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Three ways to eat spaghettis: amoebae from two Amoebozoa lineages are able to feed on cyanobacteria of the genus *Oscillatoria*

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Summary

Cyanobacteria are widely distributed in all habitats. It makes them an attractive food source for many organisms, including Amoebozoa. We tested 23 strains of naked lobose amoebae belonging to the classes Tubulinea and Discosea for the ability to feed on the filaments of a widely distributed cyanobacterium *Oscillatoria* sp. Three strains were found to be able to feed on this organism. We documented and described the processes of feeding and discussed different ways that amoebae used to ingest cyanobacterial filaments. The present study experimentally shows that average-sized species of Amoebozoa are able to consume and digest filamentous cyanobacteria and disrupt cyanobacterial biofilm, preventing its formation and adherence to the substrate.

Key words: Amoebozoa, Cyanobacteria, feeding, morphology, evolution

Introduction

Amoeboid protists belonging to the supergroup Amoebozoa are heterotrophic organisms that feed on a wide range of prokaryotic and eukaryotic food items. Among their food objects are bacteria, fungi, microalgae, other protists and even smallest metazoans (Gibbs and Dellinger, 1908; Prescott and James, 1955; Page, 1972, 1988; Arndt, 1993; Gilbert et al., 2000; Geisen et al., 2016). There is data on the ability of amoebae to consume other representatives of Amoebozoa (e.g., Lapage, 1922; Mesentsev et al., 2022). Some amoebae species eat diatoms and other algae (e.g., Penard, 1902; Page and Baldock, 1980).

Cyanobacteria are widely distributed in all habitats. Biomass of these autotrophic bacteria can reach up to 24.5 g/m^{-2} of dry weight in soils (Garcia-Pichel et al., 2003) and on average they constitute over 90% of the biomass in freshwater ecosystems (Heathcote et al., 2016). It makes them an attractive food source for many organisms, including Amoebozoa. Early observations showing the consumption of "blue-green algae" by amoebae *Thecamoeba verrucosa* and *Amoeba proteus* were provided by Leidy (1879), Rhumbler (1898) and Doflein (1911). Further, it was found that those species are consumers of *Oscillatoria* sp. (Picken, 1937). Filaments of these cyanobacteria can also be

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eaten by *Thecamoeba sphaeronucleolus* (Haberey, 1973a, 1973b) and *Acanthamoeba* spp. (Lazcano et al., 1977; Wright et al., 1981); the latter can also feed on *Anacystis nidulans* and *Nostoc* sp. (Ho and Alexander, 1974). Detailed overview of amoeboid consumers of cyanobacteria was given by Dryden and Wright (1987). Laybourn-Parry et al. (1987) demonstrated that *Mayorella* sp. was also feeding on *Anabaena* sp. and *Nostoc* sp. Bamforth (2004) noted that the species, isolated from the desert soils of the Grand Canyon in northern Arizona, and identified as *Vannella simplex*, occurred only in microbial crusts containing cyanobacteria. In the same study, he showed rather abundant amoebae fauna in cyanobacterial crusts; however, no specific observations on food spectra of these species were made (op. cit., Table 3). The species *Vannella ebro*, isolated from microbial mats in Ebro Delta (Spain), is able to consume filamentous cyanobacteria (Smirnov, 2001). Nishibe et al. (2004) showed that a testate amoeba *Penardochlamys* sp. successfully consumed cells of bloom-forming *Microcystis* sp., which also was a food source for at least 8 naked amoebae species, among them – *Vannella* and *Korotnevella* spp. (Van Wichelen et al., 2016). Meisterfeld and Badewitz (2006) showed that a species *Amphizonella violacea* could ingest unidentified filamentous cyanobacteria.

Despite numerous observations, few papers contain detailed documentation of the process of feeding (e.g., Haberey 1973a, 1973b). In particular, this concerns the pattern of the ingestion of filamentous cyanobacteria by Amoebozoa. These cyanobacteria constitute the majority of photosynthetic organisms in some types of microbial mats, so the ability to digest cyanobacterial filaments means the competence of an amoeba to use photosynthetic components of a microbial mat as a food source thus influencing its structure and integrity.

In our current study, we tested 23 strains of naked lobose amoebae belonging to the classes Tubulinea and Discosea for the ability to feed on the filaments of a widely distributed cyanobacterium *Oscillatoria* sp. Three strains were found to be able to feed on this organism. We documented and described the processes of feeding and discussed different ways that amoebae used to ingest cyanobacterial filaments.

Material and methods

Amoebae strains used in this study were isolated from various substrates and locations and identified to genus or species level using the appropriate sets of light- and electron-microscopic and molecular data. In total, we tested 23 strains of lobose amoebae belonging to two major phylogenetic lineages of Amoebozoa – Tubulinea and Discosea (Table 1). The food object was the cyanobacteria *Oscillatoria* sp. The culture of cyanobacteria was obtained from the collection of the core facility center "Culturing of microorganisms" of the St. Petersburg University Research Park. The reference number for the culture is *Oscillatoria* sp. strain Titova CALU-1415.

For the feeding experiment, ca. 20 amoebae cells were collected using tapered-tip Pasteur pipette, washed in PJ solution two or three times and placed into Petri dishes filled with Gromov's culture medium number 6 (Gromov and Titova, 1983). Half a milliliter of the suspension containing cyanobacterial filaments was added to each dish. Then the dishes were gently shaken to distribute the inoculated filaments and amoebae cells uniformly. Exact count of cyanobacteria was not performed, but the control and the experimental dish in a pair were always inoculated from the same portion of cyanobacterial suspension. Every experiment was performed in duplicate. The experimental dish contained amoebae and cyanobacteria and the control dish contained cyanobacteria only. Further, dishes were placed under the JBL ReptilJunge full spectrum lamp (70W) at a distance of about 1 meter, the lamp was on for 14 hours per day and off for 10 hours.

We observed dishes from day 3 to day 30 (if amoebae remained alive) and photographed the feeding cells and the process of feeding using the inverted microscope Leica DMI3000. Overall images of dishes with LS2 strain were taken using a cell phone Redmi Note 9 Pro (camera 64 Mp, 1/1, 72", f/1, 89). If a strain showed the ability to consume cyanobacteria, the experiment (inoculation and observations) was replicated 7 times under the stereotype conditions.

Results

Of the 23 strains studied, 18 were unable to feed on cyanobacteria (Table 1). Most of them died after a few days; however, some strains of the genera *Leptomyxa* and *Cochliopodium* formed cysts.

The ability to phagocytize cyanobacterial filaments was noted in five amoeba strains. Three of them, namely *Leptomyxa regia* strain LS2 (Fig. 1, A-D), *Endostelium* sp. strain A1.2 (Fig. 1, E-H) and

Table 1. List of strains tested for the ability to feed on *Oscillatoria* sp. Green color indicates strains that successfully fed on cyanobacteria for 20 day, yellow – for 5 days, pink –did not consume *Oscillatoria* sp.

Strains	Isolation source	Cultivation medium	Size (length, width; μm)
Thecamoeba sp. strain Ta51	River bottom sediment, Tiber River (Rome, Italy)	wMY agar	70-100, 50-70
Leptomyxa regia strain LS2	Pond bottom sediment, Izmailovo Park (Moscow, Russia)	0.025% WG on PJ medium	78-104, 16-26
Endostelium sp. strain A1	Pond bottom sediment, "Apothecaries' Garden" (Moscow, Russia)	wMY agar	92-193, 72-132
Thecamoeba cf. *aesculea* strain Val	Lake bottom sediment (Valaam Island, Russia)	wMY agar	72-122, 52-80
Thecamoeba vumurta strain 130	Pond bottom sediment (Izhevsk, Russia)	wMY agar	45-75, 30-60
Mayorella sp. strain O1	Pond bottom sediment, Sokolniki Park (Moscow, Russia)	0.025% WG on PJ medium	37-53, 17-22
Mayorella sp. strain S1	Pond bottom sediment, Izmailovo Park (Moscow, Russia)	0.025% WG on PJ medium	100-156, 29-56
Cochliopodium sp. strain S2	Pond bottom sediment, Izmailovo Park (Moscow, Russia)	0.025% WG on PJ medium	35-53, 44-69
Vannella simplex strain KRKA39733	River bottom sediment, Krka natural reserve (Lozovac, Croatia)	0.025% WG on PJ medium	32-59, 44-72
Thecamoeba sp. strain Ta15	Leaf litter, Komi Respublica (Kuratovo, Russia)	wMY agar	15-28, 13-19
Amoeba sp. strain AO	Moss, forest near Yashchera river (Leningrad region, Russia)	PJ medium with rice grain added	183-230, 26-36
Thecamoeba aesculea strain Ta4	Steppe felt, "Worskla Forest" natural reserve (Borisovka, Russia)	wMY agar	50-120, 60-80
Thecamoeba foliovenanda strain Ta72	Leaf litter, Mshinskaya station (Leningrad region, Russia)	wMY agar	34-90, 31-83
Thecamoeba quadrilineata strain Ta24	Leaf litter, Khanty-Mansi Autonomous Okrug (Listvennichniy, Russia)	wMY agar	26-65, 20-36
Thecamoeba sp. strain Ta91	Leaf litter, Astrakhan natural reserve (Astrakhan region, Russia)	wMY agar	60-90, 30-55
Vannella primoblina strain Van79	Dead plant remnants (Kislovodsk, Russia)	wMY agar	28-50, 32-49
Thecamoeba sp. strain KG	Moss (Pushkin, Russia)	wMY agar	75-120, 55-90
Leptomyxa sp. strain DV1	Soil, Pervomayskiy Park (Blagoveshchensk, Russia)	0.025% WG on PJ medium	106-361, 69-186
Leptomyxa sp. strain LD2	Soil, Primorskiy district (Saint-Petersburg, Russia)	0.025% WG on PJ medium	78-185, 143-276
Leptomyxa sp. strain LChMor2	Soil, Chukotka Autonomus Okrug (Meinypilgyno, Russia)	0.025% WG on PJ medium	141-810
Leptomyxa sp. strain LVal	Lake bottom sediment (Valaam Island, Russia)	0.025% WG on PJ medium	47-100, 21-75
Cochliopodium vestitum strain Kup	Lake bottom sediment (Keret Archipelago, Sredniy Island, Russia)	0.025% WG on PJ medium	48-106, 53-103

Cultivation media: 0.025% WG on PJ medium: Geisen et al. (2014); wMY agar: Spiegel et al. (1995); PJ medium: Prescott and James (1955).

Thecamoeba sp. strain 51 (Fig. 1, I-L), were able to feed and multiply on this food source for more than two weeks. The remaining two strains, *Thecamoeba* cf. *aesculea* strain Val and *Thecamoeba vumurta* strain 130, died in experimental cultures by the fifth day. In the course of observations, we found several cells of *Thecamoeba aesculea* containing digestive vacuoles with spirally twisted cyanobacteria inside (Fig. 1, M), as well as floating cells containing filaments at different stages of lysis (Fig. 1, N). In cultures of *Thecamoeba vumurta*, we found cells containing short half-destroyed fragments of cyanobacteria in the digestive vacuoles (Fig. 1, O, P).

Three amoeba strains survived in the experimental dishes for more than two weeks and demonstrated the ability to live and reproduce using cyanobacteria as a food item. Each of these strains showed its own particular way of feeding on cyanobacterial filaments.

The most curious way of feeding was demonstrated by the *Leptomyxa regia* strain LS2 (Kulishkin et al., 2022). At the beginning of the process, the

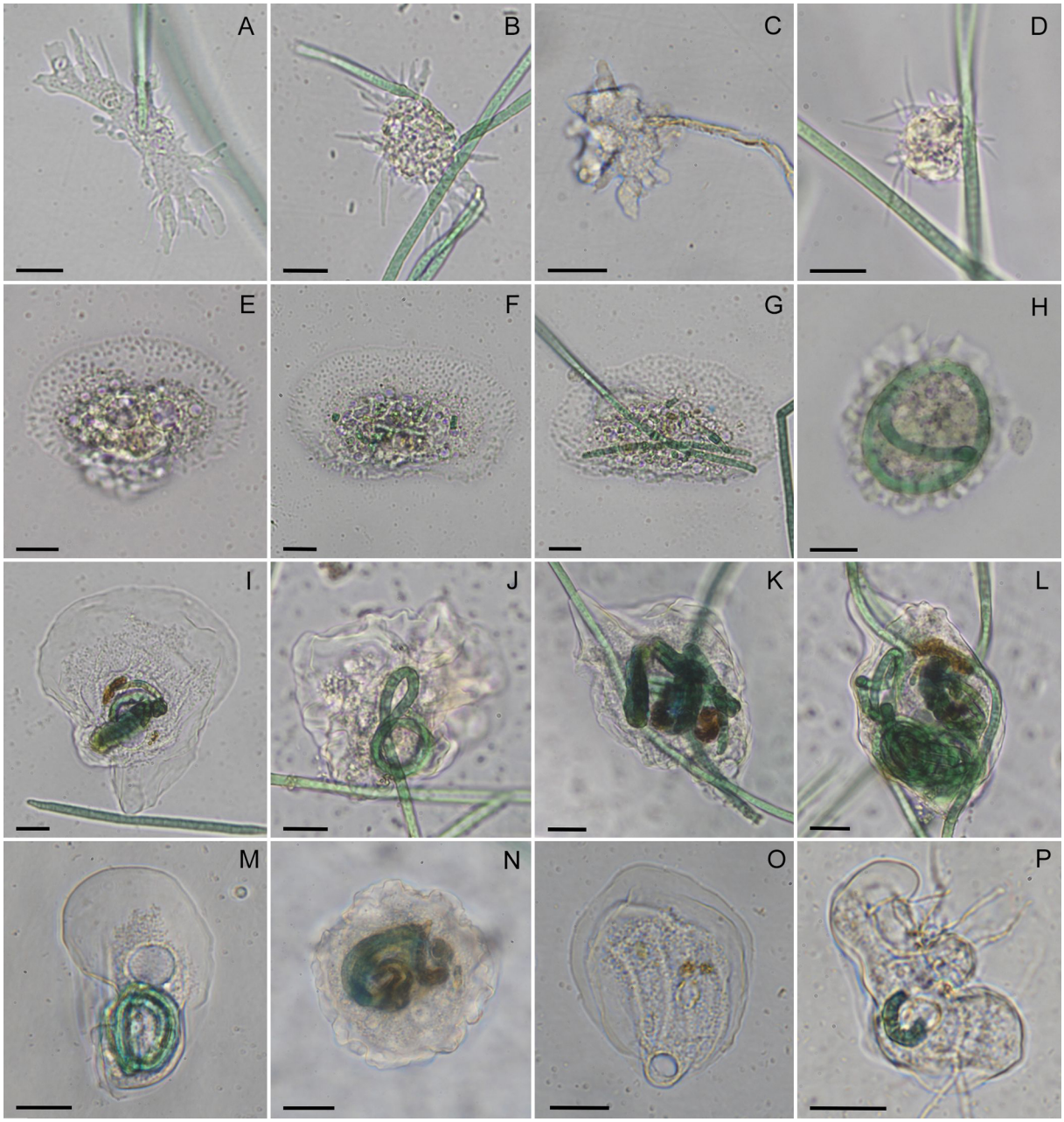


Fig. 1. Amoebae species, found to be able to feed on filaments of *Oscillatoria* sp. A-D – *Leptomyxa regia* strain LS2; A-B – flattened forms, ingesting floating cyanobacterial filaments; C – floating amoeba, excreting empty remnant of cyanobacterial filament; D – floating amoeba adhered to the filament. E-H – *Endostelium* sp.; E – locomotive form of the cell; F – locomotive forms containing digestive vacuoles with bacterial filaments in the granuloplasm; G – locomotive cell ingesting a cyanobacterial filament; H – floating amoeba with coiled filament in the cytoplasm. I-L – *Thecamoeba* sp. strain 51; I – locomotive cell containing digestive vacuoles with cyanobacteria; J-L – various stages of phagocytosis and coiling of cyanobacterial filaments. M-N – *Thecamoeba aesculea* strain Val; M – locomotive cell with a coiled filament in the cytoplasm; N – floating amoeba containing a coiled cyanobacterium. O-P – *Thecamoeba* sp. strain 130; O – locomotive cell showing residual digestive vacuoles; P – floating amoeba containing a small fragment of cyanobacterial filament in the granuloplasm. Scale bars: 20 μm.

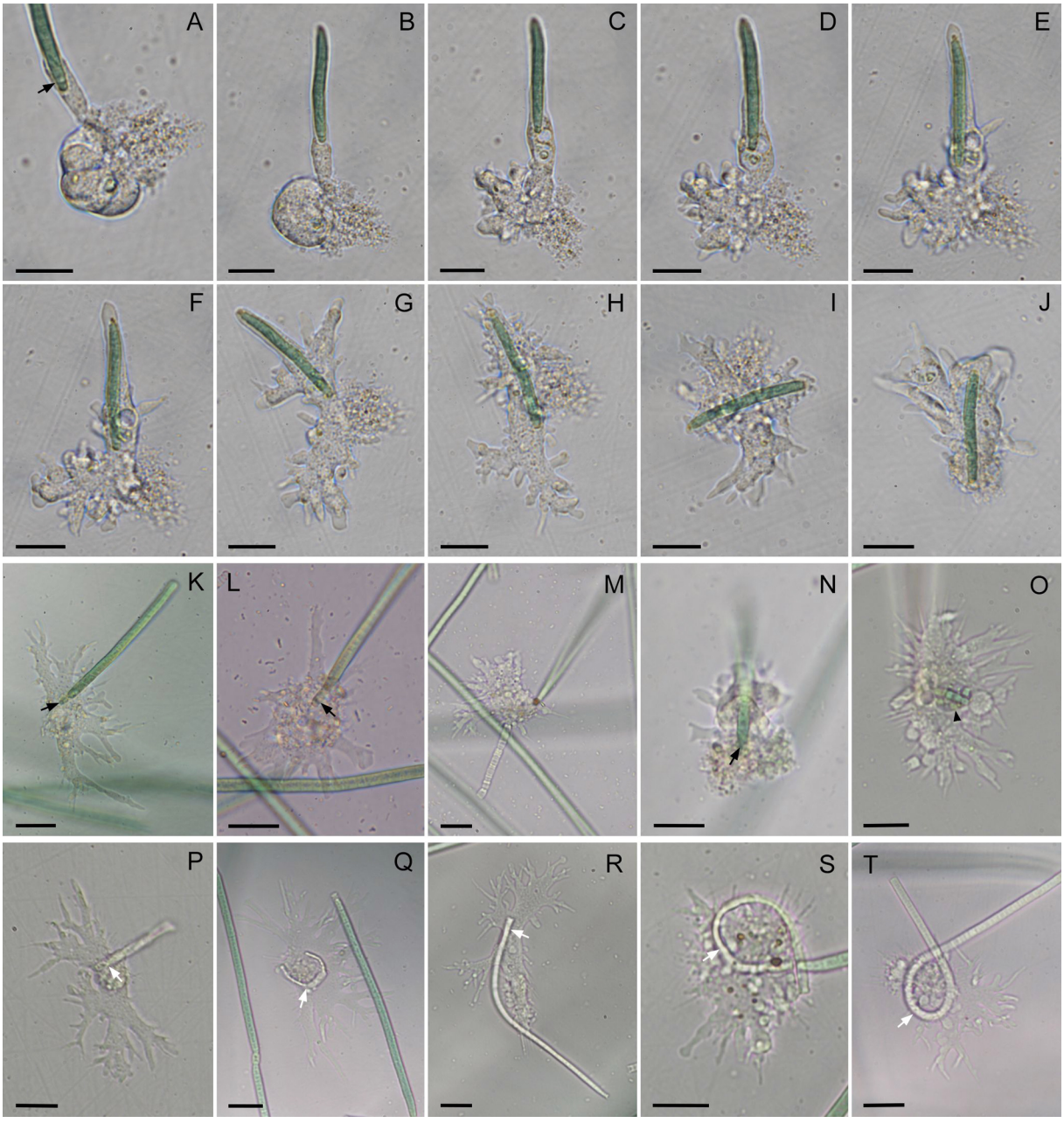


Fig. 2. Feeding of *Leptomyxa regia* strain LS2 on *Oscillatoria* sp. A-J – Subsequent stages of phagocytosis of a cyanobacterial filament; K-M – flattened forms of *L. regia* holding and ingesting a long cyanobacterial filament from one end; N – visible part of a filament inside the digestive vacuole; O – small part of cyanobacterium inside the digestive vacuole; P-T – empty remnants of cyanobacterial filaments of various length. Scale bars: 20 μm.

amoeba cell approached a fragment of cyanobacterial filament and formed a pseudopodium, contacting it. After this contact, the filament from one end began to sink into the digestive vacuole, which the cell formed inside this pseudopodium (Fig. 2, A). During the process, the cell extended the pseudopodium along the cyanobacterial filament until it reached its distal end (Fig. 2, B-D). As a result, the cyanobacterium was completely enclosed in the digestive vacuole (Fig. 2, E-F). After ingesting the cyanobacterium, the amoeba began to draw in the pseudopodium containing the digestive vacuole, and after this, the cell reverted to its usual flattened shape. The digestive vacuole containing the cyanobacterium appeared inside the cell body (Fig. 2, H-I). Despite the presence of a relatively large

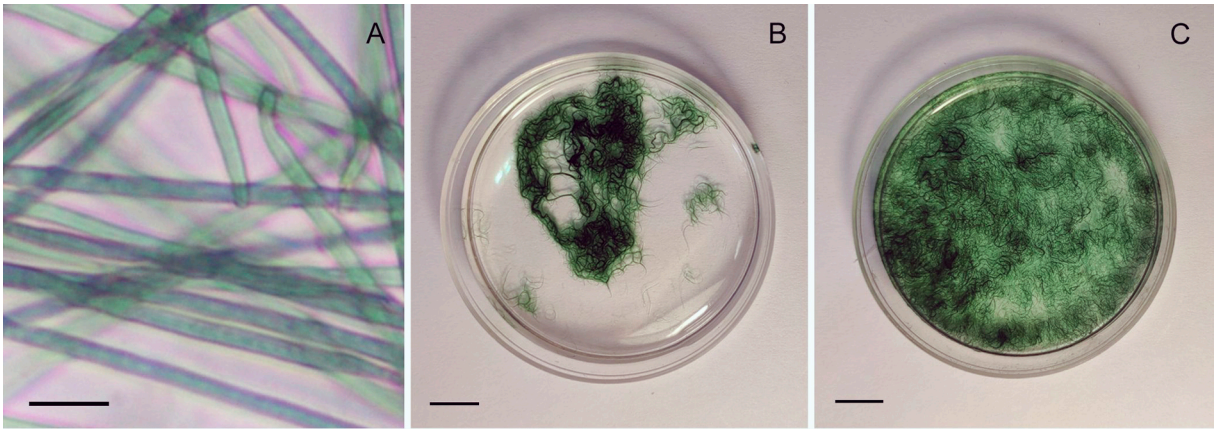


Fig. 3. Comparison of Petri dishes inoculated with *Leptomyxa regia* and *Oscillatoria* sp. and *Oscillatoria* sp. only. A – Cyanobacterial filaments under microscope; B – photo of the Petri dish containing both *L. regia* and *Oscillatoria* sp. after 30 days of cultivation; C – photo of the Petri dish containing *Oscillatoria* sp. only after 30 days of cultivation. Scale bars: A – 20 μm; B, C – 1 cm.

food vacuole inside the cell, the amoeba retained the ability to move by forming wide short flattened pseudopodia (Fig. 2, J). In culture, many cells contained empty shells of cyanobacterial filaments and sometimes excreted them (Fig. 2, P-Q).

L. regia strain LS2 was able to feed not only on short filaments and their fragments, but also on long filaments of cyanobacteria. Long filaments were never fully ingested by the amoeba, and in this strain, we have never seen coiled filaments inside vacuoles, unlike in the other two strains (Fig. 2, L-M). The cell digested long filaments by crawling over them and gradually moving from one end of the filament to the other. Because of this process, numerous empty shells of long cyanobacterial filaments remained in the experimental cultures (Fig. 2, R-T). At the same time, we observed the process when some amoebae, during feeding, detached shorter pieces of long filaments and captured them in the digestive vacuole (Fig. 2, O). During this process, the cell used to "bite off" a piece of the filament during the formation of the digestive vacuole due to the closing of the vacuole with only partly ingested cyanobacterium. The remaining part of the filament was then discarded, but could be attacked by the same cell in the future.

The cells of the *L. regia* strain LS2 lived and multiplied in the experimental culture for all 30 days of the experiment. During this time, they completely destroyed the lower part of the biofilm formed by cyanobacteria adhered to the substrate. The first differences in cyanobacteria cell density between the experimental and control dishes were found by the 10th day of the experiment. Over time, the number of cyanobacterial filaments decreased in the experimental dish until day 30, when we stopped the observation. By the end of the experiment, only the floating part of the biofilm remained in the dish. Meanwhile in the control dishes, the biofilm developed normally and tightly adhered to the bottom of the dish (Fig. 3, A-C). When the number of *Oscillatoria* filaments attached to the bottom of the dish was greatly reduced, we noticed in cultures floating amoeba cells adhered to cyanobacteria from the upper part of the biofilm, floating in the Petri dish (Fig. 1, D). However, we have never seen feeding of the floating amoebae on these cyanobacteria.

The second mode of feeding on cyanobacteria was demonstrated by amoebae of the *Endostelium* sp. strain A1.2 (Surkova et al., 2022). These cells slowly moved along the dish until the contact of the hyaloplasm with the cyanobacterial filament occurred (Fig. 4, A). Further, in the place of this contact, the amoeba formed a triangular or trapezoidal pseudopodium (Fig. 4, L), covering the bacterial cell, and further enclosed it in the digestive vacuole (Fig. 4, C-E). After some time, the pseudopodium started retraction, and the phagocytized filament moved into the granuloplasm of the cell. Short filaments and fragments of filaments were arranged in the digestive vacuole almost intact, long filaments were coiled into a spiral during this process (Fig. 4, G-H). In the digestive vacuole, the cyanobacteria gradually decreased in size and their color changed from green to brown (Fig. 4, J-K). In experimental cultures, we found a large number of floating cells of *Endostelium*, containing vacuoles

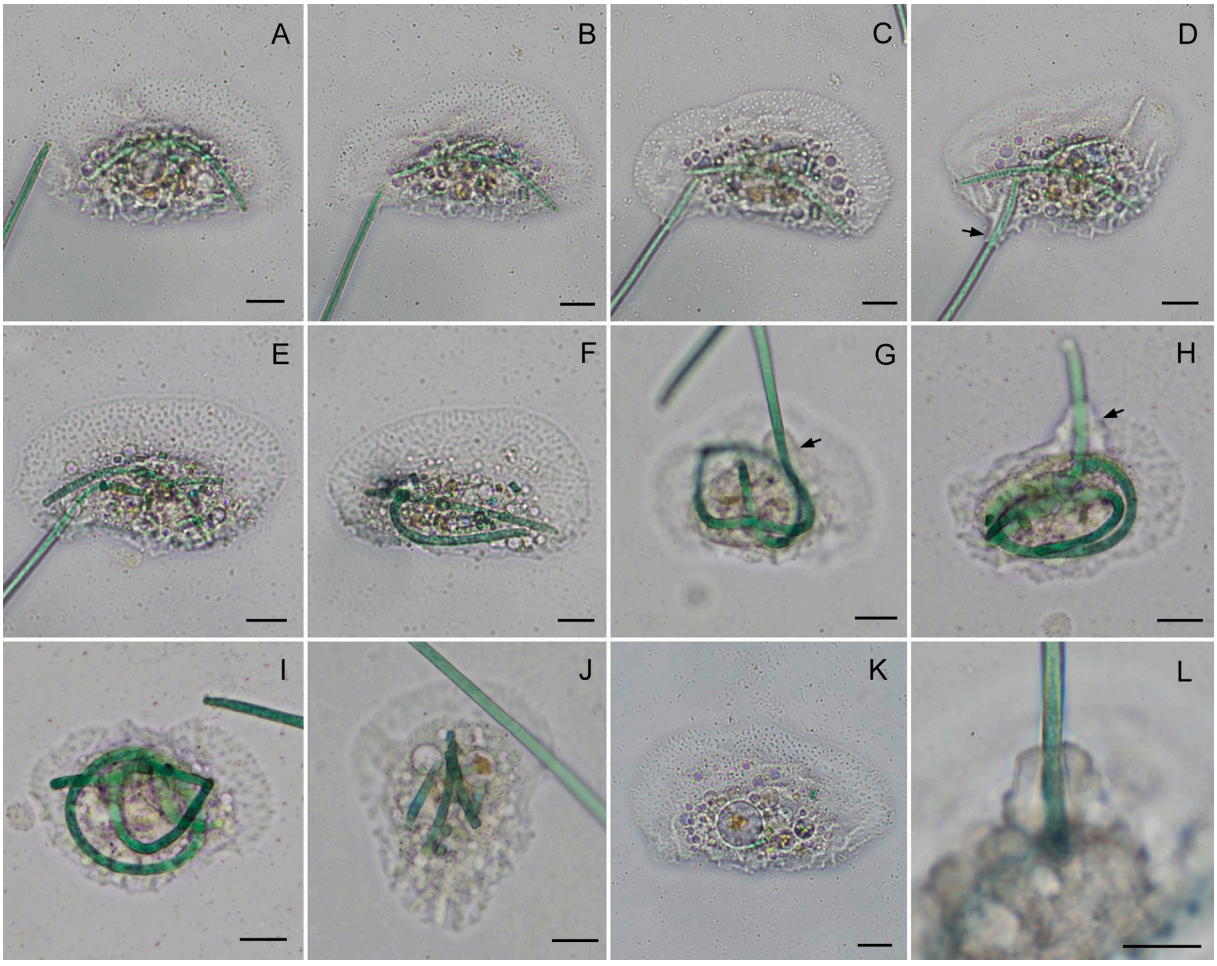


Fig. 4. Feeding of *Endostelium* sp. strain A1.2 on *Oscillatoria* sp. A-B – Contact between amoeba and cyanobacterium; C, G – beginning of the formation of pseudopodium; D, H – elongation of pseudopodium; E – absorption of a filament into the cytoplasm of the cell; F, I – the final stage of the process of filament absorption; J – fragments of *Oscillatoria* sp. in the cell cytoplasm; K – almost digested bacterium in the food vacuole; L – feeding pseudopodium under higher magnification. Scale bars: 20 μm.

with spirally coiled cyanobacterial filaments in the cytoplasm (Fig. 1, H). Visual differences in the density of cyanobacteria in the experimental and control dishes were not found by the 30th day of the experiment. The cyanobacterial biofilm remained attached to the bottom of the dish in both experimental and control dishes.

The third way of feeding was demonstrated by *Thecamoeba* sp. strain 51. Cyanobacteria in experimental dishes formed a network of filaments intertwined at the bottom, from which numerous filaments extended into the water layer. One end of these filaments was fixed in this biofilm. The other end was free. In experimental cultures, we found a large number of floating cells attached to the free ends of cyanobacterial filaments. Further, the amoeba "stretched" along the filament, placing it into the elongated digestive vacuole formed during this process, and enveloped the filament (Fig. 5, A, B). When the digestive vacuole with the filament inside reached the posterior end of the cell, the amoeba began to coil the cyanobacterial filament in its posterior part into a tight spiral (Fig. 5, E-H), gradually increasing the number of turns (Fig. 5, I-K). When a cell completely consumed one filament, it usually attacked another one and repeated the process. We have seen amoebae containing multiple coiled cyanobacterial filaments in the cytoplasm (Fig. 5, M-O). The digested filaments slowly decreased in size and became

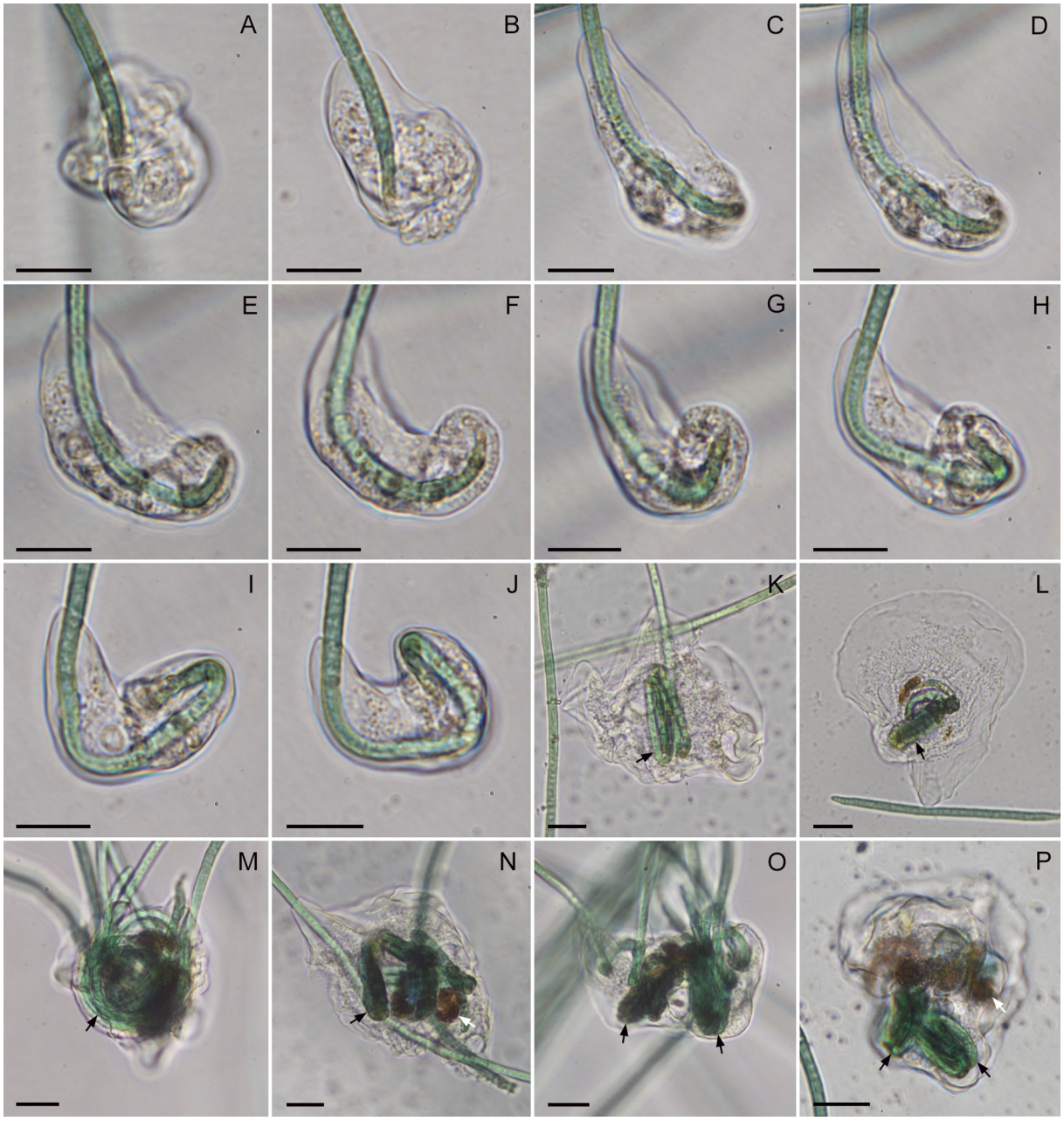


Fig. 5. Feeding of *Thecamoeba* sp. strain Ta51 on *Oscillatoria* sp. A – Contact between amoeba and cyanobacterium; B – absorption of filament into cell cytoplasm; C-J – gradual folding of a filament inside the cell; K – spiral-folded filament inside the amoeba; L – fully ingested cyanobacterium; M-P – feeding and non-feeding cells containing spiral-folded filaments of *Oscillatoria* sp. in food vacuoles. Scale bars: 20 μm.

brownish (Fig. 5, N-P). By the 19th-21st day of the experiment, all amoebae cells in all dishes died. This happened in all replicates of the experiment; the reason for this outcome remained unclear. We found no visible differences in the density of cyanobacterial filaments in the experimental and control dishes on the day 20 of observation on this strain.

Discussion

Among the studied strains, belonging to two different amoebozoan lineages (Tubulinea and Discosea), we have found three that demonstrated different ways to ingest filaments of *Oscillatoria* sp. The difference in the pattern of feeding could

be related to the morphological features of these amoebae. Amoebae of the genus *Leptomyxa*, including the species *Leptomyxa regia*, are characterized by a thin flattened body and a tendency to form comet-shaped or branched cells on the bottom of the culture dish. We suggest that species like *L. regia* are physically unable to keep spiral-folded filaments inside the cell body and therefore its feeding strategy is to seek for short fragments of cyanobacterial filaments or gradually digest longer filaments from one end. A similar feeding strategy is used by a heterolobosean *Euhyperamoeba fallax* (Seravin and Goodkov, 1987). This organism also demonstrated digestion of the filament from one end or from its mid-part, followed by break-up and destruction of the filament in the food vacuole. The processes, resulting in the cut-off of small pieces of filaments is to the certain extent similar to the filament break-up by *Nuclearia thermophila* (Nucleariidae, Opisthokonta), feeding on filamentous cyanobacterium *Planktothrix rubescens* (Dirren et al., 2017). Mechanisms of the food uptake in such species are poorly understood and require further studies.

Alternatively, the cells of *Thecamoeba* and *Endostelium* spp. are thicker and more compact, and they are able to twist the filament and coil it in the digestive vacuoles, retaining mobility with such a food vacuole inside. This is congruent with observations of other authors on such organisms (Leidy, 1879; Rhumbler, 1898; Haberey, 1973a, 1973b). Thick glycocalyx of *Endostelium* sp. can be moved apart and short subpseudopodia with rounded ends can protrude through it for locomotion and feeding. The same is known for species of the genus *Pellita* (Smirnov and Kudryavtsev, 2005; Kudryavtsev et al., 2014). Possibly, the formation of the "hole" in glycocalyx in the place of contact of subpseudopodia with cyanobacterial filaments warrants the formation of characteristic trapezoidal "feeding" pseudopodium, seen only in this species.

The species *L. regia* strain LS2 was the only one capable to destroy cyanobacterial biofilm. This may be due to its specific way of feeding, in particular – the ability to digest a filament starting from its mid-part. Under the natural conditions, the upper free-floating part of the biofilm could be easily washed away with the local water currents, while disrupted remnants of the mat may serve as food for other protists and micrometazoans. Consequently, such amoebae species may slow down or even prevent the formation of the biofilm under natural condition.

It is notable that *Thecamoeba aesculea* strain Val isolated from lake's bottom sediment was able to feed on cyanobacteria (however, it did not survive for a long time on this food), while *Thecamoeba* cf. *aesculea* strain Ta4 from steppe felt avoided feeding on it (Table 1). This result might be related to the differences in natural food ration of these amoebae strains, living under very different environmental conditions. However, this observation deserves attention, since the food specialization could indicate the beginning of a speciation process.

The ability of naked amoebae to feed on cyanobacteria has an important evolutionary implication. Recently, we proposed a hypothesis relating the origin of Amoebozoa with the domination of photosynthetic microbial mats in the Mid-Proterozoic period (Tekle et al., 2022). Specifically, we suggested that Amoebozoa evolved in microbial mats and the direction of the evolution was the increment in size linked to the loss of flagellar motion due to its low efficiency for larger cells. Larger size allowed ancient amoebozoans to disrupt biofilms and digest cyanobacterial filaments, thus providing them an access to the most abundant and widespread food source at that time (Tekle et al., 2022). The present study experimentally shows that modern average-sized species of Amoebozoa are able to consume and digest filamentous cyanobacteria and disrupt cyanobacterial biofilm, preventing its proliferation and adherence to the substrate. Both these observations support our hypothesis mentioned above. In addition, we are aware that modern species might have evolved in many aspects rather far from their mid-proterozoic ancestors.

A recent study aimed to reconstruct the evolution of the Earth atmosphere suggest that the increment in O_2 concentration was not stepwise, but a series of relatively rapid oscillations of the O_2 concentration occurred in the Proterozoic period (Krause et al., 2022). If this hypothesis is valid, the ability of Amoebozoa to inhabit and feed in cyanobacterial mats could be their extra advantage. The photosynthetic cyanobacterial mat is an oxygen producer, so the local O_2 concentration within a mat and in its close surrounding probably was higher and more stable than in the environment, as it is in modern photosynthetic mats (Stal, 1995; Wieland and Kühl, 2006; Gingras et al., 2011; Kaplan, 2011). This could provide ancient Amoebozoa a stable niche allowing them to retain the relatively large size necessary to feed on cyanobacteria (Tekle et al., 2022).

Finally, we can make a speculative (though logical enough) suggestion that Amoebozoa, being capable to destruct cyanobacterial biofilms, together with other organisms might contribute to the sunset of stromatolites. Amoebae not just eat the biofilm; they destroy and disrupt it, making it available for other organisms. Thus, amoebae act as edificators and form suitable environment for a variety of smaller unicellular organisms, efficiently consuming the available biomass of cyanobacteria. From this point of view, amoebae not only consume the biofilm but also promote further evolution by favoring the diversification of organisms populating this kind of environment. Over time, these historical developments have probably resulted in the decrement in the number and biovolume of microbial mats, while the appearance of larger multicellular animals completed this process.

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