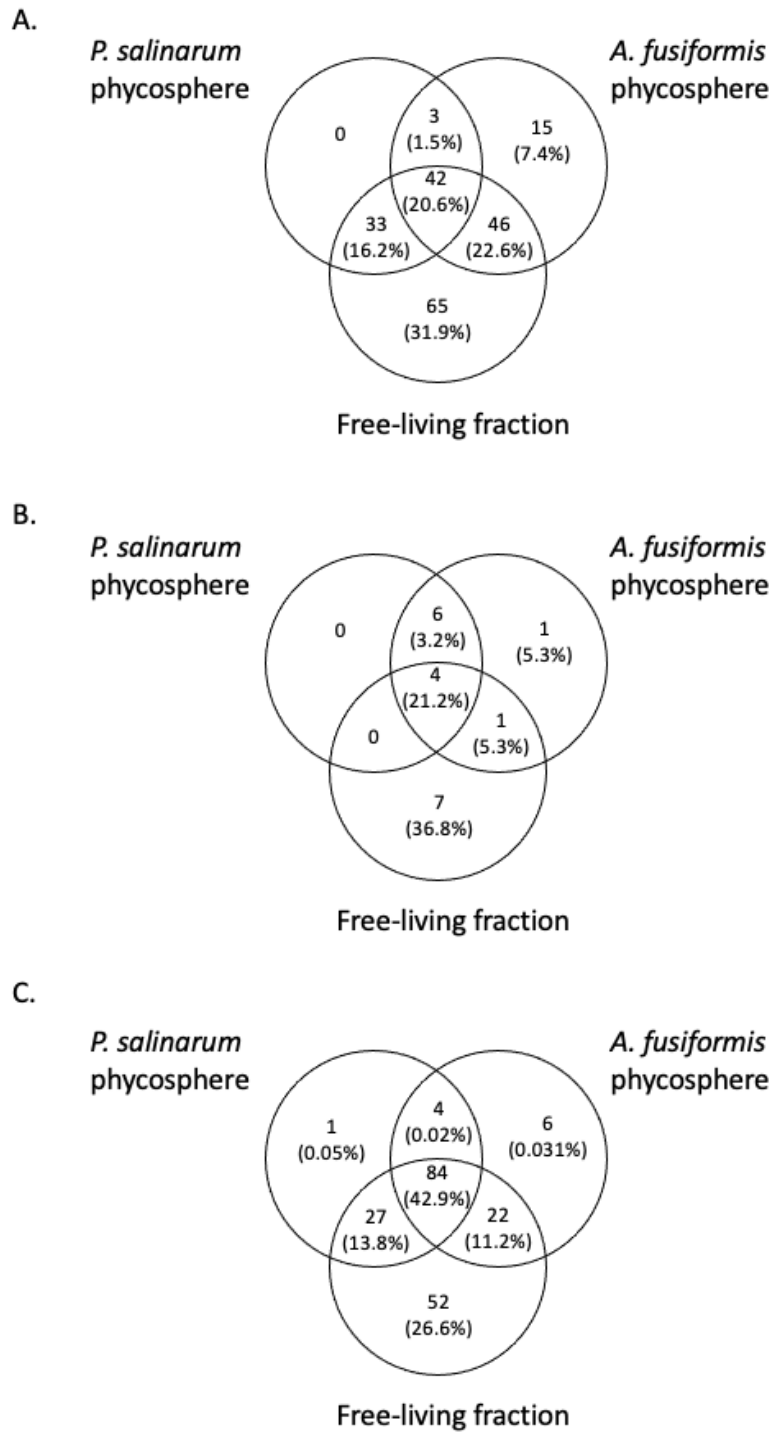
909 **communities' structure, evaluated at the OTUs level.** NMDS ordination was performed

910 on Jaccard matrices based on presence-absence data. Jaccard indices were compiled

911 considering upstream (UP\_C) and downstream controls (DS\_C), *A. fusiformis* (Af\_P) and  
912 *P. salinarum* (Ps\_P) phycospheres and the free-living (FL) microbiome (eight samples per  
913 condition). To fully compare the OTUs composition in each condition, indices were  
914 computed from the bacterial dataset normalized to 101 sequences, the microeukaryotic  
915 dataset normalized to 196 sequences and the 4,000 sequences archaeal dataset.



917 **Figure 3. Relative OTUs abundance in *A. fusiformis* and *P. salinarum* phycospheres**  
918 **compared to free-living microbiome.** Relative abundance of OTUs were declined for  
919 bacteria (A), microeukaryotes (B) and archaea (C).

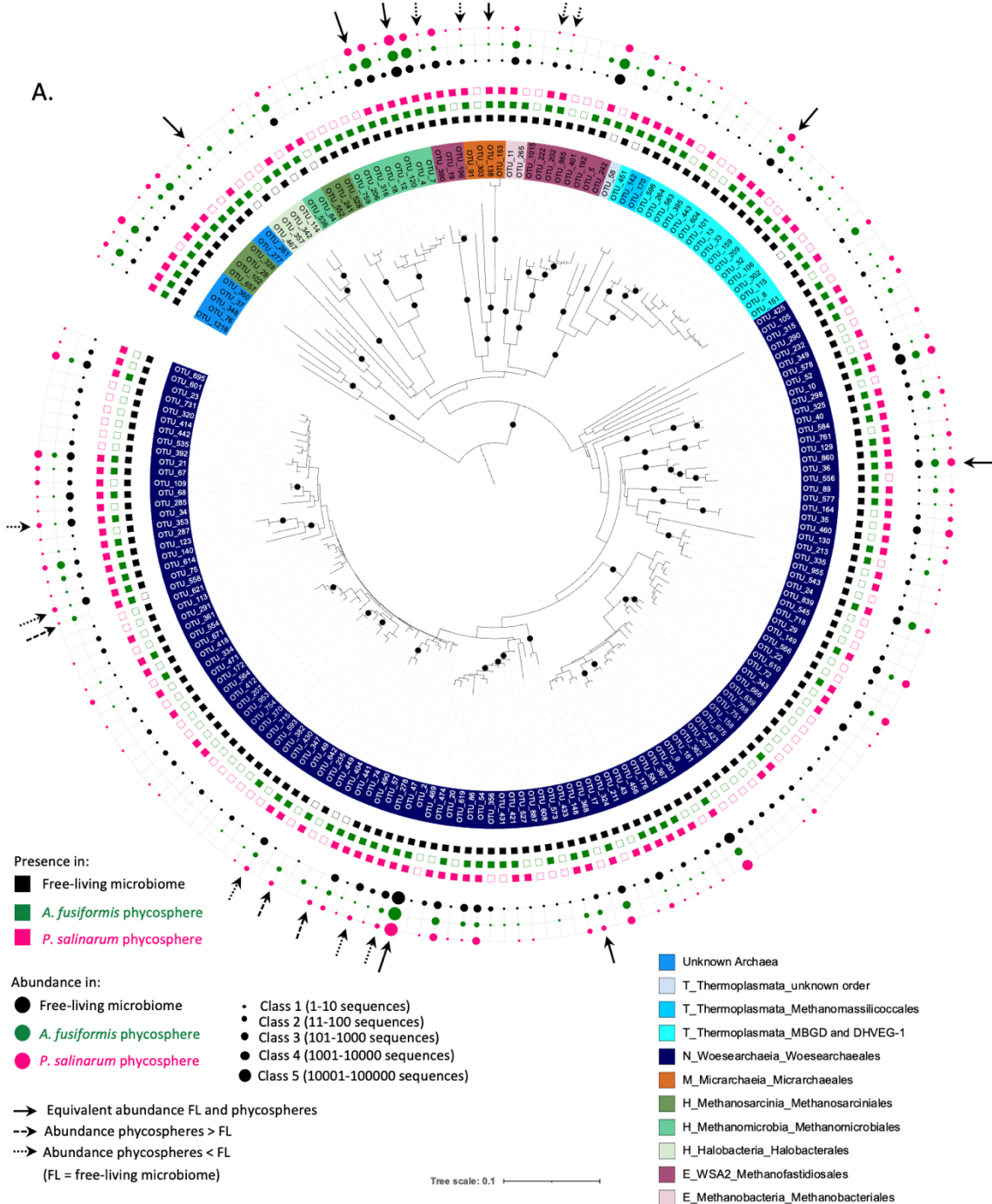


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921 **Figure 4. Distribution of OTUs among the *A. fusiformis* and *P. salinarum***

922 **phycospheres, and the free-living microbiome.** Venn diagrams presenting shared and

923 specific bacterial (A), microeukaryotic (B) and archaeal (C) OTUs.



**B.**

	All	Common	Specific <i>A. fusiformis</i> phycosphere	Specific <i>P. salinarum</i> phycosphere	Specific Free-living fraction	Common <i>A. fusiformis</i> and <i>P. salinarum</i> phycospheres	Common Free-living fraction and <i>P. salinarum</i> phycospheres	Common Free-living fraction and <i>A. fusiformis</i> phycospheres
E_Methanobacteria_Methanobacteriales	2	1						1
E_WSA2_Methanofastidiosales	11	5	1		3		1	1
H_Halobacteria_Halobacteriales	4	1	2		1			
H_Methanomicrobia_Methanomicrobiales	12	7	1					4
H_Methanosarcinia_Methanosarcinales	6	3		1				2
M_Micrarchaeia_Micrarchaeales	4	3			1			
N_Woearchaeia_Woearchaeales	129	47	1		43	3	26	9
T_Thermoplasmata_MBGD and DHVEG-1	18	9	1		4	1		3
T_Thermoplasmata_Methanomassilicoccales	2	2						
T_Thermoplasmata_unknown order	1	1						
Unknown Archaea	7	5						2
	196	84	6	1	52	4	27	22

925 **Figure 5. (A) Phylogenetic analysis of archaeal 16S rRNA genes retrieved in *A.***  
926 ***fusiformis* and *P. salinarum* phycospheres and in the free-living microbiome and (B)**  
927 **core and specific OTUs among phycospheres and free-living microbiome.** The tree was  
928 constructed with ~421bp positions and rooted at mid-point. Bootstraps > 70 are presented  
929 as black circles. Occurrence of OTUs associated to the *A. fusiformis* phycosphere were  
930 presented as green squares, to the *P. salinarum* phycosphere as pink squares, while OTUs  
931 from the free-living microbiome are indicated with black squares. Abundance of OTUs was  
932 ranked as follows: 0 sequence: class 0, between 1-10 sequences: class 1, between 11-100:  
933 class 2, between 101-1000: class 3, between 1001-10000: class 4, between 10001-100000:  
934 class 5.

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