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1 **Influence of aphotic haloclines and euxinia on organic biomarkers and microbial**  
2 **communities in a thalassohaline and alkaline volcanic crater lake**

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34

35 **Running title:** Organic biomarkers and microbial diversity in a thalassohaline lake

36

37 **Abstract**

38 Studies on microbial communities, and their associated organic biomarkers, that are found  
39 thriving in the aphotic euxinic waters in modern stratified ecosystems are scarce  
40 compared to those undertaken in euxinic photic zones. The Dziani Dzaha (Mayotte, Indian  
41 Ocean) is a tropical, saline, alkaline crater lake that has recently been presented as a  
42 modern analogue of Proterozoic Oceans due to its thalassohaline classification (having  
43 water of marine origin) and specific biogeochemical characteristics. Continuous intense  
44 photosynthetic production and microbial mineralization keeps most of the water column  
45 permanently aphotic and anoxic preventing the development of an euxinic (sulfidic and  
46 anoxic) photic zone despite a high sulfide/sulfate ratio and the presence of permanent or  
47 seasonal haloclines. In this study, the molecular composition of the organic matter in Lake  
48 Dziani Dzaha was investigated and compared to the microbial diversity evaluated through

49 16S rRNA gene amplicon sequencing, over two contrasting seasons (rainy vs. dry) that  
50 influence water column stratification. Depth profiles of organic biomarker concentrations  
51 (chlorophyll-a and lipid biomarkers) and bacterial and archaeal OTU abundances  
52 appeared to be strongly dependent on the presence of aphotic haloclines and euxinia.  
53 OTU abundances revealed the importance of specific haloalkaliphilic bacterial and  
54 archaeal assemblages in phytoplanktonic biomass recycling and the biogeochemical  
55 functioning of the lake, suggesting new haloalkaline non-phototrophic anaerobic microbial  
56 precursors for some of the lipid biomarkers. Uncultured Firmicutes from the family  
57 Syntrophomonadaceae (Clostridiales), and Bacteroidetes from the ML635J-40 aquatic  
58 group, emerged as abundant chemotrophic bacterial members in the anoxic or euxinic  
59 waters, and were probably responsible for the production of short-chain *n*-alkenes, wax  
60 esters, diplopterol and tetrahymanol. Halocline-dependent euxinia also had a strong  
61 impact on the archaeal community which was dominated by Woesearchaeota in the  
62 sulfide-free waters. In the euxinic waters, methanogenic Euryarchaeota from the  
63 Methanomicrobia, Thermoplasmata and WSA2 classes dominated and were likely at the  
64 origin of common hydrocarbon biomarkers of methanogens (phytane, pentamethyl-  
65 eicosenes, and partially hydrogenated squalene).

66  
67 **Key words:** Haline and aphotic euxinia, lipid biomarkers, bacterial and archaeal diversity,  
68 Syntrophomonadaceae, Bacteroidetes ML635J-40 aquatic group, WSA2 methanogenic  
69 Euryarchaeota.

70

## 71 **1. INTRODUCTION**

72 The reliability of lipid biomarkers that are assigned to specific biological origins in the  
73 environment is dependent on previous characterization of similar lipids from isolated and  
74 cultured (micro)organisms. The use of lipid biomarkers to reconstruct the paleo-biosphere  
75 and paleo-environments through the analysis of fossil sedimentary records is essentially  
76 based on the principle of actualism (uniformitarianism), that assumes that biosynthetic  
77 pathways have not evolved drastically over time within the different domains of the tree of  
78 life (Brocks & Pearson, 2005). However, the number of (micro)organisms isolated from  
79 the environment and available in pure cultures is still limited, and over the past few  
80 decades, the use of lipid biomarkers has largely benefited from phylogenetic trees and  
81 culture-independent techniques applied to natural communities (Brocks & Banfield, 2009).  
82 Great advances have been made with comparative and environmental genomics, which  
83 have demonstrated that certain classes of lipids can be produced by a much greater  
84 diversity of organisms than previously envisaged (Pearson, Flood Page, Jorgenson,  
85 Fischer, & Higgins, 2007; Brocks & Banfield, 2009; Pearson, 2014; Banta, Wei, &  
86 Welander, 2015). As an alternative, the comparison of lipid biomarker abundance with the  
87 composition of the microbial community, as determined by genetic methods, can  
88 potentially help to elucidate the biological origin of certain lipids (Villanueva et al., 2014).  
89 Investigating the possible origin(s) of lipid biomarkers can be particularly challenging in  
90 specific environments such as saline and/or stratified water bodies including soda lakes,  
91 meromictic lakes, and closed basins, where a significant part of the microbial community  
92 is generally composed of, as-yet-uncharacterized, or non-isolated organisms (Overmann,  
93 Beatty, & Hall, 1996; Sorokin et al., 2014; Hamilton et al., 2016). These ecosystems are  
94 characterized by seasonal or permanent physico-chemical gradients which divide the

95 water column into at least two or more physically- and chemically- contrasting  
96 compartments hosting diverse microbial communities that are adapted to specific  
97 ecological niches. Typically, phototrophic organisms such as (pico)cyanobacteria deliver  
98 fixed carbon to vertically organized chemolitho- and chemoorgano-trophic bacterial and  
99 archaeal communities that then recycle a major part of the sinking organic matter (Sorokin  
100 et al., 2014; Sorokin, Banciu, & Muyzer, 2015). In stratified sulfur-rich environments where  
101 light reaches anoxic waters, the presence of an euxinic (sulfidic and anoxic) photic zone  
102 further allows dense populations of purple and green sulfur anoxygenic phototrophic  
103 bacteria to develop and impact the carbon and sulfur cycles (Overmann et al., 1996;  
104 Hamilton et al., 2014). These conditions resemble, at least regionally, those that have  
105 prevailed over several periods of the Earth's history, such as the Mesoproterozoic and  
106 Phanerozoic biotic crises, when parts of the ocean were oxygen-depleted and partly  
107 euxinic (Grice et al., 2005; Lyons, Anbar, Severmann, Scott, & Gill, 2009). For this reason  
108 contemporaneous stratified and euxinic ecosystems are often considered to be good  
109 models for the past ocean (Meyer & Kump, 2008). The structure and lipid biomarkers of  
110 the microbial communities thriving in such euxinic ecosystems have been regularly  
111 investigated, and lipid biomarkers specific to photic zone euxinia and associated  
112 chemoclines have been characterized and used for paleo-environmental reconstructions  
113 (Grice et al., 2005; French, Rocher, Zumberger, & Summons, 2015). Surprisingly, much  
114 less is known about the microbial communities thriving in aphotic euxinic waters (i.e.,  
115 below the euxinic photic zone or in euxinic ecosystems deprived of an euxinic photic zone)  
116 or their potential specific organic biomarkers, although such ecological niches often  
117 represent a major part of euxinic ecosystems (Klepac-Ceraj et al., 2012; Hamilton et al.,  
118 2016).

119 Lake Dziani Dzaha (Mayotte, Indian Ocean) is a tropical volcanic crater lake formed ca.  
120 6-7 kyr ago. It has recently been presented as a new contemporaneous analogue of  
121 Proterozoic oceans based on its thalassohaline classification (having water of marine  
122 origin), microbial functioning, biogeochemical characteristics, and the presence of one  
123 deep permanent and one shallow seasonal halocline (Leboulanger et al., 2017; Bernard  
124 et al., 2019; Cadeau et al., 2020; Sarazin et al., 2020). The apparent absence of  
125 hydrological connections with the surrounding Indian Ocean makes it a very peculiar  
126 saline lake, functioning as an endorheic basin, where the water composition has been  
127 modified through time by biological and climatic/environmental processes to attain its  
128 current characteristics (Sarazin et al., 2020). These include strong alkalinity, elevated pH,  
129 low sulfate content, and continuous massive rates of organic matter production and  
130 mineralization which limit light penetration and maintain a permanently anoxic and aphotic  
131 water column below ca. 1 m depth (Leboulanger et al., 2017; Hugoni et al., 2018; Bernard  
132 et al., 2019; Sarazin et al., 2020). Depending on the season (rainy vs. dry), a strong  
133 halocline develops at ca. 2 m depth, i.e., below the limited photic zone, and sulfidic  
134 conditions intensify in the anoxic waters below. However, the combination of the sun-  
135 shielding impact of the huge cyanobacterial biomass and the strong halocline precludes  
136 the photic zone overlapping with euxinic waters and thus prevents the development of an  
137 euxinic photic zone.

138 Previous studies on the thriving microbial diversity in Lake Dziani Dzaha have shown that  
139 the photosynthetic community is strongly dominated by the cyanobacteria *Arthrospira*  
140 *fusiformis* and the picoeukaryote green algae *Picocystis salinarum* which are responsible  
141 for the permanent green colour of the surface waters (Leboulanger et al., 2017; Bernard  
142 et al., 2019). A metabarcoding approach has demonstrated that the bacterial and archaeal

143 diversity is shaped by the contrasting physico-chemical conditions observed in the  
144 different compartments of the water column, showing both typical and atypical features  
145 when compared to other saline alkaline ecosystems (Hugoni et al. 2018). In addition to *A.*  
146 *fusiformis* and *P. salinarum*, the archaeal phylum Woesearchaeota dominates the  
147 microbial assemblage in the shallow oxygenated surface waters. This phylum remains  
148 dominant in the anoxic waters deprived of sulfides, whereas methanogenic Euryarchaeota  
149 and the chemotrophic bacterial phyla Firmicutes and Bacteroidetes take over the microbial  
150 diversity in anoxic and sulfidic (euxinic) waters. In line with the low sulfate content of the  
151 lake waters (<3mM in non-sulfidic waters and undetected in euxinic waters) and the  
152 absence of an euxinic photic zone, sulfate-reducing bacteria and anoxygenic phototrophic  
153 sulfur bacteria do not develop significantly in this ecosystem (Leboulanger et al., 2017;  
154 Hugoni et al., 2018).

155 In this study, the composition of the organic matter (OM) in Lake Dziani Dzaha was  
156 investigated and depth concentration profiles of selected biomarkers present in the  
157 suspended OM were compared to a more in-depth analysis of the bacterial and archaeal  
158 diversity (down to the class or order levels) for the two contrasting seasons that influence  
159 water column stratification and euxinia (rainy vs. dry). The microbial organic signature and  
160 community structure of the different compartments of the water column, suggest an  
161 uncommon microbial origin for certain lipid biomarkers and highlight the importance of  
162 specific bacterial and archaeal assemblages that develop below seasonal or permanent  
163 aphotic and euxinic haloclines, in the biogeochemical functioning of this saline and  
164 alkaline lake.

165

## 166 **2. MATERIALS AND METHODS**



## 167 **2.1 Site description, water column geochemistry and sampling**

168 Dziani Dzaha is a crater lake located on the French 'Petite Terre' island of the Mayotte  
169 Comoros archipelago, in the western Indian Ocean (Figs. 1A, B). Situated close to the  
170 ocean (ca. 0.2 km; Fig. 1C), it is likely that this inland lake was formed during the most  
171 recent phreatomagmatic eruption (between 7.5 and 4 ky BP; Zinke, Reijmer, &  
172 Thomassin, 2003). Lake Dziani Dzaha has been defined as a thalassohaline ecosystem  
173 because its dissolved mineral composition originates predominantly from seawater  
174 (Leboulanger et al., 2017; Sarazin et al., 2020), in contrast to athalassohaline ecosystems  
175 where the dissolved mineral composition originates from the evaporation of continental  
176 water. The unique seawater chemistry of the lake has evolved through time, in a closed  
177 system, to attain its current (poly)extremophilic characteristics, with alkalinity 100 times  
178 that of seawater, elevated pH (9.1-9.4), saline to hypersaline conditions (34-71 g L<sup>-1</sup>), low  
179 sulfate concentrations (0-3 mM) and high dissolved organic carbon (DOC) concentrations  
180 (4-8 mmol L<sup>-1</sup>) (Sarazin et al., 2020). The water temperature ranges between 27 and 33°C  
181 all year round. The seasonality, the huge cyanobacterial production (with *Arthrospira*  
182 *fusiformis* representing ca. 98% of the biomass) and recycling by the microbial  
183 communities are the main drivers of the present-day dynamics of the water chemistry of  
184 Lake Dziani Dzaha (Leboulanger et al., 2017; Bernard et al., 2019). Due to its small  
185 catchment area (ca. 0.5 km<sup>2</sup>) relative to its size (ca. 0.24 km<sup>2</sup>; Figs. 1C, D) and the  
186 absence of connected rivers, the lake is only marginally influenced by watershed inputs.  
187 During the rainy season, from November to March, the lake receives intense rainfall, which  
188 rapidly induces strong water column stratification characterized by a seasonal halocline  
189 at ca. 2 m depth and euxinic waters below, where sulfide concentrations reach up to 6.5  
190 mM. During the dry season, from April to October, rainfall is scarce, and strong

191 evaporation from the lake induces a 0.7-1 m decrease in the water level and, ultimately  
192 (3-4 months after the end of precipitations), the disappearance of the seasonal halocline.  
193 For simplicity, both periods are referred to as 'with' and 'without' seasonal stratification,  
194 respectively. However, whatever the period, lake waters become rapidly anoxic below the  
195 surface due to limited wind mixing, owing to the steep crater slopes, and the intense  
196 mineralization of the huge phytoplanktonic productivity which prevents light penetration  
197 and photosynthetic O<sub>2</sub> production below a depth of 1 m (Leboulanger et al., 2017). The  
198 maximum water depth of Lake Dziani Dzaha ca. 18 m, is found in a pit to the east of the  
199 lake (Fig. 1E), where an additional (permanent) halocline is observed (Fig. 2).

200 Sampling was performed at the pit site (Fig. 1E) during two field surveys, one conducted  
201 during October 2014 (around the end of the dry season, i.e., without seasonal  
202 stratification) and the other, in April 2015 (around the end of the wet period, i.e., with  
203 seasonal stratification). Prior to water sampling, pH, dissolved oxygen, temperature, and  
204 conductivity depth profiles were recorded using a YSI 6600XLM probe. These profiles  
205 enabled the sampling strategy to be adapted for organic biomarkers and microbial  
206 diversity analyses depending on the depth of the seasonal and permanent haloclines.

207 Water was then sampled using a horizontal Niskin bottle to provide an appropriate vertical  
208 resolution. H<sub>2</sub>S/HS<sup>-</sup> concentrations (i.e., soluble sulfide concentrations (ΣS(-II))), were  
209 determined in the field by colorimetry using a AQUALYTIC SpectroDirect  
210 spectrophotometer and Merck reagent kits. For lipid analyses, 500 to 1000 mL of lake  
211 water was filtered onto pre-combusted GF/F filters, and for total chlorophyll-a (Chl-a)  
212 analysis 5 mL water samples were conditioned similarly. For microbial diversity  
213 determination, 20 mL water samples were first filtered through 3 µm polycarbonate filters  
214 before being filtered again onto 0.2 µm polycarbonate filters. All filters were stored at -

215 20°C until analysis. The total phytoplankton abundance was determined by flow cytometry  
216 and cell counting as described in Bernard et al. (2019).

217

## 218 **2.2. Chlorophyll-a and lipid biomarker analyses**

219 Chl-a concentration was determined, on the sampling day, by spectrophotometry as  
220 described by Ritchie (2006). The samples were placed into polystyrene tubes with 3 mL  
221 96% ethanol and ultrasonified for 30 min in an ice bath. The closed tubes were then stored  
222 overnight at 4°C to complete pigment extraction in the dark. The extracts were filtered and  
223 transferred into spectrophotometric optical glass cuvettes, and absorption was measured  
224 over the visible light range (400-800 nm) using an Aqualytic AL800 spectrophotometer.

225 Lipids were extracted by ultrasonication with methanol-dichloromethane (MeOH-DCM 2:1,  
226 v/v, ×2), methanol-DCM (1:1, v/v, ×2), and DCM (×1). The combined extracts were  
227 concentrated by rotary evaporation and, following the removal of elemental sulfur using  
228 activated copper curls, the total lipid extract (TLE) was evaporated to dryness under a N<sub>2</sub>  
229 flux. The TLE was then chromatographed over a column of silica gel (60 Merck silica gel)  
230 and fractions of increasing polarity were eluted using n-hexane (hydrocarbons), 5% ethyl  
231 acetate in hexane (wax esters), and 20 % ethyl acetate in hexane (alcohols). Each fraction  
232 was concentrated, spiked with an internal standard, and alcohols were silylated using a  
233 mixture of pyridine/BSTFA (1:1, v/v, 45 min at 50°C) prior to gas chromatography (GC)  
234 and GC-mass spectrometry (GC-MS) analyses.

235 GC analyses were performed on a HP-6890 Series gas chromatograph equipped with a  
236 cool on-column injector and a flame ionization detector. GC-MS analyses were performed  
237 on a HP 6890 Series Plus gas chromatograph equipped with a cool on-column injector  
238 and coupled to an Agilent 5975C (VL MSD) mass spectrometer. Compound separation

239 was achieved with a fused silica capillary column (30 m × 0.25 mm) coated with HP-5MS  
240 (0.25 µm film thickness) using helium as the carrier gas (constant flow, 1 mL min<sup>-1</sup>). The  
241 injector temperature was programmed as follows: 60°C (held 0.5 min), 60 to 300°C (200°C  
242 min<sup>-1</sup>), 300°C (held 1 min). The oven temperature program was set as: 60°C (held 0.5  
243 min), 60 to 130°C (20°C min<sup>-1</sup>), 130 to 300°C (4°C min<sup>-1</sup>), and 300°C (held 55 min).  
244 Electron impact mass spectra were recorded at 70 eV in full scan mode in the m/z range  
245 50-700. The different lipid biomarkers were identified based on their characteristic mass  
246 spectral fragmentations, mass spectra libraries from the manufacturer, and comparison  
247 with data in the literature (e.g., ten Haven, Rohmer, Rullkötter, & Bisseret, 1989).

248

### 249 **2.3. DNA extraction, sequencing, and bioinformatics analyses**

250 Methods employed here are detailed in Hugoni et al. (2018). Briefly, for each sampling  
251 depth, DNA extractions were conducted on both 3 µm and 0.2 µm filters using the Power  
252 Water DNA isolation kit according to the manufacturer's instructions (MoBio laboratories).  
253 Bacterial and archaeal 16S rRNA genes were amplified in triplicate using 357F/926R and  
254 519F/915R primers, respectively (Hugoni et al., 2018), and amplicons were sequenced  
255 on an Illumina HiSeq 2500 (300bp PE, GATC Biotech, Konstanz, Germany).

256 Bioinformatic analyses were conducted using FROGS (Escudié et al., 2018) and  
257 consisted of merging the 3µm and 0.2µm fractions, removing low quality sequences,  
258 dereplication, clustering using SWARM, removing chimeras using VSEARCH, filtering  
259 sequences representing less than 0.005%, and affiliated against the Silva database  
260 (v.128). To compare samples with each other, we applied a normalization threshold of  
261 124,779 sequences per sample for Bacteria and 37,529 for Archaea.

262 OTU richness and diversity was estimated using Chao1 and Shannon indexes,

263 respectively. Canonical correspondence analysis (CCA) was performed to assess the  
264 relationships between the bacterial and archaeal taxonomic groups and environmental  
265 parameters. Sequences were considered at the «Phylum» level and pooled for each  
266 sampling date. CCA was performed using 5 environmental factors (temperature, salinity,  
267 oxygen, H<sub>2</sub>S/HS<sup>-</sup> concentration and pH) with the taxonomic phyla abundance using the  
268 VEGAN package (<http://cran.r-project.org/web/packages/vegan/index.html>) in R.

269

### 270 **3. RESULTS**

#### 271 **3.1. Geochemistry of the water column at the time of sampling**

272 The physico-chemical characteristics of the water column in Lake Dziani Dzaha at the end  
273 of the dry and rainy seasons (i.e., without and with seasonal stratification; Fig. 2) were  
274 consistent with previous observations made at similar times of the year (Leboulanger et  
275 al., 2017). The lake water temperature remained stable (around 30°C) from the bottom of  
276 the oxic-anoxic transition zone, down to the water-sediment interface, but within the first  
277 two meters below the lake surface a thermal gradient was present (Fig. 2A). The pH of  
278 the water was constant with depth (average pH ~9.2) and only showed a systematic  
279 decrease below the seasonal and the permanent haloclines (ca. -0.4 and -0.2 pH,  
280 respectively). Water salinity was more dependent on the season (Fig. 2A) and in the  
281 absence of seasonal stratification, the salinity was 64 psu from the surface down to 14 m  
282 depth increasing to 71 psu below the deep halocline. In the presence of the seasonal  
283 halocline, the upper part of the water column (i.e., above the halocline) was less salty (43-  
284 45 psu) due to rainfall dilution (Fig. 2A). Below this halocline, the salinity shifted back to  
285 ca. 65 psu down to the deep halocline at ca. 14 m depth, then below this, increased again  
286 reaching the same salinity as observed during the dry season (Fig. 2B). Whatever the

287 season, the oxygen concentration rapidly decreased from the oversaturated surface  
288 waters to the completely anoxic waters. The oxygen penetration depth fluctuated daily,  
289 but oxygen was never present below ca. 1.5 m depth (Fig. 2B). The oxygen penetration  
290 depth, therefore, did not coincide with the seasonal halocline set at a depth of between  
291 1.8 and 2.2 m during the rainy period. The photic zone was also limited permanently as  
292 1% Photosynthetic Active Radiation (PAR) was reached at a depth of less than 0.5 m.  
293 During the rainy season, euxinic conditions developed below the seasonal halocline, with  
294  $\text{H}_2\text{S}/\text{HS}^-$  concentrations reaching  $>2$  mM down to the sediment interface (Fig. 2B) and  
295  $\text{CH}_4$  concentrations approaching saturation (ca. 1.7 mM; Sarazin et al., 2020). During the  
296 dry season, sulfides were present in much lower concentrations ( $\text{H}_2\text{S}/\text{HS}^- < 0.16$  mM) in  
297 the anoxic waters, except below the deep permanent halocline where  $\text{H}_2\text{S}/\text{HS}^-$   
298 concentrations were higher ( $>2.8$  mM) than those measured between 2 and 14 m depth  
299 in the presence of the seasonal halocline. Both seasonal and permanent haloclines were  
300 light-deprived and thus considered aphotic.

301

### 302 **3.2. Chlorophyll-a and lipid biomarkers**

303 Due to the permanent hypereutrophic status of the lake, Chl-a concentrations in the  
304 surface oxic waters (0-1m) ranged between 600 and 750  $\mu\text{g L}^{-1}$  irrespective of season  
305 (Fig. 3). During the dry season, in the absence of the seasonal halocline, Chl-a  
306 concentrations remained roughly stable to a depth of 7 m and then dropped significantly  
307 deeper down, especially across the deep halocline (Fig. 3). Conversely, in the presence  
308 of the seasonal halocline (the wet season), Chl-a concentrations decreased by ca. 80%  
309 within the first 6 m, and then remained stable down to the water-sediment interface. The  
310 concentration profile of free phytol (Fig. 3), the hydrolyzed form of the isoprenoid chain of

311 Chl-*a*, closely followed that of Chl-*a* during both sampling periods but higher values were  
312 reached (ca. 250  $\mu\text{g L}^{-1}$ ) in the surface waters during the rainy season than during the dry  
313 season (ca. 55  $\mu\text{g L}^{-1}$ ).

314 The GC-MS analysis of the different lipid fractions revealed the presence of lipid  
315 biomarkers which, based on their non-isoprenoidal structures and depth concentration  
316 profiles, could be attributed to the anaerobic bacterial populations thriving in the lake (see  
317 discussion). Mono-unsaturated *n*-alkenes with 19 or 21 carbon atoms were systematically  
318 detected in the anoxic and/or euxinic layers of the water column. Their concentration  
319 remained low (maxima of 0.24  $\mu\text{g L}^{-1}$  for C<sub>19:1</sub> and 2.5  $\mu\text{g L}^{-1}$  for C<sub>21:1</sub>) but, whatever the  
320 sampling period, both compounds were absent from the oxygenated water layer with  
321 concentrations generally rising in the euxinic waters (Fig. 4A). A range of long chain wax  
322 esters (WE) was also observed throughout the water column. Trace amounts of saturated  
323 C<sub>30</sub> and C<sub>32</sub> WE were detected in the surface oxygenated waters but the concentration  
324 and structural diversity of WE significantly increased in the sulfidic waters below (Figs. 4B;  
325 S1). Here, WE were composed of linear or methyl-branched C<sub>14</sub>-C<sub>20</sub> acyl chains esterified  
326 to linear or methyl-branched C<sub>14</sub>-C<sub>18</sub> alcohol chains and contained 0 to 3 double bonds  
327 (Fig. S1). The concentration of C-odd WE reached ca. 13  $\mu\text{g L}^{-1}$  in euxinic waters where  
328 the systematic appearance of C-odd WE with pentadecyl and heptadecyl (sometimes  
329 branched) moieties was striking (Figs. 4B; S1). During the rainy season, C-odd WE  
330 concentrations were at a maximum below the seasonal halocline, dropping sharply with  
331 depth, before increasing again below the deep halocline. Most of the alkyl chains  
332 constitutive of the C-odd WE were retrieved in their free form in the alcohol fractions (i.e.,  
333 C-odd possibly branched *n*-alkanols). Like C-odd WE, these alcohols systematically

334 appeared below the (seasonal or deep) halocline, but their concentrations did not  
335 decrease between the two haloclines during the stratified period (Fig. 4B).

336 In addition to free phytol and C-odd *n*-alkanols, the pentacyclic triterpenoids diplopterol  
337 and tetrahymanol were among the main alcohols observed in the water column of Lake  
338 Dziani Dzaha. Remarkably, these triterpenols were mainly observed in the sulfidic part of  
339 the water column (Fig. 4C) regardless of seasonal stratification. This was especially true  
340 for tetrahymanol whose concentration systematically rose below the seasonal and/or  
341 permanent halocline (Fig. 4C). Diplopterol was also present in most compartments of the  
342 water column but, unlike tetrahymanol, its concentration only increased below the deep  
343 halocline in both sampling periods, suggesting a distinct biological precursor for these two  
344 triterpenols. As for most of the bacterial biomarkers, the maximum concentrations of  
345 diplopterol and tetrahymanol were observed at the bottom of the pit during the stratified  
346 period, reaching ca. 20 and 11  $\mu\text{g L}^{-1}$ , respectively.

347 Few GC-amenable archaeal lipid biomarkers were observed in the lipid fractions  
348 investigated, and their occurrence seemed to depend on the sampling period and/or the  
349 physico-chemical characteristics of the water. Archaeol, and to a lesser extent phytane,  
350 were detected during both sampling periods (Fig. 5). In the absence of a seasonal  
351 halocline, phytane was only detected in the euxinic waters below the deep halocline  
352 whereas archaeol was present in low concentrations at all depths but increased in the  
353 deep euxinic waters (Fig. 5B). Phytane and archaeol concentrations were much higher  
354 during the rainy season and their occurrence was correlated with the presence of  $\text{H}_2\text{S}/\text{HS}^-$   
355 . At this time, their concentration profiles were similar, showing an increase below the  
356 seasonal halocline, and an even stronger increase below the deep halocline, where they  
357 reached concentrations of ca. 1 and 12  $\mu\text{g L}^{-1}$ , respectively (Fig. 5).



358 A range of pentamethyl-eicosenes (PMI) and partially hydrogenated squalenes  
359 (hydrosqualenes) with 3 to 4 and 1 to 4 double bonds respectively, were also detected in  
360 the euxinic waters during the stratified period. Hydrosqualenes were present in much  
361 higher concentrations than PMI, the sum of all isomers reaching up to  $8.2 \mu\text{g L}^{-1}$  and  $0.9$   
362  $\mu\text{g L}^{-1}$ , respectively. The depth concentration profiles of both classes of compounds  
363 mirrored those of phytane and archaeol, showing an increase below the seasonal and/or  
364 permanent halocline (Fig. 5).

365

### 366 **3.3. Bacterial and archaeal diversity**

367 In order to get an in-depth analysis of the microbial diversity in Lake Dziani Dzaha,  
368 sequencing data acquired previously from whole microbial community at a high taxonomic  
369 level (Hugoni et al., 2018) were reinvestigated separately (Bacteria and Archaea) and  
370 down to the class or order taxonomic level (Figs. 6; 7). Whatever the sampling period (end  
371 of the dry or the rainy season), cyanobacterial OTUs strongly dominated from the surface  
372 down to the first halocline, accounting for 87% of the bacterial sequences above the  
373 permanent deep halocline during the dry season, and for 71% of the bacterial sequences  
374 above the seasonal halocline during the rainy season (Fig. 6A). Apart from cyanobacteria,  
375 the most abundant bacterial phyla were Bacteroidetes and Firmicutes, and to a lesser  
376 extent Alphaproteobacteria and Actinobacteria. Non-affiliated bacterial sequences  
377 represented at most 5% of the total sequences regardless of season or depth (Fig. 6A).  
378 Interestingly, the bacterial community structure did not seem to be modified across the  
379 oxic-anoxic transition zone whereas a strong shift was systematically observed across the  
380 transition to euxinic waters both with or without the seasonal halocline. Bacteroidetes and  
381 Firmicutes became strongly dominant in the euxinic waters where together they

382 represented 65% of the bacterial sequences below the deep halocline during the dry  
383 season and 73% of bacterial sequences below the seasonal halocline during the rainy  
384 season (Fig. 6A). A further insight into Firmicutes diversity showed the overwhelming  
385 dominance of an unknown genus of anaerobic Clostridiales from the family  
386 Syntrophomonadaceae at all depths during both sampling periods (Figs. 6B; S2). A small  
387 decrease in the proportion of this genus was however observed in euxinic waters.  
388 Bacteroidetes appeared to be more diversified than Firmicutes and were significantly  
389 influenced by the presence of haloclines (Fig. 6C). In the absence of  $H_2S/HS^-$ ,  
390 Bacteroidales belonging to the ML635J-40 aquatic group, Flavobacteriales,  
391 Sphingobacteriales and two non-cultivated orders (III and ML602M-17) were the dominant  
392 Bacteroidetes whereas, in the euxinic waters, the ML635J-40 aquatic group systematically  
393 took over the whole Bacteroidetes abundance during both seasons, representing on  
394 average 90% of the Bacteroidetes sequences.

395 Whatever the season, the archaeal community was essentially composed of  
396 Woesearchaeota and Euryarchaeota, which together represented between 91 and 97%  
397 of the total archaeal sequences (Fig. 7A). Woesearchaeota dominated the archaeal  
398 assemblages above the deep halocline during the dry season (64 to 74% of archaeal  
399 sequences) and above the seasonal halocline during the rainy season (66 to 69% of  
400 archaeal sequences), whereas Euryarchaeota became systematically dominant in the  
401 euxinic waters below (70-80% of archaeal sequences). Further analysis of Euryarchaeota  
402 diversity showed the predominance of the methanogenic class WSA2 in all depths  
403 irrespective of the sampling season, representing between 40 and 95% of the total  
404 sequences of Euryarchaeota (Fig. 7B). Methanobacteria, Methanomicrobia and  
405 Thermoplasmata were the other lineages of Euryarchaeota represented in the non-sulfidic

406 compartments of the water column contributing <10 to 50% of the total euryarchaeal  
407 sequences depending on the presence of the seasonal halocline. The number of  
408 sequences related to Methanobacteria significantly decreased in the euxinic waters  
409 whereas Methanomicrobia and Thermoplasmata were the predominant classes of  
410 Euryarchaeota along with WSA2.

411 Using a Canonical Correspondence Analysis, the influence of the main physico-chemical  
412 parameters (pH, salinity, temperature, dissolved oxygen, and H<sub>2</sub>S/HS<sup>-</sup>) on the distribution  
413 of bacterial and archaeal classes along the water column was further investigated. The  
414 first two axes represented more than 95% of the total variance (ca. 89 and 7%  
415 respectively; Fig. 8). Samples grouped into two well-separated groups highlighted the  
416 strong impact of the presence of sulfides and a significant influence of salinity on the  
417 composition of the bacterial and archaeal communities. Cyanobacteria,  
418 Alphaproteobacteria, Actinobacteria and Woesearchaeota appeared to be mostly related  
419 to pH, temperature, and dissolved oxygen, while Firmicutes, Bacteroidetes and  
420 Euryarchaeota were mostly related to H<sub>2</sub>S/HS<sup>-</sup> and salinity (Fig. 8).

421

## 422 **4. DISCUSSION**

423

### 424 **4.1 Chemocline-dependent microbial community composition**

425 The microorganisms that thrive in saline-alkaline lakes are considered as a special  
426 category of extremophiles. They are haloalkaline microorganisms and their biodiversity  
427 depends on the specific physico-chemical characteristics of the ecosystem encountered.  
428 Salinity, oxygen, and pH have been established as important factors for shaping the  
429 microbial communities in soda lakes, while striking differences in diversity have also been

430 reported between the oxic mixolimnion and the euxinic hypolimnion/monimolimnion of  
431 alkaline and non-alkaline stratified ecosystems (Humayoun, Bano, & Hollibaugh, 2003;  
432 Klepac-Ceraj et al., 2012; Lanzén et al., 2013; Hamilton et al., 2016). This is also the case  
433 for Lake Dziani Dzaha where microbial diversity, evaluated through changes in OTU  
434 abundances, appears significantly constrained by the presence of aphotic and euxinic  
435 haloclines<sup>-</sup> (Figs. 6; 7; 8). However, unlike most stratified ecosystems including soda  
436 lakes, the peculiarity of Lake Dziani Dzaha is i) the absence of euxinic photic zone, where  
437 light and sulfides meet and support the development of anoxygenic phototrophic sulfur  
438 bacteria and, ii) the apparent absence of sulfate-reducing bacteria due to the low sulfate  
439 content of the lake waters.

440 The massive phytoplanktonic biomass of Lake Dziani Dzaha is mainly composed of the  
441 filamentous cyanobacterium *Arthrospira fusiformis* (representing 97 to 99% of the total  
442 biomass) and, to a much lesser extent, the unicellular eukaryote *Picocystis salinarum* and  
443 non-heterocytous filamentous cyanobacteria from the orders Oscillatoriales and  
444 Synechococcales (Leboulanger et al., 2017; Bernard et al. 2019). Despite the permanent  
445 hypereutrophic status of the lake, a much lower number of 16S rRNA gene sequences  
446 affiliated with cyanobacteria were observed below the (seasonal) halocline (Fig. 6A)  
447 regardless of the sampling period. This was accompanied by lower phytoplanktonic cell  
448 density (Fig. S3) suggesting an efficient anaerobic biodegradation of the settling  
449 phytoplanktonic biomass. Whilst the anaerobic digestion of OM under saline and alkaline  
450 conditions has been shown to be very effective, it is a very complex and poorly understood  
451 process (Sorokin et al., 2015; Nolla-Ardèvol, Strous, & Tegetmeyer, 2015), and little is  
452 known about the biodegradation processes that occur in the presence of high  
453 concentrations of sulfides in the absence of sulfate and light. This case is of particular

454 interest since H<sub>2</sub>S/HS<sup>-</sup> is present at low concentration (or absent) in the biogas that is  
455 produced during the anaerobic digestion of OM under alkaline conditions (Van Leerdam  
456 et al., 2008; Nolla-Ardèvol et al., 2015).

457 Apart from cyanobacteria, the marked differences in bacterial and archaeal diversity  
458 observed on either side of the seasonal or permanent halocline, indicates the occurrence  
459 of distinct haloalkaliphilic microbial populations adapted to specific ecological niches that  
460 require specific conditions to develop (Fig. 8). Dominant in Lake Dziani Dzaha, Firmicutes,  
461 Bacteroidetes and to a lesser extent Alphaproteobacteria are bacterial phyla commonly  
462 observed in alkaline environments (Jones et al., 1998; Wani et al., 2006; Sorokin et al.,  
463 2014; Rojas et al., 2018; Vavourakis et al., 2018). Their role in OM degradation under  
464 saline and alkaline conditions has been recognized (Sorokin et al., 2015; Nolla-Ardèvol et  
465 al., 2015 and references therein). However, a number of peculiar features characterize  
466 the bacterial community of Lake Dziani Dzaha. First, the overwhelming dominance among  
467 Firmicutes of a single (yet unknown) genus of anaerobes belonging to the family  
468 *Syntrophomonadaceae* among Clostridiales (Figs. 6B; S2A). Prevailing at all depths, this  
469 unknown haloalkaliphile genus of Firmicutes did not appear significantly hampered by the  
470 presence of high concentrations of H<sub>2</sub>S/HS<sup>-</sup>, and was sometimes accompanied by other  
471 members of the same family from the genera *Dethiobacter* and '*Candidatus*  
472 *contubernalis*', especially in the euxinic waters (Fig. S2A). Clostridiales are primary or  
473 secondary hydrolytic anaerobes regularly observed in alkaline and soda lakes (Sorokin et  
474 al., 2014; 2015), belonging to the family *Syntrophomonadaceae*. They are known to use  
475 carboxylic acids as a source of energy, and have been increasingly found in haline and  
476 non-haline alkaline sediments. For example, several phylotypes of Clostridiales belonging  
477 to, or phylogenetically close to, the *Syntrophomonadaceae* were predominantly retrieved

478 from stratified sediments from Russian hypersaline soda lakes (Sorokin et al., 2014;  
479 Vavourakis et al., 2018) and have been reported as a major constituent of the bacterial  
480 community inhabiting the alkaline, low-salt, shoreline anaerobic sediments of Mono Lake  
481 (Rojas et al., 2018). *Synthrophomonadaceae* from the genus *Dethiobacter* have also been  
482 recovered from a mixture of soda lake sediments (Sorokin, Tourova, Mussmann, &  
483 Muyzer, 2008) and this genus has been shown to contribute to carbon cycling in the hyper-  
484 euxinic waters of the stratified Mahoney Lake (British Columbia; Hamilton et al., 2016).  
485 Interestingly, different cultured and uncultured members of the family  
486 *Synthrophomonadaceae* including the genera *Dethiobacter* and '*Candidatus*  
487 *contubernalis*' have been reported to oxidize acetate anaerobically through syntrophic  
488 acetate oxidation (SAO) with hydrogenotrophic sulfate-reducing bacteria (SRB) or  
489 methanogenic Archaea in several Russian hypersaline soda lakes (Timmers et al., 2018).  
490 In Lake Dziani Dzaha, a syntrophic association between acetate oxidizing  
491 *Synthrophomonadaceae* and hydrogenotrophic methanogenic archaea appears to be a  
492 likely scenario based on 1) the strong similarities in *Synthrophomonadaceae*  
493 assemblages observed here and in soda lakes where SAO occurs and, 2) the co-  
494 occurrence of potential adequate archaeal partners (Figs. 6; 7 and discussion below). An  
495 association between *Synthrophomonadaceae* and hydrogenotrophic SRB in Lake Dziani  
496 Dzaha appears less likely, due to the near absence of sulfates and SRB in the anoxic  
497 waters where (poly)sulfides prevail. This is particularly interesting when considering a  
498 possible analogy between Lake Dziani Dzaha and the Early Proterozoic Oceans where  
499 low oxygen concentrations in the atmosphere and surface waters are thought to have  
500 prevented sulfate production by oxidative weathering, thus strongly limiting microbial  
501 sulfate reduction to the benefit of other sulfur cycling processes (Philippot et al., 2007).

502 Another remarkable characteristic of the bacterial community of Lake Dziani Dzaha is the  
503 dominant proportion, among Bacteroidetes, of the uncultured ML635J-40 aquatic group  
504 from the family Bacteroidales. From a general point of view, this family is part of the  
505 Cytophaga-Flavobacterium-Bacteroides group (FCB group now called Sphingobacteria)  
506 which is more commonly observed in soda lakes and alkaline environments where it  
507 participates in the degradation of macromolecules such as carbohydrates and proteins  
508 (Humayoun et al., 2003; Wani et al., 2006). Interestingly enough, Bacteroidetes closely  
509 related to the ML635J-40 aquatic group have been shown to dominate the bacterial  
510 community in alkaline anaerobic reactors that have been inoculated with soda lake  
511 sediments and fed with the cyanobacterium *Arthrospira* (Nolla-Ardèvol et al., 2015).  
512 Based on metagenomic and metatranscriptomic analyses, the ML635J-40 aquatic group  
513 was further demonstrated to play a major role in the breakdown of the phytoplanktonic  
514 substrate (Nolla-Ardèvol et al., 2015). This same order of Bacteroidales has also been  
515 reported in different soda lakes (Humayoun et al., 2003; Wani et al., 2006; Lanzén et al.,  
516 2013) and in ultrabasic serpentinite spring (Brazelton et al., 2017), where they appear to  
517 be among the most important bacterial taxa. The ecological role of the ML635J-40 aquatic  
518 group is not yet fully understood but, in addition to being involved in the hydrolysis and  
519 degradation of complex OM, this group might supply hydrogen gas to methanogens and  
520 catalyze the oxidation of formate to CO<sub>2</sub> and of glycine to NH<sub>3</sub> (Nolla-Ardèvol et al., 2015;  
521 Brazelton et al., 2017). Such primordial biogeochemical implications of the ML635J-40  
522 aquatic group can be envisaged beneath the haloclines of Lake Dziani Dzaha where the  
523 highest numbers of OTUs related to this Bacteroidales group were observed (Fig. 6C),  
524 along with a sharp decrease in cyanobacterial biomass (Figs. 3; 6A) and CH<sub>4</sub> and NH<sub>3</sub>  
525 production (Sarazin et al., 2020).

526 The archaeal diversity in Lake Dziani Dzaha is mainly composed of Woesearchaeota and  
527 Euryarchaeota and, like bacterial diversity, also presents some striking features. The  
528 strong dominance of Woesearchaeota in the non-euxinic waters (Fig. 6A) is in accordance  
529 with the widespread distribution of this phylum in anoxic and saline aquatic environments  
530 and sediments (Casamayor, Triadó-Margarit, & Castañeda, 2013). In Lake Dziani Dzaha,  
531 however, Woesearchaeota appear to be poorly adapted to high concentrations of  $H_2S/HS^-$   
532 , confirmed by their drastic decrease in euxinic waters. Woesearchaeota belong to the  
533 DPANN superphylum of Archaea which groups together ecologically and has genetically  
534 diverse archaeal lineages (Castelle et al., 2015). Most DPANN Archaea have a small  
535 genome size with limited metabolic capacities, and Woesearchaeota are predicted to have  
536 saccharolytic and fermentative and/or symbiotic lifestyles (Castelle et al., 2015) that are  
537 consistent with the conditions prevailing in the water column of Lake Dziani Dzaha.  
538 Putative bacterial hosts of Woesearchaeota and ecological interaction networks in Lake  
539 Dziani Dzaha still remain to be investigated, but it is noteworthy that the abundance and  
540 distribution of this uncultured archaeal phyla perfectly mirrors those of cyanobacteria  
541 (Figs. 6A; 7A).

542 A second remarkable feature of the archaeal community of Lake Dziani Dzaha was the  
543 high abundance of methanogenic Euryarchaeota found at all depths, and their systematic  
544 prevalence in euxinic waters (Fig. 7). This possibly reflects limited competition for growth  
545 substrates (acetate, pyruvate, lactate,  $H_2$ , etc.) due to the near absence of SRB in the  
546 lake. The diversity of methanogenic Euryarchaeota seems partly influenced by the  
547 absence of sulfate and the presence of strong concentrations of  $H_2S/HS^-$  below the  
548 chemoclines (Fig. 7B). Shifts in diversity were only observed in the proportions of  
549 Methanobacteria and Thermoplasmata, which respectively decreased and increased in



550 the euxinic waters, where members of Methanomicrobia and WSA2 constituted the major  
551 part (>85%) of the methanogenic community (Fig. 7B). The hydrogenotrophic genus  
552 *Methanocalculus* was the second most abundant genus of Methanomicrobia in Lake  
553 Dziani Dzaha (following an unknown genus) at all depths (Fig. S2B). *Methanocalculus* is  
554 a common and occasionally dominant producer of CH<sub>4</sub> in haline and/or alkaline  
555 environments (Ollivier et al., 1998; Lanzén et al., 2013; Nolla-Ardèvol et al., 2015; Rojas  
556 et al., 2018). This archaeon is able to use CO<sub>2</sub> or formate for the production of CH<sub>4</sub> and  
557 has been recently reported as a possible hydrogenotrophic partner in SAO (Timmers et  
558 al., 2018 and references therein). Although the number of sequences related to  
559 *Methanocalculus* did not increase systematically in the euxinic waters of Lake Dziani  
560 Dzaha (Fig. S2B), where CH<sub>4</sub> concentration approaches saturation (Sarazin et al., 2020),  
561 the presence of this genus along the water column suggests a major biogeochemical role,  
562 especially in CH<sub>4</sub> production and, possibly, in acetate oxidation through syntrophic  
563 associations with *Synthrophomonadaceae* (Figs. 6; 7; Timmers et al., 2018).  
564 *Methanocalculus* has been shown to be an active methanogen during the anaerobic  
565 incubation in alkaline conditions of bioreactors containing soda lake sediments and  
566 *Arthrospira* biomass (Nolla-Ardèvol et al., 2015). A remarkable distinction with Lake Dziani  
567 Dzaha, however, is that the CH<sub>4</sub>-rich biogas produced under such conditions was almost  
568 sulfide-free, whereas strong concentrations in H<sub>2</sub>S/HS<sup>-</sup> and CH<sub>4</sub> co-existed below the  
569 halocline and the deep chemocline in Lake Dziani Dzaha (Sarazin et al., 2020) indicating  
570 that strong concentrations of sulfides may not hamper methanogenesis under haloalkaline  
571 conditions.

572 Another striking characteristic of the Euryarchaeota community structure in Lake Dziani  
573 Dzaha was the systematic dominance of the WSA2 class in all depths whatever the

574 structure of the water column. Based on metagenomic data from different WSA2  
575 populations, the methanogenic potential of this enigmatic new class of Euryarchaeota is  
576 thought to be restricted to methylated thiol reduction (Nobu, Narihiro, Kuroda, Mei, & Liu,  
577 2016). The absence of pathways for CO<sub>2</sub>-reducing and acetoclastic methanogenesis in  
578 the investigated genomes and their heterotrophic, auxotrophic, and ammonia-dependent  
579 nature, suggests that WSA2 can only inhabit anaerobic eutrophic environments with other  
580 active methanogens, but that they have an essential ecological role for syntrophy and for  
581 linking the carbon and sulfur cycles (Nobu et al., 2016). Such an unusual lifestyle perfectly  
582 fits the biogeochemical characteristics of the euxinic waters of Lake Dziani Dzaha,  
583 explaining the overwhelming presence of WSA2 (Fig. 7B). Methanogenesis by WSA2  
584 through methylated thiol reduction may be especially efficient when CO<sub>2</sub>-reducing  
585 methanogenesis (notably performed by *Methanocalculus*) becomes unfavorable at low H<sub>2</sub>  
586 concentrations.

587

#### 588 **4.2 Inferences on the microbial origin of lipid biomarkers**

589 As a great part of the bacterial and archaeal populations thriving in Lake Dziani Dzaha  
590 are uncharacterized and isolated populations are absent, it is difficult to assign the  
591 biological origin of some of the lipid biomarkers detected in the particulate OM. Possible  
592 precursors can be inferred by comparing the quantitative distribution of these lipid  
593 compounds with the composition of the microbial community as a function of water-depth  
594 and physico-chemical characteristics of the water column. This approach remains indirect  
595 and requires caution due to possible biases (e.g., differential degradation rates of lipids  
596 and DNA, choice of primers, genes present but not expressed, multiple origins of lipid

597 biomarkers, varying lipid content of cells, etc.), but can help to clarify the biological origin  
598 of some of the compounds.

599 Linear unsaturated hydrocarbons (*n*-alkenes) are commonly observed in lacustrine  
600 settings (De Mesmay, Grossi, Williamson, Kajula, & Derenne, 2007). Short-chain  
601 homologues (<*n*-C<sub>22</sub>) are typically used as biomarkers of microalgae and of some  
602 prokaryotes, typically cyanobacteria (Volkman et al., 1998 and references therein). The  
603 depth profiles of the C<sub>19</sub> and C<sub>21</sub> mono-unsaturated *n*-alkenes in the water column of Lake  
604 Dziani Dzaha however, point towards a prokaryotic origin distinct from cyanobacteria  
605 (Figs. 4A; 6A). Non-isoprenoid unsaturated hydrocarbons are produced within the  
606 bacterial domain, but there are few reports in the literature possibly because such lipids  
607 are regularly overlooked due to their low cell concentration and/or to the use of inadequate  
608 analytical procedures (Nichols, Nichols, & McMeekin, 1995; Cario, Grossi, Schaeffer, &  
609 Oger, 2015). C<sub>22</sub> to C<sub>31</sub> alkenes with an eventual iso- or anteiso- methyl branch have been  
610 reported in members of the family Micrococcaceae (Finnerty, 1989) whereas C<sub>29</sub> to C<sub>33</sub> *n*-  
611 alkenes with one to three unsaturation have been characterized in the phototrophic green  
612 non-sulfur bacterium *Chloroflexus aurantiacus* in Hot Spring microbial mats (Shiea et al.,  
613 1990). Also, *n*-C<sub>23</sub> monoenes are important biomarkers in the anaerobic microbial mats  
614 developing in Black Sea methane seeps (Thiel, Peckmann, Schmale, Reitner, &  
615 Michaelis, 2001) and a *n*-C<sub>31:9</sub> polyene has been reported in unidentified psychrophilic  
616 bacterial strains isolated from Antarctic Sea ice (Nichols et al., 1995). Remarkably, the  
617 occurrence of *n*-alkenes in Bacteria often appears to be associated with extreme  
618 environmental conditions, which may indicate an adaptive role of these lipids as has been  
619 proposed for extremophilic Archaea (Cario et al., 2015; Tourte, Schaeffer, Grossi, & Oger,  
620 2020). This is supported in Lake Dziani Dzaha where the systematic increase in the

621 concentration of the C<sub>19</sub> and C<sub>21</sub> *n*-alkenes in the aphotic and anoxic waters suggests it  
622 originates from non-phototrophic haloalkaline anaerobic bacteria, that are adapted to  
623 euxinic conditions. The comparison of *n*-alkenes depth profiles with the number of  
624 bacterial sequences, further suggests a new biological precursor for these lipids within  
625 (yet unknown) Firmicutes or the Bacteroidales uncultured ML635J-40 aquatic group (Figs.  
626 4A; 6).

627 Long chain wax esters (WE) can be produced by bacteria, plants, zooplankton, and some  
628 other eukaryotes (Bianchi, 1995; Albers, Kattner, & Hagen, 1996; Alvarez, 2016). In Lake  
629 Dziani Dzaha, the sharp increase in concentration and structural diversity of C-odd WE  
630 observed in the euxinic waters over both seasons (Fig. 4B) supports a significant WE  
631 production by haloalkaline non-phototrophic anaerobic bacteria specifically adapted to the  
632 presence of sulfides. WE have been reported to be produced by different genera of Gram  
633 negative (hydrocarbon-degrading) Gammaproteobacteria, Gram positive Actinobacteria  
634 and filamentous anoxygenic phototrophic bacteria (van der Meer et al., 2010; Alvarez,  
635 2016 and references therein). Among these families, Actinobacteria were detected at all  
636 depths during both seasons, but their minor contribution to the bacterial community and  
637 their proportional decrease below the aphotic and euxinic chemoclines did not support  
638 this bacterial class as a potential source of C-odd WE in the lake. The comparison of WE  
639 concentration profiles with the number of bacterial sequences, would rather suggest a  
640 new biological source of these lipids within the Firmicutes or the uncultured ML635J-40  
641 aquatic group from the family Bacteroidales, potentially linked to the S cycle. Interestingly,  
642 a recent survey of 8 282 bacterial genomes showed that the key enzyme involved in WE  
643 synthesis in bacteria, WS/DGAT (Wax Ester Synthase/Acyl Coenzyme A:Diacylglycerol  
644 Acyltransferase), is present in 673 species, mostly belonging to the phylum Actinobacteria

645 and the phylum Proteobacteria, but also in a few members from the FCB group (Wang et  
646 al., 2019). The investigated Firmicutes did not have WS/DGAT enzymes (Wang et al.,  
647 2019). This would make the uncultured ML635J-40 aquatic group from the family  
648 Bacteroidales a candidate for the origin of WE in the aphotic and anoxic waters of Lake  
649 Dziani Dzaha. The production of WE by non-phototrophic strict anaerobic bacteria is  
650 unprecedented, which would further extend the potential biological precursors and  
651 environmental significance of such lipids. The decrease in concentration in C-odd WE  
652 observed in between the two haloclines during the rainy season (Fig. 4B) was not mirrored  
653 in the structure of the bacterial community (Fig. 6). This may either reflect the existence  
654 of two distinct populations of WE-producing bacteria each associated with a specific  
655 halocline, or a WE production/consumption dependent on the varying conditions  
656 encountered with depth. Indeed, bacterial WE generally constitute storage compounds  
657 that can have diverse functions in cells, mostly related to a metabolic adaptation to  
658 environmental stress or fluctuating environmental growth conditions (Grossi et al., 2010;  
659 Alvarez, 2016). The alkyl building blocks of the C-odd WE were also retrieved in their free  
660 form (i.e., potentially branched C-odd alkanols) in the euxinic waters but, unlike WE, their  
661 concentration remained roughly stable downwards in the presence of the seasonal  
662 halocline. Short-chained *n*-alkanols can be produced by both eukaryotes and bacteria  
663 (Meyers, 2003), but their depth profiles and carbon chain length distribution in Lake Dziani  
664 Dzaha clearly support a bacterial precursor. Whether or not C-odd alkanols and WE share  
665 a common source is difficult to predict since bacterial WE can be produced either  
666 autotrophically, or heterotrophically from available alkyl substrates (Grossi et al., 2010).  
667 Hopanoids are specific membrane constituents of some bacteria, though such  
668 compounds have also been reported in plants (Volkman, 2005). Based on the study of

669 pure bacterial strains, the biosynthesis of hopanoids has long been considered restricted  
670 to oxygen-respiring microorganisms and a limited number of facultative anaerobes  
671 (Rohmer, Bouvier-Nave, & Ourisson, 1984; Neunlist, Bissere, & Rohmer, 1988; Ourisson  
672 & Rohmer, 1992), but the capacity of some strict anaerobic strains to produce simple (e.g.,  
673 diploptene, diplopterol) and elongated (hopanepolyols) bacteriohopanoids was later  
674 demonstrated. So far, the anaerobes known to synthesize hopanoids are sulfate-reducing  
675 Deltaproteobacteria from the genus *Desulfovibrio* (Blumenberg et al., 2006), iron-oxidizing  
676 deltaproteobacteria from the genus *Geobacter* (Härtner, Straub, & Kannenberg, 2005)  
677 and Planctomycetes performing the anaerobic oxidation of ammonium (Sinninghe Damsté  
678 et al., 2004). However, environmental gene surveys have shown that in natural systems,  
679 most bacteria producing hopanoids come from phyla, orders, or classes that have not  
680 been identified or characterized metabolically (Pearson, 2014). In Lake Dziani Dzaha, the  
681 constant occurrence of diplopterol from the surface waters down to the pit deep halocline,  
682 whatever the sampling period, does not distinguish between aerobic or (facultative)  
683 anaerobic production of this compound in the upper part of the water column. But the  
684 marked increase in the concentration of this hopanol below the deep halocline (Fig. 4C),  
685 supports the existence of a new anaerobic bacterial source of hopanoids able to thrive  
686 under strong euxinic and haloalkaline conditions, potentially belonging to Bacteroidetes.  
687 Since the first identification of tetrahymanol in ciliates from the genus *Tetrahymena*, and  
688 its subsequent report from different marine and freshwater ciliates (Harvey & McManus,  
689 1989), the occurrence in the sedimentary record of this pentacyclic triterpenol or its  
690 molecular fossil gammacerane, has generally been assigned as originating from  
691 bacterivorous Protozoa, feeding at microbially active oxic-anoxic interfaces and defined  
692 as an indication of a stratified water column, typical for settings with photic zone euxinia

693 (ten Haven et al., 1989; Sinninghe Damsté et al., 1995). However, tetrahymanol has also  
694 been observed in anoxygenic phototrophic Alphaproteobacteria (Kleemann et al., 1990),  
695 and the presence of a gene encoding for a tetrahymanol synthase (Ths) was recently  
696 identified in 104 bacterial genomes belonging to Alphaproteobacteria, aerobic  
697 methanotrophic Gammaproteobacteria and sulfate-reducing Deltaproteobacteria,  
698 confirming a more widespread potential to produce tetrahymanol in the bacterial domain  
699 than previously thought (Banta et al., 2015). The presence of tetrahymanol in Lake Dziani  
700 Dzaha is in line with its regular report in alkaline, anoxic, evaporative, and stratified  
701 environments (e.g., ten Haven et al., 1989; Thiel et al., 1997; Reinhardt et al., 2019).  
702 Flagellated unicellular eukaryotes (ciliates) belonging to the phyla Excavata and  
703 Ciliophora are present in Lake Dziani Dzaha (Hugoni et al., 2018) and potentially account  
704 for the presence of tetrahymanol in some parts of the water column, especially in the non-  
705 sulfidic waters. However, the number of sequences assigned to ciliates collapses and  
706 tends toward zero below the halocline during the stratified period (Hugoni et al., 2018)  
707 when tetrahymanol concentrations show a three-fold increase (Fig. 4C), making ciliates  
708 an unlikely source of this lipid biomarker in the euxinic compartment of the lake. There,  
709 tetrahymanol most likely originates from non-phototrophic anaerobic bacteria, capable of  
710 developing at high salinity, high alkalinity and potentially involved in the S cycle. The  
711 strong co-variation of Firmicutes and Bacteroidetes OTUs with tetrahymanol  
712 concentration suggests that this triterpenol originated from one of these two bacterial  
713 lineages (Figs. 4 and 6). Genes encoding for Ths have not been detected within genomes  
714 of Firmicutes but are present in one Bacteroidetes genome (Banta et al., 2015), supporting  
715 yet unknown anaerobic haloalkaline members of this phylum as a potential source of  
716 tetrahymanol in Lake Dziani Dzaha.

717  
718 The diversity of GC-amenable archaeal lipid biomarkers detectable in the particulate OM  
719 appeared limited relative to bacterial diversity in Lake Dziani Dzaha, but fit with the  
720 apparent simplicity of the archaeal biodiversity of the lake. Archaeol is a common archaeal  
721 biomarker which does not exhibit strong species specificity, and is widely present in  
722 methanogens and halophiles (Russell, 1989; Koga, Nishihara, Morii, & Agakawa-  
723 Matsushita, 1993; Tourte et al., 2020). The comparison of archaeol concentration profile  
724 with archaeal OTUs indeed suggested a major production of this lipid by Euryarchaeota  
725 in Lake Dziani Dzaha (Figs 5A; 7B). Although the lipidome of Woesearchaeota has not  
726 yet been investigated due to the lack of available cultures, our data does not support  
727 archaeol as a major lipid constituent in this newly described archaeal phylum. Phytane,  
728 saturated and unsaturated PMI and partially hydrogenated squalenes are more specific  
729 of methanogens (Tornabene et al., 1979; Risatti, Rowland, Yon, & Maxwell, 1984;  
730 Schouten, van der Maarel, Huber, & Sinninghe Damsté, 1997). The detection of these  
731 compounds only in the euxinic waters of Lake Dziani Dzaha agree with the distribution of  
732 methanogenic Euryarchaeota OTUs and suggests the production of these isoprenoid  
733 hydrocarbons by members of Methanomicrobia, Thermoplasmata and WSA2. PMI and  
734 partially hydrogenated squalenes, occurred only when the seasonal halocline was  
735 present, further indicating the development of a specific methanogenic assemblage in the  
736 euxinic waters during the rainy season characterized by enhanced CH<sub>4</sub> production  
737 (Sarazin et al., 2020).

738

## 739 **5. CONCLUSIONS**



740 The stratified saline and alkaline ecosystem Dziani Dzaha, exhibits a unique microbial  
741 community mostly composed of uncultured bacterial and archaeal lineages. Apart from  
742 the massive development of the cyanobacteria *Arthrospira* in the restricted oxygenated  
743 water layer, Firmicutes and Bacteroidetes dominate the bacterial community while  
744 Woesearchaeota and methanogenic Euryarchaeota represent most of archaeal lineages.  
745 The diversity of the microbial community in this hypereutrophic and polyextremophilic lake  
746 appears to be shaped by the physico-chemical composition of the water column, and more  
747 specifically, by the presence of aphotic haloclines and euxinic waters. In the latter case,  
748 the microbial diversity is much lower than in non-sulfidic waters and is strongly dominated  
749 by an unknown genus of Syntrophomonadaceae (affiliated with Firmicutes\_Costridiales)  
750 and the ML635J-40 aquatic group of Bacteroidetes, associated with three groups of  
751 methanogenic Euryarchaeota from the Methanomicrobia, Thermoplasmata and WSA2  
752 classes. Such dominance suggests essential roles of these microbial groups in anaerobic  
753 carbon and sulfur cycling likely to be promoted by specific (yet still uninvestigated)  
754 ecological interactions. This is in accordance with the suspected strong recycling of the  
755 cyanobacterial biomass across aphotic sulfide gradients whatever the seasonally driven  
756 structure of the water column (presence/absence of the seasonal halocline). Our results  
757 however do not support anoxygenic photosynthesis as a significant biogeochemical  
758 process in Dziani Dzaha, contrary to many past and present sulfidic environments.

759 The sharp changes in bacterial and archaeal diversity induced by the presence of aphotic  
760 and euxinic haloclines in Lake Dziani Dzaha were generally mirrored by changes in  
761 specific lipid biomarker concentrations, questioning the biological origin of some of these  
762 compounds , and supporting an unprecedented microbial origin for some of them. C<sub>19</sub> and  
763 C<sub>21</sub> *n*-alkenes, WE and pentacyclic triterpenes including diplopterol and tetrahymanol on

764 one hand, and phytane, saturated and unsaturated PMI and partially hydrogenated  
765 squalenes on the other hand appeared to be produced by haloalkaline non-phototrophic  
766 anaerobic bacteria and archaea, respectively. These anaerobes appear to be adapted to  
767 and/or are dependent on strong euxinic conditions. The precise biological precursors of  
768 these lipid biomarkers in Lake Dziani Dzaha remain to be characterized by enrichment  
769 cultures or strain isolation. It is, however, likely that the aforementioned bacterial  
770 compounds are produced by yet unknown members of Syntrophomonadaceae within  
771 Firmicutes and/or the Bacteroidales uncultured ML635J-40 aquatic group, and the  
772 archaeal biomarkers by yet unknown methanogenic Euryarchaeota notably belonging to  
773 the recently proposed WSA2 class. The possibility for tetrahymanol to be produced by  
774 non-phototrophic anaerobic bacteria in Lake Dziani Dzaha does not hamper its possible  
775 use (or of its molecular fossil gammacerane) as a marker of stratification in the  
776 sedimentary record. A better understanding of the specific environmental conditions linked  
777 to its production in stratified ecosystems would however improve our understanding of its  
778 occurrence and biogeochemical significance throughout Earth's history.

779

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789

## 790 **Data Availability Statement**

791 The sequence data generated in this study have been deposited on the European  
792 Nucleotide Archive (ENA) browser (<http://www.ebi.ac.uk/ena/data/>) under the accession  
793 number: PRJEB24947.

794

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1013

1014

## FIGURE CAPTIONS

- 1015
- 1016
- 1017 **FIGURE 1** Lake Dziani Dzaha location (**A & B**), size (**C & D**), and bathymetry (**E**).
- 1018
- 1019 **FIGURE 2** Water column physico-chemical characteristics of Lake Dziani Dzaha (pit) at
- 1020 the time of sampling at the end of the dry (November 2014) and rainy (April 2015) seasons.
- 1021 **A)** pH, temperature and salinity; **B)** diurnal dissolved O<sub>2</sub> and H<sub>2</sub>S/HS<sup>-</sup>. Note that following
- 1022 the end of the rainy season, the seasonal halocline may last for 2 to 3 additional months
- 1023 before disappearing.
- 1024
- 1025 **FIGURE 3** Depth concentration profiles of chlorophyll-a and free phytol in Lake Dziani
- 1026 Dzaha (pit) as a function of seasonal stratification.
- 1027
- 1028 **FIGURE 4** Depth concentration profiles of **A)** C<sub>19:1</sub> and C<sub>21:1</sub> *n*-alkenes, **B)** summed C-odd
- 1029 *n*-alkanols and C-odd wax esters and **C)** diplopterol and tetrahymanol in Lake Dziani
- 1030 Dzaha (pit) as a function of seasonal stratification.
- 1031
- 1032 **FIGURE 5** Depth concentration profiles of phytane and summed PMI **A)** and archaeol and
- 1033 summed hydrosqualenes **B)** in Lake Dziani Dzaha (pit) as a function of seasonal
- 1034 stratification.
- 1035
- 1036 **FIGURE 6** Relative abundance (% of sequences) of bacterial phyla **A)** and among
- 1037 Firmicutes **B)** and Bacteroidetes **C)** orders in Lake Dziani Dzaha (pit) as a function of
- 1038 seasonal stratification.

1039  
1040 **FIGURE 7** Relative abundance (% of sequences) of archaeal phyla **A**) and among  
1041 euryarchaeal classes **B**) in Lake Dziani Dzaha (pit) as a function of seasonal stratification.

1042  
1043 **FIGURE 8** Canonical Correspondence Analysis (CCA) between physico-chemical  
1044 characteristics of Lake Dziani Dzaha (pH, salinity, temperature, O<sub>2</sub> and H<sub>2</sub>S/HS<sup>-</sup>) and the  
1045 most abundant (>1% of pooled sequences) bacterial and archaeal phylum. NS = without  
1046 seasonal halocline, S = with seasonal halocline.

1047  
1048 **Supplementary figures**

1049  
1050 **FIGURE S1** Partial GC-MS chromatograms (TIC) of the wax esters (WE) present in Lake  
1051 Dziani Dzaha particulate organic matter collected at 0.2m (oxic), 6m (anoxic and non-  
1052 sulfidic) and 17.4m (anoxic and sulfidic) at the end of the dry season (absence of seasonal  
1053 halocline).

1054  
1055 **FIGURE S2** Relative abundance (% of sequences) of genera present among the family of  
1056 Syntrophomonadaceae **A**) and the class of Methanomicrobia **B**) in Lake Dziani Dzaha  
1057 (pit) as a function of seasonal stratification.

1058  
1059 **FIGURE S3** Depth concentration profiles of phytoplankton abundance in Lake Dziani  
1060 Dzaha (pit) as a function of seasonal stratification.

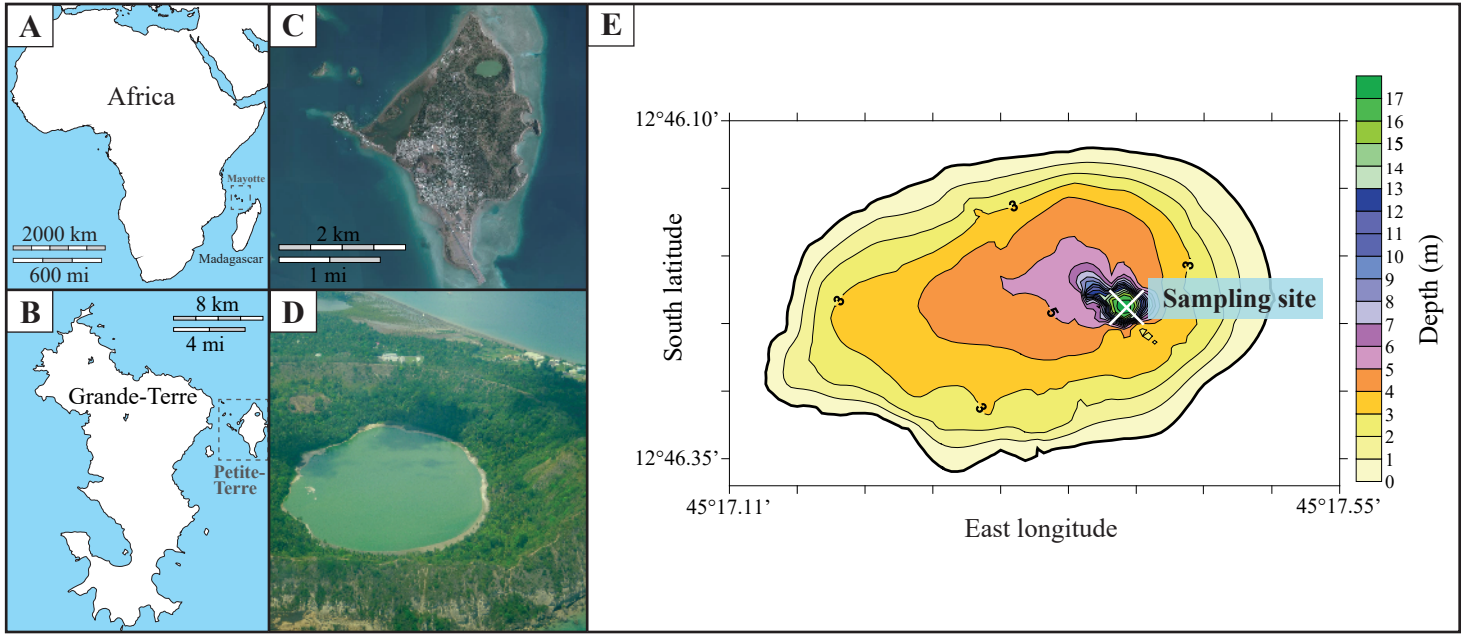
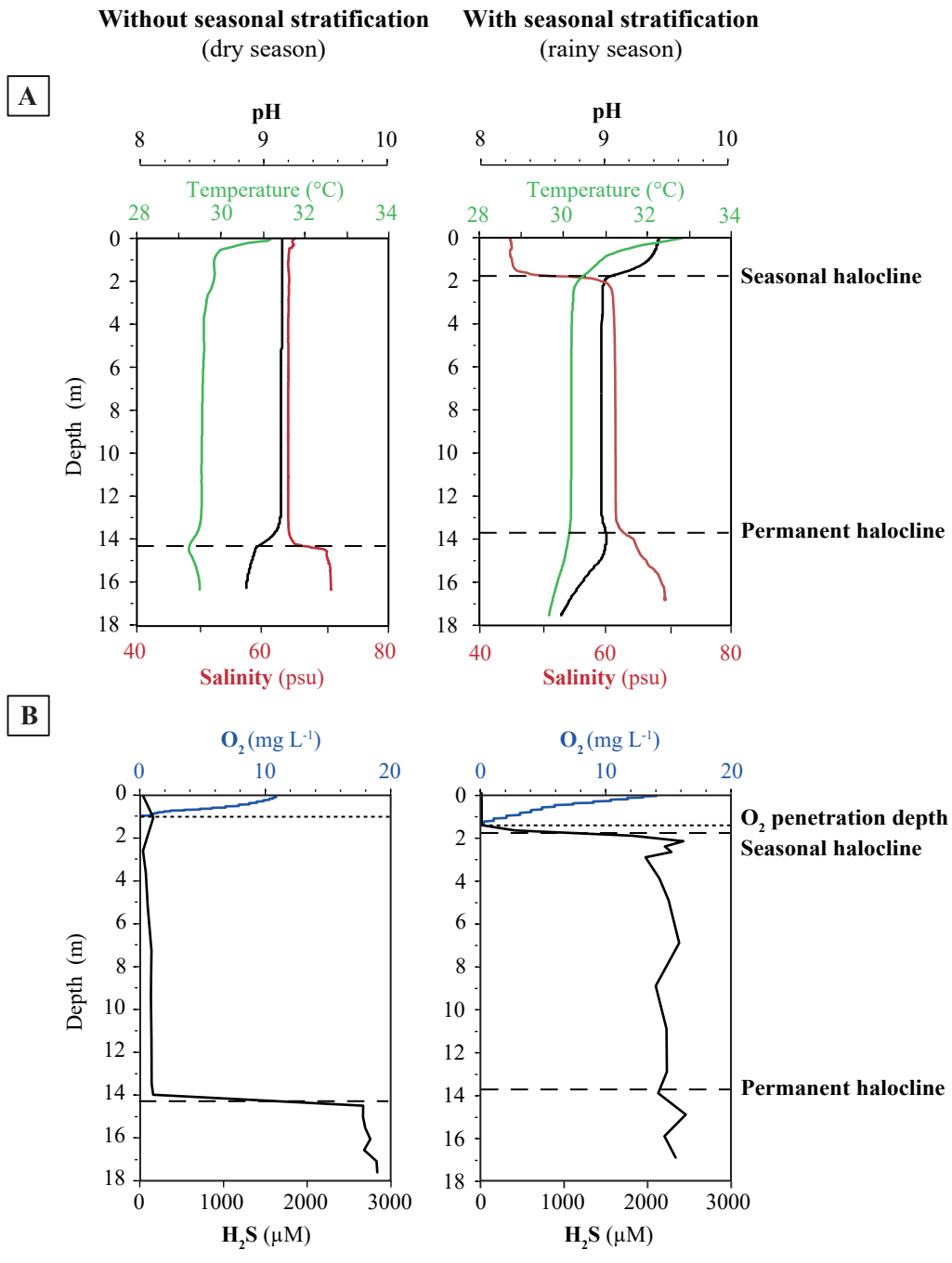


Fig. 1

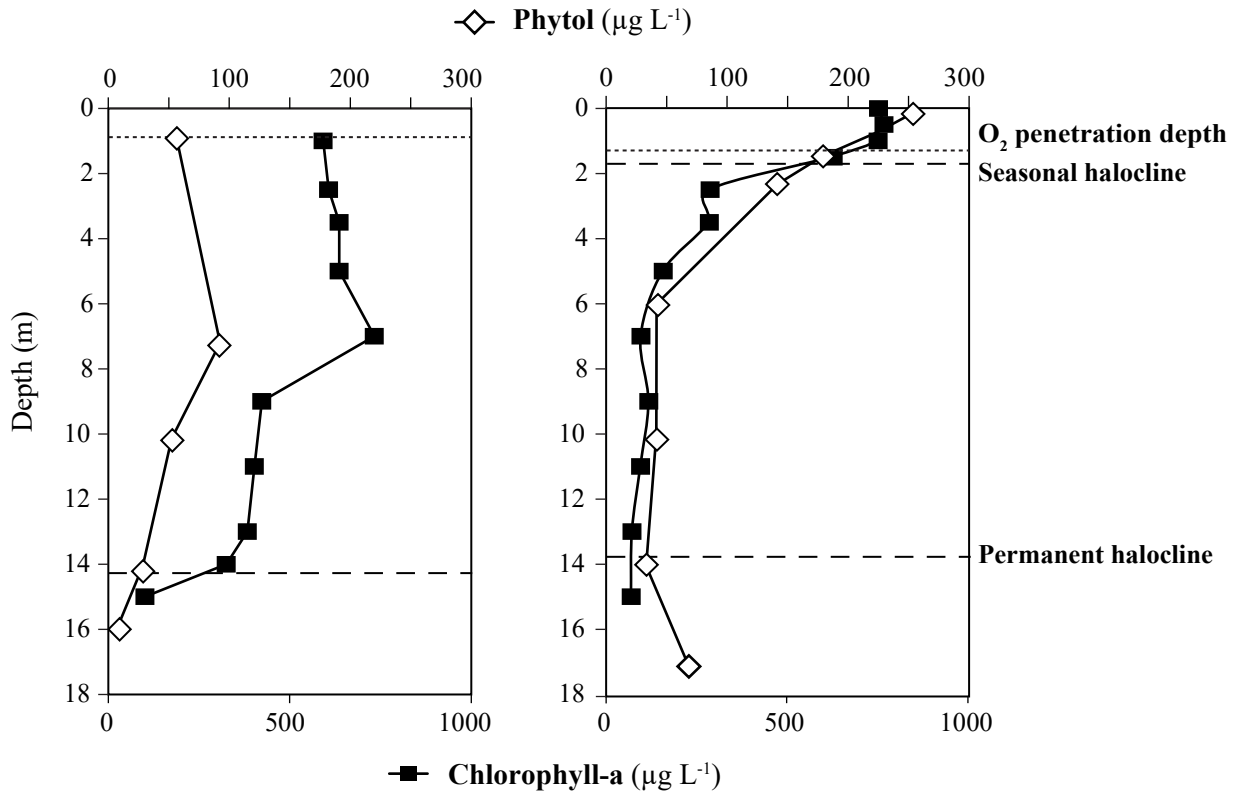


**Fig. 2**



**Without seasonal stratification**  
(dry season)

**With seasonal stratification**  
(rainy season)

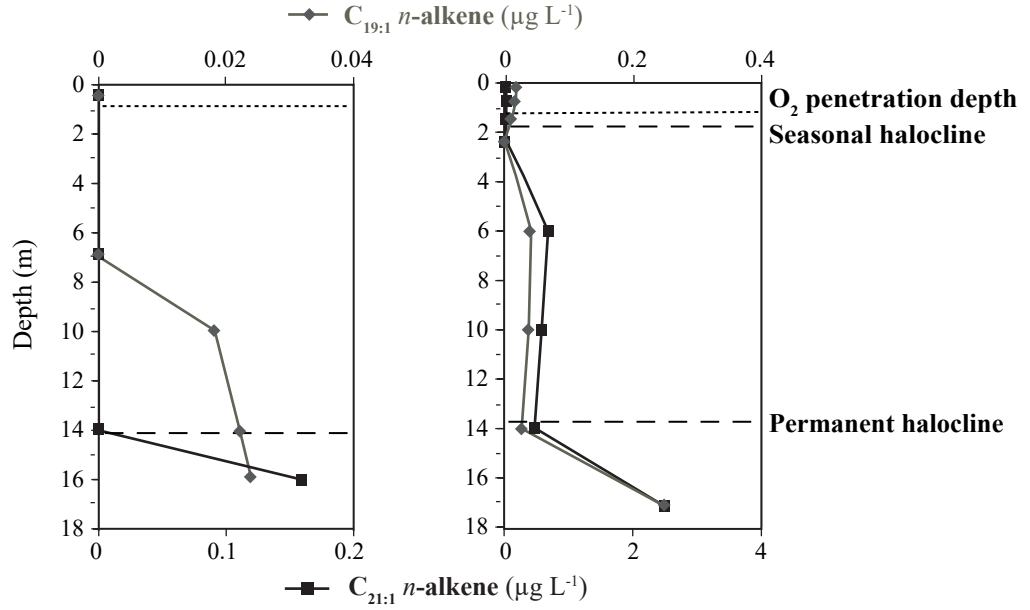


**Fig. 3 revised**

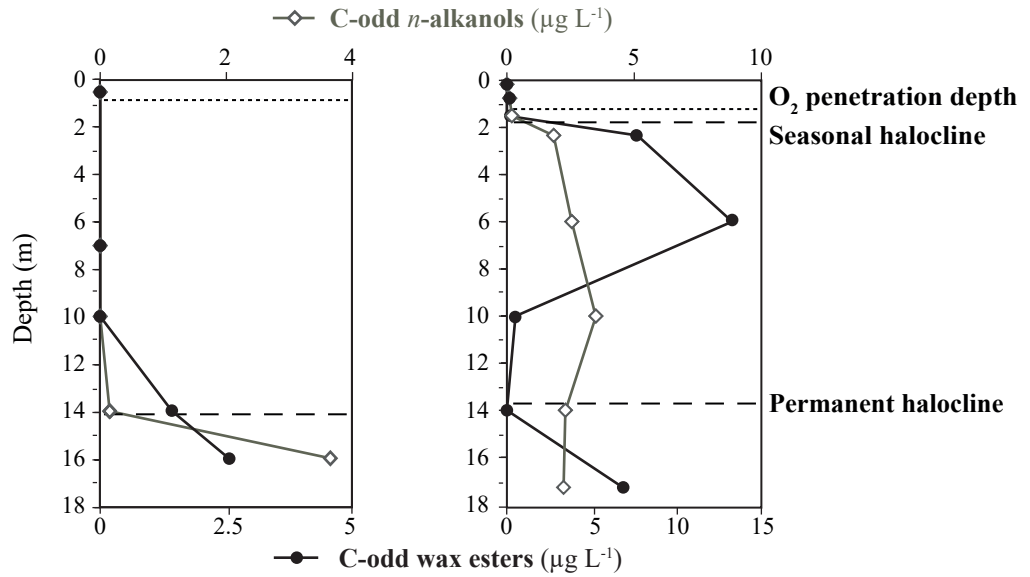
**Without seasonal stratification**  
(dry season)

**With seasonal stratification**  
(rainy season)

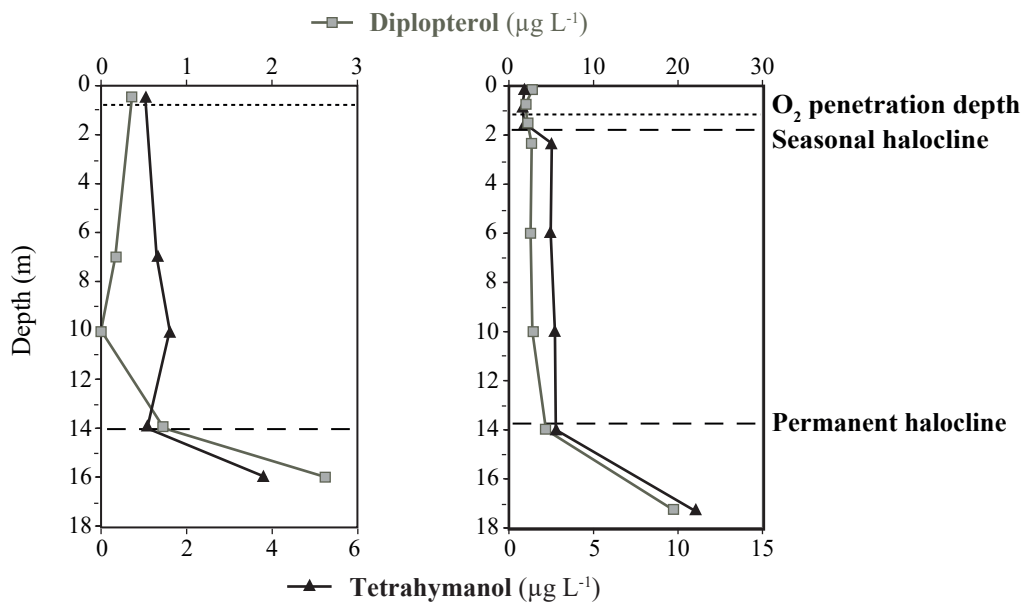
**A**



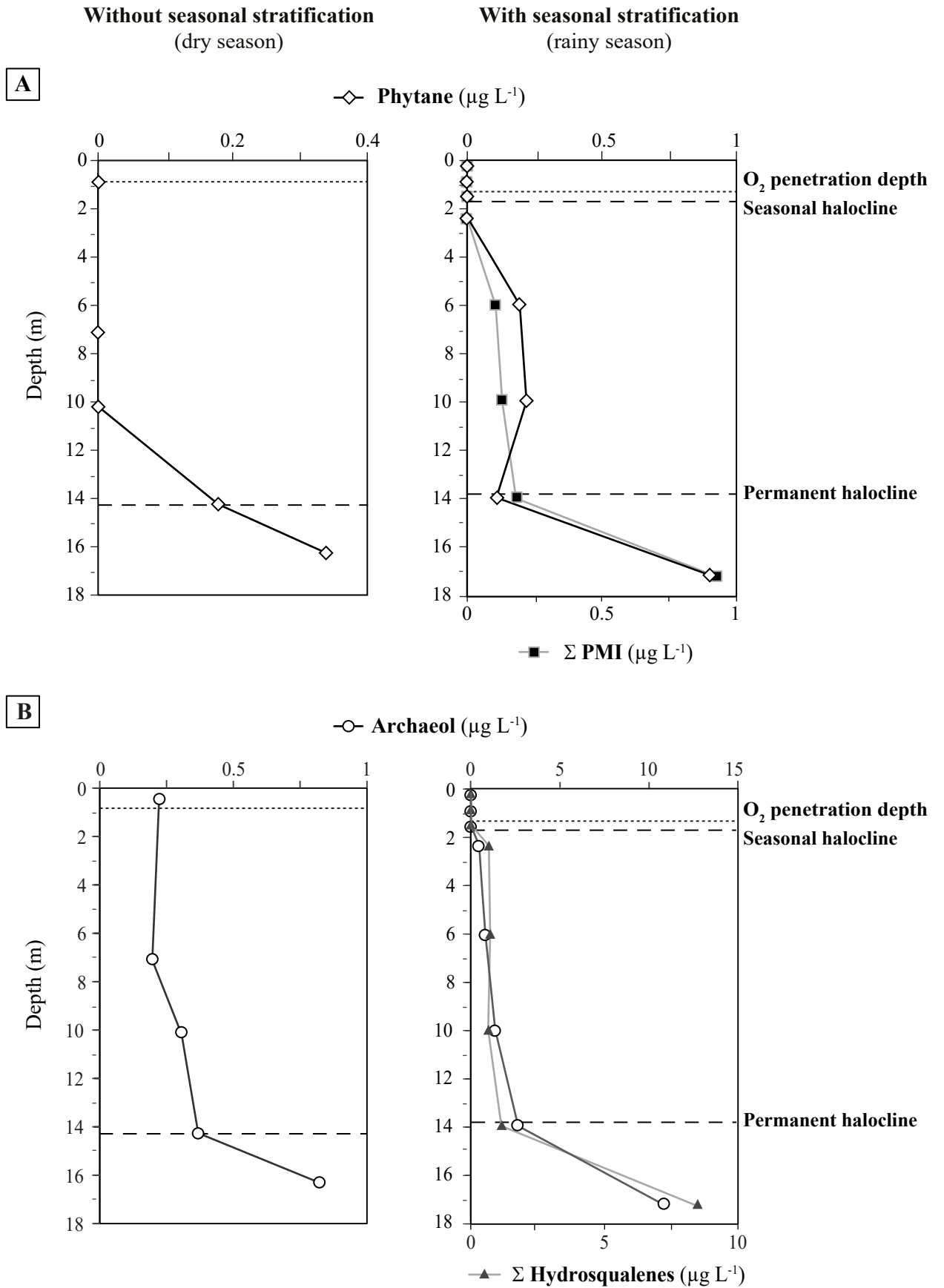
**B**



**C**



**Fig. 4**

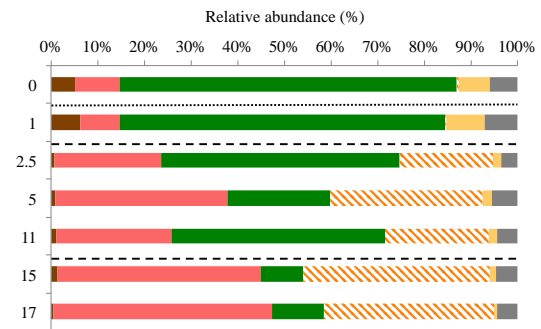
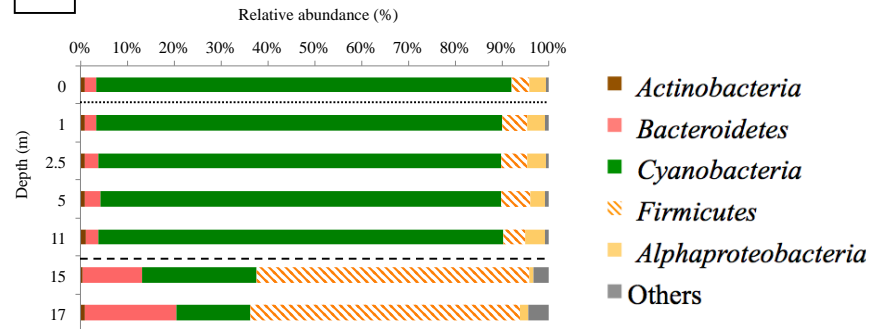


**Fig. 5**

**With seasonal stratification**  
(dry season)

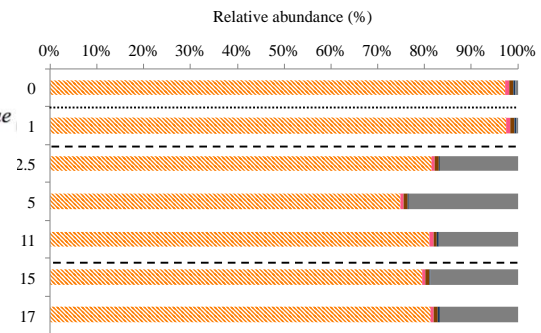
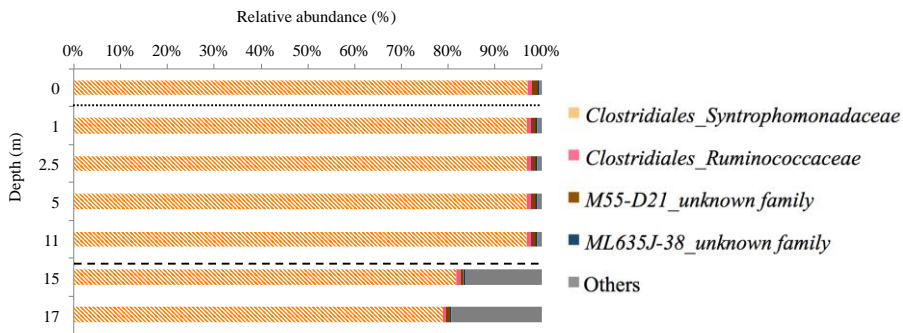
**Without seasonal stratification**  
(rainy season)

**A**



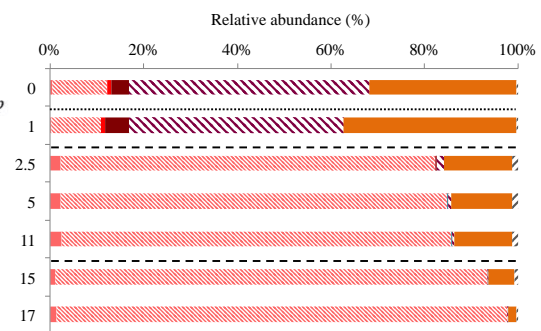
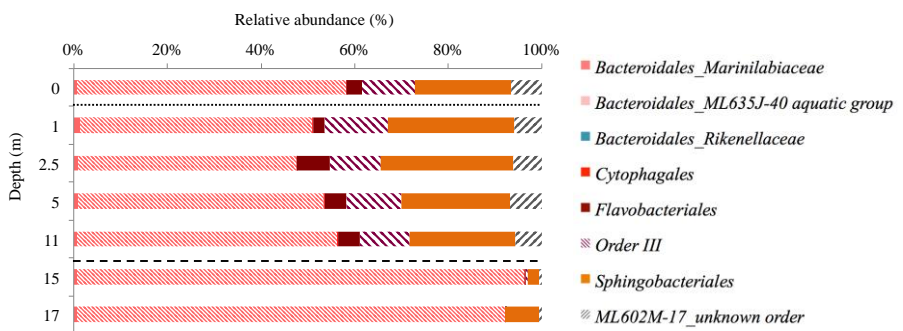
**B**

among Firmicutes (Order level)



**C**

among Bacteroidetes (Order level)

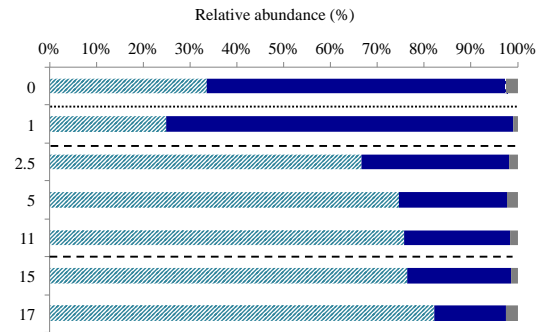
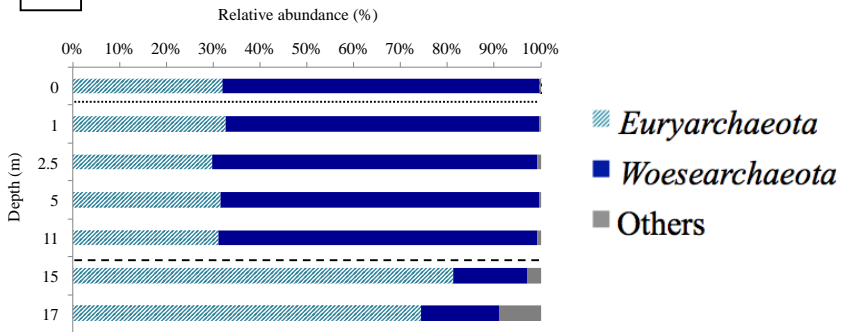


**Fig. 6**

**With seasonal stratification**  
(dry season)

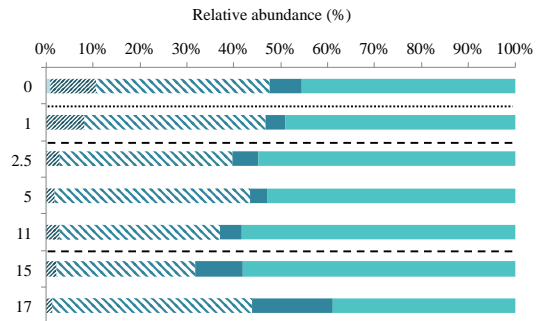
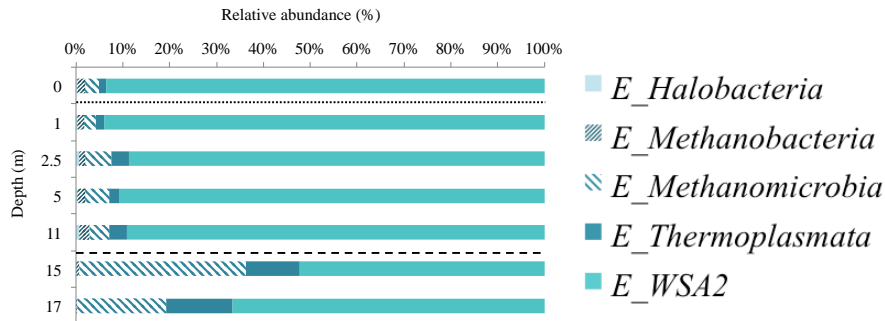
**Without seasonal stratification**  
(rainy season)

**A**



**B**

among Euryarchaeota (Class level)



**Fig. 7**

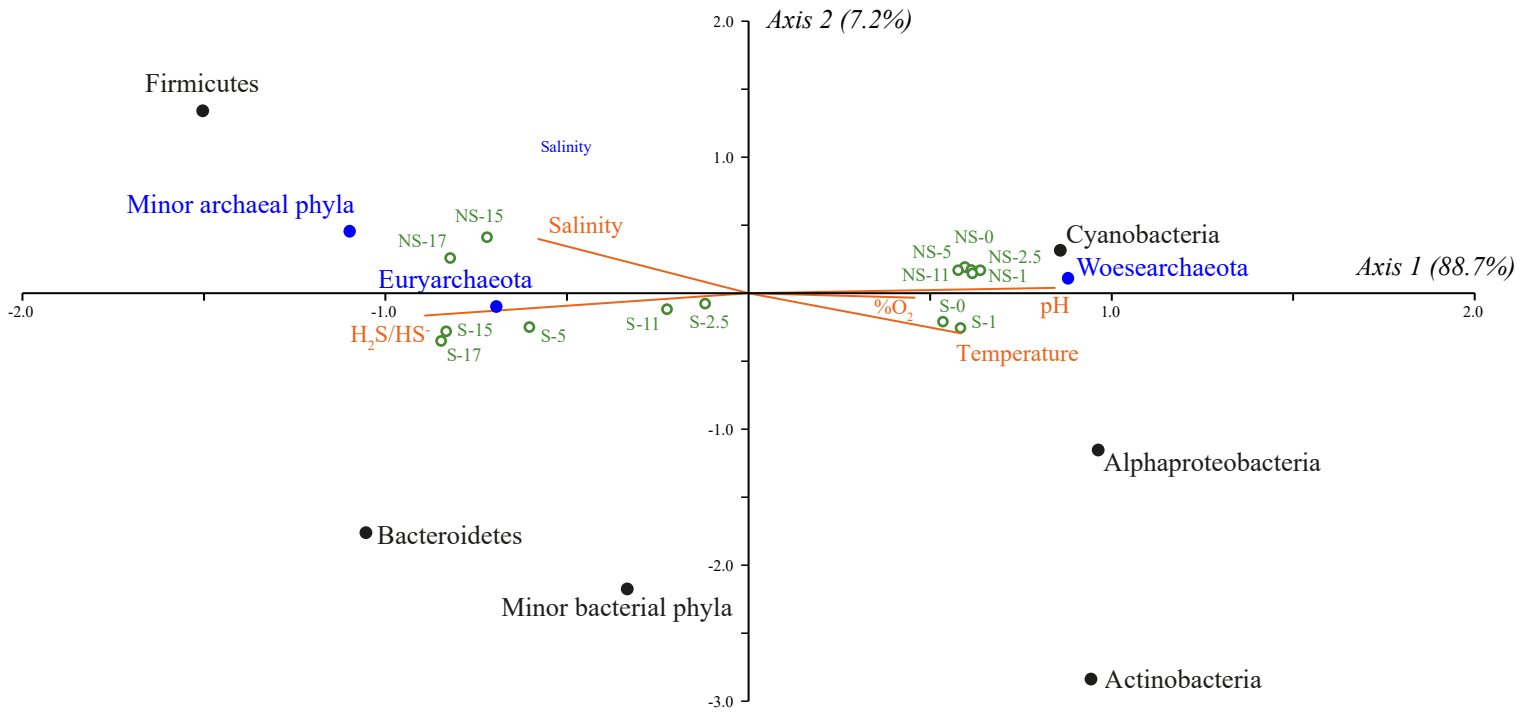
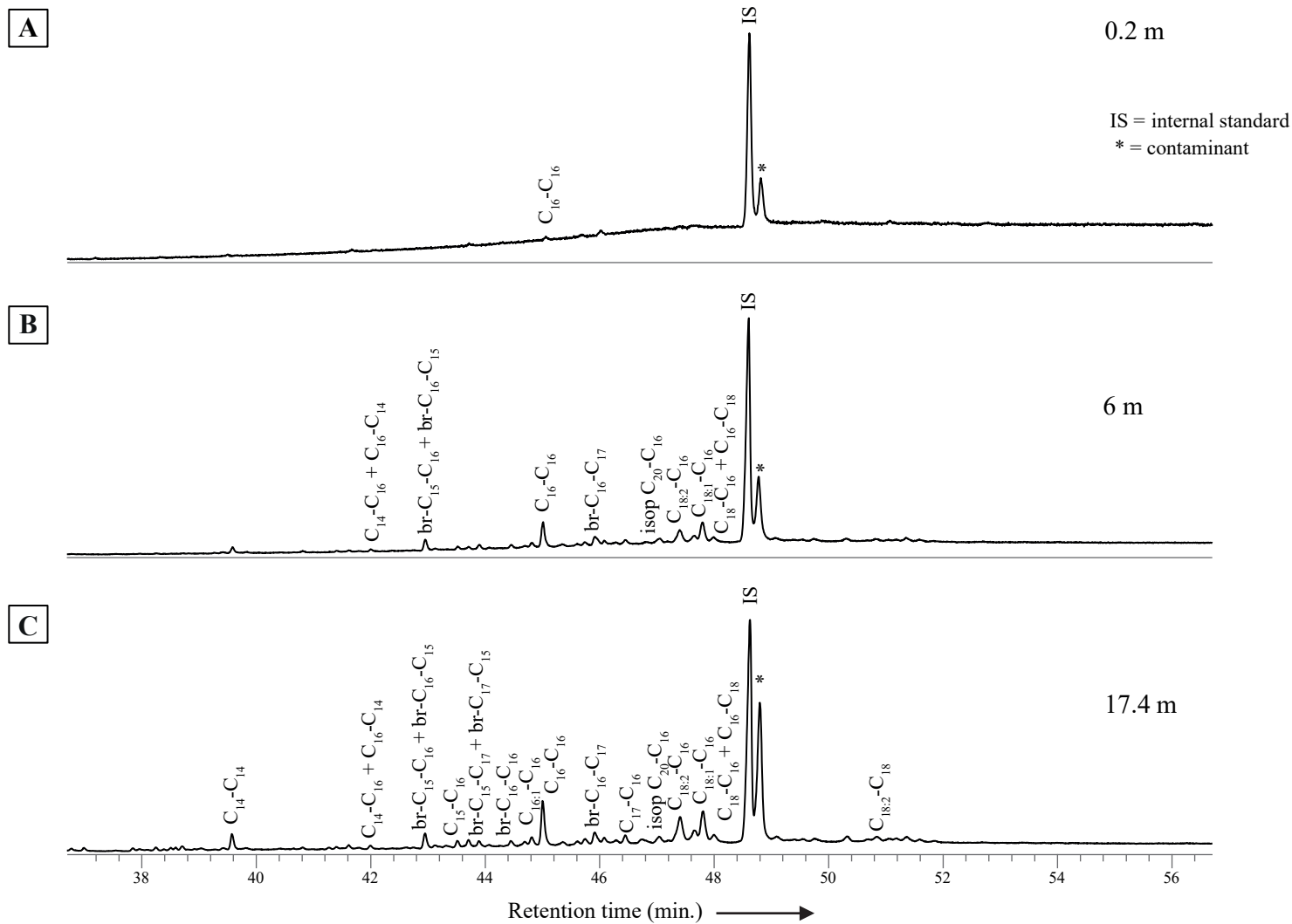


Fig. 8



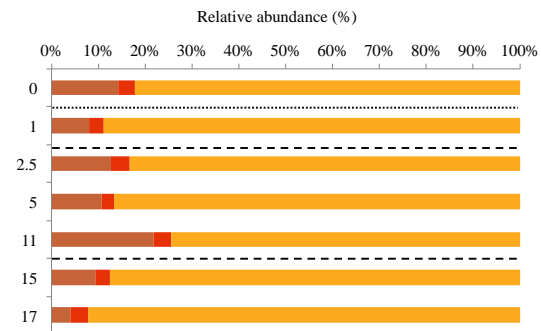
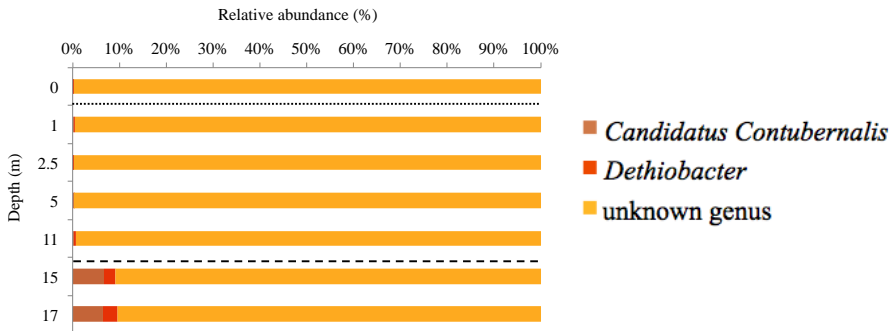
**Fig. S1**

**With seasonal stratification  
(dry season)**

**Without seasonal stratification  
(rainy season)**

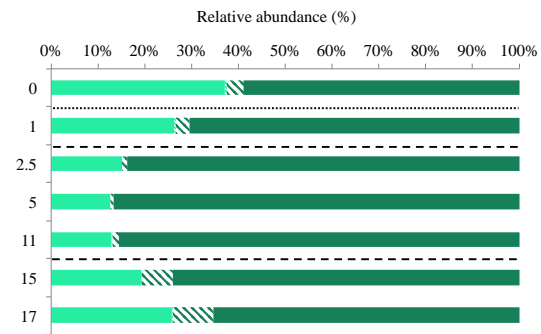
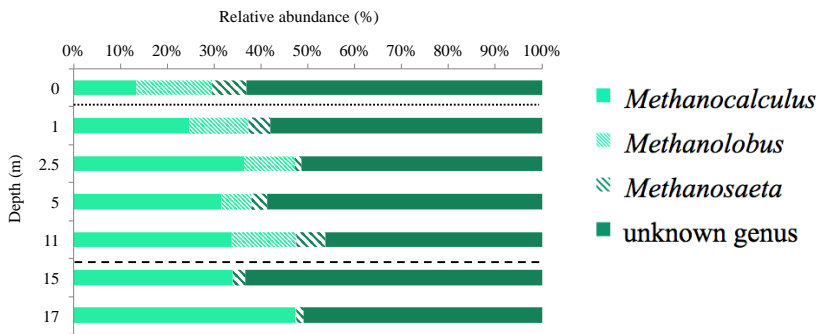
**A**

among Syntrophomonadaceae (Genus level)



**B**

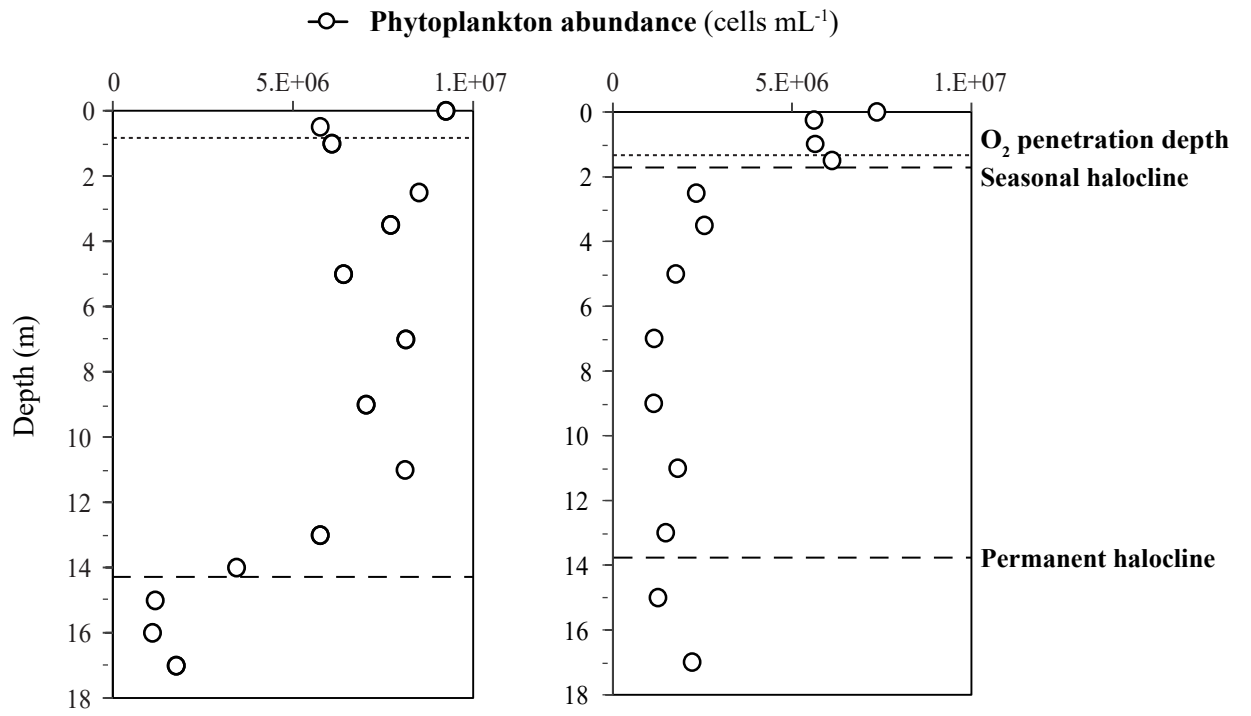
among Methanomicrobia (Genus level)





**Without seasonal stratification**  
(dry season)

**With seasonal stratification**  
(rainy season)



**Fig. S3 new**