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1 **Morphology and ecology of two new amoebae, isolated from a**
2 **thalassohaline lake, Dziani Dzaha.**

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14 **Abstract**

15 Dziani Dzaha is a hypersaline lake (Mayotte island), whose microbial community is dominated
16 by photosynthetic microorganisms. Here, we describe two new free-living heteroloboseans.
17 One belonging to the *Pharyngomonas* genus and the other, whose 18S rRNA gene sequence
18 shares only 85% homology to its closest relatives *Euplaesiobystra hypersalinica*, was proposed
19 as a new species of this genus being called *Euplaesiobystra dzianiensis*. Both strains were salt
20 tolerant to 75‰ and grew between 25 and 37°C. Their distribution patterns varied seasonally
21 and depended also on depth. Noticeably, both free-living amoebae isolates were able to graze
22 on *Arthrospira* filaments, which are found within the same water layer. In conclusion, we
23 document for the first time the presence and ecology of free-living amoebae in the
24 thalassohaline lake Dziani Dzaha, and describe a new species of the *Euplaesiobystra* genus.

25 **Key words: Arthrospira, grazing, halotolerant, Heterolobosea, thalassohaline, water.**

26 **Introduction**

27 Thalassohaline ecosystems are hypersaline lacustrine environments whose water is of marine
28 origin, unlike soda lakes which are filled with water originating from surrounding watersheds
29 and concentrated by solar evaporation, thus qualifying as athalassohaline. The lake Dziani
30 Dzaha, located in a tropical area (Mayotte island, Southwestern Indian Ocean) is a *bona fide*
31 thalassohaline lake (Leboulanger et al., 2017) formed in a crater likely resulting from an
32 eruptive event that occurred during the late Pleistocene/early Miocene era (Nougier et al., 1986;
33 Zinke et al., 2003). The lake reaches a maximum depth of 18 m, presents a permanent anoxic
34 zone below 1.5 m and a periodic chemocline around 14 m. During the rainy period (December-
35 April), the upper layer is diluted creating a seasonal pycnocline, while during the dry period
36 (June-November) salinity is constant over depth. Lake water is warm (29-35°C) over the whole
37 year and depth; pH is alkaline, classically between 9 and 9.5 depending on the season. The lake
38 Dziani Dzaha microbial planktonic community has recently been described via a
39 metabarcoding approach (Hugoni et al., 2018). Bacterial and archaeal diversity was influenced
40 mainly by the sampling depth, whereas dry and rainy seasons alternations drove changes in
41 vertical segregation of microorganisms. Photosynthetic microorganisms were the most
42 abundant and were typically present in surface layers, while chemotrophic taxa and
43 methanogenic archaea were more abundant in the deeper layers. Eukaryotic diversity was
44 highly influenced by the season, and to a lower extent by the depth. Overall, the diversity was
45 dominated by photosynthetic organisms: the cyanobacteria *Arthrospira fusiformis* representing

46 on average 48% of the total sequences in most layers, especially during the dry season, and the
47 eukaryotic chlorophyte *Picocystis salinarum* representing 15% of the total sequences, which
48 was by far the most abundant eukaryotic taxon. Apart from *Picocystis*, various eukaryotes were
49 detected belonging to Fungi, Stramenopiles, and ciliates. Noticeably, no amoebae have been
50 detected, although such microorganisms have been found in hypersaline environments before
51 (Park and Simpson, 2015).

52 Free-living amoebae (FLA) are chemotrophic protists found in various environments, such as
53 water and soil. They belong mainly to the Amoebozoa, Excavata (Heterolobosea) and Rhizaria
54 groups (Samba-Louaka et al., 2019). Heterolobosean were previously described in hypersaline
55 environments, such as soda lakes and solar salterns (Finlay et al., 1987; Park et al., 2009; Park
56 and Simpson, 2011; Plotnikov et al., 2015). Heterolobosea have a diversity of life stages: some
57 genera are known as pure flagellates, such as *Percolomonas* (Nikolaev et al., 2004),
58 *Pleurostomum* (Park et al., 2007) and *Aurem* (Jhin and Park, 2019), while others, such as
59 *Naegleria* (De Jonckheere, 2014), *Tetramitus* (Baumgartner et al., 2009), *Tulamoeba* (Park et
60 al., 2009), *Euplaesiobystra* (Park et al., 2009) and *Pharyngomonas* (Harding et al., 2013),
61 switch from an amoeba stage to a flagellate one, which make them refer to as
62 amoeboflagellates. Heterolobosea possess characteristic eruptive cytoplasmic flow that allow
63 them to move and feed. They mainly graze on bacteria by phagocytosis (Pánek et al., 2017).
64 Many of them are also able to differentiate into a cyst (dormant stage).

65 The aim of our study was to assess the presence of amoebae in the lake Dziani Dzaha by a
66 culture-based approach. We hereby characterize two new heterolobosean isolates affiliated to
67 *Pharyngomonas* and *Euplaesiobystra* genera. Their presence was researched throughout
68 different seasons and along the water column. Experiments were performed to explore whether
69 the isolated amoebae could graze filamentous cyanobacteria, which constitutes the highest
70 biomass in the lake Dziani Dzaha.

71 **Results**

72 *Pharyngomonas* sp. DD1

73 **Morphology and Ultrastructure**

74 Light microscopic observations of *Pharyngomonas* sp. DD1 showed a typical morphology for
75 this genus, most notably root-like extensions (Fig. 1). *Pharyngomonas* sp. DD1 trophozoites
76 ($27 \pm 12.9 \mu\text{m}$ long (between 14 and 64 μm), $12 \pm 2.5 \mu\text{m}$ wide (between 7.5 and 19.4 μm))
77 presented a variety of shapes, including elongated forms that are present in older cultures, as

78 previously observed in other *Pharyngomonas* strains (Harding et al., 2013; Plotnikov et al.,
79 2015). It can switch from long crenulated forms (Fig. 1A and 1D) to flabellate shapes or to
80 more ovoid ones (Fig. 1B and 1C). When cells are elongated, they can deploy finger-like
81 pseudopodia as well as very long root-like extensions (which can represent half to two-thirds
82 of the cell length), these being more visible before encystment. This FLA isolate formed round
83 or ovoid-shaped cysts ($10.5 \pm 1 \mu\text{m}$ diameter) that appeared doubled-layered. The ectocyst
84 thickness is quite variable, the cyst being surrounded by "bead-like" structures, or crypts as
85 previously referred to (Park and Simpson, 2016). No ostiole could be clearly distinguished.
86 Encystment of a trophozoite culture is very synchronous for this strain and takes place in less
87 than 24 hours, as soon as bacterial substrate has been depleted. Despite numerous observations,
88 we were unable to see a flagellate form, which was described for all other *Pharyngomonas*
89 strains except *P. turkanaensis* (Park and Simpson, 2016).

90 Transmission electron microscopy was performed to describe the ultrastructure of this isolate
91 (Fig. 2). The nucleus was clearly visible, and the nucleolus can be distinguished (Fig. 2B, 2C
92 and 2D). Numerous elongated mitochondria were present in the whole cytoplasm (Fig. 2B and
93 2C). No endoplasmic reticulum envelopment was present around mitochondria, which is
94 characteristic of some heterolobosean genera such as *Pharyngomonas*, *Stephanopogon*,
95 *Percolomonas* or *Pleurostomum* (Supplementary Fig. 3; Pánek et al., 2017) No dictyosomes
96 could be clearly distinguished. One can observe large digestive vacuoles that seem to
97 correspond to different maturation stages (Fig 2A and 2D), from the phagosome containing
98 bacteria to multilamellar bodies (MLB) (Hohl, 1965). MLB have been already described for
99 the social amoeba *Dictyostelium discoideum* (Paquet et al., 2013) as well as for the ciliate
100 *Tetrahymena pyriformis* (Trigui et al., 2016), both of which secrete them when fed on bacteria.
101 This isolate produced round-shaped multilayered cysts (Fig. 2E and 2G). The thickness of the
102 different layers did not appear regular and small vesicles were clearly apparent between the
103 endo- and ectocyst (Fig. 2F and 2H). Vesicles surrounding the cysts (crypts), observed in
104 optical microscopy, seem to be in fact expelled MLB that remain attached to the cyst wall.

105 *Euplaesiobystra dzianiensis* DD2

106 **Morphology and Ultrastructure**

107 *Euplaesiobystra dzianiensis* trophozoites ($22 \pm 4.2 \mu\text{m}$ long (between 16.7 and 34 μm), $13 \pm$
108 $3.4 \mu\text{m}$ wide (between 8 and 20.6 μm) displayed a canonical heterolobosean morphology, being
109 limax-shaped, monopodial and forming eruptive pseudopodia (Fig. 3A). Sometimes, a bulbous
110 uroid with fine filaments (Fig. 3B and 3C) can be observed when trophozoites are in motion.

111 This FLA formed pleomorphic cysts which were mostly spherical ($9.5 \pm 0.9 \mu\text{m}$ diameter), but
112 some showed irregular outlines (Fig. 3D-F). A distinct outer layer, corresponding to the
113 ectocyst, was visible (Fig. 3E). Encystment of this strain wasn't homogenous in our growth
114 conditions: even after several days of starvation, trophozoites were still present. Cysts shown
115 on figure 3 originate from a one-month old culture and are surrounded by cellular debris,
116 suggesting that a portion of the initial trophozoite population lysed. In addition, despite the
117 documented ability of *Euplaesiobystra hypersalinica* (Park et al., 2009) to switch to a transient
118 flagellated stage, we were unable to observe such stage in our growth conditions.

119 *Euplaesiobystra dzianiensis* DD2 (Fig. 4) trophozoites contained one nucleus which displays
120 a central nucleolus (Fig 4A). Electron-dense round shaped mitochondria (Fig. 4B) can be
121 observed as well as numerous vacuoles, some of which contain bacteria probably undergoing
122 digestion. No dictyosomes could be clearly distinguished, but some vacuoles displayed
123 structural features resembling those of MLB, which are also present within the extracellular
124 environment (Fig. 4C). Cysts harbored an irregular shape and thin but distinct ectocyst
125 compared to a thick fibrillar endocyst (Fig. 4D). Within cysts, lipid droplets can be
126 distinguished as well as some vesicles. A plugged ostiole was clearly observable (Fig. 4G), and
127 some cysts displayed two pores (Fig. 4E). These observations are in line with previous work
128 which documented the possible presence of 2 or more pores per cyst for *Euplaesiobystra*
129 *hypersalinica* (Park et al., 2009). Within crescent-shaped cysts, cytoplasm in the vicinity of the
130 cyst wall was highly vesiculated, hinting at export towards the cyst wall, while the plasma
131 membrane was clearly distinguishable in the "rounded" cyst (Fig. 5D). These elements
132 suggested that crescent cysts could be still encysting compared to rounded ones which would
133 be more mature.

134 **Molecular phylogeny of *Pharyngomonas* sp. DD1 and *Euplaesiobystra dzianiensis* DD2**

135 Two morphotypes of amoebae were isolated from the lake water after plate cultivation and
136 liquid subcultures. To identify these isolates, DNA was extracted and 18S rRNA gene was
137 sequenced. The 18S rRNA gene sequence of the first isolate DD1 (3136 bp) was compared to
138 the nr database with BLAST, showing that the best hits (E-value = 0.0) corresponded to two
139 *Pharyngomonas* sequences (i.e. *Pharyngomonas turkanaensis* LO and *Pharyngomonas* sp. RL)
140 with 96% and 94% identity, respectively. A 983 bp group I intron was identified in the 18S
141 rRNA sequence at position 583. Phylogeny confirmed this isolate belongs to the
142 *Pharyngomonas* genus and is consequently referred to as *Pharyngomonas* sp. DD1 (Fig. 5).
143 The helix 17-1 of 18S V3 region, a characteristic feature of Tetramitia was not present in

144 *Pharyngomonas* sp. DD1 rRNA gene sequence, consistent with its phylogenetic placement
145 within *Pharyngomonas* (Supplementary Fig. 1 ; Nikolaev et al., 2004).

146 The 18S rRNA gene sequence of the second isolate DD2 (1846 bp) was confronted to the nr/nt
147 database with BLAST, showing that the best hits (E-value = 0) corresponded to a partial 18S
148 sequence from an uncultured heterolobosean isolate CBN AP20 (92% identity) and
149 *Euplaesiobystra hypersalinica* strain A2 (85% identity). In addition to this moderate sequence
150 homology, the determined phylogenetic positioning of this isolate suggests that it represents a
151 new, distinct species related to *Euplaesiobystra hypersalinica*, the closest matching organism
152 identified using 18S rRNA gene sequence (Fig. 5). It is therefore likely to represent a new
153 species that we propose to name *Euplaesiobystra dzianiensis* sp. nov. (see taxonomic summary).
154 Because the CBN AP20 sequence is significantly shorter than the other *Euplaesiobystra* ones,
155 we excluded it from the main phylogenetic analysis. Inclusion of this sequences confirmed that
156 isolate CBN AP20 is the closest relative to *E. dzianiensis* (Supplementary Fig. 2). As expected
157 from its phylogenetic position, the *E. dzianiensis* DD2 rRNA gene sequence does include the
158 ‘helix 17-1’ element characteristic of Tetramitia (Supplementary Fig. 1).

159 *Ecology*

160 **Amoeba isolates distribution in the lake**

161 In previous studies, it has been reported that the season and/or the depth in the lake can
162 influence its microbial diversity (Bernard et al., 2019; Hugoni et al., 2018). To see whether the
163 two FLA isolates were influenced by these parameters, we performed a specific qPCR on DNA
164 isolated from the water lake sampled in April (end of the rainy season) and November (dry
165 season) 2015. *Pharyngomonas* sp. DD1 concentration increased in November as compared to
166 April (Fig. 6). This FLA concentration decreased with the depth, with the same pattern as
167 photosynthetic microorganisms (e.g. cyanobacteria) below 5 m depth (Hugoni et al., 2018). *E.*
168 *dzianiensis* DD2 showed an even more marked behaviour regarding seasonality since this
169 isolate was almost not detected in April while it was present in all samples in November (Fig.
170 6). It is also clear that its concentration decreased with the lake depth. These two isolates follow
171 a similar trend of seasonal and depth distributions, being found in higher concentrations during
172 dry season in shallow waters of lake Dziani Dzaha.

173 **Salt and temperature tolerance**

174 The two FLA strains presently described were isolated from a thalassohaline lake showing
175 constant high salinity (40-60 ‰) and temperatures up to 35°C (Leboulanger et al., 2017). Thus,

176 we performed in vitro experiments to evaluate their salt and temperature tolerance for growth
177 as compared to the in situ environmental conditions. *Pharyngomonas* sp. DD1 growth was not
178 affected by temperature between 25 and 37°C, while it was clearly inhibited at 43°C (Table 1).
179 With respect to salinity, *Pharyngomonas* sp. DD1 grew faster within the range from 15 to 75
180 ‰. Growth was diminished at 100 ‰ and totally inhibited at 150 ‰ in our conditions.

181 *Euplaesiobystra dzianiensis* DD2 growth was inhibited at 43°C (Table 1). Regarding salinity,
182 *E. dzianiensis* DD2 grew faster within the 15 to 40 ‰ range. The growth was hampered at 75
183 ‰ and fully inhibited at 100 ‰ in our conditions. Ultimately, the two isolates displayed a
184 similar behaviour regarding temperature, with a growth optimum ranging between 25 and
185 37°C, while *E. dzianiensis* was less halotolerant than *Pharyngomonas*.

186 ***Euplaesiobystra dzianiensis* DD2 and *Pharyngomonas* sp. DD1 are able to phagocytose**

187 **Arthrospira**

188 The spirulina *Arthrospira fusiformis* is the most abundant cyanobacterium in the lake,
189 accounting for 95 % of the total photosynthetic biomass (Bernard et al., 2019). Its presence has
190 been positively correlated with the temperature (Bernard et al., 2019). As the highest
191 temperatures, as well as highest light intensity, were found in the upper layer of the water
192 column (first three meters), *A. fusiformis* is mainly found within this level. Because amoebae
193 were shown in this study to follow the same distribution pattern, grazing capacity of the two
194 isolates on cyanobacterial prey was addressed. Grazing experiments, using culture of *A.*
195 *fusiformis* isolated from the lake, were performed by mixing cyanobacteria and FLA in a single
196 culture. *Euplaesiobystra dzianiensis* grazing on *Arthrospira* was observed (Fig. 7). It appeared
197 that *E. dzianiensis* was able to ingest an *Arthrospira* filament, of almost the same size as itself
198 (Fig. 7A). *A. fusiformis* parts could be observed in the majority of screened trophozoites. The
199 same experiment was performed with *Pharyngomonas* sp. DD1 and *Arthrospira* pieces were
200 also clearly observed in several trophozoite cells (Fig. 8).

201 **Discussion**

202 The thalassohaline lake of Dziani dzaha is considered an extreme environment of interest as it
203 mimics environmental conditions that could be found in Precambrian oceans (Bernard et al.,
204 2019). It could thus provide a window into early adaptation of microorganisms to such
205 environmental conditions. Free-living amoebae belonging to the Heterolobosea have been
206 repeatedly isolated in a variety of extreme environments with physicochemical properties that

207 are somewhat similar to what is found in the lake Dziani dzaha (Pánek et al., 2017). This
208 prompted us to look for amoebae in this thalassohaline lake.

209 The two amoebae presently described were not detected by the previous metabarcoding study
210 of the lake (Hugoni et al., 2018). This result might be partly due to a low and fluctuating
211 abundance of these amoebae within the eukaryotic community of the lake or by the use of the
212 universal 18S primers (515F and 951R) (Hugoni et al., 2018), which might not match the 18S
213 rRNA gene sequence of the amoebae isolates. To check this hypothesis, the primers were tested
214 with the SILVA database allowing three mismatches, without any in the last three nucleotides,
215 to inspect the sequence coverage among eukaryotes. The primer pair matched 92% of the
216 eukaryotic sequences within the SILVA database, showing their universality, however they did
217 not match any heterolobosean sequences. Our analysis confirmed that heteroloboseids are
218 highly likely to be missed using the 515F-951R 18S rRNA gene primers.

219 One amoeba isolated belongs to the *Pharyngomonas* genus and displayed morphological as
220 well as genetic features that are hallmarks of this genus. Appending the size data collected by
221 Park and Simpson (Park and Simpson, 2016 ; Supplementary Table 1), it appears that our strain
222 is closer to *Pharyngomonas turkanaensis* in terms of size and shape of trophozoites and cyst
223 than from *P. kirbyi* and other isolates. Such observations were supported by the phylogeny
224 reconstructed from 18S rRNA gene sequences, showing that *P. turkanaensis* is the closest
225 relative to *Pharyngomonas* sp. DD1. The comparison of morphological features highlighted
226 that endocysts and ectocysts seemed thicker for *Pharyngomonas* sp. DD1 than for other
227 described genus members. This might be due to vesicles/crypts, surrounding the cysts,
228 observed in optical microscopy. Such vesicles seem in fact to be expelled multilamellar bodies
229 (MLB) which stay attached to the cyst. They look different from previous observations which
230 reported the presence of bacteria and/or fungi within these crypts (Park and Simpson, 2016).
231 These authors also mentioned “scrolling feature within ectocyst (presumed fixation artifact)”
232 which resemble what we consider as MLB surrounding *Pharyngomas* sp. DD1 cysts, but
233 probably not an artefact. The moderate sequence divergence and the morphological difference
234 of cyst structure between *P. turkanaensis* and *Pharyngomonas* sp. DD1 are not sufficient, in
235 our opinion, to formally propose a new species.

236 The other isolated amoeba represents a new species of the *Euplaesiobystra* genus. The
237 morphology and ultrastructure of this isolate is consistent with previous account of
238 *Euplaesiobystra hypersalinica* (Park et al., 2009). Sequence comparison and phylogenetic

239 analyses highlighted a marked genetic divergence, with only a moderate identity to
240 *Euplaesiobystra hypersalinica* strain A2 (85% identity). While morphology and phylogeny
241 confidently group this isolate with *Euplaesiobystra*, it also clearly highlights a marked
242 divergence from all other isolates, thus motivating the proposal of the novel species *E.*
243 *dzianiensis*. Sequence comparison to the nr/nt database of the NCBI also revealed a high
244 identity (92%) to a short, partial sequence of an heterolobosean isolate designated as CBN
245 AP20. Phylogenetic inferences based on these short fragments confirmed that isolate CBN
246 AP20 is the closest relative of *E. dzianiensis*. The CBN AP20 isolates was recently reported as
247 a contaminant in spirulina production cultures (Yuan et al., 2018).

248 Our inability to identify a flagellate stage for both isolates and especially for *Pharyngomonas*,
249 for which such a stage has been well described may be attributed to the way the strains were
250 isolated (Park and Simpson, 2011; Plotnikov et al., 2015). Other authors used clonal liquid
251 dilutions from environmental samples; in this study, we used agar plate isolation method, thus
252 starting from trophozoites. After that, strains were propagated in flasks in ASW supplemented
253 with a high density of *E. coli*: it may be possible that these conditions, while allowing quick
254 and efficient growth of the amoeba stage, are not well suited to promote flagellation as has
255 been reported for other Heterolobosea such as *Naegleria* (De Jonckheere, 2011). Some
256 heterolobosea like *Marinamoeba* (De Jonckheere et al., 2009), only possess an amoebae stage,
257 while others, like *Percolomonas* and *Stephanopogon* (Cavalier-Smith and Nikolaev, 2008) are
258 only described as flagellates. Taking this into account, the recent characterization of
259 *Pharyngomonas turkanaensis* suggested this isolate might be unable to adopt a flagellate stage
260 (Park and Simpson, 2016). Ultimately, it might be that the ability to adopt a flagellate stage is
261 not a conserved trait within the *Pharyngomonas* genus.

262 Because *Pharyngomonas* and *Euplaesiobystra* spp. were previously described as being able to
263 grow in high salinity conditions, we tested this ability on both new isolates. *Pharyngomonas*
264 sp. DD1 is able to grow between 15 and 100 ‰, which is consistent with its natural
265 environment whose salinity varies between 34 and 71 ‰ (Hugoni et al., 2018). Interestingly,
266 this result on salinity is quite similar to those observed with *P. turkanaensis* LO with a highest
267 growth rate between 15 and 30 ‰ salinity and a cessation of growth by 125 ‰, although the
268 latter strain has been isolated from the Turkana lake presenting a low salinity of about 4 ‰
269 (Park and Simpson, 2016). These values are substantially lower than the ones observed for
270 *Pharyngomonas kirbyi* SD1A and AS12B (75-250 ‰) (Park and Simpson, 2011), and
271 *Pharyngomonas* sp. RL (50-250 ‰) (Harding et al., 2013). *Pharyngomonas* sp. DD1 thus

272 seems also far less halophilic than these strains. *Pharyngomonas* sp. DD1 is another example
273 of a halotolerant isolate in the clade of Pharyngomonada, as *Tulamoeba bucina* (Kirby et al.,
274 2015) is within the Tulamoebidae.

275 Compared to its closest relative in the literature, *i.e.* *Euplaesiobystra hypersalinica*, whose
276 optimal growth salinity is around 150-200 ‰ (Park et al., 2009), *E. dzianiensis* DD2 was
277 clearly less halotolerant. The two *Euplaesiobystra hypersalinica* isolates which have been
278 described in the literature, originated from a hypersaline solar saltern (Park et al., 2009) (growth
279 between 100 and 300 ‰) or from a lagoon (Park and Simpson, 2015) (290 ‰ in the originating
280 sample). The third, which was first named *Plaesiobystra hypersalinica*, also originates from a
281 hypersaline (140 ‰) environment (Park et al., 2009). The salinity tolerances of *Euplaesiobystra*
282 *dzianiensis* and its closest relative, the heterolobosean CBN AP20, which has been isolated
283 from cultures in Zarrouk medium (Zarrouk, 1966) whose salinity is about 20 ‰, suggests that
284 these isolates are adapted to mesohaline rather than hypersaline environments.

285 qPCR assays were designed in order to investigate the presence and relative abundance of both
286 amoebae in the lake Dziani Dzaha. Overall, they were found in higher relative abundance
287 during the dry season (November), as compared to the rainy season (April). This pattern was
288 particularly marked for *E. dzianiensis*, which was mostly undetected by qPCR in the rainy
289 season. Additionally, the relative abundance of both amoebae decreased along an increasing
290 water depth gradient. Thus, both amoebae seemed to be more abundantly found in surface
291 water, regardless of the season. Such distribution patterns coincide with those of photosynthetic
292 microorganisms of the lake Dziani Dzaha, such as the cyanobacterium *Arthrospira fusiformis*.
293 Amoebae usually feed on smaller microorganisms that are ingested through phagocytosis.
294 However, a few examples of amoebae feeding on larger preys are documented, such a
295 *Vampyrella* spp. and *Leptophrys vorax*, (Rhizaria, Endomyxa) which feed on algal preys larger
296 than themselves (Hess et al., 2012). Based on this, we investigated the ability of *E. dzianiensis*
297 DD2 and *Pharyngomonas* sp. DD1 to feed on *A. fusiformis*, which are characterized as being
298 straight or slightly wavy cylindrical trichomes whose size ranged from 170 to 2390 µm
299 (Cellamare et al., 2018) and represent the most abundant cyanobacteria in the lake Dziani
300 dzaha. Microscopic observations confirmed that both amoebae were able to feed on *A.*
301 *fusiformis*, despite the fact that those bacteria are elongated and cannot be internalized as a
302 whole by the amoebae. One of the closest matching sequences in the nr database for *E.*
303 *dzianiensis* is that of the uncultured heterolobosean isolate CBN AP20, which has recently been
304 reported as a contaminant in spirulina production cultures (Yuan et al., 2018). Further, it was

305 shown that isolate CBN AP20 was able to graze on the spirulina *Arthrospira*. Thus, such
306 observation suggest that these amoebae may feed on cyanobacteria in natural conditions, using
307 a mechanism that remains to be elucidated.

308 In conclusion, our study described a new FLA species among the Heterolobosea,
309 *Euplaesiobystra dzianiensis* (DD2), and a new isolate of *Pharyngomonas* sp. (DD1), from the
310 hypersaline lake Dziani Dzaha. Their growth in vitro was optimal at 25-37°C and around 40
311 psu salinity, with a better tolerance to higher salinity for *Pharyngomonas*. Their abundances
312 varied depending on the season and on the depth. Finally, we show that both isolates were able
313 to phagocytose *A. fusiformis*, which is the predominant photosynthetic microorganism found
314 in the lake. It would be interesting to examine whether other amoebae could be found in the
315 lake and to better characterize the trophic interactions between these organisms and their preys.

316 **Taxonomic summary**

317 Assignment: Eukaryota; Excavata; Discoba; Discicristata; Heterolobosea; Tetramitia;
318 *Euplaesiobystra*

319 *Euplaesiobystra dzianiensis* n. sp

320 Diagnosis: Heterolobosean, limax-shaped amoeba, capable of forming cysts. Single nucleus.
321 Found in hypersaline habitat. Trophozoites: 17-33 µm (average: 22 µm) and 8-20 µm (average:
322 13 µm) in length and width; sometimes with uroid and uroidal filaments during motion.
323 Frequent eruptive movements. Cyst: spherical or crescent shaped; 9.5 µm in diameter, 1-2
324 observed plugged pores, wall with distinct ectocyst and endocyst. Growth in salinities ranging
325 from 15 to 75 ‰. Flagellate phase unknown.

326 Type material: two samples have been deposited as the name-bearing type (an hapantotype) in
327 the protist collection of the National Museum of Natural History, Paris. The first is a culture of
328 isolate DD2, preserved in ethanol 70 % (accession number MNHN-IR-2020-03), the second is
329 a cyst suspension (accession number MNHN-IR-2020-04).

330 Type locality: type culture isolated from the thalassohaline lake Dziani Dzaha located in the
331 Petite Terre island in Mayotte (12°46'15.6" S; 45°17'19.2" E).

332 Etymology: The species epithet “*dzianiensis*” refers to the lake Dziani Dzaha from which the
333 sample is originating (-ensis).

334 Gene sequence. The 18S rRNA gene sequence from *Euplaesiobystra dzianiensis* isolate DD2
335 has the Genbank Accession number MN969059.

336 Zoobank registration Described under the Zoological Code; Zoobank registration
337 urn:lsid:zoobank.org:act:53D12014-6E7B-4D87-8080-691FB443CB1F

338 This work has been registered with Zoobank as urn:lsid:zoobank.org:pub:FAEA9268-8E01-
339 4E33-AF36-039AF9436616

340 **Materials and methods**

341 *Isolation and cultivation of amoebae*

342 Water samples were taken from the lake Dziani Dzaha, located in the Petite Terre island in
343 Mayotte (12°46'15.6" S; 45°17'19.2" E). The lake was sampled in April and November 2015,
344 along a depth profile (0.25 m, 1 m, 2.5 m, 5 m, 11 m, 15 m and 17 m depth) located above the
345 deepest point of the lake, using a horizontal 1.2-L Niskin bottle (Hugoni *et al.* 2018). The lake
346 Dziani Dzaha presents peculiar features such as high salinity (around 52 psu), high pH (9 to
347 9.5) and high temperature (27 to 35°C).

348 A water sample (100 ml) was filtered on a sterile Nucleopore polycarbonate filter (3 µm pore
349 size; 47 mm diameter) using a vacuum pump. The filter was cut in two pieces and deposited
350 onto saline non-nutrient agar (NaCl 60 g/L, Sodium Citrate tribasic 0.8 g/L, MgSO₄ 4 mM,
351 Na₂HPO₄ 2.5 mM, KH₂PO₄ 2.5 mM, CaCl₂ 0.5 mM) seeded with 100 µL of an *Escherichia*
352 *coli* suspension (*E. coli* were grown overnight in Lysogeny Broth - peptone 10 g/L, yeast
353 extract 5 g/L, NaCl 10 g/L - , centrifuged for five minutes at 6000 g and resuspended in ASW
354 to reach an optical density of about 50 when measured at 600 nm) (NNA-Eco plate). The plates
355 were sealed with parafilm and incubated at 30°C until a migration front was observed. Fronts
356 were excised from plates and used for re-isolation on an NNA-Eco plate or to inoculate flasks
357 containing Artificial Sea Water (ASW: 26.29 g NaCl, 0.74 g KCl, 0.99 g CaCl₂, 6.09 g
358 MgCl₂•6H₂O, 3.94 g MgSO₄•7H₂O L⁻¹, pH 7.8) seeded with a population of *E. coli* (the
359 previously described suspension was diluted to one hundredth in the amoebae growth medium
360 so that the optical density measured at 600 nm was approximately 0.5). Isolated amoebae were
361 routinely grown in ASW-*E. coli* at 30°C.

362 *Cultivation of Arthrospira fusiformis*

363 *Arthrospira fusiformis* suspensions were a kind gift from Charlotte Duval. They have been
364 grown at 25°C with a light intensity of 15 µmol photons m⁻² s⁻¹ with a 16 hours light/8 hours

365 dark cycles in Z8 Mayotte medium (per 1 L: 20 g NaCl, 0.467 g NaNO₃, 59 mg
366 Ca(NO₃)₂•4H₂O, 25 mg MgSO₄•7H₂O, 31 mg K₂HPO₄, 21 mg Na₂CO₃, 10 mL Fe-EDTA
367 solution (A: dissolve 2.8 g FeCl₃•6H₂O in 100 mL 0.1 N HCl, B: dissolve 3.9 g EDTA-Na₂ in
368 0.1 M NaOH, add 10 ml solution A and 9.5 ml solution B plus water to 1 L), 1 mL trace solution
369 (for 1L: 0.31 g H₃BO₃, 0.223 g MnSO₄•4H₂O, 0.89 mg V₂O₅, 3.3 mg Na₂WO₄•2H₂O, 8.8 mg
370 (NH₄)₆Mo₇O₂₄•4H₂O, 12.1 mg KBr, 8.3 mg KI, 28.7 mg ZnSO₄•7H₂O, 15.5 mg
371 Cd(NO₃)₂•4H₂O, 14.6 mg Co(NO₃)₂•6H₂O, 12.5 mg CuSO₄•5H₂O, 19.8 mg
372 (NH₄)₂Ni(SO₄)₂•6H₂O, 4.1 mg Cr(NO₃)₃•9H₂O, 47.4 mg Al₂(SO₄)₃K₂SO₄•24H₂O), Cultures
373 were not axenic but have been passed in aseptic conditions.

374 *Microscopy*

375 Cultures of FLA at different developmental stages were transferred into 35 mm dishes with
376 glass coverslips (μ-dish, Ibidi). The cells were left to adhere at room temperature and observed
377 using an inverted microscope equipped with differential interference contrast (DIC)
378 (Olympus). Images' contrast and brightness were further adjusted using ImageJ. DIC
379 micrographs were used to evaluate cell sizes, which are expressed at mean values ± standard
380 deviation (40 trophozoites and 20 cysts were measured for each strain). For cyanobacterial
381 grazing assays, images were acquired using an inverted brightfield microscope. For
382 transmission electron microscopy, FLA cultures in their trophozoite or cyst stages were fixed
383 by incubating the culture in ASW or 60‰ artificial salinity medium (see “Salinity and
384 temperature tolerance” for composition) containing a final concentration of 2.5%
385 glutaraldehyde for 1 hour at 4°C. Cells were rinsed three times in phosphate buffer (0.1 M, pH
386 7.2) and post-fixed in a 1% osmium tetroxide solution, for 1 hour at 4°C. Cells were rinsed,
387 embedded in Histogel for further handling. Cells were dehydrated in increasing acetone bath
388 (50%, 70%, 90%, 100%) and embedded, first in a mix of acetone and araldite resin (1:1) for
389 one hour, then in pure araldite and left to harden for approximately 24 h. Ultrathin section (70
390 nm) were stained with 4% uranyl acetate and lead citrate, and observed using JEOL 1010
391 transmission electron microscope operating at 75 kV.

392 *Molecular identification*

393 Nucleic acids were extracted from cultivated isolates using Microbial Genomic DNA kit
394 (Macherey-Nagel), according to manufacturer's recommendations. Primers ACA-5'
395 (CTGGTTGATCCTGCCAGTAGTC) and ACA-3' (TGATCCTTCCGCAGGTTTAC) were
396 used to amplify 18S rRNA gene with Phusion DNA polymerase (ThermoFisher) with the
397 following parameters: 98°C for 30 sec, followed by 35 cycles comprising: 98°C for 10 sec,

398 65°C for 30 sec, 72°C for 1 min, then a final elongation step of 5 min at 72°C. The amplicons
399 were sequenced by the Sanger method. The sequences have been deposited within GenBank
400 under the accession numbers MN969060 and MN969059 for isolates DD1 and DD2
401 respectively.

402 *Phylogenetic analysis*

403 A collection of full length or near full-length sequences belonging to Heterolobosea were
404 retrieved from the NCBI nucleotide database, along with Euglenozoa sequences for
405 constituting an outgroup. Sequences were aligned using MUSCLE (Edgar 2004). Alignment
406 was manually inspected, in order to eliminate regions corresponding to intronic sequences and
407 to trim sequences to begin and end on same positions. Sequences alignment was further cleaned
408 using BMGE, resulting in an alignment spanning 1671 nucleotides, comprising 46 sequences
409 (Criscuolo and Gribaldo 2010). Phylogeny inference was performed using IQ-Tree (Nguyen et
410 al. 2015). The best substitution model was defined using ModelFinder, based on the highest
411 Bayesian information criterion, which was found to correspond to the TIM2+R5 model. During
412 tree inference, branch support was assessed using bootstrapping (1000 iterations) and an SH-
413 like approximate likelihood ratio test. The resulting tree was rendered and further annotated
414 using iTOL (Letunic and Bork 2019).

415 *Quantitative PCR*

416 Quantitative PCR (qPCR) analysis was performed to detect *Pharyngomonas* sp. DD1 and
417 *Euplaesiobystra dzianiensis* DD2, using primer pairs PharF1 5'-
418 CGTATTACTGGGCGAGAGGTG-3', PharR1 5'-CGTTCCTGATTGACGGGAGAG-3' and
419 GargF1 5'-CAGCGATCAAAGCGTAAGGAAG-3', GargR1 5'-
420 CGTGCAGCCCAAGACATATTAG-3', respectively (primers were designed from 18S rRNA
421 genes using Clone manager). Sampling of water from the lake Dziani Dzaha was performed in
422 April and November 2015 and at various depths (Hugoni *et al.* 2018). DNA extraction was
423 performed as described previously (Hugoni *et al.* 2018). qPCR was performed using the
424 LightCycler 480 apparatus and FastStart DNA Master plus SYBR Green I according to
425 manufacturer instructions (Roche Applied Science). The reactions were performed under the
426 following conditions: an initial denaturation step of 95°C for 10 min, followed by 45 cycles:
427 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. To verify the specificity of the amplicon for
428 each primer pair, a melting curve analysis was performed ranging from 65°C to 95°C.

429 *Salinity and temperature tolerance*

430 Salinity and temperature tolerance of both strains were investigated by inoculating 50 µl of
431 actively growing stock cultures in ASW (trophozoites were dividing, no cysts were observable)
432 in 1 mL of various salinity media based on 300‰ artificial salinity medium (272 g NaCl, 7.6
433 g KCl, 17.8 g MgCl₂, 1.8 g MgSO₄·7H₂O, 1.3 g CaCl₂ l⁻¹ water (Park 2012)) in a 24-well
434 plate. Dilutions in ASW, 15‰, 40‰, 75‰, 100‰ and 150‰ were seeded with *E. coli* (~5.10⁸
435 cells). *E. coli* (~5.10⁸ cells) were added every 48 or 72h for ten days to limit encystment.
436 Growth was microscopically monitored every 24 or 48h at 25, 30, 37 or 43°C, by looking for
437 actively moving amoebae and increase of the populations density. After ten days, growth was
438 confirmed by transferring 50 µL of each culture into 1 ml of the same medium and subsequent
439 observations for ten days (adding *E. coli* (~5.10⁸ cells) every 48 or 72h. All tests were
440 performed twice.

441 *Arthrospira grazing tests*

442 Suspensions of *Arthrospira fusiformis* were kindly provided by Cécile Bernard. Bacteria were
443 diluted 1/10th in 1 mL of ASW (in 24-wells plate) and inoculated 1/100th with an actively
444 growing culture of amoebae. Mixtures were grown at 30°C and observed under an Olympus
445 IX51 microscope equipped with an Olympus DP26 camera.

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450 currently a protected water body with free public access but restricted activities, under the
451 control of the French agency for littoral ecosystems conservation ([http://www.conservatoiredu-](http://www.conservatoiredu-littoral.fr/)
452 [littoral.fr/](http://www.conservatoiredu-littoral.fr/)).

453 **Authors contribution**

454 Willy Aucher: Conceptualization, Methodology, Validation, Investigation, Visualization,
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457 analysis, Visualization, Writing

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462 Marc Bouvy: Resources, Writing
463 Yann Héchard: Conceptualization, Supervision, Writing

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579 **Figure legends**

580 **Figure 1:** Differential interference contrast micrographs of *Pharyngomonas* sp. DD1 grown in
581 Artificial Sea Water (ASW) liquid medium. The trophozoite stage can show a variety of shapes
582 (A-D) while the cyst stage consistently shows round-shaped cells with a multilayered cell wall
583 (E-F), surrounded by spherical, “bead-like” structures. Scale bar represents 10 μm for all
584 panels.

585 **Figure 2:** Transmission electron micrographs of *Pharyngomonas* sp. DD1. Trophozoite cells
586 harbor numerous vacuoles, possibly arising from phagocytosis (A, B, C (detail from B), D).
587 Cysts ultrastructure (E-H) confirms the presence of a spacious, thin multilayered wall, in which
588 are embedded “bead-like” structures that appear to be host-derived multi-layered bodies
589 (details F and H). Scale bar is 2 μm for all panels.

590 **Figure 3:** Differential interference contrast micrographs of *Euplaesiobystra dzianiensis* DD2
591 grown in ASW. *Euplaesiobystra dzianiensis* trophozoites are monopodial (A) and tend to
592 branch when changing movement direction (B, C), showing rapid eruptive deployment of large
593 pseudopodia. Cysts show an irregular, round-shaped morphology (D-F) with a double wall
594 clearly observable (E). Scale bar represents 10 μm for all panels

595 **Figure 4:** Transmission electron micrographs of *Euplaesiobystra dzianiensis* DD2.
596 Trophozoites cells display a single nucleus with a large nucleolus, as well as numerous
597 vacuoles seemingly indicating various stages of phagocytosis (A). Round shaped mitochondria
598 with discoidal cristae (B; detail from A). Some intracellular vacuoles contain multi-layered
599 bodies, which are then expelled in the extracellular medium (C). Cysts morphology of *E.*
600 *dzianiensis* is variable, ranging from round to crescent shapes (D-F), always doubled-layered
601 with a well-defined ectocyst. Prominent ostioles (D-G) can be easily seen on cyst cell walls.
602 Scale bar is 2 μm for all panels.

603 **Figure 5:** Phylogenetic tree of the Heterolobosea group, reconstructed using maximum
604 likelihood (TIM2+R5 model). The FLA isolates newly described in the present study are
605 indicated in bold. Branch supports, assessed using bootstrap (1000 iterations; left value) and
606 an SH-like approximate likelihood ratio test (SH-ALRT; right value), are indicated at nodes
607 with branches length > 0.05 . Euglenozoa sequences were used as an outgroup. Scale bar shows
608 the substitution rate per site. Environmental origin for sequences is indicated by coloured
609 circles, when available.

610 **Figure 6:** Molecular quantification of FLA isolates in the lake Dziani Dzaha, using specific
611 quantitative PCR assays. Absolute DNA concentrations in samples were determined at depths
612 ranging from 0.25 m to 17 m, in either April (rainy season) or November (dry season) 2015.
613 The black thick line represents the lower limit of quantification.

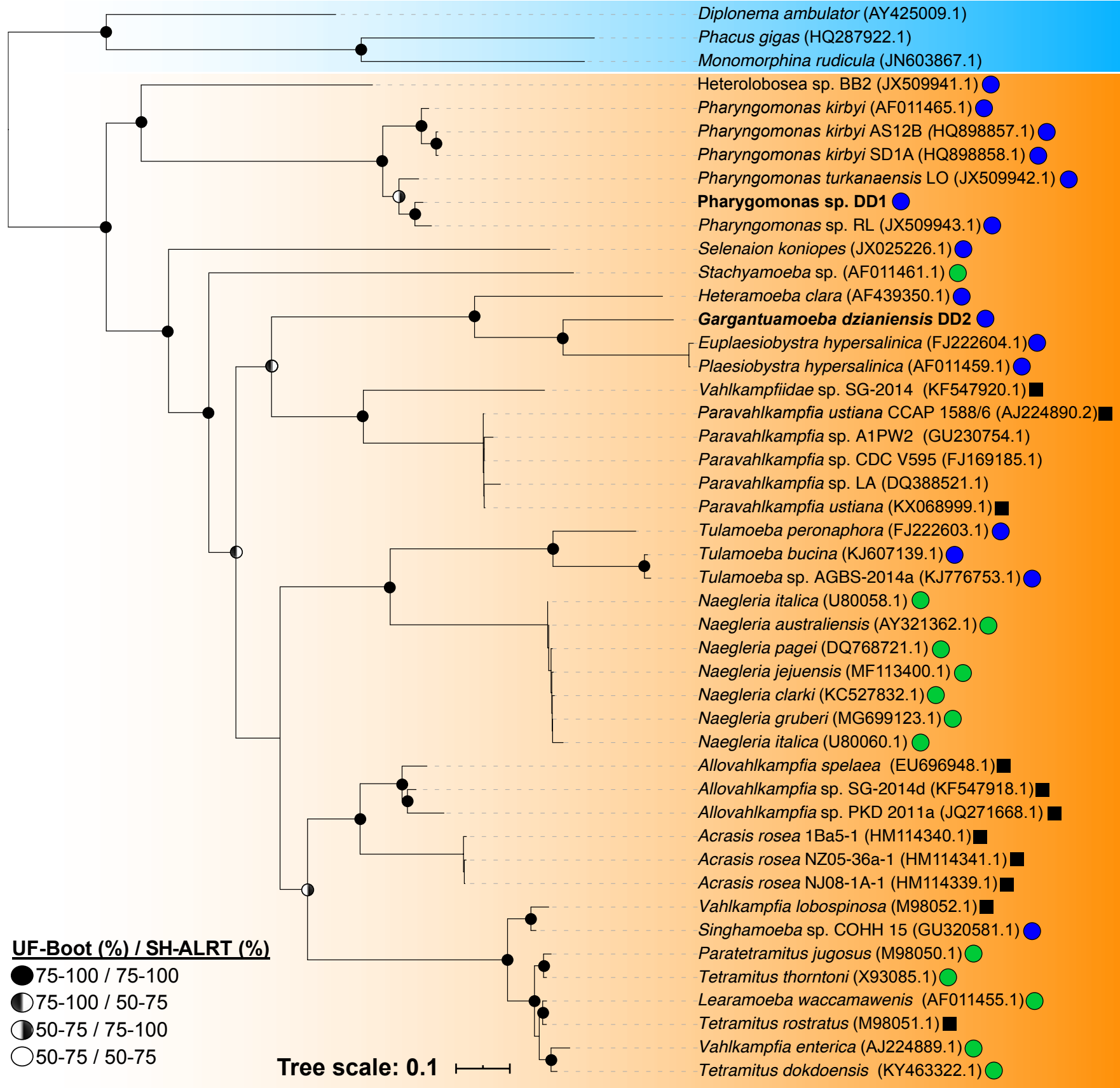
614 **Table 1:** Qualitatively estimated growth response to salinities and temperatures of
615 *Pharyngomonas* sp. DD1 and *Euplaesiobystra dzianiensis* DD2. Table note: + = moderate
616 growth, ++ = growth to high densities, - = no growth

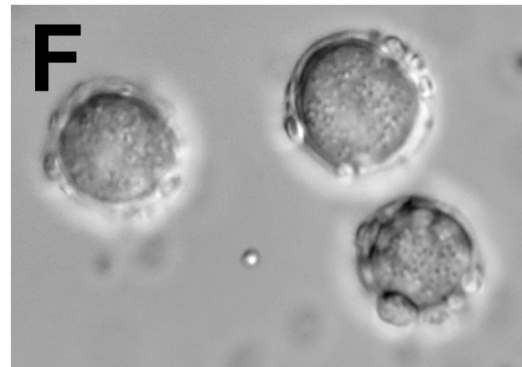
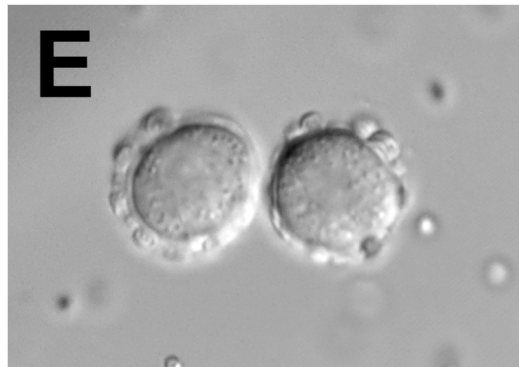
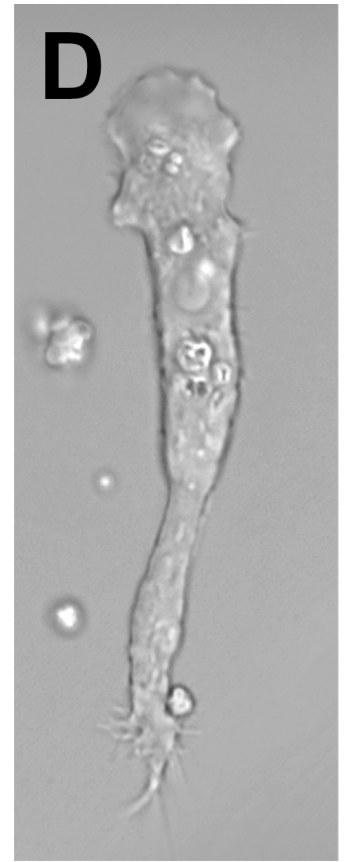
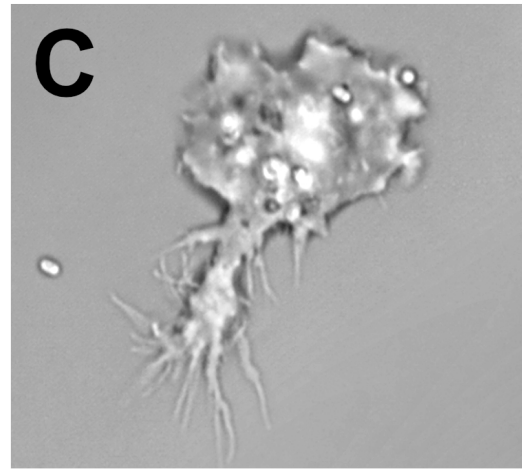
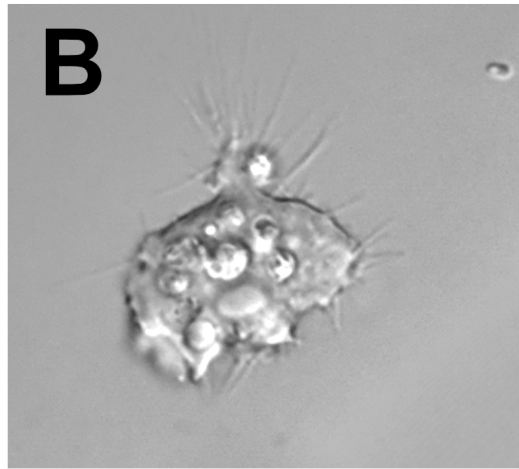
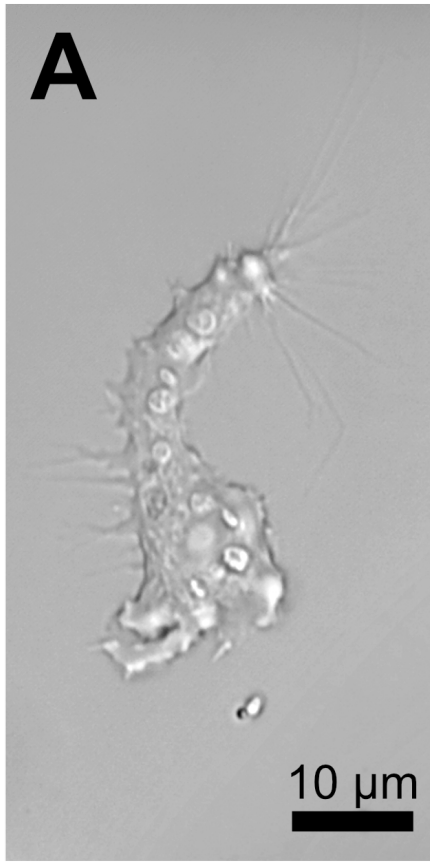
617 **Figure 7:** Light micrographs of *Euplaesiobystra dzianiensis* DD2 grazing on *Arthrospira*
618 *fusiformis*. Whole cyanobacterial filaments (A) or vacuoles filled with cyanobacterial-derived
619 contents (B) were observed in trophozoite cells of *E. dzianiensis*. Scale bar is 20 µm for both
620 panels.

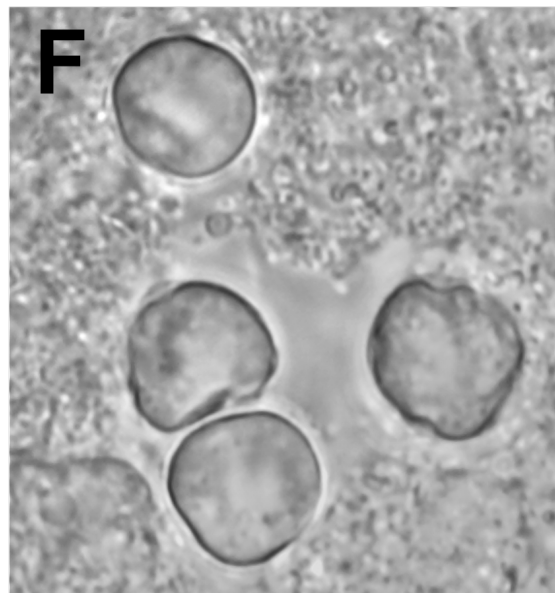
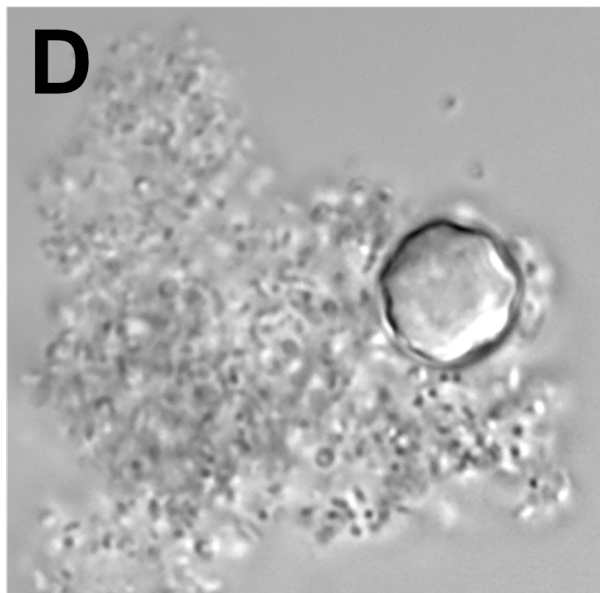
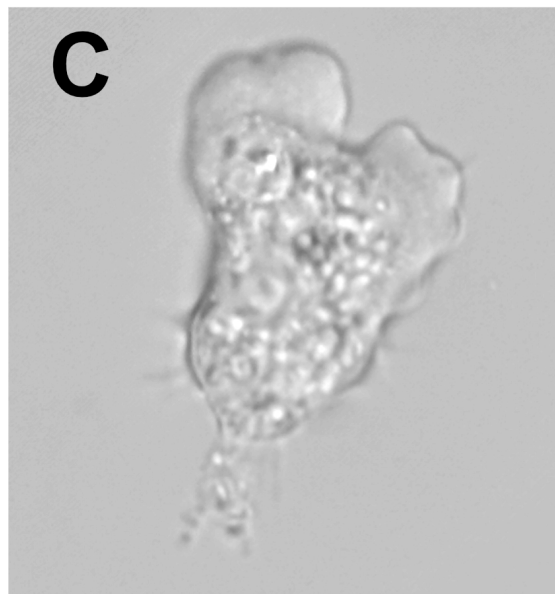
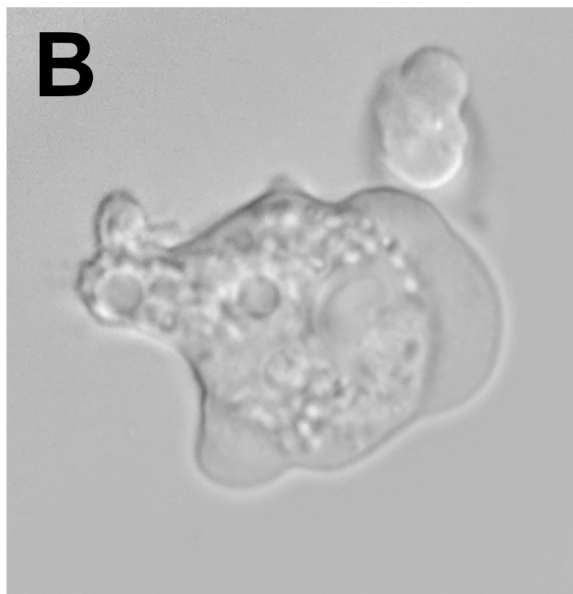
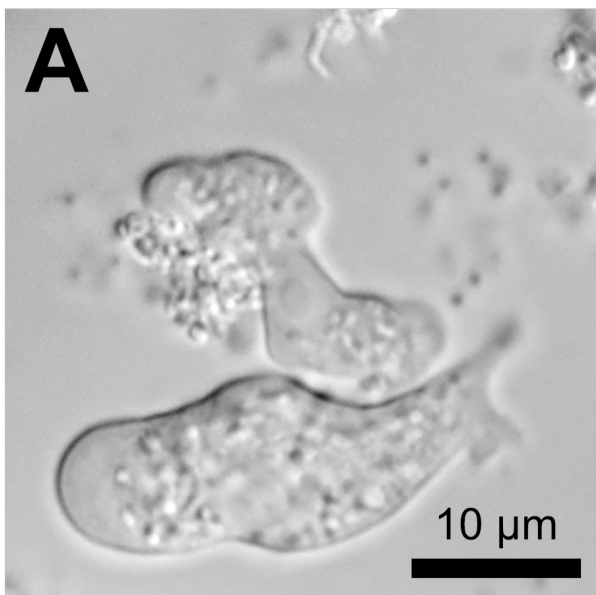
621 **Figure 8:** Light micrographs of *Pharyngomonas* sp. DD1 grazing on *Arthrospira fusiformis*.
622 Numerous trophozoite cells were observed in the vicinity of cyanobacterial filaments. Large
623 (A) and smaller (B) vacuoles filled cyanobacterial-derived contents could be observed in the
624 majority of *Pharyngomonas* sp. DD1 trophozoites. Scale bar is 20 µm for both panels.

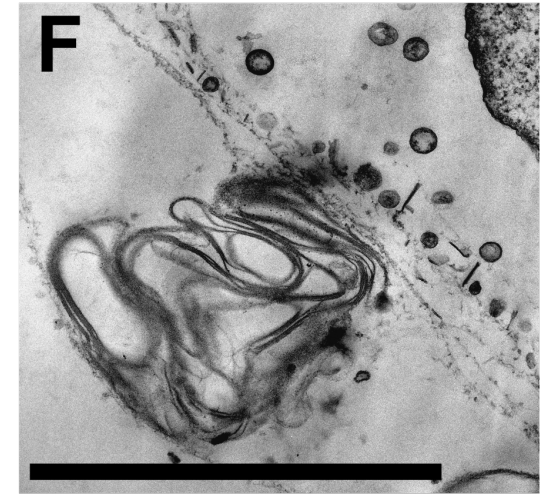
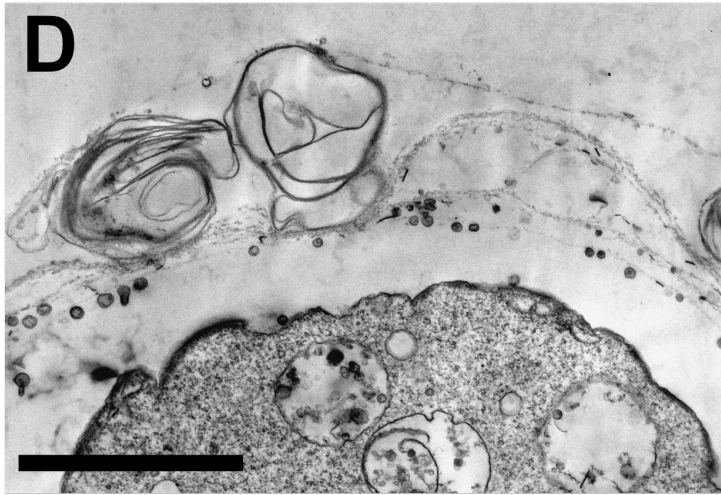
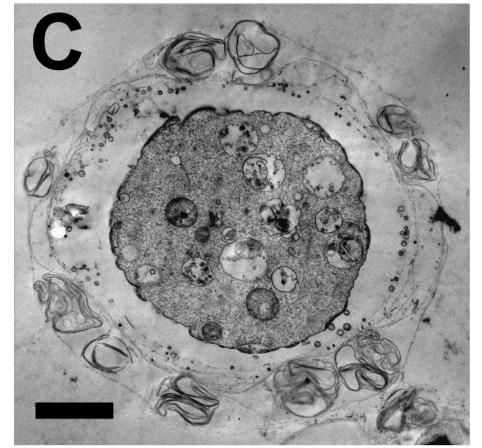
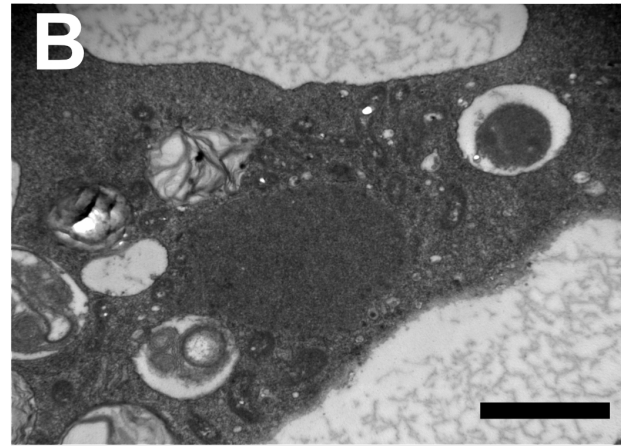
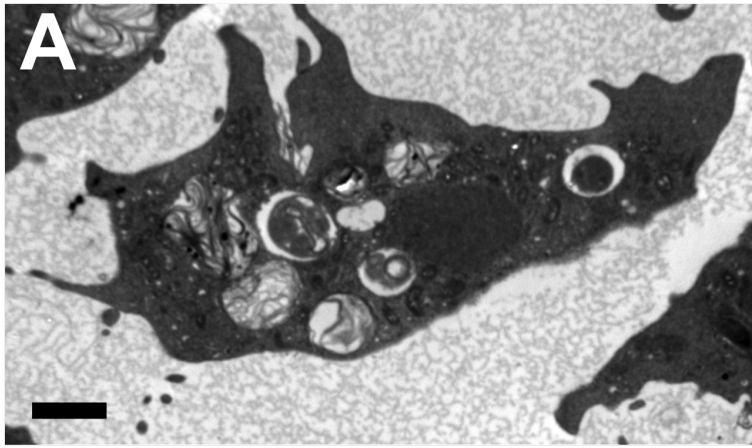
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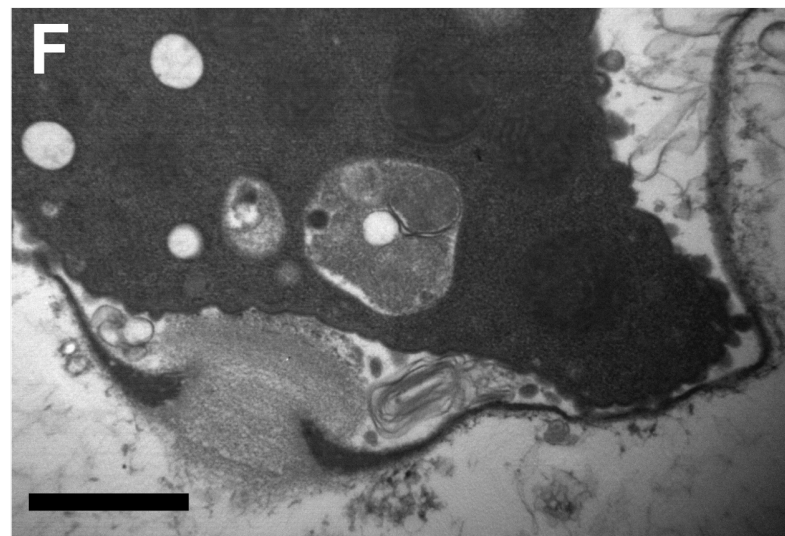
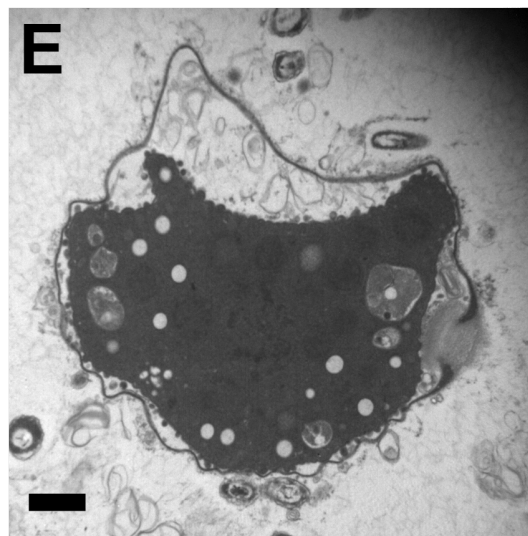
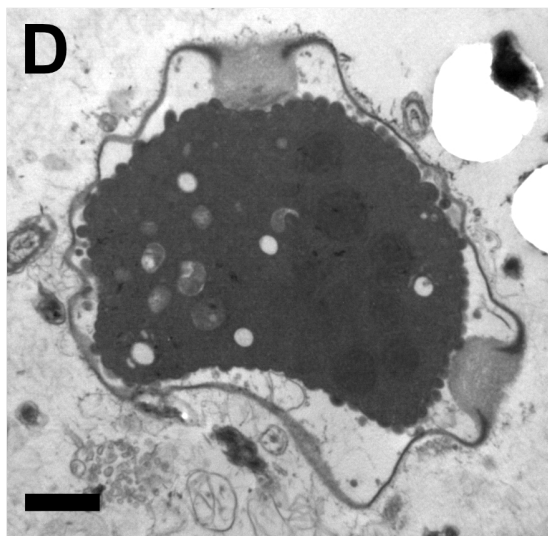
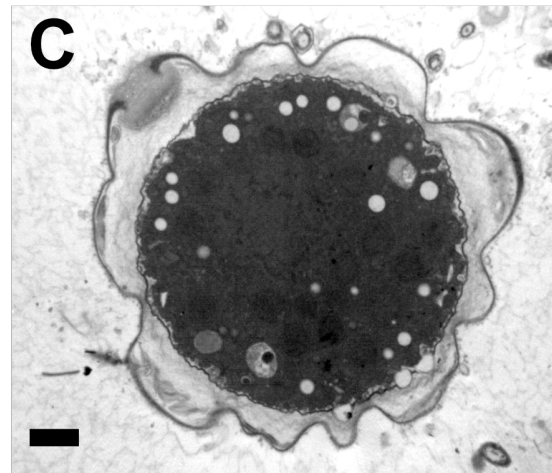
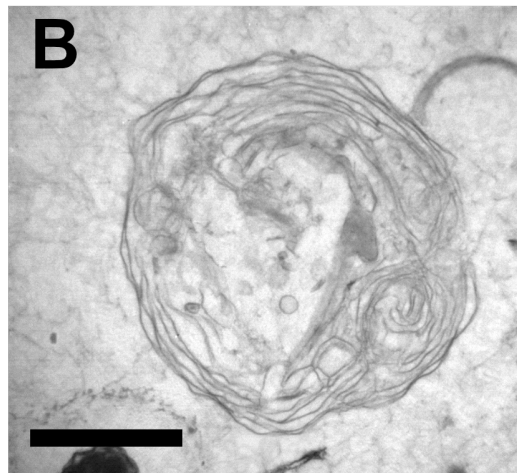
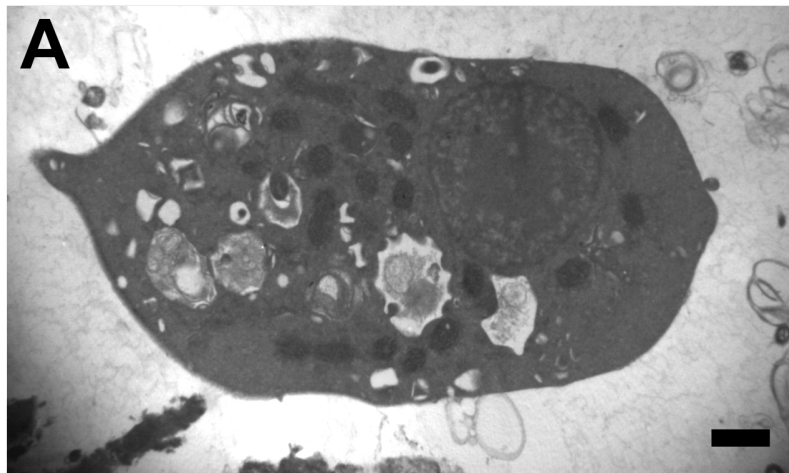
Euglenozoa





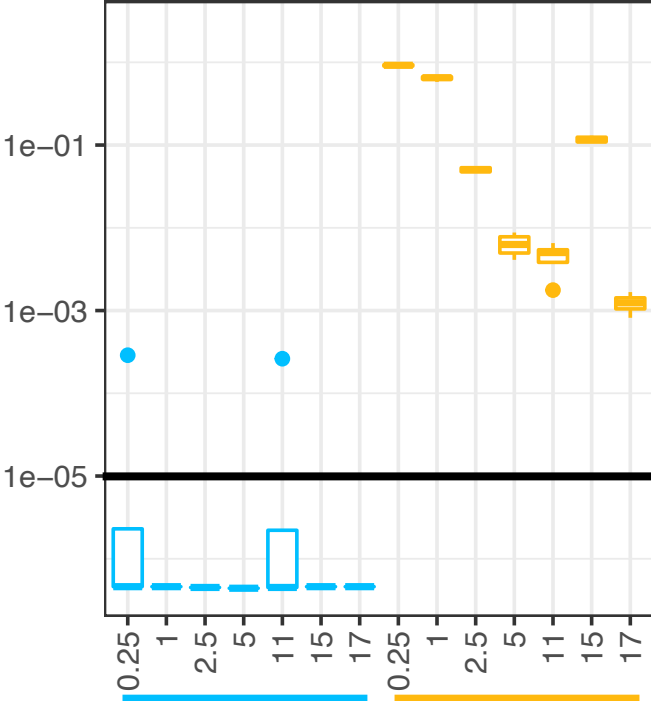




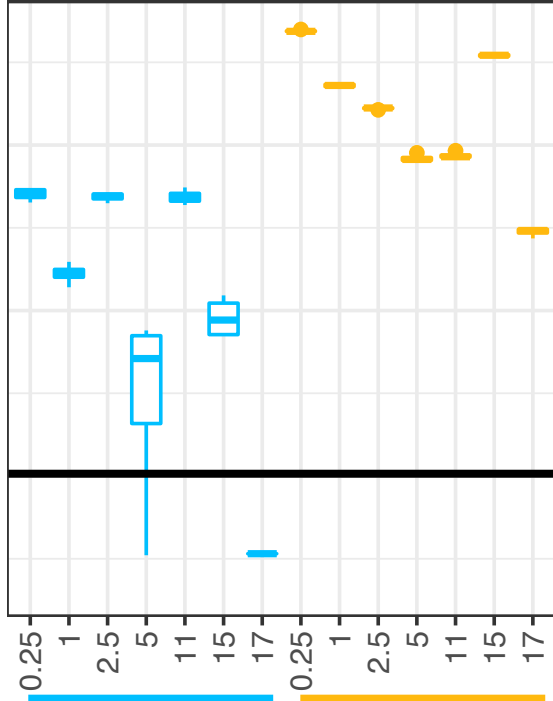


DNA Concentration (ng/mL)

Gargantuamoeba dzianiensis DD2



Pharyngomonas sp. DD1



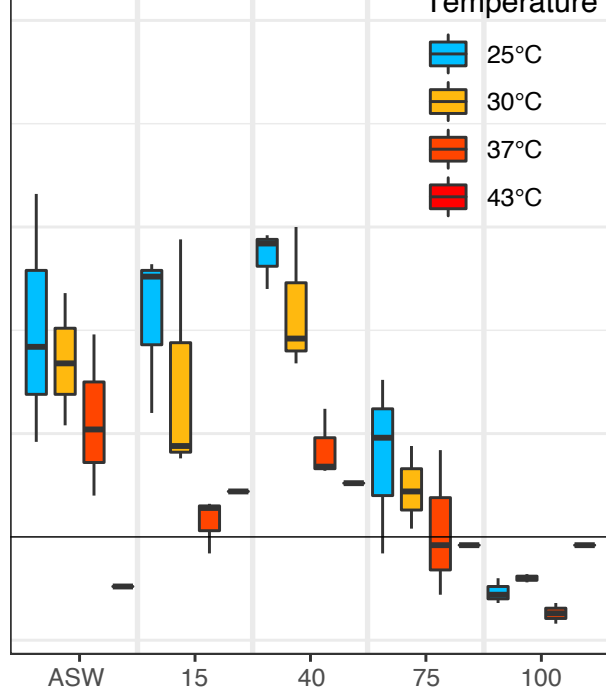
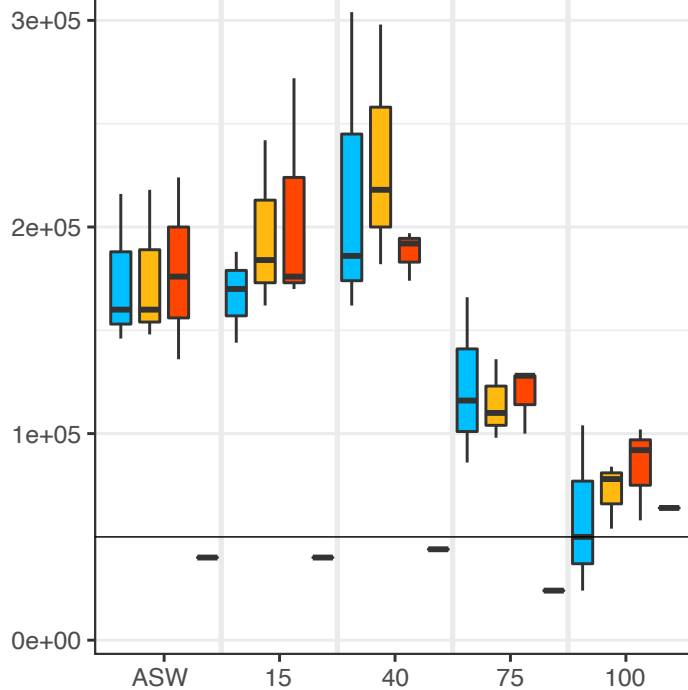
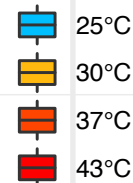
Depth (meters)

Concentration (cells/mL)

Pharyngomonas sp. DD1

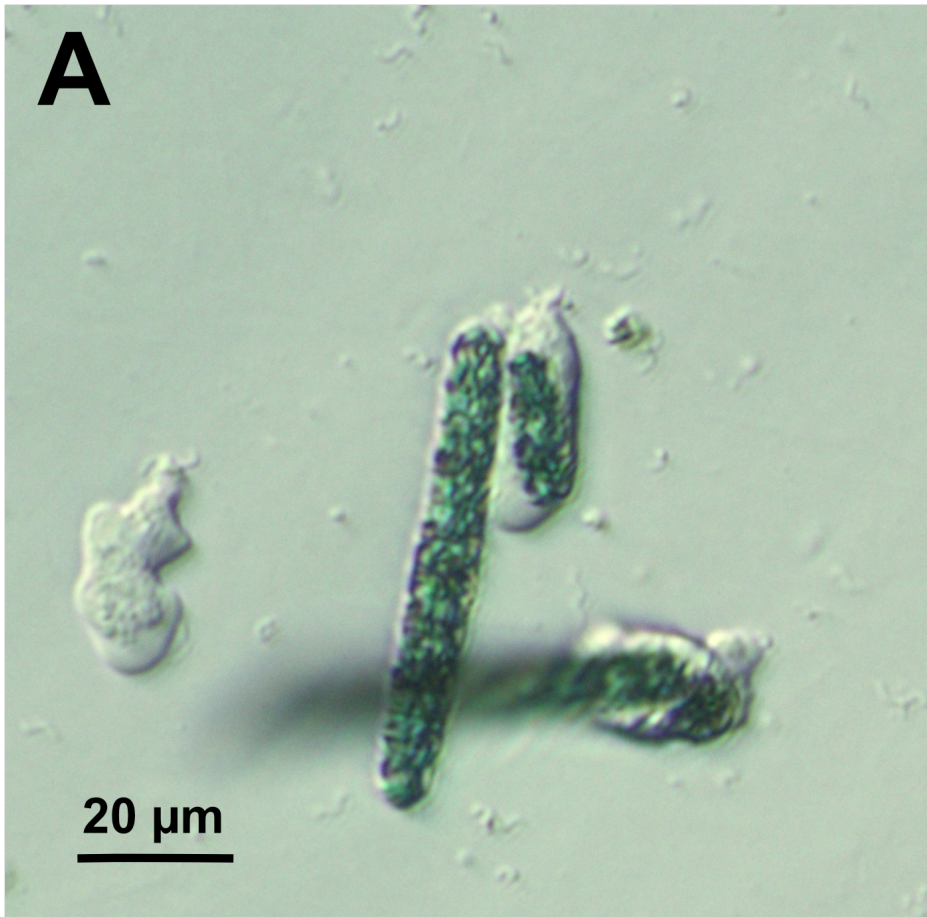
Gargantuamoeba dzianiensis DD2

Temperature



Salinity (‰)

A



B

