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Diversity of thraustochytrid protists isolated from brown alga, *Sargassum cinereum* using 18S rDNA sequencing and their morphological response to heavy metals

Running head: Algal thraustochytrids-diversity, metal effect / *Sargassum*-associated thraustochytrids

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ABSTRACT

Thraustochytrids, the exclusively marine organisms of kingdom Stramenopila and a source of essential fatty acids in the marine milieu, possess osmoheterotrophic mode of nutrition and are therefore affected by type and source of available organic matter and pollution. To study their response to heavy metal pollution, they were isolated from the brown alga *Sargassum cinereum* from the coastal waters of Dona Paula, Goa, India. A total of 22 isolates were obtained from two samples collected during February and March 2012. Based on their 18S rRNA gene sequencing, majority of the isolates were identified as *Thraustochytrium kinnei*. The rest were identified to be *Sicyoidochytrium minutum*, *Ulkenia visurgensis* and species of *Thraustochytrium* and *Aurantiochytrium*. Six isolates were screened for various enzymatic activities. Characteristic and distinctive enzyme profile was obtained from isolates of different genera. All isolates were also screened for their tolerance to heavy metals. They showed good growth in the presence of Mn^{2+} . The other metals that were tolerated by most of the isolates were in the order $Ni^{2+} > Cr^{6+} > Zn^{2+}$. Seven isolates grew in the presence of Cu^{2+} , and six in the presence of Cd^{2+} . The isolates growing on metals showed vast differences from their normal morphology such as small colony size, shrunken cells etc. Scanning electron micrographs revealed holes or depressions in the cell wall in the presence of metals. On the whole, the isolates belonging to *Ulkenia visurgensis* and *Aurantiochytrium* sp. showed tolerance to more metals than *Thraustochytrium kinnei*. Cluster analysis showed no peculiar trend of metal tolerance to any particular genus as the characters were scattered in the clusters.

KEYWORDS: thraustochytrids, marine, Labyrinthulomycota, Stramenopila, *Sargassum*, brown alga, heavy metals

INTRODUCTION

Marine algae and their products are the focus of medical and biochemical research due to the abundance of useful bioactive compounds that can be isolated and unearthed by various purification techniques (Bazes *et al.*, 2009; Badrinathan *et al.*, 2012). The surfaces of algae nurture diverse communities of organisms which also contribute to various bioactive compounds (Soria-Mercado *et al.*, 2012). The brown algal genus *Sargassum*, being invasive for their capacity of adaptation and their high growth rate, supports a diverse community of marine organisms including micro- and macro-epiphytes (see Coston-Clements *et al.*, 1991). The microorganisms associated with the algae represent a source of secondary metabolites with an array of biological activities originally attributed to the host (Armstrong *et al.*, 2001; Penesyan *et al.*, 2009; Hollants *et al.*, 2013). Drifting algae facilitate redistribution of organisms in pelagic environment (Turner & Rooker, 2006). When grown in the vicinity of heavy metal contaminated site, *Sargassum* has been found to be effective in binding heavy metals (Krishnakumar *et al.*, 1990; Yang & Volesky, 1999).

Thraustochytrid protists are microorganisms commonly found on algal surfaces (Miller & Jones, 1983; Raghukumar, 2002) in addition to bacteria (Soria-Mercado *et al.*, 2012). These belong to phylum Labyrinthulomycota of the kingdom Stramenopila. These unicellular eukaryotes range from 2-20 μm in diameter and are a common component of marine microbial consortia. There are various reports on the association of these organisms with other macroorganisms like the zooplankton. These serve as prey to the zooplankton and hence supply them with essential nutrients such as polyunsaturated fatty acids (Damare *et al.*, 2013). They have been found to be endolithic in mollusk shell fragments (Porter & Lingle, 1992) and have been isolated from surface mucus of hermatypic corals (Harel *et al.*, 2008). They are also known to be pathogenic to abalone, certain fishes and seagrasses (see Raghukumar, 2002). These protists have been rarely found to be associated with living phytoplankton or plants (Raghukumar, 2002) but have been found abundantly in the detritus of *Sargassum* (Sathe-Pathak *et al.*, 1993).

In the present study, thraustochytrid protists were isolated from drifting pieces of *Sargassum cinereum* and identified based on 18S rDNA sequencing. Though *Sargassum* has the capacity to accumulate heavy metals from solution, it is not known if the thraustochytrid isolates derived from this brown alga have the ability to tolerate them. Hence a preliminarily screening was done to determine the effect of heavy metals on the morphology of these isolates.

MATERIALS AND METHODS

Isolation of thraustochytrids

Leaf blades of the brown alga *Sargassum cinereum* floating in the surface waters at Dona Paula Bay, Goa, India were collected, washed with sterile seawater and plated on Modified Vishniac (MV) Agar medium supplemented with antibiotics (Ampilox-C 500 mg – trade name; 0.25 mg ml⁻¹ each of ampicillin and cloxacillin final concentration added in MV). The plates were incubated at room temperature and observed for growth every day for one week. The colonies of thraustochytrids were confirmed microscopically and sub-cultured on MV agar plates without antibiotics. Additionally, the washed leaf blades were cut into small pieces under sterile conditions and incubated in sterile seawater baited with pine pollen (baiting technique for isolation). After 2-3 days when thraustochytrid cells were seen (microscopically) growing on the pollen, they were transferred to MV agar plates with antibiotics and sub-cultured thereafter.

Molecular identification of thraustochytrid isolates

Biomass of all isolates was obtained by growing each in MV broth for 5 days and centrifuging at 8000 rpm/15 min. DNA was extracted from the cell pellets using ZR Fungal/Bacterial DNA MiniPrep™ kit (Cat. No. D6005, Zymo Research). Further 18S rRNA gene amplification was carried out using the two sets of primers viz., (i) 18S001 and NS4 and (ii) NS3 and 18S13 (White *et al.*, 1990; Honda *et al.*, 1999) with the same conditions as previously described by Damare and Raghukumar (2010). The PCR products were purified using AxyPrep™ PCR Cleanup Kit supplied by Axygen Biosciences (Cat. No. AP-PCR-250). The quality of the purified PCR product was also checked by electrophoresis. The purified PCR products were sequenced using a 3130xl Genetic Analyzer (ABI Sequencer 2200), Applied Biosystems by capillary electrophoresis. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) provided by NCBI. Phylogenetic tree based on the 18S rDNA sequence was generated by the neighbor-joining (NJ) method using MEGA version 4 (Tamura *et al.*, 2007). Apart from molecular identification, identity of these isolates was confirmed by microscopic analysis.

Enzymatic profiles

Seven isolates were tested for enzymatic profiles. These included one isolate belonging to *Thraustochytrium* sp. (DPSgF18), two isolates of *T. kinnei*: one from February sample (DPSgF11) and the other from March sample (DPSgMr6), *Aurantiochytrium mangrovei* (DPSgF16), *A. limacinum*

(DPSgF17), *Sicyodochytrium minutum* (DPSgF25) and *Ulkenia visurgensis* (DPSgMr10). These seven isolates (five from February sampling and two from March sampling) were grown in MV broth at 28° C for 3 days and harvested under sterile conditions by centrifuging in sterile centrifuge tubes at 8000 rpm for 10 min. The cells were washed with sterile seawater and then resuspended in API Suspension Medium bringing the turbidity of the suspension between 5-6 McFarland Standard. Each of this was tested for the presence/absence of 19 different enzymes using the semi-quantitative API-ZYM system (Biomérieux; Ref 25200) (Bongiorni *et al.*, 2005). The strips containing wells were inoculated with small sub-aliquots (65 µl) and incubated at 28° C for 6 h after which the enzyme activities were checked as per the manufacturer's instructions and intensity of all reactions were noted. Cluster analysis was carried out in Statistica v 5.0 software.

Morphological response to heavy metals

Morphological response of all the isolates to 7 different heavy metals was tested: manganese (Mn^{2+} as $MnCl_2$), zinc (Zn^{2+} as $ZnCl_2$), nickel (Ni^{2+} as $NiSO_4$), copper (Cu^{2+} as $CuSO_4$), cadmium (Cd^{2+} as $CdCl_2$), chromium (Cr^{6+} as $K_2Cr_2O_7$) and lead (Pb^{2+} as $Pb(NO_3)_2$). Stock solutions (1 M or 500 mM) of these metals were prepared. Each metal was incorporated in MV agar medium at a final concentration of 10 mM (Dinu *et al.*, 2011). The isolates were streaked on each of these plates. Growth was monitored on the plates for 15 days by measuring the colony diameter under the microscope (Olympus BX60). Tolerance to Pb^{2+} was also examined by incorporating $Pb(NO_3)_2$ in MV agar at 1, 2, 3 and 5 mM in addition to 10 mM. The growth of the isolates in presence of metals as compared to control (MV without any metal) was monitored for one month. The capacity of all the isolates to tolerate 10 mM of each metal was analyzed by cluster analysis using unweighted pair group average method in Statistica v 5.0 software. Plating and microscopy was repeated twice before proceeding for scanning electron microscopic (SEM) analysis.

The isolates with tolerance to metals were grown in MV broth supplemented with respective metal solutions separately and the biomass obtained after 10 days of growth were treated for SEM analysis according to protocol described by Washington State University (http://sbs.wsu.edu/fmic/protocol/fixindex/sem_standard_fix.html). In brief, the cells were washed with sterile seawater, primarily fixed in 1 % glutaraldehyde and filtered over pretreated 0.4 µm white polycarbonate filter paper (Millipore). The filter papers were pretreated with 0.1 % polylysine hydrobromide (Sigma) and air dried (Kalab *et al.*, 2008). The cells were then washed with sterile

distilled water and treated with 1 % osmium tetroxide for 1 h at RT. The filter papers bearing the cells were passed through an increasing series of dehydration steps (30, 50, 70, 95 and 100 % ethanol) of 10 minutes each. The filter papers were mounted on stubs, freeze-dried (Thermo – SAVANT, Model MICRO MODUL YO-230) and sputter coated with gold. They were then observed with JEOL, JSM-5800LV Scanning Electron Microscope.

RESULTS

Isolation, identification and enzymatic profiles of thraustochytrid isolates

Altogether 35 isolates were obtained in culture from *Sargassum* samples collected at 2 different times and only 22 survived repeated sub-culturing. Out of the 22, 19 isolates were attained during February 2012 and 3 isolates during March 2012. The isolates were designated as DPSgF- for February samples and DPSgMr- for March samples. The topology of the phylogram showed that 22 strains obtained from *Sargassum* belonged to 6 species, *Thraustochytrium kinnei* A. Gaertn., *Thraustochytrium* sp. ATCC 26185, *Aurantiochytrium limacinum* (D. Honda & Yokochi) R. Yokoyama & D. Honda, *A. mangrovei* (Raghuk.) R. Yokoy. & D. Honda, *Sicyoidochytrium minutum* (Raghuk.) R. Yokoy., Salleh & D. Honda and *Ulkenia visurgensis* (Ulken) A. Gaertn., among which *T. kinnei* A. Gaertn. occupied majority of the isolates. Fifteen isolates namely, DPSgF1-3, DPSgF5-6, DPSgF8-9, DPSgF11-15, DPSgF22-23 and DPSgMr6 were *T. kinnei*. DPSgF10 and DPSgF18 were identified at molecular level (from sequence data) as *Thraustochytrium* sp. ATCC 26185 and their morphological characteristics resembled the same species as well. Dense lipid bodies in the cytoplasm, cell size between 6-14 µm and lack of clear ectoplasmic net elements were characteristics of *Thraustochytrium* sp. ATCC 26185 (Weete *et al.*, 1997).

Isolate DPSgF16 and DPSgF17 were identified at molecular level to be species of *Aurantiochytrium*. Microscopic analysis of the two confirmed their identity to be *Aurantiochytrium mangrovei* (earlier *Schizochytrium mangrovei*) and *Aurantiochytrium limacinum* (earlier *S. limacinum*) respectively. Release of limax-shaped amoebae by DPSgF17 confirmed its identity as *A. limacinum*. DPSgF16 showed characteristics similar to *A. mangrovei* (Raghukumar, 1988) such as production of amoebae in MV agar. Isolate DPSgF25 was identified as *Sicyoidochytrium minutum*. Two isolates from March namely, DPSgMr2 and DPSgMr10 were identified as *Ulkenia visurgensis*.

Enzyme profile of the isolates representing each genus/species was distinct from each other (Table 1). *Thraustochytrium* sp. ATCC 26185 (DPSgF18) was different from that of *T. kinnei* (DPSgF11 and DPSgMr6). The former produced α -mannosidase and no activity with trypsin, cysteine arylamidase and N-acetyl- β -glucosaminidase as compared to *T. kinnei*. Profiles of the two species of *Aurantiochytrium* were similar. However, *A. limacinum* (DPSgF17) differed from *A. mangrovei* (DPSgF16) by absence of trypsin and β -glucuronidase. These 2 differed from *S. minutum* (DPSgF25) and *U. visurgensis* (DPSgMr10) by the presence of esterase and lipase. DPSgMr10 differed from the rest by the absence of α -chymotrypsin and β -glucosidase. It produced the least number (9) of enzymes whereas isolate DPSgF17 (*A. limacinum*) produced the maximum number (18).

Phylogenetic analysis distributed the isolates into two clusters, one containing isolates belonging to *Aurantiochytrium* sp. and the other comprising the rest of the genera. The inclusion of several isolates of *Thraustochytrium*, *Sicyoidochytrium* and *Ulkenia* species to clustering algorithm established these thraustochytrids as a highly supported monophyletic group that was distinct from *Aurantiochytrium* spp. (Figure 1). This major cluster of thraustochytrids consisted of a subcluster of all *T. kinnei* isolates and of *Ulkenia* sp. that formed sister lineage to *Thraustochytrium* sp. ATCC 26185. On the other hand, in the cluster analysis based on enzymatic profiles, isolates of *Aurantiochytrium* sp. (DPSgF16 and DPSgF17) grouped with isolates of *T. kinnei* (DPSgF11 and DPSgMr6) and *Sicyoidochytrium* sp. (DPSgF25) and the isolate belonging to *Ulkenia* sp. (DPSgMr10) separated out in a distinct clade (Figure 2).

Morphological response to heavy metals

When compared with the control plate (i.e. MV agar without any metals), there was either poor growth or absolutely no growth of thraustochytrid isolates in the agar incorporated with different heavy metals (Table 2). All the isolates grew in the presence of Mn^{2+} but to varying extent. There was no difference in colony size in the presence of Mn^{2+} in 4 out of 22 isolates (DPSgF5, 17, 25 and DPSgMr10) as compared to control, but the cells were loosely packed inside the colony in the case of DPSgF5 and occasionally amoeboid cells were formed in case of DPSgMr10. In DPSgF17 and DPSgF25, there was no difference in cellular morphology as compared to the control (MV without any metal). Colony size was reduced by 25-70 % in other 18 isolates studied i.e. from an average diameter of 175-200 μm under normal MV, the colony size was reduced to 100-150 μm in the presence of Mn^{2+} . Tolerance of the isolates to Mn^{2+} was followed by Ni^{2+} , Cr^{6+} and then Zn^{2+} . In 91 % of the isolates (20 out of 22) there was tolerance to 10 mM $NiSO_4$ (Table 2). Twelve out of 22 isolates (55 % of the total tested) tolerated

Cr⁶⁺. Nine isolates (41 % of the total tested) showed tolerance to Zn²⁺. Cu²⁺ was tolerated by 7 isolates (32 % of the total) and Cd²⁺ by 6 (27 %). There was slight tolerance to 1 mM Pb²⁺ in two isolates DPSgMr2 and DPSgMr10 as marked by very poor growth and shrunken cells. Colony size reduced by 72% (200-225 µm to 50-60 µm average diameter) in these two isolates. No isolates grew in the presence of 2, 3, 5 and 10 mM Pb²⁺.

There were differences in morphological characteristics of cells of all isolates in presence of various metals as compared to normal cells. A few of these isolates are showed in Figures 3-6. No distinct colony formation was observed in some; instead cells were seen spread all over the line of inoculation (as seen in the presence of Cu²⁺ and Ni²⁺ in Figure 3, Zn²⁺ in Figure 4 and 5). Distinct changes in the cell wall in the presence of metals as compared to the normal cells were evident in the electron micrographs. The presence of metals resulted in either depressions in the cells or holes on their cell wall (Figure 6). Significant amount of cell shrinkage was noticed due to the presence of metals as compared to normal healthy cells (e.g. panel 5 and 8 in Figure 6). Average cell size was reduced from 20 µm to 5 µm in the presence of a few metals like Cu²⁺, Ni²⁺.

Cluster analysis based on the metal tolerance capacity of each isolate divided all the isolates into two main clades, one comprising isolates that were sensitive to only one out of six metals (clade I) and second comprising isolates that were sensitive to one or many metals (clade II). Overall the characters of tolerance to different metals were scattered throughout the clusters (Figure 7). Isolates DPSgF16 and DPSgF17 belonging to *Aurantiochytrium* sp. and DPSgF18 classified as *Thraustochytrium* sp. ATCC 26185 clustered in clade I. The rest of the isolates belonging to *T. kinnei*, *Ulkenia* etc. grouped together in clade II similar to NJ tree.

DISCUSSION

The fast-growing brown alga *Sargassum* is spread by currents or by ships and provides crucial habitat for a wide variety of marine animals. The polyunsaturated fatty acid (PUFA) signatures of particulate organic matter from the habitats surrounding *Sargassum* are known to contain high levels of PUFAs like 22:6 n-3 (Turner & Rooker, 2006). As the macroalgae contain very low quantities of long-chain PUFAs, especially 22:6n-3, the organic matter appears to have originated from other living organisms associated with *Sargassum*. Thraustochytrid protists are known to produce abundant amount of this fatty acid intracellularly (Raghukumar, 2008). Thraustochytrids could therefore be the source of 22:6n-3 in the

organic matter. This suggests the prevalence of these compounds in these habitats and probable association of these organisms with the alga. This was confirmed by the successful isolation of these protists from *Sargassum cinereum* during both sampling times in the present study.

Thraustochytrids are generally found to be substrate-specific in occurrence rather than free-living in the water column. This substrate-specific nature helps these protists to grow on *Sargassum* as epibionts (Raghukumar, 2002). Though the brown alga produces tannins on distal growing tips that have an inhibitory effect on colonizing epibionts, its effect lasts for a short time after which a succession of other organisms like bacteria, hydroids, bryozoans, etc. rapidly follow (see Coston-Clements *et al.*, 1991). This explains the prevalence of thraustochytrids with the algae and their subsequent isolation in the present study. *Aplanochytrium minuta* (S.W. Watson & Raper) C.A. Leander & D. Porter, a member of sister group of thraustochytrids, called aplanochytrids, was regularly found to be associated as an endobiont with *S. cinereum* (Sathe-Pathak *et al.*, 1993). But in the present study, this species was not recovered from the samples. Rather all isolates obtained were found to be thraustochytrids.

Thraustochytrids are also common inhabitants of the detritus originating from *Sargassum* (Sharma *et al.*, 1994). Marine detritus appears to be an excellent substrate for growth of these protists (Raghukumar & Damare, 2011). Owing to their absorptive mode of nutrition and production of various degradative enzymes, these organisms are thought to be the important remineralizers in the marine ecosystem (Raghukumar & Damare, 2011). In order to explain this role, studies on extracellular enzyme production were carried out. Amongst the 19 enzymes tested, the isolates in the present study showed positive activity with most of them. All the isolates produced alkaline as well as acid phosphatase indicating their ability to dephosphorylate organic molecules for uptake during PO_4^{3-} replete conditions and also to grow under PO_4^{3-} starvation as PO_4^{3-} is an important limiting nutrient for growth of the algae (Kuenzler & Perras, 1965). Except for *Sicyoidochytrium minutum* and *Ulkenia visurgensis*, most of the isolates produced esterases and lipases that help to breakdown short and long chain fatty acids respectively and as a consequence utilize wide range of substrate molecules possessing amide or ester bonds (Bourne *et al.*, 2000). Various proteolytic enzymes like arylamidases, trypsin, chymotrypsin were produced by most of the test isolates. Amongst the carbohydrate-degrading enzymes, none of the isolates produced fucosidase, an enzyme that breaks down the sugar generally present in the cell wall of the brown algae as well as in the extracellular polysaccharides secreted by the phytoplankton suggesting that thraustochytrids may not be able to degrade the algal cell wall and hence may not initiate an infection.

Production of N-acetyl- β -glucosaminidase by all the isolates calls attention towards the ability of thraustochytrids to breakdown chitin from exoskeleton of marine invertebrates that may inhabit the *Sargassum* mats (Yu *et al.*, 1991). This would protect the mats against invaders/consumers (Turner & Rooker, 2006). Thraustochytrids isolated from seagrasses and green algae that form mats in the water column similar to *Sargassum* also produce this enzyme (Bongiorni *et al.*, 2005).

The production of various enzymes facilitates absorption of nutrients from the surfaces of *Sargassum* by breaking down complex organic compounds. β -glucuronidase produced by DPSgF16 and DPSgF25 helps in digesting uronic acid rich polysaccharides secreted by algae (Dodgson *et al.*, 1953). Thus enzyme production by these isolates confirms not only the osmoheterotrophic mode of nutrition of thraustochytrids but also their role as remineralizers in the food chain (Raghukumar & Damare, 2011). Production of limited number of enzymes by isolate belonging to *U. visurgensis* indicates low variety of substrate preference or utilization of more easily available substrates than breaking down of refractory matter.

Thraustochytrids occasionally show ability to withstand and grow in the presence of heavy metals (Raghukumar *et al.*, 2008). Generally seaweeds have a high capacity to bind heavy metals. Biosorption of copper, gold, cadmium, zinc, manganese and even radioactive metal such as uranium on *Sargassum* biomass has been studied and is therefore recommended as an excellent biomaterial for accumulating and recovering metals from industrial effluents (Krishnakumar *et al.*, 1990; Volesky & Holan, 1995; Yang & Volesky, 1999; Antunes *et al.*, 2003). Biosorption on *Sargassum* biomass may induce metal tolerance in its associated microorganisms (Wagner-Döbler *et al.*, 2002). Hence thraustochytrids from *Sargassum* might be a good subject to study metal-tolerance. There are only two reports that deal with tolerance of thraustochytrids to heavy metals (Raghukumar *et al.*, 2008; Lin *et al.*, 2010). In their study Lin *et al.* (2010) observed that *Schizochytrium* cells were tolerant to Cu^{2+} and Zn^{2+} to a certain extent. In the present study the *Aurantiochytrium* spp. were tolerant to both of these metals along with manganese, nickel and chromium. Deformities in cellular structure were seen as an effect of metals on the cells. Lin *et al.* (2010) observed leakage of cellular contents due to cell lysis when exposed to high levels of Cu^{2+} and Zn^{2+} . In the present study the high metal concentration resulted in holes or depressions in the cells as also observed by Singh *et al.* (2013) in yeasts. Raghukumar *et al.* (2008) studied the effect of heavy metals including iron, manganese and lead on one thraustochytrid isolate which they identified as *Ulkenia* sp. They isolated this species from shallow water hydrothermal vent where heavy metals occur

in high concentrations, thus suggesting its adaptation to the environment. The *Ulkenia* sp. in the present study was tolerant to all the metals studied except cadmium. This was the only species that exhibited tolerance to 1 mM Pb²⁺. Resistance to heavy metals is usually specific to species (Miersch *et al.*, 1997). Generally all the isolates showed wide tolerance to Mn²⁺ which is a micronutrient and required as a cofactor for several metalloenzymes. In eukaryotes, when manganese is amply available the polypeptides that transport this nutrient in the cell are directed to the lumen of cellular vacuole where they are degraded by vacuolar proteases, thereby minimizing cellular uptake and preventing cell damage (Culotta *et al.*, 2005). This would have probably resulted in greater tolerance to manganese than other metals.

The biosorbing capacity of the cells depends not only on the type and concentration of metal ions but also on the type of cell wall that comes in contact with the metals. The functional groups present in the cell wall are mostly responsible for binding heavy metals due to the net charge on them which helps in binding charged metal ions (Das *et al.*, 2008). The cell walls of thraustochytrids are made of sulphated polysaccharides containing galactose, glucose, xylose, mannose and also proteins (Chamberlain, 1980). In addition to that these organisms secrete extracellular polysaccharides (EPS) that contain uronic acids and sulphates, conferring them an overall negative charge (Jain *et al.*, 2005). The polyanionic nature of the EPS exhibits high binding affinity for cations as well as trace metals and acting as ion-exchanger (Poli *et al.*, 2010). EPS also helps in biofouling and enhances the capacity of source organisms to adhere to marine substrata, for example *Sargassum* in the present study (Jain *et al.*, 2005).

Microorganisms living in association with many marine macroorganisms are mainly responsible for their capability to produce bioactive compounds earlier ascribed to the hosts (Armstrong *et al.*, 2001; Penesyan *et al.*, 2009; Hollants *et al.*, 2013). Moreover, these might also cause biosorption of heavy metals from contaminated seawater. Correspondingly the microorganisms living in association with *Sargassum*, for example thraustochytrids, may be contributing towards its potential to accumulate heavy metals from contaminated seawater. Detailed studies on biosorption of heavy metals on thraustochytrid biomass will shed more light on this aspect.

In conclusion, this study showed that thraustochytrids are commonly associated with *Sargassum* and could be isolated from this brown alga. By producing various enzymes, these epibiotic organisms survive on the nutrients released from *Sargassum* or other algal surface-associated organisms and might serve to protect the alga from colonization by parasites. Their tendency to tolerate heavy metals may be

used for bioremediation as these organisms contribute to a large extent to microbial biomass carbon owing to their great abundance in marine environment and hence can be harnessed for this purpose (Damare & Raghukumar, 2008). The heavy metal tolerant capacity of thraustochytrids could be further studied for its bioremediation potential in future.

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FIGURE LEGENDS

- Fig. 1.** Neighbour-joining (NJ) tree of 18S rDNA of thraustochytrids isolated in the present study along with other thraustochytrid sequences from NCBI database using MEGA 4.0.1. The 18S rDNA sequence of dinoflagellate *Prorocentrum micans* (Accession no. DQ485144) is used as an outgroup. Some sequences that were too divergent were not considered for construction of the tree. *Schizochytrium mangrovei* and *S. limacinum* have been emended as *Aurantiochytrium mangrovei* and *A. limacinum* respectively according to [Yokoyama and Honda \(2007\)](#).
- Fig. 2.** Cluster analysis of representative thraustochytrid isolates based on their enzyme profiles using unweighted pair group average method in Statistica v 5.0 software.
- Fig. 3.** Microphotographs of the colonies of isolate DPSgF12 growing on Modified Vishniac (MV) agar plates incorporated with different metals. Bar represents 100 μm .
- Fig. 4.** Microphotographs of the colonies of isolate DPSgF18 growing on Modified Vishniac (MV) agar plates incorporated with different metals. Bar represents 100 μm .
- Fig. 5.** Microphotographs of the colonies of isolate DPSgMr2 growing on Modified Vishniac (MV) agar plates incorporated with different metals. Bar represents 100 μm .
- Fig. 6.** Scanning electron microscopy photographs of three isolates grown in the presence of metals; DPSgMr10 isolate in the presence of normal MV (1) and MV supplemented with Ni^{+2} (2 and 3), DPSgF22 isolate in the presence of normal MV (4) and MV supplemented with Mn^{+2} (5) and DPSgF16 isolate in the presence of normal MV (6) and MV supplemented with Ni^{+2} (7) and Cu^{+2} (8). The arrows pointing leftwards show the EPS surrounding or released by the cells. Cell shrinkage due to metal ions is evident in 5 and 8. The arrow pointing towards the right in 3 shows distinct holes formed in the cell wall. The rightward pointing arrow in 7 shows the distorted cell shape as compared to the spherical cell (normal) in 6. The arrow pointing towards the right in 8 shows depression in the cell wall as an effect of metal.
- Fig. 7.** Cluster analysis of all thraustochytrid isolates based on their metal tolerance capacity using unweighted pair group average method in Statistica v 5.0 software. Clade I and II are marked in the figure. Distribution of a few metals in the tree clearly indicates scattering of these characters.

Table 1. Enzymatic profiles of representative thraustochytrid isolates from the present study. ('+' sign indicates positive activity and '-' sign indicates absence of activity).

solates							
Enzymes	<i>Thraustochytrium kinnei</i>		<i>Thraustochytrium</i> sp. ATCC26185	<i>Aurantiochytrium mangrovei</i>	<i>A. limacinum</i>	<i>Sicyoidochytrium minutum</i>	<i>Ulkenia visurgensis</i>
	DPSgF11	DPSgMr6	DPSgF18	DPSgF16	DPSgF17	DPSgF25	DPSgMr10
alkaline phosphatase	+	+	+	+	+	+	+
esterase	+	+	+	+	+	-	-
esterase lipase	+	+	+	+	+	-	-
lipase	+	+	-	+	+	-	-
leucine arylamidase	+	+	+	+	+	+	+
valine arylamidase	+	+	+	+	+	+	+
cystine arylamidase	+	+	-	+	+	+	-
trypsin	+	+	-	+	-	+	-
α -chymotrypsin	+	+	+	+	+	+	-
acid phosphatase	+	+	+	+	+	+	+
naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+
α -galactosidase	-	-	-	+	+	+	+
β -galactosidase	+	+	+	+	+	+	+
β -glucuronidase	-	-	-	+	-	+	-
α -glucosidase	-	-	-	+	+	+	-
β -glucosidase	+	+	+	+	+	+	-
N-acetyl- β -glucosaminidase	+	+	-	+	+	+	+
α -mannosidase	-	-	+	+	+	+	+
α -fucosidase	-	-	-	-	-	-	-

Table 2. Ability of thraustochytrid isolates to grow on Modified Vishniac (MV) agar incorporated with various metal solutions. ('+' sign indicates growth or tolerance to that metal and '-' sign indicates absence of growth and hence zero tolerance to that metal).

	Mn	Zn	Ni	Cu	Cd	Cr
DPSgF1	+	-	+	-	-	+
DPSgF2	+	-	-	-	-	+
DPSgF3	+	-	-	-	-	+
DPSgF5	+	+	+	-	-	-
DPSgF6	+	-	+	-	-	-
DPSgF8	+	-	+	-	-	-
DPSgF9	+	+	+	-	-	+
DPSgF10	+	-	+	-	+	-
DPSgF11	+	-	+	-	-	+
DPSgF12	+	+	+	+	-	+
DPSgF13	+	-	+	-	-	+
DPSgF14	+	+	+	-	-	+
DPSgF15	+	-	+	-	+	-
DPSgF16	+	+	+	+	+	-
DPSgF17	+	+	+	+	+	-
DPSgF18	+	+	+	+	+	-
DPSgF22	+	-	+	-	+	+
DPSgF23	+	-	+	-	-	-
DPSgF25	+	-	+	+	-	+
DPSgMr2	+	+	+	+	-	+
DPSgMr6	+	-	+	-	-	-
DPSgMr10	+	+	+	+	-	+

Fig. 1

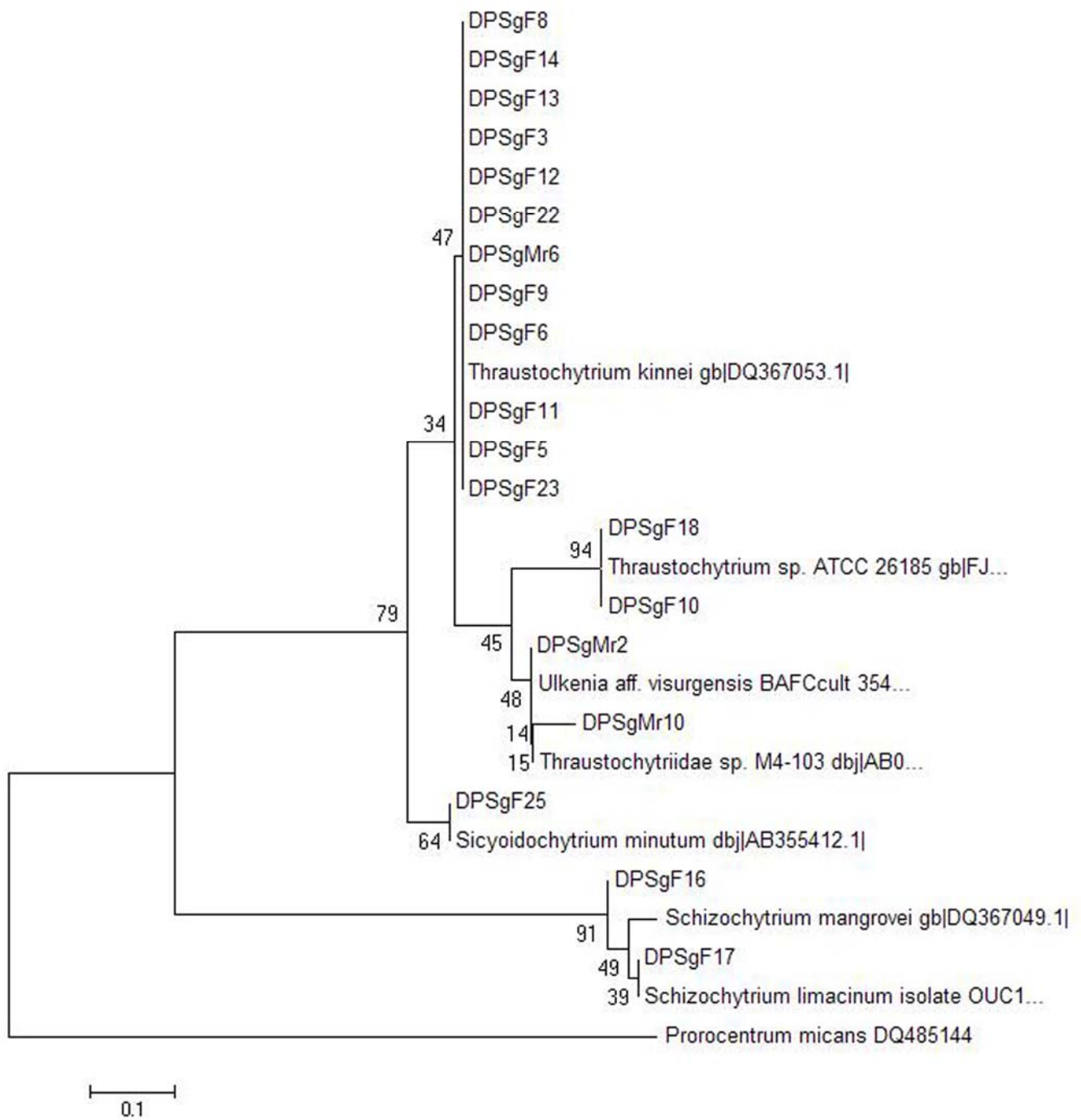


Fig. 2

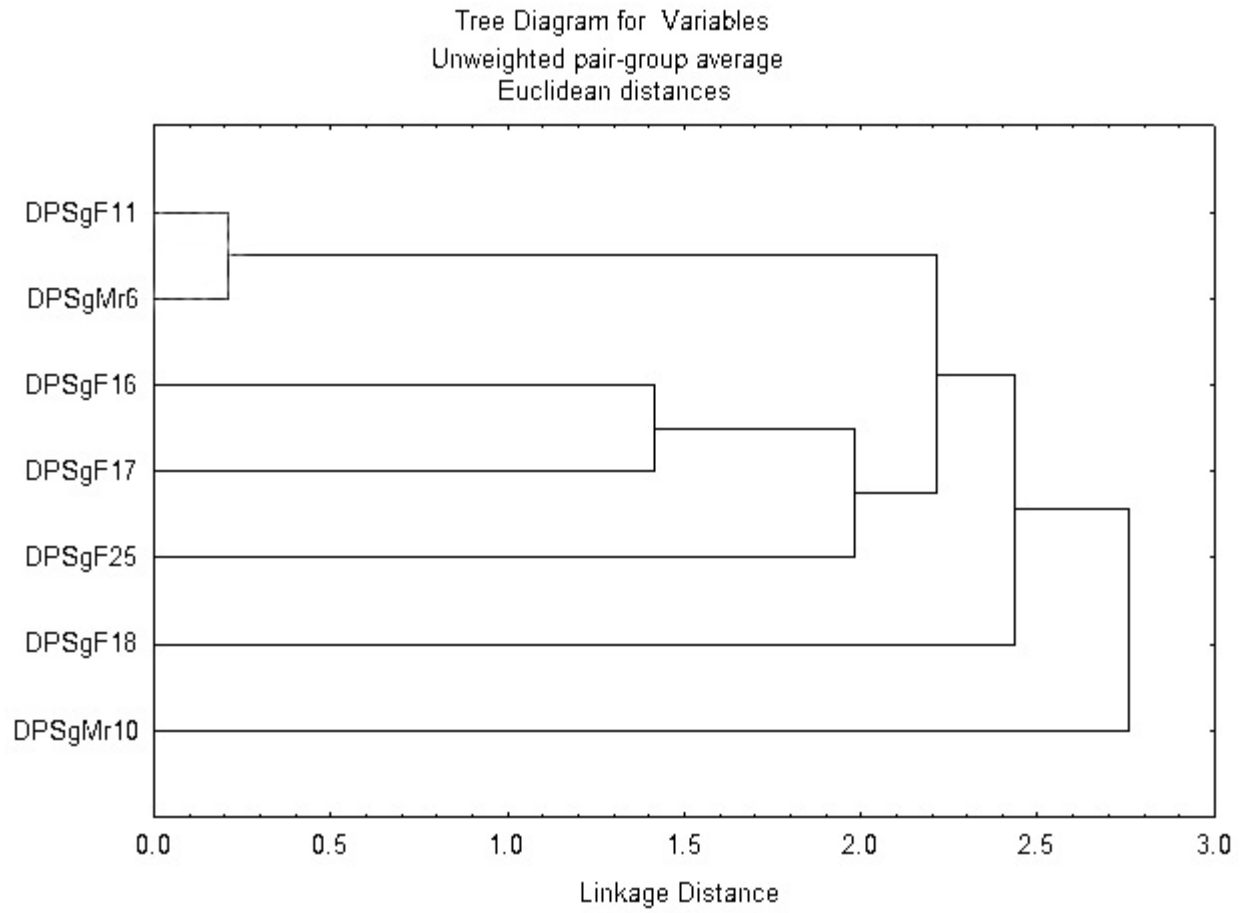


Fig. 3.

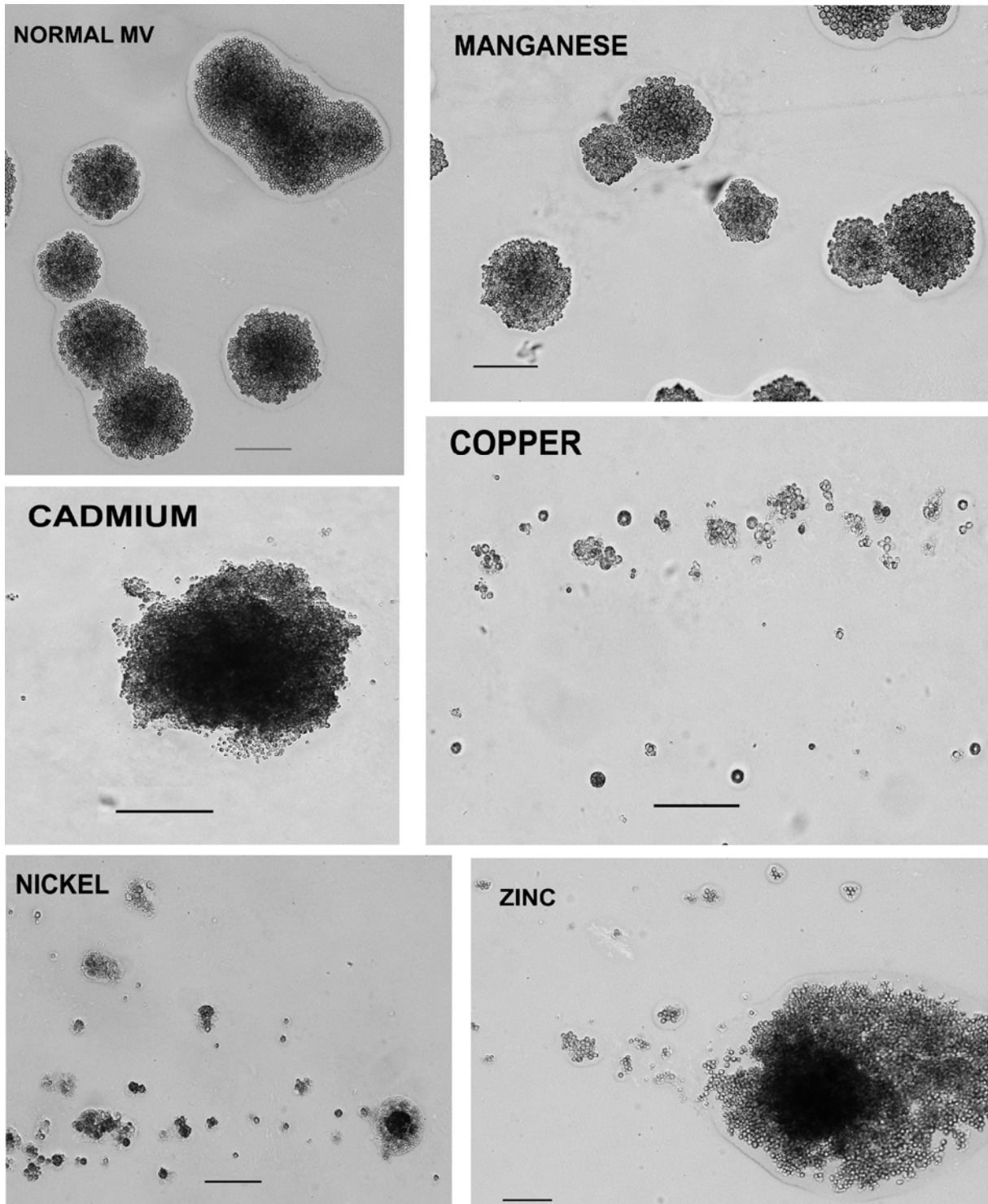


Fig. 4.

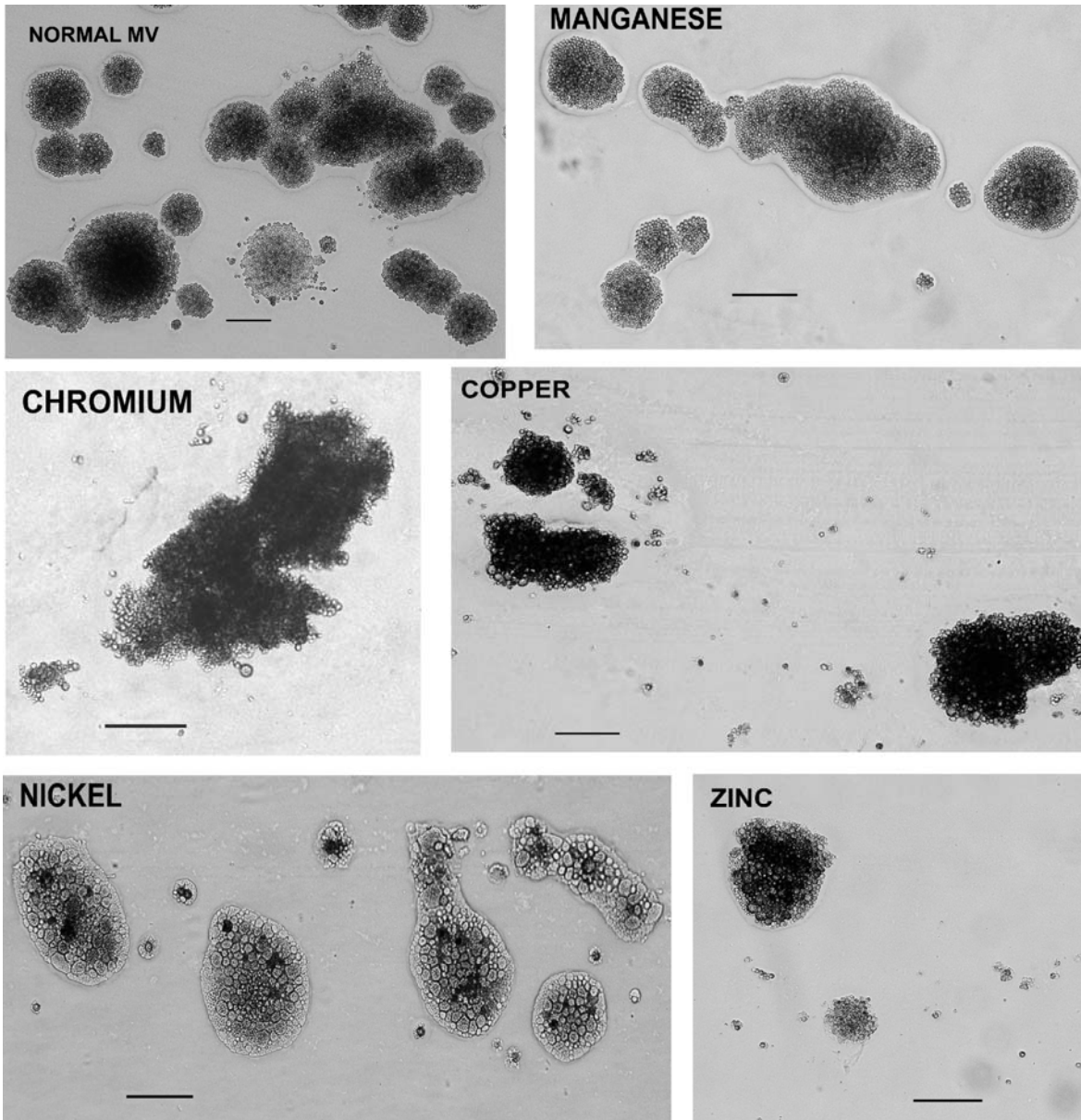


Fig. 5.

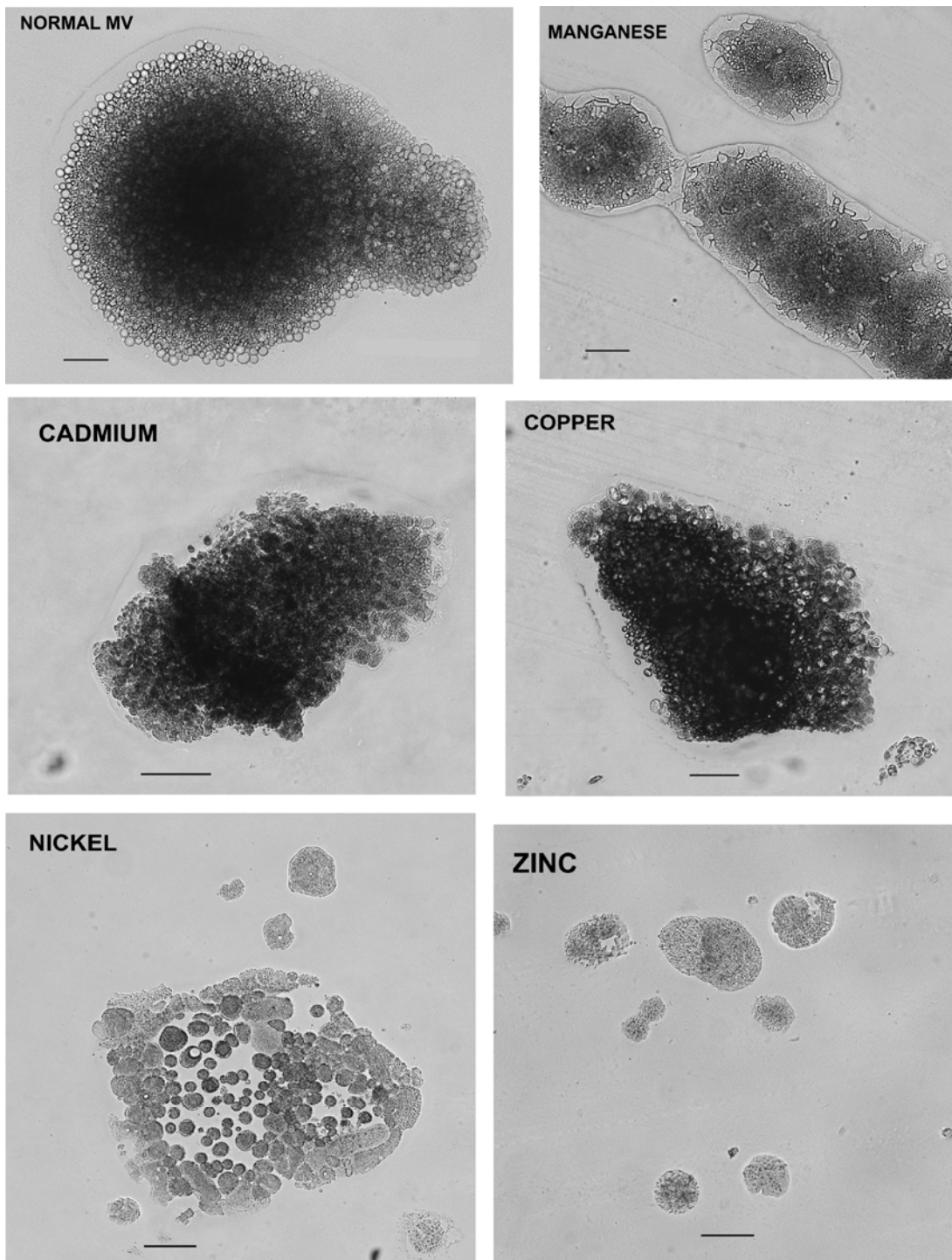


Fig. 6.

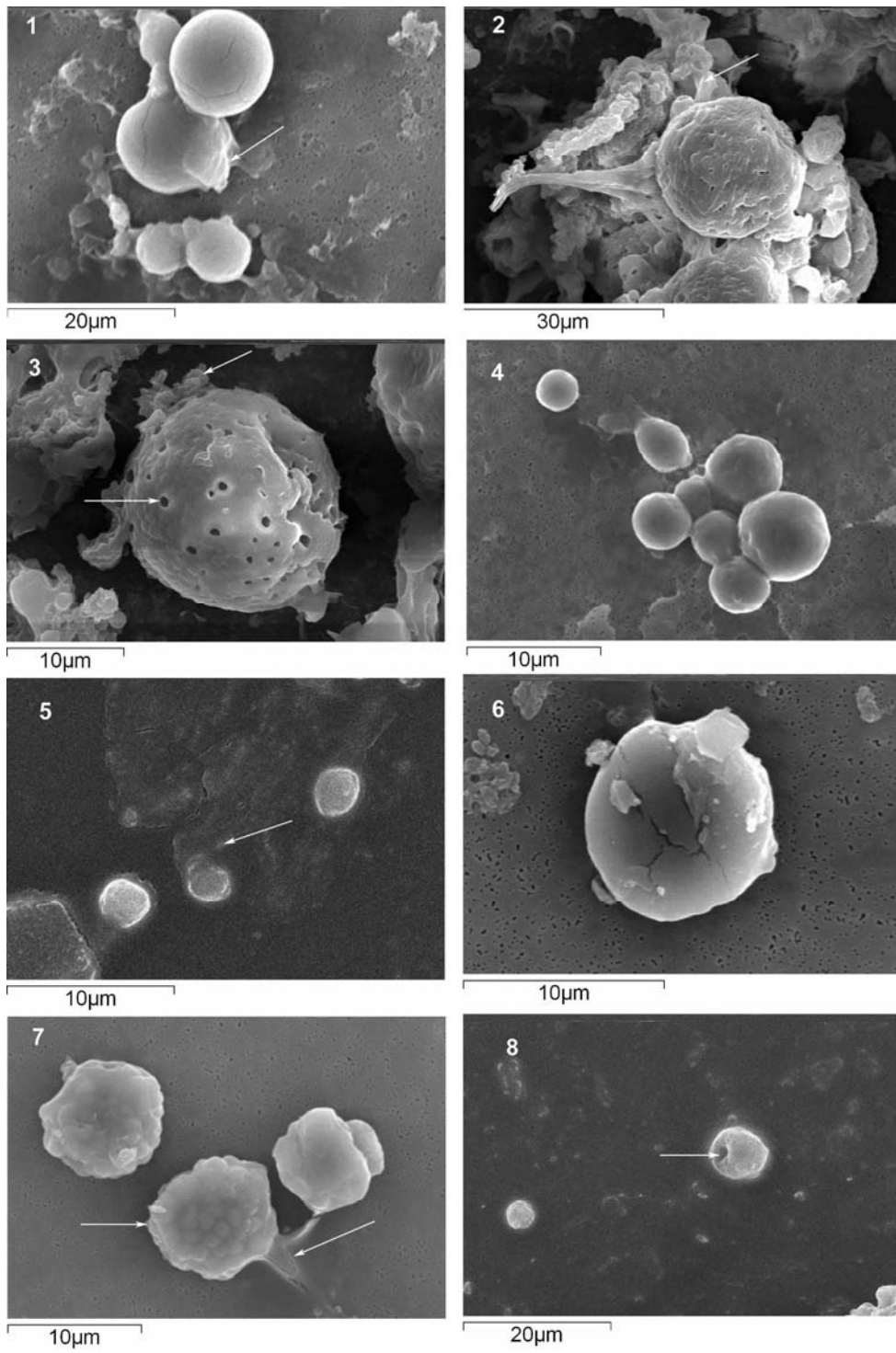


Fig. 7.

