Morphology and Ecology of Two New Amoebae, Isolated From a Thalassohaline Lake, Dziani Dzaha

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Abstract

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

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

Introduction

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

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

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

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

Results

Pharyngomonas sp. DD1

Morphology and Ultrastructure

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

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

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

Euplaesiobystra dzianiensis DD2

Morphology and Ultrastructure

Euplaesiobystra dzianiensis trophozoites (22 ± 4.2 µm long (between 16.7 and 34 µm), 13 ± 3.4 µm wide (between 8 and 20.6 µm) displayed a canonical heterolobosean morphology, being limax-shaped, monopodial and forming eruptive pseudopodia (Fig. 3A). Sometimes, a bulbous uroid with fine filaments (Fig. 3B and 3C) can be observed when trophozoites are in motion.

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

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

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

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

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

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

Ecology

Amoeba isolates distribution in the lake

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

Salt and temperature tolerance

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

Taxonomic summary

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

Euplaesiobystra dzianiensis n. sp

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

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

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

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

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

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

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

Materials and methods

Isolation and cultivation of amoebae

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

A water sample (100 ml) was filtered on a sterile Nucleopore polycarbonate filter (3 µm pore size; 47 mm diameter) using a vacuum pump. The filter was cut in two pieces and deposited onto saline non-nutrient agar (NaCl 60 g/L, Sodium Citrate tribasic 0.8 g/L, $MgSO_4$ 4 mM, Na_2HPO_4 2.5 mM, KH_2PO_4 2.5 mM, $CaCl_2$ 0.5 mM) seeded with 100 µL of an *Escherichia coli* suspension (*E. coli* were grown overnight in Lysogeny Broth - peptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L - , centrifuged for five minutes at 6000 g and resuspended in ASW to reach an optical density of about 50 when measured at 600 nm) (NNA-Eco plate). The plates were sealed with parafilm and incubated at 30°C until a migration front was observed. Fronts were excised from plates and used for re-isolation on an NNA-Eco plate or to inoculate flasks containing Artificial Sea Water (ASW: 26.29 g NaCl, 0.74 g KCl, 0.99 g $CaCl_2$, 6.09 g $MgCl_2 \bullet 6H_2O$, 3.94 g $MgSO_4 \bullet 7H_2O$ L^{-1}, pH 7.8) seeded with a population of *E. coli* (the previously described suspension was diluted to one hundredth in the amoebae growth medium so that the optical density measured at 600 nm was approximately 0.5). Isolated amoebae were routinely grown in ASW-*E. coli* at 30°C.

Cultivation of Arthrospira fusiformis

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

dark cycles in Z8 Mayotte medium (per 1 L: 20 g NaCl, 0.467 g $NaNO_3$, 59 mg $Ca(NO_3)_2$●$4H_2O$, 25 mg $MgSO_4$●$7H_2O$, 31 mg K_2HPO_4, 21 mg Na_2CO_3, 10 mL Fe-EDTA solution (A: dissolve 2.8 g $FeCl_3$●$6H_2O$ in 100 mL 0.1 N HCl, B: dissolve 3.9 g EDTA-Na_2 in 0.1 M NaOH, add 10 ml solution A and 9.5 ml solution B plus water to 1 L), 1 mL trace solution (for 1L: 0.31 g H_3BO_3, 0.223 g $MnSO_4 \cdot 4H_2O$, 0.89 mg V_2O_5, 3.3 mg Na_2WO_4●$2H_2O$, 8.8 mg $(NH_4)_6Mo_7O_{24}$●$4H_2O$, 12.1 mg KBr, 8.3 mg KI, 28.7 mg $ZnSO_4 \cdot 7H_2O$, 15.5 mg $Cd(NO_3)_2$●$4H_2O$, 14.6 mg $Co(NO_3)_2$●$6H_2O$, 12.5 mg $CuSO_4$●5H2O, 19.8 mg $(NH_4)_2Ni(SO_4)_2$●$6H_2O$, 4.1 mg $Cr(NO_3)_3$●$9H_2O$, 47.4 mg $Al_2(SO_4)_3K_2SO_4$●$24H_2O$), Cultures were not axenic but have been passed in aseptic conditions.

Microscopy

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

Molecular identification

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

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

Phylogenetic analysis

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

Quantitative PCR

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

Salinity and temperature tolerance

Salinity and temperature tolerance of both strains were investigated by inoculating 50 µl of actively growing stock cultures in ASW (trophozoites were dividing, no cysts were observable) in 1 mL of various salinity media based on 300‰ artificial salinity medium (272 g NaCl, 7.6 g KCl, 17.8 g MgCl2,1.8 g MgSO4·7H2O, 1.3 g CaCl2 l−1 water (Park 2012)) in a 24-well plate. Dilutions in ASW, 15‰, 40‰, 75‰, 100‰ and 150‰ were seeded with *E. coli* (~5.10^8 cells). *E. coli* (~5.10^8 cells) were added every 48 or 72h for ten days to limit encystment. Growth was microscopically monitored every 24 or 48h at 25, 30, 37 or 43°C, by looking for actively moving amoebae and increase of the populations density. After ten days, growth was confirmed by transferring 50 µL of each culture into 1 ml of the same medium and subsequent observations for ten days (adding *E. coli* (~5.10^8 cells) every 48 or 72h. All tests were performed twice.

Arthrospira grazing tests

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

Acknowledgments

The authors thanks Cécile Bernard and Charlotte Duval for providing the cyanobacteria culture for the grazing study. The field permit was granted by the Conservatoire du Littoral et des Rivages Lacustres, Antenne Océan Indien, due to the fact that the lake Dziani Dzaha is currently a protected water body with free public access but restricted activities, under the control of the French agency for littoral ecosystems conservation (http://www.conservatoiredu-littoral.fr/).

Authors contribution

Willy Aucher: Conceptualization, Methodology, Validation, Investigation, Visualization, Writing

Vincent Delafont: Conceptualization, Investigation, Data Curation, Formal analysis,Visualization, Writing

Elodie Ponlaitiac: Investigation

Aurélien Alafaci: Investigation

Hélène Agogué: Resources

Christophe Leboulanger: Resources

Marc Bouvy: Resources, Writing

Yann Héchard: Conceptualization, Supervision, Writing

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Figure legends

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

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

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

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

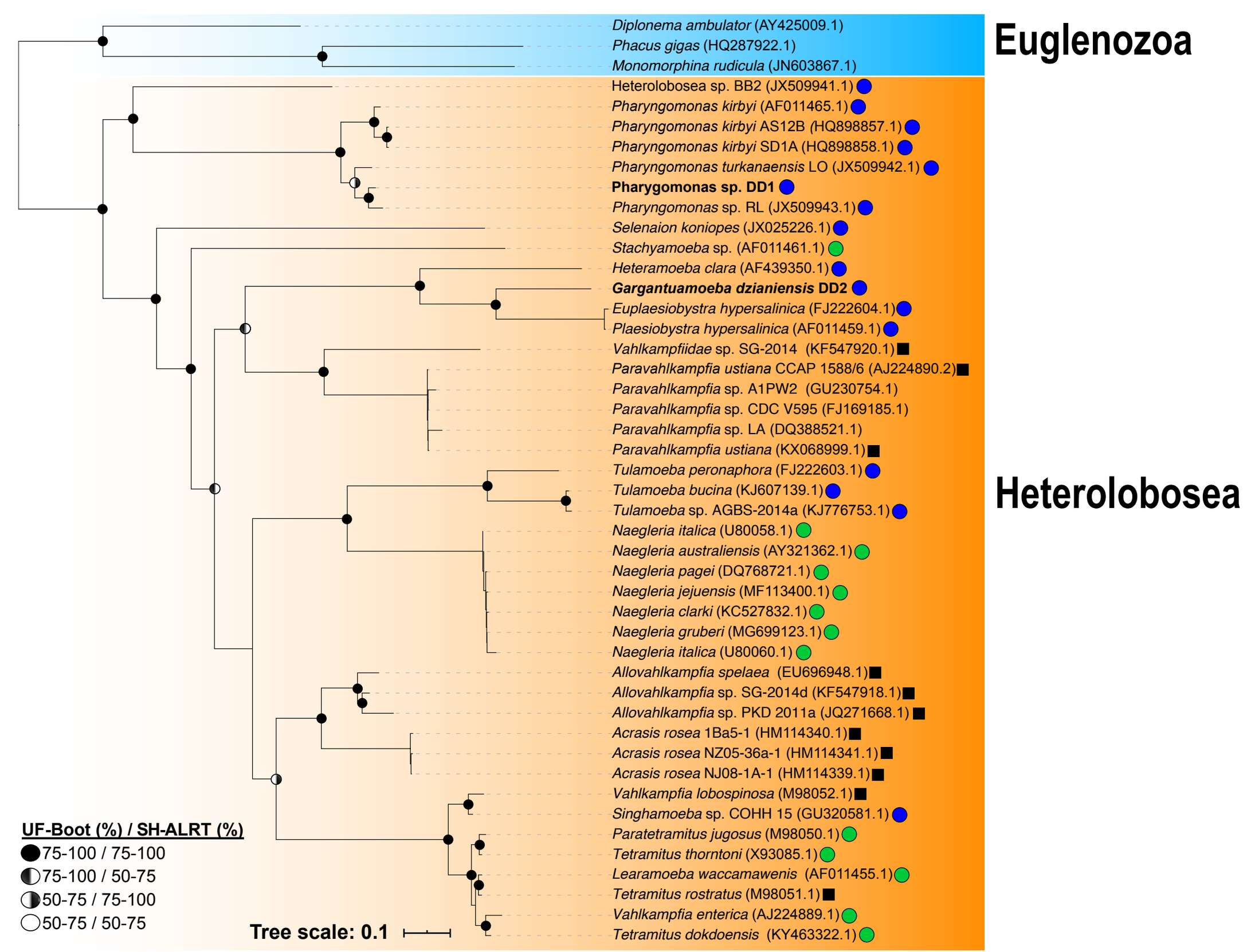
Figure 5: Phylogenetic tree of the Heterolobosea group, reconstructed using maximum likelihood (TIM2+R5 model). The FLA isolates newly described in the present study are indicated in bold. Branch supports, assessed using bootstrap (1000 iterations; left value) and an SH-like approximate likelihood ratio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

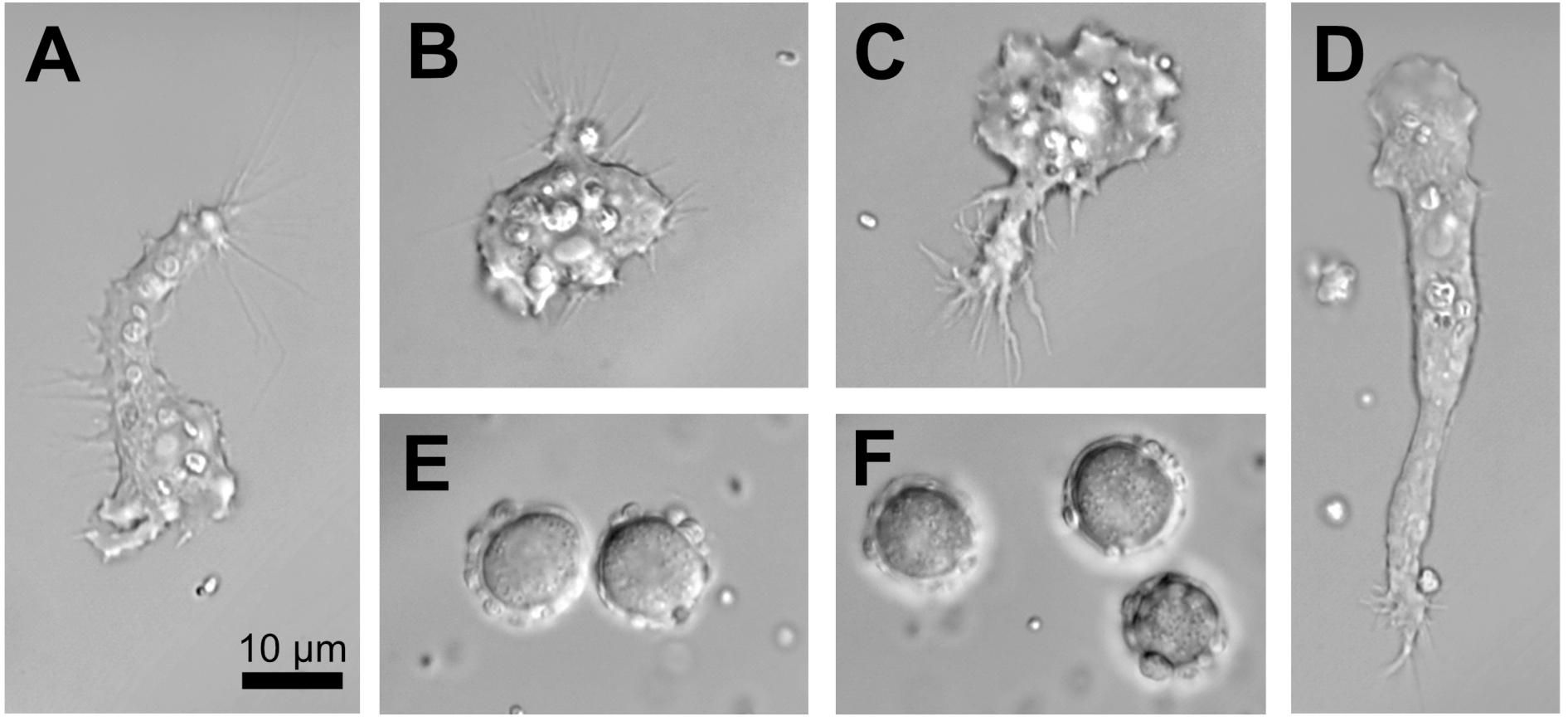
Figure 6: Molecular quantification of FLA isolates in the lake Dziani Dzaha, using specific quantitative PCR assays. Absolute DNA concentrations in samples were determined at depths ranging from 0.25 m to 17 m, in either April (rainy season) or November (dry season) 2015. The black thick line represents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

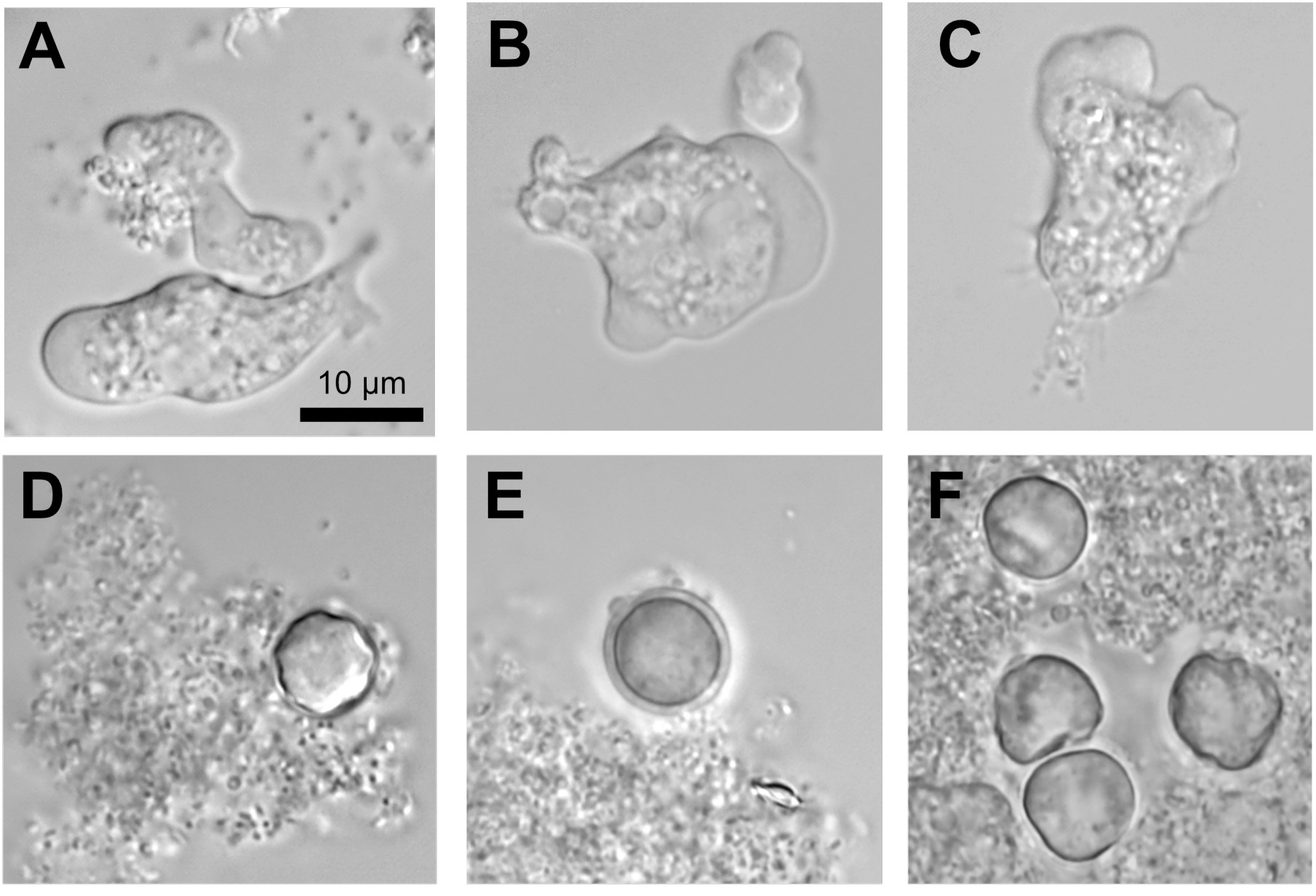
Table 1: Qualitatively estimated growth response to salinities and temperatures of *Pharyngomonas* sp. DD1 and *Euplaseiobystra dzianiensis* DD2. Table note: + = moderate growth, ++ = growth to high densities, - = no growth

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

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


Euglenozoa
Heterolobosea
Diplonema ambulator (AY425009.1)
Phacus gigas (HQ287922.1)
Monomorphina rudicula (JN603867.1)
Heterolobosea sp. BB2 (JX509941.1)
Pharyngomonas kirbyi (AF011465.1)
Pharyngomonas kirbyi AS12B (HQ898857.1)
Pharyngomonas kirbyi SD1A (HQ898858.1)
Pharyngomonas turkanaensis LO (JX509942.1)
Pharygomonas sp. DD1
Pharyngomonas sp. RL (JX509943.1)
Selenaion koniopes (JX025226.1)
Stachyamoeba sp. (AF011461.1)
Heteramoeba clara (AF439350.1)
Gargantuamoeba dzianiensis DD2
Euplaesiobystra hypersalinica (FJ222604.1)
Plaesiobystra hypersalinica (AF011459.1)
Vahlkampfiidae sp. SG-2014 (KF547920.1)
Paravahlkampfia ustiana CCAP 1588/6 (AJ224890.2)
Paravahlkampfia sp. A1PW2 (GU230754.1)
Paravahlkampfia sp. CDC V595 (FJ169185.1)
Paravahlkampfia sp. LA (DQ388521.1)
Paravahlkampfia ustiana (KX068999.1)
Tulamoeba peronaphora (FJ222603.1)
Tulamoeba bucina (KJ607139.1)
Tulamoeba sp. AGBS-2014a (KJ776753.1)
Naegleria italica (U80058.1)
Naegleria australiensis (AY321362.1)
Naegleria pagei (DQ768721.1)
Naegleria jejuensis (MF113400.1)
Naegleria clarki (KC527832.1)
Naegleria gruberi (MG699123.1)
Naegleria italica (U80060.1)
Allovahlkampfia spelaea (EU696948.1)
Allovahlkampfia sp. SG-2014d (KF547918.1)
Allovahlkampfia sp. PKD 2011a (JQ271668.1)
Acrasis rosea 1Ba5-1 (HM114340.1)
Acrasis rosea NZ05-36a-1 (HM114341.1)
Acrasis rosea NJ08-1A-1 (HM114339.1)
Vahlkampfia lobospinosa (M98052.1)
Singhamoeba sp. COHH 15 (GU320581.1)
Paratetramitus jugosus (M98050.1)
Tetramitus thorntoni (X93085.1)
Learamoeba waccamawenis (AF011455.1)
Tetramitus rostratus (M98051.1)
Vahlkampfia enterica (AJ224889.1)
Tetramitus dokdoensis (KY463322.1)
UF-Boot (%) / SH-ALRT (%)
75-100 / 75-100
75-100 / 50-75
50-75 / 75-100
50-75 / 50-75
Tree scale: 0.1


A
B
C
D
E
F
10 µm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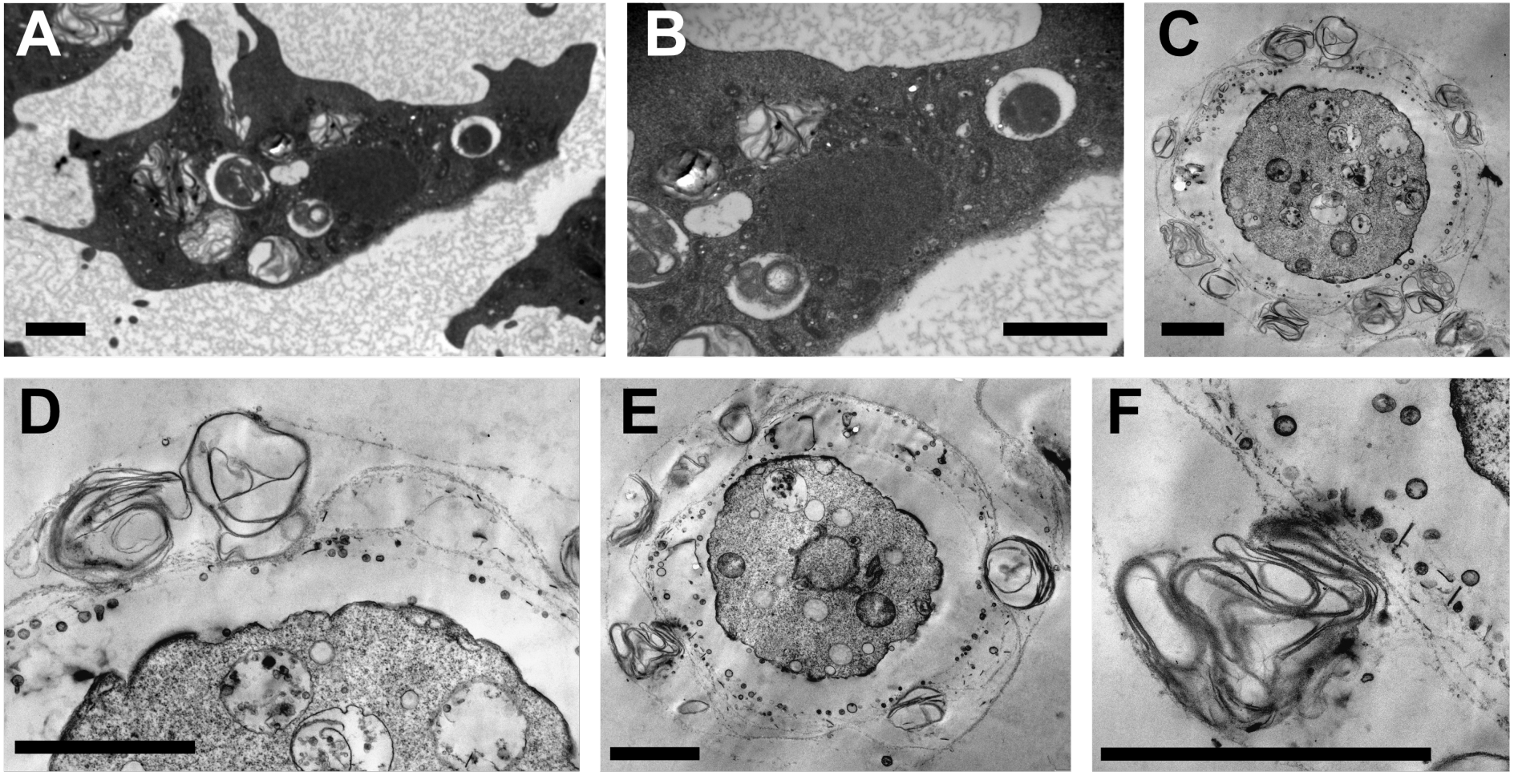
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B
C
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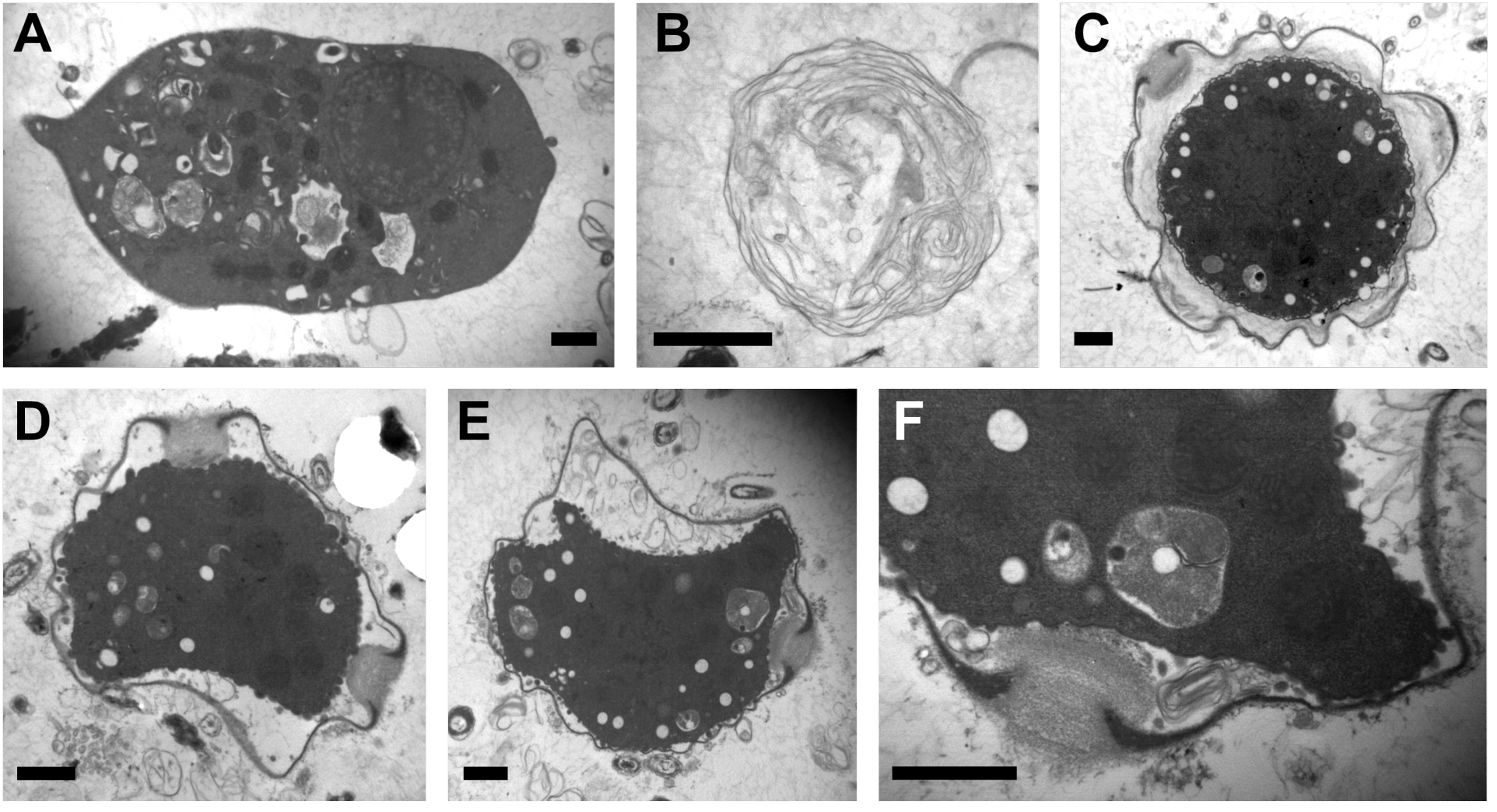
A
B
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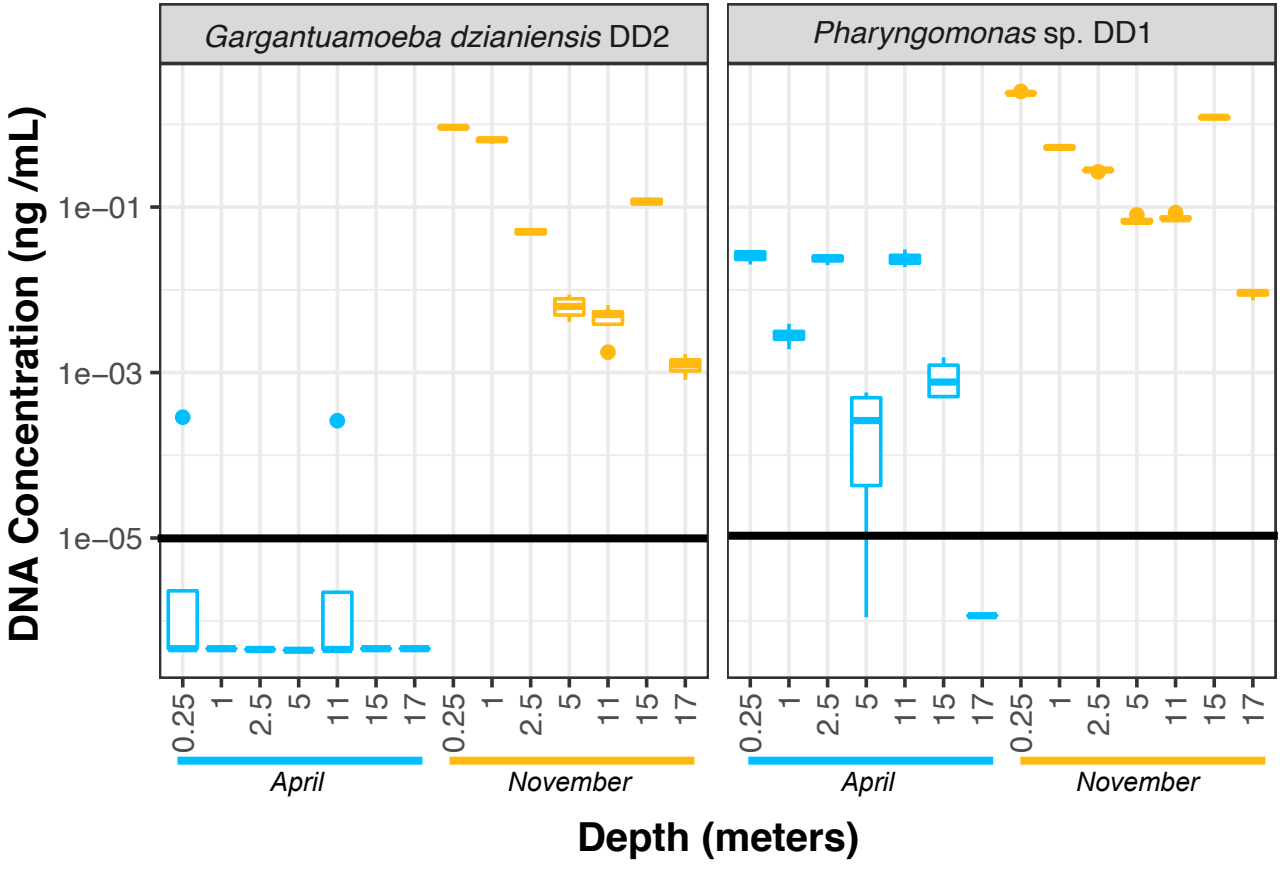
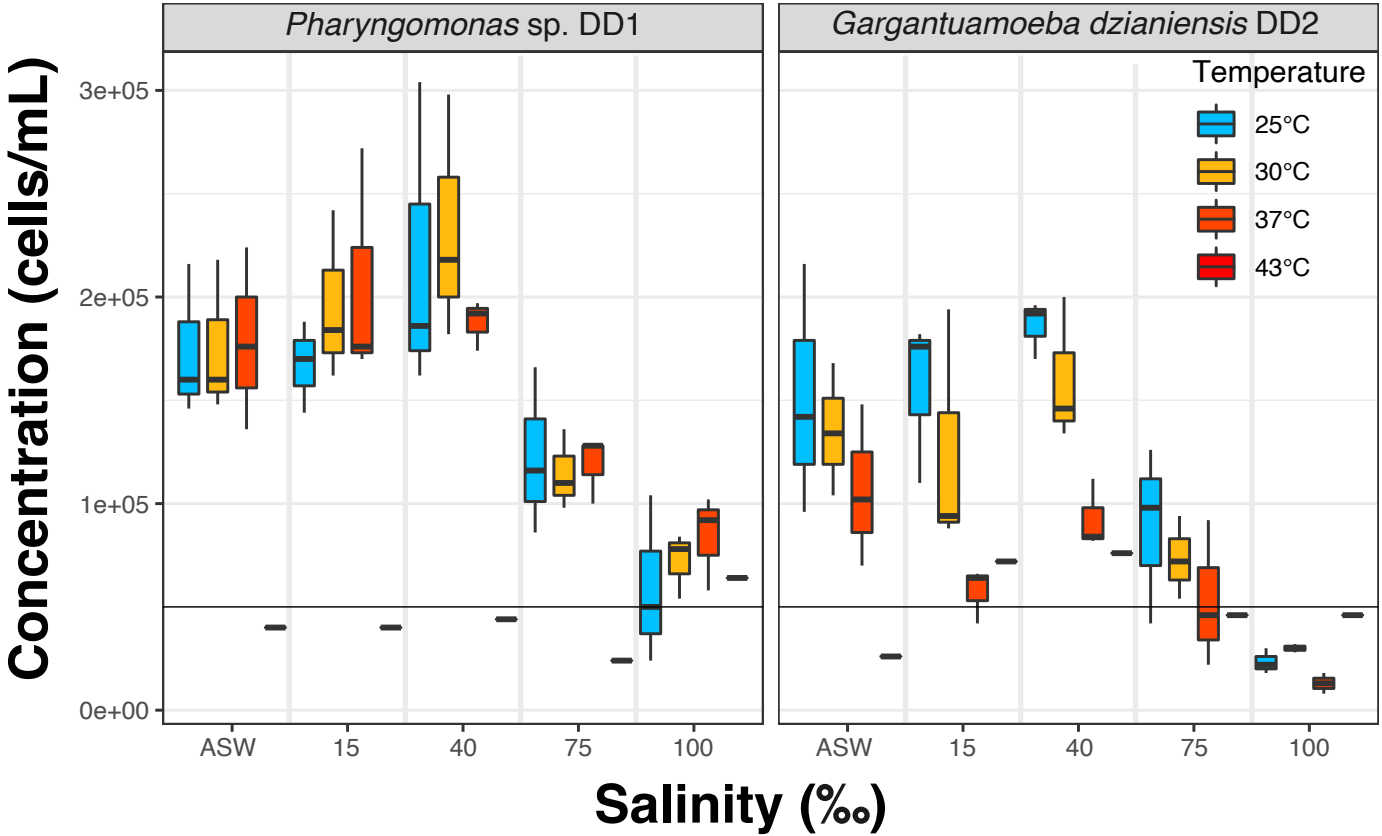
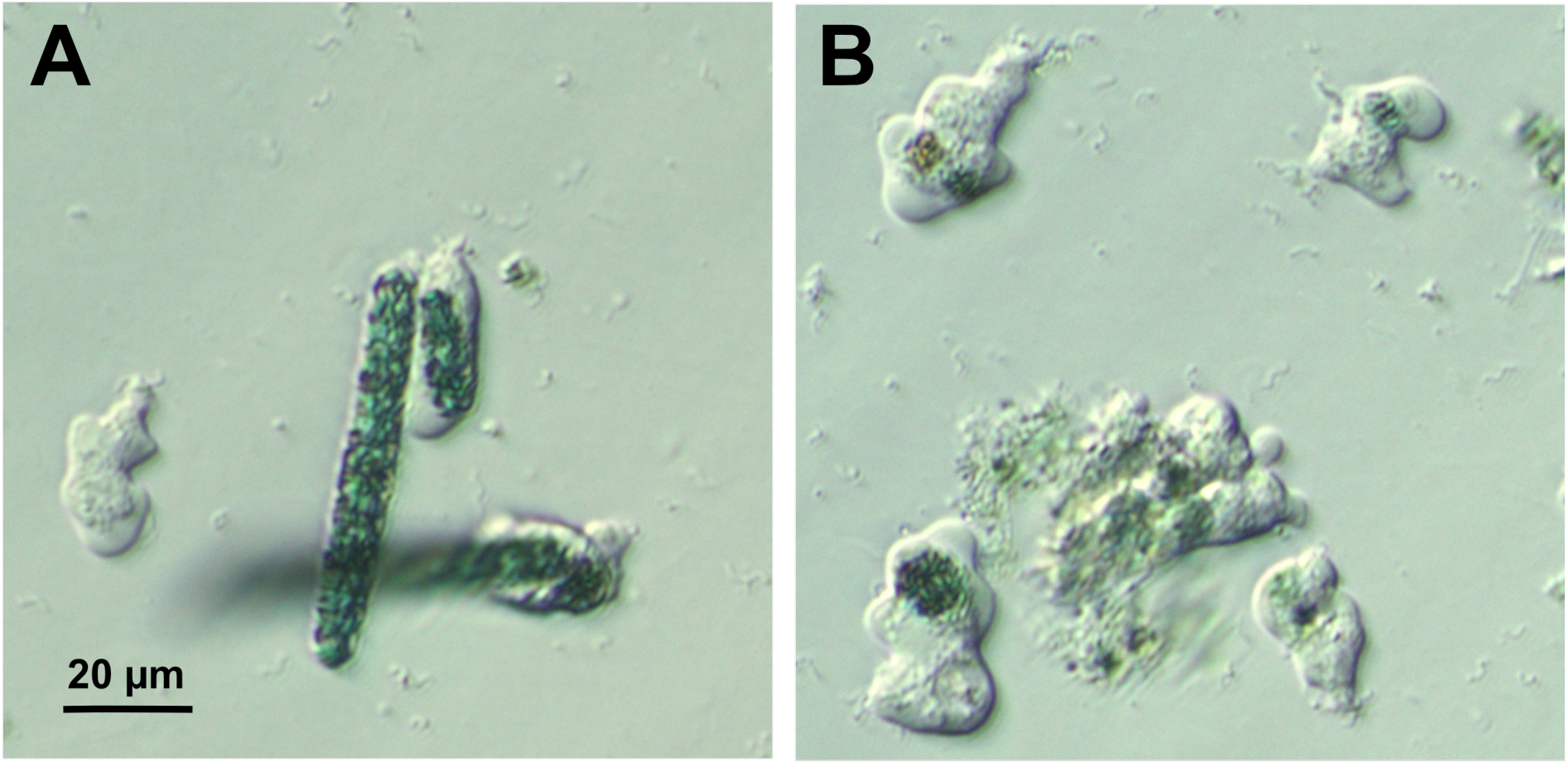
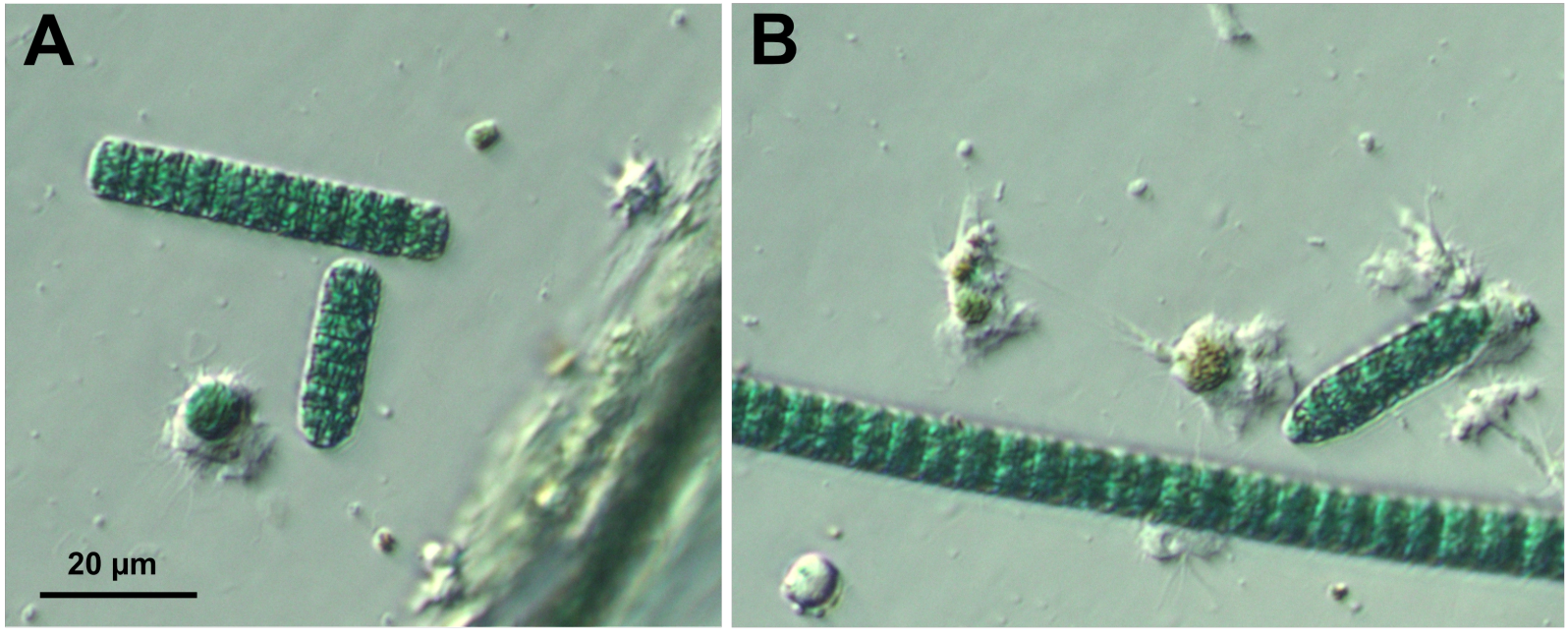

Gargantuamoeba dzianiensis DD2
Pharyngomonas sp. DD1
DNA Concentration (ng /mL)
1e−01
1e−03
1e−05
0.25
1
2.5
5
11
15
17
April
November
Depth (meters)


Pharyngomonas sp. DD1
Gargantuamoeba dzianiensis DD2
Temperature
25°C
30°C
37°C
43°C
Concentration (cells/mL)
3e+05
2e+05
1e+05
0e+00
ASW
15
40
75
100
Salinity (‰)


A
20 µm
B


A
20 µm
B