Global Implications of the Nitrogen Cycle

Edited by

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CHAPTER 9

THE ROLE OF EUKARYOTIC MICROBES IN THE MARINE NITROGEN CYCLE

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Abstract

The marine nitrogen cycle is important ecologically as nitrogen is a nutrient required by phytoplankton, which as primary producers, form the basis of the marine food chain. Nitrate and nitrite ions are utilised as terminal electron acceptors for respiration by microorganisms during the unavailability of oxygen. The anaerobic processes of nitrogen transformation, such as denitrification and anammox are prolific in the oxygen minimum zones of the marine environment. Eukaryotic microorganisms or protists form a major part of picoplankton and nanoplankton in the pelagic water column of the oceans and seas. These also thrive and flourish in the marine sediments and are involved in various biogeochemical activities along with bacteria. These are known to be involved in the nitrogen cycle by various enzymes involved in denitrification, nitrification, and ammonification. The fungi, diatoms, dinoflagellates, and foraminifera are the most widely known eukaryotes to be involved in nitrogen cycling. These eukaryotic microorganisms belong to various supergroups in the tree of life and contribute to the marine nitrogen cycle in some way or the other. This chapter summarises the various eukaryotic microorganisms involved in the different processes of the nitrogen cycle, as well as those whose involvement is still ambiguous.

Introduction

The marine environment sustains nitrogen (N) for the survival and growth of organisms in the form of organic and inorganic N. Organic and inorganic N moves through the environment undergoing various biotransformations from one form to another, either to be assimilated by the organisms or to be lost to the environment. The latter can occur in many ways, one of which is via the transport of marine snow into the deep sea. Marine snow (marine aggregates present in the pelagic water column) constitutes a rich habitat for microbial growth including protists (eukaryotic unicellular microorganisms), and hence are deemed hotspots for microbial activity (Azam and Long 2001, 496; Glud et al. 2015, 2026). The diversity of protists inhabiting an aggregate and the spatial distribution of protists over an aggregate (surface and inner parts of the aggregate, and aggregate-water interphase) depend upon the substrate availability, and in turn, on the age of the aggregate (Artolozaga et al. 2000, 191, 193-94; Thornton 2002, 155-56). Marine aggregates and their plumes are rich sources of nutrients, owing to the continuous biological processes occurring within them. These processes involve decay of organic matter formed due to the death of organisms associated with the aggregates, as well as the release of dissolved organic matter (DOM) by the living organisms within the aggregate (Azam and Long 2001, 497). Thus, the labile DOM that is utilised by microorganisms gets converted into the semi-labile form and further into the recalcitrant form which is buried into the deep sea for thousands of years and reaches the surface of the ocean only during thermohaline circulation (Jiao et al. 2010, 593). The sinking of the recalcitrant organic matter in the form of aggregates results in the sequestration of organic carbon in the deep sea and also serves as a medium for the loss of N from the pelagic water column into the greater depths. Furthermore, the action of various microbes that convert the organic or inorganic nitrate (NO_3^-) into nitrous oxide (N_2O) or dinitrogen (N_2) via dissimilatory nitrate reduction to ammonium (DNRA) and denitrification, acts as another means for N loss through the water-air interface (Isobe and Ohte 2014, 4-12).

For decades it has been thought that the processes that sustain the N cycle in the marine environment were carried out only by prokaryotes. Bacteria are known to be involved in every biotransformation process that governs the N cycle: N fixation, ammonification, nitrification, assimilatory nitrate reduction, DNRA, denitrification, and anaerobic ammonium oxidation (anammox) (Thamdrup 2012, 411). Ammonium-oxidising archaea and denitrifying archaea have emerged as significant contributors of the marine N cycle and are responsible for the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and reduction of NO_3^- to N_2, respectively (Cabello et al. 2004, 3527, 3533-36). But the role of microeukaryotes in N cycling was not reviewed until their presence was detected in the oxygen-deficient zones of the marine water column which led researchers to speculate their probable involvement in the marine N cycle. Moreover, some of these harbour endosymbiotic N fixing prokaryotes that provide their eukaryotic host with bioavailable organic N (Kneip et al. 2007, 1).

Protists are an important component of the microbiota of the redoxclines, which form nitrate-sulphide interfaces at an intermediate layer in the water column possessing strong vertical redox gradients generated due to stratification which prevents the mixing of the upper oxygenated and anoxic bottom waters (Hannig et al. 2007, 1336, Wylezich and Jürgens 2011, 2939). This has been proved by classical microscopy and 18S rDNA surveys. These eukaryotes regulate the abundance and productivity of the prokaryotes in these regions (Anderson et al. 2013, 1580). They form an important constituent of the biota in marine waters representing almost half of the biomass in surface waters and about a quarter in subsurface waters (Fukuda et al. 2007, 203; Suttle 2007, 803). These microorganisms, though collectively commonly known as protists or protozoa, fall into various supergroups in the tree of life (Adl et al. 2012, 429-93). The classification of these microorganisms into different eukaryotic supergroups is under continuous revision, mainly due to their diverse characteristics (Adl et al. 2012, 429; Pawlowski 2013, 40; Burki 2014, 1). An overview of their classification is shown in Figure 9-1 (Adl et al. 2012). The intracellular storage of NO_3^- has been observed in many of the microeukaryotes, such as foraminifera, gromiids, diatoms, dinoflagellates, fungi, haptophytes, and chlorophytes. The intracellular concentration of NO_3^- even exceeds the concentration in the surrounding pore water (Kamp and Stief 2017, 1). More than 60 mmol L^{-1} NO_3^- is accumulated by free-living, as well as aggregate-associated diatom *Skeletonema marinoi* (Kamp et al. 2016, 1). Foraminifera store up to 463 mM, gromiids up to 567 mM, haptophytes up to 14 mM, chlorophytes up to 4.9 mM, dinoflagellates up to 1.8 mM, and fungi up to 0.4 mM NO_3^- intracellularly. The NO_3^- uptake rates in six different species of diatoms and dinoflagellates range from 18 to 310 fmol $cell^{-1}$ h^{-1} at ambient NO_3^- concentrations of < 40 µM (Lomas and Gilbert 2000, 905). This intracellular NO_3^- is used up when denitrification is triggered due to anoxic conditions. Diatoms also utilise NO_3^- for assimilatory NO_3^-

reduction (Lomas and Glibert 2000, 909; Kamp et al. 2011, 5649-50). The intracellular NO_3^- is either reduced nutritionally for growth and biosynthesis, or non-nutritionally releasing NO_2^-, NH_4^+, or dissolved organic nitrogen (DON) in the surrounding waters (Lomas and Gilbert 2000, 908). Denitrification in these systems is generally mediated by the bacterial endosymbionts, mostly the Gamma-Proteobacteria, within these eukaryotes (Bernhard et al. 2004, 955). Nitrate respiration in eukaryotes was first reported in a protozoan *Loxodes* found in freshwater lakes. This was catalysed by the nitrate reductase (NAR) enzyme found in their mitochondria. Certain diatoms and dinoflagellates also accumulate NH_4^+ intracellularly (Lomas and Gilbert 2000, 906).

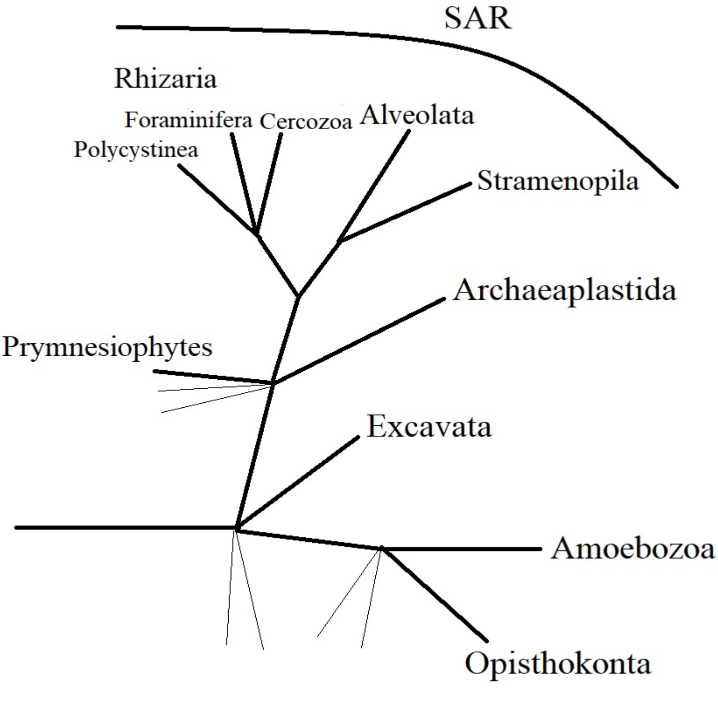

Figure 9-1: The tree of life for the protists as modified from Adl et al. (2012). The thick lines show the supergroups and the groups within. The thin lines are the *incertae sedis* groups as reported by Adl et al. (2012). The tree only shows phylogenetic placement irrespective of the time of evolution. Hence the length of the lines may be ignored.

The contribution of microorganisms belonging to the various eukaryotic supergroups to the N cycle is discussed further (Burki 2014, 1) (Tables 9-1 and 9-2).

Opisthokonta

This supergroup consists of choanoflagellates, ichthyosporeans, microsporidians, and fungi. Choanoflagellates are heterotrophic nanoflagellates (HNF) that are bacterivorous in nature. These are the normal inhabitants of the oxic water column (Vørs et al. 1995, 590, 598; López-García et al. 2001, 604; Leakey et al. 2002, 333), but of late, are found in hypoxic and sulphidic water masses too (Wylezich and Jürgens 2011, 2939; Wylezich et al. 2012, 1). Earlier, these had been detected by targeted environmental sequencing in the oxygen minimum zones (OMZ) of the Arabian Sea (Jebaraj et al. 2010, 406). Ichthyosporeans normally inhabit the digestive tract of marine invertebrates and are also known to be pathogenic to fishes. These can survive under anoxic conditions owing to the presence of the *pfl* gene which codes for an oxygen-sensitive enzyme, pyruvate formate lyase, which generates formate and acetyl-CoA non-oxidatively from pyruvate and CoA. Microsporidians are obligate intracellular parasites of both marine and freshwater organisms. They infect organisms while switching between habitats, such as fish, crustaceans, nematodes, etc. from shallow sediments to methane seeps in the deep sea (Ironside et al. 2008, 355; Ardila-Garcia and Fast 2012, 1544; Sapir et al. 2014, 1; Stentiford et al. 2016, 336). These amitochondriate microorganisms are characterised by the presence of a highly reduced organelle, the mitosome that is devoid of enzymes for oxidative phosphorylation and the Kreb's cycle thus, rendering these microorganisms anaerobic (Burri et al. 2006, 15916). Microsporidians are now classified under Phylum Microsporidia of Kingdom Fungi.

Fungi are ubiquitous in the terrestrial environment and are now also known to be occurring in the freshwater and marine environments. These saprophytes can survive harsh conditions by their spores. Marine environments harbour yeast, as well as filamentous forms of these microorganisms (Raghukumar 2006, 388, 396; Kutty and Philip 2008, 465). They are also found in the OMZ of the water column and hence thought to be playing a role in denitrification (Cathrine and Raghukumar 2009, 100). Fungi are capable of reducing NO_2^- to NO (nitric oxide), N_2O, and N_2 under varying oxygen levels (Shoun et al. 1992, 277). This is mediated by *nirK* [Cu containing nitrite reductase (NIR)] and *p450nor* [cytochrome P450 nitric oxide reductase (NOR)] located within the mitochondria (Shoun et al. 2012, 1186). The genes for these denitrifying enzymes were first identified in *Fusarium oxysporum* and *Cylindrocarpon tonkinense* (Morozkina and Kurakov 2007, 544). Fungi also carry out ammonia (NH_3) fermentation during complete anaerobic conditions (Zhou et al. 2002, 1892) which is similar to the DNRA pathway seen in some bacteria (Giblin et al. 2013, 124). The NAR in fungi is similar to that of *E. coli* and other denitrifying bacteria and is also involved in the DNRA pathway (Morozkina and Kurakov 2007, 546). Many denitrifying fungi have the capacity for co-denitrification, that is, to produce N_2 or N_2O from NO_2^- along with other N substrates under denitrifying conditions. Nitrogen is produced from amines, and N_2O is produced from imines or azide (Shoun et al. 2012, 1188).

Table 9-1: The various pathways of the nitrogen cycle observed in different eukaryotic groups

Supergroup	Pathway	Examples (with significance)
Opisthokonta	Denitrification, DNRA, Co-denitrification	Fungi (saprophytes, pathogens)
Amoebozoa	Ammonia oxidation, Denitrification	Amoebae (shape microbial communities)
Stramenopiles (of SAR)	Intracellular nitrate storage, DNRA	Diatoms (phytoplankton)
	Nitric oxide formation from nitrite, Denitrification	Oomycetes (infectious zoosporic organisms)
Alveolates (of SAR)	Nitrate assimilation in cytosol, Nitrate reduction	Dinoflagellates (causal agents of harmful algal blooms-HABs)
Rhizaria (of SAR)	Store nitrate intracellularly, Denitrification by endobiotic bacteria	Gromiids under Cercozoa (dominant component of benthos in the deep sea)
	Denitrification	Radiolarians, Foraminifera (important for micropaleontology studies)

Table 9-2: The various pathways of the nitrogen cycle proposed in different eukaryotic groups

Supergroup	Group	Evidence	Probable pathway	Examples
Opisthokonta	Choanoflagellates	Hypoxic, sulphidic waters; Fewer mitochondria with tubular cristae, use alternate electron acceptors (fumarate, nitrate)	Denitrification	*Monosiga, Choanoeca, Diaphanoeca, Codosiga*
	Ichthyosporeans	Survive anoxic conditions due to pyruvate formate lyase	Denitrification	*Pseudoperkinsus, Sphaeroforma, Anurofeca, Ichthyophonus, Amoebidium*
	Microsporidians	Presence of mitosome	Denitrification	*Microgemma, Microsporidium, Thelohania, Nadelospora, Perezia*
Excavata	Euglenozoa, kinetoplastids, bodonea, heteroloboseids	Presence of hydrogenosomes	Denitrification	*Euglena, Trypanosoma, Naegleria, Pharyngomonas*
Archaeaplastida	Prasinophytes	Assimilate ammonia through multiple ammonium transporters	Ammonification	*Micromonas*
Stramenopiles	Oomycetes	Nitric oxide formation by nitrite reductase	Denitrification	*Eurychasma, Ectrogella, Lagenisma, Pythium*
	Raphidophytes	Blooms lead to oxygen depletion	Denitrification	*Heterosigma, Chattonella*
	Labyrinthulomycetes	Anoxic waters and suboxic to anoxic sediments	Denitrification	*Schizochytrium, Thraustochytrium, Aplanochytrium, Labyrinthula, Aurantiochytrium*
	Bicosoecids	Oxygen-depleted waters and sediments; synthesise ectoine that accelerates denitrification	Denitrification	*Cafeteria, Halocafeteria, Caecitellus, Rictus*
Prymnesiophytes	Coccolithophores, haptophytes	Harmful blooms lead to loss of oxygen and sediment nitrate	Denitrification	*Emiliana, Phaeocystisi*

Amoebozoa

Organisms belonging to this supergroup are found in the NH_3-oxidising activated sludge systems and hence, can carry out the process of NH_3 oxidation (Moreno et al. 2010, 2203, 2205). The gene involved in denitrification, *nirK*, has been identified in organisms belonging to Amoebozoa. Genus *Hartmannella* is a widespread organism in anoxic sediments of waterlogged agricultural fields (Wang et al. 2017, 2). These organisms are thus, quite likely to be involved in denitrification and NH_3 oxidation in anoxic marine systems like the OMZ.

Excavata

The heteroloboseids in this supergroup effectively switch between the amoeba and flagellate stages. Some of them are obligate flagellates. This group generally consists of aerobic organisms but also comprises obligately anaerobic or microaerophilic organisms. These can be found in brackish habitats and inland salt marshes (Pánek et al. 2014, 2281). This supergroup includes euglenozoa, kinetoplastids, bodonea, and other flagellates which are either, free-living in freshwater and marine habitats or occur as parasites or symbionts to marine organisms/vertebrates. The organisms possess hydrogenosomes which are membrane-bound organelles similar to mitochondria. Hydrogenosomes produce hydrogen and ATP by substrate-level phosphorylation and help generate energy under anaerobic conditions, (Mentel and Martin 2008, 2719).

Archaeaplastida

This group comprises plants, green algae, and red algae. The sequences belonging to protists in this supergroup have been identified by metagenomic analyses of samples from extreme habitats, such as the subglacial lakes at Antarctica, and the glacial cryoconite holes in the Arctic glaciers and Antarctica (Cameron et al. 2012, 254; Rogers et al. 2013, 629). In addition to the sequences of these protists, the sequences belonging to nitrifying, NO_3^- reducing, and anammox bacteria were also revealed, indicating the occurrence of these activities in that environment. *Micromonas pusilla* is the picoeukaryotic green alga (0.2-3 µm cell size) under prasinophytes belonging to this supergroup. It is one of the most abundant picoplankton found in the surface waters of oceans worldwide and more often in the temperate coastal environments (Vaulot et al. 2008, 808). Their chloroplasts possess a peptidoglycan cell wall (Van Baren et al. 2016, 2) and they show a mixotrophic mode of nutrition during conditions of low nutrient availability (McKie-Krisberg and Sanders 2014, 1953). A *Micromonas*-like genome was found to be associated with the *Phaeocystis* blooms in the Amundsen Sea, Antarctica and these were found to assimilate NH_3 through multiple NH_4^+ transporters almost twice as much as that of NO_3^- or urea (Cochlan and Harrison 1991, 129; Delmont et al. 2015, 1).

SAR (Stramenopiles, Alveolates, and Rhizaria) group

Stramenopiles

This group was formerly classified as Kingdom Chromista and includes diatoms, brown algae, golden algae, oomycetes, raphidophytes, and labyrinthulomycetes. Brown algae and golden algae are multicellular seaweeds seen on tropical and temperate beaches. During certain stages of their growth, they occur as unicellular forms, such as spores or gametes that spread throughout the water.

Diatoms

These form the basis of the marine food chain owing to their ability to carry out primary production and are thus, important ecologically (Fig. 9-2). Therefore, they are found abundantly in surface waters to fulfil their requirement of sunlight. They also occur in large numbers in the subsurface region forming the secondary chlorophyll maxima. Oxygen minimum zones prevail below the photic zone where diatoms would have been thought not to exist due to the absence of light which is the essential requirement for primary production. But diatoms are found in many OMZ all over the world (McCreary Jr. et al. 2013, 15; Parris et al. 2014, 4-5). These tend to form blooms in the surface waters during favourable conditions, such as the abundance of nutrients, favourable light, and temperature. When these blooms senesce, their cells sink and are exposed to decay. Therefore, they also form an important component of marine snow (Thornton 2002, 149). Fossilised diatoms are used to construct the paleoclimate and are also found in the polar region (Miettinen et al. 2018). Diatoms store NO_3^- intracellularly as they grow to use it up later in case of stress conditions (Kamp et al. 2011, 5649). Stress could be due to the depletion of surface nutrients leading to the decay in phytoplankton blooms and thus, their sinking to the deep. As the diatom cells start sinking, they pass from the surface oxic waters to suboxic and anoxic waters. Their transition between these waters also includes phases of darkness. Such stress conditions force their conversion to the resting stage to survive. In this instance, they use up the intracellular NO_3^- pool to sustain cell metabolism during the transition and temporary stay in anoxic layers. The diatoms are also capable of DNRA (Kamp et al. 2013, 1) which was earlier thought to be an exclusively bacterial process. This may be possible due to the incorporation of prokaryotic genes within the diatom genome by horizontal gene transfer (Bowler et al. 2010, 333, 337). The DNRA activity of diatoms also releases NH_4^+ in the OMZ. This NH_4^+ in the anoxic waters is the substrate for anammox which is responsible for up to 50% of the N loss from the ocean (Kamp et al. 2016, 1, 4; Stief et al. 2016, 2, 8).

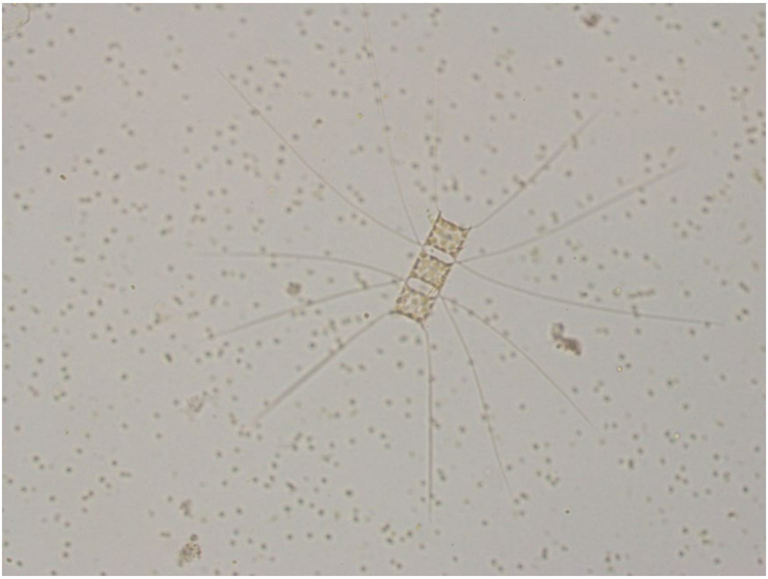

Figure 9-2: A microphotograph of the diatom *Chaetoceros* sp. as viewed under a bright field microscope with a 10X magnification. (Picture courtesy: Dr. Ravidas Naik)

Oomycetes

These are the water molds that cause infection in fish, crustaceans, nematodes, and algae (Beakes 2009, 11, 15, 16; Strittmatter et al. 2011, 259). These produce NO as an offensive strategy in plants during an infection (Arasimowicz-Jelonek and Floryszak-Wieczorek 2016, 1). The NO leads to plant cell death and accelerates the spread of infection. The production of NO is known to occur from NO_2^- by the action of NIR produced by the *nir* gene (Arasimowicz-Jelonek and Floryszak-Wieczorek 2014, 407). Cell lysis during viral infections or natural death may release this NO in the environment to be utilised by other microorganisms. Under anoxic or suboxic conditions, these organisms may also cause complete denitrification to N_2.

Raphidophytes

These are unicellular algae found in marine and freshwater systems. Some species produce blooms on surface waters. They contribute to the red tides and subsequently, loss of fish populations (Padmakumar et al. 2012a, 1). Examples include *Heterosigma akashiwo* and *Chattonella marina*. These blooms deplete the oxygen in the underlying waters forcing these microeukaryotes to use endogenously produced electron acceptors, such as fumarate (Müller et al. 2012, 446-453). These organisms may also utilise NO_3^- as a terminal electron acceptor in the absence of oxygen, a process not yet investigated in them.

Labyrithulomycetes

These protists are known to be obligately marine and aerobic (Fig. 9-3). However, environmental sequencing has revealed their occurrence in the anoxic Cariaco Basin (off the north-central coast of Venezuela) at 270 and 340 m depth and in the anoxic Berkeley Aquatic Park, California (Stoeck et al. 2003, 5661-62; Danovaro et al. 2011, 8325). Recently, they were isolated from the sediments of the Arabian Sea where the dissolved oxygen concentrations approached near-zero levels. These occurred as frequently as 6-81% in the suboxic to anoxic sediments (Cathrine and Raghukumar 2009, 102). These sediments were identified to harbour anaerobic denitrification by fungi. Hence, it could be speculated that these protists too may have the capability to carry out denitrification to survive in such regions. These protists are generally called thraustochytrids, with labyrinthulids and aplanochytrids as their sister groups. These characteristically produce polyunsaturated fatty acids especially ω-3 docosahexanoic acid (DHA) and eicosapentanoic acid (EPA), intracellularly, and in large amounts. Since the fatty acid biosynthetic pathways require oxygen, the presence of these organisms in regions devoid of oxygen, was mystifying. It is now known that the synthesis of polyunsaturated fatty acids (PUFA) occurs by two distinct pathways, one aerobic and the other anaerobic involving PUFA synthase enzyme (Matsuda et al. 2012, 1210). Hence these organisms can survive anaerobic conditions.

Figure 9-3: A microphotograph of a thraustochytrid isolate grown on an agar medium (Modified Vishniac medium). The photograph shows spherical cells of 5-15 µm size and with biflagellate zoospores (marked by a black arrow). The zoospores are ellipsoidal to oval in shape depending on the plane in which they were captured during their flagella-aided motion. The thraustochytrid cells produce rhizoid-like structures called ectoplasmic net elements (marked by a white arrow) which are extensions of the plasma membrane. These structures aid in the attachment to the substratum and the secretion of extracellular enzymes and are a characteristic feature of Labyrinthulomycetes.

Biocosoecids

These are heterotrophic nanoflagellate voracious bacterivores and include *Cafeteria*, *Halocafeteria*, *Caecitellus*, and *Rictus*. These are mostly found in the coastal waters, hypersaline habitats, and sediments with low oxygen (Park and Simpson 2010, 1173; Yubuki et al. 2010, 264; Raja et al. 2017, 266). Their sequences have also been identified from the oxygen-depleted waters of various estuaries and fjords (Zuendorf et al. 2006, Kolodzieg and Stoeck 2007, 2723; 485; Stoeck et al. 2010, 27). Their presence in anoxic waters and their tendency to phagocytose bacteria may, therefore, prove important in N cycling in such anoxic regions which harbour various nitrifying/denitrifying bacteria as these may fall prey to the bicosoecids thus, causing an imbalance in the cycle. They produce ectoines which act as stress protectants and as nutrients that mediate various ecophysiologically important processes in food webs when released in the environment (Czech et al. 2018, 39, 42).

Alveolates

Dinoflagellates, which are one of the main players of the harmful algal blooms, belong to this group (Fig. 9-4). They take up NO_3^- and store it in the cytosol, as well as reduce it via NAR (Dortch and Maske 1982, 299). They never fall devoid of N due to diurnal vertical migration. In the presence of NH_4^+, they do not take up NO_3^- (Dagenais-Bellefeuille and Morse 2013, 4).

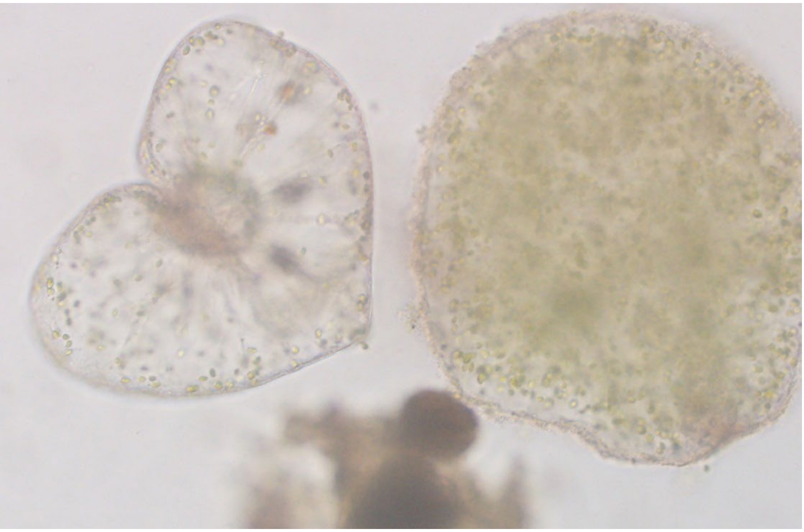

Figure 9-4: A microphotograph depicting the aboral side view and the top view of the dinoflagellate *Noctiluca* sp. as observed under a bright-field microscope with a 10X magnification. (Picture courtesy: Dr. Ravidas Naik)

They form symbiotic associations with corals, to whom they supply N rich organic matter fixed autotrophically within their cells. They also absorb the N lost by the animal host. These endosymbionts, also called zooxanthellae thus, drive the microscale N cycle in the reef ecosystem (Tanaka et al. 2018, 1). Warm temperatures result in their expulsion from the corals causing coral bleaching and considerable loss of the N reserves from the ecosystem (Tanaka et al. 2018, 7). Some dinoflagellates also live in association with denitrifying bacteria, such as *Labrenzia* which possess *nos* genes for nitrous oxide reductase (NOS) (Wyman et al. 2013, 2670-72). Phylum Chromerida also includes photosynthetic protozoa that are endosymbiotic within a few corals and hence, may play a role in the N cycle (Cumbo et al. 2013, 237).

Rhizaria

This group of SAR includes cercozoans, radiolarians, and foraminifera (Fig. 9-5). Sequences of cercozoans which include amoebae and flagellates have been observed in the anoxic basins of Gotland Deep in the central Baltic Sea, the Mediterranean Sea, and the OMZ of the Arabian Sea (Marie et al. 2006, 403; Cathrine and Raghukumar 2009; Stock et al. 2009, 267, 273-278). Hence, they are likely to carry out denitrification under anoxic conditions. The gromiids under Cercozoa store NO_3^- intracellularly and possess endobiotic bacteria that carry out denitrification (Høgslund et al. 2017, 1). These resemble the foraminifera in producing organic tests (hard external shells). The radiolarians consisting of Polycystinea and Acantharea form the majority of microfossils; the former containing silica and the latter a strontium sulphate skeleton. Their tests are a robust source of organic N and thus, N isotope studies of these microfossils help to extract the palaeoclimate which reflects the prevailing conditions at the sea surface during the period of their survival. Based on such studies the radiolarians were thought to have witnessed denitrification in the equatorial Pacific and the Southern Ocean in the past (Robinson et al. 2007, 201; Robinson et al. 2015, 912). Benthic foraminifera are also important in palaeoceanographic studies due to their high potential for fossilisation and the presence of calcareous tests. They occur in a wide range of oxygen concentrations including OMZ and anaerobic conditions. The varying oxygen levels determine their varying distribution (Panchang et al. 2006, 235). They are also infaunal and show higher assimilation within the sediments on par with the meiofauna under suboxic conditions.

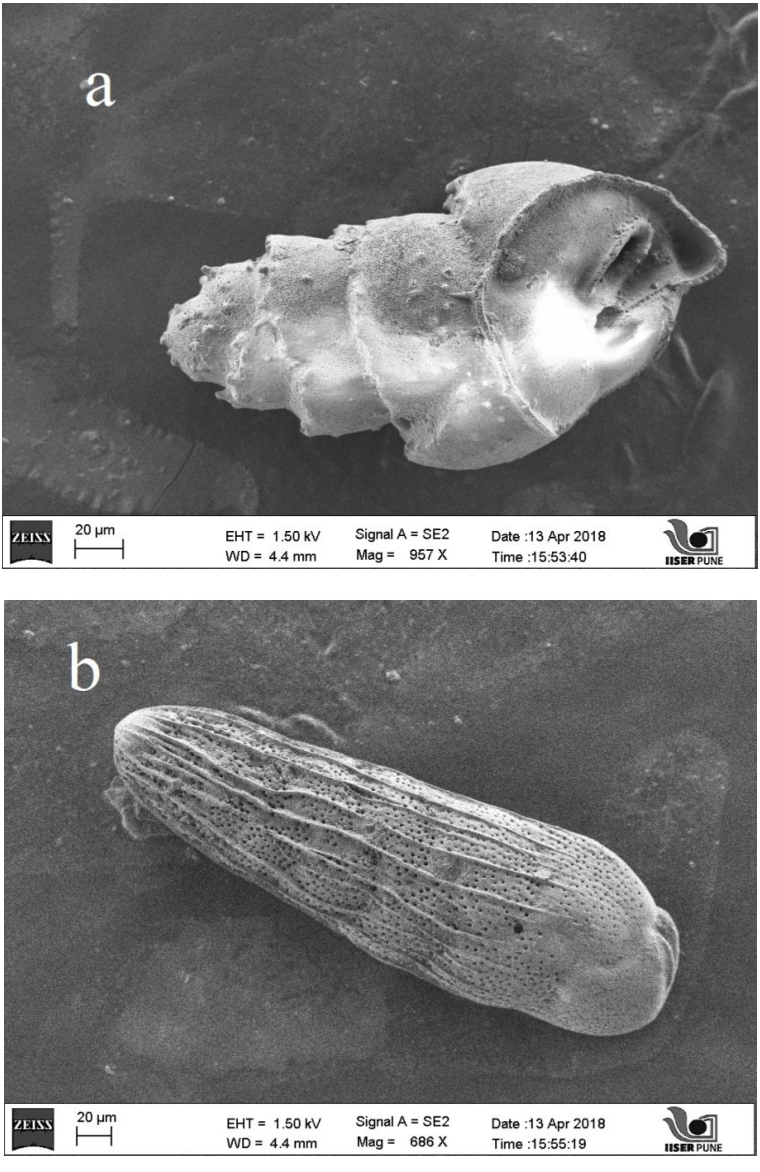

Figure 9-5: Scanning electron microphotographs of foraminifera (a) *Bulimina marginata* and (b) *Brizalina striata*. (Picture courtesy: Dr. Rajani Panchang)

They were previously not known to tolerate anoxic conditions. But, of late, they have also been found to survive in anoxic-dysoxic environments by respiring NO_3^- and forming N_2 by denitrification (Piña-Ochoa et al. 2010a, 1148, 1150). Their denitrification rates were found to be very significant in the Peruvian OMZ. Up to 50% of benthic denitrification was attributed to these organisms in certain regions in the OMZ (Glock et al. 2013, 4767, 4772). *Globobulimina pseudospinescens* was the first foraminifer to be found to carry out denitrification. This species produces a shell with multiple chambers and hence deemed multilocular. *Allogromid foraminifera* are unilocular tectinous species that also perform denitrification. They are known to store NO_3^- intracellularly, either transporting from the outside environment or producing it intracellularly (Risgaard-Petersen et al. 2006, 94; Bernhard et al. 2012, 967). Recently, it has been discovered that they survive anoxic-dysoxic conditions by their symbiotic association with denitrifying bacteria, archaea, or kinetoplastidy (by sequestering algal plastids). These even calcify in an anoxic environment (Nardelli et al. 2014, 4029; Meilijson et al. 2016, 78). The denitrification ability of the foraminifera is attributed to their endobionts and this was identified by GeneFISH using the *nirK* probe. The number of endobionts required to give rise to measurable denitrification rates is in the order of 6-23 x 10^3 cells (Risgaard-Petersen et al. 2006, 94; Bernhard et al. 2012, 964-968).

Prymnesiophytes

Also called haptophytes, these organisms are responsible for harmful ocean blooms which adversely affect marine productivity and consequently, the global carbon cycle and climate. The blooms of *Phaeocystis* spp. are particularly a nuisance due to the ensuing foam formation on the beach. *Emiliana huxleyi* is a coccolithophore having a calcium carbonate skeleton, whose blooms are so big that they can be seen from space. The blooms of both these organisms are significant from an ecological point of view because as the blooms grow they block sunlight penetration and cause oxygen depletion, thereby instigating a series of events leading to eutrophication and subsequent death of the water body. *E. huxleyi* has been found to utilise N sources other than NO_3^-, such as amino acids, amides, urea, purines (like hypoxanthine), and especially NH_4^+ produced by the blooms and hence, can bloom even in NO_3^--limited waters (Lessard et al. 2005, 1020-22). *Phaeocystis* spp. and *E. huxleyi* both affect the sulphur cycle due to the production of dimethylsulphoniopropionate (DMSP) which is converted to dimethyl sulphide (DMS). DMS is volatile and cloud-inducing. Moreover, *E. huxleyi* causes an upsurge in calcium carbonate deposition in sediments. Over the past millions of years, this has given rise to chalk cliffs, such as those seen on the island of Rügen in the Baltic Sea (Tyrrell et al. 2008, 486). When these blooms collapse due to viral infection, they significantly affect the marine ecosystem because of the increase in microbial activity which depletes the oxygen in the water column (Danovaro et al. 2011, 997-1000). The formation of suboxic to anoxic conditions during bloom decay may prove important to the N cycle. Under the conditions of N depletion, the production of *E. huxleyi* viruses is delayed and the burst size of *P. pouchetii* viruses is consequently reduced (Danovaro et al. 2011, 1001). The loss of sediment NO_3^- due to denitrification is a common phenomenon as *P. pouchetii* competes with the spring diatom blooms (Jiang et al. 2014, 36-37).

The contribution of microeukaryotes to the major processes in the nitrogen cycle

Ammonification

Ammonia is found to be assimilated by organisms belonging to Archaeaplastida through multiple NH_4^+ transporters. The DNRA activity of the diatoms also gives rise to NH_3 from NO_3^- in the water column. Nitrogen fixation also gives rise to NH_4^+.

Nitrogen fixation

The colony-forming cyanobacteria fix atmospheric N_2, and 50% of the nitrogen is released as NH_4^+ in the surrounding water and transferred to the diatoms and copepods. This transfer takes place in a short turnover time. Thus, the newly fixed N may reach the shallow sediments by fast export through faecal pellets and marine snow. Thus, cyanobacteria may contribute to the transfer of N into the food web and impact the biogeochemical processes in short time scales (Adam et al. 2016, 450, 457).

Solenicola setigera is a stramenopile living in a symbiotic association with the diatom *Leptocylindrus mediterraneus* in the open ocean waters and even in suboxic waters (Padmakumar et al. 2012b, 97). The cyanobacterium, *Synechococcus,* which also fixes N_2, forms an endosymbiotic relationship with the stramenopile *Solenicola* (Carpenter and Foster 2007, 14). The cyanobacterium, *Richelia intracellularis* is symbiotic with the diatoms *Rhizosolenia* and *Hemiaulus* and gets passed on to the next generation of the host during the formation of auxospores, which are specialised cells for the enlargement of vegetative cells that usually have undergone size reduction due to successive mitotic divisions (Villareal 1989, 357, 361). *Calothrix* is symbiotic and attaches externally to *Chaetoceros* (Hilton et al. 2013, 2, 4). The cyanobacterium *Cyanothece* is an endosymbiont of the diatom *Rhopalodia gibba* (Kneip et al. 2008, 2). Thus, the symbiotic diatom populations are an equally important source of NH_4^+ as the free-living colonial cyanobacteria (Foster et al. 2011, 1489-91). Dinoflagellates, such as *Ornithocercua*, *Histoneis*, and *Citharistes*, possess cyanobacterial symbionts. The radiolarian *Dictyocoryne truncatum* also possess cyanobacterial symbionts (Carpenter and Foster 2002, 15). The uncultured N fixing unicellular cyanobacterium group A (UCYNA) grows endosymbiotically within many prymnesiophytes, such as *Braarudosphaera bigelowii* and *Chrysochromulina parkeae* (Hagino et al. 2013, 1).

Nitrification

Fungi possess the nitric oxide dioxygenase gene (*nod*) which converts NO back into NO_3^- in their cytoplasm, which then enters the mitochondria where it is reduced to NO_2^- (Morozkina and Korokov 2007, 547). Many protists, such as the diatoms, dinoflagellates, radiolarians, and foraminifera store NO_3^- intracellularly via assimilation from the environment or an internal nitrification process.

Dissimilatory nitrate reduction to ammonium

Fungi convert NO_3^- to NH_4^+ by DNRA. The process involves the reduction of NO_3^- to NO_2^- by the *nar* genes followed by the reduction to NH_4^+ by the *nrfA* gene. In addition to fungi, the diatoms also reduce intracellular NO_3^- and produce NH_4^+ by the dissimilatory pathway.

Denitrification

Denitrification is a very common phenomenon carried out by many eukaryotes. It is an anaerobic process and hence, takes place in the absence of oxygen as organisms utilise alternate sources of electron acceptors, such as NO_3^- or NO_2^-. Although N_2O is formed as an intermediate, the end product of denitrification is N_2. Microeukaryotes of different groups are found to be involved in this process. Fungi produce all the enzymes involved in denitrification, such as NAR, NIR, NOR, and NOS (Morozkina and Kurakov 2007, 544-46). They are also involved in the co-denitrification process from amines and imines (Shoun et al. 2012, 1188, 1191). Amoebae under Amoebozoa also possess genes for denitrification but they are not as significant as the bacterial or fungal denitrifiers. The diatoms have an intracellular stock of NO_3^- which they reduce during harsh conditions necessary for their survival, such as those experienced in anoxic or suboxic environments. Diatoms reduce NO_3^- via DNRA. Thus, though denitrification by diatoms occurs under anoxic conditions, it is not very significant as compared to bacteria or fungi. Diatoms generally have shown to have either no or little effect on denitrification in microcosm experiments and very rarely, they enhance denitrification (Stock et al. 2014, 1, 4). However, the aggregates of diatoms formed after the senescence of the bloom may become anaerobic internally as they sink to greater depths. This occurs due to the excessive microbial activity within the aggregates. Thus, these aggregates are active spots for denitrification by denitrifying bacteria (Thornton 2002, 149, 156). The oomycetes also possess the denitrifying ability to cause nitrosative stress caused by NO derived molecules called reactive nitrogen species (RNS) in their host plant tissues for the spread of infection (Arasimowicz-Jelonek and Floryszak-Wieczorek 2014, 410-11). The presence of these organisms in oxygen-deficient regions of the water column or in shallow water sediments, where they may cause infection to the algae living in low light regions, may contribute to denitrification in the surrounding environment. The capacity of Labyrinthulomycetes (fungi-like protists) to withstand oxygen-deficient conditions in the OMZ and survive there may bestow them with the denitrifying ability that is similar to fungi (Leaño and Damare 2012, 215-17). However, no study reports this ability which leaves the door open for future investigation. Dinoflagellates too contribute to denitrification owing to the presence of symbiotic denitrifying bacteria in association with them (Wyman et al. 2013, 2676-77). The protists belonging to Rhizaria too contribute to denitrification by the endobiotic denitrifying bacteria. Foraminifera are the most noteworthy amongst these (Risgaard-Petersen et al. 2006, 93; Glock et al. 2013, 4767).

Concluding remarks

The occurrence of eukaryotic microorganisms in anoxic marine waters gives them the ability to survive in such environments using alternate electron acceptors, such as NO_3^-, in the absence of oxygen. Thus, these organisms significantly contribute to the marine N cycle. They carry out processes, such as denitrification and DNRA. The NO_3^- stored within some of these cells comes to their use during stress conditions or aids prokaryotes when released from the cells. Thus, marine eukaryotes, including fungi, diatoms, dinoflagellates, and foraminifera, have tremendous untapped potential in N cycling, especially in anoxic waters and sediments.

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