Ecological Studies and Molecular Characterization of Thraustochytrids and Aplanochytrids from Oceanic Water Column

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

in

MARINE SCIENCES

to the

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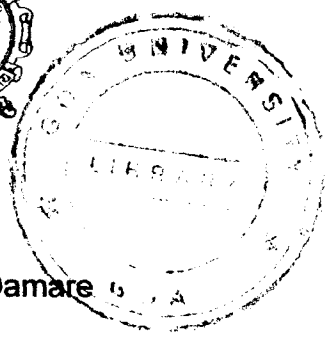

by

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August 2009

Statement

As required under the University Ordinance 0.19.8 (vi), I state that the present thesis entitled "**Ecological Studies and Molecular Characterization of Thraustochytrids and Aplanochytrids from Oceanic Water Column**" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities and suggestions have been availed of.

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Certificate

This is to certify that the thesis entitled "**Ecological Studies and Molecular Characterization of Thraustochytrids and Aplanochytrids from Oceanic Water Column**" submitted by **Varada S. Damare** for the award of the degree of Doctor of Philosophy in Department of Marine Science is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any degree or diploma in any University or Institution.

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All the corrections indicated by the referees have been incorporated at appropriate places in the thesis.

Varada Damare
7th Aug, 2009.

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Varada S. Damare

Dedicated

To

My Family

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Chapter 1.

Introduction to the taxonomy and ecology of thraustochytrids and aplanochytrids

1 General Introduction

Thraustochytrids and aplanochytrids are eukaryotic, unicellular organisms belonging to the Kingdom Chromista or Stramenipila (also called Stramenopila), which also includes diatoms, brown algae, the oomycetan fungi and a variety of flagellates (Mozley *et al.*, 2003) (Fig. 1). They are further classified under this Kingdom in the Phylum Heterokonta and Class Labyrinthulomycota or Labyrinthulomycetes. The Labyrinthulomycetes includes three groups, namely the thraustochytrids, aplanochytrids and labyrinthulids (Leander & Porter, 2000). The aplanochytrids were earlier called the labyrinthuloids. All three groups are osmoheterotrophic in their mode of nutrition.

Members of the Kingdom Straminipila are characterized by zoospores in which the two flagella are of unequal length, being termed the heterokont flagella (Fig. 2). The longer anterior flagellum is of the whiplash type, while the shorter posterior one is of the tinsel type. The whiplash, anterior flagellum is covered with tripartite tubular hairs (TTH). The TTH is composed of a cone-like base, a tubular shaft and usually two unequal diverging terminal fibres. Only one terminal fibre has been found in labyrinthulids and aplanochytrids (see Dick, 2001). Members of Labyrinthulomycetes typically reproduce by such straminipilan zoospores.

A major distinguishing feature of the Labyrinthulomycetes is the presence of the ectoplasmic net elements or EN. The EN are branched extensions of the plasma membrane and arise from an organelle called

the sagenogenetosome or bothrosome (Porter, 1990) (Fig. 3). Besides helping them in absorbing nutrients from the environment, the EN also help cells to attach to a substratum (Raghukumar, 2002).

Fig. 1. Diagrammatic representation of various groups of organisms in the tree of life.

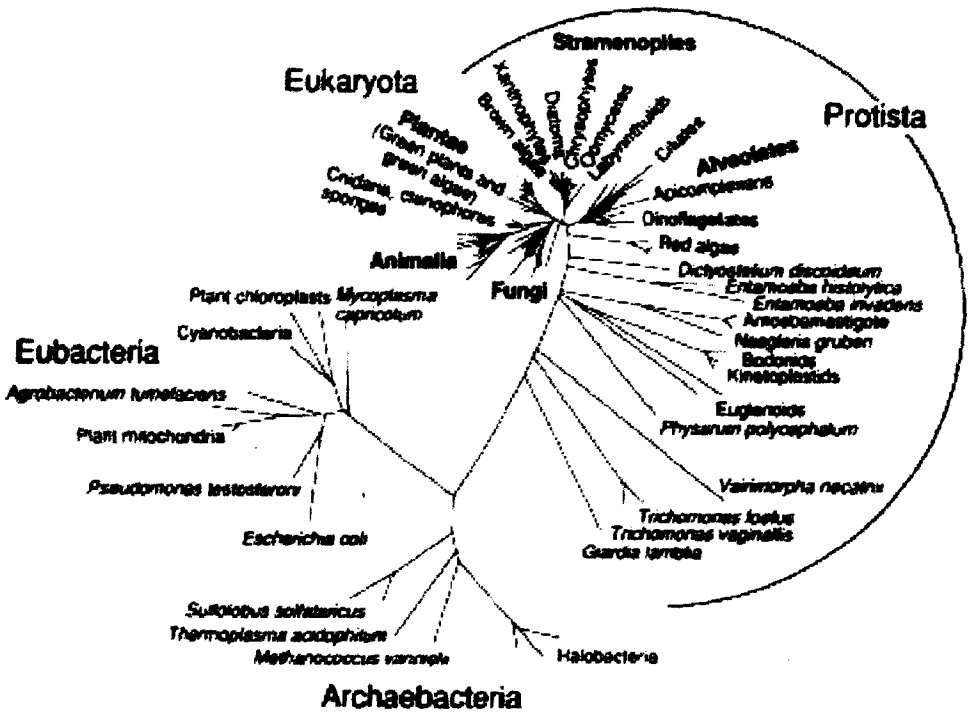


Fig. 2. (a) A typical, straminipilan, heterokont zoospore characteristic of Labyrinthulomycetes, showing a whiplash and a tinsel flagellum. (b) Arrangement of tripartite hairs on the tinsel flagellum in other straminipiles (i) and Labyrinthulomycetes (ii) (From Dick, 2001)

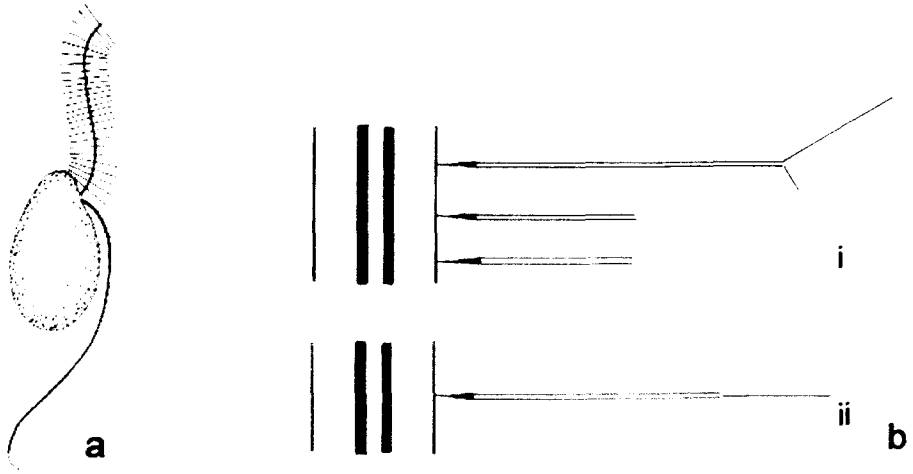
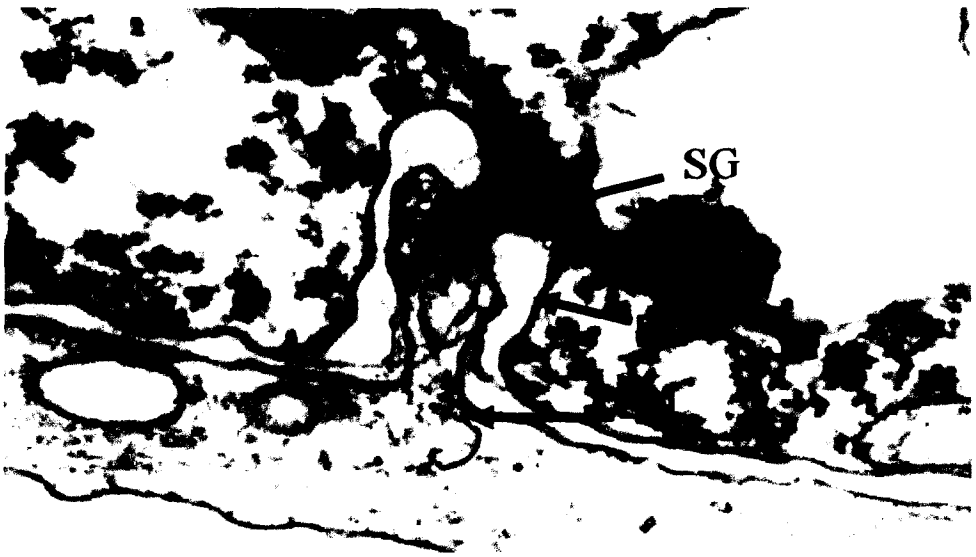


Fig. 3. Electron micrograph of sagenogenetosome or bothrosome of Labyrinthulomycetes (SG) and the invaginated plasmalemma (PL) that extends out as the ectoplasmic net element (EN). (From http://www.botany.uga.edu/zoosporicfungi/amgz.htm)



1.1 Characteristics of the three Labyrinthulomycetes groups

Labyrinthulids, thraustochytrids and aplanochytrids are distinguished by their general morphology (Fig. 4), as well as cell wall chemistry. Labyrinthulids are characterized by cells that are enrobed by and glide through the ectoplasmic net elements. Thraustochytrids are characterized by cells that are not interconnected by EN. All thraustochytrids except for the genus *Althornia* possess EN. Aplanochytrids are characterized by cells that are capable of a crawling movement by virtue of EN that do not completely enrobe the cells. Thraustochytrids reproduce exclusively by production of biflagellate zoospores. The labyrinthulids reproduce by fission, as well as through biflagellate zoospores. The aplanochytrids reproduce typically by the production of non-flagellate spores that move by gliding on their EN, but also are capable of producing biflagellate zoospores. The scales of the multilayered cell wall are circular and composed predominantly of galactose in case of labyrinthulids and thraustochytrids and fucose in case of aplanochytrids (Leander *et al.*, 2004).

Fig. 4. General characteristics of the three major groups of Labyrinthulomycetes, the thraustochytrids, aplanochytrids and labyrinthulids.

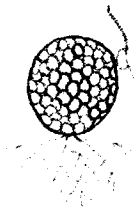
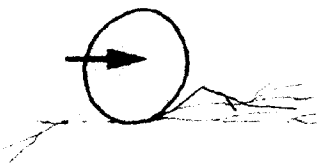
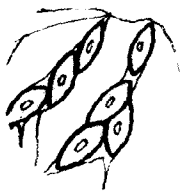
Kingdom Straminipila

↓

Phylum Heterokonta

↓

Class Labyrinthulomycetes

Order Thraustochytriales	**Order Aplanochytriales**	**Order Labyrinthulales**
		
Unicellular, Non-colonial, Reproduction by Zoospores	Unicellular or semi-colonial; Reproduction by motile spores gliding on ectoplasmic net elements or by zoospores	Unicellular, colonial, Reproduction by fission or by zoospores; Enrobed by the ectoplasmic net elements
Genera: *Thraustochytrium;* (~16 species) *Japonochytrium;* (1 species) *Schizochytrium;* (~ 5 species) *Ulkenia;* (~ 6 species) *Althornia* (1 species)	**Genera:** *Aplanochytrium* (~8 species)	**Genus:** *Labyrinthula* (~13 species)

The labyrinthulids consist of a single genus *Labyrinthula*. Thraustochytrids are comprised of five genera, *Althornia*, *Japonochytrium*, *Schizochytrium*, *Thraustochytrium* and *Ulkenia* (Porter, 1990; Raghukumar, 2002). However, the current classification of these genera based on morphology does not correspond with the molecular phylogenetic relationships based on the 18S rRNA gene sequences (Honda *et al.*, 1999). The genus *Schizochytrium* has been emended and two new genera *Aurantiochytrium* and *Oblongichytrium* have been included in it (Yokoyama & Honda, 2007). The aplanochytrids like labyrinthulids comprise a single genus *Aplanochytrium*. Five species from the genus *Labyrinthuloides* and one species from *Labyrinthula* were transferred to *Aplanochytrium* by Leander & Porter (2000).

1.2 A brief history of the classification of the Labyrinthulomycetes

The labyrinthulids were originally regarded as Myxomycota (slime molds) from the first observations made by Cienkowski (see Yokochi *et al.*, 2001) whereas thraustochytrids were included in the oomycetan fungi after their first isolation by Sparrow (see Raghukumar, 2002), based on their biflagellate zoospores. The aplanochytrids (earlier called the labyrinthuloids) were also placed under the thraustochytrids. Based on the chemical composition, the formation of the cell walls and ultrastructural features of the ectoplasmic net elements, Olive (1975) placed the two into the subphylum Labyrinthulina of the phylum Labyrinthulomycota.

Based on zoospore ultrastructure, especially of the flagellar apparatus and molecular phylogenetic studies, thraustochytrids and labyrinthulids were found to possess a close relationship with the heterokont algae (Porter, 1990). Based on further evidence derived from the 18S rDNA sequences, Cavalier-Smith (1994) and Cavalier-Smith *et al.* (1994) classified the two groups under the families Thraustochytridae and Labyrinthulidae, respectively, of the Class Labyrinthulea, Subphylum Labyrinthista and Phylum Heterokonta, of the Kingdom Chromista.

Dick (2001) classified Labyrinthulomycetes under the Phylum Heterokonta and Class Labyrinthista under the Kingdom Straminipila (a term coined by D. J. Patterson in 1989). Thraustochytrids were placed in the family Thraustochytriaceae of the Order Thraustochytriales and the labyrinthulids in the family Labyrinthulaceae of the Order Labyrinthulales. Thus, the classification of the thraustochytrids and labyrinthulids were thoroughly revised, having been placed earlier under the protozoans and fungi. The Kingdom Stramenipila in which they are currently placed represents an extremely diverse group of organisms that include brown algae, chrysophyte algae, xanthophyte algae, diatoms etc.

Honda *et al.* (1999) suggested that the Labyrinthulomycetes consisted of two distinct phylogenetic groups, the Thraustochytrid Phylogenetic Group and the Labyrinthula Phylogenetic Group. Leander & Porter (2001), based on the SSU ribosomal sequences showed that the genus *Aplanochytrium* was distinct from the labyrinthulids and

thraustochytrids and that the Labyrinthulomycetes actually comprised of three genetically distinct clades, the labyrinthulids, thraustochytrids and aplanochytrids based on the 18S rDNA phylogenies.

1.3 Important physiological characteristics of the Labyrinthulomycetes

Labyrinthulomycetes are exclusively marine and prefer salinities of 20-34 ppt. They have an absolute requirement for Na^+ ions which cannot be replaced by K^+. They are absorptive or osmoheterotrophic in their mode of nutrition, similar to bacteria and fungi. An important characteristic of the Labyrinthulomycetes is the presence of docosahexaenoic acid (DHA) as the signature fatty acid. DHA is an omega-3 polyunsaturated fatty acid (ω-3 PUFA). Several species often accumulate lipids up to 50 % of their body weight, DHA constituting 25 % or more (Bajpai *et al.*, 1991; Singh & Ward, 1997; Yaguchi *et al.*, 1997; Yokochi *et al.*, 1998). DHA is a commercially important fatty acid and has numerous benefits for human health and is particularly important as infant nutrition. In addition, crustaceans cannot synthesize this PUFA, which is essential for their growth and reproduction. Therefore, they derive this PUFA from their diet. Labyrinthulomycetes are one source of DHA for crustaceans in the marine ecosystem.

1.4 Distribution and ecology

Labyrinthulomycetes are ubiquitous in the marine environment (Table 1) (Raghukumar, 2002). They are predominantly saprotrophic, although a few have also often been reported to be parasitic in a number of invertebrates, particularly mollusks.

The labyrinthulid, *Labyrinthula zosterae* is well known as the causal agent of wasting disease of eelgrass which devastated large populations of the plant during early 19th century (Short *et al.*, 1993; Ralph & Short, 2002). They are also prevalent with decaying leaves of mangroves (Perveen *et al.*, 2006; Yokochi *et al.*, 2001).

Species of *Aplanochytrium* were originally described from sub-Antarctic waters, sediments, oyster mantle and gastropod. They are commonly associated with marine algae and seagrasses (see Leander *et al.*, 2004).

Thraustochytrids are extremely common in a wide variety of marine habitats (see Table 1). They have been isolated from living and decaying algae, as well as estuarine, coastal and oceanic waters and sediments (Raghukumar & Gaertner, 1980; Bongiorni & Dini, 2002; Santangelo *et al.*, 2000; Raghukumar, 2002). Thraustochytrids are found in large numbers in mangrove detritus, where they are believed to play an important role in the derital food web. The detrital food web is of major importance in the coastal marine ecosystem (Raghukumar *et al.*, 1994).

Table 1. Some examples from literature on the occurrence of Labyrinthulomycetes in the marine environment.

Location	Reference
Thraustochytrids in decaying seaweed, *Fucus serratus*, England	Miller & Jones, 1983
Labyrinthula in the seagrass *Zostera marina*	Muehlstein *et al.*, 1988
Aplanochytrids in marine algae	Leander *et al.*, 2004
Thraustochytrids and aplanochytrids in brown algal detritus of *Sargassum cinereum*, India	Sathe-Pathak *et al.*, 1993
Labyrinthulomycetes in decaying mangrove leaves	Findlay *et al.*, 1986
Thraustochytrids in phytoplankton detritus, North Sea	Raghukumar & Schaumann, 1993
Thraustochytrids in faecal pellets of salp *Pegea confoederata*, Arabian Sea	Raghukumar & Raghukumar, 1999
In tissue culture of tunicates	Rinkevich, 1999
Calcareous shells of mollusks	Porter & Lingle, 1992
Coelenteron of hydroids	Raghukumar, 1988
Water column, North Sea, Germany	Gaertner, 1967
Sediments, at Pachino Bay, Sicily, Italy	Bongiorni *et al.*, 2005
Thraustochytrids in water column, of the Arabian Sea	Raghukumar *et al.*, 2001
Water column, Seto Inland Sea, Japan and Hyuga-nada area, Japan	Kimura *et al.*, 2001
Water column, Seto Inland Sea, Japan and Hyuga-nada area, Japan	Kimura *et al.*, 1999
Water column, Seto Inland Sea, Japan	Naganuma *et al.*, 1998

Microorganisms present in the detritus, namely bacteria, fungi and thraustochytrids produce several degradative enzymes that break down the complex organic constituents present in the detritus and mediate major biochemical changes therein. The microbially transformed detritus supports a variety of detritus-feeding animals that are important not only in ecosystem dynamics, but also in aquaculture (see Raghukumar *et al.*, 1994; Sharma *et al.*, 1994).

Thraustochytrids have been shown to secrete a wide variety of extracellular enzymes, such as protease, lipase, cellulase, amylase and xylanase (Raghukumar *et al.*, 1994; Sharma *et al.*, 1994; Bremer & Talbot, 1995; Bongiorni *et al.*, 2005). Thraustochytrids and aplanochytrids have also been found associated with a wide variety of invertebrates, often occurring as parasites. Parasitic associations have commonly been reported in many mollusks (Polglasse, 1980; Jones & O'Dor, 1983; Bower, 1987; Bower *et al.*, 1989). Non-parasitic associations have been found in hydroids, corals and tunicates (Raghukumar, 2002). Thraustochytrids in the latter have often been found to be a nuisance in cell cultures of the invertebrates (Rinkevich, 1999). However, it is not clear if such symbiotic associations are merely commensalistic or mutualistic in nature.

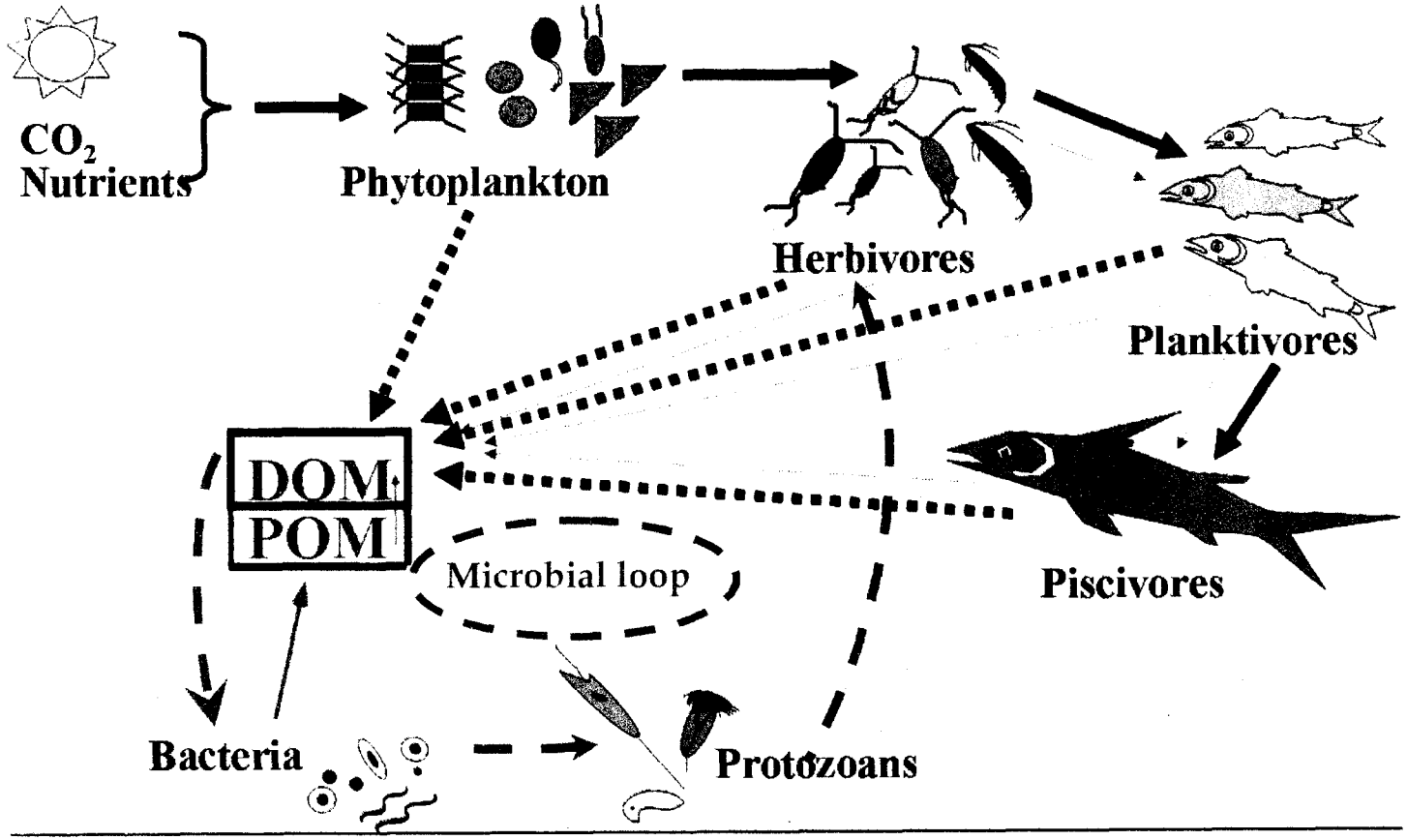
1.5 Occurrence in the water column

Thraustochytrids and aplanochytrids are common in coastal and oceanic water column. These studies, pioneered by Dr Alwin Gaertner

from Germany have depended on culture methods for enumeration of these organisms in the water column and sediments (Raghukumar, 2002). Subsequently, the development of a direct epifluorescence microscopy-based enumeration technique by Raghukumar and Schaumann (1993) enabled more accurate estimations of their populations in natural environments (see Naganuma *et al.*, 1998; Kimura *et al.*, 1999 Raghukumar *et al.*, 2001). These studies have shown that their numbers in the water column vary from below detection levels to a few thousand cells L^{-1}. At their maxima, their biomass in the water column can occasionally be equivalent to that of bacteria (Raghukumar *et al.*, 2001). Although cells detected through the AfDD method have been termed as thraustochytrids, it is also quite likely that many of these belong to the aplanochytrids, since the latter stain in the same manner as the thraustochytrids. Hence, it would be more appropriate to term such cells as those belonging to the 'Labyrinthulomycetes'.

Studies based on direct enumeration suggest that thraustochytrids may have an important role in microbial dynamics of the sea (Raghukumar, 2002). Bacterial dynamics in the marine ecosystem have been studied in great detail. In order to understand the role of thraustochytrids in the ocean, it is necessary to review the role of bacteria and other heterotrophic microorganisms in the ocean (Fig. 5).

Fig. 5 Schematic representation of energy flow in planktonic food web and microbial loop.



Energy flow and nutrient cycling in oligotrophic oceanic ecosystems is significantly influenced by picoplanktonic microorganisms (diameter < 2 μm), primarily bacteria, cyanobacteria, prochlorophytes and some protozoa (Sherr & Sherr, 1991). Bacteria play a main role of converting DOC into POC which enters the planktonic food webs (Ducklow *et al.*, 1986). They utilize the DOC which accumulates as their biomass, thus converting dissolved matter into particulate. Bacterial biomass in the sea is often related to phytoplankton concentration and bacteria utilise 10 to 50 % of carbon fixed by photosynthesis (Azam *et al.*, 1983). Heterotrophic flagellates too are ubiquitous in marine environment and are the main consumers of these prokaryotes. The flagellates in turn are preyed upon by microzooplankton returning the energy from microbial loop to conventional planktonic food chain (Azam *et al.*, 1983; Massana *et al.*, 2002). The microbial loop is an essential link for both oceanic secondary productivity by zooplankton and regeneration of essential rate-limiting nutrients (Meyers, 2000). Bacteria which act as decomposers in any food web are now seen as transformers of organic nutrients and dissolved organic matter (DOM) into living biomass and particulate carbon (Meyers, 2000).

Despite several studies on abundance of Labyrinthulomycetes in water column, their role in the planktonic food web is not very clear. Kimura *et al.* (1999) and Raghukumar (2002) have discussed the role that these microorganisms might play in the food web. Owing to their

osmoheterotrophic mode of nutrition similar to that of bacteria and production of various degradative enzymes, they might play a role of decomposers similar to bacteria in the detrital food web. Since they produce the omega-3 PUFA, DHA essential for growth and reproduction of crustaceans, they may also serve as an important source of food to these higher organisms, thus entering the microbial loop in the water column.

Most of the studies on these organisms deal with coastal waters. Though a few species of Labyrinthulomycetes have been isolated from oceanic waters of Antarctica, their abundance and distribution in oceanic waters is poorly known. Not much is known about their associations with bacteria and zooplankton of oceanic water column. While they have often been noticed to be associated with coastal invertebrates, such an association in oceanic waters has not been examined.

The present study was carried out keeping the above lacunae in mind and with the following objectives:

1. To isolate thraustochytrids and aplanochytrids from zooplankton in oceanic waters, to characterize them with respect to their life cycle in order to identify them and to group them based on their carbon and nitrogen nutrition and production of extracellular enzymes.
2. To study the abundance of thraustochytrids and bacteria in oceanic water column to understand their relations.

3. To carry out molecular characterization of isolates obtained from zooplankton based on their 18S ribosomal RNA gene and ITS sequences;
4. To design molecular probes for a few isolates to enable their detection in natural samples.

Chapter 2.

Aplanochytrids from Zooplankton of Equatorial Indian Ocean

2.1 Introduction

Most of the known species belonging to the Labyrinthulomycetes so far, including thraustochytrids, labyrinthulids and aplanochytrids have been discovered and taxonomically described from coastal marine environments. The labyrinthulid genus, *Labyrinthula,* has been known as a parasite of seagrass for more than 80 years now (Young III, 1943; Short *et al.*, 1987; Muehlstein *et al.*, 1988) and many species of this genus are regularly found in seagrasses or in decaying mangrove leaves (Bigelow *et al.*, 2005; Steele *et al.*, 2005). The first thraustochytrid, *Thraustochytrium proliferum* was described by Sparrow (1936) from marine algae in the vicinity of Woods Hole (Atlantic coastal waters). Many other species of *Thraustochytrium* and the other genera of thraustochytrids, namely *Japonochytrium, Schizochytrium* and *Ulkenia* were subsequently described from coastal regions (see Raghukumar, 2002). These include littoral waters bounding Woods Hole, Massachussets (Goldstein, 1963a; Iida *et al.*, 1996), New Haven, Connecticut (Goldstein, 1963b; Goldstein & Belsky, 1964), hypersaline waters of Great Salt Lake, USA (Amon, 1978), North Sea (Gaertner & Raghukumar, 1980; Gaertner, 1982), seawater from mangrove area of Yap Islands in west Pacific Ocean (Honda *et al.*, 1998) and mangrove waters of Chorao Islands in India (Bongiorni *et al.*, 2005c). The genus *Labyrinthuloides* (Watson and Raper) Perkins (now

synonymous with *Aplanochytrium* Bahnweg and Sparrow) was described from coastal waters of York River, Virginia (Perkins, 1973). Several other species of *Aplanochytrium* were subsequently described in association with marine algae (see Raghukumar, 2002).

In contrast to coastal waters, the diversity of Labyrinthulomycetes in oceanic waters and sediments is poorly known. The first member of Labyrinthulomycetes to be described from oceanic waters was *Aplanochytrium kerguelensis* from the Antarctic waters by Bahnweg and Sparrow (1972). These authors also described several new species of *Thraustochytrium* from these waters.

Attempts to culture and identify Labyrinthulomycetes have also been mostly confined to a variety of substrates in coastal ecosystems (Table 1).

Table 1. Examples of studies to culture Labyrinthulomycetes from various substrates from all over the world.

Organisms	Substrate	Association	Area of isolation	References
Thraustochytrids	Algae	Saprotrophic	Maine; Southsea Castle, Hampshire; Dona Paula Bay & Zuari river, Goa	Booth & Miller, 1968; Miller & Jones, 1983; Sathe-Pathak *et al.*, 1993; Raghukumar *et al.*, 1992; Raghukumar, 1988
Thraustochytrids, Labyrinthulids	Mangrove leaves or detritus	Saprotrophic	Orda, Goa; Okinawa Prefecture, Japan; sub-tropical mangroves (25-27°N); Coastal area of Takehara in Seto Inland Sea, Japan; Futial National Nature Reserve, Shenzhen, China; Hong Kong; Indonesia, Palombina & Poetto Beach, Goro Lagoon, Ancona Port (Italy)	Raghukumar, 1988; Perveen *et al.*, 2006; Yokochi *et al.*, 2001; Bowles *et al.*, 1999; Kumon *et al.*, 2006; Wong *et al.*, 2005; Fan *et al.*, 2002 a,b; Fan *et al.*, 2007; Kamlangdee & Fan, 2003; Unagul *et al.*, 2005; Bongiorni *et al.*, 2005c
Thraustochytrids, Aplanochytrids	Seagrasses	Saprotrophic	Florida, Puerto Rico; Eastern Canadian coastal sites from Nova Scotia, Newfoundland, Labrador	Leander *et al.*, 2004; Burja *et al.*, 2006
Labyrinthulids	Seagrasses	Parasitic	US, west coast of Europe; Japan	Short *et al.*, 1987; Muehlstein *et al.*, 1988; Ralph & Short, 2002; Short *et al.*, 1993; Bigelow *et al.*, 2005
Thraustochytrids, Aplanochytrids	Sediments	Saprotrophic	59-61°N, 50-51°N; Fladenground area of North Sea; Eastern	Bowles *et al.*, 1999; Raghukumar, 1980; Burja *et al.*,

			Canadian coastal sites from Nova Scotia, Prince Edward Island, New Brunswick, Newfoundland, Labrador; South coast of Madeira, Portugal; Indonesia, Palombina & Poetto Beach, Goro Lagoon, Ancona Port (Italy)	2006; Jakobsen *et al.*, 2007; Bongiorni *et al.*, 2005b
Thraustochytrids, Labyrinthulids, Aplanochytrids	Invertebrates	Saprotrophic, Endolithic	Coasts of Maine, Georgia, Jamaica; York river, Virginia; Northern Arabian Sea; Estuarine region in southern Portugal	Porter & Lingle, 1992; Perkins, 1973; Raghukumar & Raghukumar, 1999; Azevedo & Corral, 1997; Naganuma *et al.*, 1996 (Kimura *et al.*, 1999)
Thraustochytrids, Labyrinthulids, Aplanochytrids	Invertebrates	Parasitic	Eastsound & Bellingham Bay, Washington; Buckingham Canal; Canada; India; Vancouver Island; Gulf of St. Lawrence; Northern Adriatic Sea;	McLean & Porter, 1982; Loganathan *et al.*, 1988; Jones & O'Dor, 1983; Anderson *et al.*, 2003; Polglase, 1980; Raghukumar, 1987a & b; Bower, 1987; Bower *et al.*, 1989; Whyte *et al.*, 1994; Schärer *et al.*, 2007
Thraustochytrids	Coral and coral mucous	Saprotrophic	Gulf of Eilat, Israel; India	Harel et al., 2008; Raghukumar & Balasubramanian, 1991
Thraustochytrids	Cell lines of coral, tunicates, sponges, mollusks, grass prawn	Contaminant	Israel, Japan	Frank *et al.*, 1994; Rinkevich, 1999; Rinkevich & Rabinowitz, 1997; Rinkevich *et al.*, 1998; Hsu *et al.*, 1995; Kawamura & Fujiwara, 1995

Thraustochytrids and labyrinthulids have frequently been isolated from decaying mangrove leaves. Numerous parasitic, as well as non-parasitic associations with invertebrates have been reported. Among the important parasitic associations are the QPX- quahog parasite unknown in the clam *Mercenaria mercenaria*, the yellow spot disease in the skin of the nudibranch *Tritonia diomedea* and skin ulcers in the octopus *Eledone cirrhosa* (Polglasse, 1980; McLean & Porter, 1982; Whyte *et al.*, 1994; Smolowitz & Leavitt, 1997; Anderson *et al.*, 2003). *Schizochytrium* sp. was found to infect gills of squid *Illex illecebrosus* and cause fin rot and ulcerative lesions in estuarine fishes *Lates calcerifer* and *Etroplus suratensis* (Loganathan *et al.*, 1988; Jones & O'Dor, 1983). *Labyrinthuloides haliotidis*, now known as *Aplanochytrium haliotidis*, causes a pathogenic infection in abalone *Haliotis kamtschatkana* (Bower, 1987; Bower *et al.*, 1989).

Non-parasitic, saprophytic associations of thraustochytrids include their dense presence in faecal pellets of the salp *Pegea confoederata*, the occurrence of *Ulkenia visurgensis* in coelenteron and hydranth of a hydroid and their presence in gut contents and faecal material of the sea urchin *Lytechinus variegates* (Raghukumar & Raghukumar, 1999; Wagener-Merner *et al.*, 1980). When present in corals and coral mucus, they may provide nutritional sources to corals helping them survive during

bleaching events (Raghukumar & Balasubramanian, 1991; Harel *et al.*, 2008). Particularly interesting are thraustochytrids that are common contaminants in cell cultures of sponges, cnidarians, crustaceans, molluscs, echinoderms and tunicates (Rinkevich, 1999). These examples suggest a definite role for saprophytic thraustochytrids in association with these invertebrates.

Members of Labyrinthulomycetes have not often been brought into culture and identified from the oceanic environment. An exception is the isolation and description of *Aplanochytrium kerguelensis* and four *Thraustochytrium* species (Bahnweg & Sparrow, 1972 & 1974), from Antarctic waters. Therefore, we have no clear picture of their association with invertebrates in the oceanic environment, unlike what we know for coastal habitats. The dominant invertebrates in the oceanic water column are crustacean mesozooplankton.

The following objectives were formulated in light of the above.

(1) To investigate the occurrence of Labyrinthulomycetes in zooplankton of the equatorial Indian Ocean water by isolating them in culture.

(2) To taxonomically identify isolates of Labyrinthulomycetes, from zooplankton by studying their life cycle and morphology.

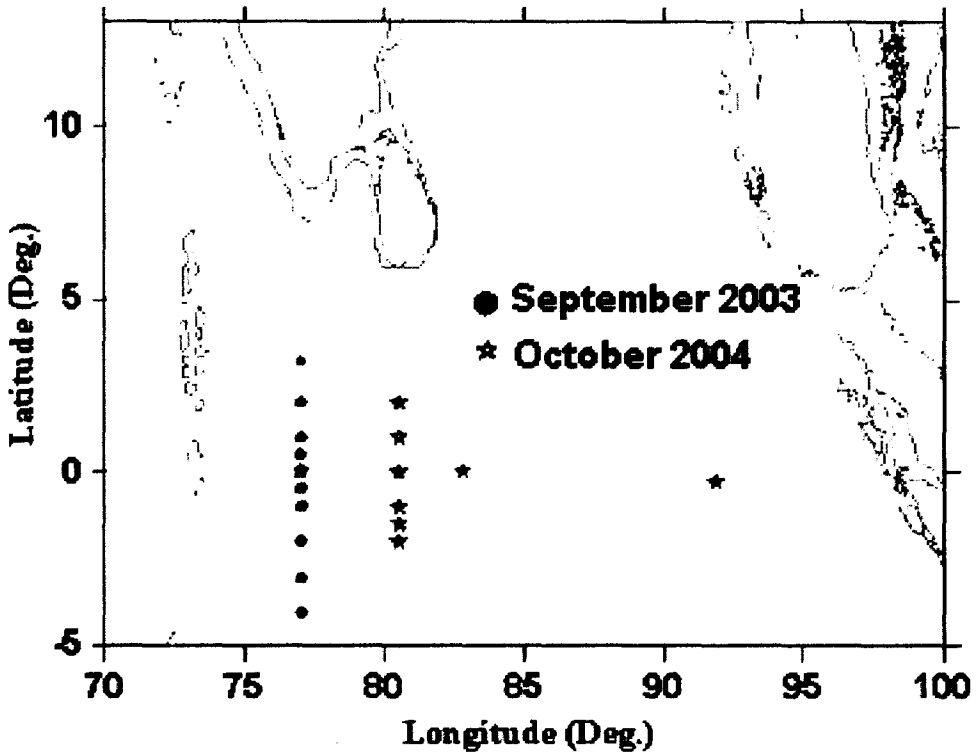
(3) To study a few physiological characteristics of the Labyrinthulomycetes isolated from zooplankton by studying their carbon and nitrogen requirements.

(4) To analyze the morphological and physiological groupings of the Labyrinthulomycetes isolated from zooplankton by carrying out Cluster analysis.

2.2 Materials and Methods.

2.2.1. Isolation of Labyrinthulomycetes: Labyrinthulomycetes were isolated from mesozooplankton from the equatorial Indian Ocean on board ORV Sagar Kanya during Cruise # SK 196 in September 2003 and Cruise # SK 212 in October 2004. Zooplankton were collected using a Multiple Plankton Net (MPN, MultiNet Type Midi with 200 µm mesh nets, Hydrobios, Germany) from different depths ranging from surface to 1000 m . A total of 76 samples were collected from 10 stations in September 2003 and 9 stations in October 2004 (Fig. 1).

Fig. 1 ORV Sagar Kanya station locations in the equatorial Indian Ocean during cruises SK 196 (September 2003) and SK 212 (October 2004).



Zooplankton specimens were thoroughly washed in sterile seawater and individual animals were plated on to Modified Vishniac (MV) Agar plates (Porter, 1990) with the following composition (ingredients added as w/v).

- Dextrose: 1.0 %
- Peptone: 0,15 %
- Yeast extract: 0.01 %
- Liver infusion broth: 0.001 %;
- Agar: 0.8 %
- Seawater: 100 mL

- Fetal bovine serum: 1 % (Added separately to the autoclaved medium)
- Procaine penicillin: 40000 (added separately to the autoclaved medium)
- Streptomycin (Ambistryn): 0.075 g (Added separately to the autoclaved medium).

In addition to zooplankton specimens, attempts were made to isolate thraustochytrids and aplanochytrids from faecal pellets of zooplankton during October 2004. Faecal pellets were collected using the method modified from the one used by Raghukumar & Raghukumar (1999). Freshly collected zooplankton specimens were placed in 500 ml beakers containing 0.22 µm filter-sterilized seawater in such a way that they were suspended over a 100 µm mesh bolting silk. After 1 hour the animals were removed and the material that had passed through the mesh and collected at the bottom of the beakers were plated on MV medium. The plates were incubated for 7 – 14 days till colonies of thraustochytrids or aplanochytrids appeared. Colonies of these organisms were recognized by their general morphology. They resemble yeast colonies. However, unlike the shiny texture of yeasts, colonies of thraustochytrids and aplanochytrids possess rough textures. In addition, cells of thraustochytrids vary largely in size, ranging from 2 – 20 µm. The identity of the colonies was further confirmed by examining the colonies under the lower power of a microscope. Bacteria-free colonies were then sub-

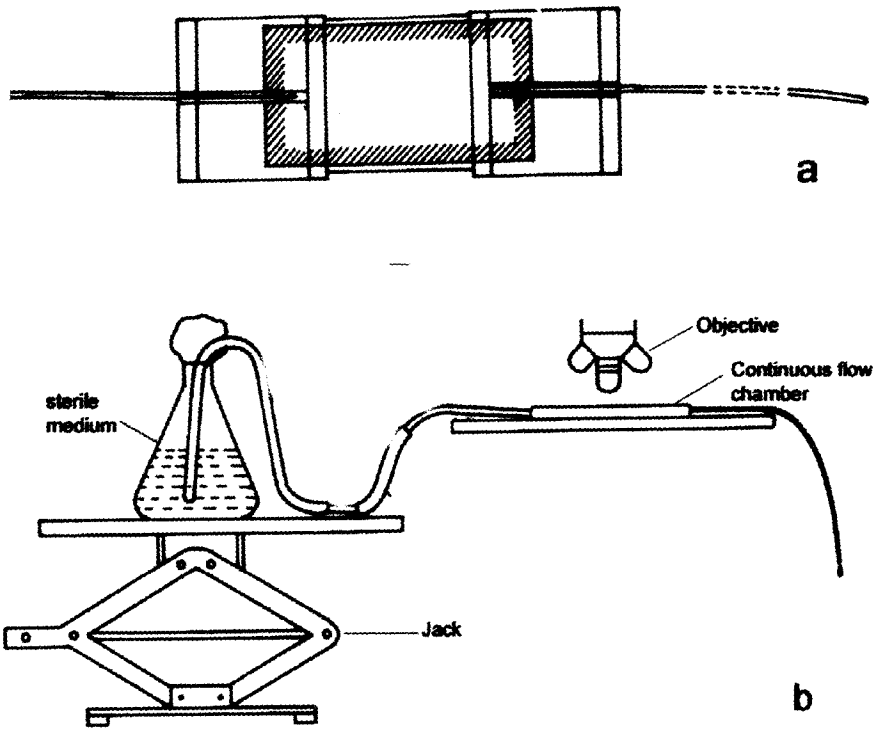
cultured on to sterile MV agar plates without antibiotics. In addition, they were also sub-cultured onto sterile seawater containing autoclaved *Artemia* larvae, obtained using standard methods of hatching *Artemia* cysts in seawater (Bahnweg & Sparrow, 1974). Growth on MV plates and *Artemia* larvae was observed after about three days of inoculation. Cultures were maintained by routine sub-culturing on MV agar tubes containing 0.4 % agar, instead of 0.8 % agar as in the isolation plates.

2.2.2. Morphology and Life Cycle: Colony and cell morphology of all the isolates was examined by streaking cultures on MV agar plates. A cover slip was gently placed over seven day old colonies which were then examined microscopically using a Carl Zeiss 'Axioskop' 2 plus microscope. Colony characteristics described by Leander *et al.* (2004) were used to distinguish the isolates.

Life cycle of 14 isolates was examined using a modified continuous flow chamber described by Raghukumar (1987). Basically, this chamber consists of a small piece of glass slide (3 x 2.3 cm) fixed on an aluminium slide containing groove of the same size as that of the glass piece (Fig. 2). A small drop of inoculum is placed in the centre of the slide. A coverslip of 24 mm^2 is placed on a firm base and silicon vacuum grease is applied on all edges, using a syringe. The coverslip is lowered on the slide and gently

pressed at the edges to make the grease spread thinly and evenly to seal the inoculum drop in the middle. At the two ends of the glass slide a silicon tubing (ID 0.8 mm, OD 2 mm) is fixed for the supply of nutrients or seawater. One end of the tubing is inserted into sterile seawater or liquid medium and the other end serves as an outlet for the same medium to ensure supply of fresh medium and removal of used medium. The flow of the water or medium is gentle to ensure that the inoculum is not washed off. The flow is regulated by adjusting the height of the jack on which the flask containing the liquid medium is rested. This device allows continuous microscopic observation and photomicrographic recording of cells for a period of 2 to 3 days. Life cycle and development of the isolates were photographed using a Zeiss AxioCam digital camera.

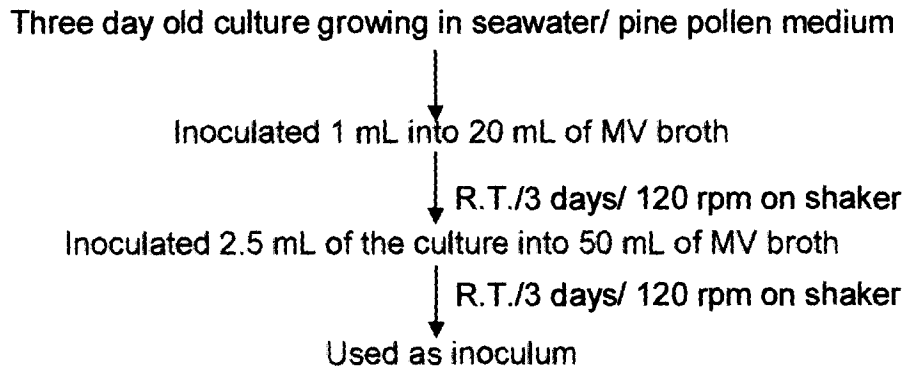
Fig. 2. Diagrammatic representation of continuous flow chamber (a) and assembly of the chamber for observation under microscope (b). (From Raghukumar, 1987).



***2.2.3. Physiological characterization*:** Fourteen isolates were characterized in terms of their carbon and nitrogen requirement, as well as the production of extracellular, hydrolytic enzymes.

2.2.3.1. Carbon requirements: Inoculum for experiments on carbon requirements was prepared as given in Figure 3. Experimental flasks contained 20 ml of MV medium using different sugars, in place of glucose. The sugars were autoclaved separately and added at a final concentration of 1 % to the MV medium excluding glucose. The inoculum volume corresponded to 5 % (1 ml).

Fig. 3 Flow diagram of preparation of inoculum of aplanochytrid isolates for the experiment on carbon utilization



A total of 16 sugars as follows were tried, based on Bahnweg (1979b).

- Hexoses: Glucose, Galactose, Rhamnose, Fructose
- Pentoses: Ribose, Xylose, Arabinose
- Disaccharides: Lactose, Sucrose, Maltose, Cellobiose, Trehalose, Mellibiose
- Trisaccharides: Raffinose
- Polysaccharides: Xylan, Starch

Growth was checked after 3 days of inoculation by measuring the increase in dry weight.

2.2.3.2. Nitrogen requirements: Fourteen isolates were examined. Since many thraustochytrids and aplanochytrids do not use inorganic nitrogen, 16 amino acids were tested as follows, based on Bahnweg (1979a).

- Aliphatic: Glycine, Alanine, Leucine
- Aromatic: Tryptophan, Phenylalanine
- Acidic: Glutamic acid
- Basic: Histidine, Lysine, Arginine
- Amide: Asparagine
- Hydroxyl: Serine, Threonine
- Cyclic: Proline
- Sulphur-containing: Cysteine, Cystine
- Others: Ornithine

Growth of different nitrogen sources was compared in terms of colony diameter, since biomass differences in a liquid medium were not clear-cut. The following medium was used for these experiments (all values w/v).

- Dextrose: 1.0 %.
- KH2PO4: 0.025 %.
- Agar: 0.6 %
- Seawater: 100 ml
- Fetal bovine serum (added after autoclaving the medium).
- Individual amino acids as above (filter-sterilized and added after autoclaving the medium): 1.0 %.
- Mixture of 0.005 % riboflavin and 0.005 % cyanocobalamin: 0.1 %
- Procaine penicillin: 20000 U
- Streptomycin (Trade name Ambystrin-S): 0.0375 g.

The serum, amino acids, vitamins, penicillin and streptomycin were added sterile after autoclaving the rest of the medium.

The cultures were streaked on the agar plates and the colony diameter was measured after 5 days of growth at R.T.

2.2.3.3. *Production of extracellular enzymes:* Qualitative assays were carried out on 14 isolates for the production of protease, lipase, amylase and chitinase. All isolates were inoculated from a 3 day old cultures growing in seawater/ pine pollen medium into MV agar plates supplemented with 1 % w/v of various substrates for the enzymes, as follows and the enzyme activity was examined after growth for 5 days.

- Protease (Molitoris, 2000): 1 % skimmed milk (Trade name, Sagar, India). Presence and extent of the zone of clearance around the colony indicated enzyme activity.
- Lipase (Molitoris, 2000): 1 % Tween 80 with 0.1 g L^{-1} calcium chloride hydrous. Production of white precipitate indicates enzyme activity.
- Amylase (Molitoris, 2000): 1 % Starch. Blue colouration of the medium after the addition of Lugol (iodine) solution indicates absence of amylase activity and a colourless halo surrounding a colony indicates its presence.

- Chitinase: Chitin (Hi Media) was dissolved in 50 % sulphuric acid and precipitated with cold distilled water by diluting it 15 fold. The precipitate was washed with distilled water till the pH was close to 7.0. The precipitate was collected by centrifugation and weighed (Austin, 1988). The required amount was then autoclaved separately prior to adding to the autoclaved medium. Presence and extent of the zone of clearance around the colony indicated enzyme activity.

2.2.3.4. ***Cluster analysis:*** Cluster analysis using unweighted pair group average method in Statistica v 5.0 software was carried out between all the isolates to analyse the similarities between them. The characteristics used for the analysis are presented in Table 2. A total of 27 each of morphological as well as physiological characters were used for the analysis amounting to a total of 54 characters. Each character used for the analysis was designated plus or minus based on its presence or absence in that particular isolate. Likewise, characteristics of all the isolates were described as plus or minus. Phenograms were plotted based on the analysis in Statistica v 5.0 software.

Table 2. Characters used for cluster analysis of all the 14 isolates from equatorial Indian Ocean.

No.	Morphological characters	Physiological characters (the best C or N source)
1	Colony with agar penetration	Glucose
2	Colony with rays of continuous band of cells	Galactose
3	Colony with rays of disjoint patches of cells	Rhamnose
4	Colony with rays sprawling from centre outwards	Fructose
5	Colony with rays sprawling from periphery of colony	Ribose
6	Colony with clumps	Xylose
7	Young cell 3-6 μm	Arabinose
8	Young cell 6-8 μm	Cellobiose
9	Young cell 8-10 μm	Trehalose
10	Mature cell <10 μm	Lactose
11	Mature cell 10-20 μm	Maltose
12	Mature cell 20< μm	Sucrose
13	Sporangium 10-18 μm	Mellibiose
14	Sporangium 18-26 μm	Raffinose
15	Sporangium 26-34 μm	Xylan
16	Spore 3-4 μm	Starch
17	Spore 4-5 μm	Proline
18	Spore 5-6 μm	Glutamic acid
19	Spore 6-7 μm	Asparagine
20	Circular spores	Lysine
21	Cuniform spores	Arginine
22	Circular and cuniform spores	Glycine
23	Ellipsoidal spores	Alanine
24	Oval spores	Leucine
25	Intact cell wall during spore release	Serine
26	Disintegrating cell wall during spore release	Threonine
27	Presence of amoeboid cells	Ornithine

2.3. Results

2.3.1. Isolation of Thraustochytrids and Aplanochytrids

A total of 2100 zooplankton specimens, collected from 76 multiple plankton net samples and obtained from 19 stations during two cruises were plated onto MV medium for isolating thraustochytrids and aplanochytrids (Table 3). Out of this, a total of 171 specimens, corresponding to around 8 % were positive for the protists.

Out of the total 171 colonies obtained, a total of 14 isolates were subcultured and maintained. Five of these were isolated during September 2003 and the remaining 9 during October 2004 (Table 4). Three further isolates were obtained from the plates on which the filtrate containing fecal pellets was plated.

Table 3. Details of CTD stations where Multiple Plankton Net operations were carried out and sampling depths. Depth ranges given in bold indicate samples from which aplanochytrids were recovered in culture.

Sampling period	Time (hr)	Latitude	Longitude	Depths (m)
September 2003 (Cruise SK 196)	0750	3° N	77° E	500- 300, **300- 200, 200- 40, 40-0**
	0230	02° N	77° E	500- 300, **300- 200, 200- 40, 40-0**
	1100	01° N	77° E	500- 300, 300- 200, **200- 40**, 40-0
	1900	0.5° N	77° E	500- 300, 300- 200, **200- 70, 70-0**
	0730	0° Eq	77° E	**500- 300, 300- 200, 200- 40, 40-0**
	0115	0.5° S	77° E	500- 300, 300- 200, 200- 30, 30-0
	0350	1° S	77° E	500- 300, 300- 200, 200- 40, **40-0**
	0115	2° S	77° E	500- 300, 300- 200, 200- 30, 30-0
	0600	3° S	77° E	500- 300, 300- 200, **200- 40**, 40-0
	1700	4° S	77° E	500- 300, 300- 200, 200- 30, **30-0**
October 2004 (Cruise SK 212)	2130	0° Eq	77° E	500- 300, **300- 200, 200- 60, 60-0**
	0020	2° N	80.5° E	500- 300, 300- 200, 200- 20, 20-0
	2330	1° N	80.5° E	**500- 300,** 300- 200, **200- 30**, 30-0
	1430	0° Eq	80.5° E	**1000-500**, 500- 200, **200- 38, 38- 0**
	1600	0° Eq	83° E	1000-500, 500- 300, 300- 30, 30- 0
	0830	1° S	80.5° E	**1000-500, 500- 200, 200- 60, 60- 0**
	1340	1.5° S	80.5° E	500- 300, 300- 200, **200- 60, 60-0**
	2215	2° S	80.5° E	**500- 300**, 300- 200, **200- 60, 60-0**
	1400	0° Eq	93° E	1000-500, 500- 300, 300- 30, **30- 0**

Table 4. Depth and location in the equatorial Indian Ocean from which isolates were obtained.

Date	Latitude	Longitude	Depth (m)	Isolate No.
05 Sep.2003	02° 59.5' N	77° 01.4' E	300- 200	S1961
05 Sep.2003	02° 59.5' N	77° 01.4' E	200- 40	S1962
05 Sep.2003	02° 59.5' N	77° 01.4' E	40- 0	S1963
06 Sep.2003	02° 00.1'N	77° 00.3' E	40- 0	S19610
07 Sep.2003	01° 00.2' N	77° 00.0' E	200- 40	S19615
28 Oct.2004	00° 59.893' S	80° 30.317' E	1000- 500	S2121
28 Oct.2004	00° 59.893' S	80° 30.317' E	60- 0	S2122
29 Oct.2004	01° 28.917' S	80° 30.629' E	60- 0 (faecal pellets)	S2123, S2124
30 Oct.2004	01° 55.932' S	80° 39.926' E	200- 60	S2125, S2126, S2127
30 Oct.2004	01° 55.932' S	80° 39.926' E	500- 300	S2128
06 Nov.2004	00° 00.539' S	92° 58.552' E	30- 0 (faecal pellets)	S2129

2.3.2. Morphology and Life Cycle

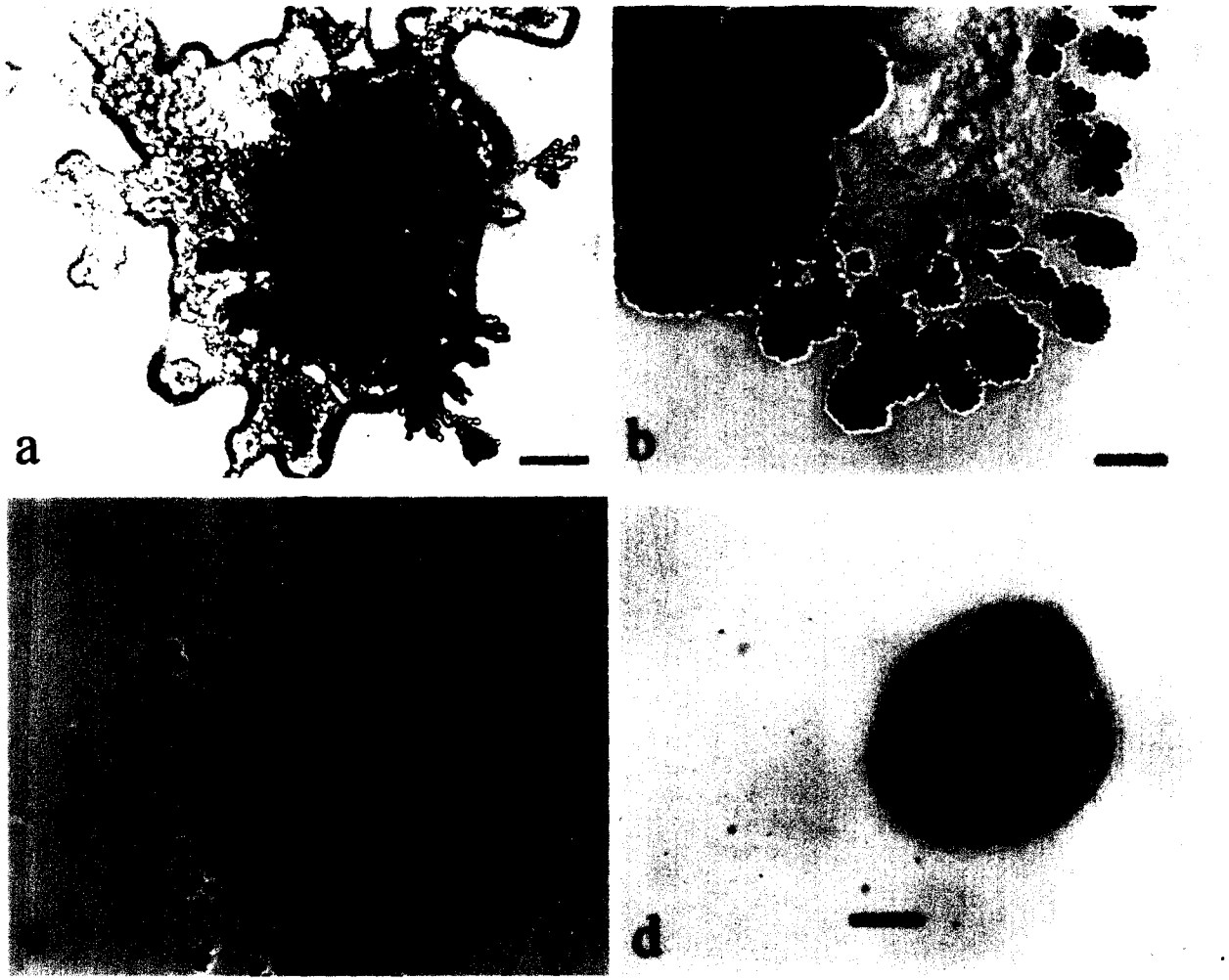
Colony morphology of the isolates could be broadly classified into two categories (Fig. 3). In the first type, cells of the organism penetrated the agar and grew within (Fig. 3a), while such behaviour was absent in the other category (3b-d). These two groups could be further subdivided into two subgroups each, namely those in which the colonies produced distinct

broad rays of continuous band or patches of cells (Fig. 3a,b,c) and those in which the rays were absent (Fig. 3d). The characteristics of individual isolates in terms of colony morphology are presented in Table 5.

Table 5. Colony characteristics of the 14 isolates from Indian Ocean.

Colony Type			Isolate No.
Agar penetration present	Colonies forming broad rays sprawling from the center outwards		S1961, S1963, S19615
	Colonies forming clumps of cells on the agar surface	Radial pattern, wavy margin	S1962
	Colonies forming clumps of cells on the agar surface along with distinct rays	Radial pattern, wavy margin	S19610
		Radial pattern absent	-
No agar penetration	Colonies forming broad rays sprawling from the center outwards		S2123, S2125
	Colonies forming clumps of cells on the agar surface	Radial pattern, wavy margin	S2126
		Radial pattern absent	S2122, S2129
	Colonies forming clumps of cells on the agar surface along with distinct rays	Radial pattern	S2127
		Radial pattern absent	S2121, S2124, S2128

Fig. 3. Examples of different colony morphologies of various isolates from the equatorial Indian Ocean. Bar represents 100 µm. (a) Colony of isolate S1961 producing rays of continuous band of cells with agar penetration; (b) colony of S2123 with same type of rays without agar penetration; (c) clump-like colony of S2127 producing rays of disjointed patches of cells from the edge of the colony; (d) clump-like colony of S1962.



The ectoplasmic net elements (EN) showed three kinds of morphology. Isolates S1962, S1963, S2123, S2125, S2127 and S2129 produced fine filaments of EN, that were less than 0.5 µm in width (Fig. 4a). EN elements of isolate S2125 had a very broad base of 3 µm or more (Fig. 4b). Isolates S1961, S19610, S19615, S2121, S2126 and S2128 also produced fine filaments, but the filaments often showed broad areas in between, that were 0.5 to 1 µm wide (Fig. 4c). Isolates S2122 and S2124 produced only broad filaments, that were 0.5 to 1 µm wide (Fig. 4d).

All 14 isolates showed a similar development and life cycle pattern, as follows (Fig 5). Motile spores, ranging in size from 3.1 to 7.0 µm settled down and grew into mature vegetative cells. These cells were globose to subIglobose in size and ranged in size from 7.8 to 27.8 µm in diameter. Mature vegetative cells transformed into sporangia, whereby the cytoplasmic contents divided simultaneously into 12 to 32 spores. The spores were enclosed within the cell wall of the sporangium. Alternatively, the mature vegetative cell divided into a diad and then a tetrad and subsequently into a number of small cells, the spores, all enclosed within the sporangial wall. The spores were later released from the fully mature sporangium either by dissolution of its cell wall or through a tear or break at any point on the cell wall (Fig. 6). In the former case, the cell wall disappeared and the spores moved away from each other by gliding

Fig. 4. Examples of the different morphologies of the ectoplasmic net elements (EN) (arrows) of various isolates from the equatorial Indian Ocean. Bar represents 100 µm. (a) Cells possessing very fine filaments of EN; (b) cells possessing EN with a broad base; (c) cells possessing fine EN filaments with broad areas in between; (d) cells with broad filaments of EN.

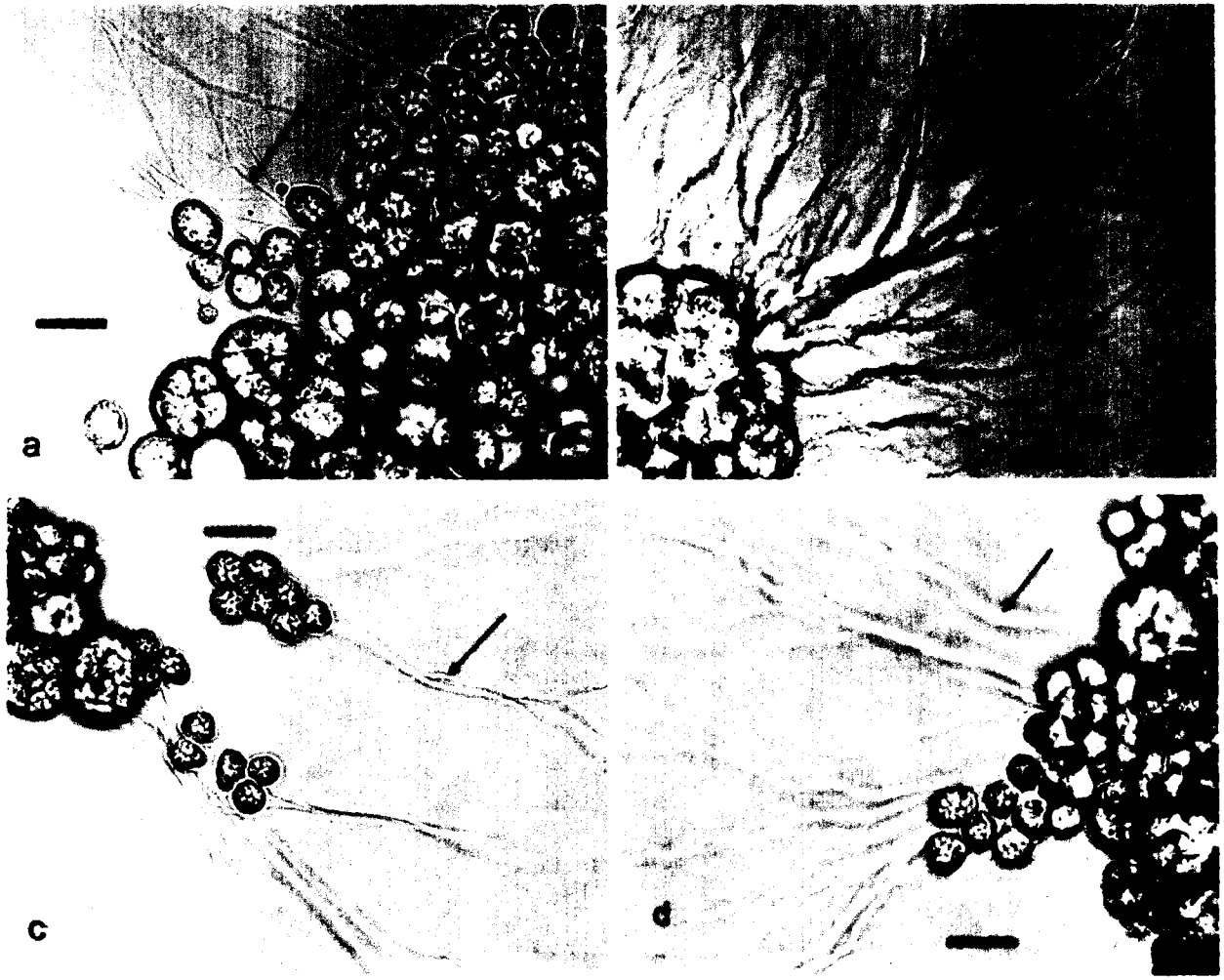
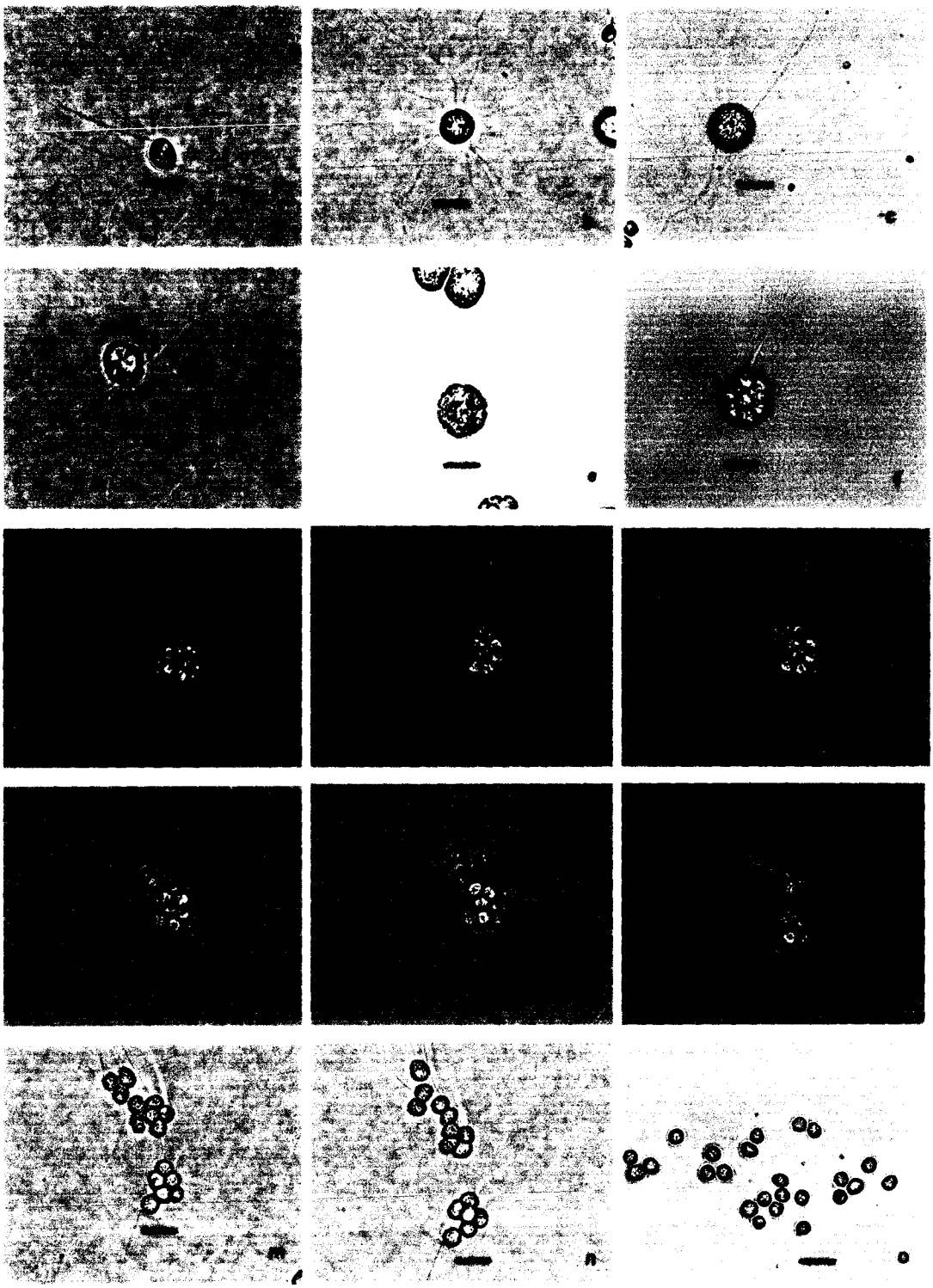


Fig. 5. Representative development and life cycle pattern based on different isolates of *Aplanochytrium yorkensis* isolated from the equatorial Indian Ocean. Bar represents 10 μm.



motion over the ectoplasmic net elements. In the latter case, the spores were released through one or two points of break in the cell wall. The spores when released from the sporangium moved away from the same by gliding over the ectoplasmic net elements. The spores were cuniform, circular, oval or ellipsoidal in shape (Fig. 7). The isolates were identified as *Aplanochytrium yorkensis* (Perkins) Leander and Porter (syn.: *Labyrinthuloides yorkensis* Perkins (1973), based on their developmental cycle and presence of a large eccentric vacuole in the cell that was obvious in case of larger cells (Fig. 8). Amoeboid cells were produced occasionally. These either divided into non-motile vegetative cells (Fig. 9) or motile spores (Fig. 10).

None of the 14 isolates corresponded to thraustochytrids. The morphology of all colonies that grew out of zooplankton also resembled those of *Aplanochytrium* as described above. Although all the isolates corresponded to *Aplanochytrium yorkensis*, they differed widely in cell shape and size, the number, shape and size of spores formed, the mode of liberation of the spores and the presence or absence of amoeboid cells. These differences are tabulated in Table 6.

Fig. 6. Release of spores by dissolution of cell wall (a,b,c) and break at two points (d) and one point in cell wall (e,f). Bar represents 10 μm.

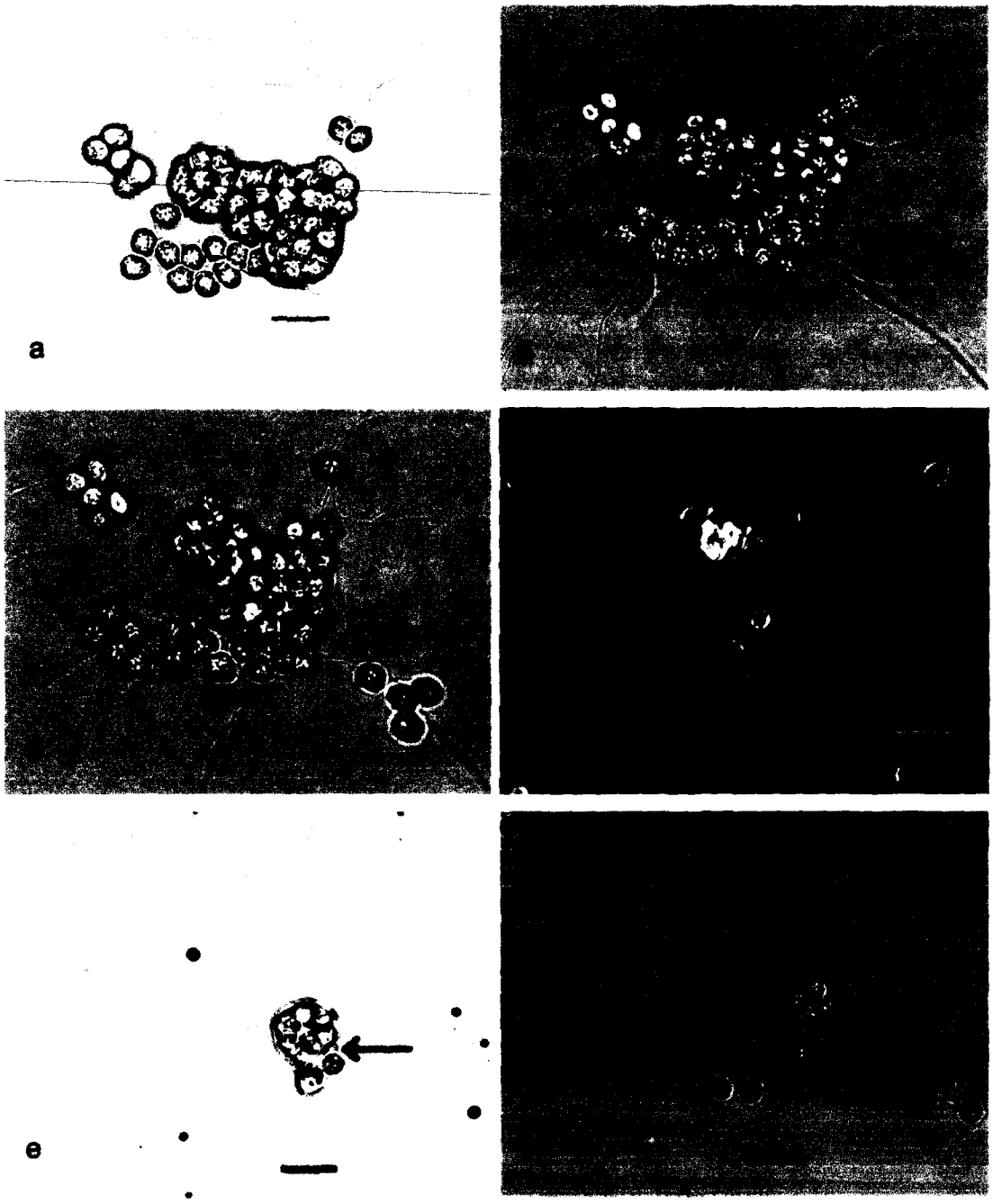


Fig. 6. Release of spores by dissolution of cell wall (a,b,c) and break at two points (d) and one point in cell wall (e,f). Bar represents 10 μm.

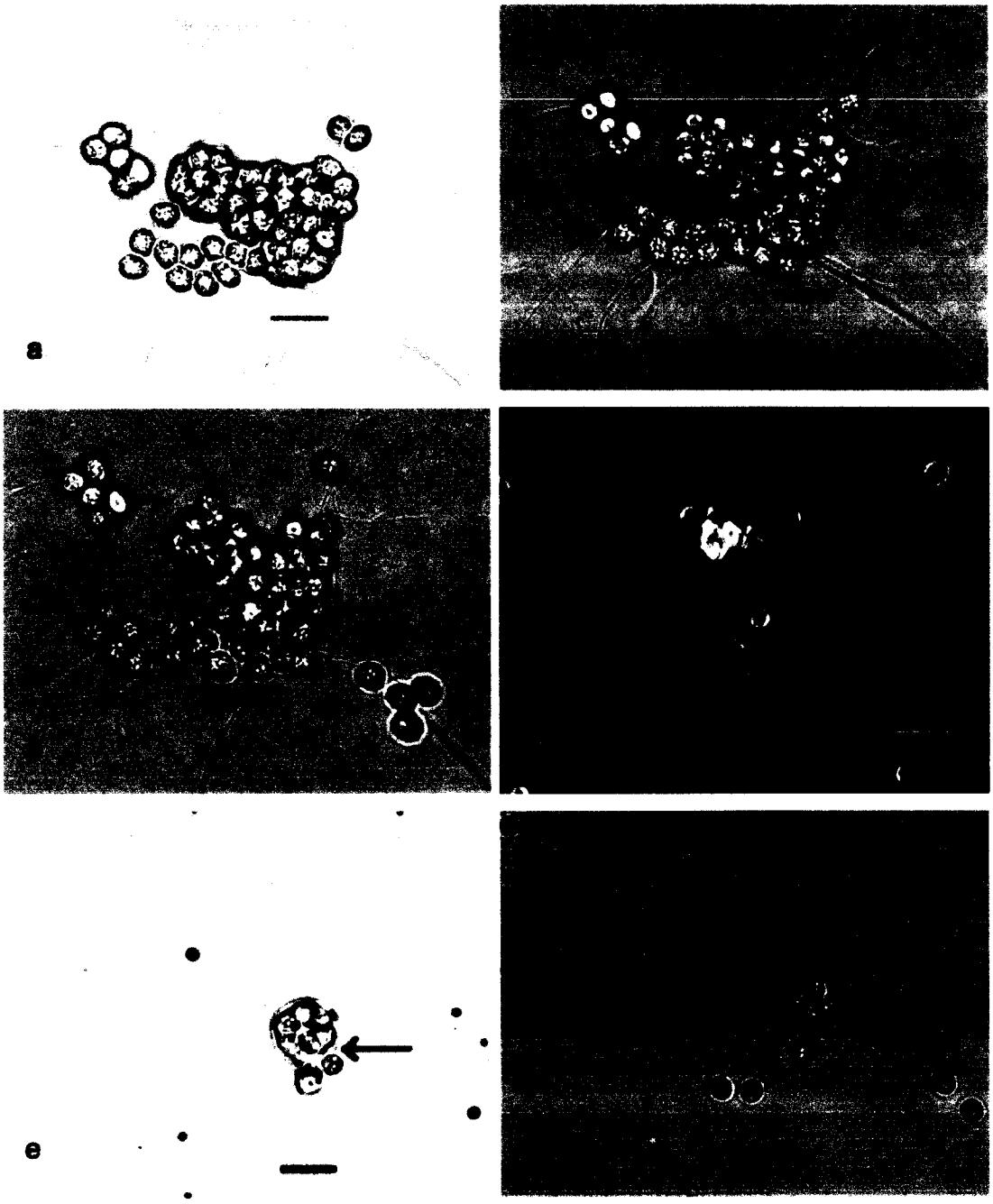


Fig. 7. Various spore shapes (a) cuniform, (b) circular, (c) ellipsoidal (marked by arrow) and (d) oval (marked by arrow) (Bar represents 10 μm).

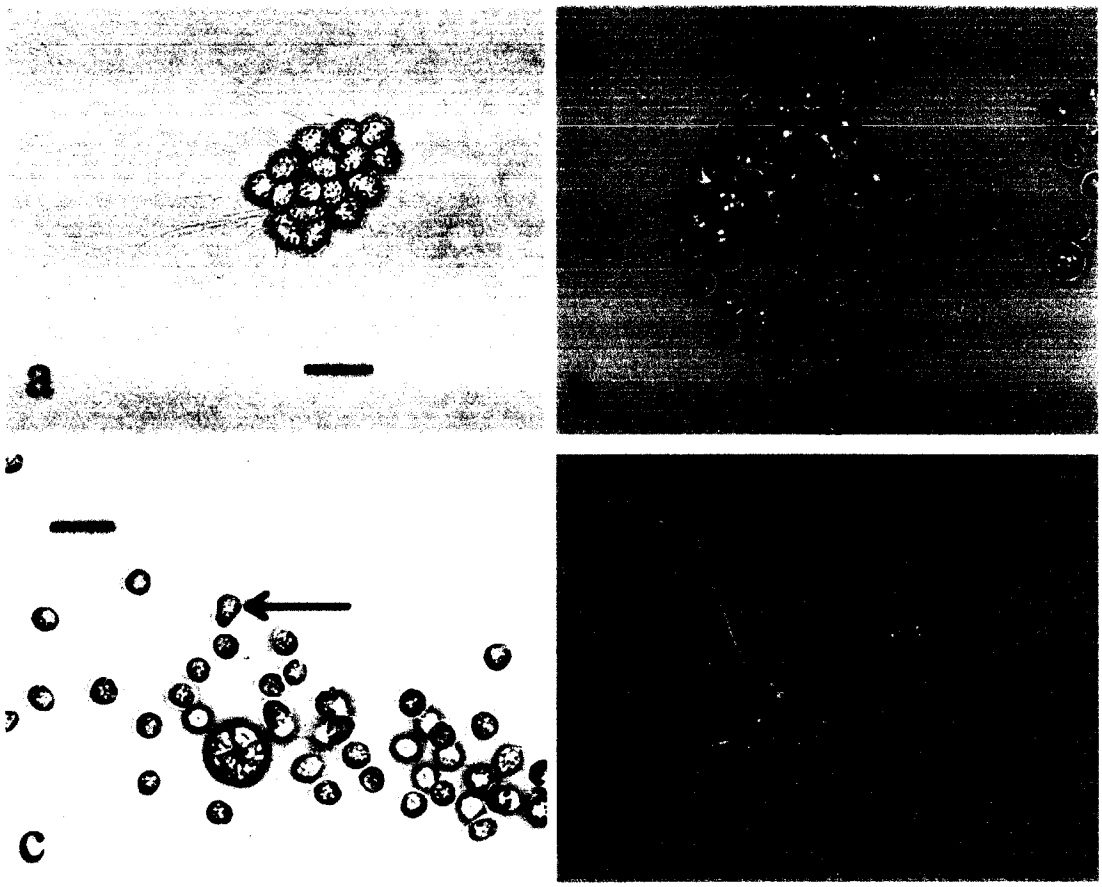


Fig. 8. Various developmental stages of aplanochytrid isolate S2127 displaying large eccentric vacuoles inside the cells (Bar represents 20 μm).

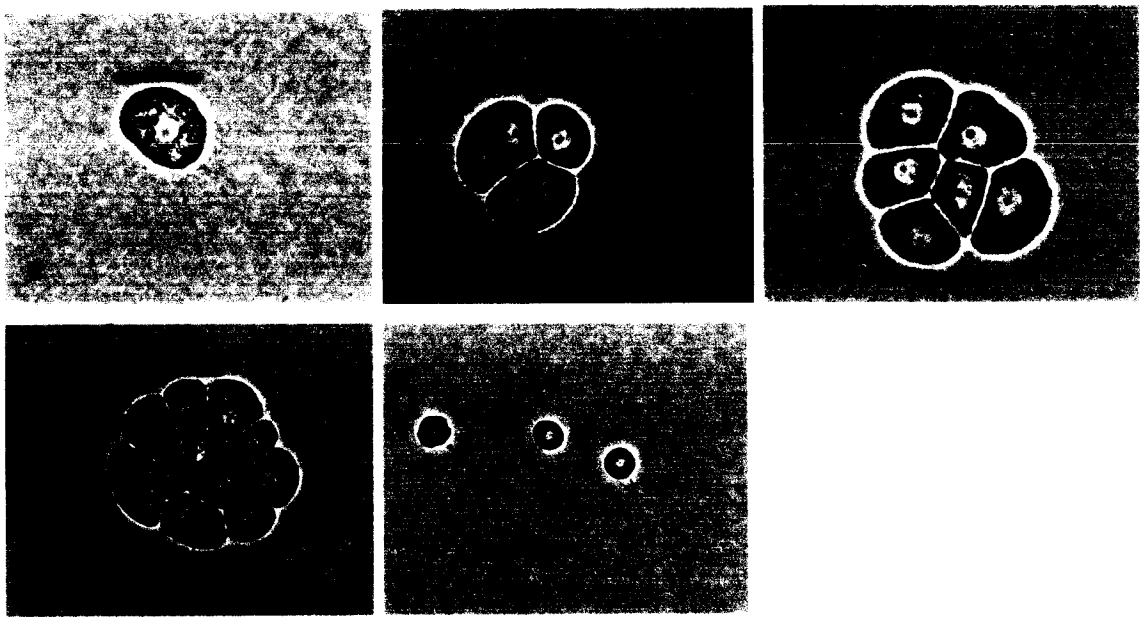


Fig. 9. Fragmentation of amoeboid cells into vegetative cells. (Bar represents 10 μm in a-e, 5μm in f)

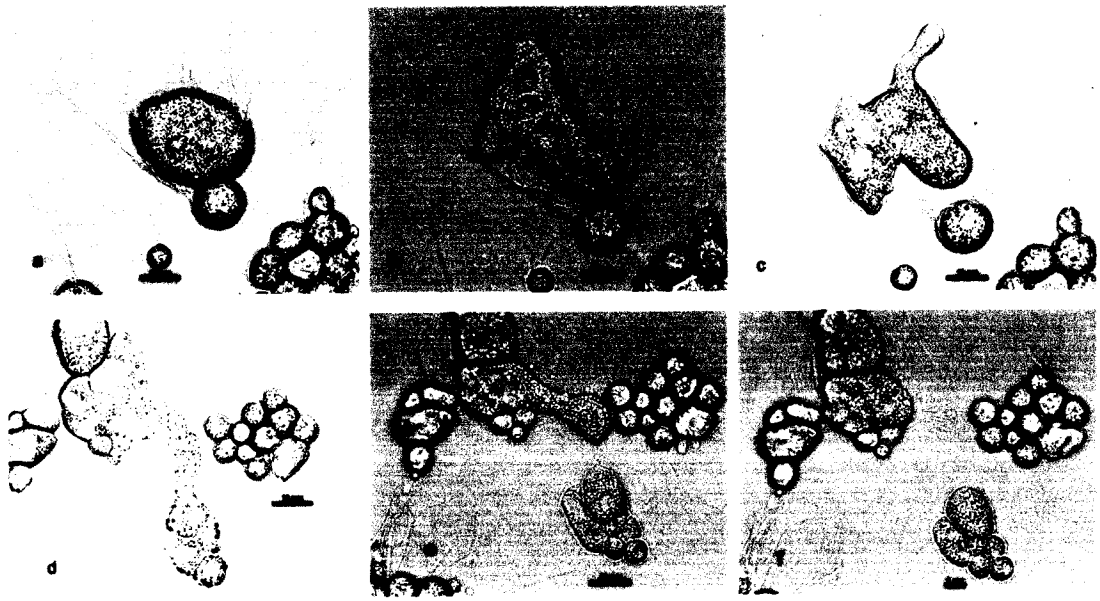


Fig. 10. Successive binary fission of amoeboid cells into spores. (Bar represents 10 μm in a-c, e, g-l and 5 μm in d and f)

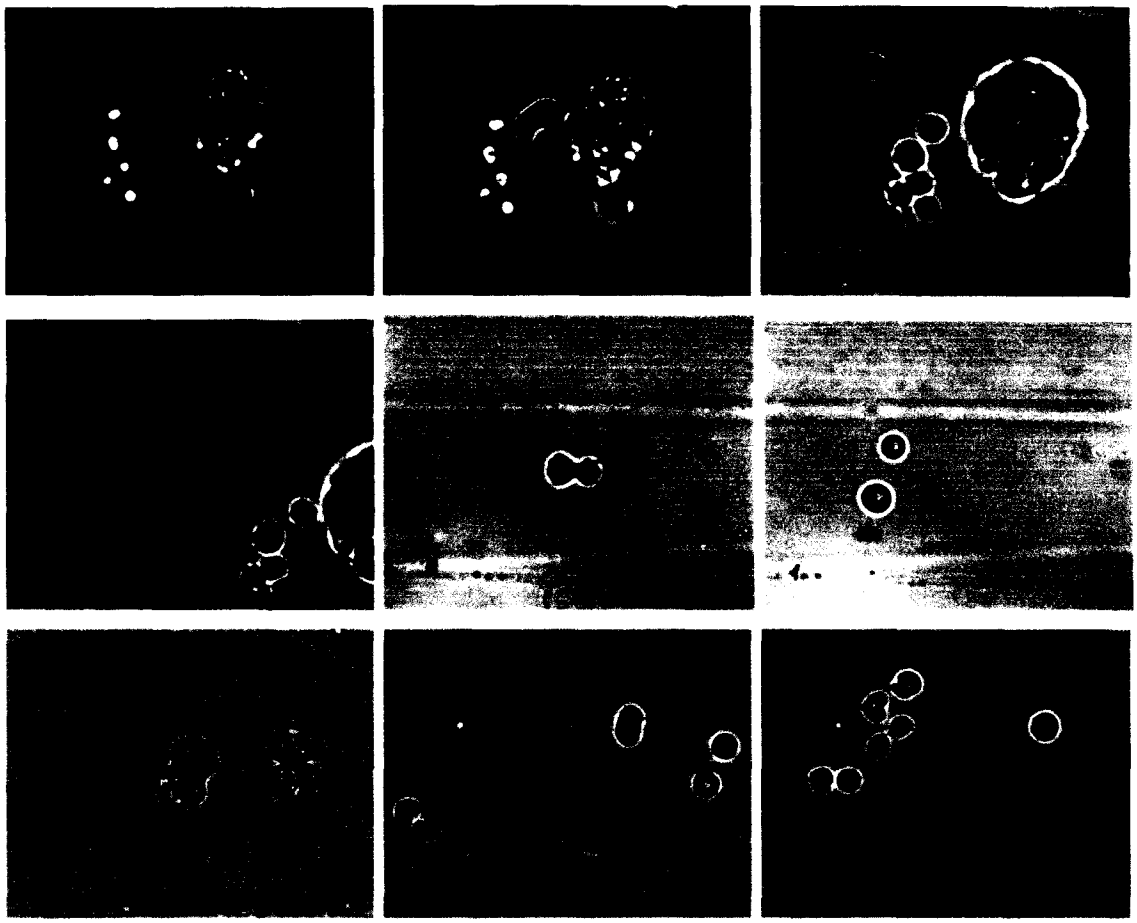


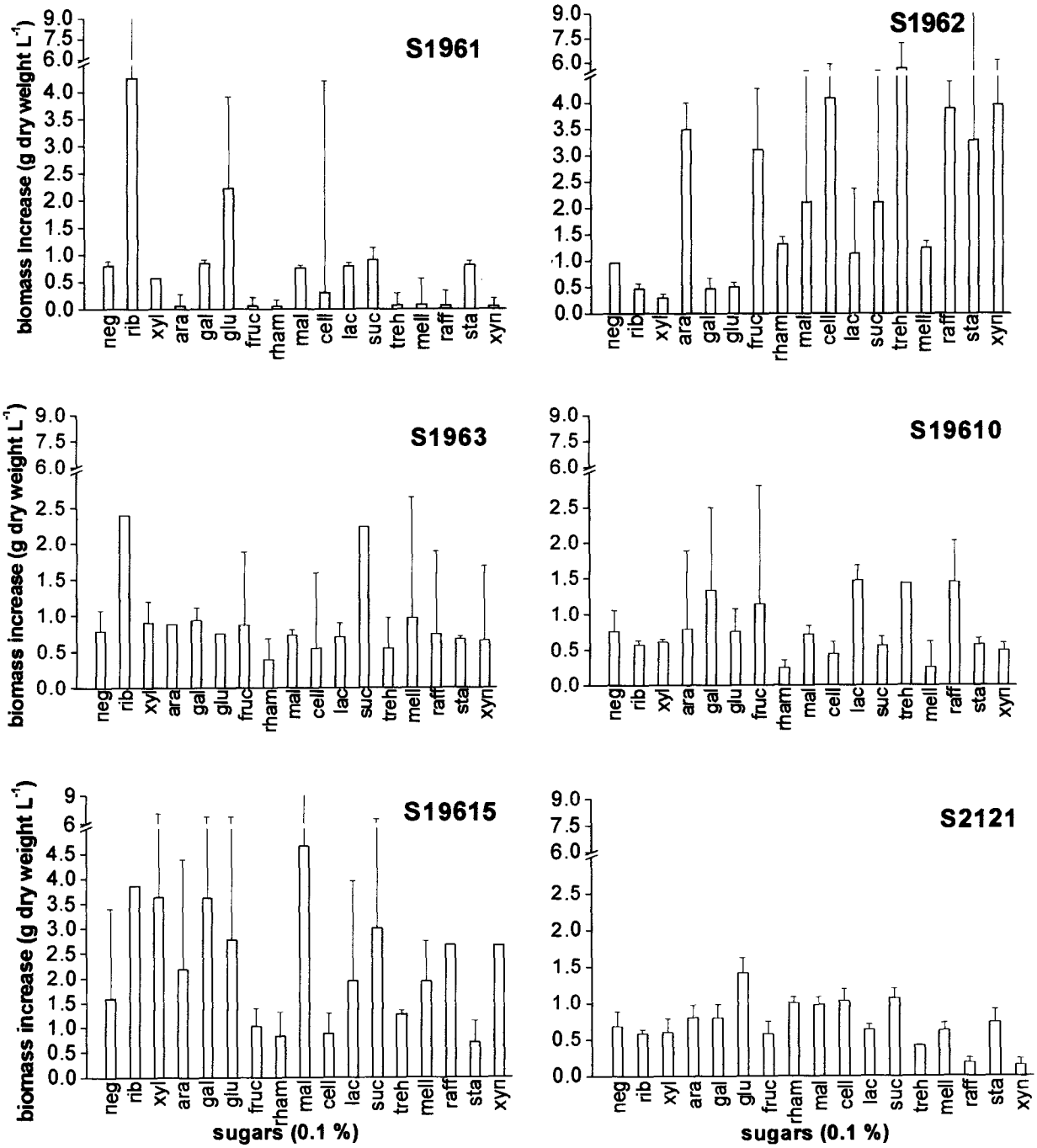
Table 6. Differences in cell morphology of the 14 isolates isolated from equatorial Indian Ocean

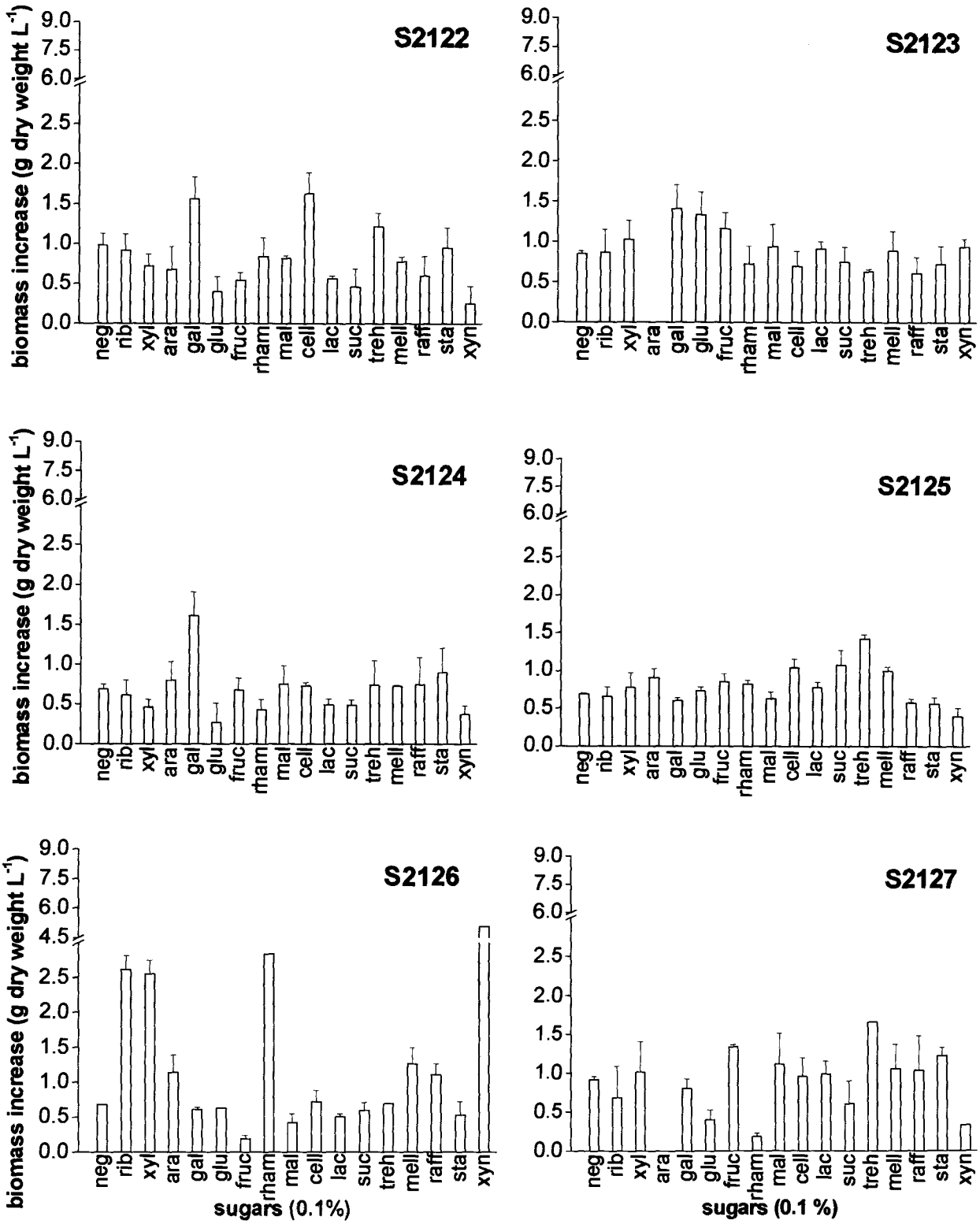
Isolate No.	Size (μm) of			No. of spores	Released spore shape	Presence of ectoplasmic net	Presence of intact cell wall	Presence of amoeboid cells
	Mature cell before formation of spores	Sporangium containing fully mature spores	Spore					
S1961	19.1- 27.8	19.4- 35.0	5.0- 5.8	23- 28	Cuniform	Fine with broad areas	disintegrating	Present sometimes
S1962	9.4- 13.5	13.8- 14.9	3.8- 4.6	-	Circular, cuniform	Fine filaments	-	Nil
S1963	10- 15.4	13.3- 16.9	3.1- 4.5	26- 32	Circular,cuniform, ellipsoidal	Fine filaments	Present (break at two points)	Nil
S19610	15.7- 20	20.0- 20.2	3.1- 5.8	17- 21	Circular	Fine with broad areas	disintegrating	Nil
S19615	10.6- 13.3	10.6- 17.6	3.3- 3.8	16- 26	Cuniform, ellipsoidal	Fine with broad areas	Present (break at one point or disintegrating)	Nil
S2121	10.7- 12.0	14.5-16.4	3.9- 5.0	15- 22	Cuniform	Fine with broad areas	disintegrating	Nil
S2122	7.8-13	13.3-17.5	3.8- 4.9	16- 18	Circular, cuniform	Broad	Present (break at one point)	Present
S2123	10.3- 15.7	12.5- 23.6	4.0- 5.7	16- 22	Circular, cuniform	Fine filaments	Present (break at one point)	Nil
S2124	12.5-15	13.3- 16.7	3.8- 4.9	12- 15	Circular, cuniform	Broad	Present (break at one point)	Present
S2125	9.4- 11.4	16.0- 22.5	4.4- 4.7	12- 13	Cuniform	Fine filaments broad base	Present (break at one point)	Present
S2126	13.5- 15.2	18.0- 27.6	4.8- 7.0	~ 28	Circular, oval	Fine with broad areas	disintegrating	Present
S2127	12.7- 27	18.3- 32	4.4- 6.5	-	Circular, cuniform	Fine filaments	disintegrating	Present
S2128	13.0- 15.2	15.0- 21.6	3.6- 5.0	21- 22	Circular, cuniform	Fine with broad areas	disintegrating	Nil
S2129	10.0- 12.5	13.8- 25	3.8- 5.1	12- 18	Cuniform	Fine filaments	Present (break at one point)	Nil

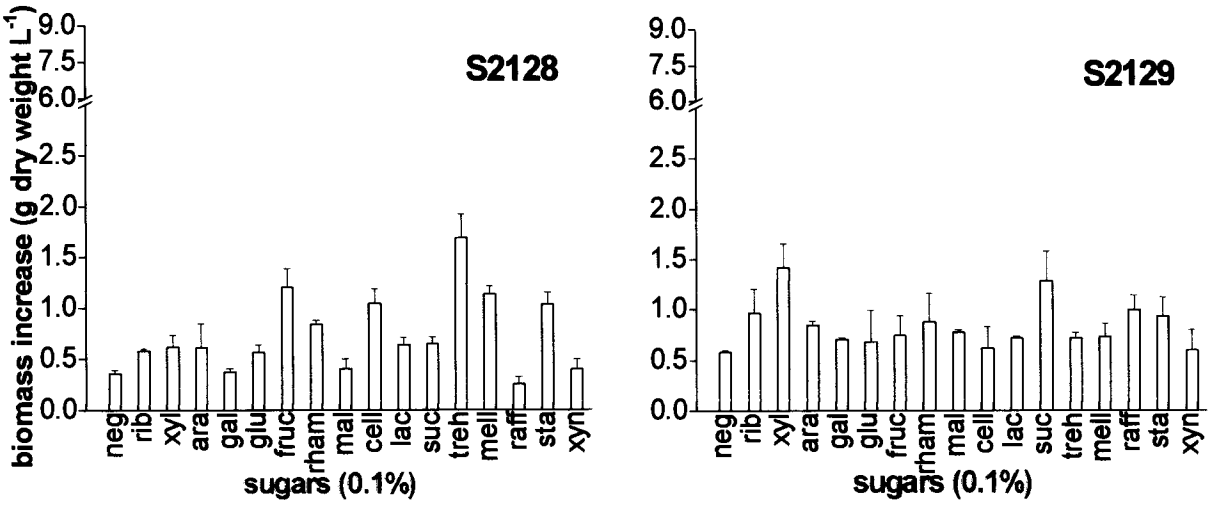
2.3.3 Physiological characterization

Most isolates utilized a broad range of sugars (Fig. 11). However, individual isolates varied in their preference to the different sugars. Three isolates, namely S1961, S1963 and S2129 grew best in the presence of the pentoses, ribose and xylose respectively. S2121, S2123 and S2124 utilized hexoses best. Of these, isolate S2121 preferred glucose, while S2123 and S2124 showed maximum growth in the presence of galactose. Eight isolates showed a preference for the disaccharides maltose, trehalose, rhamnose, cellobiose and lactose. Isolate S2122 also showed best growth in presence of cellobiose followed by galactose. Isolate S2126 exhibited best growth in the presence of the polysaccharide xylan followed by isolates S1962 and S19615.

Fig. 11. Growth of the 14 aplanochytrid isolates on various carbon sources. (Key: neg- negative control, rib- ribose, xyl- xylose, ara- arabinose, gal- galactose, glu- glucose, fruc- fructose, rham- rhamnose, mal- maltose, cell- cellobiose, lac- lactose, suc- sucrose, treh- trehalose, mell- mellibiose, raff- raffinose, sta- starch, xyn- xylan)

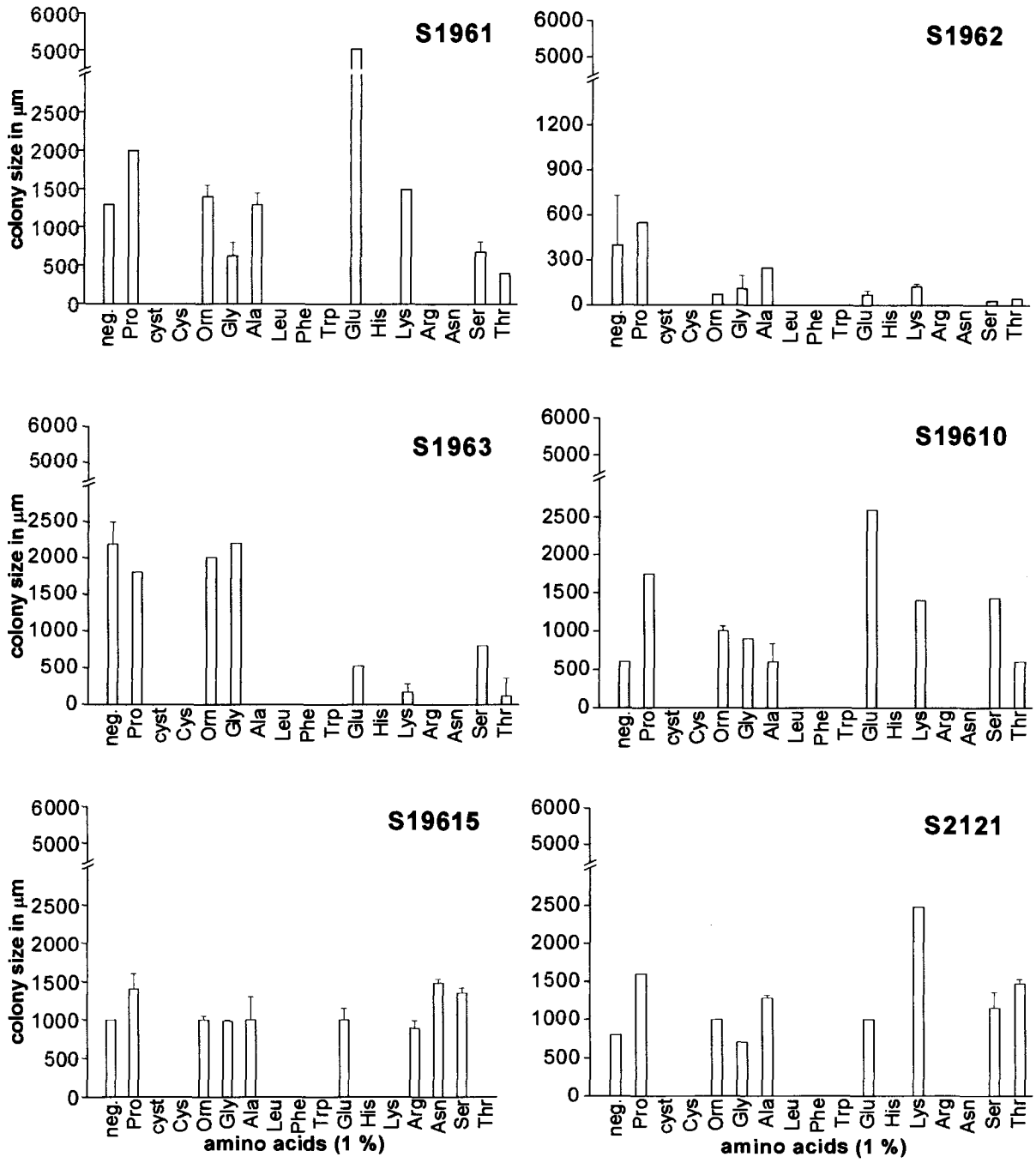


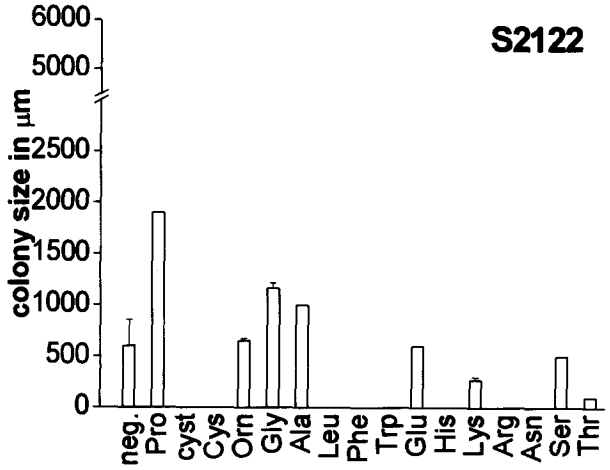

S2122
S2123
S2124
S2125
S2126
S2127
biomass increase (g dry weight L-1)
sugars (0.1%)
sugars (0.1 %)
neg
rib
xyl
ara
gal
glu
fruc
rham
mal
cell
lac
suc
treh
mell
raff
sta
xyn

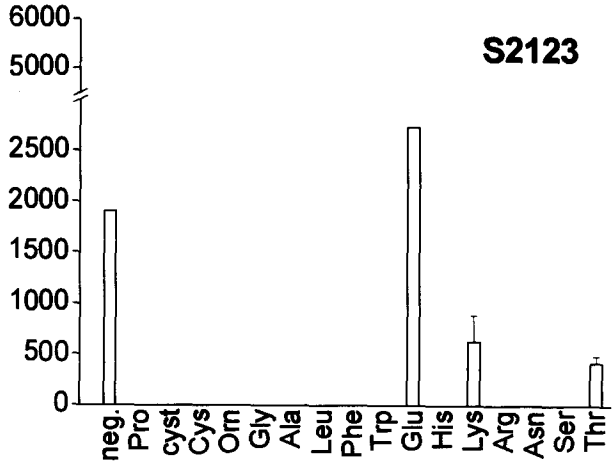


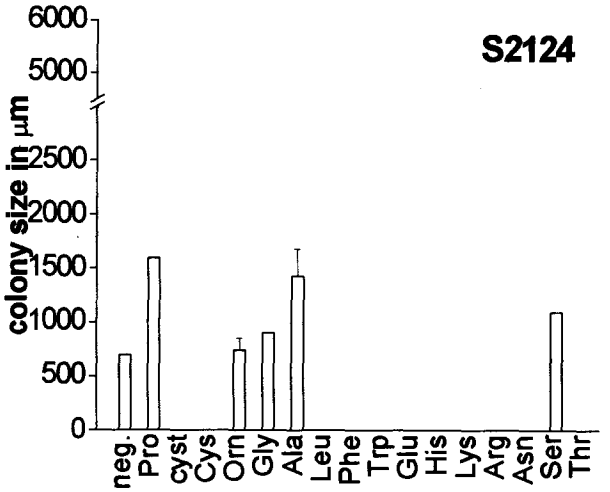
All isolates utilized glutamate. S1961 and S2126 isolates grew best in the presence of this amino acid, compared to others. All isolates except for S2123 and S2127 utilized proline and S2122 and S2124 preferred this amino acid as the nitrogen source to others. None of the isolates utilized the aromatic amino acids phenylalanine and tryptophan as well as others like cystine, cysteine, histidine and leucine.

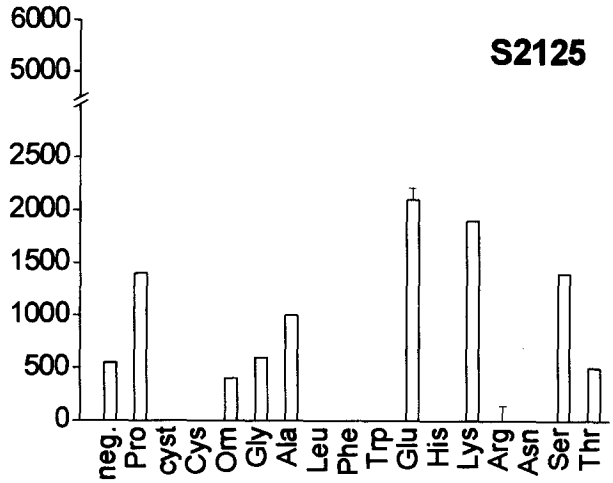
Fig 12. Growth of the 14 isolates on various nitrogen sources. (Key: neg- negative control, Pro- proline, cyst- cystine, Cys- cysteine, Orn- ornithine, Gly- glycine, Ala- alanine, Ser- serine, Lys- lysine, Arg- arginine, Asn- asparagine, Thr- threonine, Glu- glutamic acid, His- histidine, Leu- leucine, Phe- phenylalanine and Trp- tryptophan)

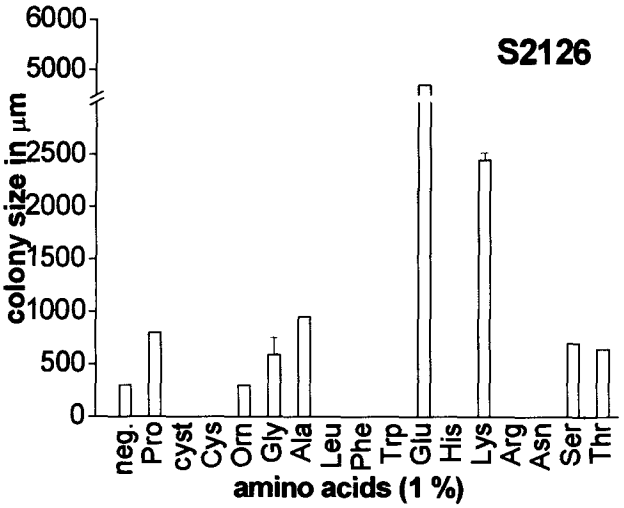


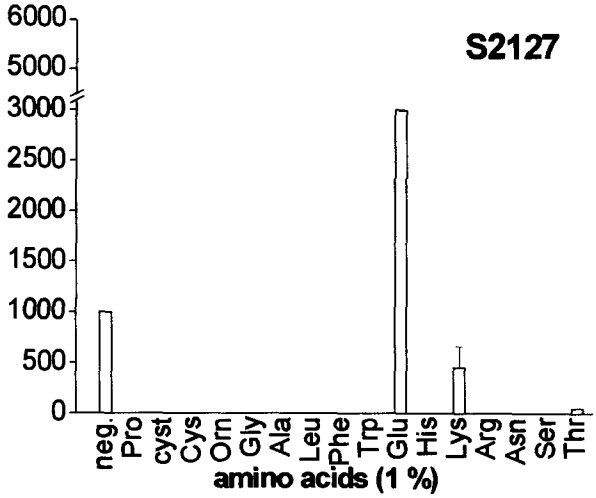

S2122
colony size in µm
6000
5000
2500
2000
1500
1000
500
0
neg.
Pro
cyst
Cys
Orn
Gly
Ala
Leu
Phe
Trp
Glu
His
Lys
Arg
Asn
Ser
Thr

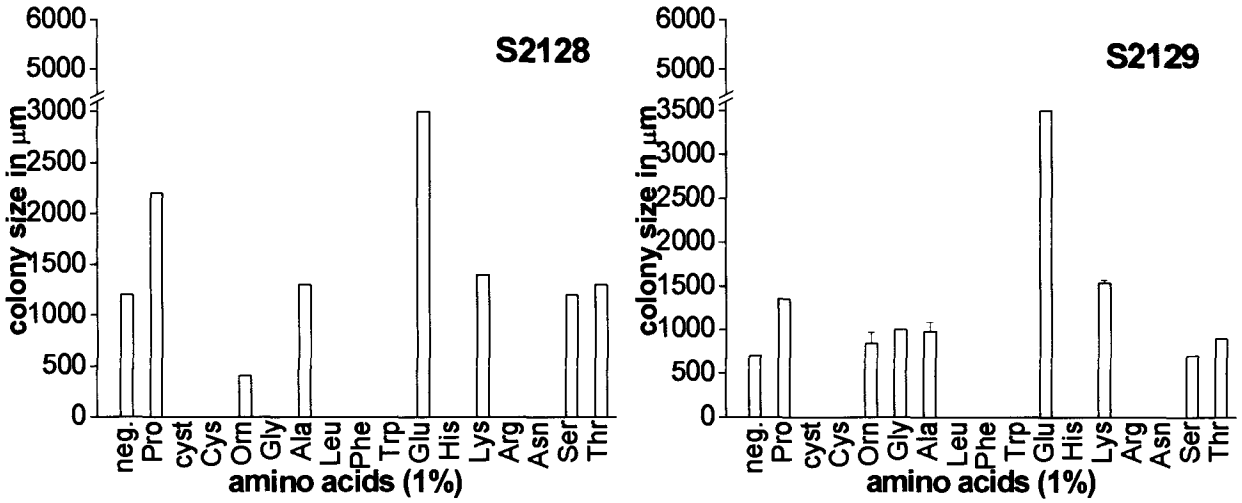

S2123
6000
5000
2500
2000
1500
1000
500
0
neg.
Pro
cyst
Cys
Orn
Gly
Ala
Leu
Phe
Trp
Glu
His
Lys
Arg
Asn
Ser
Thr


S2124
colony size in µm
6000
5000
2500
2000
1500
1000
500
0
neg.
Pro
cyst
Cys
Orn
Gly
Ala
Leu
Phe
Trp
Glu
His
Lys
Arg
Asn
Ser
Thr


S2125
6000
5000
2500
2000
1500
1000
500
0
neg.
Pro
cyst
Cys
Orn
Gly
Ala
Leu
Phe
Trp
Glu
His
Lys
Arg
Asn
Ser
Thr


S2126
colony size in µm
6000
5000
2500
2000
1500
1000
500
0
neg.
Pro
cyst
Cys
Orn
Gly
Ala
Leu
Phe
Trp
Glu
His
Lys
Arg
Asn
Ser
Thr
amino acids (1 %)


S2127
6000
5000
3000
2500
2000
1500
1000
500
0
neg.
Pro
cyst
Cys
Orn
Gly
Ala
Leu
Phe
Trp
Glu
His
Lys
Arg
Asn
Ser
Thr
amino acids (1 %)



Ten of the 14 isolates produced protease. None produced amylase, lipase or chitinase. Isolate S1963 showed the largest clearance zone on skimmed milk medium, while isolate S2123 showed the least (Table 7).

Table 7. Protease activity of 14 aplanochytrid isolates from equatorial Indian Ocean, as observed by the zone of clearance of milk protein.

Isolate no.	Zone of clearance of milk protein (mm)
S1961	16.5
S1962	nil
S1963	18.125
S19610	10.75
S19615	-
S2121	nil
S2122	nil
S2123	2
S2124	13.25
S2125	11.5
S2126	2.625
S2127	3.5
S2128	10.75
S2129	7.75

Cluster analysis was carried out using three different combinations of characters. (1) Morphological and life cycle characters; (2) physiological characters and (3) both morphological and physiological characters. Two distinct clusters were found in the phenogram done only with morphological characters (Fig. 13), one containing isolates with sporangia

> 25 μm size and the second containing those with < 25 μm. All other characters appeared in both clusters.

The phenogram generated using carbon and nitrogen requirements (Fig. 14) separated out S2121 in a cluster. This isolate preferred glucose as a carbon source and lysine and threonine as a nitrogen source. Within the second cluster, isolates S1961, S1963 and S19615, which utilized the pentose sugar ribose were clearly separated from the rest. Trehalose-utilizing isolates, S1962, S2125, S2127 and S2128 also stood out as a separate cluster.

The phenogram generated using a combination of both morphological as well as physiological characters placed a greater emphasis on the cell size (Fig. 15). Here again two distinct clusters were formed, one containing isolates with cell size up to 27.8 μm and sporangium size >25 μm and second containing isolates with smaller cell size up to 15.4 μm and smaller sporangium <25 μm. Rest all characters appeared to be scattered throughout the phenogram.

Fig. 13. Phenogram made by using 27 different morphological characters of fourteen isolates of *Aplanochytrium yorkensis*, isolated from the equatorial Indian Ocean.

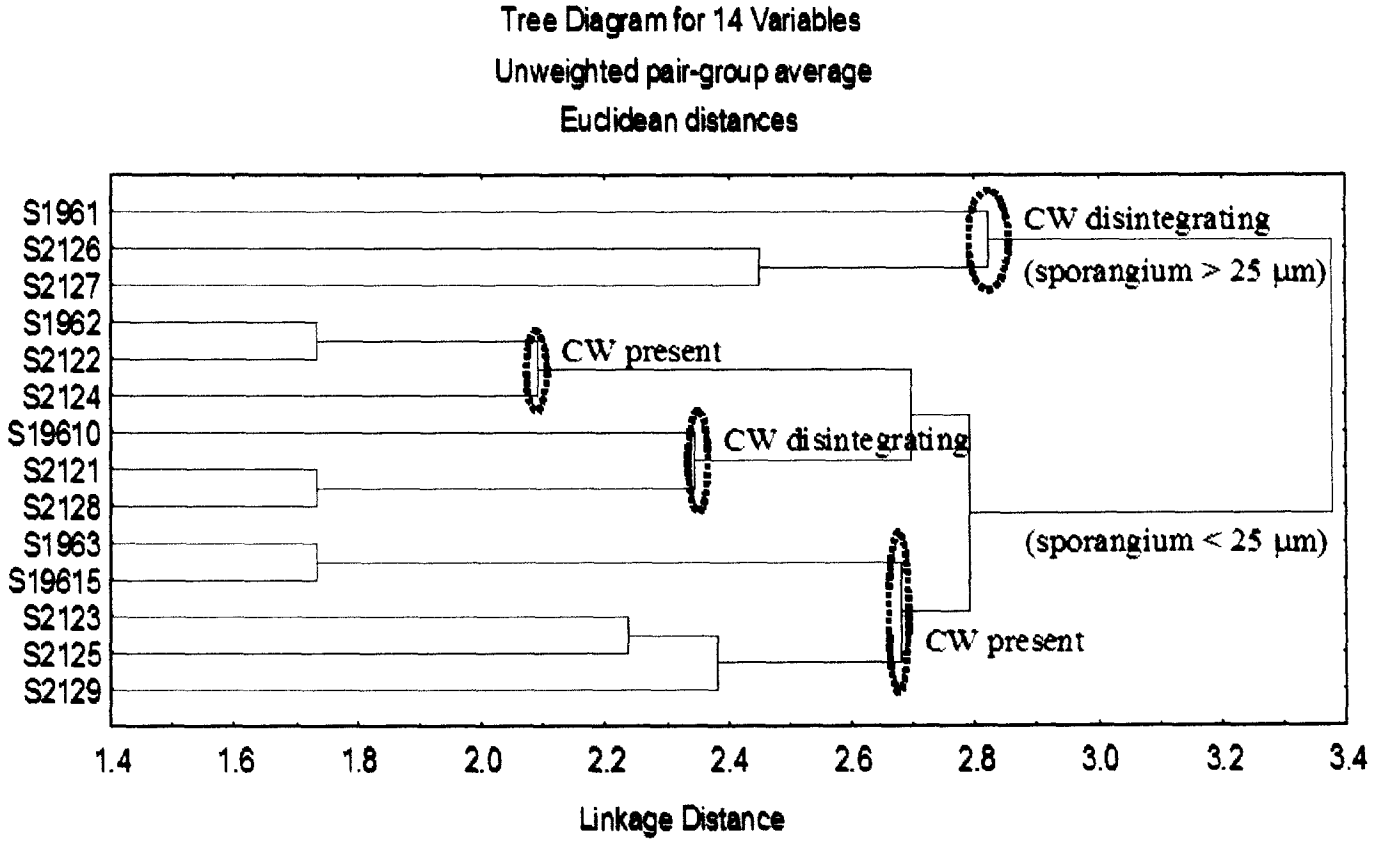


Fig. 14. Phenogram made by using 27 different physiological characters based on carbon and nitrogen nutrition of fourteen isolates of *Aplanochytrium yorkensis*, isolated from the equatorial Indian Ocean.

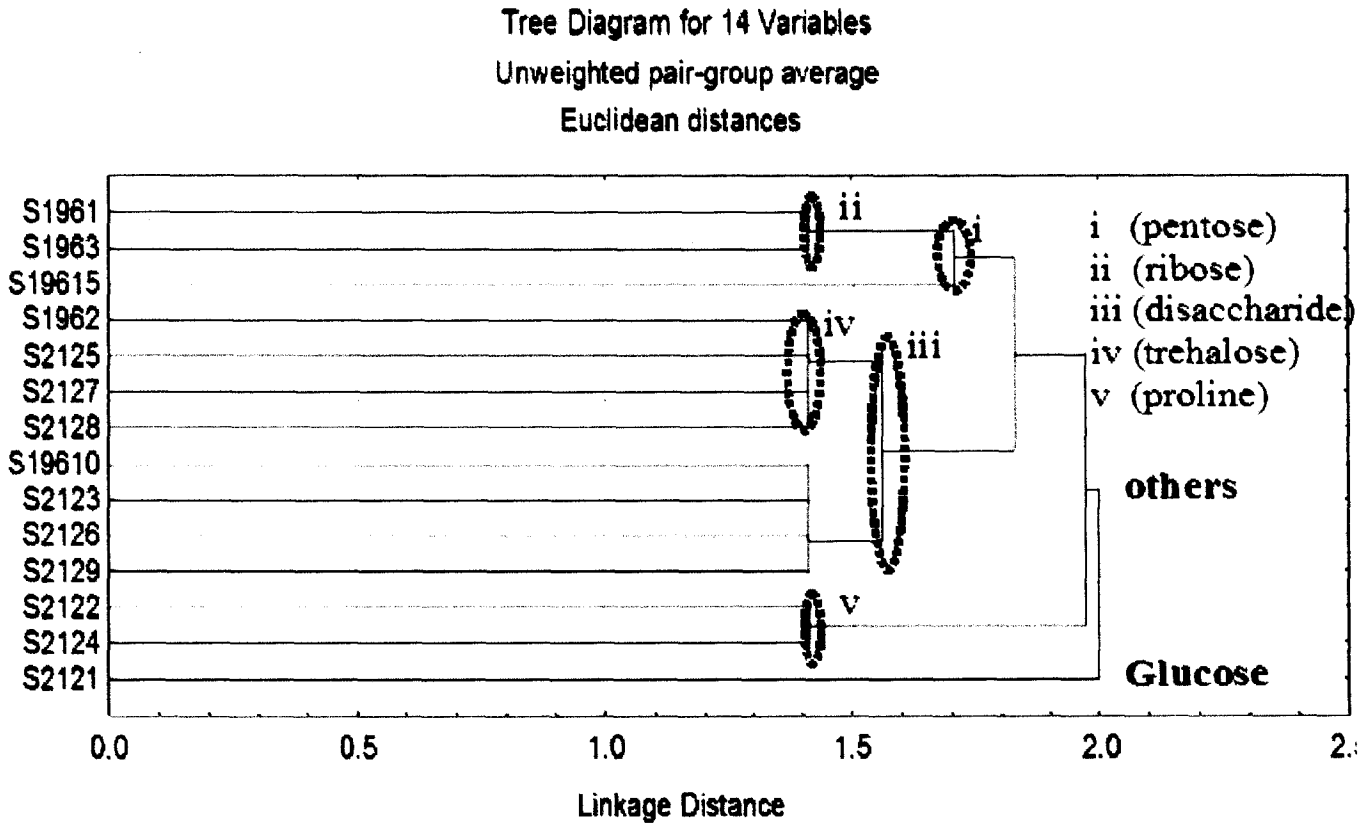
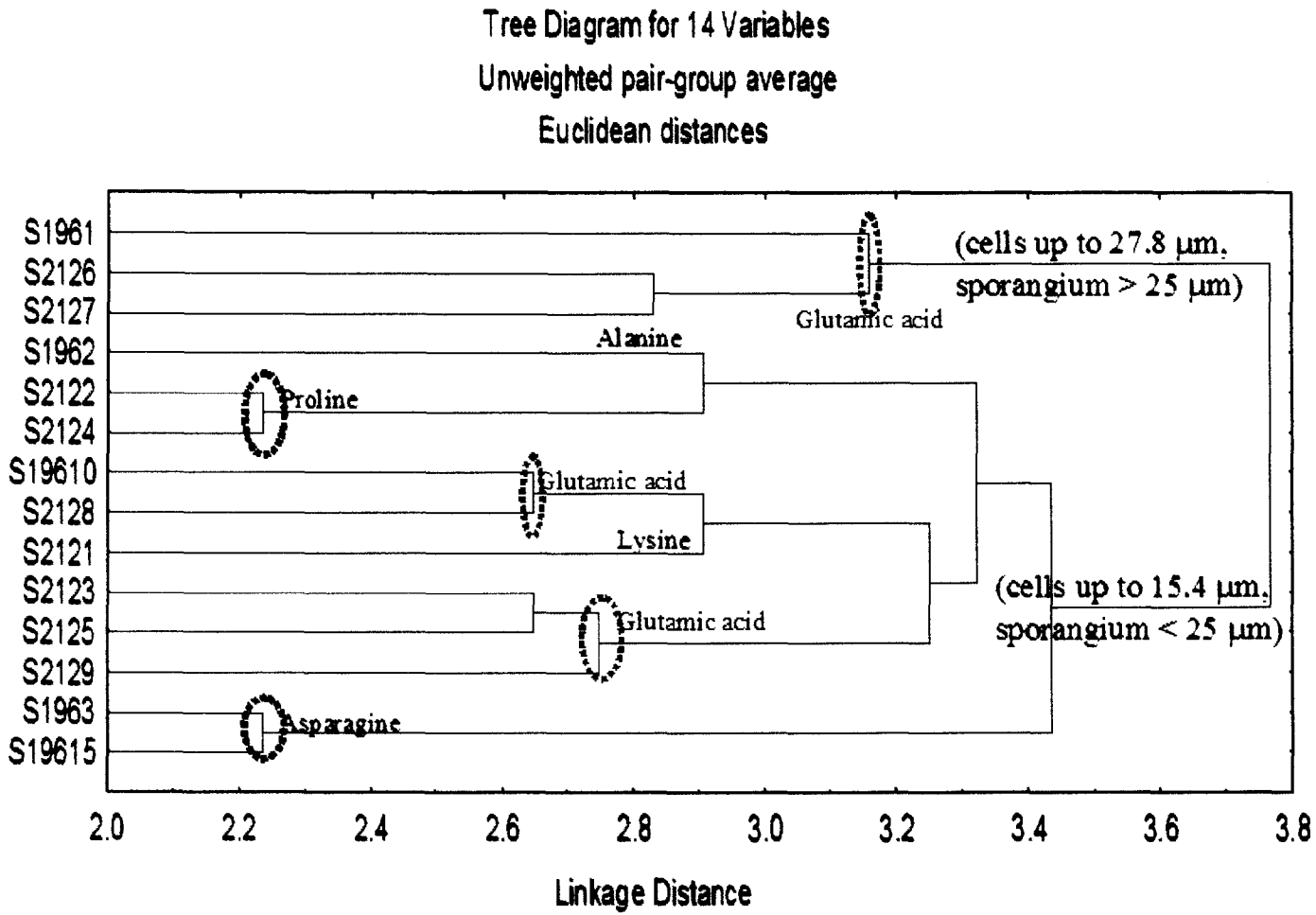


Fig. 15. Phenogram made by using 27 morphological and 27 physiological characters of fourteen isolates of *Aplanochytrium yorkensis*, isolated from the equatorial Indian Ocean.



2.4 Discussion

Taxonomic identity of the organisms: All 14 isolates cultured from zooplankton and identified up to species level based on morphology and life cycle corresponded to *Labyrinthuloides yorkensis* Perkins, originally described from Virginia waters in 1973 (Perkins, 1973). *Labyrinthuloides yorkensis* was described with the following features.

- Uninucleate vegetative cells capable of gliding motility utilizing EN
- EN contain no cytoplasmic organelles, only membrane-bound cisternae
- Cells move independently and may reverse direction of movement
- EN do not enrobe cells
- Vegetative multiplication by binary fission or sporangial formation as a result of successive bipartition or progressive cleavage of the protoplast
- Zoospores, if formed, are biflagellate
- Plasmodia and amoebae may be formed

Except for the absence of biflagellate zoospores, the life cycle of the present isolates fully corresponded to *Labyrinthuloides yorkensis* with regards to the pattern of formation of gliding spores by either successive bipartition or progressive cleavage and formation of motile amoebae and plasmodia on agar surfaces as described by Perkins (1973).

While reviewing the aplanochytrids based on a number of morphological, as well as molecular characteristics, Leander and Porter (2000) concluded that the genus *Labyrinthuloides* was synonymous with *Aplanochytrium.* The type species of the latter genus, *Aplanochytrium kerguelensis* Bahnweg and Sparrow, was originally described from the Antarctic waters near Kerguelen Islands (Bahnweg and Sparrow, 1972). Since *Aplanochytrium* has precedence over *Labyrinthuloides,* all species described under the latter genus have now been placed under *Aplanochytrium.* The 14 isolates obtained in the present study, therefore, were identified as *Aplanochytrium yorkensis* (Perkins) Leander and Porter.

Leander *et al.* (2004) found that *A. yorkensis* and *A. kerguelensis* consistently grouped together in a clade based on 18S rDNA sequences and they named this as '*A. yorkensis* complex'. *A. kerguelensis* is very similar to *A. yorkensis* in all respects, except for the fact that Bahnweg and Sparrow reported that the spores of *A. kerguelensis* were non-motile and crawled out of the sporangium. It is likely that the authors did not notice the spores gliding using the ectoplasmic net elements.

There are several possible reasons for the predominance of *Aplanochytrium yorkensis* in the present study.

1. *Aplanochytrium yorkensis* might be the most dominant species in oceanic waters. For example, Raghukumar (1985) attempted to culture Labyrinthulomycetes from oceanic waters of the Arabian Sea

and succeeded in isolating only *A. yorkensis*. No thraustochytrids could be isolated in his samples.

2. All isolations in this study were made from mesozooplankton. It is likely that aplanochytrids have a definite association with mesozooplankton. This needs to be verified by future studies dealing with isolation of thraustochytrids and aplanochytrids from zooplankton in different waters.
3. Thraustochytrids might have been associated with the mesozooplankton but might not have been amenable to culture using the medium adopted in this study. This is a likely possibility, since Raghukumar *et al.* (2002) observed that while up to 1313 x 10^3 cells of thraustochytrids were present per litre seawater of the Arabian Sea, culture methods yielded only 10.5 x 10^3 cells L^{-1}. being a mere 0.08 % of the total numbers. It is well known with bacteria that culturable numbers are far less than the actual numbers, a phenomenon termed as 'the plate count anomaly' (Staley & Konopka, 1985). Novel and unconventional methods and media for culturing thraustochytrids might shed more light on the diversity of thraustochytrids in the oceans.

Variations among the *Aplanochytrium yorkensis* isolates: Although all isolates in the present study corresponded to *Aplanochytrium yorkensis*,

they varied considerably from each other in a number of morphological characters. These were: colony characteristics, ectoplasmic net elements, mode of liberation of spores, presence or absence of amoebae, shape and size of the spores and organic carbon and nitrogen preferences (Tables 5-7, Figs. 3,4,6,7,9-12). Such variations among species of *Aplanochytrium* have been studied in detail by Leander *et al.* (2004). Based on colony morphology, these authors divided their *Aplanochytrium* isolates into 2 main categories, one forming distinct rays sprawling from the centre outwards and the second forming clumps without radial pattern. These two categories were observed also in the present study. However, the more important differentiation appeared to be the presence or absence of agar penetration, since this corresponded to isolates from different cruises. Thus, the isolates obtained from the first cruise in September 2003 produced colonies that penetrated the agar, while the isolates belonging to the second cruise in October 2004 did not do so.

Amoeboid cells were produced by a few isolates in the present study. Perkins (1973) found amoeboid cells in fresh cultures of *A. yorkensis*, but these were not seen to replicate. They rounded up and became sporangia after a brief period of motility. The amoeboid cells produced by the Indian Ocean isolates did not round up into vegetative cells. Instead, they either fragmented into vegetative cells which later formed sporangia or fragmented successively to form spores. The former

phenomemon is observed in *A. thasii* and *A. schizochytrops.* Amoebae are also known in many thraustochytrids (Gaertner, 1977; Raghukumar, 1982; Honda *et al.*, 1998; Bongiorni *et al.*, 2005c). However, the reason behind their production is not known. Interestingly in the present study, amoeboid cells were mostly produced by isolates that did not penetrate the agar. Out of the nine isolates which did not penetrate the agar, five produced amoebae. Only one isolate amongst the five which penetrated the agar, produced amoebae and that too occasionally. Owing to their osmoheterotrophic mode of nutrition, these organisms need to spread out over a large area to be able to attach to new substrates and absorb nutrition with the help of EN elements. This may be facilitated by the production of amoeboid cells.

Physiological characteristics: Thraustochytrids are known to produce a variety of degradative enzymes (Bongiorni *et al.*, 2005b). Aplanochytrids are also expected to play a role in degradation and mineralization of complex organic compounds in the marine environment, being heterotrophic like their sister group, the thraustochytrids (Raghukumar, 2002). Since physiological characters might throw light upon their ecological behaviour, the production of different degradative enzymes, as well as carbon and nitrogen requirements of aplanochytrids was studied.

Members of the Labyrinthulomycetes generally seem to be inefficient in degrading chitin, which is a structural part of the exoskeleton of zooplankton (Jeuniaux & Voss-Foucart, 1991). None of the Labyrinthulomycetes studied by Bahnweg (1979b) hydrolysed chitin. Likewise, none of the present isolates produced chitinase indicating that aplanochytrids may not play a significant role in degradation of zooplankton exoskeleton. They also did not produce amylase and lipase.

Members of Labyrinthulomycetes appear to be highly efficient in degrading protein. All the 23 isolates studied by Bahnweg (1979b) and 7 out of the 11 isolates studied by Bongiorni *et al.* (2005b) produced protease. Likewise, most of the isolates in the present study were capable of producing proteases. The production of protease as the sole extracellular degradative enzyme suggests that aplanochytrids might have an important role in the degradation of complex proteinaceous compounds of zooplankton cadavers.

Glucose is generally the favoured carbon source for Labyrinthulomycetes (Bahnweg, 1979b). Interestingly, the present isolates of *Aplanochytrium yorkensis* generally preferred pentoses and disaccharides to glucose. Yet, all these isolates were also capable of growing well with other carbon sources, thus being highly versatile in terms of their carbon nutrition.

In the case of amino acids, all the isolates displayed poor growth in liquid medium showing almost insignificant differences in dry weight of biomass. Therefore they were plated on solid media containing different amino acids and their response to different amino acids was measured in terms of their colony diameter. Amino acid requirements of the aplanochytrids in the present study confirmed to earlier results of Bahnweg (1979a) and others (Goldstein, 1963a,b,c; Goldstein & Belsky, 1964; Alderman & Jones, 1971; Sykes & Porter, 1973; Vishniac, 1955) on thraustochytrids and aplanochytrids, in that glutamate was the most favoured amino acid for most isolates and the second most preferred after proline, lysine and alanine for the others. In fact, glutamate can serve as an excellent carbon, as well as nitrogen source for thrausochytrids and aplanochytrids. Bahnweg (1979a) observed that *A. yorkensis* was able to grow also on leucine, isoleucine and glutamine along with glutamate and proline.

Microorganisms utilize glutamate for synthesis of glutamine, proline or aspartic acid (Cameron *et al.*, 1952; Smith, 1957). This might be true also of aplanochytrids. Since proline cannot replace glutamic acid for growth (Smith, 1957), there may be higher requirement for glutamic acid followed by proline as seen in the present study. However, Bahnweg (1979a) observed the opposite wherein growth in presence of proline was more than that in the presence of glutamic acid. Similar to his observation,

the isolate S2124 in the present study also showed maximum growth in the presence of proline. The high requirement for proline might be due to its ATP-synthesizing ability by driving mitochondrial oxidative phosphorylation (Shetty & Wahlqvist, 2004). None of the isolates in the present study showed any growth on histidine, arginine, asparagine, cystine, cysteine, leucine, phenylalanine and tryptophan.

Every individual of the fourteen *Aplanochytrium yorkensis* isolates differed in its sum total of physiological and morphological characteristics. However, cluster analyses trees made separately based either on morphology or physiology did not result in similar groupings of isolates (Figs. 13-14). Thus, for example, they could be separated into two distinct clades when physiological characters were used, the single isolate S2121 preferring glucose as carbon source separating from the rest at the first level. Among the other characters, those that preferred pentoses (S1961, S1963 and S19615) and those that preferred disaccharides (8 isolates) formed distinct clades at the third level (Fig. 14). Using morphological characters, they could be separated into two distinct clades, the ones with sporangia larger than 25 µm (S1961, S2126 and S2127) and those with smaller sporangia forming distinct clades at the first level. Since it was not clear whether the morphological or physiological characteristics were more significant, cluster analyses using both these characters together was carried out (Fig. 15). This yielded two clades, those with sporangia above

25 µm and those that were smaller, at the first level. Strains that preferred asparagine as an amino acid were distinct from those that that had other preferences at the second level. These results lead to the following conclusions.

1. All isolates belonged to a single species, *Aplanochytrium yorkensis*, but with high variability in morphological and physiological characteristics.
2. Individual isolates probably represent distinct clones.
3. The lack of grouping based on any of the characters was likely to have been the result of a high degree of mixing between the populations. The fact that all the isolates were obtained from zooplankton from the same area, namely the equatorial Indian Ocean (77° E, 80.5° E and 93° E), emphasizes this view.
4. The morphological and physiological variations observed may thus be inherent variations of a species and may not be reliable tools to distinguish species within the genus *Aplanochytrium*.
5. Such a mixing could have been the result of sexual reproduction. Although no sexual reproduction has been reported in the Labyrinthulomycetes so far, it cannot be ruled out. Perkins & Amon (1969) reported synaptonemal complexes of chromosomes in their electron microscopic studies on *Labyrinthula*, suggesting the presence of sexual reproduction in it (Porter, 1990).

6. Alternatively, it is possible that although morphologically the isolates corresponded to *A. yorkensis* complex, morphology and physiology alone might not be sufficient to resolve the taxonomy of these organisms. This is discussed further in Chapter 3.

2.5 Conclusions

No thraustochytrids were isolated from zooplankton. All isolates corresponded to the aplanochytrid *Aplanochytrium yorkensis*, suggesting either a predominance of this species in zooplankton or the presence of uncultured thraustochytrids in the samples. The fourteen isolates showed numerous variations in terms of colony morphology, life cycle and preferences to organic carbon and amino acids. Among the morphological characters, important variations include the colony margin, penetration of agar and production of amoebae. Only one isolate preferred glucose as the carbon source, all others utilizing pentoses and disaccharides more efficiently. All grew well on glutamate and proline. All isolates produced protease, while none produced lipase, amylase and chitinase. No consistent grouping of the isolates using cluster analyses was noticed using either morphological or physiological characters. It is suggested that *Aplanochytrium yorkensis* in zooplankton of the equatorial Indian Ocean exists in populations with a high variability and mixing.

Chapter 3.

Molecular characterization of Aplanochytrids from the equatorial Indian Ocean

3.1 Introduction

Conventional taxonomy has relied on phenotypic characters such as morphology and life cycle in the case of higher organisms and biochemical characteristics in the case of microorganisms. Molecular methods have provided some of the most powerful tools for identification and phylogenetic determination of organisms in recent years. Gene sequences permit the construction of phylogenetic trees that reflect the evolutionary history of the species in a given clade and thus provide a natural taxonomy (Edwards-Ingram *et al.*, 2004).

Molecular taxonomy using genetic information in the form of DNA sequences as a universal character set for identification of organisms has several advantages.

- Molecular taxonomy is important especially to those groups in which distinctive phenotypical features are difficult to observe or compare.
- The use of appropriate genes for phylogenetic study also helps in inferring evolutionary relationships.
- Molecular taxonomy avoids subjectivity that often characterizes the use of morphology.
- Molecular taxonomy and evolution requires the use of a gene sequence which can be called as a molecular chronometer, as defined by Woese in 1970. These are molecular sequences having no phenotypic expression and which can undergo changes over time that become fixed in the organisms. Such changes can be used to

measure time in relative sense and therefore these sequences can be used for constructing genealogies. However, defining a single general marker gene for the taxonomic classification of an organism on all taxonomic levels, such as species, genus, phylum and Kingdom, is challenging. Thus, a fast-evolving marker may be needed for classification on the species level, but it may be too divergent for reconstructing the phylogeny at a higher level (Schultz *et al.*, 2005). Thus not all molecular chronometers are equally useful for reconstructing genealogies.

The most useful molecular chronometers actually are ribosomal RNA molecules (Woese, 1987).

- rRNAs are large molecules and hence contain considerable information and their size also makes them less erratic chronometers than smaller molecules.
- They are present in multiple copies within a cell.
- They don't seem to be subjected to lateral gene transfer.
- They are easy to isolate in large quantities.
- They are highly conserved and contain regions that evolve at different rates and are useful at the level of species to higher phylogenetic levels.

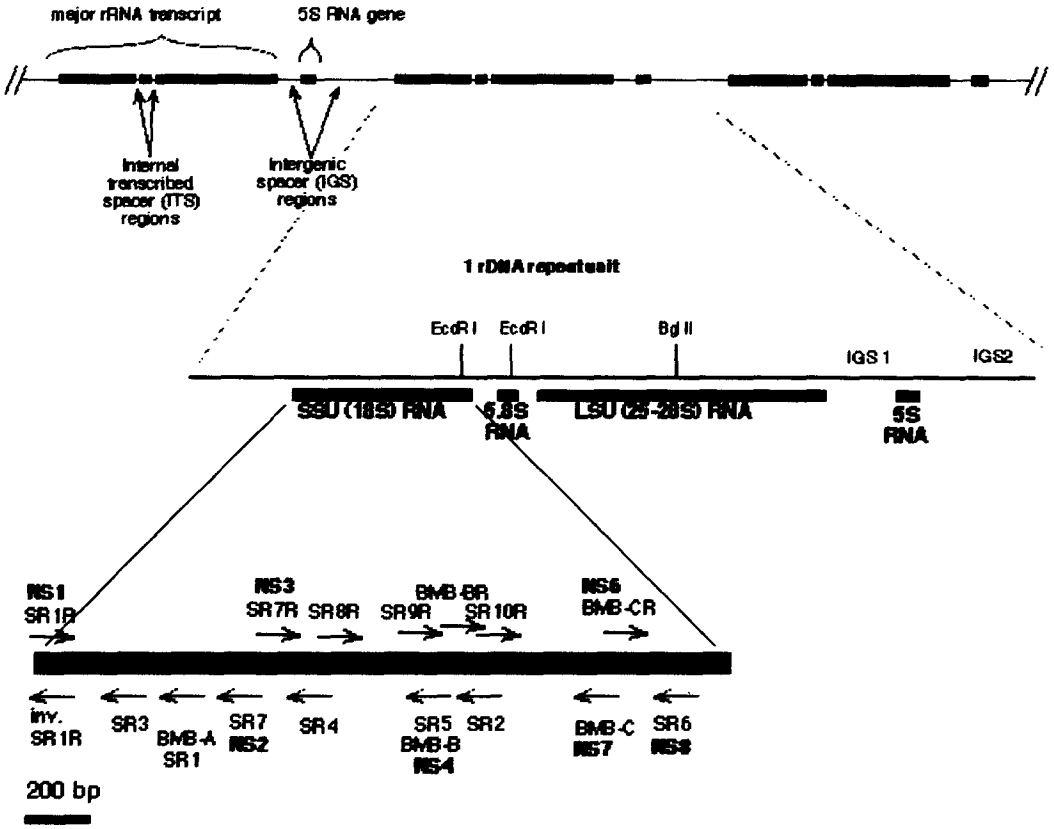
Ribosomal DNA sequences encoding the 16S for prokaryotes and 18S for eukaryotes have found increasing use in inferring

relationships among and, especially, within the major taxonomic groupings.

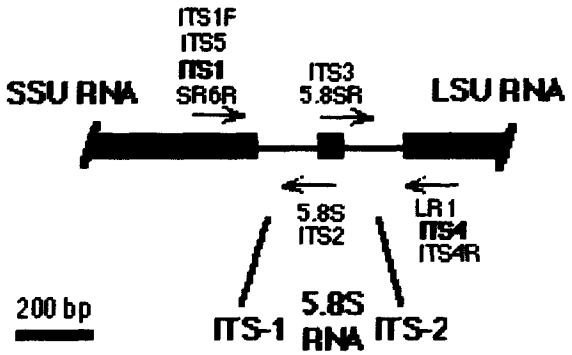
The rDNA or the nuclear-encoded ribosomal RNA genes exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8-12 kb each) arranged in a head-to-toe manner (Fig. 1). Each repeat unit has coding regions for one major transcript (containing the primary rRNAs for a single ribosome), punctuated by one or more intergenic spacer (IGS) regions. In some groups, each repeat also has a separately transcribed coding region for 5S RNA whose position and direction of transcription may vary among groups. The ITS regions are stretches of DNA between the 18S, 5.8S and 28S rRNA genes. The ITS region, a part of rDNA cistron, is highly conserved intraspecifically but variable between different species. Hence it is well suited marker for low level phylogenetic analyses (Schultz *et al.*, 2006). Thus using different markers like ITS for low level and 18S or 28S rDNA for high level classification helps in taxonomic studies.

Molecular taxonomy has aided in recognizing fundamental domains in the phylogeny of living organisms and classifying them into more natural groups. A diagrammatic tree depicting the organization of most eukaryotes into six major groups, showing their relationship with the Bacteria and Archaea is shown in Fig. 2 (Simpson & Roger, 2004).

Fig. 1. Schematic view of ribosomal DNA repeat unit (From http://www.biology.duke.edu/fungi/mycolab/primers.htm)

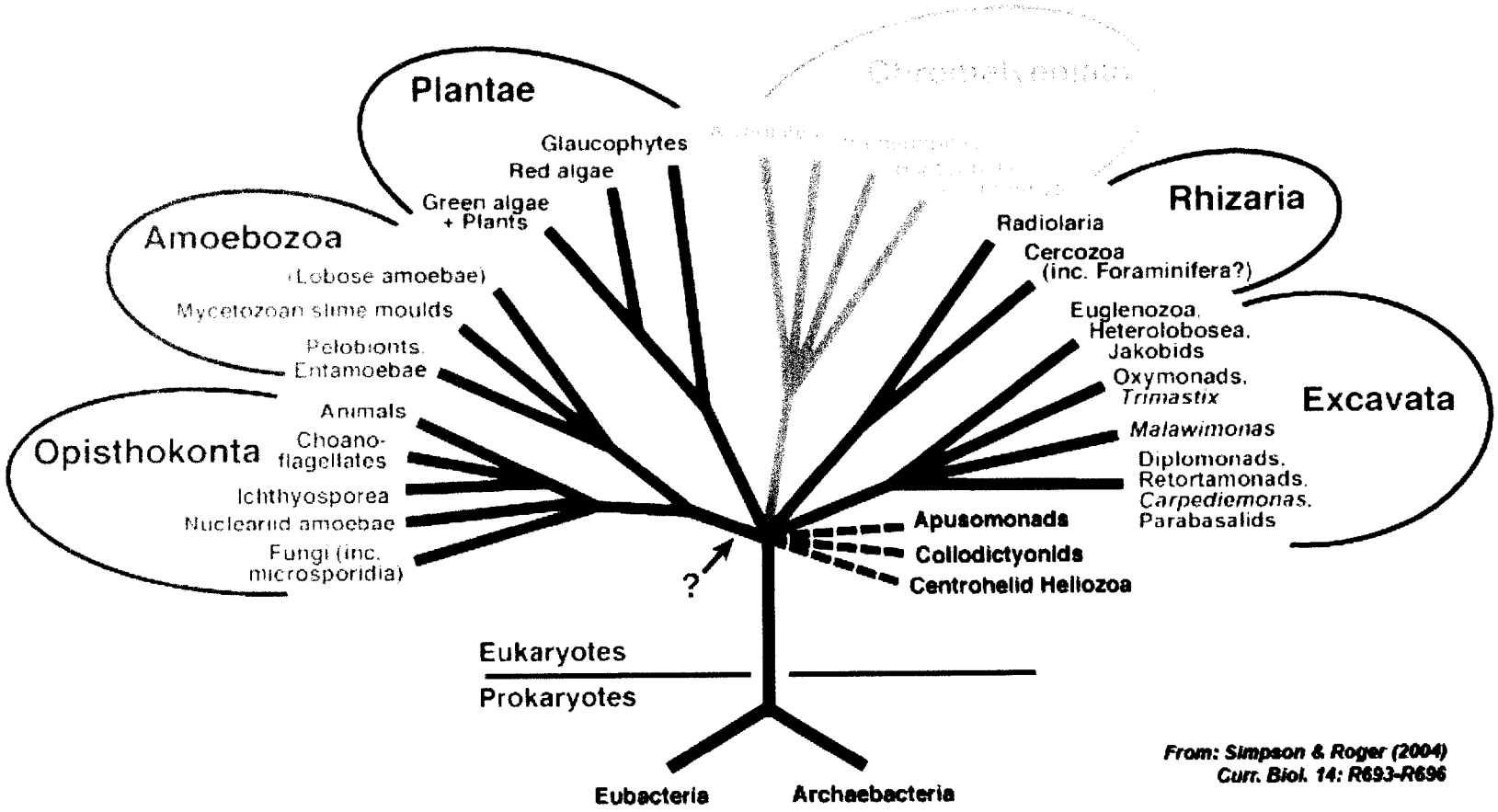


Primers most useful for routine sequencing are shown in bold



Primers for routine sequencing are shown in bold

Fig. 2. Diagrammatic representation of various groups of organisms in tree of life.



The small subunit (SSU) 18S rDNA sequences and ITS sequences are now widely applied to study the taxonomy and phylogeny of both prokaryotes and eukaryotes, both in the terrestrial and marine environment. SSU sequences have also been used to study the diversity of organisms in a given environment, without resorting to isolating or culturing them. While such studies have been widely used to analyse environmental prokaryotic diversity, it is only since about a decade that molecular tools have been used to study cultured and uncultured marine microbial eukaryotic diversity. These studies have shown that picoeukaryotes (less than 1 µm in size) are fundamental components of marine ecosystem (Massana *et al.*, 2004; Moreira & Lopez-Garcia, 2002). Deep-sea sequences from up to (2000 m depth) have shown the presence of thraustochytrids and labyrinthulids in such samples (Moreira & Lopez-Garcia, 2002). van Hannen *et al.* (1999) described the eukaryotic diversity in a detritus-derived experimental system using molecular methods. rRNA-targeted eukaryotic specific probes can be used for fluorescent in situ hybridization to enumerate small eukaryotic cells in marine samples (Lim *et al.*, 1996). Ribosomal DNA sequences are increasingly used for inferring evolutionary relationships amongst non-cultivated protists too, e.g. Radiozoa (Moreira *et al.*, 2007).

The importance of 18S r DNA sequences in understanding the taxonomy of thraustochytrids, aplanochytrids and labyrinthulids was highlighted by the work of Honda *et al.* (1999), who made a detailed study on numerous isolates of these organisms. These authors showed that the exclusive use of morphology and life cycle for classifying the Labyrinthulomycetes could be inadequate or even misleading. This study was also instrumental in revising the taxonomy of the thraustochytrid genus *Schizochytrium,* which was reorganized into 3 new genera (Yokoyama & Honda, 2007). Bongiorni *et al.*, (2005c) used the SSU sequences to describe a new species of *Thraustochytrium*. Leander *et al.*, (2004) made a detailed taxonomic and phylogenetic revision of the aplanochytrids based on the 18S rDNA sequences.

As with most microorganisms, molecular characterization has become an important tool for taxonomic identifications of thraustochytrids, in addition to conventional methods. Although the fourteen isolates from zooplankton of the equatorial Indian Ocean were characterized in terms of their morphology and life cycle, it was essential also to confirm these in terms of their 18S rDNA sequences. Since all of them corresponded to a single species, *Aplanochytrium yorkensis,* it was considered appropriate to characterize them further in terms of their ITS sequences, which shed light on intra-species

differences more effectively than the 18S sequences. In the light of the above, the following objectives were defined.

(1) To sequence and analyze the 18S rRNA gene of the isolates obtained from zooplankton of equatorial Indian Ocean in order to classify them to species level.

(2) To sequence and analyze the ITS rDNA of the isolates in order to understand their intra-species relationships.

3.2 Materials and Methods

3.2.1 Genomic DNA isolation: Isolation of whole genomic DNA from the isolates obtained from equatorial Indian Ocean was carried out by a modification of phenol-chloroform:isoamyl alcohol method (Sambrook *et al.*, 1989). The cell pellets obtained after growing the isolates for 5-7 days in MV broth, were washed with sterile seawater and subjected to repeated freeze-thaw cycles using liquid nitrogen. After 3 freeze-thaw cycles, lysis buffer was added into the tubes followed by equal amounts of phenol and chloroform:isoamyl alcohol. Sterile glass beads of 150-350 μm size (Sigma) were added and vortexed till the entire contents of the tube turned viscous in nature. The tubes were then centrifuged at 12000 rpm for 15 mins, the supernatant collected and treated with chloroform:isoamyl alcohol and centrifuged at 12000 rpm for 3 mins. The supernatant was collected

and DNA was precipitated with 2.5 times volume of cold ethanol. The tubes were stored at -20° C for better precipitation. They were then centrifuged at 12000 rpm for 10 min under cold conditions. The pellets formed were washed once with cold 70 % ethanol and dried using a centrifugal vacuum concentrator (Biotron Ecospin 314, S. No. Ec-050310). The dried DNA pellets were dissolved in sterile TE buffer and checked for purity by electrophoresis using 0.8 % agarose gel. The DNA was stored at -20° C until further analysis.

3.2.2 DNA amplification: 18S rDNA regions of the isolates were amplified by polymerase chain reaction with primers designed by Honda *et al.* (1999). Two universal internal primers (NS3 and NS4) were used (Table 1). Amplification was carried out with final concentrations of 7.5 pM primer each, 100 μM dNTP mix, 1X PCR buffer containing 1.5 mM $MgCl_2$, and 1U Taq polymerase. The reaction conditions were standardized for each primer pair [set I- 18S001 (fwd) & NS4 (rev) and set II- NS3 (fwd) & 18S13 (rev)] by carrying out gradient PCR in Peltier Thermal Cycler (MJ Research, PTC-200, S. No. AL083846). The temperature range checked for annealing of both primer sets was 55-65° C. The conditions were denaturation at 94° C/4 min, 30 cycles of denaturation 94° C/50s, annealing 59.5° C/50 s for set I and 62.5° C/50 s for set II and

extension 72° C/1 min, and final extension step of 72° C/10 min in BioRad Thermal Cycler (S. No. AL100938).

ITS rDNA regions of the isolates were amplified similarly using ITS1 and ITS4 primers (White *et al.*, 1990). Amplification was carried out with final concentrations of 7.5 pM primer each, 100 μM dNTP mix, 1X PCR buffer containing 1.5mM $MgCl_2$, and 1U Taq polymerase. The reaction conditions that were standardized for this primer pair by carrying out gradient PCR as mentioned above were 94° C/4 min, 30 cycles of 94° C/50 s, 57° C/45 s, 72° C/1 min, and final elongation step of 72° C/7 min.

Table 1. List of primers used for 18S and ITS rDNA amplification in the present study.

Primers	Type	Sequence
18S001	Forward	AACCTGGTTGATCCTGCCAGTA
18S13	Reverse	CCTTGTTACGACTTCACCTTCCTCT
NS3	Forward	GCAAGTCTGGTGCCAGCAGCC
NS4	Reverse	CTTCCGTCAATTCCTTTAAG
ITS1	Forward	TCCGTAGGTGAACCTGCGG
ITS4	Reverse	TCCTCCGCTTATTGATATGC

3.2.3 PCR product purification and sequencing: The amplified PCR products were checked by electrophoresis on 1.2 % agarose gel. The PCR products were purified using Montage PCR Clean Up Kit (Millipore) and sent for sequencing. The purified PCR products of the isolates for which sequencing failed were cloned using TOPO

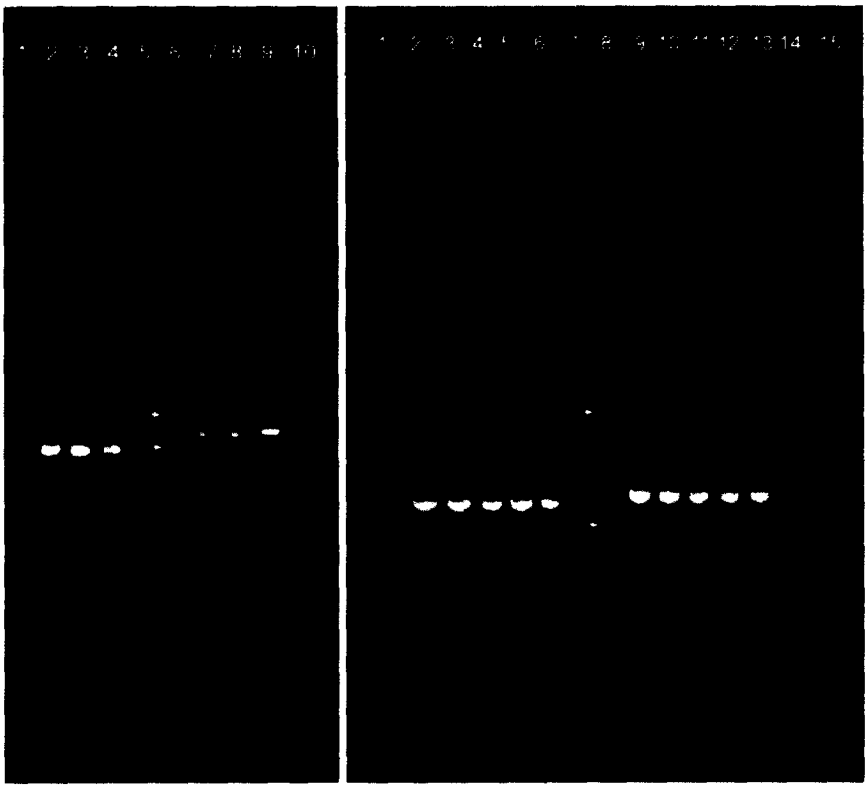
Cloning Kit (Invitrogen, USA) and the plasmid from the transformants was extracted by Alkaline Lysis method (Birnboim & Doly, 1979) and were sequenced by Microsynth AG (Switzerland) or Bangalore Genei (India).

3.2.4 Phylogenetic analysis: Phylogenetic trees based on the 18S rDNA and ITS sequences were generated using the Neighbour-Joining (NJ), Minimum-Evolution (ME) and the Maximum-Parsimony (MP) methods. The analyses were performed by using MEGA version 4. Sources of the other sequences, rooted to Stramenopiles were obtained from NCBI database. For phylogenetic trees based on ITS sequences, only those sequences generated from this study were used, since no ITS sequences for aplanochytrids are available.

3.3 Results

The 18S rDNA of 8 cultures and ITS rDNA of 7 cultures isolated from zooplankton of the equatorial Indian Ocean water column were sequenced. Figs. I and II show the amplified products of 18S rDNA and ITS rDNA respectively as visualized on agarose gel. Partial sequences of 18S rDNA and ITS rDNA were obtained. The 18S rDNA sequences ranged in size from 1353 to 1731 bp and that of the ITS region ranged from 447 to 572 bp.

Fig. I. Agarose gel electrophoresis of PCR –amplified products of 18S rDNA of 7 isolates of *Aplanochytrium yorkensis* with both the primer sets. (Upper arrow marks band of size 2 kb and lower of 1 kb).



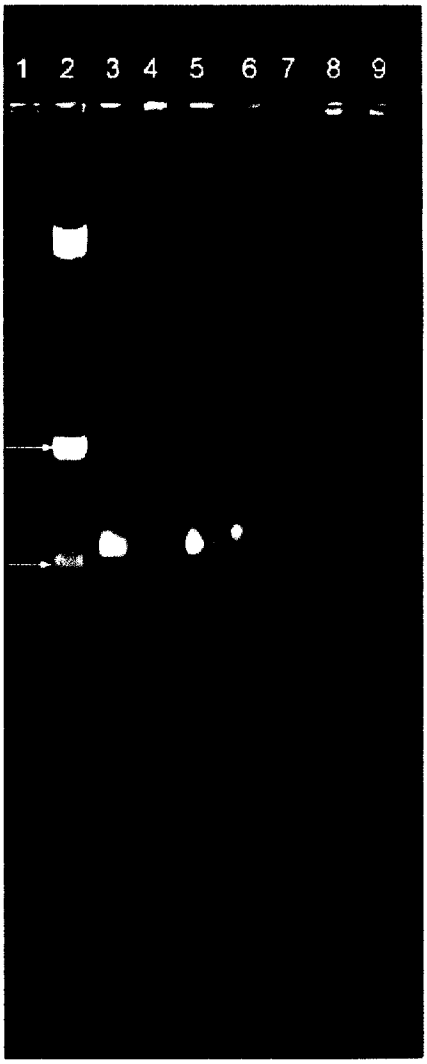
Band Size of DNA in Lane 6 (left gel) and 8 (right gel) from top to bottom

1	10 kb
2	8 kb
3	6 kb
4	5 kb
5	4 kb
6	3 kb
7	2 kb
8	1.5 kb
9	1.2 kb
10	1 kb
11	0.9 kb
12	0.7 kb
13	0.6 kb
14	0.5 kb
15	0.3 kb

Lane	Band
2	S1961 primer set I
3	S1962 primer set I
4	S19610 primer set I
5	Negative control
6	DNA ladder
7	S1961 primer set II
8	S1962 primer set II
9	S19610 primer set II
10	Negative control

Lane	Band
2	S2122 primer set I
3	S2124 primer set I
4	S2125 primer set I
5	S2128 primer set I
6	S2129 primer set I
7	Negative control
8	DNA ladder
9	S2122 primer set II
10	S2124 primer set II
11	S2125 primer set II
12	S2128 primer set II
13	S2129 primer set II
14	Negative control

Fig. II. Agarose gel electrophoresis of PCR-amplified products of ITS rDNA. (Upper arrow marks band of size 1 kb and lower of 0.5 kb)



Lane	Band
2	DNA ladder
3	S2128
4	S1963
5	S2129
6	S19610
7	S2124
8	S1961
9	S2122

Band Size of DNA in Lane 2 from top to bottom	
1	2 kb
2	1.5 kb
3	1.4 kb
4	1.3 kb
5	1.3 kb
6	1.2 kb
7	1.1 kb
8	1 kb
9	0.9 kb
10	0.8 kb
11	0.7 kb
12	0.6 kb
13	0.5 kb
14	0.4 kb
15	0.3 kb
16	0.2 kb
17	0.1 kb

BLAST analysis of the 18S rDNA sequences of the 8 isolates obtained from equatorial Indian Ocean showed 98 to 100 % similarity to *Aplanochytrium kerguelense* and *A. stocchinoi* (Table 2).

Table 2. Details of 18S rDNA sequences of 8 aplanochytrid isolates from the present study.

Isolate	Date	18S rDNA size	BLAST (% query coverage- % similarity)	Gene bank accession No.
S1961	Sep 2003	1682 bp	*A. stocchinoi* (99%) & *A. kerguelense* (100%)- 98%	EU851167
S1962	Sep 2003	1353 bp	*A. stocchinoi* (99%)- 98%	EU851174
S19610	Sep 2003	1675 bp	*A. stocchinoi* (99%) & *A. kerguelense* (100%)- 98%	EU851168
S2122	Oct 2004	1644 bp	*A. stocchinoi* & *A. kerguelense* (99%)- 98%	EU851172
S2124	Oct 2004	1683 bp	*A. stocchinoi* (99%) & *A. kerguelense* (100%)- 98%	EU851169
S2125	Oct 2004	1682 bp	*A. stocchinoi* (99%) & *A. kerguelense* (100%)- 98%	EU851170
S2128	Oct 2004	1731 bp	*A. stocchinoi* (99%)- 98% similarity & *A. kerguelense* (99%)- 97%	EU851173
S2129	Oct 2004	1681 bp	*A. stocchinoi* (99%) & *A. kerguelense* (100%)- 98%	EU851171

Phylogenetic analysis carried out using the three clustering algorithms, Neighbour-Joining, Minimum-Evolution and Maximum-Parsimony methods displayed similar topologies of the phenetic trees except for minor differences in the branch lengths (Figs. 3-5). Three distinct clades of the Labyrinthulomycetes were noticed. The first one comprised the labyrinthulid, *Labyrinthula* sp., which formed a separate clade with a high bootstrap value of 99 to 100 % from the rest of the Labyrinthulomycetes. The other members of the Labyrinthulomycetes formed two clades, the first comprising the two thraustochytrids, *Schizochytrium minutum* and *Thraustochytrium multirudimentale*, which formed a monophyletic group supported by comparatively high bootstrap value of 73 % in NJ tree, 88 % in ME tree and 64 % in MP tree, separated from the second clade comprising the aplanochytrids (Figs. 3-5). Within the *Aplanochytrium* clade, the Indian Ocean isolates and also *Aplanochytrium* DQ367045 formed a distinct clade separated from *A. kerguelensis*, with a bootstrap value of 62 % in NJ tree, 61 % in ME tree and 66 % in MP tree. The Indian Ocean isolates were themselves clustered in two distinct clades, those isolated during SK 212 cruise in October 2004 forming one clade (bootstrap value of 98, 97 and 78 % in NJ, ME and MP trees respectively), while those isolated during SK 196 cruise of September 2003 formed another distinct clade in all 3 trees (63, 72 and 56 % bootstrap values in NJ, ME and MP trees respectively.

ITS sequence data are not available for Labyrinthulomycetes in data banks, except for the labyrinthulid Quahog Parasite X. The ITS sequences of the 8 isolates (Appendix 1) showed 90 % homology to the Quahog Parasite X (Table 3). The ITS rDNA tree of seven isolates obtained from equatorial Indian Ocean is shown in Fig. 6. The topologies of the 3 trees were basically similar in that isolates S19610, S1963 and S 1961, isolated during September 2003 formed a separate clade. The branch length of each of these isolates was negligible. The closest relative of these was S2124, although the bootstrap length (100 %) distinctly separated this from the others. Except for the isolate S2124, the other isolates obtained during October 2004, i.e. S2122, S2128 and S2129 were clustered into one clade.

Table 3. Details of ITS rDNA sequences of 7 aplanochytrid isolates from the present study.

Isolate	Date	ITS rDNA size	BLAST (% query coverage- % similarity)	Gene bank accession No.
S1961	Sep 2003	556 bp	Labyrinthulid quahog parasite QPX isolate clone (28%)- 90% similarity	EU872090
S1963	Sep 2003	503 bp	Labyrinthulid quahog parasite QPX isolate clone (31%)- 90% similarity	EU872091
S19610	Sep 2003	503 bp	Labyrinthulid quahog parasite QPX isolate clone (31%)- 90% similarity	EU872092
S2122	Oct 2004	552 bp	*Cladosporium cladosporioides* (100%)- 98% similarity	-
S2124	Oct 2004	447 bp	*Hyaloperonospora tribulina* (24%) - 78% similarity	EU872093
S2128	Oct 2004	572 bp	Hypocreaceae clone (99%)- 98% similarity	-
S2129	Oct 2004	562 bp	Hypocreaceae clone (99%)- 98% similarity	-

Fig. 3. Neighbour-Joining (NJ) tree of 18S rDNA of aplanochytrids of the present study with *Prorocentrum micans* and *Oxytricha granulifera* as outgroups. (Key: AYC- *Aplanochytrium yorkensis* complex, AA- all aplanochytrids, AMC- *Aplanochytrium minuta* complex, T- thraustochytrids, L- labyrinthulids)

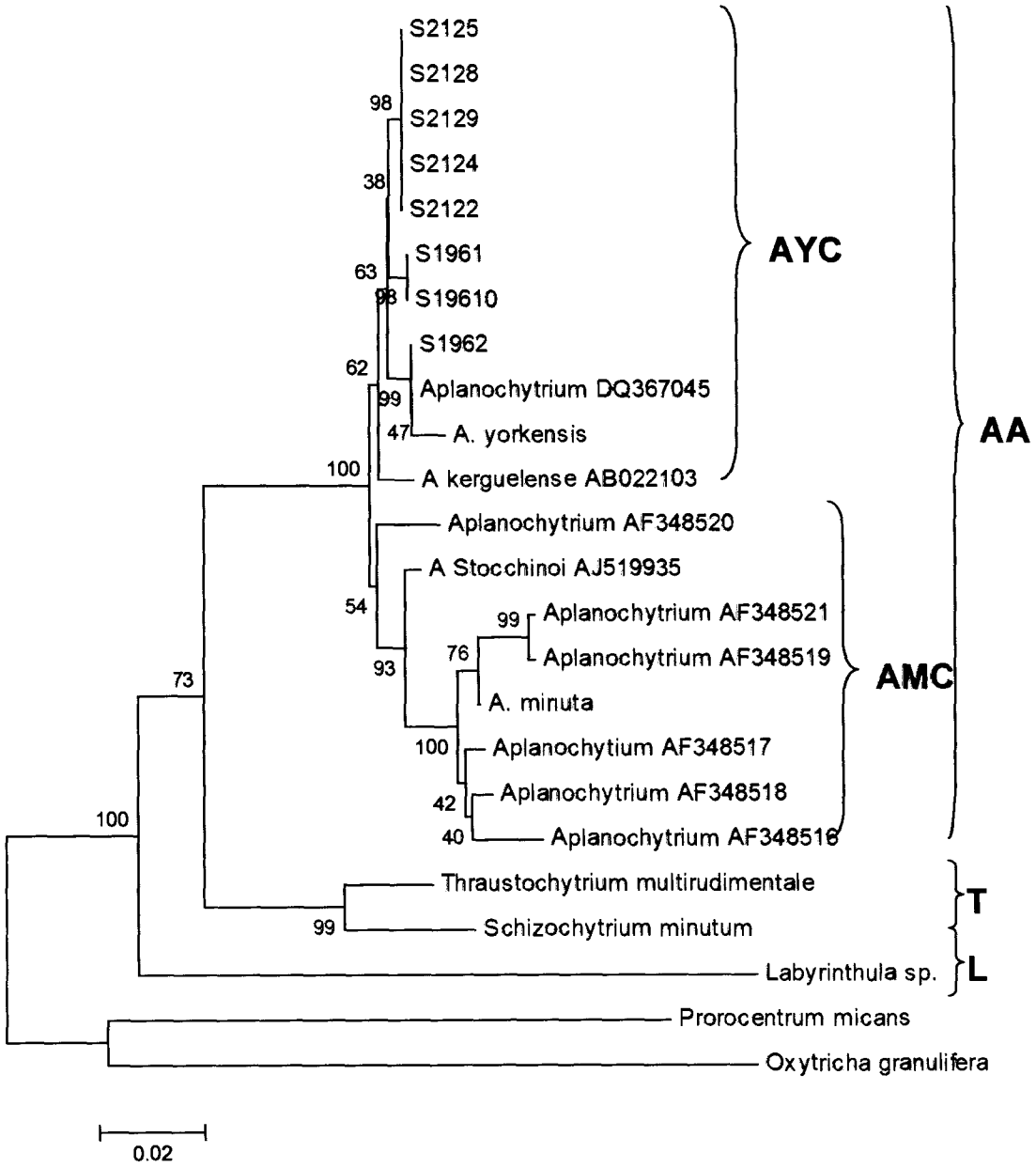


Fig. 4. Minimum-Evolution (ME) tree of 18S rDNA of aplanochytrids of the present study with *Prorocentrum micans* and *Oxytricha granulifera* as outgroups. (Key: AYC- *Aplanochytrium yorkensis* complex, AA- all aplanochytrids, AMC- *Aplanochytrium minuta* complex, T- thraustochytrids, L- labyrinthulids)

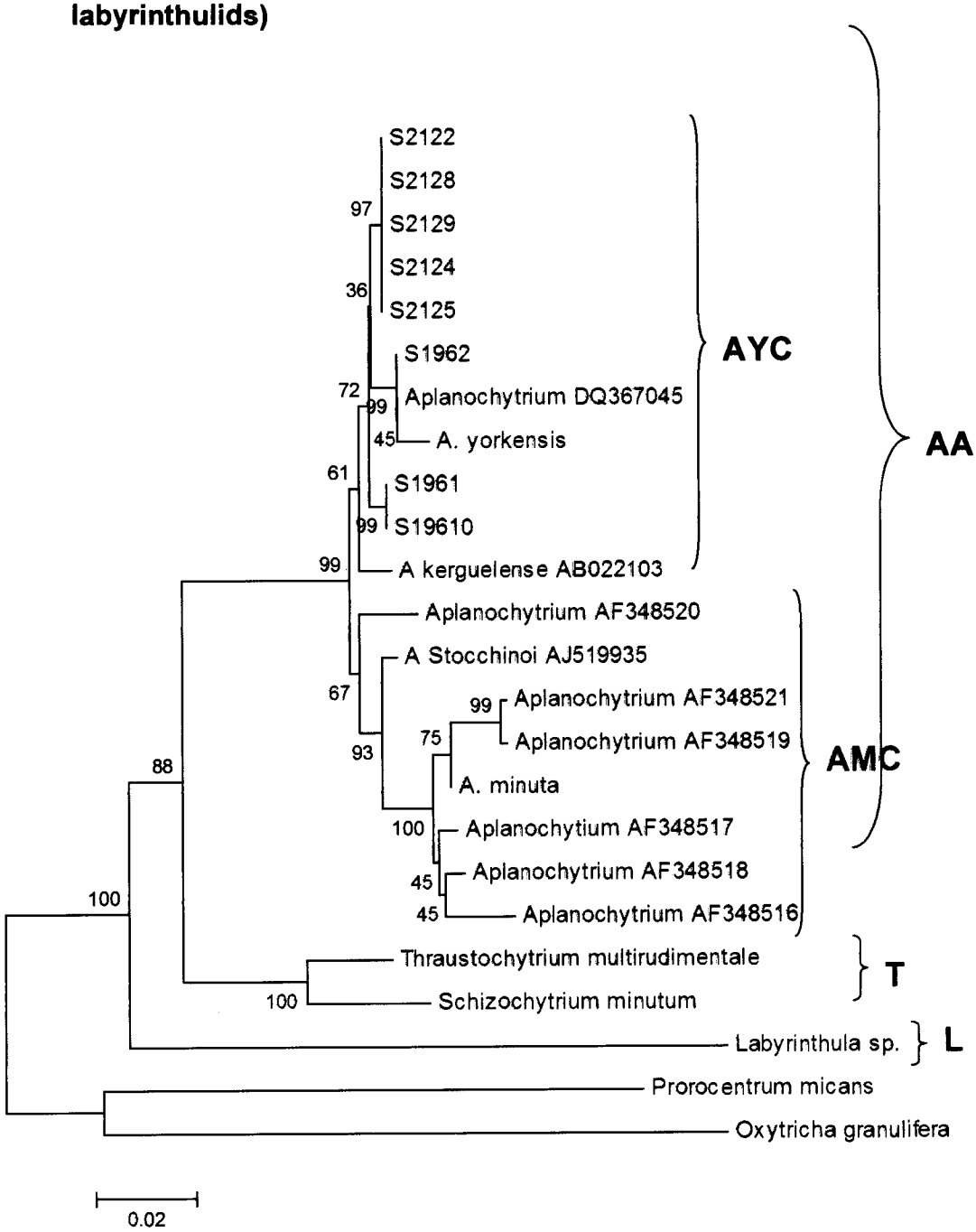


Fig. 5. Maximum-Parsimony (MP) tree of 18S rDNA of aplanochytrids of the present study with *Prorocentrum micans* and *Oxytricha granulifera* as outgroups. (Key: AYC- *Aplanochytrium yorkensis* complex, AA- all aplanochytrids, AMC- *Aplanochytrium minuta* complex, T- thraustochytrids, L- labyrinthulids)

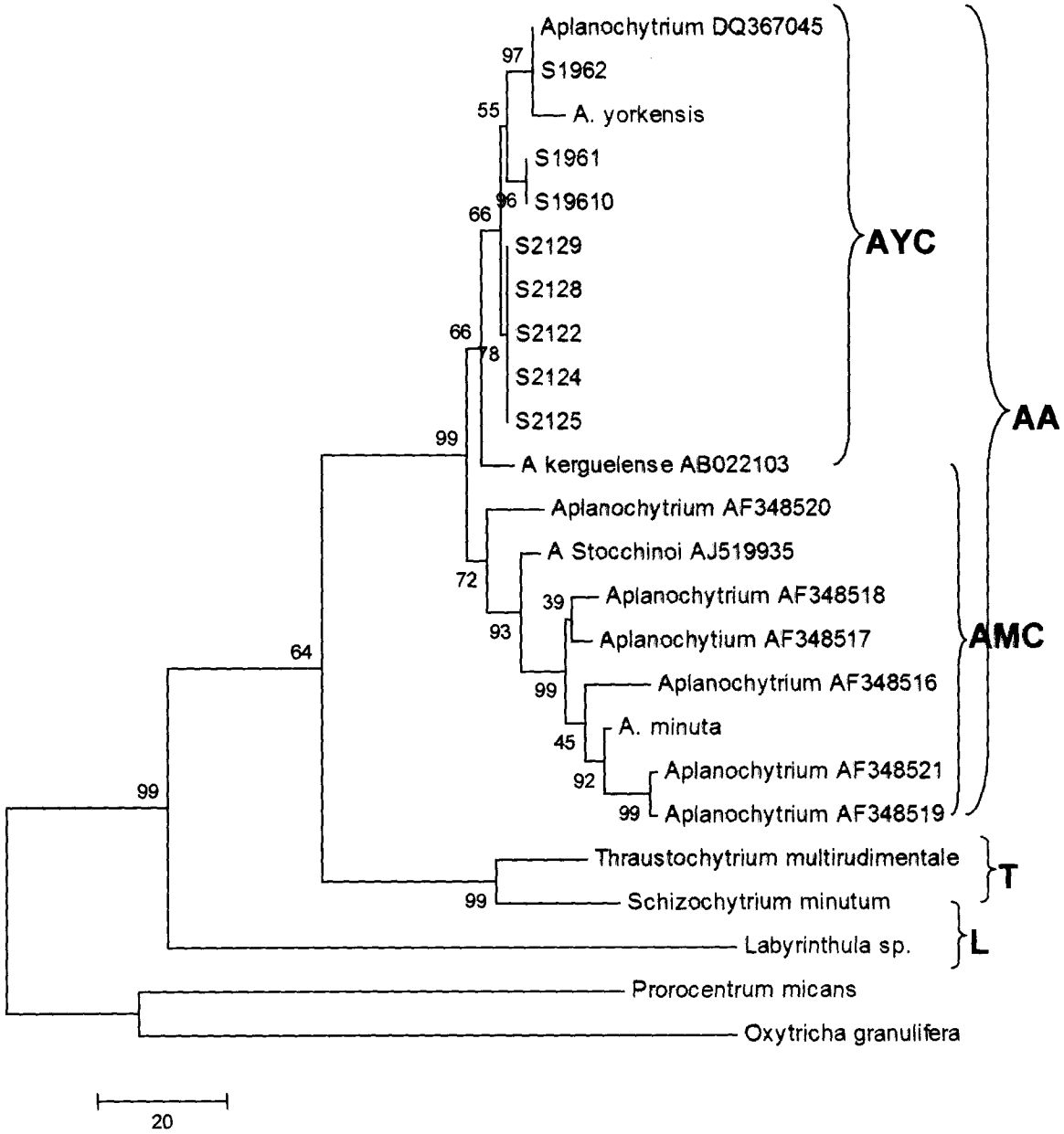
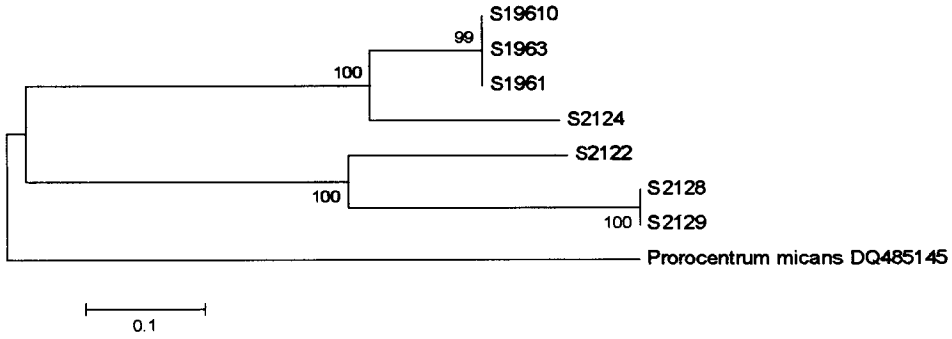
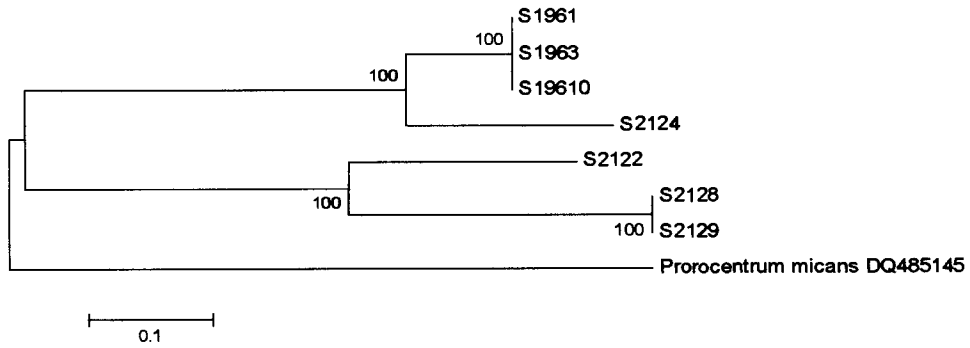


Fig. 6. Phylogenetic trees of ITS rDNA of aplanochytrids of the present study with *Prorocentrum micans* as outgroup.

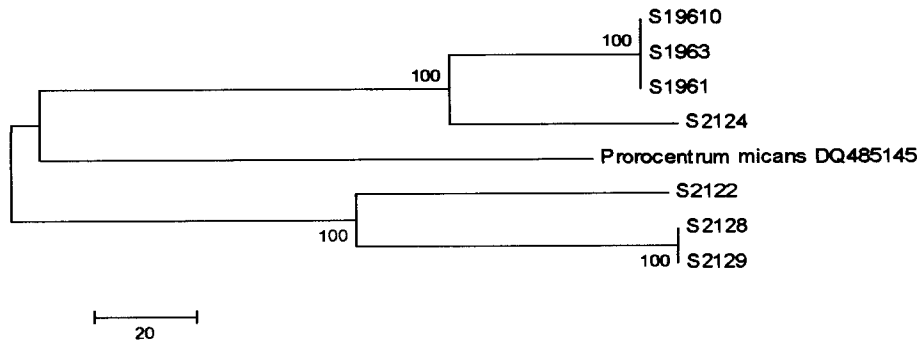
a- NJ tree



b- ME tree



c- MP tree



3.4 Discussion

Phylogenetic trees generated using the 18S rDNA sequences of the eight *Aplanochytrium* isolates, as well as other Labyrinthulomycetes confirmed the findings of Honda *et al.* (1999), Leander & Porter (2000) and Bongiorni *et al.* (2005c), that the Labyrinthulomycetes are comprised of three distinct phylogenetic groups, namely the thraustochytrids, aplanochytrids and labyrinthulids. The eight isolates of *Aplanochytrium yorkensis* of the present study, together with other species of *Aplanochytrium*, namely *A. kerguelensis, A. yorkensis, A. minuta, A. stocchinoi, A. schizochytrops, A. thaisii, A. saliens* and *A. haliotidis* formed a monophyletic group distinct from thraustochytrids and labyrinthulids.

Based on their morphological as well as molecular studies, Leander *et al.* (2004) divided the aplanochytrids into 2 groups or complexes. They found that *A. yorkensis* and *A. kerguelensis* were consistently placed together forming a clade and named it as '*Aplanochytrium yorkensis* complex'. The second clade, the '*Aplanochytrium minuta* complex' consists of the other 6 species. The eight isolates from zooplankton of the equatorial Indian Ocean fall under the '*Aplanochytrium yorkensis*". Based on the morphology and life cycle (Chapter 2), as well as their 18S rDNA sequences, it is clear that the eight isolates in the present study belonged to the '*Aplanochytrium yorkensis*' complex.

In the 18S phylogenetic trees generated in the present study, *A. kerguelensis* (the *A. yorkensis* complex) was the earliest diverging aplanochytrid followed by the isolates of the present study, together with *A. yorkensis* and *Aplanochytrium* Dq367045. Leander *et al.* (2004) also concluded that *A. kerguelensis* diverged before the rest of the aplanochytrids. Although BLAST showed that the present isolates had a high degree of homology to *A. stochhinoi*, it is clear from the 18S phylogenetic trees that they were closer to *A. yorkensis*. The genus *Aplanochytrium* is characterized by the gliding of vegetative cells via ectoplasmic net (EN) and also the formation of motile spores that too glide using the EN. The several taxonomic problems for this genus have been pointed out by Leander *et al.* (2004).

The 8 isolates of the present study were closest to 'Aplanochytrium Dq367045', *Aplanochytrium yorkensis* and *A. kerguelensis* in all three phylogenetic trees and could be placed in the *A. yorkensis* complex based on their 18S rDNA sequences (Figs. 3-5). All the 8 isolates obtained in the present study formed a clade distinct from *A.kerguelensis*, with a bootstrap value of 63, 61 and 66 % in NJ, ME and MP trees respectively. These results suggest that the present isolates might constitute a new taxon, even if their life cycle and morphology corresponded to those of *A. yorkensis*. The studies of Honda *et al.* (1999) have shown that conventional morphological characteristics may not be sufficient to define

species of thraustochytrids. Yokoyama & Honda (2007) recently emended the genus *Schizochytrium* that was based on morphology and life cycle and instead divided it into three different genera, *Schizochytrium* sensu stricto, *Aurantiochytrium* and *Oblongichytrium* gen. nov. based on carotenoid and fatty acid profiles. It is likely that morphology and life cycle are also not sufficient to classify aplanochytrids. It will be interesting to examine the fatty acid and carotenoid profiles of isolates from equatorial Indian Ocean and other aplanochytrids to understand their taxonomy better. No detailed work on these aspects has been carried out so far.

The eight isolates from the Indian Ocean formed a separate clade both from *Aplanochytrium kerguelensis* and *A. yorkensis*. However, these isolates themselves were distinctly separated into those isolated during September 2003 (cruise # SK 196; isolates S1961, S19610, S1962) and those isolated during October 2004 (cruise # SK 212; isolates S2122, S2124, S2125, S2128 and S2129). The two groups showed a significant bootstrap value of 99 % in the 18S sequences. Trees based on ITS sequences confirmed the distinctiveness of the two groups, except for isolate S2124. Thus the two groups showed a 100 % bootstrap value using these sequences (Fig. 6). The ITS sequences clearly showed that the Indian Ocean isolates from the two seasons differed significantly, but not among themselves. This leads to the interesting question as to

whether genetically distinct populations appear in association with zooplankton during different seasons.

3.5 Conclusions: Phylogenetic trees based on the 18S rDNA sequences confirmed morphological observations that the eight isolates of the present study, obtained from zooplankton of the equatorial Indian Ocean were closest to *Aplanochytrium yorkensis*. The eight isolates could be classified as belonging to the '*Aplanochytrium yorkensis* complex' that comprises *A. yorkensis* and *A. kerguelensis* and distinct from the thraustochytrid and labyrinthulid complexes. Among the eight isolates, those obtained from each of the two cruises clustered together, indicating that distinct populations of the aplanochytrids could be present at different times. Among these five isolates from October 2004 cruise formed a distinct clade from the rest of the '*A. yorkensis* complex', thus raising the possibility of being new species. This grouping of the eight isolates was also confirmed by trees based on ITS sequences.

Chapter 4.

Relation of Thraustochytrids and Aplanochytrids to Bacteria and Transparent Exopolymeric Particles (TEPs) in the water column of the equatorial Indian Ocean

4.1 Introduction

Information on spatial and temporal occurrence and population densities of any given group of organisms in different habitats provides an insight into their ecological niche. Early studies on thraustochytrids focused on their presence in coastal habitats, including marine algae, intertidal regions and nearshore waters (Goldstein & Belsky, 1964; Perkins, 1973). Alwin Gaertner in Germany pioneered studies on the occurrence and population densities of thraustochytrids in offshore waters (Gaertner, 1982; Gaertner & Raghukumar, 1980; Raghukumar & Gaertner, 1980). Gaertner carried out extensive studies on the abundance of thraustochytrids in the North Sea waters and the Atlantic, using the Most Probable Number (MPN) culture techniques, using the pine pollen baiting technique. His studies showed that these protists were present in the water column in numbers ranging from below-detectable levels to a few hundreds per liter of sea water. Their numbers in the sediments were far higher. Although these studies unequivocally showed that these protists were always present in the water column, the actual population levels cannot be accurately determined using culture methods, as was shown in the case of bacteria (Jannasch & Jones, 1959; Watson et al., 1977). The development of epifluorescence methods to directly enumerate bacteria in water (Francisco *et al.*, 1973; Hobbie *et al.*, 1977) showed that culturable numbers are far less than the actual numbers present, a phenomenon that came to be termed 'The Great Plate Count

Anomaly' (Staley & Konopka, 1985). An epifluorescence method for counting thraustochytrids in the water column was developed by Raghukumar & Schaumann (1993) to provide a more accurate estimate of their abundance and to overcome the difficulty in distinguishing these protists from others of the same size and colour. This method, using acriflavine (the acriflavine direct detection or AfDD technique), allowed their detection in natural environment by distinguishing them from the other protists through differential staining of their cell wall and cellular contents.

Studies on their abundance and biomass using the AfDD technique, namely those of Raghukumar & Schaumann (1993) in the North Sea, Naganuma *et al.* (1998) and Kimura *et al.* (1999) in the Seto Inland Sea, Japan, Raghukumar *et al.* (2001) in the Arabian Sea and Bongiorni *et al.* (2005a) in the Mediterranean, have demonstrated that thraustochytrids often attain densities of a few hundred thousand cells per liter seawater.

Bacteria are the most abundant living organisms in the sea and play an extremely significant role in the biogeochemistry of the oceans (Azam *et al.*, 1983). Since the Labyrinthulomycetes, including thraustochytrids, aplanochytrids and labyrinthulids are also osmoheterotrophic in nutrition like bacteria (Porter, 1990; Raghukumar, 2002), they are likely to play a role similar to that of bacteria in remineralization of particulate and dissolved organic matter. However, the

relative roles of the two groups of organisms in such ecological processes is not known. Therefore, it is important to study the relation between populations of thraustochytrids, aplanochytrids and labyrinthulids *vis a vis* bacteria in order to understand their ecological role better.

Kimura *et al.* (1999, 2001), Raghukumar *et al.* (2001) and Bongiorni *et al.* (2005a) have compared thraustochytrid biomass to that of bacteria. Their studies demonstrated that thraustochytrids occasionally attained biomass values up to 50 % of the bacteria. These few studies, dealing with these protists in coastal waters or the land-locked Arabian Sea, have noticed varying relations between thraustochytrids and bacteria.

There seems to be a significant positive relation between thraustochytrids and particulate organic carbon (Kimura *et al.*, 2001, Raghukumar *et al.*, 2001). Allochthonous material in the sea brought by riverine input seem to have significant influence on the abundance of these organisms which in turn greatly influences the coastal carbon cycling. The importance of autochthonous particles and substrates that thraustochytrids and aplanochytrids utilize in the water column is not clear.

Marine aggregates or marine snow are hot spots of microbial activity in oceanic waters. Marine aggregates comprise detrital and inorganic particles, faecal pellets and cadavers of zooplankton and microorganisms. These are entrapped in Transparent Exopolymeric Particles (TEPs) which act as the sticky matrix for marine snow (Simon *et al.*, 1990; Passow & Alldredge, 1994). TEPs are fibrillar

mucopolysaccharides formed through coagulation of the increasingly refractory dissolved organic matter left behind after the action of heterotrophic bacterial processes on the biologically labile organic carbon of dissolved polysaccharide exudates released by phytoplankton and bacteria (Alldredge *et al.*, 1993; Beauvais *et al.*, 2003). TEPs have been studied for many years and their importance in oceanic biogeochemical processes is well accepted (Simon *et al.*, 2002). TEPs too are potential substrates for microbial growth. Bacteria play a major role in remineralization of these particles (Kiorboe, 2001). Relation of thraustochytrids with POC suggests that marine aggregates could be one of the potential habitats of thraustochytrids in the water column. Raghukumar *et al.* (2001) noticed dense colonization of marine aggregates by thraustochytrids.

The occurrence and abundance of thraustochytrids and aplanochytrids in the oceanic water column removed from coastal influences have not been studied so far. Consequently, it is also not known how they are related to bacteria in this environment. Besides, the possibility of TEPs serving as a substrate for thraustochytrids in the water column has not been addressed so far.

As a step towards filling these lacunae, it was decided to carry out the following studies.

(1) To enumerate thraustochytrids in the water column of the equatorial Indian Ocean in order to understand their distribution in an oceanic environment.

(2) To enumerate bacteria from the water column to analyze the relationship between thraustochytrid and bacterial populations.

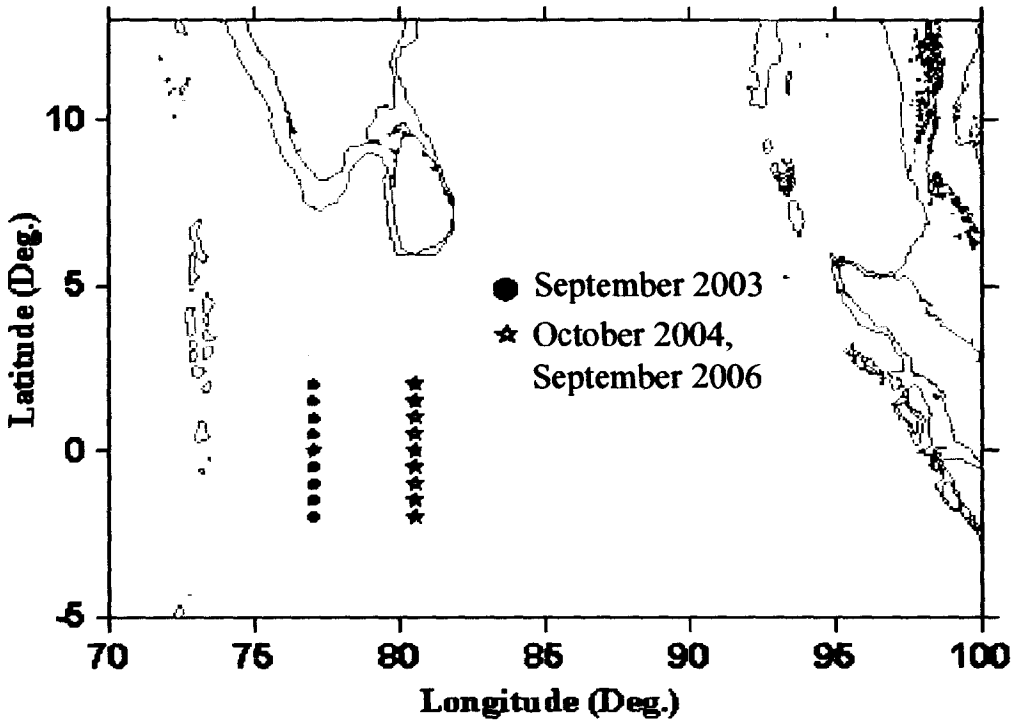
(3) To enumerate TEPs from the water column in order to study their relation to thraustochytrids and bacteria.

4.2 Materials and Methods

4.2.1 Sampling stations

Sampling was carried out in the equatorial Indian Ocean during three cruises on board *ORV Sagar Kanya* during September 2003 (Cruise # SK 196), October 2004 (Cruise # SK 212) and September 2006 (Cruise # SK 228) (Fig.1). Altogether 9 stations between 2° N and 2° S were studied for each cruise, at a longitude of 77° E during September 2003 and 80.5° E during October 2004 and September 2006.

Fig. 1. ORV Sagar Kanya station locations in the equatorial Indian Ocean during cruises SK 196 (September 2003), SK 212 (October 2004) and SK 228 (September 2006).

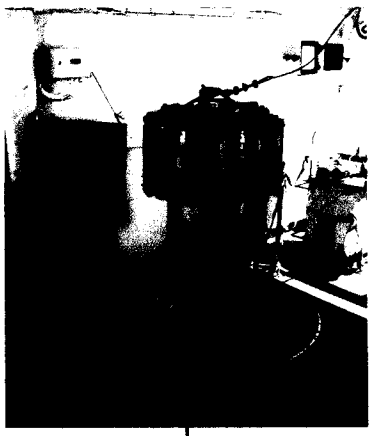


4.2.2 Sample collection

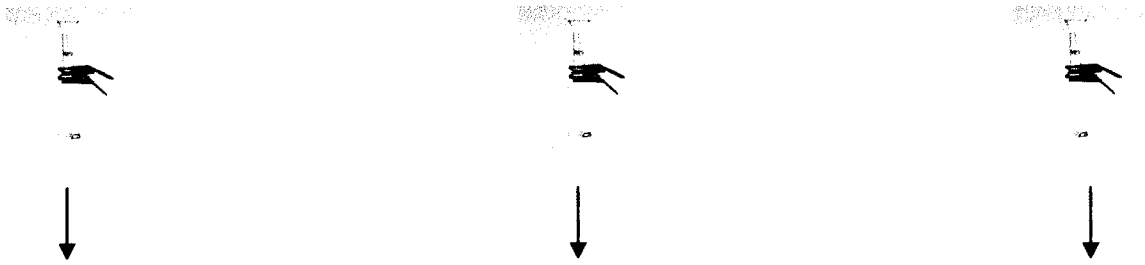
A general scheme of collection and processing the water samples is given in Fig. 2. Water samples were collected with clean 5 L or 10 L Niskin bottles attached to a Sea Bird CTD rosette sampler from 9 depths in each cruise, namely surface (3 m), 10, 20, 40, 60, 80, 100, 120 and 200 m. Two additional depths of 500 m and 1000 m were sampled during October 2004 and September 2006 (a total of 11 depths). Temperature and salinity data were obtained from the CTD readings.

Fig. 2. General scheme of collection of water samples from various depths by SeaBird CTD and processing them for analysis.

Niskin bottles fixed to SeaBird CTD for collecting water samples from various depths.



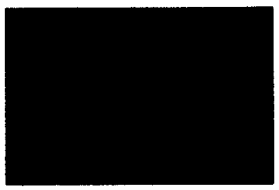
Fixation of water samples with formalin up to 2 % final concentration and refrigeration



10 mL through 0.22 μm black polycarbonate filter paper

AODC staining for bacteria

Epifluorescence microscopy



25 mL through 0.4 μm black polycarbonate filter paper

AfDD staining for thraustochytrids

Epifluorescence microscopy

10-25 mL through 0.4 μm white polycarbonate filter paper

Alcian blue staining for TEPs

H_2SO_4 digestion

Spectrophotometric estimation at 787 nm

4.2.3 Estimation of bacterial and thraustochyrid abundance

Water samples collected during the three cruises were preserved with formaldehyde at a final concentration of 2 % and stored at 5° C. Ten mL of water was filtered over 25 mm, 0.22 μm black polycarbonate Isopore membrane filters (Millipore, USA). The filter paper was stained for bacteria by the acridine orange direct count (AODC) method (Parsons *et al.*, 1984). A concentration of 0.01 % of acridine orange was overlaid over the filter paper and incubated in dark for 4 mins. After incubation, the filter paper was washed by passing sterile 0.22 μm filtered distilled water through it. The filters were mounted on microscope slides in non-fluorescent immersion oil (Olympus Optical Co. Ltd) and observed under blue excitation light using BX60 Olympus epifluorescence microscope equipped with 100 W Hg lamp. Ten different fields were observed for counting.

Thraustochytrids were enumerated by filtering 25 mL of water over 0.4 μm black polycarbonate Isopore membrane filter (Millipore, USA). The filters were then stained according to the acriflavine direct detection (AfDD) technique (Raghukumar & Schaumann, 1993). After filtering the sample water through the filter, sterile 0.22 μm filtered distilled water was passed through it and later it was overlaid with 0.05 % acriflavine (working solution) and incubated for 4 mins in dark. After incubation, the stain was drained and the filter paper was overlaid with 70 % isopropanol for 1 min. The filter paper was mounted on microscope slide in non-fluorescent

immersion oil (Olympus Optical Co. Ltd) and observed under epifluorescence microscope. Thraustochytrid cells were counted from 100 different fields under blue excitation filter and they could be distinguished from the other protists by their red cell wall and green cellular contents and were counterchecked for photosynthetic picoplankton (fluorescing red) under green filter.

4.2.4 Determination of biomass

Kimura *et al.* (1999) and Raghukumar *et al.* (2001) used a value of 20.6 pg of C per thraustochytrid cell, considering an average cell diameter of 5 µm. The same value was used as factor for converting thraustochytrid cell densities into biomass. For bacteria, a factor of 20.0 fg of C per average bacterial cell from ocean waters was used (Ducklow, 2000).

4.2.5 Determination of TEPs concentration

Transparent Exopolymeric Particles (TEPs) were estimated in 2 of the 3 cruises (during October 2004 and September 2006), following the method of Passow & Alldredge (1995). Ten to twenty five mL of water samples were filtered through 0.4 µm polycarbonate filter papers and stained with alcian blue (0.02 % wt/ vol in 0.06 % acetic acid, pH 2.5). The filter papers were then transferred into beakers and soaked in 80 % sulphuric acid for 2 hours. The absorbance of the solution was read at 787

nm against distilled water as reference. Concentration of TEPs was determined in duplicates or triplicates for all the samples and were calculated using the formula described by Passow & Alldredge (1995) as follows.

$$C_{TEP} = (E_{787} - C_{787}) \times (V_f)^{-1} \times f_x$$

where E_{787} is absorption of the sample, C_{787} is the absorption of the blank, V_f is the volume filtered in liters and f_x is the calibration factor in μg.

f_x is calculated by relating dry weight measurements of alginic acid particles retained on the filters to their staining capacity according to

$$f_x = W \times [(est_{787} - C_{787}) \times V_{st}^{-1}]^{-1}$$

where W is the dry weight of the standard (μg L^{-1}), est_{787} is its average absorption, C_{787} is the absorption of the blank, and V_{st} is the volume in liters filtered for staining.

C_{TEP} is expressed as milligram equivalent of alginic acid (AA) per liter [mg eq AA L^{-1}] (Ramaiah *et al.*, 2000).

The relationship of bacteria, thraustochytrids and TEPs to each other during the cruises were analyzed using correlation matrix (Statistica 5.0).

4.3 Results

4.3.1 Hydrography of study area

During September 2003, the isothermal mixed layer was present up to 40 m depth at the equator and north, becoming shallower (20 m) towards the south (Fig. 3). The thermocline was deeper at all the locations in October 2004 and September 2006 as compared to September 2003 (60 – 80 m). Salinity profiles showed the presence of a halocline at 20 m depth in September 2003 and October 2004. The halocline during September 2006 was present at about 40 m at the equator and deeper at about 60 m on either side of it. A subsurface salinity maximum at 40 to 80 m was observed during the three cruises.

4.3.2 Distribution of thraustochytrids and bacteria during SK 196 (September 2003)

Thraustochytrid numbers showed clear vertical distribution profiles in 8 of 9 stations (Fig. 4). Two peaks of abundance were present, one above the thermocline in the surface waters from 0 to 20 m, and the other below it at 40 to 100 m. Likewise, bacteria were abundant at 0 to 20 m, but showed a prominent peak also at 100 m in most of the stations. Thraustochytrid numbers were low at the thermocline.

Fig. 3. Thermohaline profiles of 3 stations during cruises September 2003 (SK 196), October 2004 (SK 212) and September 2006 (SK 228).

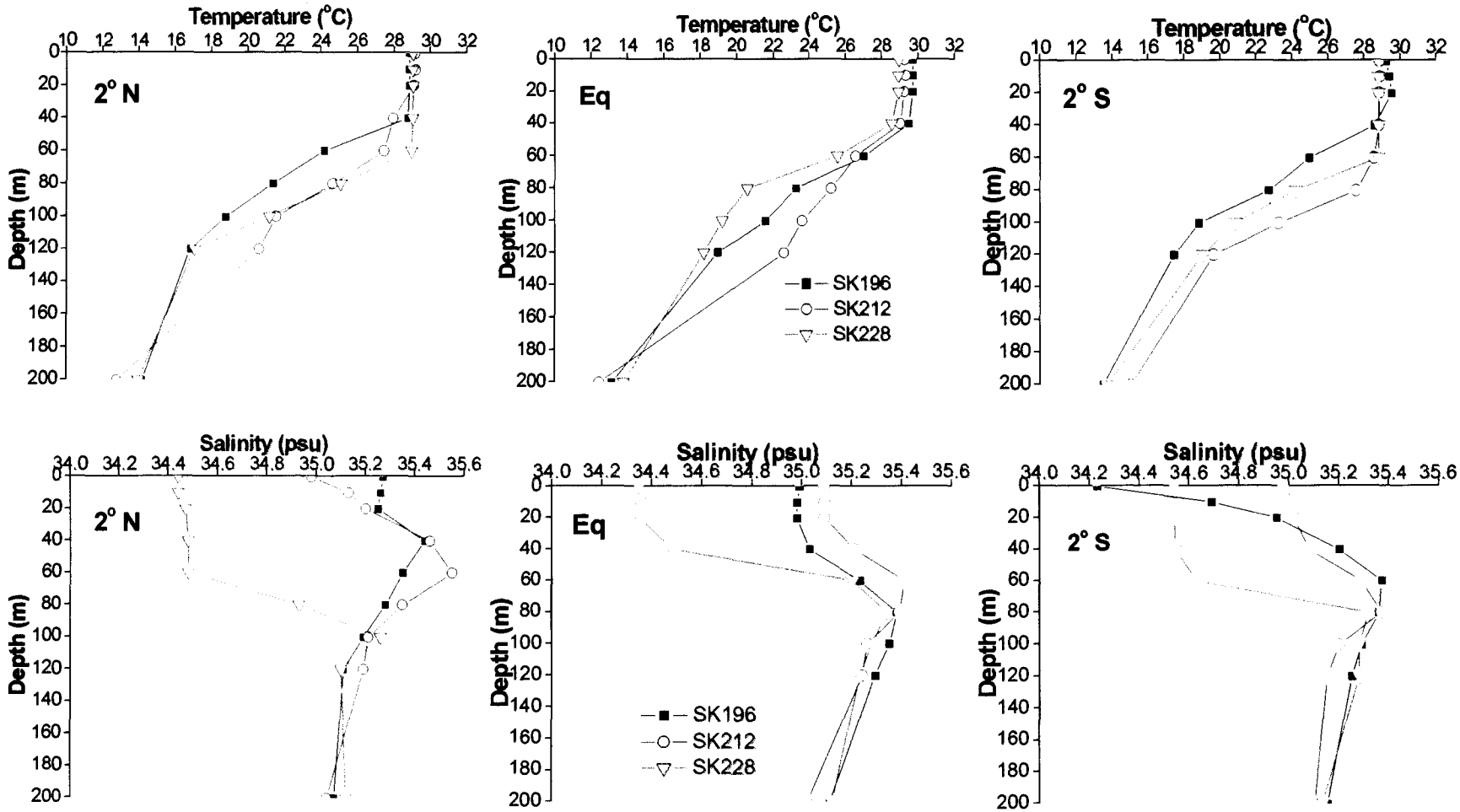
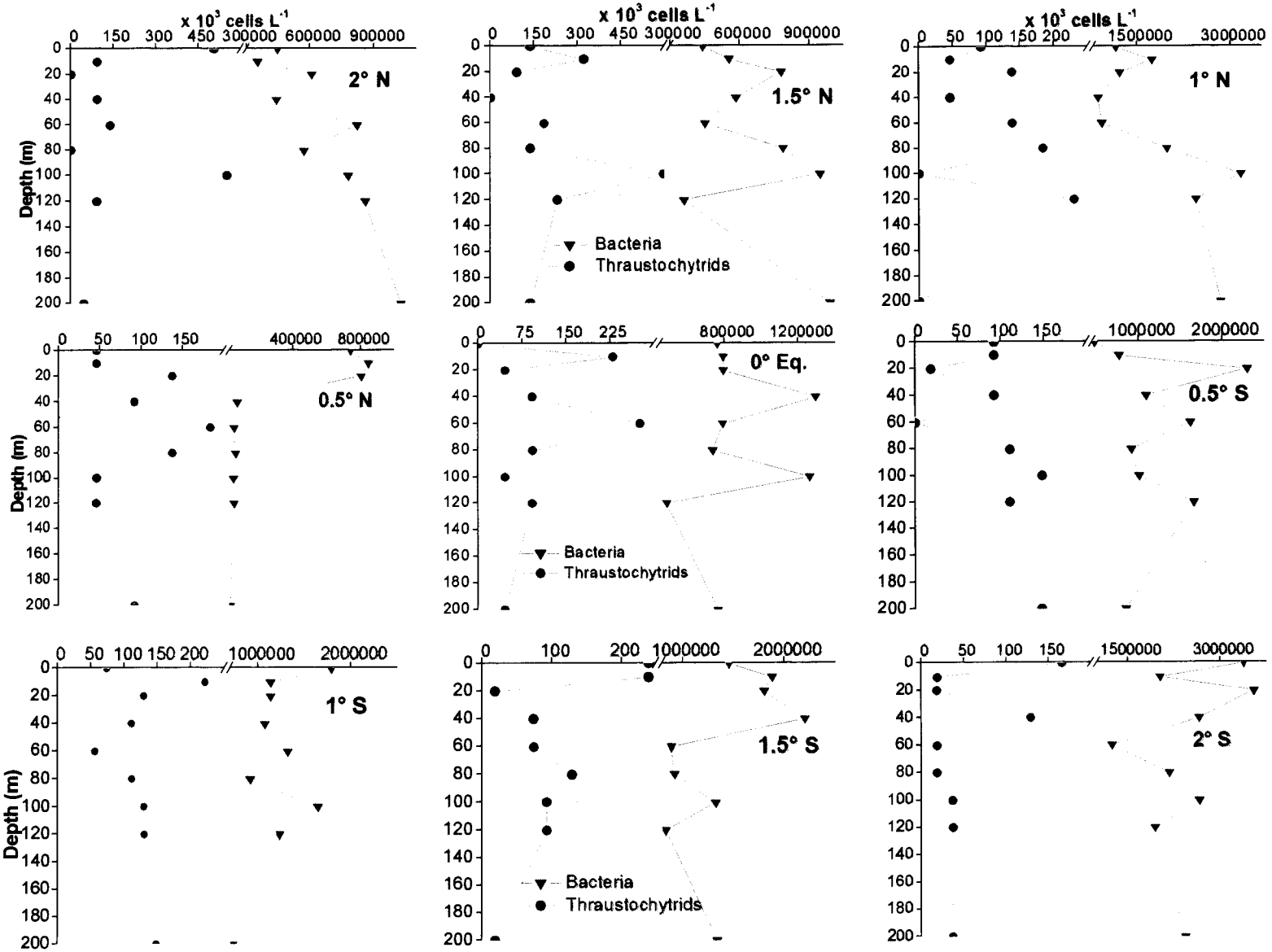


Fig. 4. Distribution of thraustochytrids and bacteria with depth in equatorial Indian Ocean during September 2003 (Cruise SK 196).



Thraustochytrid populations in the water column above the thermocline ranged from values below detection levels to 506 x 10^3 cells L^{-1}, while bacteria ranged from 69 to 3533 x 10^6 cells L^{-1} (Fig. 5). The maximum density of thraustochytrids was found at the surface at 2° N, while the highest bacterial density occurred at 20 m at 2° S. Thraustochytrid numbers below the thermocline ranged from values below detection to 598 x 10^3 cells L^{-1} and bacterial numbers ranged from 33 to 3151 x 10^6 cells L^{-1}. Highest density of thraustochytrids was found at 100 m at 1.5° N. No distinct relationship between bacteria and thraustochytrids were discernible when all the stations were considered (Fig. 5). However, when the relationship between the two were analyzed for individual stations, a statistically negative correlation was noticed in one station, namely at the latitude of 0.5°S (Table 1).

Fig. 5. Scatter plots of thraustochytrids against bacteria during September 2003.

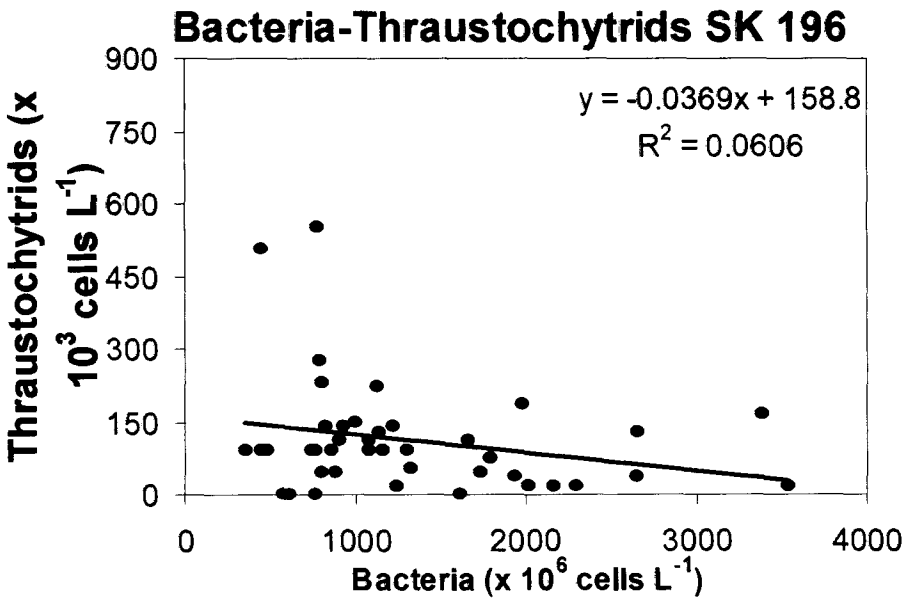


Table 1. Results of correlation analyses ('r') values between abundance of thraustochytrids and bacteria at different stations during SK Cruise # 196 in September 2003. Significant values are given in bold.

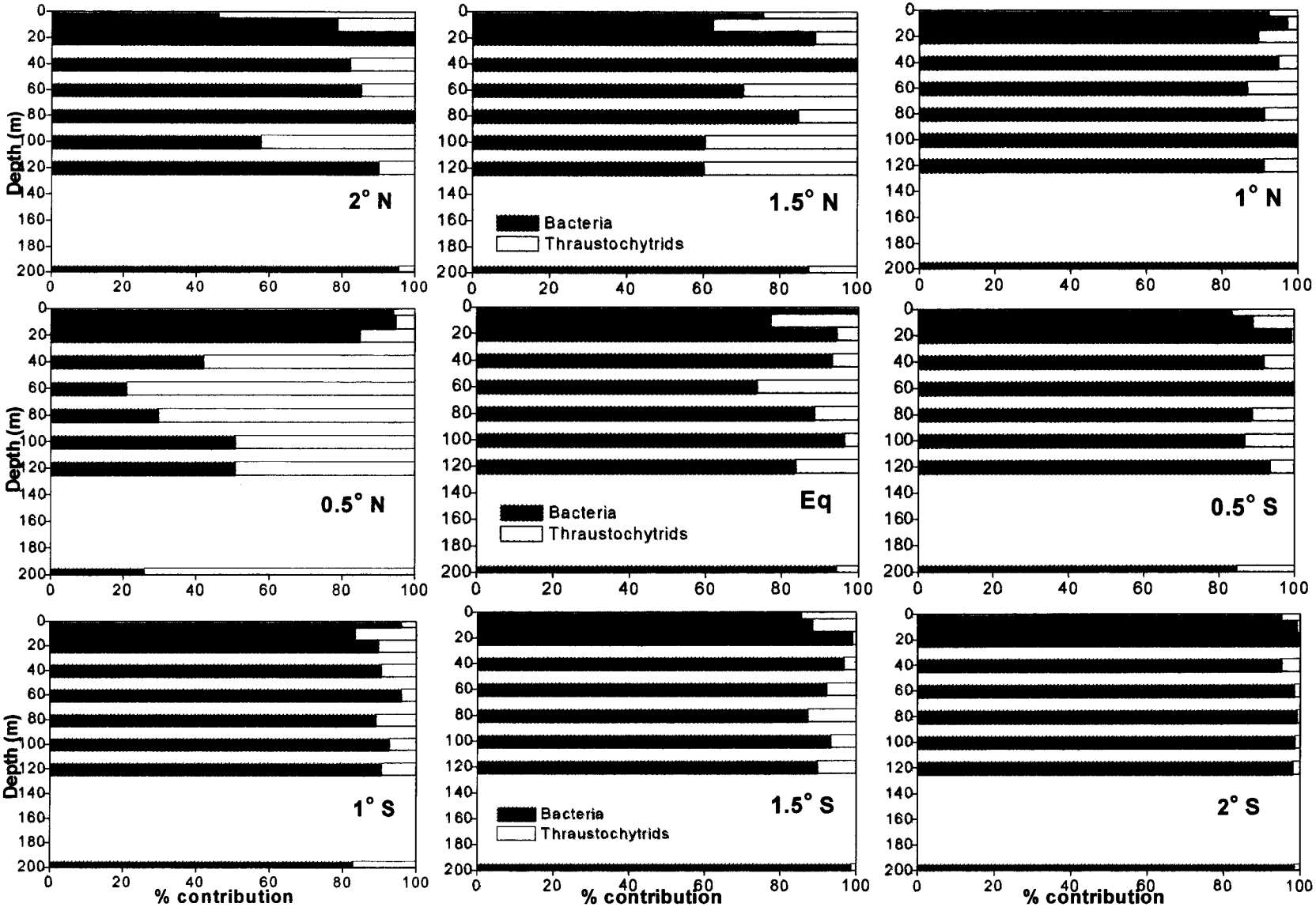
Parameters	2°N	1.5° N	1°N	0.5°N	0°Eq.	0.5°S	1°S	1.5° S	2°S
Thraustoch-ytrids vs. bacteria	-0.07; p=0.85	0.26; p=0.5	-0.25; p=0.5	-0.22; p=0.6	-0.12; p=0.75	**-0.64; p=0.06**	-0.38; p=0.31	0.1; p=0.8	0.49; p=0.2

Table 2. Contribution of thraustochytrid/ bacterial biomass to microbial carbon in terms of μg C L^{-1} in the water column of equatorial Indian Ocean during September 2003.

Depth (m)	2°N	1.5°N	1°N	0.5°N	0°Eq	0.5°S	1°S	1.5°S	2°S
0	10.4/8.9	2.8/8.8	1.9/23.2	0.9/14.8	0.001/15	1.9/9.6	1.5/35.8	4.9/29.2	3.4/67.5
10	1.9/7	6.6/11	0.9/34.7	0.9/16.9	4.7/15.9	1.9/15.3	4.5/22.5	4.9/37.4	0.4/40.2
20	0.001/12.2	1.9/15.5	2.8/24.4	2.8/16	0.9/15.8	0.4/45.9	2.7/22.6	0.4/35.9	0.4/70.7
40	1.9/8.8	0.001/12	0.9/17.5	1.9/1.4	1.9/25.9	1.9/21.6	2.3/21.4	1.5/44	2.7/53
60	2.8/16.4	3.8/9	2.8/18.5	3.8/1	5.7/15.8	0.001/32	1.1/26.3	1.5/17.8	0.4/24.7
80	0.001/11.4	2.8/15.7	3.8/39.5	2.8/1.2	1.9/14.7	2.3/18	2.3/18.1	2.7/18.2	0.4/43.3
100	11.4/15.5	12.3/18.9	0.001/63	0.9/1	0.9/25.2	3/19.9	2.7/32.8	1.9/26.4	0.8/52.8
120	1.9/17.1	4.7/7.2	4.7/48.8	0.9/1	1.9/9.7	2.3/33.1	2.7/24.6	1.9/16.7	0.8/38.6
200	0.9/20.5	2.8/19.8	0.001/57	1.9/0.7	0.9/15.3	3/16.8	3/14.4	0.4/26.8	0.8/48.4

Thraustochytrids contributed 0 to 10.4 μg C L^{-1} to the upper 40 m water column, while bacterial biomass amounted to 1.3 to 70.7 μg C L^{-1} (Table 2). Similarly thraustochytrids contributed up to 12.3 μg C L^{-1} below the thermocline and bacteria up to 63 μg C L^{-1}. The relative contribution of thraustochytrid biomass to the total microbial biomass carbon (bacteria + thraustochytrids) amounted to 0.002 - 42.3%, except at one station (depth 60 m, 0.5° N, 77° E) where thraustochytris contributed 79.1 % (Fig. 6).

Fig. 6. Relative percentage contribution of the biomass of thraustochytrids and bacteria in the water column of equatorial Indian Ocean during September 2003.



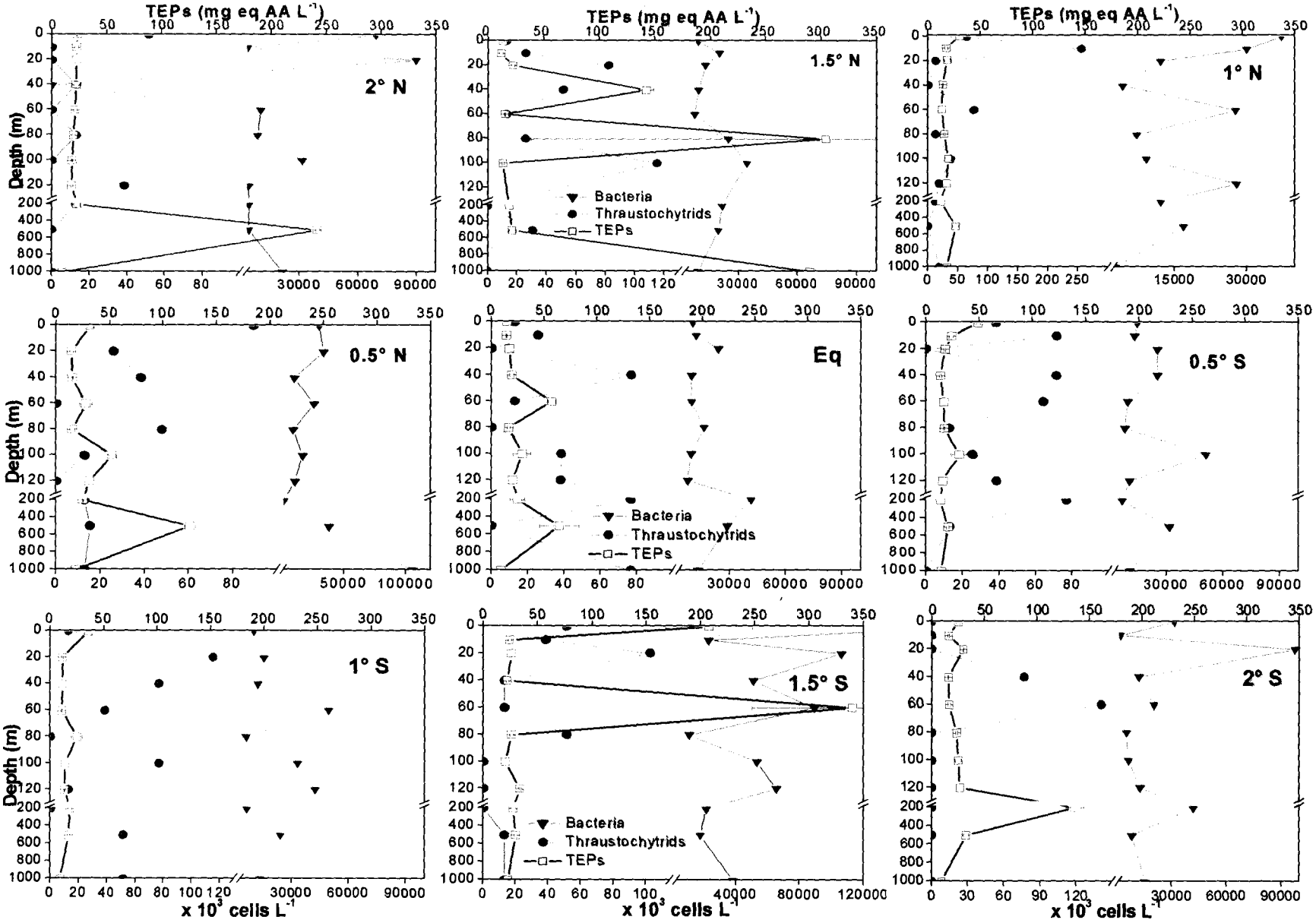
4.3.3 Distribution of thraustochytrids, bacteria and TEPs during SK 212

Thraustochytrids and bacteria generally showed 2 peaks of distribution, one above the thermocline between 0 – 40 m and the other below it at 100 - 120 m from equator northwards. Maximum densities of the two groups was found below the thermocline at 120 m at 1° N. Both groups showed a prominent peak between 20 and 40 m southwards of the equator. TEPs showed several peaks in the water column, many of these occurring below 100 m.

Thraustochytrids ranged from below detection levels to 674.6 x 10^3 cells L^{-1} (Fig. 7). Bacterial densities were lower, ranging from 1.84 to 807.3 x 10^6 L^{-1}. Numbers of both thraustochytrids and bacteria were less below 120 m, the former ranging from negligible to 73.6 x 10^3 cells L^{-1} and the latter from 19.32 to 103.73 x 10^6 cells L^{-1}. TEPs ranged from 5.3 to 451.1 mg equivalent AA L^{-1} in the water column during this period (Fig. 7).

The combined data for thraustochytrids and bacteria at all depths for all the stations showed a strong positive correlation between the two groups (Fig. 11). Correlation analysis carried out between the two at the individual stations showed that bacteria and thraustochytrids were positively related in 5 of the 9 stations (Table 3). No significant overall correlation was found between TEPs and bacteria or thraustochytrids (Fig. 11; scatter diagram). However, when individual stations were analyzed, a

Fig. 7. Distribution of thraustochytrids and bacteria with depth in equatorial Indian Ocean during October 2004 (Cruise SK 212).



significantly positive correlation of TEPs to thraustochytrids and bacteria was noticed at 2 stations (at 1° N and 2° S) and a negative correlation at one (0° Eq; Fig. 3; Table 3). The vertical profiles of TEPs at many stations was very similar to that of bacteria and thraustochytrids but displaced by 20 m upwards or downwards.

The biomass carbon contributed by thraustochytrids ranged from negligible to 15.8 μg C L^{-1} (Table 4). Organic carbon from bacteria ranged from 0.04 to 17.02 μg C L^{-1}. Bacteria and thraustochytrids contributed almost equal amounts of carbon to the water column at most of the stations during this period. At some of the locations, the biomass carbon contributed by thraustochytrids exceeded that of bacteria, *eg.* at 2° N and 0.5° S. Thus, thraustochytrids contributed 0.1 - 99.4 % of the total bacterial and thraustochytrid biomass carbon during October 2004 (Fig. 8).

Table 3. Results of correlation analyses ('r') values between abundance of thraustochytrids, bacteria and TEPs at different stations during SK Cruise # 212 in October 2004. Significant values are given in bold.

Parameters	2°N	1.5°N	1°N	0.5°N	0°Eq.	0.5°S	1°S	1.5°S	2°S
Thraustochytrids vs. bacteria	0.17; p=0.63	**0.70; p=0.02**	**0.9; p<0.001**	0.54; p=0.11	**0.82; p=0.01**	0.28; p=0.4	**0.92; p<0.001**	0.13; p=0.75	**0.8; p=0.003**
Thraustochytrids vs. TEPs	-0.54; p=0.11	0.09; p=0.78	**0.85 p<0.001**	-0.11; p=0.75	**-0.85; p=0.007**	-0.27; p=0.42	-0.11; p=0.74	-0.2; p=0.6	**0.67; p=0.02**
Bacteria vs. TEPs	-0.14; p=0.69	0.29; p=0.42	**0.93; p<0.001**	-0.51; p=0.1	**-0.75; p=0.03**	-0.027; p=0.94	0.07; p=0.83	-0.19; p=0.63	**0.73; p=0.01**

Fig. 8. Relative percentage contribution of the biomass of thraustochytrids and bacteria in the water column of equatorial Indian Ocean during October 2004.

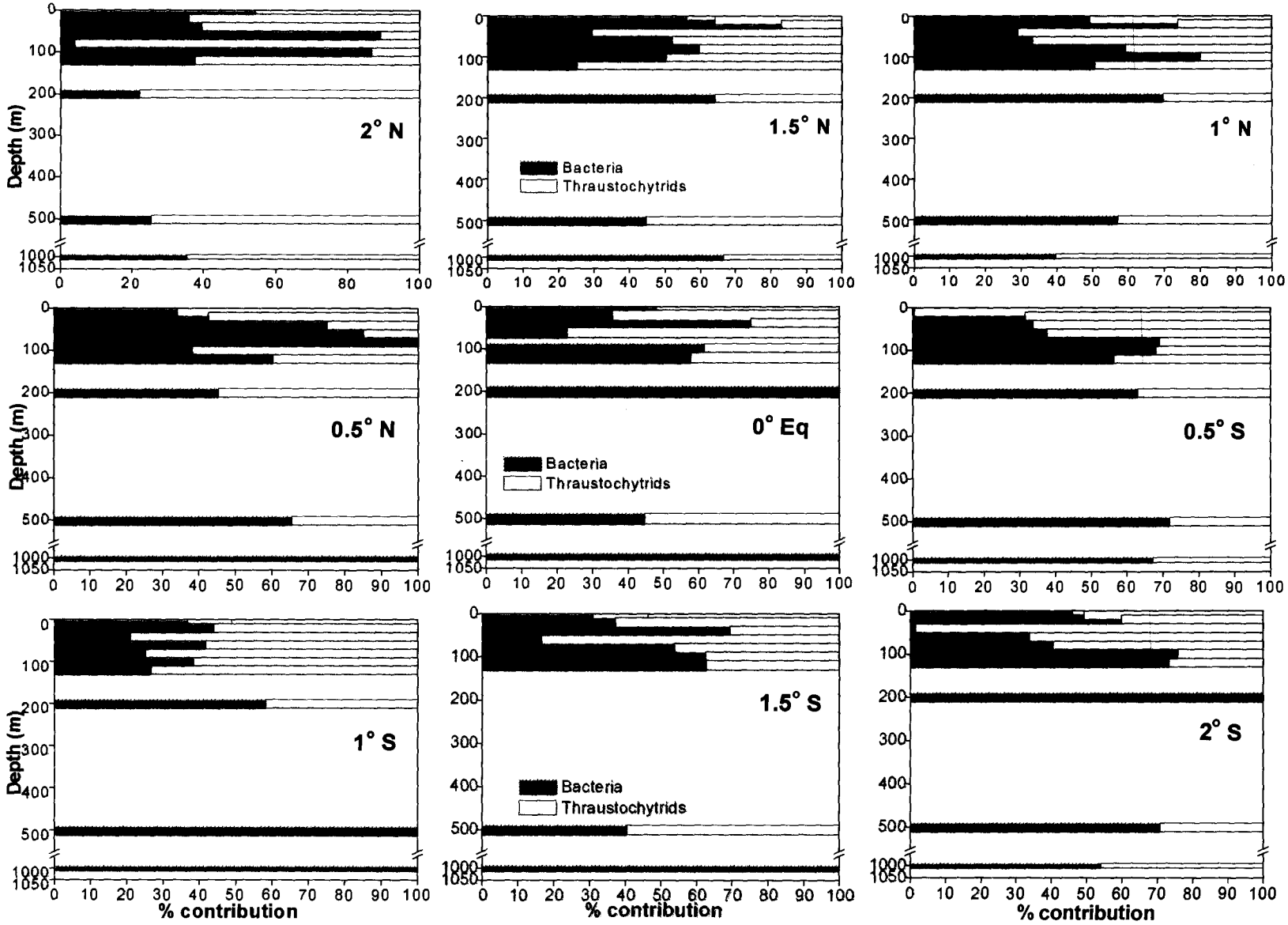


Table 4. Contribution of thraustochytrid/ bacterial biomass to microbial carbon in terms of μg C L^{-1} in the water column of equatorial Indian Ocean during October 2004. NS= Not sampled.

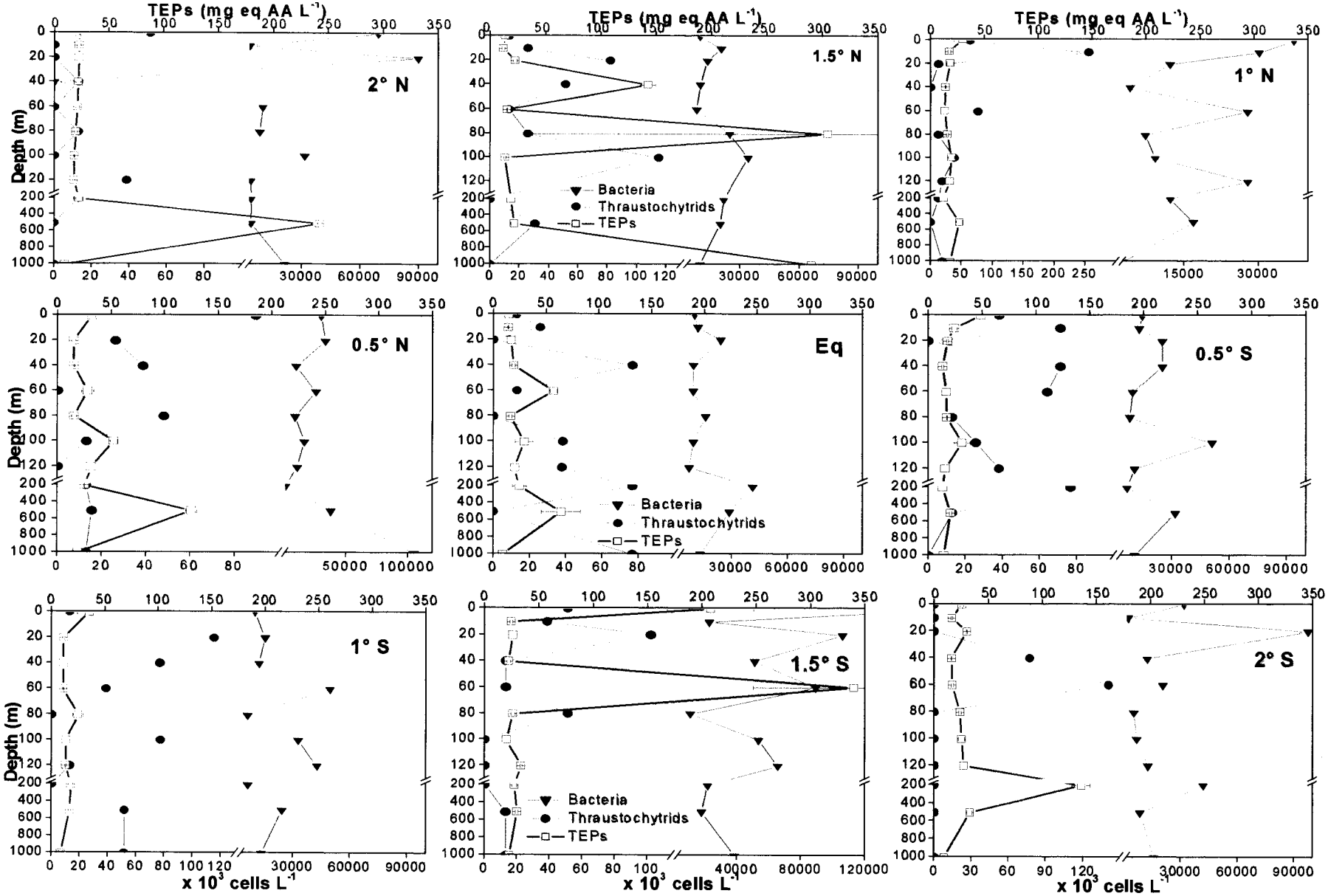
Depth (m)	2°N	1.5°N	1°N	0.5°N	0°Eq	0.5°S	1°S	1.5°S	2°S
0	1.3/2.0	5.1/6.5	2.7/5.1	1.1/1.6	5.1/4.7	1.9/1.5	8.2/4.8	3.4/1.5	2.3/1.9
10	2.7/3.2	1.5/2.7	3.8/3.7	8.8/4.6	NS	5.7/0.04	1.5/1.5	2.7/2.3	4.2/4.1
20	6.8/3.8	0.4/1.8	1.5/4.3	2.3/1.7	4.5/2.5	8.8/4.1	13.9/10.9	4.9/2.9	11.4/17.0
40	5.7/3.7	4.5/1.9	8.0/3.2	5.1/15.2	1.1/3.4	3.8/1.9	5.7/1.5	2.3/5.1	5.7/0.1
60	0.4/3.1	1.1/1.2	1.9/0.9	0.4/2.1	4.5/1.3	3.8/2.3	6.9/4.9	8.8/1.7	4.2/2.1
80	1.9/0.1	0.8/1.1	1.5/2.2	<0.4/1.1	NS	1.1/2.5	3.8/1.3	0.8/0.9	2.7/1.8
100	0.4/2.4	1.5/1.5	0.8/3.1	1.9/1.2	7.6/12.1	1.5/3.2	1.5/0.9	1.3/2.1	0.8/2.4
120	4.5/2.7	13.9/4.7	15.8/16.1	1.1/1.7	1.1/1.6	1.5/2.0	2.7/1.0	1.5/2.5	1.9/5.2
200	5.7/1.6	0.8/1.4	0.8/1.8	2.3/1.9	<0.4/1.1	1.5/2.6	1.1/1.6	NS	0/1.6
500	1.1/0.4	0.8/0.6	0.8/1.0	0.8/1.4	0.8/0.6	0.4/1.0	<0.4/1.4	1.5/1.0	0.4/0.9
1000	1.5/0.8	0.4/0.8	0.8/0.5	<0.4/1.7	<0.4/0.5	0.8/1.6	<0.4/0.8	<0.4/0.6	0.8/0.9

4.3.4 Distribution of thraustochytrids, bacteria and TEPs during SK 228

Thraustochytrids frequently revealed two peaks of distribution, one within and the other below the mixed layer, between 0 and 80 m at all stations (Fig. 9). High densities were also noticed at 200 m at the equator and 0.5°S. The vertical distribution of bacteria was very similar at stations south of equator, a prominent peak often occurring well below the mixed layer at 100 to 200 m in the high salinity maximum layer. High amounts of TEPs were noticed below 200 m at many stations.

Thraustochytrids in the mixed layer up to 60 m ranged from below detection levels to 255.6 x 10^3 cells L^{-1}, while bacteria varied from 0.81 to 183.4 x 10^6 cells L^{-1} (Fig. 9). Thraustochytrid abundance below the thermocline ranged from below detection levels to 76.7 x 10^3 cells L^{-1}

Fig. 9. Distribution of thraustochytrids and bacteria with depth in equatorial Indian Ocean during September 2006 (Cruise SK 228).



and that of bacteria from 3.9 to 104.9 x 10^6 cells L^{-1}. The maximum density of thraustochytrids was found at 10 m at 1° N. Bacterial numbers at this station were also some of the highest noticed. Both were also often abundant at 100 to 1000 m, thraustochytrids ranging from below detection levels to 76.7 x 10^3 cells L^{-1} and bacteria from 2.6 to 104.9 x 10^6 cells L^{-1}. TEPs ranged from 7.5 to 339.3 mg equivalent AA L^{-1} (Fig. 9). Maximum levels of TEPs were often observed below 200 m depth.

Thraustochytrids and bacteria showed dissimilar trends of distribution in the water column during September 2006 on an overall basis (Fig. 11). No significant relations between their numbers was found also when the 9 stations were analyzed individually (Table 5). Thraustochytrids contributed 0.26 to 2.9 μg C L^{-1} biomass to the water column and bacteria contributed 0.03 to 2.1 μg C L^{-1} (Fig. 10, Table 6). Photographs of thraustochytrid cell and bacteria as seen under the microscope after AfDD and AODC staining respectively are shown in Fig. 12.

Table 5. Results of correlation analyses ('r') values between abundance of thraustochytrids, bacteria and TEPs to each other during SK Cruise # 228 in September 2006.

Parameters	2°N	1.5°N	1°N	0.5°N	0°Eq.	0.5°S	1°S	1.5°S	2°S
Thraustochytrids vs. bacteria	0.19; p=0.58	0.5; p=0.15	0.56; p=0.07	-0.08; p=0.82	0.07; p=0.85	-0.27; p=0.42	0.09; p=0.80	0.41; p=0.22	-0.1; p=0.78
Thraustochytrids vs. TEPs	-0.22; p=0.52	-0.27; p=0.46	-0.09; p=0.79	-0.20; p=0.57	-0.31; p=0.35	-0.22; p=0.52	-0.57; p=0.09	-0.009; p=0.98	-0.23; p=0.5
Bacteria vs. TEPs	-0.2; p=0.57	-0.08; p=0.82	0.05; p=0.89	0.18; p=0.61	0.21; p=0.54	0.21; p=0.54	-0.51; p=0.13	0.59; p=0.06	0.29; p=0.4

Fig. 10. Relative percentage contribution of the biomass of thraustochytrids and bacteria in the water column of equatorial Indian Ocean during September 2006.

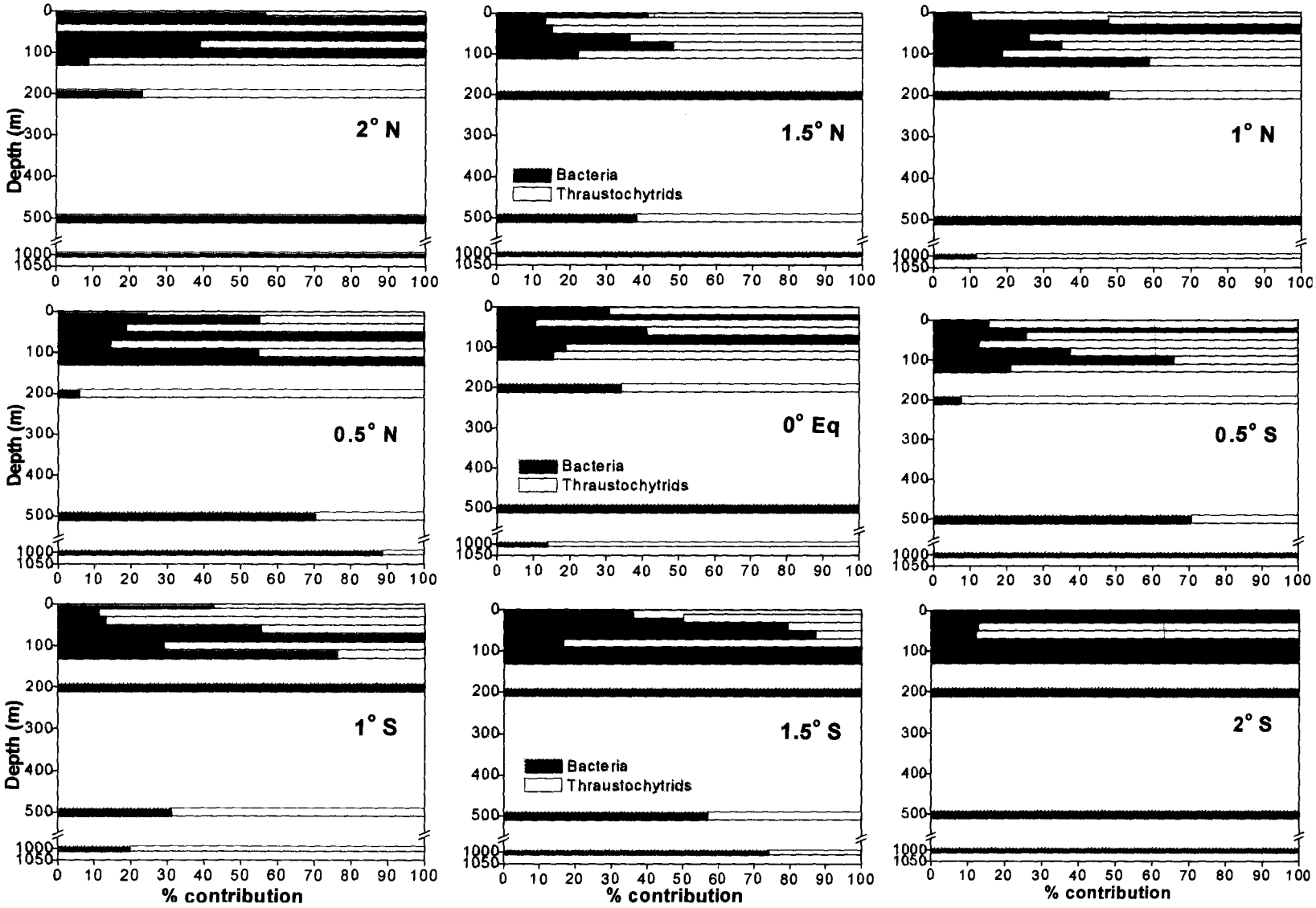


Fig. 11. Scatter plots of thraustochytrids, bacteria and TEPs during October 2004 (SK 212) and September 2006 (SK 228).

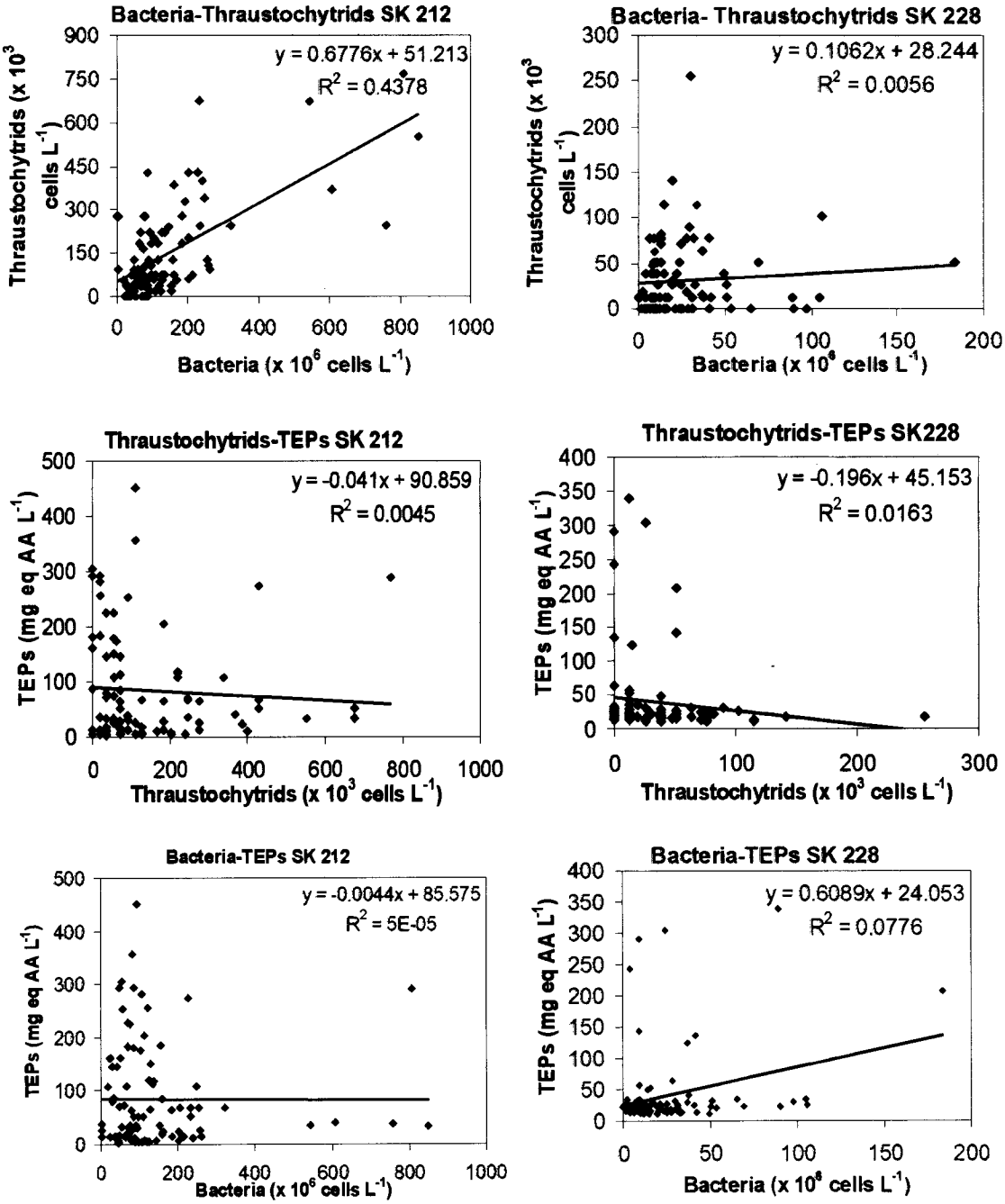
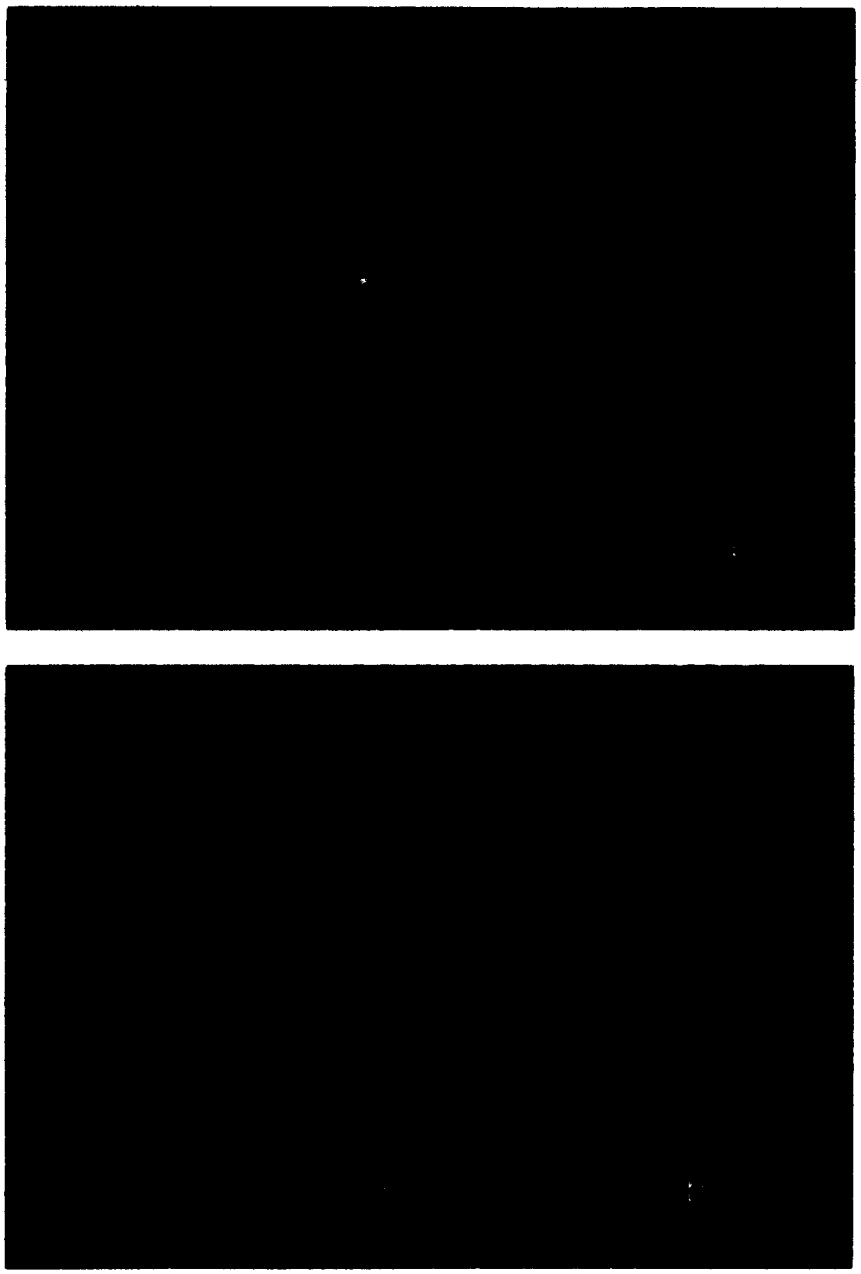


Table 6. Contribution of thraustochytrid/ bacterial biomass to microbial carbon in terms of μg C L^{-1} in the water column of equatorial Indian Ocean during September 2006. NS= Not sampled, ND= Not determined.

Depth (m)	2°N	1.5°N	1°N	0.5°N	0°Eq	0.5°S	1°S	1.5°S	2°S
0	1.05/1.38	0.26/0.19	1.32/0.74	1.8/0.59	0.26/0.2	0.79/0.29	0.26/0.2	1.05/3.7	<0.2/0.63
10	<0.2/0.06	0.53/0.4	5.3/0.6	NS	0.53/0.24	1.47/0.26	NS	0.79/0.45	<0.2/0.03
20	<0.2/1.8	1.7/0.26	0.26/0.24	0.54/0.66	<0.2/0.48	<0.2/0.5	2.4/0.3	2.1/2.1	<0.2/1.95
40	0.26/ND	1.1/0.19	<0.2/0.08	0.79/0.18	1.6/0.19	1.5/0.5	1.6/0.24	0.26/1.0	1.6/0.24
60	<0.2/0.2	0.26/0.15	1.6/0.55	<0.2/0.5	0.26/0.18	1.32/0.19	0.79/0.99	0.26/1.8	2.9/0.4
80	0.26/0.17	0.53/0.49	0.2/0.14	0.99/0.17	<0.2/0.32	0.26/0.16	<0.2/0.12	1.05/0.21	<0.2/0.1
100	<0.2/0.63	2.4/0.68	0.79/0.18	0.26/0.32	0.79/0.18	0.53/1.02	1.6/0.65	<0.2/1.1	<0.2/0.12
120	0.79/0.08	NS	0.39/0.56	<0.2/0.2	0.78/0.14	0.79/0.21	0.26/0.84	<0.2/1.3	<0.2/0.24
200	0.26/0.08	<0.2/0.43	0.26/0.24	0.26/0.02	1.6/0.82	1.6/0.13	<0.2/0.12	<0.2/0.4	<0.2/0.83
500	<0.2/0.08	0.63/0.39	<0.2/0.34	0.32/0.75	<0.2/0.57	0.26/0.63	1.05/0.48	0.26/0.35	<0.2/0.16
1000	<0.2/0.43	<0.2/0.18	0.39/0.05	0.26/2.1	1.6/0.26	<0.2/0.21	1.05/0.26	0.26/0.76	<0.20/0.3

Fig. 12. Photomicrographs of thraustochytrid cell (arrow) in water sample stained by acriflavine direct detection (AfDD) technique (a) and bacteria stained by acridine orange direct count (AODC) method (b) as seen under blue light. Bar represents 10 μm.



4.4 Discussion

The surface mixed layer of the ocean is a biologically dynamic region. When adequate mineral nutrients and illumination are available, primary productivity by photosynthetic organisms in the mixed layer is high, microbial activity is considerable and vigorous nutrient recycling takes place within it (Sarkar *et al.*, 2004). Microbial populations are therefore much higher in the mixed layer compared to deeper layers. Correspondingly, both thraustochytrid and bacterial abundance were high in the mixed layer of all the stations in the 3 cruises studied. The highest bacterial count of 3533 x 10^6 cells L^{-1} amongst all the three cruises was seen in this layer during September 2003 (cruise # SK 196). Thraustochytrids also reached values up to as high as 506 to 674.7 x 10^3 cells L^{-1} during September 2003 and October 2004 respectively.

The present study demonstrates the abundant occurrence of thraustochytrids in oceanic waters. Most of the earlier studies had addressed these protists in coastal waters or land-locked seas, such as the Arabian Sea. (Raghukumar *et al.*, 1990; Raghukumar *et al.*, 2001; Ramaiah *et al.*, 2005). Kimura *et al.* (1999) even hypothesized that thraustochytrids might be more dependent on allochthonous nutrients that are washed into the sea, more than autochthonous material. This study clearly shows that thraustochytrids are present abundantly in waters far removed from any land influence.

Densities of thraustochytrids in the present study corresponded to earlier reports of up to a few hundred thousand cells per liter seawater (Naganuma *et al.*, 1998; Kimura *et al.*, 1999; Kimura *et al.*, 2001; Raghukumar *et al.*, 2001; Bongiorni *et al.*, 2005a). Their numbers reached a maximum of 766.6 x 10^3 cells L^{-1}, a value that was almost equivalent to those found in the adjacent Arabian Sea by Raghukumar *et al.* (2001). These values exceeded those observed by Kimura *et al.* (1999, 2001) and Naganuma *et al.* (1998) in the Seto Inland Sea, Japan. Likewise bacterial densities corresponded to values recorded by Wiebinga *et al.* (1997) for the North West Indian Ocean (300-1600 x 10^6 cells L^{-1}), Prasanna Kumar *et al.* (2001) in the Northern Arabian Sea during the North East Monsoon (66-1555 x 10^6 cells L^{-1}), Pomeroy & Joint (1999) in the Arabian Sea during the southwest monsoon (620-1630 x 10^6 cells L^{-1}), Ramaiah *et al.* (1996) in the central and eastern Arabian Sea at the end of Southwest Monsoon (340-1500 x 10^6 cells L^{-1}) and Ducklow (1993) in the Northwest Indian Ocean (>1000 x 10^6 cells L^{-1}). The occurrence of high densities of thraustochytrids and bacteria in this study suggests that the equatorial Indian Ocean might be a biologically productive region.

Thraustochytrids in the equatorial Indian Ocean often contributed significantly to the organic carbon pool like that of bacteria (Fig. 6, 8 and 10). Their biomass exceeded that of bacteria at certain stations. A similar observation was made by Raghukumar *et al.* (2001), who noticed that thraustochytrid biomass occasionally exceeded that of the bacterial

biomass during certain seasons and locations in the Arabian Sea. Ramaiah *et al.* (2005) also observed that thraustochytrid exceeded bacterial biomass during winter in Northern Arabian Sea.

Abundance, biomass and productivity are important indicators of the ecological role of bacteria in the water column and have been extensively studied for numerous geographical regions (Ducklow, 2000; Morán *et al.*, 2004). High densities of these organisms would result in enhanced rates of organic matter remineralization and will support active feeding and multiplication of microbivorous protists, thus sustaining the microbial loop (Azam *et al.*, 1983; Lenz, 1992). Thraustochytrids being similar to bacteria in their mode of nutrition may also play a role in remineralization of organic matter. They may also often play a major role as a feed for microbivorous protists. Thraustochytrid cells are characterized by the ω-3 fatty acid, docosahexaenoic acid, a key essential fatty acid in the growth and maturation of crustaceans (Veloza *et al.*, 2006). Zooplankton are believed to obtain their DHA from their prey and several studies have attempted to trace the source of DHA in the food web (Veloza *et al.*, 2006; Alonzo *et al.*, 2005). Hence, the involvement of thraustochytrids in the microbial loop may have far reaching implications in the food web (Naganuma *et al.*, 1998; Kimura *et al.*, 1999).

Standing biomass of microorganisms is the end result of their production and grazing by microzooplankton. One of the present constraints in understanding the ecological role of thraustochytrids is the

lack of a suitable technique to measure their productivity in natural samples. In contrast to the thymidine-incorporation method for bacteria (Fuhrman & Azam, 1982) and ergosterol synthesis rates in fungi (Newell, 2001; Newell *et al.*, 2000), no specific biochemical targets for estimating productivity of thraustochytrids has been found to date. Future studies are required to understand their productivity in natural environment.

Thraustochytrids were patchily distributed in the water column of the equatorial Indian Ocean. Unlike bacteria, which were always present in the water column, thraustochytrid populations varied widely, from levels below detection to hundreds of thousand cells per liter even at a single location. This suggests that they might be associated with particles or aggregates. Raghukumar *et al.* (2001) had earlier noticed dense populations of thraustochytrids in association with diatom mucus particles in the water column at the end of the southwest monsoon in the Arabian Sea. Lyons *et al.* (2005) reported the dense presence of the thraustochytrid pathogen QPX in marine aggregates and suggested that aggregates might provide a means for survival and transport of the pathogen. Kimura *et al.* (2001) and Raghukumar *et al.* (2001) found that thraustochytrid abundance was significantly related to POC concentration in the water column. This prompted the study of TEPs in the equatorial Indian Ocean during October 2004 (cruise # SK 212) and September 2006 (cruise # SK 228). The two groups were occasionally, but not always related to TEPs in the water column. Bacteria and thraustochytrids, which

were significantly related to each other in 5 of the 9 stations during October 2004 also showed a positive relation to TEPs; thraustochytrids in 4 of the 9 and bacteria in 3 of the 9 stations (Table 3). Since thraustochytrids produce a variety of degradative enzymes and may decompose refractory organic substrates (Bongiorni *et al.*, 2005b), they might act upon or survive on the refractory material present in TEPs and marine aggregates. Even in those stations where no positive relation to TEPs was observed, the distribution patterns of bacteria and thraustochytrids were similar, however being displaced by about 20 m downwards in the water column (Fig. 6). This could be explained as follows. Although bacteria attached to marine snow cause a rapid enzymatic hydrolysis of the material, their carbon demand is low. Most of the dissolved organic matter (DOM) therefore diffuses into the surrounding waters as a plume trailing the increasingly refractory sinking particles (Alldredge & Youngbluth, 1985; Cho & Azam, 1988; Simon *et al.*, 1990; Smith *et al.*, 1992; Kiørboe, 2001; Engel *et al.*, 2004). High abundance of bacteria and thraustochytrids nearly 20 m above peak TEPs concentrations at some of the stations might reflect their active growth in such plume.

In contrast to October 2004 (SK 212), no relation between TEPs on the one hand and bacteria and thraustochytrids on the other were seen during September 2006 (SK 228). Such varying relationship between TEPs, bacteria and thraustochytrids suggests that the chemistry of TEPs

might be important, rather than the total amount of it. TEPs may be formed as a result of bacterial activity on phytoplankton exudates (Sugimoto *et al.*, 2007), or even without bacterial intervention directly from diatom exudates (Grossart *et al.*, 2006). These processes would have an influence on the chemistry of TEPs, which will have to be addressed to gain a greater understanding of the role of microbes attached to them.

4.5 Conclusions

Thraustochytrids were present in high abundance in the oceanic waters of the equatorial Indian Ocean. Their numbers frequently reached levels of several hundred thousands of cells per litre water, similar to those found earlier in the Arabian Sea and Japan Sea. The high densities of thraustochytrids and bacteria in the equatorial Indian Ocean suggest that the equatorial Indian Ocean region might be biologically productive. Since their population varied considerably during different seasons, they appear to be seasonally variable. Bacteria and thraustochytrids showed distinct vertical stratification in their abundance, mostly being confined to the mixed layer. However, high numbers were also occasionally seen below 200 m. Bacteria and thraustochytrids revealed varying relationships between them. They were highly positively related during October 2004, but not at other times. Thraustochytrids were distributed patchily in the water column, suggesting their possible dependence on particulate organic matter or TEPs. TEPs were studied in two cruises

during October 2004 and September 2006. Thraustochytrids and bacteria were positively related to TEPs in some, but not all the stations sampled. This varying relationship with TEPs suggest that the chemistry of TEPs might be an important factor that decides the growth of thraustochytrids and bacteria on them.

Chapter 5.

In vitro studies on the relation between thraustochytrids, aplanochytrids, bacteria and Transparent Exopolymeric Particles

5.1 Introduction

The discovery of an amazing diversity of living organisms in the deep sea during HMS Challenger Expedition in 1872-1876 led by Sir Charles Wyville Thompson stunned the late 19th century scientific community, who had believed that no life could exist in deep ocean. However, the mechanisms and the driving force that sustained life at such depths were still a mystery till scientists at Hokkaido University beamed a search light after diving in the ocean in the submersible Kuroshio and saw the raining of particles that resembled snow flakes. They named these 'snow flakes' as marine snow (Hunjo, 1997). Marine snow, or marine aggregates as they are more recently called, sink the carbon from the ocean's euphotic layer to the ocean depths and regulate the global carbon cycle (Azam & Long, 2001; Engel *et al.*, 2004).

Transparent Extracellular Polysaccharides or TEPs play a major role in the formation of marine aggregates, as follows. Several aquatic organisms including prokaryotes and microalgae in the water column and macroalgae in coastal waters release copious amounts of dissolved organic carbon in the form of extracellular polymeric substances (EPS) (Aaronson 1971; Painter 1983; Hoagland *et al.* 1993). Mucilaginous carbohydrates surrounding the cells of flagellates also contribute to EPS. Heterotrophic bacterial processes on the biologically labile organic carbon of the EPS gradually convert them into increasingly refractory material, which then coagulate to form fibrillar, sticky mucopolysaccharides. These

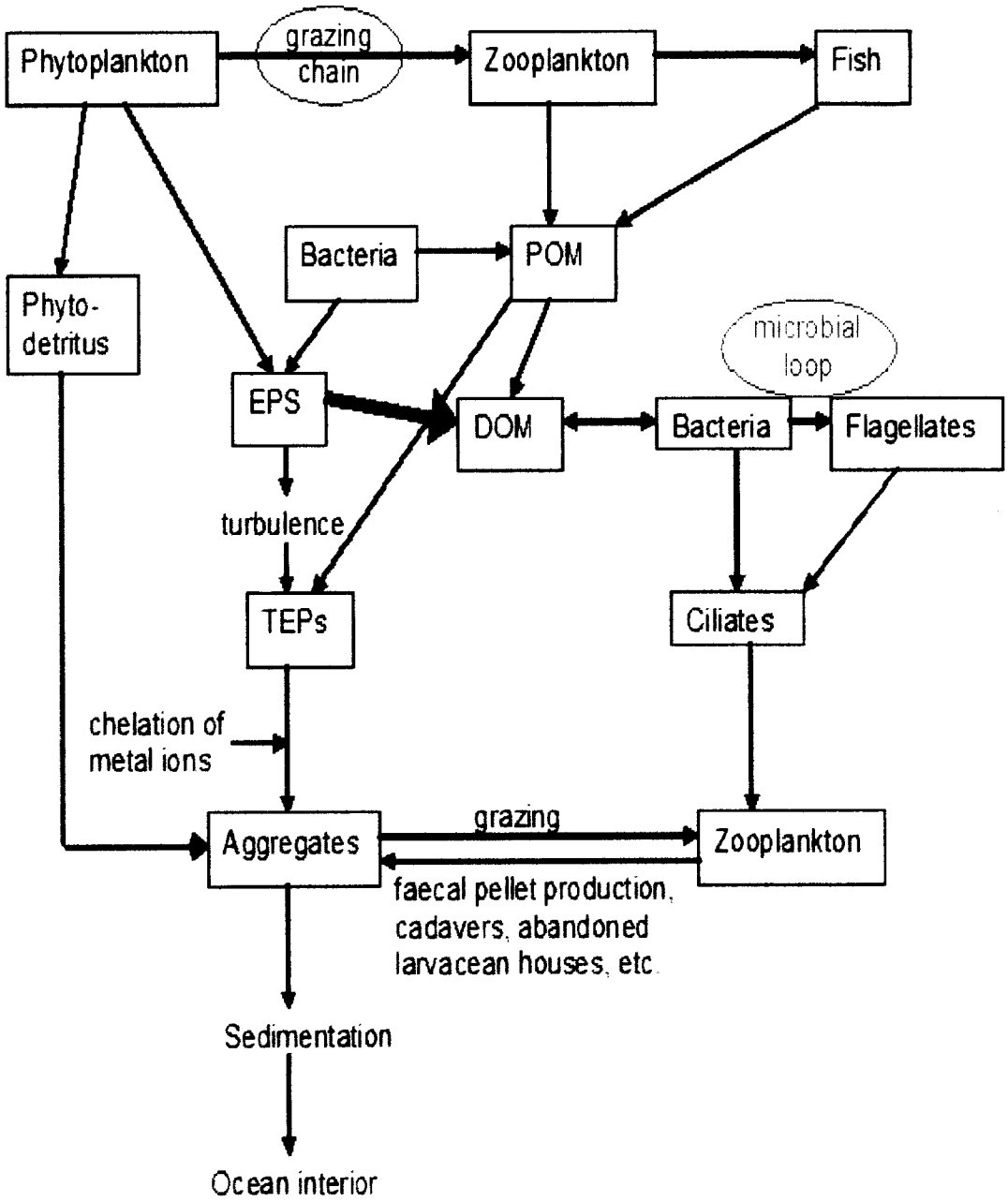
are light and transparent in nature and therefore called "transparent exopolymeric particles" or TEPs (Alldredge *et al.*, 1993; Beauvais *et al.*, 2003). Bacteria, phytoplankton and also zooplankton contribute directly or indirectly to formation of TEPs via the excretion of dissolved organic matter (Alldredge *et al.*, 1993; Strom *et al.*, 1997; Stoderegger and Herndl, 1999; Passow, 2002). Bacteria attached to TEPs stabilize the physical structure of TEPs possibly by releasing refractory DOM into the TEPs matrix (Schuster & Herndl, 1995). Possibly bacteria also enhance the production of TEPs by phytoplankton (Passow *et al.*, 2001). The hydrodynamics and environmental conditions in the water column influence the spatial and temporal variability of TEPs. Turbulence of the water column is equally important like bacteria and phytoplankton in the formation of TEPs (Schuster & Herndl, 1995; Prieto *et al.*, 2006). Turbulence facilitates colloidal fibrils and TEPs to coagulate resulting in significantly larger mean areas per TEP than in stagnant treatments. Divalent cation concentration is also important in formation of TEPs as they act as ionic bridges for polysaccharides in gels and TEPs are mostly made of acidic polysaccharides (Thornton, 2004). TEPs can adsorb iron and manganese and can act as scavenger of dissolved elements (Beauvais *et al.*, 2004).

Owing to their stickiness and surface-reactive nature, TEPs easily adhere to each other and entrap a variety of particulate matter, such as faecal pellets and cadavers of zooplankton and larvacean houses. This

leads to the formation of large aggregates, the marine snow (Passow *et al.*, 2001; Engel *et al.*, 2004; Verdugo *et al.*, 2004). The formation of marine snow or aggregates is schematically represented in Fig. 1. Because TEP are formed directly from DOC, they represent a potentially highly significant pathway by which DOC can be transformed into POC and sequestered via sedimentation. As an aggregate begins to sink, it attracts more suspended particles which are added to it making it heavier and faster moving. An aggregate may break apart, spilling its contents into the water, but soon the spilled particles are picked up or "scavenged" by other falling aggregates.

TEPs harbour 0.5 to 90 % of the total bacterial community of the water column and provide them physical refuges from predators unable to feed on surfaces (Alldredge *et al.*, 1993; Mari & Rassoulzadegan, 2004). Some zooplankton, especially copepods do not actively feed on TEPs (Prieto *et al.*, 2001; Dutz *et al.*, 2005). Yet organisms such as protists and benthic suspension feeders can feed on TEPs and thus bypass the microbial loop (Thornton, 2004).

Fig. 1. Schematic representation of formation and fate of marine snow or aggregates.



TEPs in the water column are fairly transparent, but can be made visible by staining with an acidic stain alcian blue (Alldredge *et al.*, 1993). TEPs abundance, sizes and distribution from natural environments have been studied by the alcian blue staining method (Garcia *et al.*, 2002; Ramaiah & Furuya, 2002; Radić *et al.*, 2005). They have also been generated in laboratory using devices like rolling tables and Couette flocculators for experimental purposes (Shanks & Edmondson, 1989; Weiss *et al.*, 1996; Grossart *et al.*, 1997; Unanue *et al.*, 1998; Artolozaga *et al.* 2000; Passow, 2000; Bhaskar *et al.*, 2005). They have been generated from various sources like bacteria, diatoms, macroalgal detritus, organic matter derived from seaweeds, fishes and benthic suspension feeders (Biddanda, 1985; Schuster & Herndl, 1995; Unanue *et al.*, 1998; Mari, 1999; Ramaiah *et al.*, 2001; Thornton, 2004; Bhaskar *et al.*, 2005; McKee *et al.*, 2005; Radić *et al.*, 2006; Heinonen *et al.*, 2007).

Various studies have been carried out on microbial dynamics, microbial degradation of organic carbon and nitrogen on TEPs (Passow, 2000; Grossart & Plough, 2001). All of them mostly focus on the role of bacteria in degradation. Little is known about the role of other microbes in degradation. Raghukumar *et al.* (2001) found dense numbers of thraustochytrids on aggregates in Arabian Sea. They as well as Kimura *et al.* (2001) observed increasing thraustochytrid numbers with increasing POC concentration. Thraustochytrids are known to produce a wide spectrum of enzymes involved in the hydrolysis of several classes of

organic compounds, and thus degrading a large variety of substrates. (Bahnweg, 1979; Sharma *et al.*, 1994; Bremer & Talbot, 1995; Bongiorni *et al.*, 2005b). It is therefore likely that thraustochytrids and aplanochytrids also play a role in degradation of TEPs. There is a void in our knowledge about the role of thraustochytrids in TEPs dynamics. In this study, a high abundance of thraustochytrids and aplanochytrids was found in the water column of the equatorial Indian Ocean (Chapter 4). A positive correlation of thraustochytrids with TEPs and bacteria was noticed in some stations, but not all. It was felt that experiments to study these relationships would shed more light on their nature.

In view of the above, the following objectives were formulated in order to obtain an insight into the relationship of thraustochytrids with TEPS.

(1) To study the interaction of thraustochytrids and bacteria with the abundance and chemistry of TEPs generated in the laboratory from oceanic waters.

(2) To study the interaction of thraustochytrids and bacteria with the abundance and chemistry of TEPs generated in the laboratory from coastal waters.

(3) To study the interaction of thraustochytrids and bacteria with the abundance and chemistry of TEPs generated in the laboratory from diatom and bacterial culture filtrates containing extracellular polysaccharides.

5.2 Materials and Methods

The relationship between thraustochytrids, bacteria and TEPs was studied in a total of six experiments. TEPs for these experiments were generated from water samples using a roller table (plankton wheel) based on the design of Shanks & Edmondson (1989). Two of the experiments were carried out using surface seawater collected at 2° N 80.5° E during cruise # SK 228 in September 2006, two using water collected from the coastal waters of Dona Paula Bay, Goa (Lat 15.47; Long 73.87), one using the culture filtrate from a diatom and one using seawater containing extracellular polysaccharides of a bacterium.

5.2.1.1 Design of roller table: The roller table design (Fig. 2) consisted of a 600 mm long cage made up of 4 aluminium rods of diameter 12.5 mm mounted horizontally on 2 PVC end flanges of diameter 140 mm and 20 mm thick in which the sample bottles were housed. The entire cage was mounted on steel frame with the help of pillow block bearings (P208) which were coupled to the end flanges by 10 mm x 100 mm long aluminium shafts. An electric motor was bolted on one end of the frame which drove the cage using a pulley and belt arrangement. A flange was also provided in the centre of the cage to firmly secure the bottles and minimize sagging. A single phase 230 V, 50 Hz, 0.1 HP reversible synchronous motor was used which had a fixed rpm of 60. A reduction of 1:3 in speed was achieved with the help of small pulley of 25 mm diameter

coupled to the motor shaft and a big pulley of 75 mm diameter coupled to the shaft connected to the pillow block and driven by a belt (FHP2180).

5.2.1.2 Assembly of roller table: The sample bottles of predetermined size holding around 1 L of water with head space were laid in the cage in horizontal manner and the end flanges were tightened using nuts. Care was taken to prevent over-tightening. The cage was then mounted on the shaft connected to pillow block.

For the experiments, four bottles of 1 L each were filled with the required water sample and incubated at 24 rpm in the laboratory for the required number of days. Water samples collected from the roller table experimental bottles for each of the experiments were analyzed for total bacteria, thraustochytrids and TEPs, using the acridine orange direct count (AODC), acriflavine direct detection (AfDD) and alcian blue staining technique respectively, as given in Chapter 4. Samples were analyzed in duplicate.

Fig. 2a. Schematic diagram of the roller table (plankton wheel).

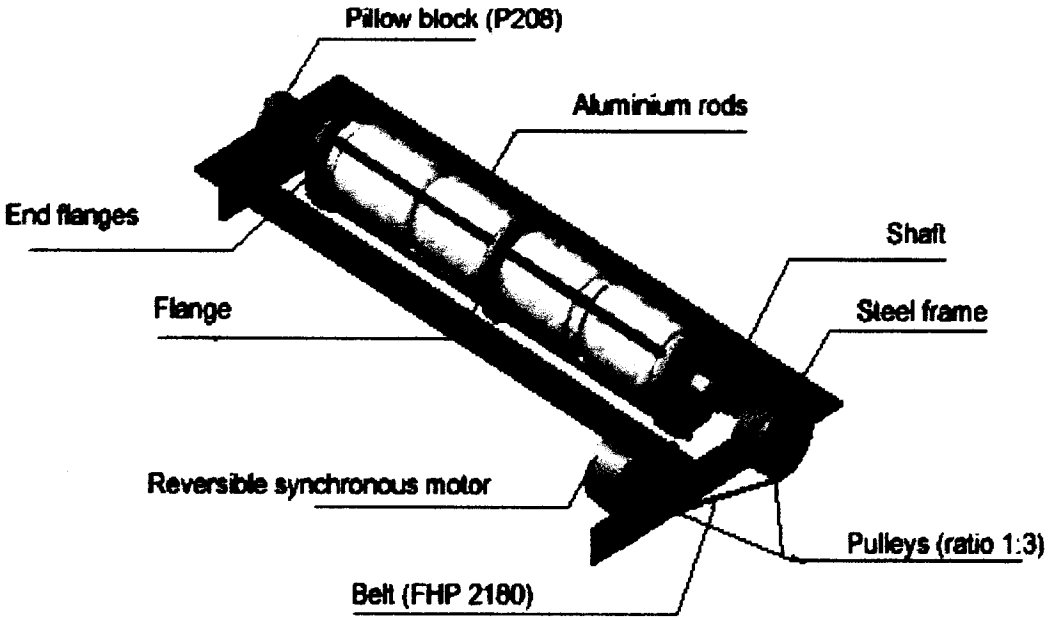


Fig. 2b. Photograph of bottles mounted on the roller table (plankton wheel)

Carbohydrate chemistry of the TEPs was studied in some experiments using FITC-labelled lectins (Sigma-Aldrich), as follows.

1. Concanavalin A for D-mannose, D-glucose and fructofuranose residues;
2. Limulin for n-acetylneuraminic acid, glucuronic acid and phosphorycholine analogs;
3. Lectin from *Ulex europaeus* for fucose.

Stock solutions of the lectins were prepared according to Khandeparker *et al.* (2003). Concanavalin A at a concentration of 2.5 mg mL^{-1}, Limulin at a concentration of 1 mg mL^{-1} and Ulex europaeus lectin at a concentration of 1 mg mL^{-1} were prepared. In order to analyze for the presence of the carbohydrates, 1 mL of water sample was taken in 2 mL Eppendorf tubes and 50 µl of Concanavalin A or 25 µL of Limulin or 50 µL of *Ulex europaeus* lectin was added separately. The samples were incubated for 30 minutes at a room temperature of 25 to 30°C. They were then filtered over 0.22 µm black polycarbonate Isopore membrane filters and examined using an epifluorescence microscope at 450-490 nm (blue excitation filter). A total of 100 microscope fields were examined and the number of samples positive for each lectin were calculated.

5.2.2 Experiment 1. TEPs from the equatorial Indian Ocean water containing microzooplankton, bacteria and thraustocytrids: Water collected from the equatorial Indian Ocean and filtered through a 20 µm

mesh to remove mesozooplankton and phytoplankton, but retaining microzooplankton, bacteria and thraustocytrids was used for the experiments. The four experimental bottles were incubated using the roller table for 15 days at 24 rpm in the laboratory on board under diffuse light and at a temperature of 25–27° C, similar to sea surface temperature (SST) observed during sampling. Fifteen mL samples were withdrawn from the bottles every 3-4 days and fixed with formalin at a final concentration of 2 % and kept under refrigeration till analysis in the laboratory on land, within 3 months. The amount of water withdrawn from the bottles for the analysis was replaced with 0.22 μm filtered seawater collected from the same location and kept refrigerated till use. Total bacteria, thraustochytrids and TEPs were analyzed.

5.2.3 Experiment 2. TEPs from the equatorial Indian Ocean water containing phytoplankton, microzooplankton, bacteria and thraustochytrids: Water collected from the equatorial Indian Ocean and filtered through a 200 μm mesh to remove mesozooplankton, but retaining phytoplankton, microzooplankton, bacteria and thraustochytrids was used for the experiments. Experimental bottles were incubated using the roller table for 2 weeks on board under diffuse light and at a temperature of 25–27 °C, similar to sea surface temperature (SST). The bottles were divided into two sets of two each. Samples from one set were drawn on Days 0, 5 and 9 and from the other on days 3, 7 and 14. A total of 50 mL sample

was drawn each time. The volume of the bottles was maintained constant by replacing the sample amount of 50 mL with 0.22 μm filtered seawater collected from the same station and kept refrigerated till use. The samples were fixed with formalin at a final concentration of 2 % and kept under refrigeration till analysis in the laboratory on land, within 3 months. Total bacteria, thraustochytrids and TEPs were analyzed.

5.2.4 Experiment 3. TEPs from the coastal waters of Goa, containing microzooplankton, bacteria and thraustochytrids: Water collected from Dona Paula Bay and filtered through a 20 μm mesh to remove mesozooplankton and phytoplankton, but retaining microzooplankton, bacteria and thraustochytrids was used for the roller table experiments. The four experimental bottles were incubated using the roller table for 15 days at 24 rpm in the laboratory on board under diffuse light and at a temperature of 25–27° C, similar to sea surface temperature (SST) as observed during sampling. The bottles were divided in two sets. Samples from set I were withdrawn on day 1, 3, 5 and 13 and from set II on day 2, 4, 7, 9 and 15. Total bacteria, thraustochytrids and TEPs were analyzed.

***5.2.5 Experiment 4. TEPs from the coastal waters of Goa, in the presence and absence of bacteria and phytoplankton*:** The four bottles were divided into two sets each. One set of two (A and B) was filled with 20 μm- filtered seawater, thus excluding phytoplankton, but

retaining microzooplankton, bacteria and thraustochytrids. The other set (C and D) was filled with 200 μm-filtered seawater to exclude mesozooplankton, but to retain phytoplankton, microzooplankton, bacteria and thraustochytrids. One bottle of each set (B and D) was amended with antibiotics (400,000 U Penicillin and 0.7 g Streptomycin) to suppress bacteria. All bottles were incubated in 12 hrs cycle of light-dark. Samples were withdrawn from each bottle on day 1, 3, 4, 5, 6, 7, 9, 11, 13 and 15.

Total bacteria, thraustochytrids and TEPs were analyzed. Chemistry of the TEPs was studied using Concanavalin A, Limulin and *Ulex europaeus* lectins.

5.2.6 Experiment 5. TEPs from the culture filtrate of a diatom: A diatom culture belonging to *Chaetoceros* sp. was grown in 2 L of F/2 medium (Guillard & Ryther, 1962) for 8 days. After the incubation period, the medium containing cells was centrifuged and the supernatant was filtered through sterile 0.22 μm filter paper under sterile conditions to obtain the culture filtrate. The culture filtrate was diluted 2.5 times with 0.22 μm filtered seawater and then filled in 4 bottles. The remaining culture filtrate was stored under refrigeration.

A thraustochytrid culture isolated from water of Dona Paula Bay was grown in MV broth for 5 days. The cells were harvested by centrifuging at 8000 rpm for 15 mins. The cells were washed twice with sterile seawater, suspended in 0.22 μm filtered seawater and enumerated

using haemocytometer. Three bottles were inoculated with this culture and the fourth bottle was maintained as control and contained no thraustochytrid cells. Samples of 50 mL each were withdrawn every alternate day till day 7. After withdrawal of the sample from the bottles, the volume of each bottle was maintained constant by replacing with the culture filtrate of the diatom that was diluted 2.5 times with 0.22 μm filtered sterile seawater of Dona Paula to maintain the concentration initially present in each bottle. Total bacteria, thraustochytrids and TEPs were analyzed.

5.2.7 Experiment 6. TEPs from the Extracellular Polysaccharides (EPS) of bacteria associated with diatom culture: EPS derived from bacteria associated with culture of the diatom *Chaetoceros* sp. was used in this experiment. The EPS was obtained as follows. A total of 1 mL of the diatom culture was inoculated in 100 mL Zobel Marine Broth and checked for bacterial growth after 24 hours. This was used as inoculum to inoculate 2 L of Zobell Marine Broth. The inoculum concentration used was 5 %. After 48 hours of incubation period, bacteria were harvested by centrifugation at 10,000 rpm at 4° C. The supernatant was filtered through 0.22 μm filter paper, concentrated using a 3 kDa MW nitrocellulose membrane filter, attached to a tangential flow filtration unit (Amicon, USA). EPS was precipitated at 4° C overnight by adding absolute ethanol to a final concentration of 70 %. The precipitate was then redissolved in small

quantities of deionised water and dialyzed under cold conditions for 48 hours through 12 kDa MW dialysis bags (Sigma). During the dialysis, the water was replaced every 8 hours. The final volume of the EPS suspension after dialysis was 55 mL. It was stored at -20° C till used for the experiment. After thawing, 12 mL was dispensed in each of the 1 L roller table bottle and the remaining was stored frozen in aliquots in sterile bottles to be used for replacing the sample volume after removal for analysis. Three bottles were inoculated with the thraustochytrid culture used in the earlier experiment and an aliquot was removed for enumeration of bacteria, thraustochytrids and TEPs on day 0. The fourth bottle was maintained as control and was not inoculated with the thraustochytrid.

Fifty mL samples were withdrawn every alternate day till Day 7. After withdrawal of the sample from the bottles, the volume of each bottle was maintained constant by replacing with thawed bacterial EPS. The EPS was brought up to the concentration as that present initially in the bottles by taking 550 μL of the EPS (that was stored at -20°C and thawed before use) and bringing up the volume to 50 mL with the help of 0.22 μm filtered sterile seawater of Dona Paula and adding to each bottle.

5.2.8 Epifluorescence staining of aggregates generated in roller table experiment: The aggregates formed in bottles during Experiment 4 (5.2.5) were stained with acriflavine and calcoflour separately to check out

for thraustochytrids and fungi. In case of thraustochytrids, AfDD staining (see Chapter 4) was carried out on the aggregate that was placed in a watch glass. After incubation in acriflavine for 4 mins, the stain was drained and the aggregate was covered with 70 % isopropanol for 1 min. The aggregate was blotted dry with the help of filter paper (Whatman), mounted on a glass slide and observed under blue light.

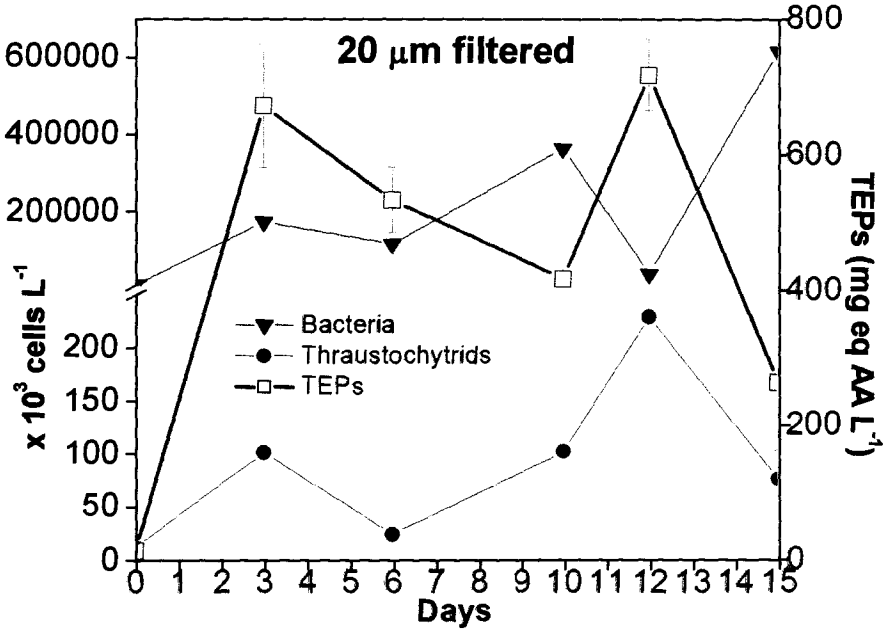
Calcofluor staining was carried out by incubating the aggregate in 0.5 % calcofluor for 1 min, draining the stain and washing by sterile distilled water to wash off excess stain (Damare *et al.*, 2006). The aggregate was then mounted on a glass slide and observed under UV by epifluorescence microscope.

5.3 Results

Experiment 1. TEPs from the equatorial Indian Ocean water containing microzooplankton, bacteria and thraustochytrids: TEPs as well as thraustochytrids displayed two peaks, one on day 3 and the other on day 12. Both groups appeared to be positively related (Fig. 3). Bacteria on the other hand displayed a trend that appeared to be negatively related to TEPs and thraustochytrids. Thus, decrease in TEPs concentration coincided with increasing bacterial numbers, especially on Day 10 and 15 and vice versa as seen on Day 12. Thraustochytrid numbers ranged from 25.5 x 10^3 cells L^{-1} on day 6 to 230 x 10^3 cells L^{-1} on

Day 12. Bacterial numbers ranged from 39.6 x 10^6 cells L^{-1}, as seen on Day 12, to 617.8 x 10^6 cells L-1, as observed on Day 15. TEPs varied from 264.4 to 718.5 mg eq AA L^{-1}.

Fig. 3. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using 20 μm filtered equatorial Indian Ocean water on board cruise # SK 228 during September 2006.



***Experiment 2. TEPs from the equatorial Indian Ocean water containing phytoplankton, microzooplankton, bacteria and thraustochytrids*:** TEPs increased steadily over time up to 12 days (Fig. 4). Thraustochytrids appeared to be positively related to TEPs and showed a similar trend of change, particularly from Day 5 onwards. Bacteria showed a much more rapid increase in numbers up to 3 days, followed by a decline. They did not show any particular trend with relation to TEPs. Thraustochytrids attained maximum densities of nearly 400 x 10^3 cells L^{-1} by Day 14, while bacteria reached values of about 200 x 10^6 cells L^{-1} by this period. The abundance of TEPs, as well as thraustochytrid numbers were lesser in this set of experiments in which phytoplankton were present than in Expt. 1 (Fig. 3), where phytoplankton were excluded.

The carbohydrate composition of the TEPs appeared to be generally uniform throughout the experiment, except on Day 5, as judged by their response to the three lectins. Positive response to lectin from *Ulex europaeus* was uniformly low, ranging from 20.7 to 28. Positive response to Concanavalin and Limulin was more or less similar. The positive percentage response to Concanavalin A ranged from 23.1 to 51.4 and that to Limulin from 24.3 to 49 (Fig. 5).

Fig. 4. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using 200 μm filtered equatorial Indian Ocean water on board cruise # SK 228 during September 2006.

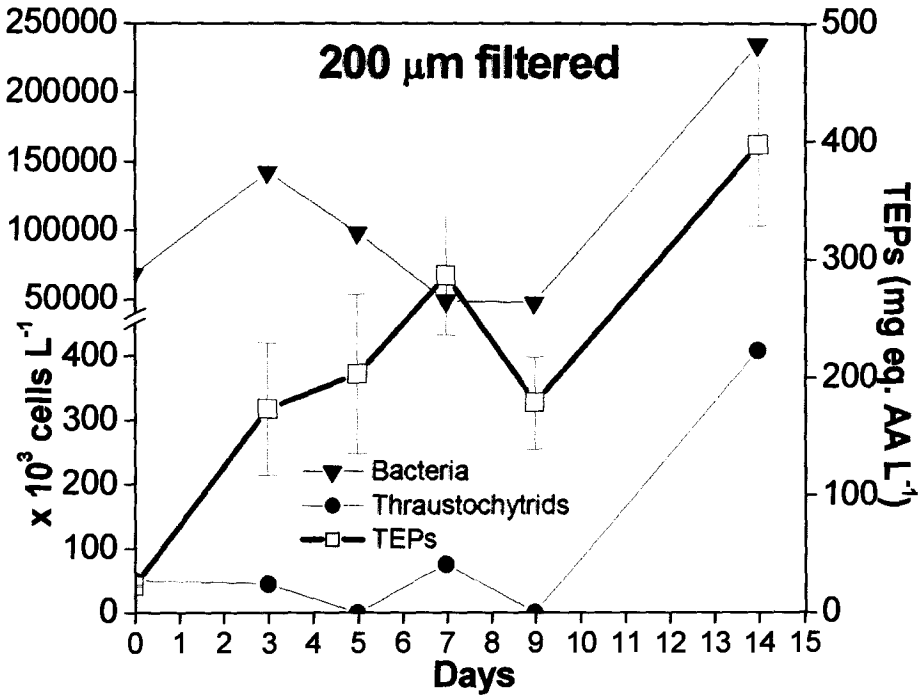
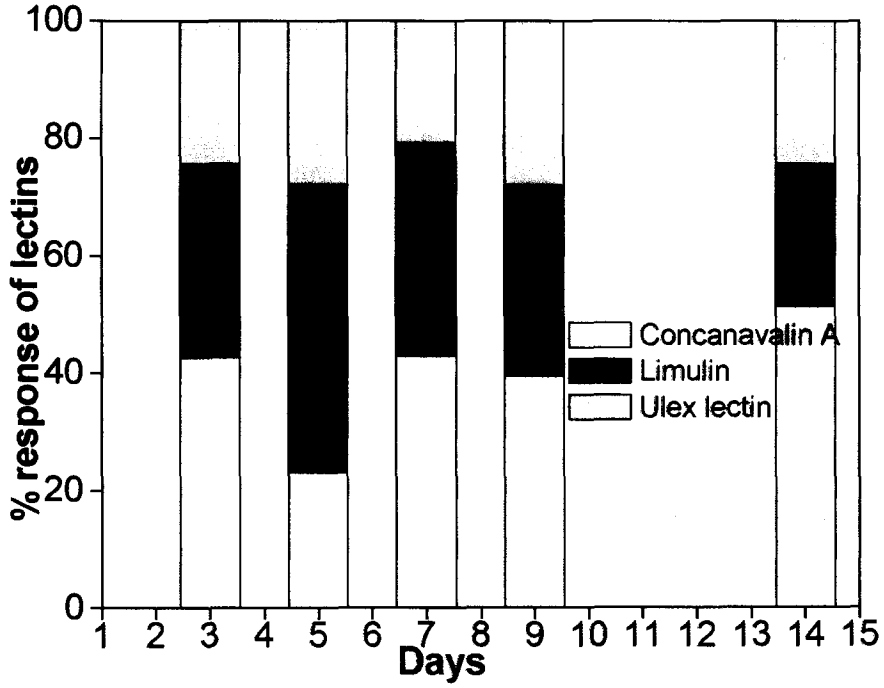


Fig. 5. Percentage response of TEPs to various lectins with respect to time in the roller table experiment conducted using 200 μm filtered equatorial Indian Ocean water on board cruise # SK 228 during September 2006.



Experiment 3. TEPs from the coastal waters of Goa, containing microzooplankton, bacteria and thraustochytrids: Thraustochytrids and TEPs showed three prominent peaks, on 1, 7 and 15 days (Fig. 6). In general, both appeared to be related positively to each other. Bacteria, on the other hand, appeared to be strongly related to TEPs till day 2 and then day 9 onwards. However, the relationship between bacteria, thraustochytrids and TEPs to each other was not significant (Table 1). Thraustochytrid attained a maximum density of 408.9 x 10^3 cells L^{-1} on Day 15, while bacterial numbers reached a peak of 1757.7 x 10^6 cells L^{-1} on Day 9. TEPs varied from 52.2 to 102.2 mg eq AA L^{-1}.

Fig. 6. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using 20 μm filtered coastal seawater.

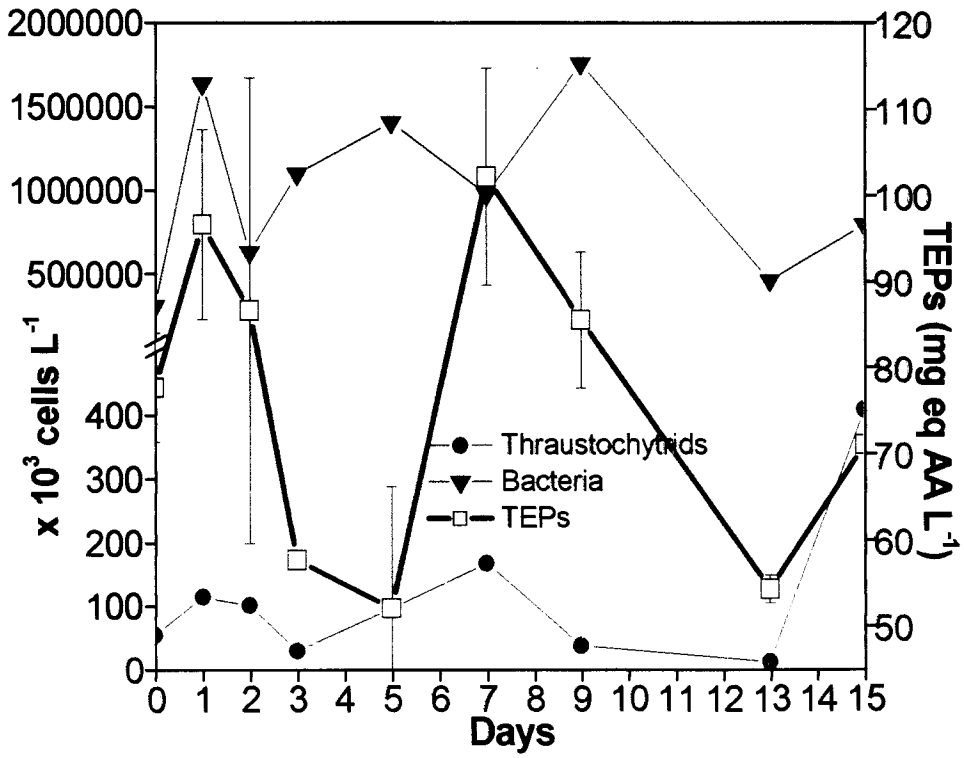


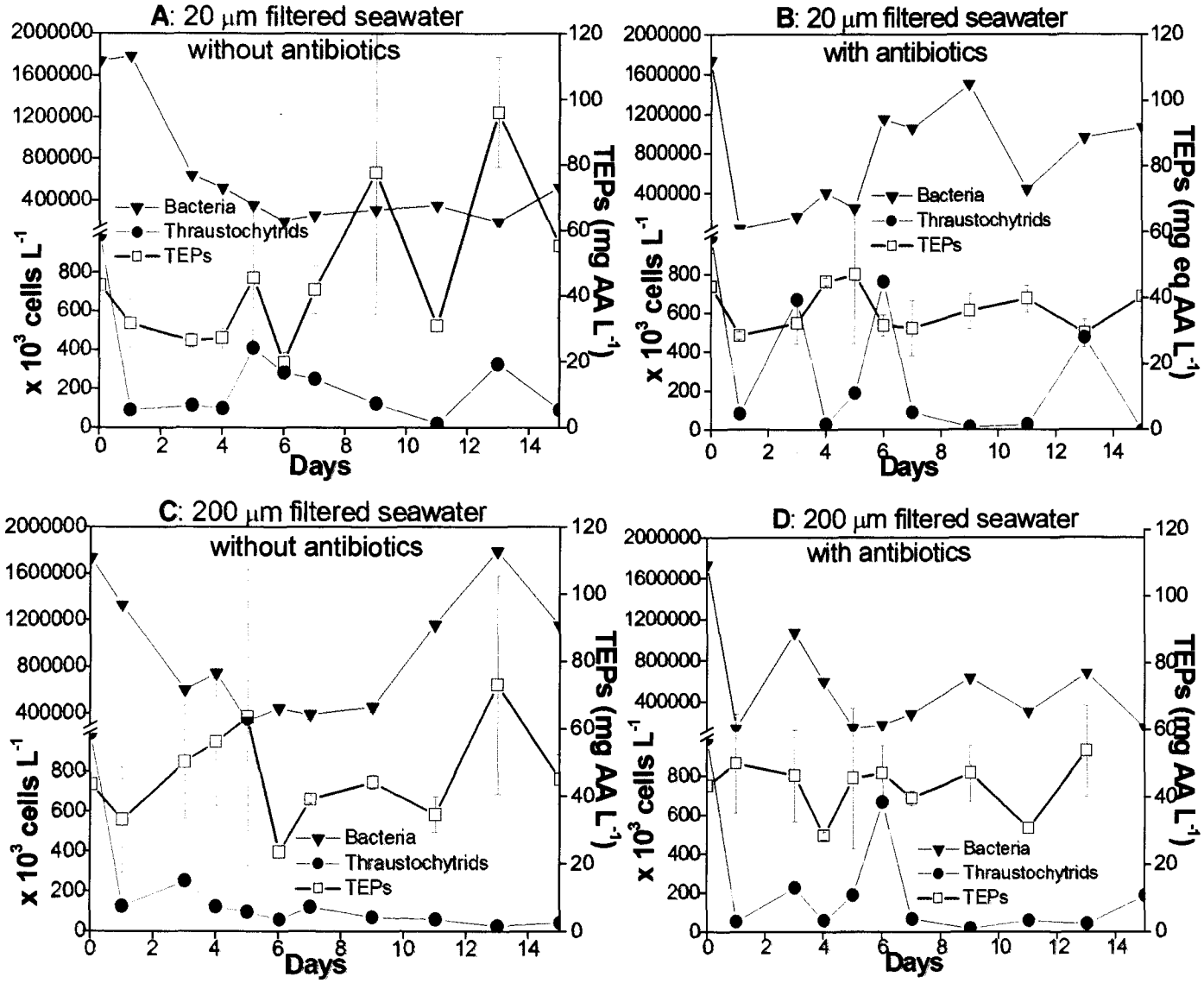
Table 1. Correlation analysis of thraustochytrids, bacteria and TEPs with each other from roller table experiment conducted using 20 μm filtered coastal seawater.

	Experiment 3 (TEPs generated from 20 μm filtered coastal seawater) (N=9)	
	R	p
Thraustochytrids-bacteria	-0.07	0.86
Thraustochytrids-TEPs	0.18	0.65
Bacteria-TEPs	0.21	0.6

Experiment 4. TEPs from the coastal waters of Goa, in the presence and absence of bacteria and phytoplankton: This experiment consisted of 4 treatments of 1 bottle each. Two of the experiments contained phytoplankton in addition to microzooplankton, bacteria and thraustochytrids and two did not. The experimental setup was as follows: (1) Microzooplankton, bacteria and thraustochytrids (20 μm filtered seawater; Bottle A); (2) Microzooplankton, bacteria and thraustochytrids, with bacteria initially suppressed by addition of antibiotics (Bottle B); (3) Phytoplankton, microzooplankton, bacteria and thraustochytrids (Botle C); (4) Phytoplankton, microzooplankton, bacteria and thraustochytrids, with bacteria initially suppressed by addition of antibiotics (Bottle D).

Thraustochytrids attained significantly higher numbers in experimental bottles A and B in which phytoplankton had been excluded (20 μm filtered seawater), than in those where phytoplankton were present (bottles C and D). Their numbers also increased when an initial dose of antibiotics was added (treatments B and D), compared to those in which antibiotics were not incorporated (treatments A and C). Thus, the highest number of thraustochytrids was found in treatment B, containing 20 μm filtered, phytoplankton-free water, where their numbers ranged from below detectable limits to 766.7 x 10^3 cells L^{-1} (Fig. 7). Least number of thraustochytrids were found in water containing phytoplankton (200 μm filtered water) without an initial dose of antibiotics (treatment C). Thraustochytrids showed one or more peaks during the entire experiment.

Fig. 7. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using coastal seawater.



Bacterial numbers were generally high in all treatments. However, their numbers were significantly higher in phytoplankton-containing water to which no antibiotics had been added (treatment C), where they attained very high densities after day 1. Initial addition of antibiotics suppressed bacteria only till the first day (treatments B and D). Bacterial numbers fluctuated widely among the different days of the experiment.

Treatments that received an initial dose of antibiotics showed the presence of large visible aggregates measuring 0.04 to 6 mm (B and D). The aggregate-free water of these bottles contained a maximum of 47.4 mg eq AA L^{-1} in bottle B on day 5 and 54.1 mg eq AA L^{-1} in bottle D on day 13. TEPs were significantly most abundant in phytoplankton-free seawater containing no initial dose of antibiotics (treatment A), where the maximum concentration of TEPs reached a value of 96.3 mg eq AA L^{-1}). The aggregates in treatments B and D were stained with acriflavine and viewed under an epifluorescence microscope to detect thraustochytrids. In addition to numerous cells of thraustochytrids, several spindle-shaped cells, resembling those of the labyrinthulid genus *Labyrinthula*, were also seen (Fig. 8). Staining of the aggregates with calcoflour, followed by epifluorescence microscopy revealed numerous fungal hyphae (Fig. 8c). However, these could not be detected under bright field (Fig. 8d). Although bacteria, thraustochytrids and TEPs varied between treatments and days, none of these was statistically related to each other (Table 2).

Fig. 8. **Photomicrographs of aggregates stained with acriflavine (a, b) under blue light and calcofluor under UV light (c) and bright light (d). Scale represents 10 μm. Thraustochytrid cells are marked by arrows in fig. a and b. Leftward arrow in fig. b points *Labyrinthula*-like cells and rightward arrow points thraustochytrid cells.**

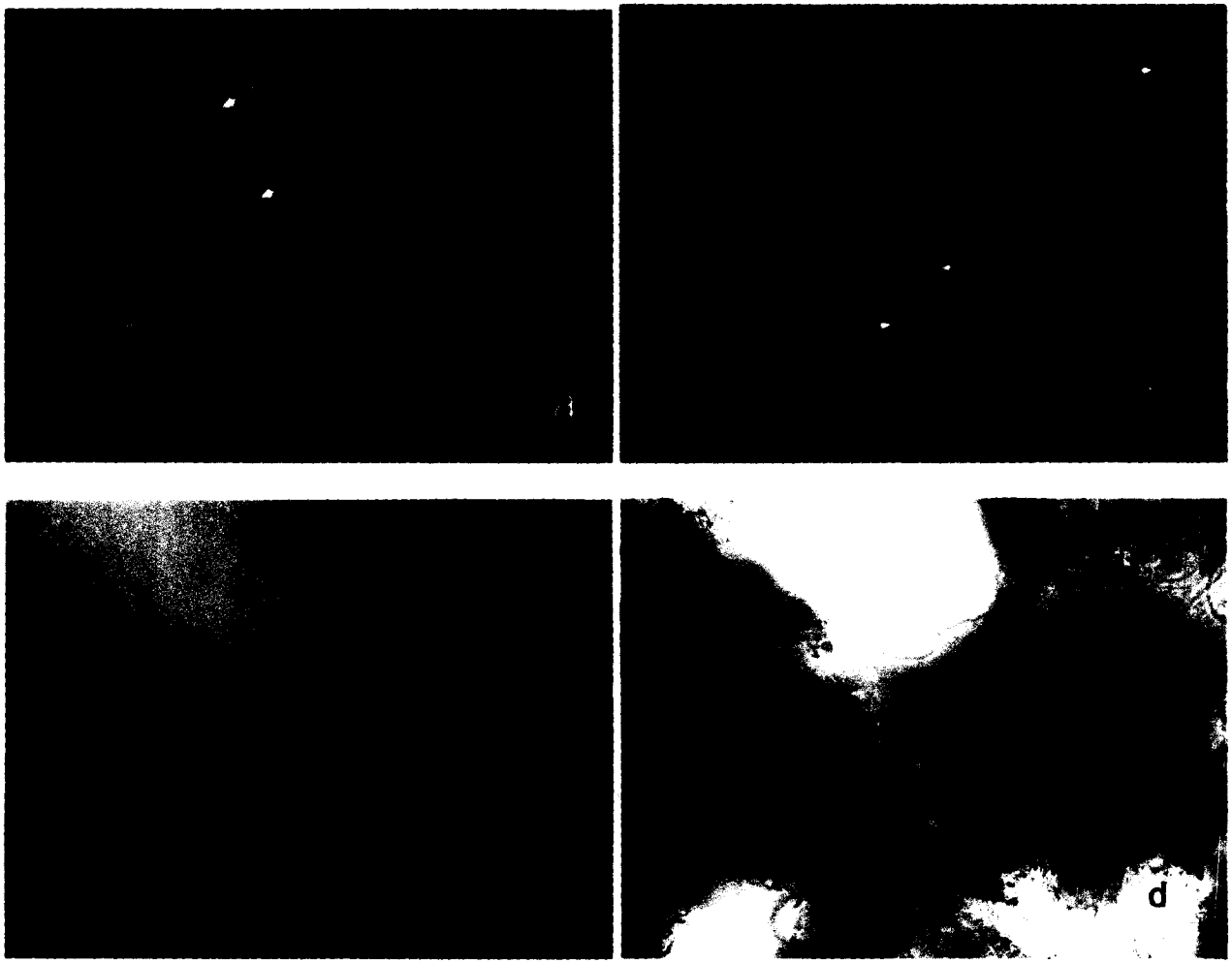


Table 2. Correlation analysis between thraustochytrids, bacteria and TEPs of experiment 4 (N=11).

	Bottle A		Bottle B		Bottle C		Bottle D	
	r	p	R	p	r	p	r	P
Thraustochytrids-bacteria	0.43	0.19	0.35	0.30	0.42	0.20	**0.58**	**0.08**
Thraustochytrids-TEPs	0.11	0.75	-0.12	0.73	-0.06	0.86	0.12	0.74
Bacteria-TEPs	-0.25	0.46	0.03	0.93	0.20	0.56	0.05	0.9

TEPs from these experiments were mostly positive for Concanavalin, the percentage response ranging from 51 to 83 % in bottle A, 50 to 95 % in bottle B, 67 to 93 % in bottle C and 58 to 86 % in bottle D (Fig. 9). Percentage positive response to lectin from *Ulex europaeus* was generally least, being distributed unevenly in all the treatments. Response to Limulin was next to that of Concanavalin. Two-way ANOVA (Table 3) showed that responses to the lectins Concanavalin and Ulex were not statistically significant between the 4 treatments or different ages of the TEPs within an experiment. However, percentage response of Limulin differed significantly between the treatments, the highest response being in bottle A which did not contain phytoplankton and the least in bottle C, which contained them (Fig. 9). The changes were not significant between the different days within a treatment.

Fig. 9. Percentage response of TEPs to various lectins with respect to time in the roller table experiment conducted using coastal water samples subjected to different treatments.

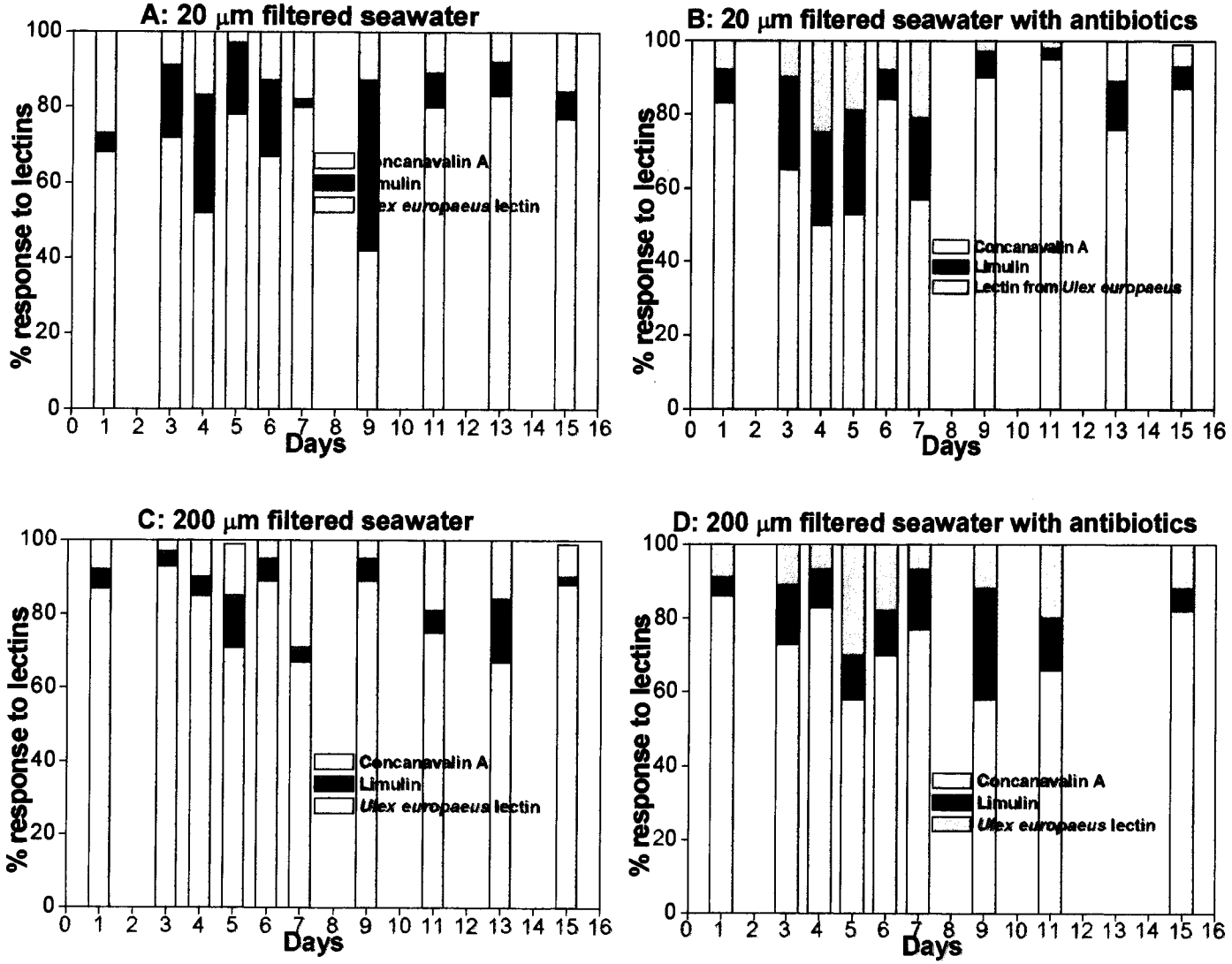


Table 3. Results of two-way ANOVA of percentage response of TEPs from coastal waters to 3 lectins. The water samples were subjected to four different treatments (presence or absence of bacteria and phytoplankton) and incubated for 16 days.

Conditions tested	% response of Concanavalin A		% response of Limulin		% response of lectin from *Ulex europaeus*	
	F	P	F	P	F	P
Variation within different treatments (A, B, C and D)	1.92	0.15	**3.04**	**0.05**	0.36	0.78
Variation within different days	1.01	0.45	1.89	0.11	0.84	0.58

Experiment 5. TEPs from the culture filtrate of a diatom: There was a significant positive correlation between TEPs and thraustochytrids in this experiment (r=0.6, p=0.02) (Table 4; Fig. 10). Both attained peak values on day 3 (Fig. 10). Bacteria too were positively correlated to TEPs, but only at 6 % confidence levels (r=0.5, p=0.06). Bacteria showed a peak on day 5 when thraustochytrid as well as TEPs numbers had started decreasing. Thraustochytrid numbers varied from 78.2 to 1705 x 10^3 cells L^{-1}. Bacteria attained maximum density of 3484 x 10^6 cells L^{-1}. The initial TEPs concentration of 28.9 reached a peak of 109.6 mg eq AA L^{-1} on day 3. The abundance of TEPs and bacteria was more in the experiment bottles than in the control to which thraustochytrids had not been added. In the absence of thraustochytrids, TEPs concentration reached a peak of only 78.5 mg eq AA L^{-1} on day 5 i.e. in the control.

Fig. 10. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using culture filtrate of diatom.

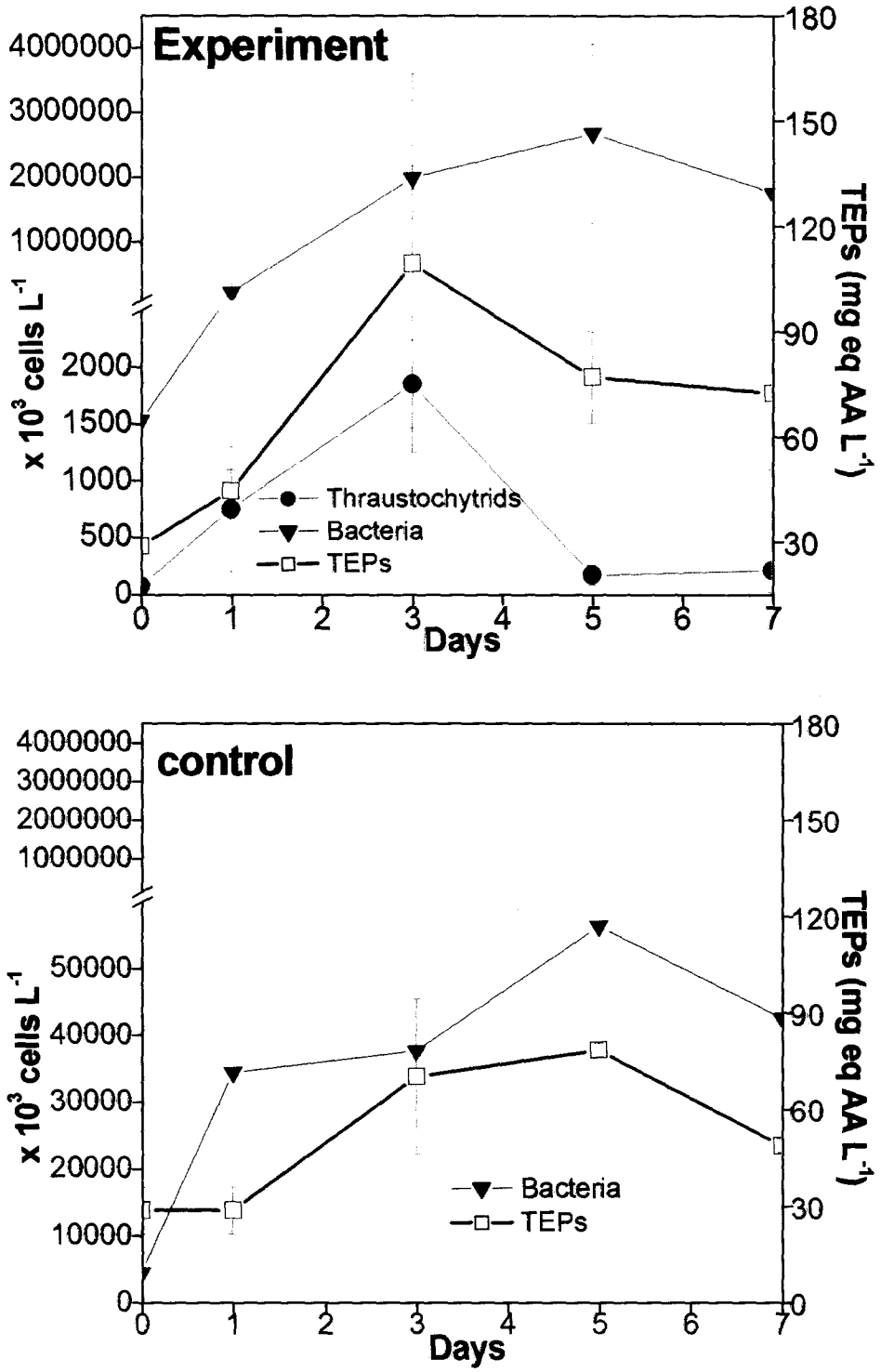


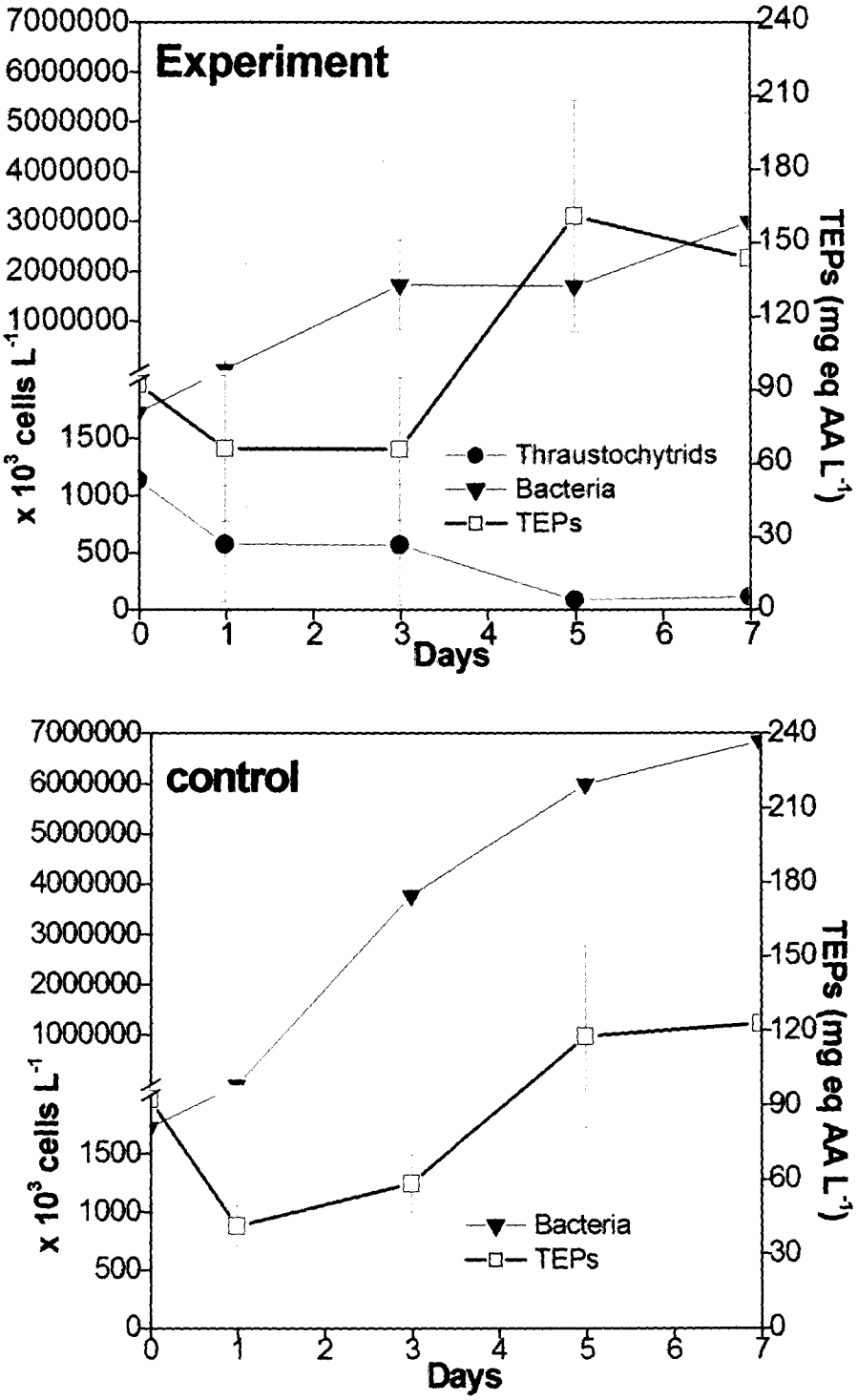
Table 4. Correlation analysis between thraustochytrids, bacteria and TEPs in the roller table experiment using diatom culture filtrates in the bottles.

	Experiment 5 (TEPs generated from the diatom Chaetoceros) (n=15)	
	R	p
Thraustochytrids-bacteria	0.18	0.51
Thraustochytrids-TEPs	**0.59**	**0.02**
Bacteria-TEPs	**0.50**	**0.059**

Experiment 6. TEPs from the Extracellular Polysaccharides (EPS) of bacteria associated with diatom: Thraustochytrid numbers steadily decreased throughout the experiment. Their numbers decreased from 1141 to 122.7 x 10^3 cells L^{-1}. (Fig.11). On the other hand, bacteria and TEPS increased throughout the experiment. Thraustochytrids were negatively related to bacteria at a statistically significant level of <1.0% (Table 5).

TEPs reached a peak of 161.2 mg eq AA L^{-1} on day 5. Bacteria on the other hand increased steadily reaching maximum density of 3003 x 10^6 cells L^{-1} on day 7. Bacterial numbers increased steadily in the control bottle where no thraustochytrids had been inoculated. These numbers were 10 times higher than those in experimental bottles. There was an initial fall in TEPs concentration in control like that in experimental bottle

Fig. 11. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using EPS of diatom-associated bacteria.



after which it increased up to 123 mg eq AA L^{-1} on day 7. TEPs levels reached a lower level when thraustochytrids were not inoculated, compared to the experimental bottles where they were.

Table 5. Correlation analysis between thraustochytrids, bacteria and TEPs of experiment 5 and 6.

	Experiment 6 (TEPs generated from a bacterium) (n=15)	
	r	P
Thraustochytrids-bacteria	**-0.67**	**0.008**
Thraustochytrids-TEPs	-0.42	0.12
Bacteria-TEPs	0.17	0.55

5.4 Discussion

The roller table used in this experiment was designed to generate TEPs even without addition of external nutrients. This takes place through coagulation and adhesion of EPS already present in the water, these processes being encouraged by the design of the instrument in which bottles containing seawater rotate in the presence of shear, thus mimicking that in oceanic waters (Shanks & Edmondson, 1989).

Thraustochytrids multiplied in the roller table bottles in all three experiments where *in situ* oceanic water from the equatorial Indian Ocean and coastal water from Dona Paula Goa were used, without any additional nutrient or inoculum (Fig. 3, 4 and 6). Two of these experiments were

carried out using oceanic or coastal water filtered through 20 μm filters, thereby excluding phytoplankton but retaining microzoooplankton and bacteria (Figs. 3 and 6). One experiment was carried out using oceanic water filtered through 200 μm, thereby retaining phytoplankton.

Although thraustochytrids increased in all experiments, they were much less in bottles containing phytoplankton as compared to those where phytoplankton were excluded (Figs. 3 and 4). Thraustochytrid growth was completely inhibited when coastal water filtered through 200 μm was used for the experiment (Fig. 7C). Yet again, thraustochytrids multiplied in the same set of experiments when the water was filtered through 20 μm filters, thus excluding phytoplankton (Fig. 7A and B). The above observations suggest that thraustochytrids are capable of multiplying in experiments that simulated natural processes of aggregate formation in the presence of microzooplankton, bacteria and thraustochytrids. They also suggest that phytoplankton might inhibit thraustochytrid growth. This is in accordance to the observations made by Raghukumar *et al.* (2001) who found low numbers of thraustochytrids during the season of high primary productivity. Gaertner (1981) too rarely observed thraustochytrids associated with living phytoplankton. Raghukumar *et al.* (1992) also observed that with respect to macroalgae, their populations increase only after senescence and death of the macroalgae. Interestingly, in the present study thraustochytrids grew well when a diatom polysaccharide was added to the experimental bottles, but

in the absence of phytoplankton cells. Therefore, while phytoplankton polysaccharides and their TEPs are probably utilized by thraustochytrids for their growth and multiplication, the presence of live phytoplankton might be inhibitory.

Bacteria would be strong competitors against thraustochytrids for nutrients. How do thraustochytrids survive in their presence? The relationship between thraustochytrids and bacteria varied between the experiments. (1) Except when phytoplankton cells were present, thraustochytrids multiplied in numbers despite dense bacterial populations, indicating a lack of competition. Both were positively related when diatom polysaccharides were added to the experimental bottles, suggesting that such substrates could be shared by both groups when in abundant supply (Fig. 10). It is likely that both bacteria and thraustochytrids coexist when nutrients are in adequate supply. (2) In an experiment where only bacterial polysaccharides were added to 0.22 μm filtered water that did not contain microzooplankton or other detritus, thraustochytrids were completely inhibited by bacteria (Fig. 12). Thus, bacteria might compete with thraustochytrids and suppress them when nutrients are limiting. The principle of competitive exclusion might be operational in this case (Mackenzie *et al.*, 2002). (3) Both thraustochytrids and bacterial populations were high when *in situ* oceanic or coastal water was used without addition of nutrients and containing only natural populations of the two groups (Figs. 3, 6, 7). Therefore, the two groups

may utilize different or the same substrate for nutrition on different occasions. These observations support our field observations on the relationship between bacteria and thraustochytrids (Chapter 4). Further experiments with individual natural substrates would throw more light on what exactly supports thraustochytrids in preference to bacteria.

TEPs are one possible source of nutrition for thraustochytrids. The two showed a positive trend in the two experiments conducted with oceanic water (Figs. 3, 4) and TEPs generated using phytoplankton polysaccharides (Fig. 11). However, TEPs and thraustochytrids showed no clear relation when coastal water was used for the experiments (Fig. 6, 7; Table 1, 2). Therefore, it appears that the source and chemistry of TEPs might be more important than total TEPS *per se*. Thraustochytrids themselves may contribute a part of TEPs, since these are known to produce substantial amounts of extracellular polysaccharides (Jain *et al.*, 2005). Therefore, a preliminary attempt to study the carbohydrate composition using lectins was carried out in this study. In natural samples, a predominance of limulin-positive TEPs appeared to be positively related to thraustochytrids (see Chapter 4). Limulin stains n-aetyl neuraminic acid, which is often produced by bacteria living on surfaces. Indeed, TEPs generated from coastal water filtered through 20 μm not only supported better growth of thraustochytrids, but were also generally more limulin positive than water filtred through 200 μm, which did not support the growth of thraustochytrids. However, thraustochytrids

did not grow on TEPs generated from bacterial polysaccharides (Fig. 12). The carbohydrate chemistry of TEPs has generally been poorly worked out in literature. Further work along these lines is required before the role of thraustochytrids in aggregate dynamics is more clearly understood.

TEPs play a major role in the formation of aggregates. Plenty of aggregates formed in experiments carried out with coastal water to which antibiotics had been added initially. Epifluorescence microscopy of these aggregates revealed not abundant bacteria, thraustochytrids, and fungi. In addition, ellipsoidal cells resembling the labyrinthulid genus *Labyrinthula* were also abundant. Fungi are known to cause macroaggregation in deep-sea sediments (Damare & Raghukumar, 2008). Initial suppression of bacteria by addition of antibiotics might have favoured the growth of fungi which might have enhanced aggregate formation, facilitating growth of thraustochytrids and labyrinthulids within them. TEPs may be contributed by phytoplankton and bacteria together or individually by phytoplankton or bacteria (Passow, 2002; Bhaskar *et al.*, 2005).

5.5 Conclusions

Roller table experiments to generate TEPS from oceanic and coastal waters showed that thraustochytrids and bacteria could multiply under conditions that stimulate TEPs and aggregate formation, even in the absence of additional nutrients. Thraustochytrids appeared to be inhibited

in the presence of phytoplankton. However, thraustochytrids grew well when diatom polysaccharides were used in the absence of living diatom cells. TEPs formed in the presence of only microzooplankton and bacteria in the water column were more favourable for the growth of thraustochytrids.. The relationship between bacteria and thraustochytrids varied in the experiments. Thus, the two may utilize the same or different substrates for their growth. Aggregates formed in water, stimulated by an initial addition of antibiotics, were extremely favourable for the growth of not only thraustochytrids, but also fungi.

Chapter 6.

Detection of aplanochytrids in zooplankton using molecular probes

6.1 Introduction

There have been many earlier reports on the association of the Labyrinthulomycetes, thraustochytrids and aplanochytrids with invertebrates in the sea. These associations may be parasitic or symbiotic (mutualistic or commensalistic). Among parasitic associations, the 'QPX quahog parasite unknown' (Whyte *et al.*, 1994; Smolowitz & Leavitt, 1997) and the abalone parasite *Aplanochytrium haliotidis* (Bower, 1987; Bower *et al.*, 1989) have been studied in detail. Symbiotic associations have been reported in oysters, mussels, tunicates, corals etc. (see Table 1, Chapter 2). Despite numerous non-parasitic invertebrate associations of Labyrinthulomcyetes, the nature of their relationship, namely whether they are commensalistic or mutualistic, is not clear. Detection of the exact location of thraustochytrids or aplanochytrids within their host tissues will provide a clue to this. For example, Raghukumar (1988) detected *Ulkenia visurgensis* in coelenteron and hydranth of hydroid using immunofluorescence, an observation that strongly suggests a mutualistic association between the invertebrate and the thraustochytrid.

Direct detection of cells of a given organism is possible by several methods, such as the use of lectins, antibodies, and molecular probes (Anderson *et al.*, 2005; Bullock, 2005).

- Lectins are carbohydrate-binding proteins which occur on cell surfaces and therefore act as recognition molecules. Because of their ability to differentiate between various carbohydrate structures, they

can be used as probes to differentiate amongst morphologically similar species (Hou *et al.*, 2008). However, some problems are associated with their use. Several different types of lectins have to be used for a single sample for comparison. This can be labor intensive when large numbers of samples have to be handled. Secondly the signal intensity depends upon the concentration of lectin receptors on the surface and therefore epifluorescence microscope for detection of the signal will not be sufficient if the signal is very low. Therefore there is a need for spectrofluorometry or flow cytometry to quantify the signal which makes their use expensive.

- Antibodies which are the host glycoproteins secreted by B cells in response to any foreign molecule, can be used for detection by conjugating with fluorophores or an enzyme which after reaction with a chromogenic substrate produces detectable colour reaction (Lipman *et al.*, 2005). Polyclonal antibodies are preferred over monoclonal antibodies as they bind to multiple epitopes and detection using immunofluorescence is widely used.

  The use of antibodies is also beset with problems like nonspecific background staining. Frequent cross reactivity is seen unless monoclonal antibodies are used in place of polyclonal antibodies.

- Oligonucleotide probes have emerged in the recent years as the most powerful tools to detect microorganisms in their natural environment. Oligonucleotide probes are short sequences of nucleotides from any

gene of an organism and are designed based on sequences that are found specifically only in that individual strain, species or other higher taxonomic levels, such that they bind to complementary sequences of RNA or DNA.

The most useful gene for designing oligonucleotide probes is the small-subunit rRNA gene. There are several advantages in using this gene for designing probes:

- The rRNA gene is present as tandem repeats in the genome. In the case of their RNA, numerous copies are present in the ribosomes. Therefore it can produce abundant signal.
- The small-subunit rRNA gene (see Chapter 3) is a highly conservative gene and sequences therein are specific to different phylogenetic levels, namely prokaryotes or eukaryotes, different kingdoms, phyla and genera. Oligonucleotide probes to detect bacteria in natural environments have extensively targeted the small-subunit, 16SrRNA gene and that for eukaryotes, the 18SrRNA gene. The ITS portion of this gene is highly variable between species and often even strains. Specific sequences from the ITS region can be used to design probes to detect at species level.

A probe should preferably be of a length of about 17-28 nucleotides with GC content of around 50-60 %. It should end in C or G and should not form primer dimers. It should not be self complementary i.e. should not form hairpins (Innis & Gelfand, 1990).

Labeled oligonucleotide probes can be used in different ways for detection of the corresponding organism.

- Dot-blot hybridization: This is done by extracting the DNA of the organism and carrying out a PCR of the small-subunit rDNA using appropriate primers. The amplified products from agarose gel are transferred on to a nitrocellulose or nylon membrane and immobilized on the membrane. Bands on the membrane containing the DNA sequence of interest are detected by hybridization with the labeled probe.
- *In situ* hybridization (ISH): This is another common method to directly detect the cells of the organism in question (Biegala *et al.*, 2003). *In situ* hybridization, first described by Gall and Pardue (1969) and John *et al.* (1969), localizes a specific DNA or RNA sequence in a portion or section of tissue or in the entire tissue with the help of a labeled oligonucleotide probe complementary to it and detecting it under a microscope (Schwarzacher, 2003).

Oligonucleotide probes, whether for dot-blot or *in situ* hybridization, can be labeled in many ways. Radioactively labeled probes have been used for many years but they have certain disadvantages like half-life of the isotopes and disposal problem of radioactive waste. Therefore non-radioactively labeled probes were brought into use. These can be labeled by direct or indirect method. Direct method involves covalent attachment of a detectable label to the probe by chemical means or enzymatic means.

Indirect method involves attachment of a hapten to the probe which is then detected by various methods depending on the choice of the hapten. Biotin or digoxigenin or fluorescein can be used as the hapten. The hapten is covalently attached to the probe. It is then detected using a specific binding protein, such as anti-biotin, avidin or streptavidin, anti-digoxigenin or anti-fluorescein, which itself is conjugated to a fluorophore or to an enzyme. In case of the latter the substrates that are used are chemiluminescent or chromogenic.

In biotin-streptavidin system, strepavidin conjugated to enzyme alkaline phosphatase binds to the hapten biotin, which itself is attached to the probe. The enzyme is then detected with the help of 5-bromo-4-chloro- 3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) substrates that gives a coloured reaction. A blue precipitate of 5,5'-dibromo-4,4'-dichloro-indigo is formed after sequential dephosphorylation of BCIP and oxidation of NBT. The advantages of this system are good signal amplifications and long-term hybridization probe stability (Paragas *et al.*, 1997).

In situ hybridization may have problems if the sequence to be determined is lower in concentration within the cell or is masked by an associated protein. A typical ISH procedure therefore comprises 5 steps.

1. Permeabilization is carried out in such a way that the structural integrity of the cell is not destroyed.

2. Prehybridization or pretreatment is carried out preferably when probe detection methods involve enzymes for visualization of the label. It reduces the background signal by neutralizing the endogenous tissue enzymes which could result in very high background.
3. Hybridization is carried out under stringent conditions for proper annealing of the probe to the target sequence (Bottari *et al.*, 2006).
4. Washing is carried out to remove the unbound probe.
5. Detection is made incubating with an appropriate substrate, followed by microscopy.

ISH has been widely used for cultivation-independent assessment of microbial communities from various aquatic and soil habitats. Not only free-living microbial systems, but also symbiotic associations between organisms have been studied by ISH. Amongst the microorganisms, bacteria and archaea are extensively studied using this technique, especially with the help of fluorescently-labeled probes. In addition to these prokaryotes, various eukaryotic microorganisms have also been studied, some examples of which are listed in Table 1.

Table 1. Examples of organisms detected using molecular probes.

Organism	Group	Detection system	Detection from	Reference
Phytophthora	Fungus	Radioactive probe	Infected potato tubers	Tooley *et al.*, 1997
Aureobasidium	Fungus	FITC probe	Apple leaf surfaces	Li *et al.*, 1997
Karenia brevis	Dinoflagellate	Fluorescein, carboxytetram-ethylrhodamine probe	Red tide samples from around Florida	Gray *et al.*, 2003
Amoebophrya	Dinoflagellate	Texas Red, fluorescein probe	Dinoflagellate *Akashiwo sanguinea*	Gunderson *et al.*, 2001
Symboidinium	Dinoflagellate	FITC probe	Corals, sea anemones	Loram *et al.*, 2007
Heterosigma	Dinoflagellate	FITC probe	Algal bloom	Chen *et al.*, 2008
Alexandrium	Dinoflagellate	FITC probe	Scottish coastal waters	John *et al.*, 2003
		FITC, Texas Red, Cy3 probe	Water samples from Gulf of Maine	Anderson *et al.*, 2005
		FITC lectin	Chinese coastal waters	Hou *et al.*, 2008
Pseudo-nitzschia	Dinoflagellate	FITC lectin	New Zealand coastal waters	Rhodes *et al.*, 1995; Rhodes, 1998

In the present study, aplanochytrids were isolated in culture from a number of zooplankton specimens collected from the water column of the equatorial Indian Ocean indicating a strong relationship between the two. But however, the knowledge about this relationship is not clear, since the actual location of the cells in relation to the zooplankton hosts cannot be studied by mere isolation in culture. The use of molecular probes can therefore be very helpful to study the associations of aplanochytrids with the zooplankton (see Chapter 2). An 18S rRNA targeted probe has recently been designed against thraustochytrids to facilitate their detection and enumeration in natural environment (Takao *et al.*, 2007). Such probes, however, work more efficiently at taxonomic levels above the genus. ITS-based probes are more useful to detect individual species in the ecosystem. Eight isolates from zooplankton were identified as belonging to the *Aplanochytrium yorkensis* complex based on morphology, life cycle and 18S rRNA genes.

In view of the above, the following objectives were defined:

(1) To design an oligonucleotide probe against some isolates of aplanochytrids isolated from zooplankton of equatorial Indian Ocean in order to use it for their detection

(2) To check the specificity of the probe in order to avoid binding to other organisms and thus avoid false positive reactions.

(3) To standardize the hybridization technique with the cell control in order to obtain high signal.

(4) To apply the probe on specimens of zooplankton collected from the equatorial Indian Ocean in order to locate the aplanochytrids in them.

6.2 Materials and Methods

***6.2.1 Alignment of ITS sequences*:** A total of 13 ITS sequences were aligned by the Multalin software, version 5.4.1. This consisted of 7 isolates of aplanochytrids obtained from zooplankton of equatorial Indian Ocean, whose ITS regions were sequenced in the present study (Chapter 3; Appendix 1) and 6 sequences of other organisms belonging to Kingdom Straminipila, downloaded from the website http://www.ncbi.nlm.nih.gov/. The list of all the 13 organisms used for the analysis are presented in Table 2.

***6.2.2 Designing of the probes*:** Based on multiple alignment of the 7 aplanochytrids and 6 other stramenopiles, sequences which showed consensus regions only among the aplanochytrids and minimal or nil similarity with other organisms were chosen for designing probes for the aplanochytrids. These consensus sequences were analysed using Vector NTI 10 software (Invitrogen, USA). This software analyses the sequences for probes based on the formation of primer dimer, melting temperatures, GC content, salt concentration for various concentrations of primers, binding regions of primer to DNA and tentative length of PCR products to be formed. The software analysis showed that it was feasible

to design probes for two isolates, namely S1961 and S2124. A total of four probes, consisting of one forward and one reverse primer for each isolate could be designed. These were designated S61F1and S61R1 for the isolate S1961 and 138F1 and 138R1 for the isolate S2124. Details of the primers are given under the 'Results' section. The primer oligonucleotides were obtained from Sigma Genosys, Bangalore. All primers were biotinylated at the 5' end.

Table 2. List of microorganism strains used for multiple alignment.

Kingdom	Phylum	Strain	Sequence length	Obtained from
Stramenopila	Labyrinthulomycota	S1961	556	Equatorial Indian Ocean (Isolated in the present study)
		S1963	503	
		S19610	503	
		S2122	552	
		S2124	447	
		S2128	572	
		S2129	562	
		Labyrinthulid quahog parasite QPX DQ641197	434 bp	NCBI database
	Bacillariophyta	*Sellaphora pupula* AJ544676	706 bp	
		Nitzschia compressa AY574377	706 bp	
Fungi	Chytridiomycota	*Spizellomyces* sp. OSU AY349099	626 bp	
		Rozella allomycis AFTO-IO297 AY997087	628 bp	
	Blastocladiomycota	*Catenophlyctis* sp. JEL298 AY997034	635 bp	

6.2.3.1 Confirmation of probe specificity: DNA was extracted from the different isolates of aplanochytrids obtained from zooplankton of equatorial Indian Ocean as well as of thraustochytrid cultures CW-1, Z7 and DP7 present in NIO culture collection, using the method described in Chapter 3. Polymerase chain reaction (PCR) amplification was carried out for each set of DNA using the forward and reverse primer probes designed for the two aplanochytrids, namely S61F1-S61R1 and 138F1-138R1 respectively. Amplification was carried out with final concentrations of 7.5 pM primer each, 100 μM dNTP mix, 1X PCR buffer containing 1.5mM $MgCl_2$, and 1U Taq polymerase in BioRad Thermal Cycler (S. No. AL100938). The annealing temperature conditions were optimized for both the primer sets by carrying out a gradient PCR in Peltier Thermal Cycler (MJ Research, PTC-200, S. No. AL083846). The temperature gradient that was checked, ranged from 45° to 65° C. The optimal conditions for S61F1-S61R1 primer set were 94° C/ 4 min, followed by 30 cycles of denaturation at 94°C/40s, annealing at 56°C/50s and extension at 72° C/ 1min, with a final elongation step of 72° C/ 6 min. Best conditions for the 138F1-138R1 primer set were 94° C/ 4 min, followed by 30 cycles of denaturation at 94° C/40s, annealing at 60°C/50s and extension at 72° C/min, with a final elongation step of 72° C/ 6 min.

6.2.3.2 Southern hybridization: PCR amplification of the DNA of the aplanochytrid isolates was carried out using both primer sets and optimal

conditions, as above. Agarose gel electrophoresis of the PCR products was carried out at 60 Volts. The gel was blotted on to a nylon membrane (Bangalore Genei) by electroblotting, using electroblotter (ETS-5, supplied with Southern Hybridization Kit, Bangalore Genei). Hybridization and post-hybridization reactions were carried out on the membrane as well as on the gel to check if all the bands had blotted over the membrane as per the instructions in the Southern Hybridization Kit (Bangalore Genei). The bands were then hybridized with the probes S61F1 for S1961, S1963 and S19610 isolates and 138F1 for S2122, S2124, S2128 and S2129 isolates. Hybridization was carried out at 45°C for 12 hours followed by 3 washing steps each with wash buffer A and B. This was followed by incubation for 1 hour in blocking buffer with continuous rocking and then incubation with streptavidin-conjugated alkaline phosphatase (Bangalore Genei) for 20 mins with rocking. Three washing steps each with wash buffer C and D were carried out followed by incubation with 1X substrate solution (BCIP-NBT) provided with the kit till blue bands appeared. The reaction was stopped by transferring the membranes in distilled water.

6.2.4 Standardization of In situ hybridization (ISH) of whole aplanochytrid cells:

Two aspects of *in situ* hybridization (ISH) were standardized using cells of isolates S1961 and S19610. These were permeabilization and the actual hybridization. Five day old cells of cultures of the isolates grown in

MV medium (see Chapter 2) were harvested and used for the experiments.

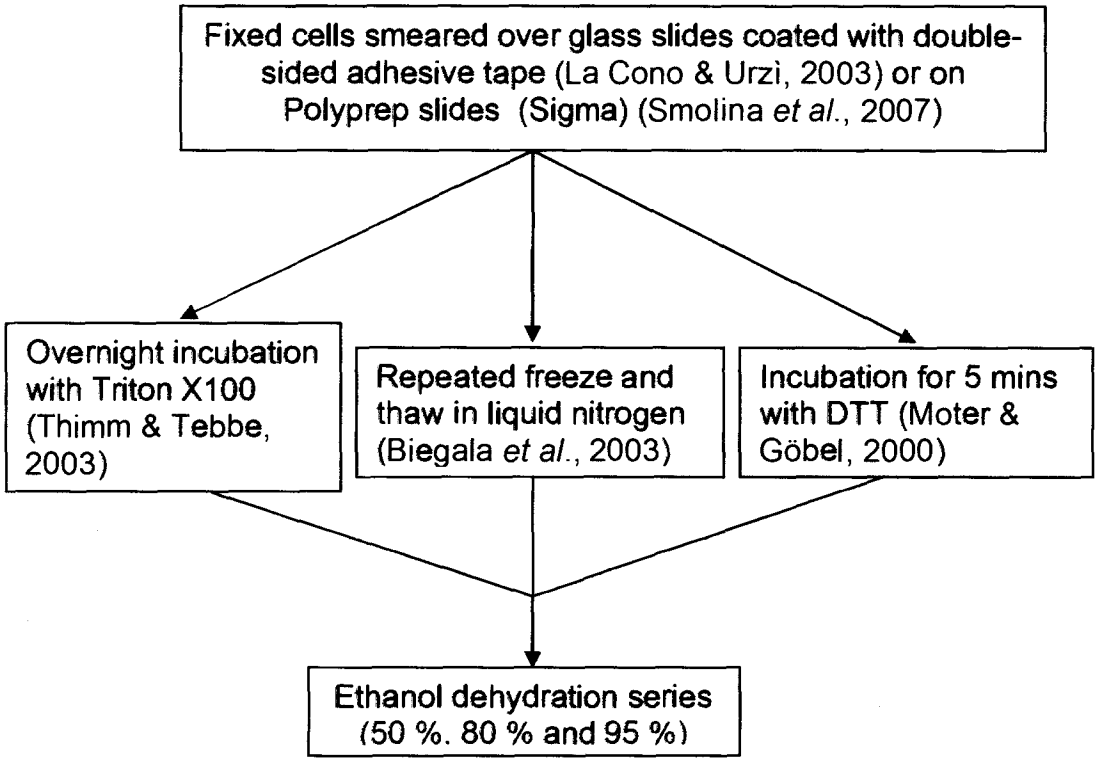
In order to optimize permeabilization, cells were suspended in phosphate-buffered saline containing formalin up to 2 % concentration and stored under refrigeration till further use. Different permeabilization techniques that were tried are shown in Fig.1.

Electroporation was carried out by transferring cells suspended in sorbitol to the cuvette and providing an electric pulse of 2500 V for ~5 milliseconds in Gene Pulser Xcell ^{TM} (BioRad, S. No. 617BR105105) as per the protocol mentioned for eukaryotic cells in the manual of Gene Pulser Xcell ^{TM}.

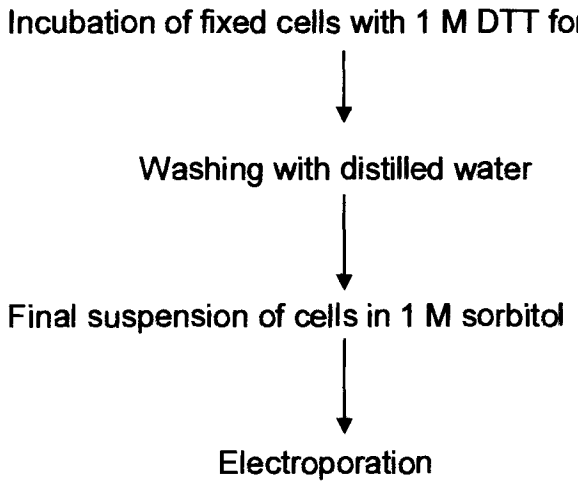
Hybridization of the permeabilized cells was optimized as follows. The Southern Hybridization Kit (Bangalore Genei) was used for the purpose. Permeabilized cells were incubated with prehybridization buffer provided with the kit at 45°C for 45 minutes. After discarding the buffer, the cells were hybridized with hybridization buffer containing S61F1 probe at a final concentration of 5 pM/μL. Two separate reactions, one containing the forward primer and one containing the reverse primer were used individually. Hybridization was carried out at 45° C for 12 hours. This was followed by the post-hybridization steps, as provided in the Southern Hybridization Kit (Bangalore Genei). The cells were washed thrice with wash buffers A and B and incubated with blocking buffer for one hour with

Fig. 1. Protocols for different permeabilization techniques carried over the cells of aplanochytrid isolates S1961 and S19610.

Protocol 1-



Protocol 2-



gentle rocking. The cells were then incubated with streptavidin-ALP conjugate for 20-30 min, washed with wash buffers C and D thrice and finally incubated in 1X substrate solution.

6.2.5 In situ hybridization of field samples using probes:

6.2.5.1 Zooplankton samples fed with aplanochytrids: In order to test if the standardized methodology on permeabilization and hybridization using aplanochytrid cells as above was also efficacious together with zooplankton samples, the following experiment was carried out. Mesozooplankton were sampled from the Dona Paula Bay using a zooplankton net of mesh size 200 μm. Approximately 7-8 live specimens of mesozooplankton were kept in 5 separate beakers each under aeration in laboratory. They were starved for a period of 6 hours. Cells of the aplanochytrid S1961 were fed to each experimental beaker. Cells were added up to twice the concentration found in natural waters of Dona Paula Bay as estimated independently using epifluorescence microscopy (232 x 10^3 cells L^{-1}). A beaker each was sampled at 4, 6, 10, 15 and 24 hours each. The entire contents of each beaker were fixed with 2 % formaldehyde to preserve the specimens and in order to check if the aplanochytrids had been grazed and were present in their guts. The specimens from each beaker were permeabilized as per protocol 2 (mentioned in Fig. 1). These were transferred to microcentrifuge tubes, treated with 1M DTT for 5 mins, washed twice with distilled water and

once with 1 M sorbitol and finally suspended in 1 M sorbitol. Electroporation was carried out using Gene Pulser Xcell ^{TM} with the same pulse conditions as that for whole aplanochytrid cells. After electroporation, *in situ* hybridization was carried out in the microcentrifuge tubes using the same procedure which was followed for the whole aplanochytrid cells as above.

6.2.5.2 *Natural zooplankton population from equatorial Indian Ocean:* Zooplankton samples collected using the multiple plankton net during the two cruises on board ORV Sagar Kanya SK 212 (October 2004) and SK 228 (September 2006) from the water column of the equatorial Indian Ocean were preserved in formalin up to 2 % concentration in refrigerator. They were subjected to *in situ* hybridization using S61F1 and 138F1 probes individually. The pretreatment as well as hybridization was carried out as mentioned above for the experiment on zooplankton specimens fed with aplanochytrids.

6.2.6 Detection and analysis: The post-hybridization steps like washing, incubation with blocking buffer, conjugate and substrate were carried out as per the protocol used for aplanochytrid cells given above. After incubation with substrate solution for 1-2 hours, the specimens were transferred to distilled water and then on to a glass slide and observed under microscope (Olympus BX60) using bright field microscopy. Specimens with blue coloration were noted as positive. The photographs

of those specimens showing positive reaction were captured using Olympus Digital DP70 camera.

6.2.7 In situ PCR of chaetognath specimens: Specimens of chaetognaths from the Indian Ocean collected and fixed as above were washed with phosphate-buffered saline (PBS) and either dried under vacuum or suspended in 1M sorbitol and electroporated before carrying out *in situ* PCR. A total of 50 µl of PCR buffer was added to each specimen in the PCR tube. *In situ* PCR using the two different sets of primers, i.e., S61F1-S61R1 and 138F1-138R1 was carried out separately, using BioRad Thermal Cycler (S. No. AL100938). PCR cycles consisted of denaturation at 94° C for 40 s, primer annealing (56°C for S61F1-R1 primer set or 60° C for the 138F1-R1 primer set) for 50 s, and extension at 72° C for 60 s. Amplification was repeated for 30 cycles with a thermal cycler. After *in situ* PCR, ISH was carried on the same specimens, following the same methods as those for the zooplankton fed with aplanochytrids as mentioned above.

6.2.8 In situ hybridization of aggregates collected from the equatorial Indian Ocean water column:

Marine aggregates that came up along with the multiple net deployed to collect mesozooplankton were fixed along with the zooplankton with formalin up to 2 % concentration and later separated

from the zooplankton using a Pasteur pipette. Two different initial methodologies were followed for *in situ* hybridization. In the first, the samples were electroporated and in the other they were not.

For electroporation, the fixed aggregates were suspended in PBS, incubated with DTT for 5 min., washed with distilled water and finally suspended in 1M sorbitol in a microcentrifuge tube. They were then transferred to a cuvette and given an electric pulse as described under standardization of ISH for aplanochytrid cells. After this pretreatment, *in situ* hybridization on those aggregates was carried out using S61F1 probe and maintaining the same conditions as mentioned above for the experiment on zooplankton specimens that were fed with aplanochytrids.

Alternatively, the aggregates were air dried over polyprep slides (Sigma) instead of subjecting them to electroporation. They were then passed through a series of ethanol dehydration (50 %, 80 % and 95 %) and dried again. ISH was performed on these aggregates over the glass slides using S61F1 probe. The protocol was the same as mentioned for the experiment on zooplankton specimens that were fed with aplanochytrids.

6.3 Results

Alignment of the ITS sequences and designing of probes

The partial-aligned ITS sequences of the 7 aplanochytrid isolates, as well as the six other stramenopiles are shown in Appendix 1. A cluster analysis of the seven aplanochytrids has been presented in Chapter 3 showing isolates S1961, S19610 and S1963 from the SK196 cruise on the one hand and isolates S2122, S2124, S2128 and S2129 from the SK212 cruise on the other clustering together. Probe design analysis using Vector NTI 10 resulted in choosing two isolates as the best possible candidates for the probe, these being S1961 from the SK196 cruise and the other S2124 from the SK212 cruise. Two sets of primers each, one forward and the other reverse, (a total of four) were designed against the two isolates (Table 3). Thus, altogether 4 probes, 2 specific for each group of aplanochytrids, were designed. Regions of binding of the probes is shown in Appendix 1.

Table 3. Sequence and type of the designed probes for the aplanochytrids from zooplankton of the equatorial Indian Ocean.

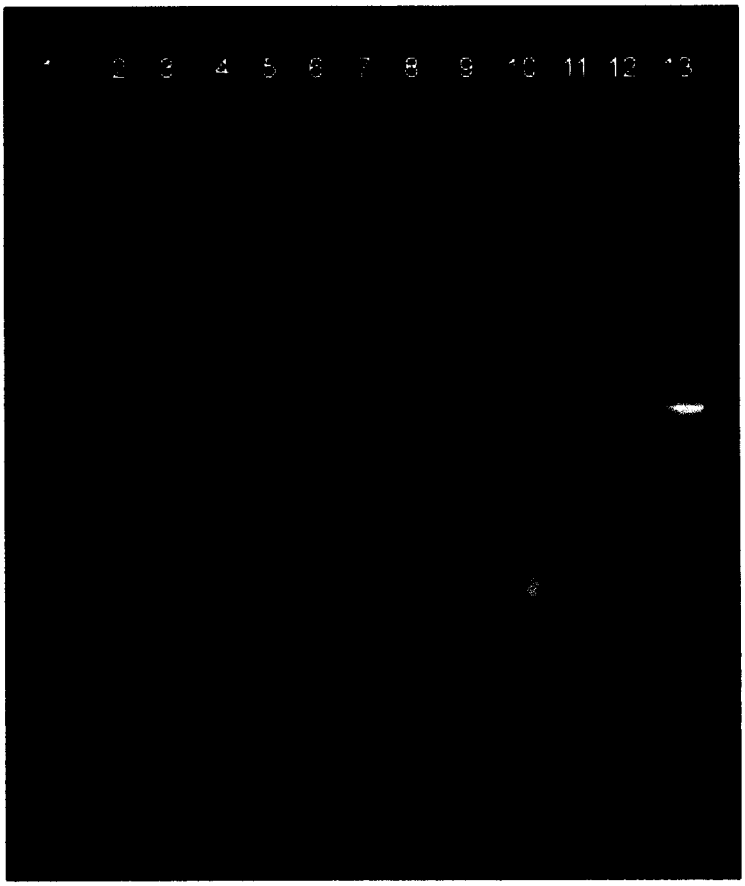
Isolate No.	Probe	Length	Sequence	Type
S1961	S61F1	19	5'-TAATCCATTTCTAATTCCG	Forward
	S61R1	19	5'-TACATAAAAACCCAAAATC	Reverse
S2124	138F1	20	5'-GCTGTCGCCTGTTCTGATTG	Forward
	138R1	20	5'-ACATACTTGCGTACATATTG	Reverse

Confirmation of probe specificity

PCR amplification with the designed set of primers showed that primer set S61F1-S61R1 amplified DNA of not only isolate S1961, but also other isolates obtained from the September 2003 cruise, namely S1963 and S19610. However, they did not amplify the DNA of the other isolates obtained from the October 2004 cruise (Fig. 2). Similarly primer set 138F1-138R1 designed for isolate S2124 amplified DNA of not only this isolate, but also that of S2122, S2128 and S2129, which had been isolated during October 2004 cruise (Fig. 3). This primer set did not amplify the DNA of the isolates obtained from the September 2003 cruise.

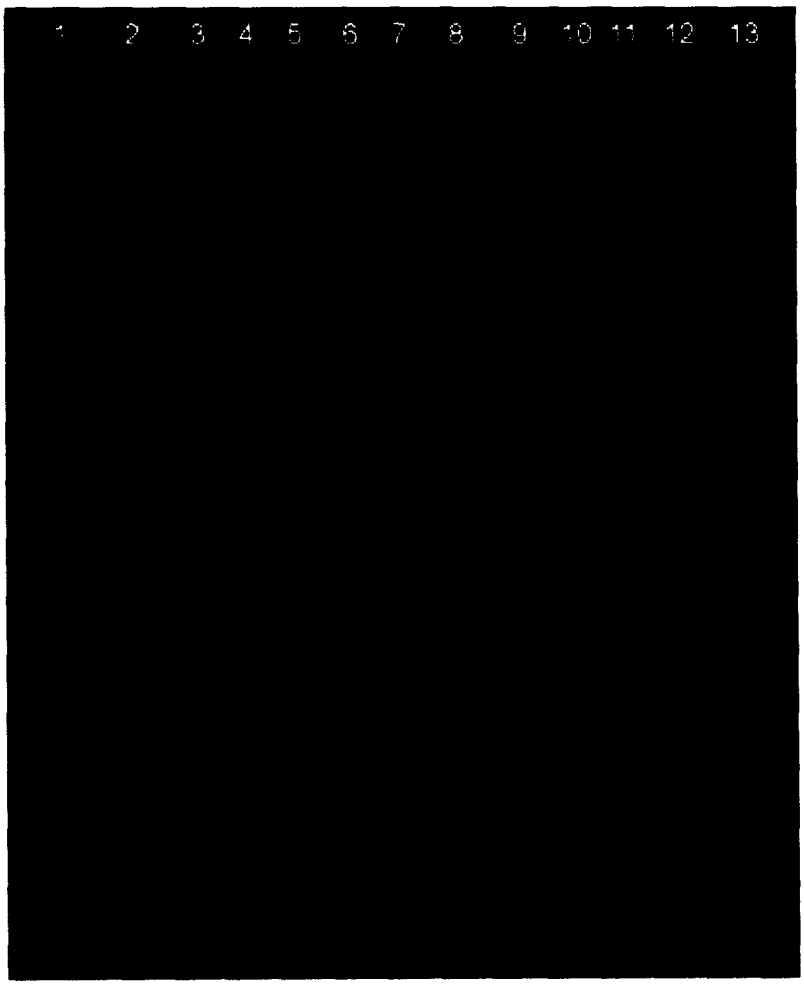
Southern hybridization carried out using S61F1 probe on the amplified product of the isolates S1961, S1963 and S19610 for the first primer set (Fig.4, 5a) and using 138F1 probe on the amplified product of the isolates S2122, S2124, S2128 and S2129 for the second primer set gave clear positive signals (Fig. 5b).

Fig. 2. Agarose gel electrophoresis of PCR-amplified products of aplanochytrids from the present study and thraustochytrids from NIO culture collection using S61F1-S61R1 primer set.



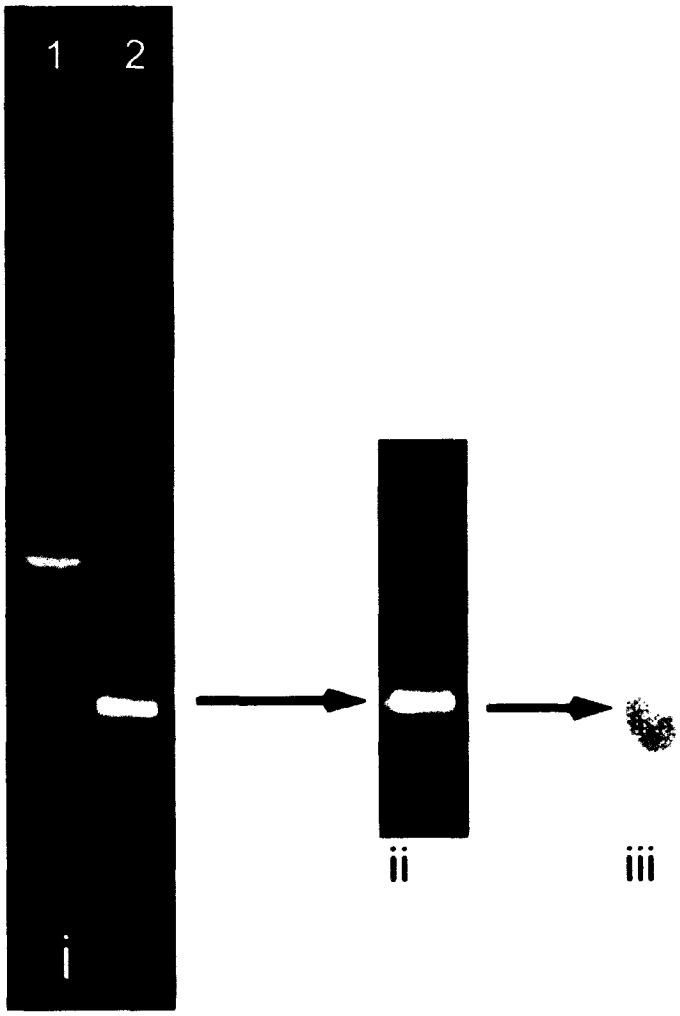
Lane	Band
1.	S1962
2.	22
3.	S2122
4.	CW-1
5.	S19610
6.	S2124
7.	S2129
8.	S2128
9.	S1963
10.	DP7
11.	S1961
12.	negative control
13.	1 kb ladder

Fig. 3. Agarose gel electrophoresis of PCR-amplified products of aplanochytrids from the present study and thraustochytrids from NIO culture collection using 138F1-138R1 primer set.



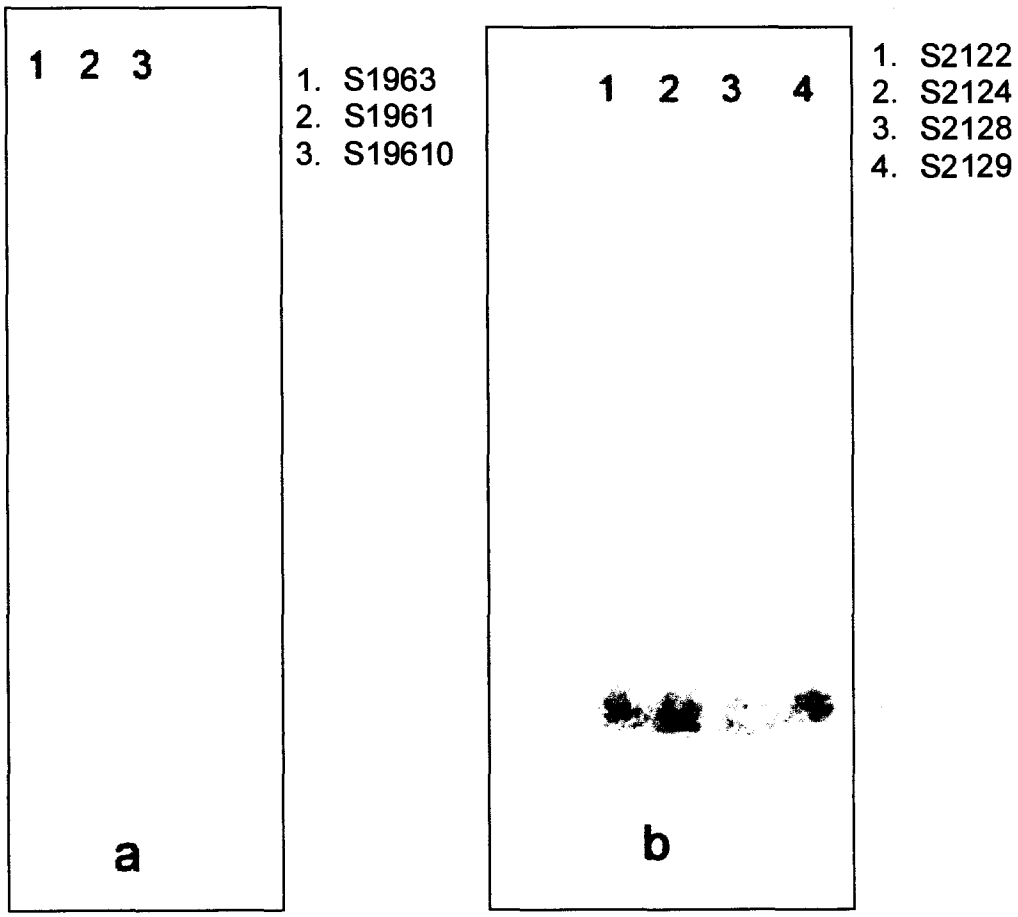
Lane	Band
1.	S1962
2.	22
3.	S2122
4.	CW-1
5.	S19610
6.	S1961
7.	S2129
8.	S2128
9.	S1963
10.	DP7
11.	S2124
12.	negative

Fig. 4. Southern hybridization of S1961 amplified product using S61F1-S61R1 primer set as seen stepwise (i) amplified PCR product of approximately 350 bp in length in agarose gel electrophoresis, (ii) portion of the gel containing the band cut prior to electroblotting and (iii) band blotted on nylon membrane visualized by hybridization reaction.



i. Lane Band
1 1 kb DNA ladder
2 S1961 PCR product using S61F1-S61R1 primers

Fig. 5. Nylon membranes as seen after performing Southern hybridization using (a) S61F1 probe and (b) 138F1 probe



Standardization of In situ hybridization (ISH) of whole aplanochytrid cells

All the different permeabilization protocols were positive for *in situ* hybridization reaction of the cells. However, the results varied depending on the protocol used. Thus, cells smeared on slides were mostly washed off. Besides, not all cells pretreated in this way showed the positive blue colour after reaction with streptavidin-conjugated alkaline phosophatase and substrate (Fig. 6a). Electroporation of cells to allow permeabilization provided more consistent results. Thus, all the cells treated in this way showed blue colour upon treatment with strepavidin-ALP. However, the cells were stained blue to different degrees (Fig. 6b). Thus, young cells stained dark as compared to older ones.

In situ hybridization of field samples using probes

Zooplankton samples fed with aplanochytrids: Zooplankton specimens that were fed with cells of the aplanochytrid S1961 for different time intervals were subjected to ISH, following permeabilization using electroporation. No reaction to ISH and, therefore, no development of blue colour that is characteristic of the alkaline phosphatase- BCIP-NBT test was detected in the tissue of the control zooplankton specimens that were not fed with aplanochytrids. On the contrary, aplanochytrid cells that reacted to ISH were detected in the hind gut of several of the zooplankton specimens towards the end of 4 hours of feeding (Fig. 7).

Fig. 6. The aplanochytrid S1961 showing a positive reaction for *in situ* hybridization using biotinylated probes and strepatividin-conjugated alkaline phosphatase. Bar represents 10 μm.

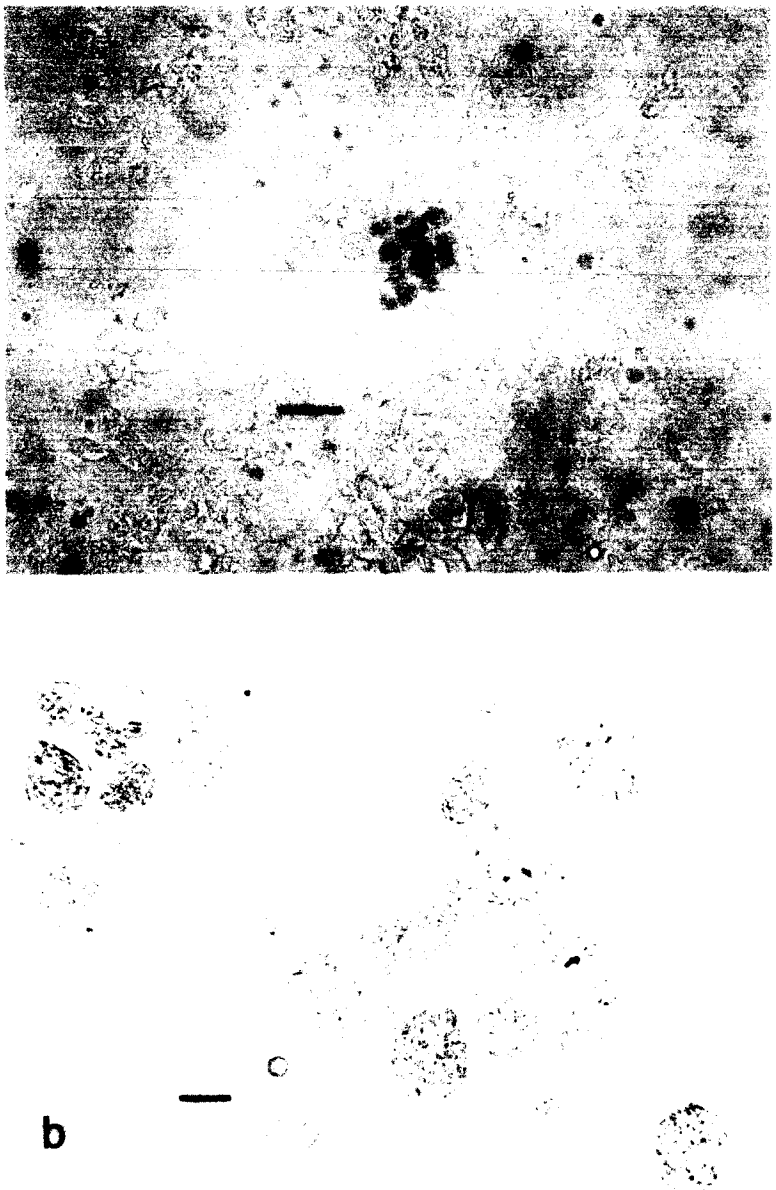


Fig. 6. The aplanochytrid S1961 showing a positive reaction for *in situ* hybridization using biotinylated probes and strepatividin-conjugated alkaline phosphatase. Bar represents 10 μm.

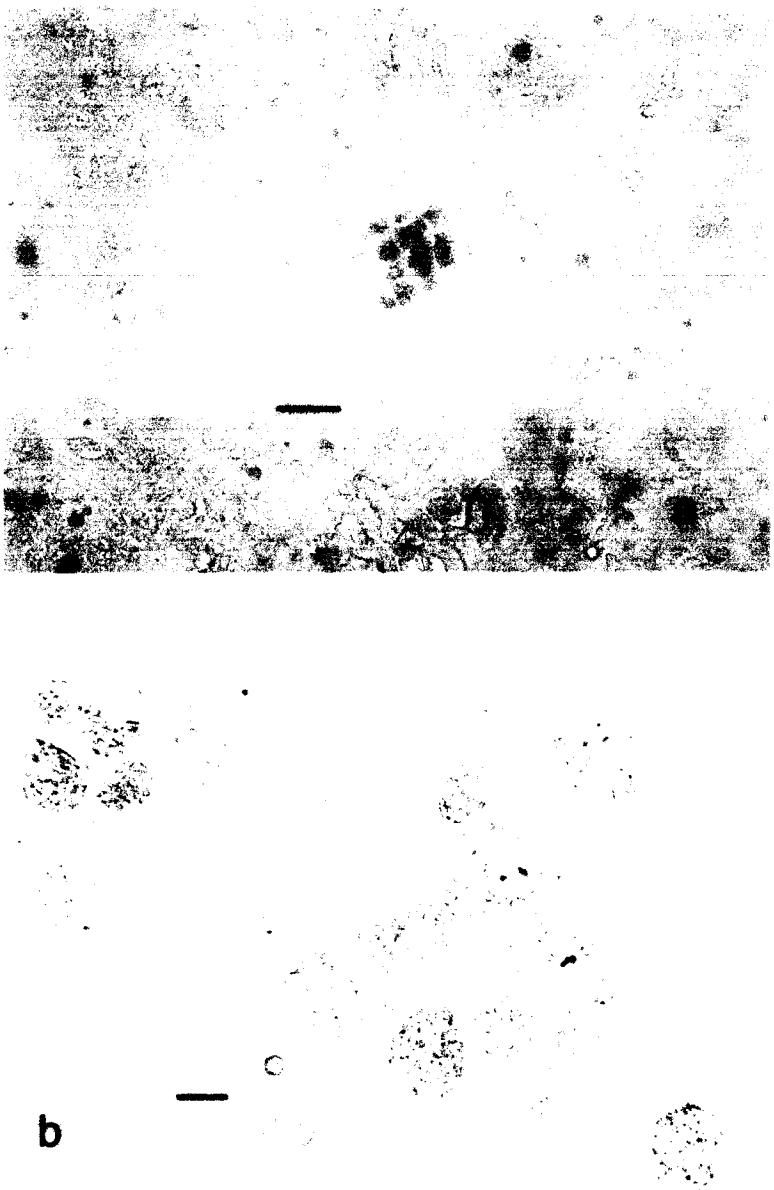
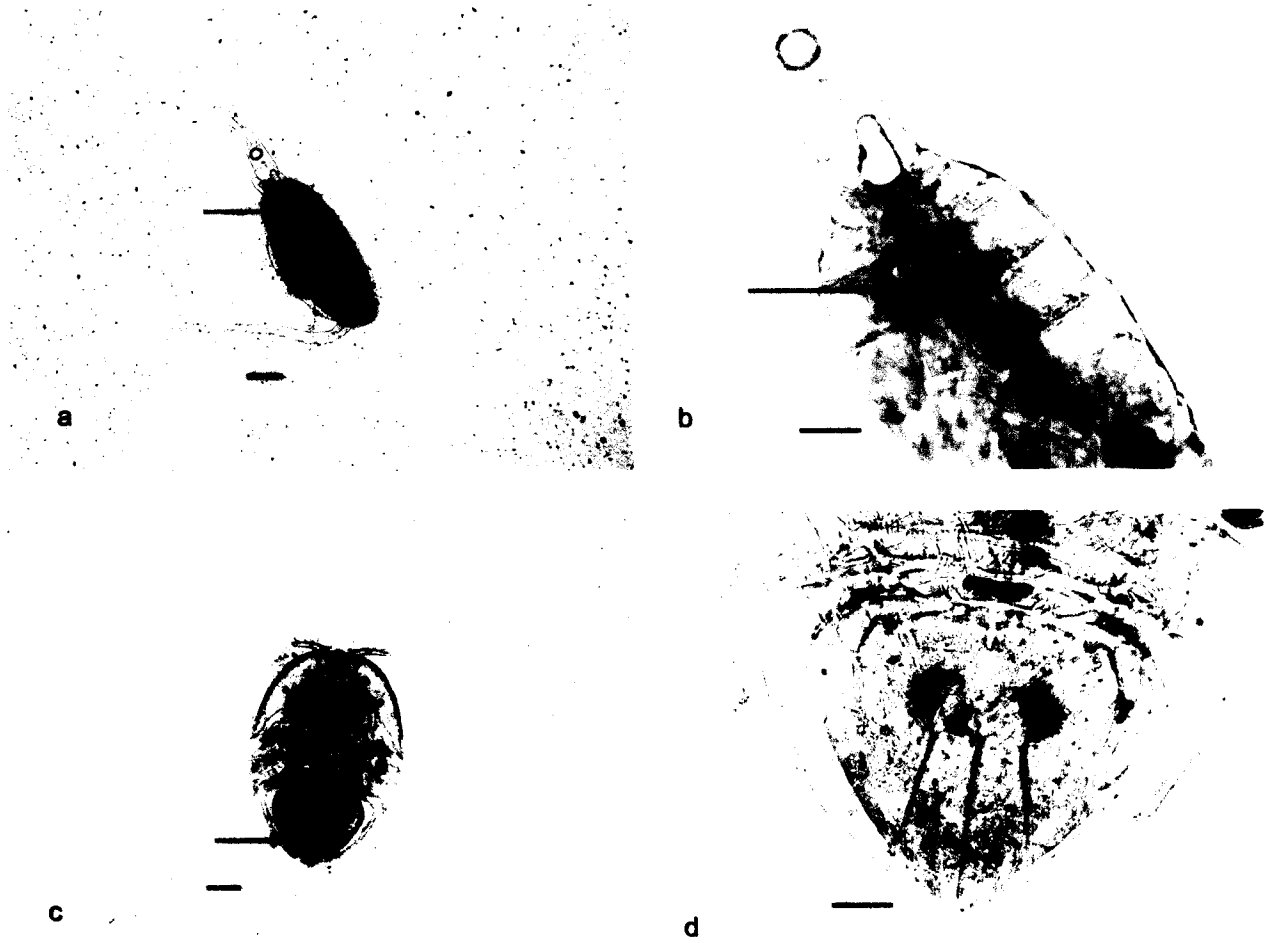


Fig. 7. Zooplankton fed with the aplanochytrid S1961 showing positive reaction in their guts for *in situ* hybridization using probes for the aplanochytrid isolate. Fig. b and d are high power photographs of a and c respectively. Bar represents 100 μm in a and c and 50 μm in b and d.



No aplanochytrid cells were detected in the specimens after 6 hours and more of feeding. However, blue colouration owing to reaction to alkaline phosphatase used in the ISH was noticed in different parts of the body of zooplankton (Fig. 8). The colour intensity decreased after 15 hours of feeding.

In situ hybridization of aggregates: Aggregates collected from the water samples of the equatorial Indian Ocean were subjected to different pretreatments, namely electroporation and air-drying over Polyprep slides prior to ISH with probes for S1961 as well as S2124. Both treatments revealed the presence of aplanochytrid probe-positive cells attached to the aggregates (Fig. 9). These cells were globose and measured 5 to 20 μm in diameter. The cells were generally present scattered in the aggregates.

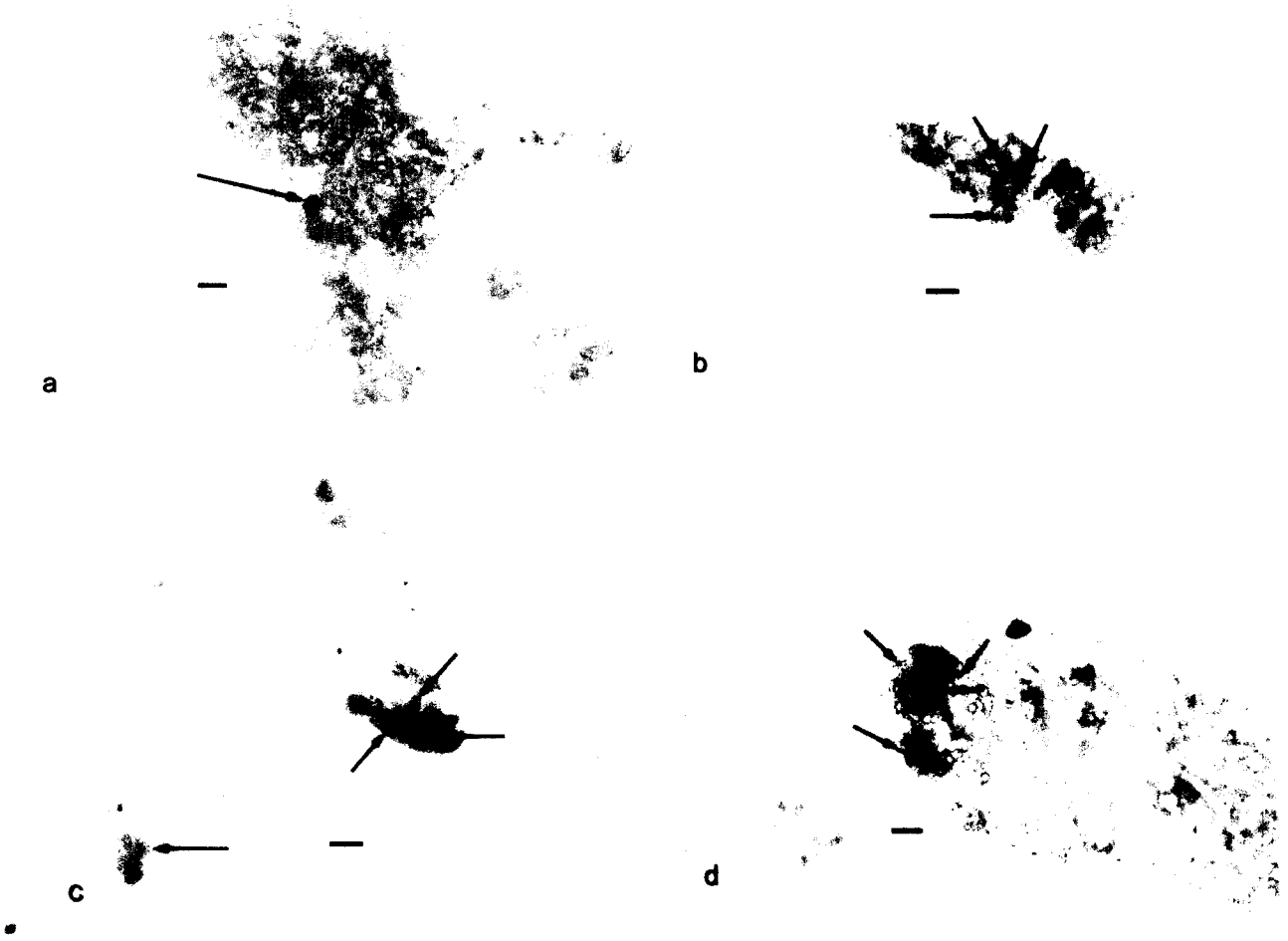
Natural zooplankton population from equatorial Indian Ocean: *In situ* hybridization using the probes developed for the aplanochytrids was carried out on zooplankton samples collected from different depths of the water column from the equatorial Indian Ocean during the October 2004 and September 2006 cruises, using electroporation and hybridization methods outlined earlier. Specimens from both the cruises showed positive reaction towards probes developed for the isolate S1961 obtained from the September 2003 cruise, as well as those designed for S2124 isolated from the October 2004 cruise (Table 4 and 5). The use of S61F1

Fig. 8. Blue reaction in different parts of the zooplankton body to the alkaline phosphatase- BCIP- NBT following *in situ* hybridization using probes for the aplanochytrid S1961.

a

b

Fig. 9. ***In situ*** **hybridization of aggregates from the equatorial Indian Ocean using biotinylated ITS-probes developed against the aplanochytrid S1961 and S2124. Bar represents 10 µm.**



probe yielded positive reactions from 1.4 - 27.6 % zooplankton specimens sampled during October 2004 cruise, while the use of 138F1 probe yielded 0 – 34.9 % positive reactions (Table 4). ISH-positive specimens of zooplankton were found in all the depths at all nine stations sampled. On the contrary, fewer depths and fewer of a total of 6 stations yielded positive specimens during the September 2006 cruise, SK 228 (Table 5). Only 0 – 12.5 % of the zooplankton specimens were positive to the S61F1 probe during the cruise, and only 0 – 9.3 % were positive to the 138F1 probe (Table 5). No distinct differential response to the two probes was noticed either between the cruises or the depths. Photographs of two specimens are shown in figure 10. Although positive reactions were seen to hybridization, no distinct aplanochytrid cells were detected in the specimens. The reaction was often generalized and found in various parts of the zooplankton.

While generally only a few copepods were positive for hybridization, all the chaetognath specimens took up the blue colour when hybridized with the two probes separately (Fig. 11). In one instance, an aggregate-like structure in the vicinity of the mouth part of the chaetognath reacted positively to ISH (Fig. 11a). Positive reactions were mostly concentrated surrounding the gut. The contents inside the mouth parts of many specimens also showed positive reaction (Fig. 11b and c). Several globose, scattered cells that reacted positively to ISH were observed within the body of the chaetognaths (Fig. 11d).

Fig. 10. ***In situ*** **hybridization of zooplankton specimens from the equatorial Indian Ocean using biotinylated ITS-probes developed against the aplanochytrid S1961 and S2124. Bar represents 10 µm.**

Fig. 11. ***In situ*** **hybridization of chaetognaths from equatorial Indian Ocean using biotinylated ITS-based probes developed against the aplanochytrid S1961 and S2124. Bar represents 100 µm in a and b and 10 µm in c and d.**

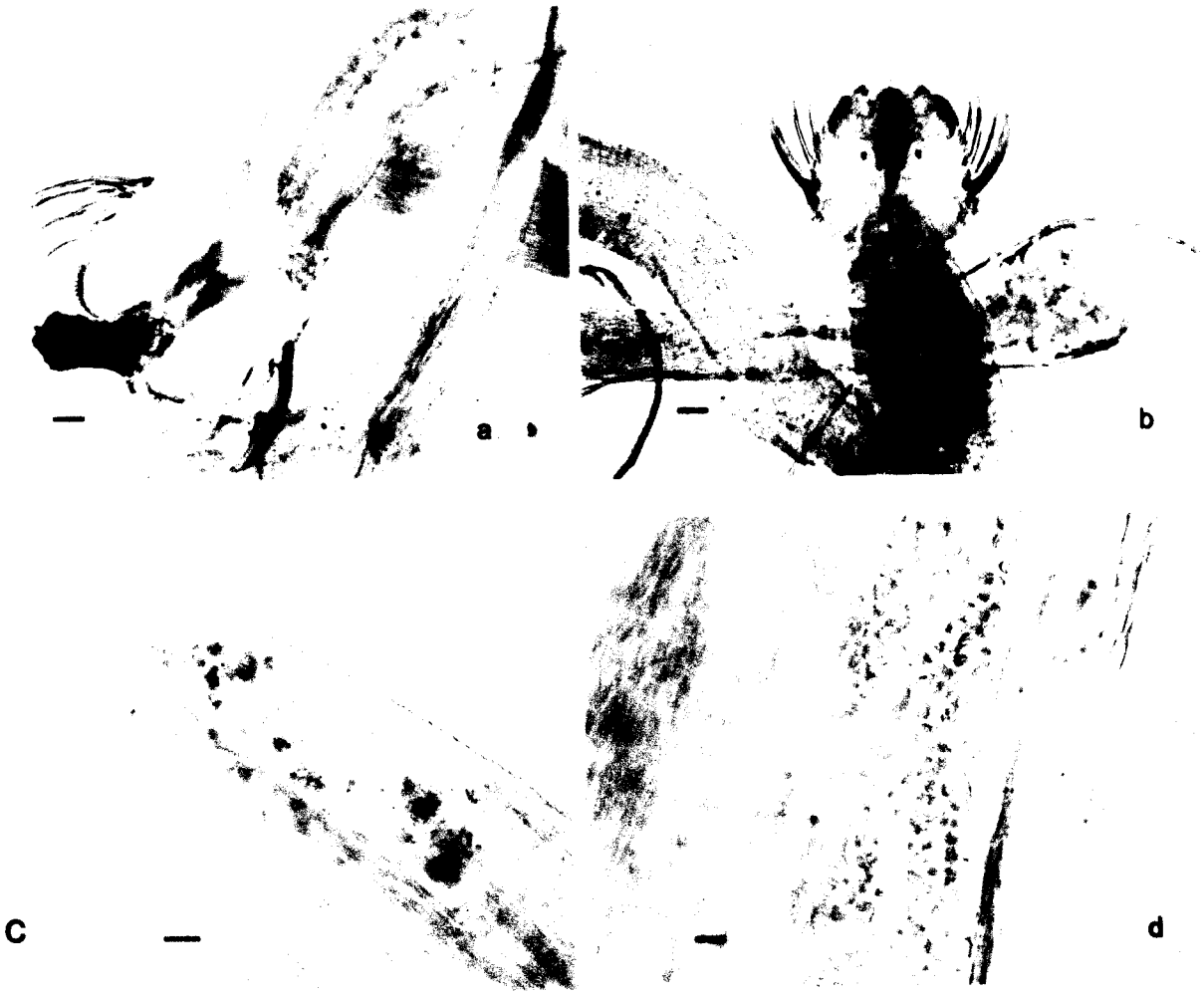


Table 4. Zooplankton from October 2004 cruise (SK 212) positive to both probes. The higher values amongst the two probes are marked in bold.

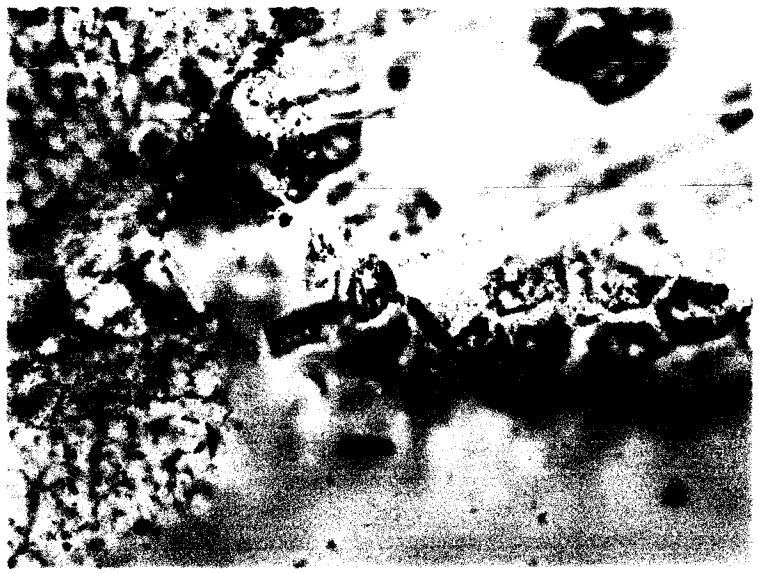
Station	Depth	S61F1 probe			138F1 probe		
		Total specimens	Total positive	% positive	Total specimens	Total positive	% positive
1°N 80.5°E	200-30	63	3	4.8	43	15	**34.9**
0°Eq 80.5°E	200-38	63	4	6.3	135	4	**9.3**
0°Eq 80.5°E	500-200	59	5	8.5	54	6	**14.0**
0°Eq 83°E	1000-500	40	3	**7.5**	64	0	0.0
0°Eq 83°E	500-300	22	3	**13.6**	29	5	11.6
0°Eq 83°E	30-0	58	16	**27.6**	77	7	16.3
1°S 80.5°E	1000-500	73	1	**1.4**	68	0	0.0
1°S 80.5°E	500-200	61	3	4.9	52	7	**16.3**
0°Eq 77°E	200-60	50	2	4.0	56	2	**4.7**

Table 5. Zooplankton from September 2006 cruise (SK 228) positive to both probes. The higher values amongst the two probes are marked in bold.

Station	Depth	S61F1 probe			138F1 probe		
		Total specimens	Total positive	% positive	Total specimens	Total positive	% positive
0°Eq 77°E	500-200	17	0	0.0	23	3	**7.0**
0°Eq 77°E	200-0	17	0	0.0	31	1	**2.3**
0°Eq 83°E	500-200	21	0	0.0	36	0	0.0
0°Eq 83°E	200-0	9	1	**11.1**	9	0	0.0
0°Eq 93°E	500-200	7	0	0.0	5	0	0.0
0°Eq 93°E	200-0	24	3	**12.5**	24	4	9.3

In situ PCR of chaetognath specimens: *In situ* PCR using primers S61F1-S61R1 and 138F1-138R1 was carried out separately on chaetognath specimens in order to increase signals, followed by electroporation and ISH using the probes S61F1 and 138F1 respectively. This procedure revealed fewer ISH-positive cells within the tissue of chaetognaths compared to the procedure where only electroporation was used prior to ISH. However, the application of *in situ* PCR resulted in cells which stained much brighter (Fig. 12). Cells that reacted positively to in situ PCR-ISH were mostly ellipsoidal to irregular in shape, similar to cells of aplanochytrids in culture (Fig. 12 a; see Chapter 2). Such aplanochytrid-positive cells were present lining the gut.

Fig. 12. ***In situ*** **hybridization of chaetognaths from the equatorial Indian Ocean following** ***in situ*** **PCR, using biotinylated ITS-probes developed against the aplanochytrid S2124. Bar represents 10 µm in fig. a and 100 µm in fig. b.**



b

6.4 Discussion

ITS-based probes for two aplanochytrid isolates from the equatorial Indian Ocean were successfully developed and used in this study. This is only the third instance where molecular probes have been used for detecting members of the Labyrinthulomycetes. Earlier Lyons *et al.* (2005) generated two 18S rRNA targeting probes against Quahog Parasite Unknown (QPX). They used these probes to detect the presence of the parasite in the marine aggregates. Takao *et al.* (2007) have also designed an 18S rRNA targeted probe against thraustochytrids, especially *Aurantiochytrium*, formerly *Schizochytrium*. Though they have optimized the FISH protocol using this probe, they have not used it for detection in natural samples.

The ITS-based probe designed here was meant to study individual strains of the aplanochytrids, belonging to the *A. yorkensis* complex. Molecular probes can be designed using different labels, such as fluorescent ones like FITC, rhodamine, enzyme-linked labels like biotin, digoxigenin using alkaline phosphatase (ALP) or hydrogen peroxidase (HRP). However, fluorescent *in situ* hybridization (FISH) probes were avoided in this study because zooplankton, on which the probes were intended to be used, have a chitinous exoskeleton, which autofluoresces and can cause severe interference in detecting the required organism. Therefore, it was decided to use a bright-field microscopy based probe which can react with chromogenic substrate to give a coloured reaction.

A biotin-labelled probe was used with alkaline phosphatase conjugated to streptavidin.

Two probes, based on two specific primers were developed for two individual isolates each in the present study. Interestingly, although both isolates apparently belonged to the *Aplanochytrium yorkensis* complex, being closest to that species, the ITS primers designed for the isolate from the SK196 cruise, S1961 was specific only to it and not to S2124 isolated form the SK212 cruise. This shows that the probes might be specific to strains or individual populations of *A. yorkensis* occurring in the waters of the equatorial Indian Ocean. ITS sequences are known to be highly variable amongst species (White *et al.*, 1990; Martin & Rygiewicz, 2005). Although the probe for S1961 did not react with S2124, it amplified the DNA of other isolates obtained from that cruise, namely S19610 and S1963. Likewise, the primers for S2124 amplified the DNA of other isolates obtained from that cruise, namely S2122, S2128 and S2129. The first three were isolated during September 2003 and the latter four during October 2004. This indicates that different populations of *A. yorkensis* might be associated with zooplankton at different periods in the equatorial Indian Ocean.

The probes could be applied successfully to detect the aplanochytrid in aggregates collected from the water column (Fig. 9). Lyons *et al.* (2005) also demonstrated that the thraustochytrid, QPX parasite, colonized marine aggregates, by using 18S rRNA probes. There

is increasing evidence to show that thraustochytrids might actively colonize and grow on marine aggregates. The use of the AfDD epifluorescence technique to detect thraustochytrids and aplanochytrids has indicated that these organisms might be related to particulate organic matter, particularly marine aggregates in the water column. Epifluorescence detection methods have a limitation because they might not be entirely specific like molecular probes. Secondly zoospores which lack the cell wall cannot be detected by epifluorescence. The use of ITS-based, species-specific probes for the aplanochytrids shows that marine aggregates are important substrates for these protists, as discussed in Chapter 4.

Microorganisms inhabiting aggregates could form an important source of nutrients for zooplankton feeding on the aggregates (Kiørboe, 2001; Grossart *et al.*, 2003). *In situ* hybridization carried out on chaetognaths often yielded positive reaction in the foregut part of the organism, suggesting the presence of the aplanochytrids in that region, probably through feeding. Thraustochytrids are now believed to be an important source of the omega-3 polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA) to zooplankton. There are no detailed reports of PUFAs in aplanochytrids. If these too are rich in DHA, they would then play an important source of the PUFA to zooplankton.

The presence of aplanochytrids in the guts of zooplankton after feeding was also verified through an experiment in which cells of the

aplanochytrid were added to living zooplankton, after which ISH using the probe for the aplanochytrid was attempted. This experiment was carried out to ensure that the aplanochytrids can be detected when associated with zooplankton. Cells added in this manner are likely to be eaten by the copepods. None of the copepods showed a positive blue reaction to alkaline phosphatase- BCIP-NBT substrate in the control set where aplanochytrid cells were not added. On the other hand, many copepods showed a blue colouration in their hind guts 4 hours after the cells of the aplanochytrid were added. This not only confirmed that the probes were effective but also that the cells were eaten and had passed into the hind gut.

ISH preceded by *in situ* PCR showed distinct, angular to amoeboid cells within the tissues of the chaetognath, typical of *A. yorkensis* on a nutrient agar medium. These results suggest that aplanochytrids might live within the body parts of these zooplankton, probably in a mutualistic association. Rinkevich & Rabinowitz (1993) have also found thraustochytrid cells circulating in blood system of tunicate *Botryllus schlosseri*. They are found in all body parts of tunicates, corals, sponges and therefore the major contaminants in cell lines of these animals (Rinkevich & Rabinowitz, 1997; Rinkevich, 1999).

The *in situ* hybridization carried out in this study often showed a positive reaction from many body parts of both copepods and the chaetognaths. For example, the setae of copepods, or the ovaries and tail

of chaetognaths also showed a positive reaction (Fig. 10c and Fig. 12c, d). There are two possible explanations for this. (1) The aplanochytrids could have been attached to the exterior of various body parts. The diffused nature of the colouring and the absence of distinct cells of the aplanochytrid probably resulted from the destruction of the aplanochytrid cells during processing. (2) Alkaline phosphatase is known to be present in the body tissues of zooplankton. The enzyme is often associated with cell surfaces (Cembella *et al.*, 1984). It is produced when phosphate concentrations are depleted intracellularly or extracellularly. Zooplankton secrete this enzyme in the environment (Jamet & Boge, 1998; Gambin *et al.*, 1999; Boge *et al.*, 2006). It is reported to be produced by adults as well as juveniles of copepods and branchipods (Lespilette *et al.*, 2007). This could be the endogenous alkaline phosphatase giving positive reaction to the substrates. Care was taken for inhibition of the endogenous ALP activity by pre-treatment with dithiothreitol (DTT), a strong reducing agent. In addition, the specimens had been fixed in formalin, which destroys endogenous alkaline phosphatase activity (Bussolati & Leonardo, 2008). Despite these precautions, false positive reactions could have occurred, owing to the presence of alkaline phosphatase produced by the zooplankton. Alternatively, the positive reactions could therefore be due to insufficient penetration of both the reagents. The use of a biotin-labelled probe for use with alkaline phosphatase, therefore, could have a handicap. The use of other labels and substrates for *in situ* hybridization,

such as the use of horse-radish peroxidase as the colour indicator, or the use of digoxigenin-labelled probes will confirm the findings of this study.

6.5 Conclusions

ITS-based probes were successfully designed against aplanochytrids isolated from mesozooplankton of the equatorial Indian Ocean. *In situ* hybridization using cells of the aplanochytrids was standardized using biotinylated probes, together with strepavidin-conjugated alkaline phosphatase for detection. Laboratory experiments using the ISH suggested that aplanochytrids added to living zooplankton were consumed by the animals. Aplanochytrids were detected in marine aggregates collected from the equatorial Indian Ocean. ISH also suggested the presence of the aplanochytrids in the guts of chaetognaths. Copepods form natural samples also reacted positively to ISH. Various body parts of the zooplankton also reacted positively to alkaline phosphatase used together with the probes during ISH, suggesting either that cells of the aplanochytrid were attached to the body surfaces or that false positive reactions occurred. Further use of the probes with other labels will help to refine the method by avoiding possible false positives and to reconfirm the results.

Chapter 7.

Summary and Conclusions

Thraustochytrids, aplanochytrids and labyrinthulids belong to the Class Labyrinthulomycetes of the Kingdom Straminipila or Chromista, which also includes diatoms, brown algae, oomycetan fungi and a variety of photosynthetic and non-photosynthetic flagellates. The Labyrinthulomycetes are heterotrophic and obligately marine. They have been found to be ubiquitous in the marine environment, occurring in a variety of habitats in both coastal and oceanic environments. A few recent studies have found thraustochytrids to be an important component of the microbial biomass in the water column. They have also been shown to be associated with a number of invertebrates in both parasitic and symbiotic modes. Owing to the production of omega-3 polyunsaturated fatty acids they also have wide ecological implications and biotechnological applications. The Labyrinthulomycetes are emerging to be an important group in the marine food web dynamics. Yet, their role in the marine environment has been poorly elucidated. They have been widely studied in coastal environment but little is known about their abundance and distribution in the oceanic environment.

The four major objectives of the present study were:

1. To isolate thraustochytrids and aplanochytrids from zooplankton in oceanic waters, to characterize them with respect to their life cycle in

order to identify them and to group them based on their carbon and nitrogen nutrition and production of extracellular enzymes.

2. To study the abundance of thraustochytrids and bacteria in oceanic water column to understand their relations.
3. To carry out molecular characterization of isolates obtained from zooplankton based on their 18S ribosomal RNA gene and ITS sequences
4. To design molecular probes for a few isolates to enable their detection in natural samples.

Most of this work was based on samples collected during three cruises on board ORV Sagar Kanya to the equatorial Indian Ocean during September 2003, October 2004, and September 2006. The following points summarize the work and conclusions.

1) Mesozooplankon collected from the equatorial Indian Ocean using a Multiple Plankton Net during the first two cruises were plated on culture media. A total of 171 specimens yielded colonies belonging to the Labyrinthulomycetes. Out of these, 14 isolates were purified and maintained for further studies. All isolates corresponded to the aplanochytrid *Aplanochytrium yorkensis* (Perkins) Leander and Porter based on morphology and life cycle. Colony morphology of all other cultures from mesozooplankton also resembled those of the species. The fourteen isolates showed numerous variations in terms of colony

morphology, life cycle and preferences to organic carbon and amino acids. Among the morphological characters, important variations include the colony margin, penetration of agar and production of amoebae. Only one isolate preferred glucose as the carbon source, all others utilizing pentoses and disaccharides more efficiently. All grew well on glutamate and proline. All isolates produced only protease among the 4 extracellular enzymes (protease, lipase, chitinase and amylase) tested. No thraustochytrids were isolated from zooplankton.

Members of thraustochytrids have often been reported from a variety of invertebrates in coastal environments. However, no studies have been carried out on their presence in zooplankton. *This study reports isolation and characterization of Labyrinthulomycetes from zooplankton for the first time.*

2) Molecular studies using 18S rDNA sequences of eight of the isolates confirmed morphological and life cycle observations that they belonged to the *Aplanochytrium yorkensis* group. The overall phylogenetic tree placed these isolates in the aplanochytrid group of the Labyrinthomycetes, distinct from thraustochytrids and labyrinthulids. Within this, they further corresponded to the *A. yorkensis* complex as suggested by Leander *et al.* (2004), being closest to that species.

The present observations confirmed the predominance of aplanochytrids in association with mesozooplankton, which might have

been the result of a definite association of this group of organisms with mesozooplankton. It is also likely that aplanochyhtrids are the most important members of Labyrinthulomycetes in oceanic waters. Yet another possibility is the presence of uncultured thraustochytrids in the samples.

3) Cluster analyses of the isolates based on morphological and physiological characters did not indicate any consistent grouping and did not provide weightage to any of the characters.

Although cluster analysis suggested that Aplanochytrium yorkensis in zooplankton of the equatorial Indian Ocean exists as populations with a high variability and mixing, phylogenetic trees based on 18S as well as ITS rRNA gene sequences grouped the isolates of the October 2004 cruise distinctly as a separate clade within the A. yorkensis complex, suggesting that these probably represented a new taxon. Use of other taxonomic characters, such as fatty acid and carotenoid profiles as has been recently used for thraustochytrids will help in the elucidation of the taxonomy of aplanochytrids. Different populations of A. yorkensis or different species of the A. yorkensis complex might be associated with mesozooplankton in different seasons or locations.

4) In order to shed light on the association of aplanochytrids with zooplankton, attempts were made to locate them on or within zooplankton

using molecular probes. Based on ITS sequences of 5 isolates obtained in the present study, four probes consisting of one forward and one reverse primer each for two isolates were designed. The oligonucleotide probes varied in length from 19 to 20 nucleotides. These probes were cross-checked for their effectiveness with the particular isolates by PCR, southern hybridization and *in situ* hybridization (ISH). The latter two were carried out with biotinylated probes, using streptavidin-conjugated alkaline phosphatase and colour detection using a chromogenic substrate for the enzyme, followed by bright field microscopy. Lack of cross-reactions with other isolates was confirmed using the same techniques. Cells of aplanochytrids fed to copepods could be detected using ISH. Aggregates collected from the water column of the equatorial Indian Ocean revealed cells of the aplanochytrid upon ISH. Likewise, the gut portions of chaetognaths reacted positively to ISH carried out. Cells resembling aplanochytrids were also detected within the tissues of chaetognaths using ISH. Copepod samples from the equatorial Indian Ocean water column reacted positively to ISH using the probes. However, no definite cells were detected in these and the reaction was diffuse, being present on various parts of the body of the copepods. A similar reaction was also often noticed with chaetognaths.

The present study confirms the presence of aplanochytrids in marine aggregates of the water column. It further suggests that aplanochytrids were consumed by zooplankton and probably were also

present within their body tissues, as has been suspected in the case of tunicates and other coral reef invertebrates. Further studies are required to confirm these findings.

5) An insight into the role of thraustochytrids and aplanochytrids in the oceanic water column can be gained by studying their abundance and distribution, as well as their relation to bacteria. Enumeration based on epifluorescence microscopy using the acriflavine direct detection method showed that they were present from below detection levels to as high as 766.6 x 10^3 cells L^{-1} at times, comparable to values obtained from studies in the Arabian Sea and the Japan Sea. They often showed distinct distribution profiles in the water column, generally being higher in the mixed layer than below it. However, their numbers were also often substantial below 200 m of the water column. Their contribution of carbon to total microbial (thraustochytrid + bacterial) biomass was very high (99.4 %, 15.8 μg C L^{-1}) at certain locations. Thraustochytrids were positively related to bacteria during October 2004, but not during the other cruises.

Thraustochytrids were patchier in their distribution compared to bacteria, probably because they were more particle- or aggregate-dependent for their growth than the latter. Thraustochytrids grow well on particulate organic matter by virtue of their ectoplasmic net elements, which help them to attach to such substrates and obtain nutrition from them through the production of various degradative enzymes.

Transparent Extracellular Particles (TEPs) form the matrix of aggregates. Therefore, the relationship between thraustochytrids, bacteria and TEPs in the water column was studied during two cruises, in October 2004 and September 2006. Thraustochytrids displayed a significantly positive correlation with TEPs in the water column at some, but not at all locations during October 2004. Their numbers often peaked about 20 m above peak TEPs concentration. This might be due to the release of DOC from the sinking particles that left a plume supporting growth of thraustochytrids.

The present study has shown that thraustochytrids and aplanochytrids are common inhabitants of the water column of oceanic waters, as they are also in coastal waters. Their high densities, occasionally corresponding to biomass values more than that of bacteria indicates that they might play an important role in the water column. The varying relations between bacteria and thraustochytrids suggests that the factors that govern the growth and populations of the two groups may vary. Likewise, the varying relations of thraustochytrids to TEPs might have been caused by the varying chemistry of TEPs, formed from different processes.

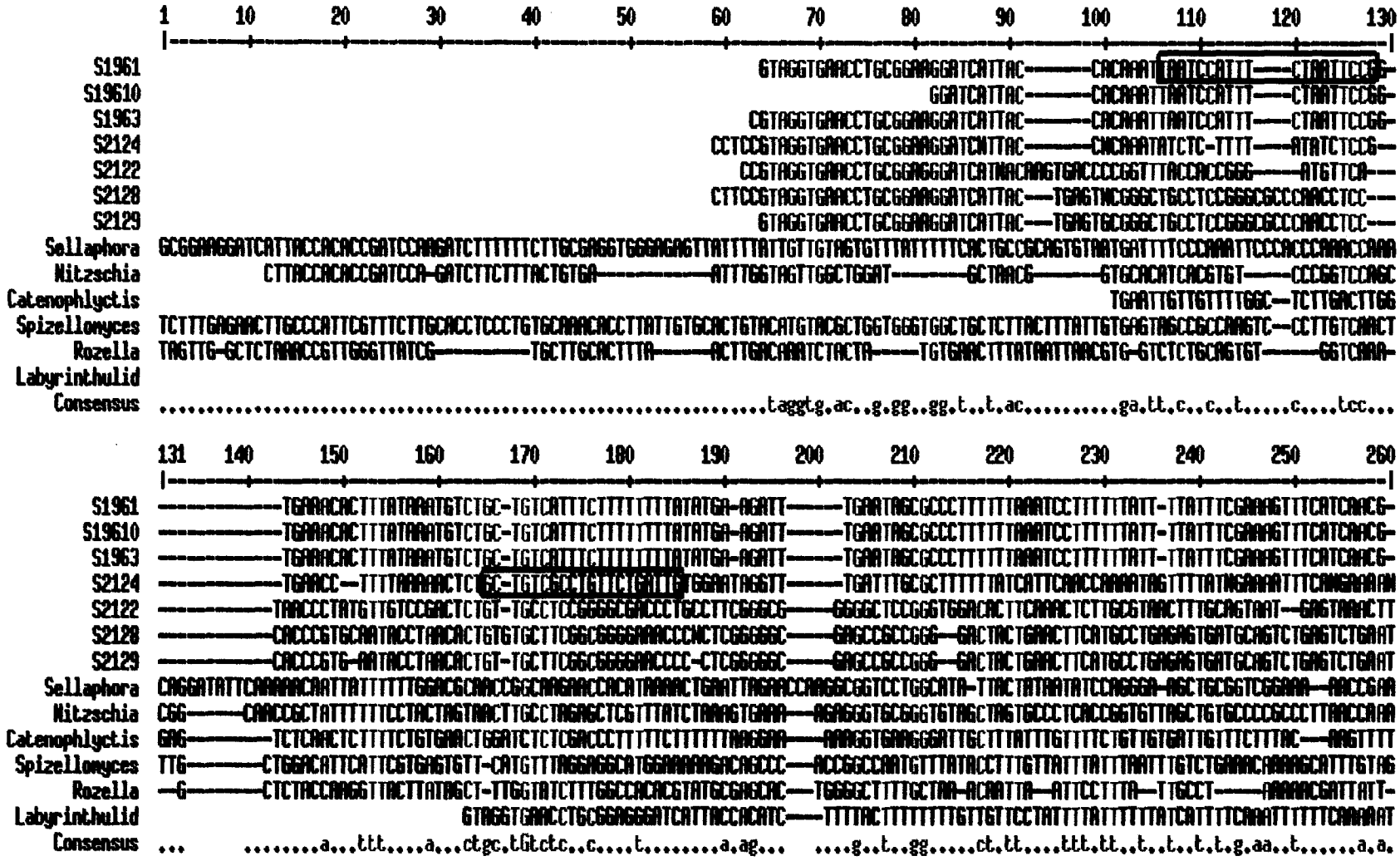
6) Experiments using roller table bottles to generate TEPs from natural seawater were carried out to understand the reasons behind the variability in relationships between thraustochytrids and TEPS. Various

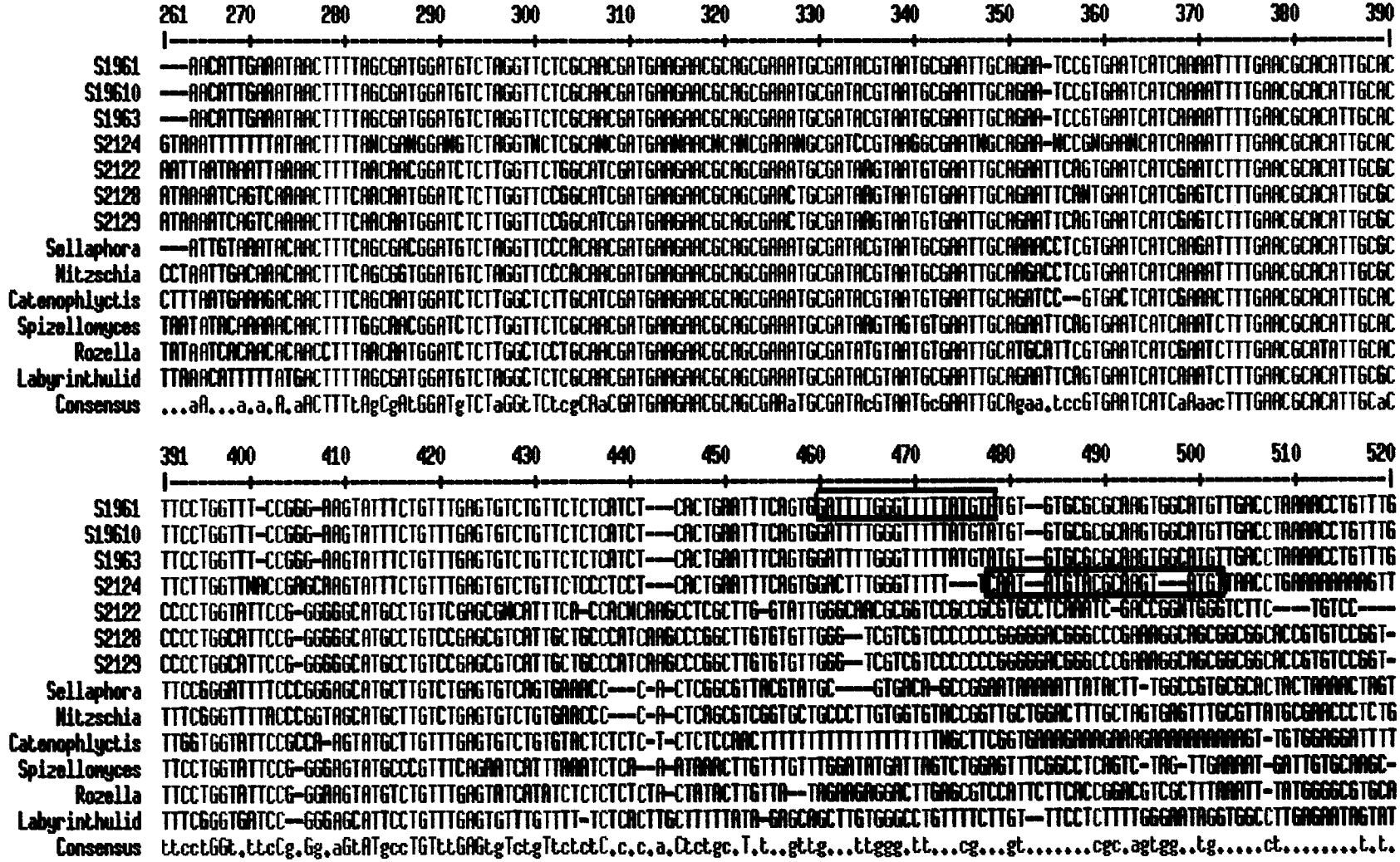
experiments were carried out using oceanic and coastal waters filtered through 200 or 20 μm, such that the waters contained phytoplankton, microzooplankton, thraustochytrids and bacteria, or in the presence of the above consortia, but without phytoplankton. The carbohydrate chemistry of the TEPs was assessed using three different lectins.

Thraustochytrids appeared to be inhibited in the presence of phytoplankton. Thraustochytrid populations increased when phytoplankton were excluded, or when only diatom polysaccharides were introduced in seawater. These studies again showed varying relationships between thraustochytrids, bacteria and TEPs. Thraustochytrids and bacteria did not appear to compete against each other in most cases. Aggregate formation seemed to be enhanced in water samples supplemented with antibiotics and favoured growth of not only thraustochytrids but also fungi.

Appendix

Appendix 1- Multiple alignment file of ITS sequences of 7 aplanochytrid isolates of the present study and 6 other organisms belonging to Kingdom Straminipila. The regions of probe design are marked as red boxes.

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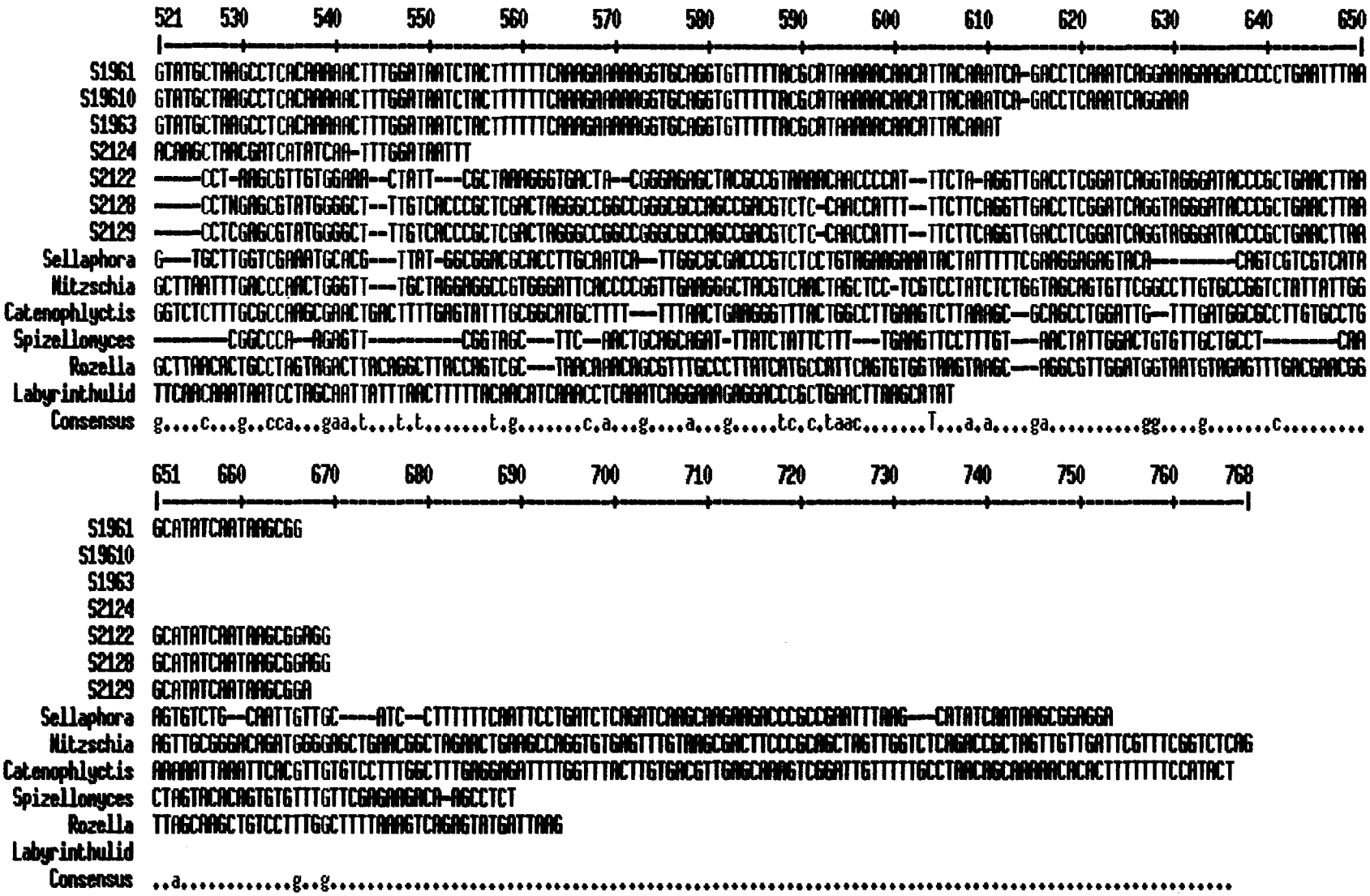
S1961
S19610
S1963
S2124
S2122
S2128
S2129
Sellaphora
Nitzschia
Catenophlyctis
Spizellomyces
Rozella
Labyrinthulid
Consensus
391 400 410 420 430 440 450 460 470 480 490 500 510 520
S1961
S19610
S1963
S2124
S2122
S2128
S2129
Sellaphora
Nitzschia
Catenophlyctis
Spizellomyces
Rozella
Labyrinthulid
Consensus


521 530 540 550 560 570 580 590 600 610 620 630 640 650
S1961
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S2128
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Sellaphora
Nitzschia
Catenophlyctis
Spizellomyces
Rozella
Labyrinthulid
Consensus
651 660 670 680 690 700 710 720 730 740 750 760 768
S1961
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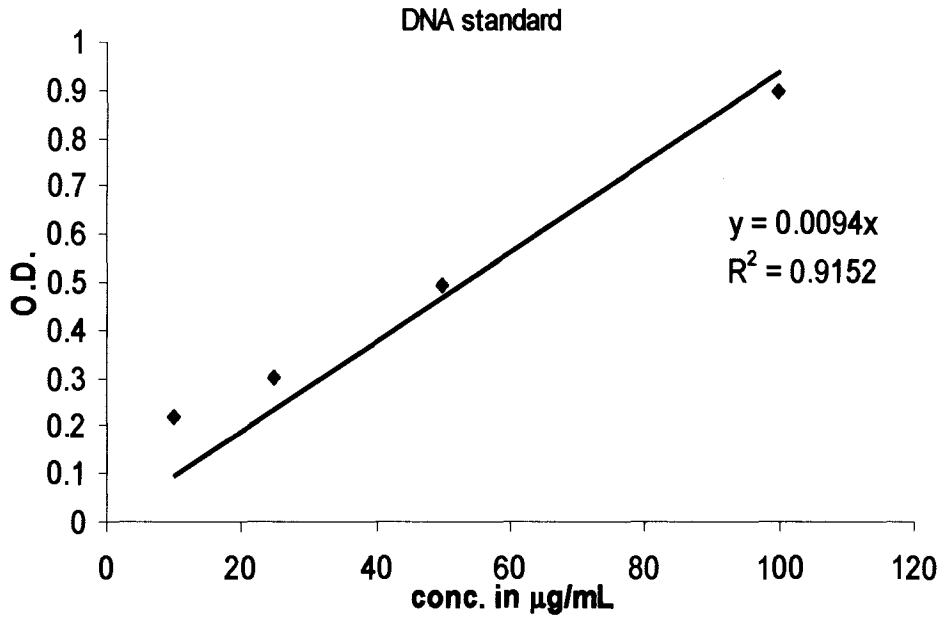
Sellaphora
Nitzschia
Catenophlyctis
Spizellomyces
Rozella
Labyrinthulid
Consensus

Appendix 2. DNA Standard

DNA (HiMedia, Cat. RM 511) was dissolved in sterile distilled water to get the desired concentration and the absorbance was read at 260 nm.



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Morphology and physiology of the marine straminipilan fungi, the aplanochytrids isolated from the equatorial Indian Ocean

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While thraustochytrids, a group of unicellular marine straminipilan protists, have been found to be abundant in the water column, little is known of aplanochytrids. These constitute one of the 3 groups belonging to the Labyrinthulomycetes. Aplanochytrids were isolated from 34 out of 76 zooplankton samples from different strata in the 0–1000 m water column in the equatorial Indian Ocean. None of the samples yielded thraustochytrids in culture, suggesting that aplanochytrids might be more prevalent in the zooplankton samples of these waters than thraustochytrids. Fourteen isolates of aplanochytrids were studied with reference to their colony and cell morphological characteristics, carbon and nitrogen nutrition and the production of four degradative enzymes. All isolates produced proteases, but not lipase, amylase or chitinase. Major interesting features of several isolates included the production of motile amoebae, preference to pentoses and disaccharides and the common preference to glutamate. Cluster analysis based on all the characters showed no clear relations to morphological or physiological traits of the isolates, thus indicating the unreliability of these characters in taxonomy of aplanochytrids. All isolates corresponded to taxon *Aplanochytrium yorkensis*. The differences observed in these isolates correspond to variations in populations of *A. yorkensis* inhabiting zooplankton in the Indian Ocean and not related to different species of the genus.

[**Key words**: Aplanochytrids, Straminipila, Labyrinthulomycetes, zooplankton, Indian Ocean]

Introduction

Aplanochytrids are fungoid, exclusively marine, unicellular protists belonging to the Phylum Labyrinthista (Labyrinthulomycetes) of the Kingdom Straminipila[1-3]. All three groups of Labyrinthulomycetes, namely labyrinthulids, thraustochytrids and aplanochytrids are characterized by the production of plasma membrane extensions called the ectoplasmic net elements (EN). Cells of labyrinthulids are enrobed in EN as a colony and move within them, while cells in the other two groups are not covered by the EN. Thraustochytrids reproduce by heterokont, biflagellate zoospores. Aplanochytrids are differentiated from thraustochytrids by the presence of spores that move in a gliding manner using the EN. In addition, cell walls of aplanochytids contain mostly fucose rather than galactose as in thraustochytrids. Aplanochytrids are comprised of the single genus *Aplanochytrium*[4]. Leander & Porter[5] redefined this genus by transferring five species of *Labyrinthuloides* Perkins and one species of *Labyrinthula* under *Aplanochytrium*.

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Aplanochytrids have so far been isolated from water samples, sediment, detritus, oyster mantle, gastropods and seagrasses[3, 6]. Although Raghukumar [7] reported their presence in the water column of the southern Arabian Sea, few detailed studies have been carried out on their occurrence in oceanic waters. This paper reports results of our studies on aplanochytrids isolated from zooplankton specimens from the equatorial Indian Ocean.

Morphological differences, G + C content of DNA, nitrogen uptake ability and molecular phylogeny of these organisms have been studied so far[6,8]. The taxonomy of aplanochytrids is beset with problems since many morphological characters that are used for their classification overlap[6]. Besides, physiological differences among various species of aplanochytrids based on their carbon and nitrogen nutrition, as well as extracellular enzyme production have not been studied in detail. In view of this, we have characterized 14 isolates obtained from zooplankton in terms of their morphology, carbon nutrition and nitrogen nutrition in an attempt to examine if such criteria would be useful in classifying species of this

group and if they would throw more light on their ecology.

Materials and Methods

Isolation of aplanochytrids–All samples were collected from the equatorial Indian Ocean during Cruise # SK 196 and SK 212 on board *ORV Sagar Kanya* during September 2003 and October 2004, respectively (Fig. 1). Zooplankton samples were collected using a Multiple Plankton Net (MPN, MultiNet Type Midi with 200 µm mesh nets, Hydrobios, Germany) from 4 depth ranges, either from 500 m to surface or 1000 m to surface

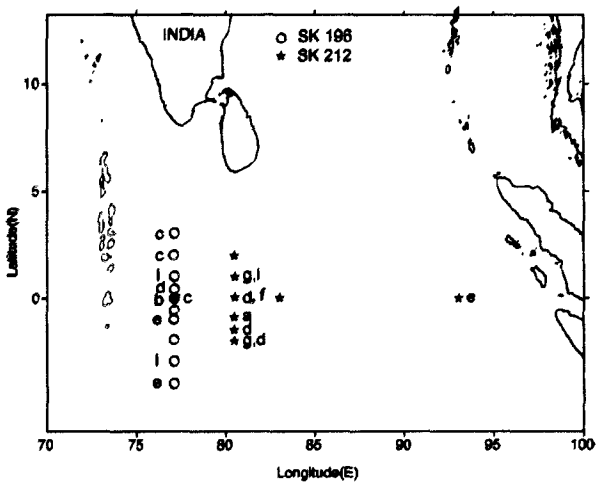

Fig. 1—*ORV Sagar Kanya* station locations in the equatorial Indian Ocean during cruises SK 196 (September 2003) and SK 212 (October-November 2004). The alphabets towards the left and right of the stations indicate depth ranges where aplanochytrids were obtained during SK 196 and SK 212 respectively. Key: a-1000 m to surface, b- 500 m to surface, c-300 m to surface, d- 200 m to surface, e- thermocline to surface, f-1000 to 500 m, g-500 to 300 m, h- 300 to 200 m and i-200 m to thermocline.

(Table 1). Zooplankton samples were washed in sterile seawater and plated on to Modified Vishniac (MV) Agar plates (0.001% liver infusion broth, 0.01% yeast extract, 0.15% peptone, 0.1% dextrose monohydrate)[9] supplemented with 40000 U Procaine Penicillin, 0.075 g Streptomycin (Trade name Ambystrin-S) and 1% fetal bovine serum under sterile conditions. During SK 212, fecal pellets of zooplankton were also plated on the same media plates one hour after MPN sampling, by sieving the seawater in which zooplankton were incubated after collection through 100 µm mesh, collecting the filtrate and plating out 0.1 ml of the filtrate. The plates were incubated for 7-14 days and the colonies obtained were sub-cultured on to sterile MV agar plates and sterile seawater plus autoclaved *Artemia* larvae. Growth on these plates and *Artemia* larvae was observed after four days of inoculation. Cultures were maintained by routine sub-culturing on MV agar tubes.

Morphological studies–Cultures were streaked on MV agar plates and their cell and colony morphology was studied under microscope after 7 days of incubation. A coverslip was gently placed over the cultures and the plates were examined using Carl- Zeiss 'Axioskop' 2 plus microscope (Sr. # 80428). Some of the cultures were studied using a modified continuous flow chamber described by Raghukumar[10]. All cultures were photographed using a Zeiss AxioCam digital camera.

Physiological characterization–Growth of the isolates was determined in presence of different carbon sources, namely lactose, galactose, sucrose, starch, ribose, glucose, maltose, xylose, rhamnose, meliobiose, arabinose, cellobiose, raffinose, fructose, trehalose and xylan. The sugars were autoclaved

Table 1—Depth and location in the equatorial Indian Ocean from which aplanochytrids were isolated.

Date	Latitude	Longitude	Depth (m)	Isolate No.
05 Sep. 2003	02° 59.5' N	77° 01.4' E	300-200	S1961
05 Sep. 2003	02° 59.5' N	77° 01.4' E	200-40	S1962
05 Sep. 2003	02° 59.5' N	77° 01.4' E	40-0	S1963
06 Sep. 2003	02° 00.1'N	77° 00.3' E	40-0	S19610
07 Sep. 2003	01° 00.2' N	77° 00.0' E	200-40	S19615
28 Oct. 2004	00° 59.893' S	80° 30.317' E	1000-500	S2121
28 Oct. 2004	00° 59.893' S	80° 30.317' E	60-0	S2122
29 Oct. 2004	01° 28.917' S	80° 30.629' E	60-0 (faecal pellets)	S2123, S2124
30 Oct. 2004	01° 55.932' S	80° 39.926' E	200-60	S2125, S2126, S2127
30 Oct. 2004	01° 55.932' S	80° 39.926' E	500-300	S2128
06 Nov. 2004	00° 00.539' S	92° 58.552' E	30-0 (faecal pellets)	S2129

separately and added at a final concentration of 0.1 % to the medium. Inoculum for all the experiments was prepared by inoculating 20 ml of MV broth with 3- day old culture growing in sea water- pine pollen. This was then used to inoculate 50 ml of MV broth and allowed to grow for 3 days. The later was used to inoculate all experimental flasks containing 20 ml of the medium. At each step 5 % of inoculum was added. The growth was checked after 3 days of inoculation by measuring the increase in dry weight.

Nitrogen requirement of the isolates was examined by growing in the presence of different amino acids, namely proline, cystine, cysteine, ornithine, glycine, alanine, serine, lysine, arginine, asparagine, threonine, glutamic acid, histidine, leucine, phenylalanine and tryptophan. The amino acids were filter-sterilized and added at a final concentration of 1 % amino acid into the medium containing 0.6 % agar, 0.1 % dextrose and 0.025 % KH_2PO_4 supplemented with 1 % fetal bovine serum, 20000 U Procaine Penicillin, 0.0375 g Streptomycin (Trade name Ambystrin-S) and 0.1 % vitamin mix (0.005 % of riboflavin and cyanocobalamin). The growth pattern on the agar plates was monitored after 5 days of inoculation. The diameter of the area over which each colony spread was measured.

Production of extracellular enzymes–The isolates were plated on MV agar plates supplemented with 1 % each of skimmed milk (Trade name Sagar, India), Tween 80 with calcium chloride, starch and chitin to observe for the production of protease, lipase, amylase and chitinase enzymes respectively. Chitin (Hi Media) was dissolved in 50 % sulphuric acid and precipitated with cold distilled water by diluting it 15 fold. The precipitate was washed with distilled water till the *p*H was close to 7.0. It was then autoclaved separately and incorporated in the media to check for the zone of clearance as an indication of positive chitinase production. Protease production was examined as the zone of clearance of milk around the colony. Production of lipase and amylase enzymes was examined according to Molitoris[11]. Growth on all the plates and production of enzymes was monitored after 5 days of inoculation.

Cluster analysis–Similarities among all the isolates were analysed by cluster analysis using unweighted pair group average method in Statistica v 5.0 software and phenograms were plotted. All the physiological characteristics, cell and colony morphological characteristics were taken into consideration for cluster analysis.

Results

Aplanochytrids were isolated from 34 out of 76 samples collected from various depths in the two cruises (Fig. 1). Samples from all depth ranges, namely 1000 m to the surface yielded aplanochytrids in culture. Out of these, a total of 14 isolates were purified. Five of these were isolated during September 2003 and the remaining 9 during October 2004 (Table 1). Three isolates were obtained from the plates on which the filtrate containing fecal pellets was plated.

Morphological studies–Two distinct types of colony morphology were observed among all the isolates (Table 2, Fig. 2A-D). These were (1) colonies producing distinct broad rays of continuous band of cells (Fig. 2A, B) or patches of cells spreading from the edge of the colony outwards (Fig. 2C) and (2) colonies forming clumps of cells on the agar surface (Fig. 2D). Five isolates produced cells that penetrated the agar, while others did not (Fig. 2A). Amoeboid cells were consistently or occasionally produced by some (Fig. 2E). Most of the isolates produced fine filaments of EN (Fig. 2F) with six isolates showing broad areas in between (Fig. 2G) and two isolates producing only broad filaments instead of fine ones (Fig. 2H). EN elements of isolate S2125 though fine in nature, had very broad base (Fig. 2I).

The differences in cell morphology of all the isolates are presented in Table 3. Cells were generally spherical in shape (Fig. 2E). Cell size ranged from a minimum of 7.8–13.0 μm to a maximum of 19–27 μm for mature cells just before differentiation into zoospores, and 13.8–14.9 μm to 19.4–35.0 μm for sporangia containing mature spores. Spores varied in size from a minimum of 3.1 to 4.5 μm to a maximum of 4.8 to 7.0 μm. The number of spores varied from a low of 12 to 13 per sporangium to a maximum of 26-32. Much overlap in cell size, spore numbers and spore size was noticed among isolates between these extremes. Spores varied in shape from circular, ovoid, ellipsoid or cuniform (Fig. 3A-D). Spores moved out of the sporangia with a gliding movement using the EN (Fig. 3E-G). During the release of spores from the sporangium, the cell wall of the sporangium either disintegrated completely (Fig. 3E, F) or a tear was produced at one or two points (Fig. 3H, I). The spores were released through these points leaving behind the cell wall intact in case of the later type of the cultures.

Table 2—Colony characteristics of 14 isolates of aplanochytrids isolated from equatorial Indian Ocean.

Isolate No.	Agar penetration	Rays of continuous band of cells	Rays of disjoint patches of cells	Rays sprawling from center outwards	Rays sprawling from periphery of colony	Clumps without rays
S1961	+	+	-	+	-	-
S1962	+	-	-	-	-	+
S1963	+	+	-	+	-	-
S19610	+	-	+	-	+	-
S19615	+	+	-	+	-	-
S2121	-	-	+	-	+	-
S2122	-	-	-	-	-	+
S2123	-	+	-	+	-	-
S2124	-	-	+	-	+	-
S2125	-	+	-	+	-	-
S2126	-	-	-	-	-	+
S2127	-	-	+	-	+	-
S2128	-	-	+	-	+	-
S2129	-	-	-	-	-	+

Key: + Present
- Absent

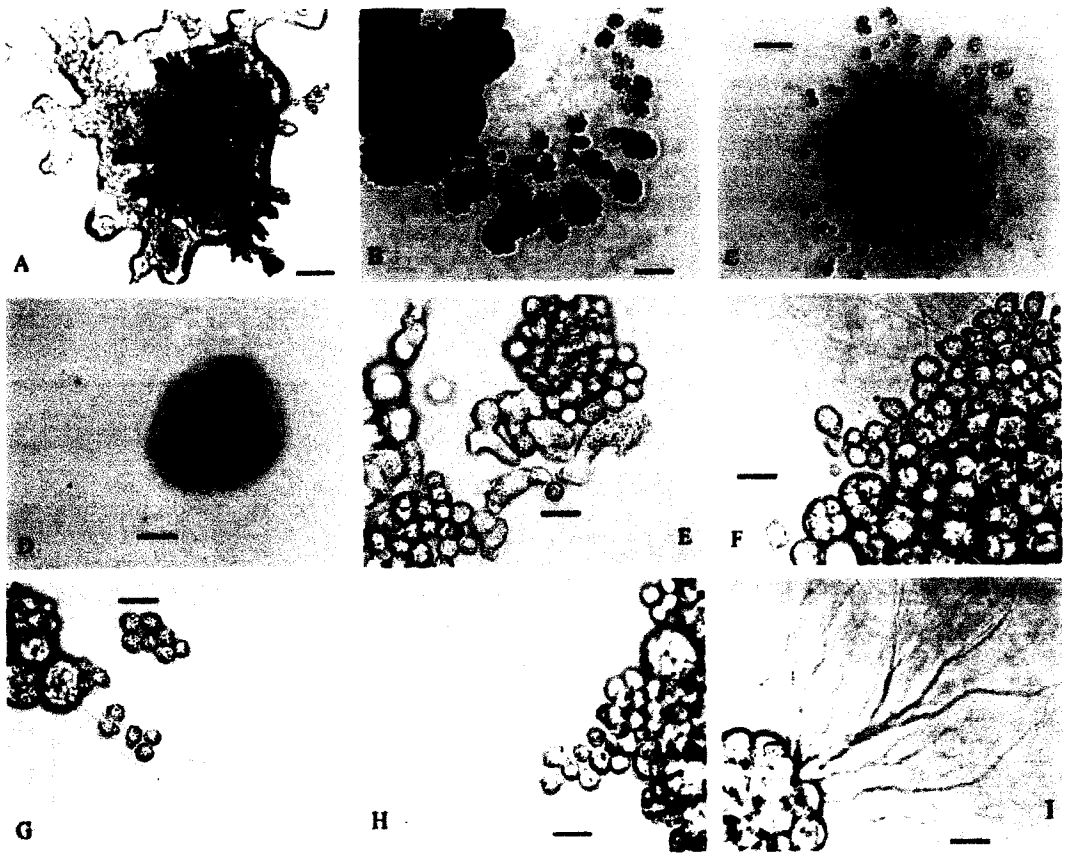

Fig. 2—Photomicrographs of aplanochytrids from equatorial Indian Ocean - A.) colony of isolate S1961 producing rays of continuous band of cells with agar penetration, B.) colony of S2123 with same type of rays without agar penetration, C.) colony of S2127 producing rays of disjointed patches of cells from the edge of the colony, D.) clump-like colony of S1962, E.) amoeboid cells amidst the spherical vegetative cells, F.) cells possessing very fine filaments of ectoplasmic net elements, G.) cells possessing fine EN filaments with broad areas in between, H.) cells with broad filaments of EN and I.) cells possessing EN with a broad base (Scale in figures A to D represents 100 μm and in figures E to I represents 10 μm).

Table 3—Difference in cell morphology of 14 isolates of aplanochytrids isolated from equatorial Indian Ocean

Isolate No.	Size (μm) of			No. of spores	Released spore shape	Presence of ectoplasmic net	Presence of intact cell wall	Presence of amoeboid cells
	Mature cell before formation of spores	Sporangium containing fully mature spores	Spore					
S1961	19.1-27.8	19.4-35.0	5.0-5.8	23-28	Cuniform	Fine with broad areas	disintegrating	Present sometimes
S1962	9.4-13.5	13.8-14.9	3.8-4.6	-	Circular, cuniform	Fine filaments	-	nil
S1963	10-15.4	13.3-16.9	3.1-4.5	26-32	Circular, cuniform, ellipsoidal	Fine filaments	Present (break at two points)	nil
S19610	15.7-20	20.0-20.2	3.1-5.8	17-21	Circular	Fine with broad areas	disintegrating	nil
S19615	10.6-13.3	10.6-17.6	3.3-3.8	16-26	Cuniform, ellipsoidal	Fine with broad areas	Present (break at one point or disintegrating)	nil
S2121	10.7-12.0	14.5-16.4	3.9-5.0	15-22	Cuniform	Fine with broad areas	disintegrating	nil
S2122	7.8-13	13.3-17.5	3.8-4.9	16-18	Circular, cuniform	Broad	Present (break at one point)	present
S2123	10.3-15.7	12.5-23.6	4.0-5.7	16-22	Circular, cuniform	Fine filaments	Present (break at one point)	nil
S2124	12.5-15	13.3-16.7	3.8-4.9	12-15	Circular, cuniform	Broad	Present (break at one point)	present
S2125	9.4-11.4	16.0-22.5	4.4-4.7	12-13	Cuniform	Fine filaments broad base	Present (break at one point)	present
S2126	13.5-15.2	18.0-27.6	4.8-7.0	~ 28	Circular, oval	Fine with broad areas	disintegrating	present
S2127	12.7-27	18.3-32	4.4-6.5	-	Circular, cuniform	Fine filaments	disintegrating	present
S2128	13.0-15.2	15.0-21.6	3.6-5.0	21-22	Circular, cuniform	Fine with broad areas	disintegrating	nil
S2129	10.0- 12.5	13.8-25	3.8-5.1	12-18	Cuniform	Fine filaments	Present (break at one point)	nil

Physiological characterization–All isolates showed varied response to utilization of different sugars (Figs. 4-6). They utilized a broad range of sugars except for a few. Eight out of the 14 isolates showed a preference for disaccharides like maltose, trehalose, rhamnose, cellobiose and lactose. Three isolates viz., S1961, S1963 and S2129 grew maximum in the presence of pentoses like ribose and xylose. In case of hexoses, most of the isolates showed preference for fructose over glucose except for the isolates S1961, S19615, S2121 and S2126, which preferred glucose and then galactose over fructose. Isolates S2122, S2123 and S2124 showed maximum growth in the presence of galactose. Isolate S2126 exhibited best growth in the presence of xylan followed by isolates S1962 and S19615 whereas the other isolates exhibited poor growth in its presence. With regards to amino acid utilization, all of them showed better growth in the presence of proline and glutamic acid followed by alanine and sometimes lysine (Fig. 7-9). Serine, glycine and ornithine too favoured some growth but lesser than that observed with the ones mentioned earlier. Histidine, arginine, asparagine, cystine cysteine and the aromatic amino acids did not induce any growth.

Enzymatic studies–Ten isolates showed degrada-tion of milk protein indicating production of protease enzyme but none of the isolates produced lipase, amylase or chitinase enzymes (Table 4).

Cluster analysis–Phenogram 1 was plotted using physiological as well as cell and colony morphology characteristics (Fig. 10A) and phenogram 2 was plotted using only the physiological characteristics (Fig. 10B). Phenogram 1 groups all the isolates in two main clusters; one containing larger cells up to 27.8 μm and larger sporangia (> 25 μm) and the other cluster containing smaller cells in the range of 7.8 to 15.4 μm and smaller sporangia (< 25 μm). The subgroupings within these clusters could not be related specifically to any morphological characters and carbon and nitrogen requirements, various characters appearing scattered among the clusters.

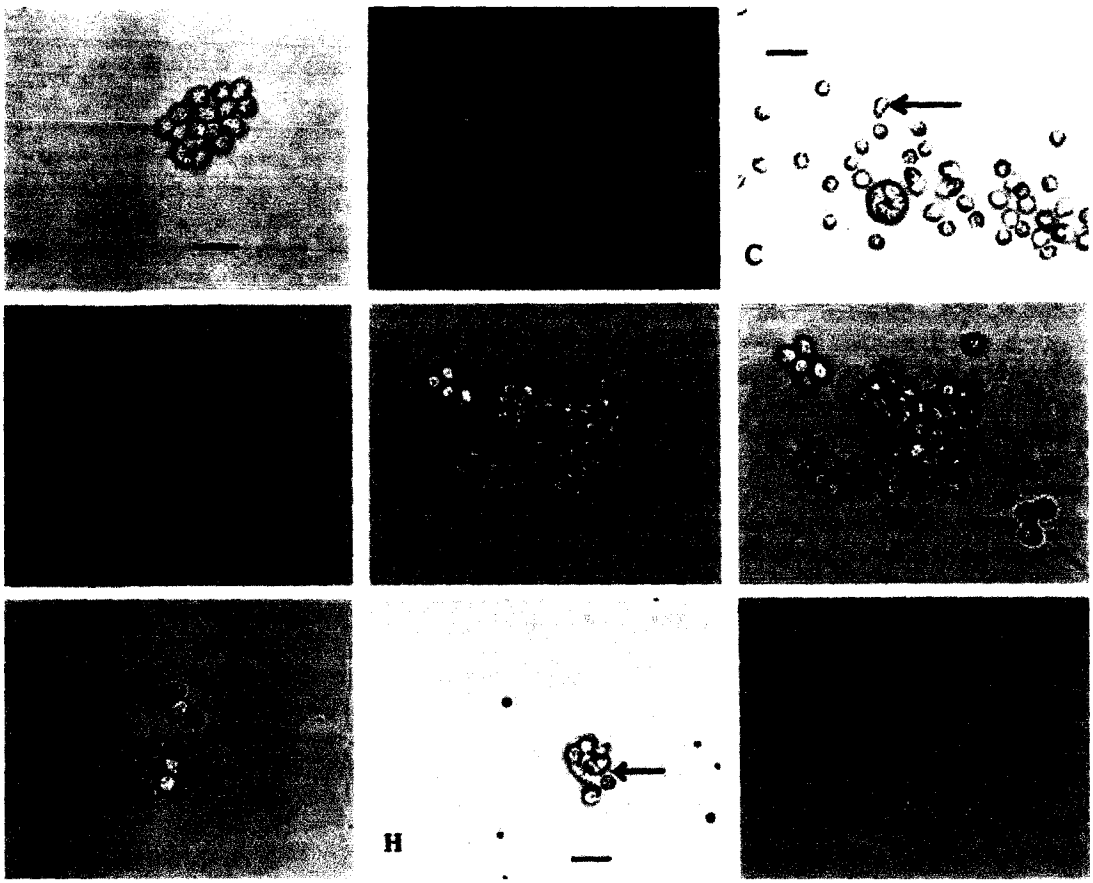

Fig. 3—Photomicrographs of aplanochytrids from equatorial Indian Ocean - A- D) different spore types, viz., (A) cuniform, (B) circular, (C) ellipsoidal (arrow), cuniform and circular and (D) oval (arrow) and circular, E- G) gliding movement of spores out of sporangia over the EN elements and (E and F) disintegration of cell wall during the release of spores, H) break at one point (arrow) in the cell wall of sporangium during the release of spores, I) same sporangium with an intact cell wall (arrow) and only 4 spores left to get released. Scale in fig. A represents 20 μm, fig. B, C, D, G, H and I represents 10 μm and in fig. E and F represents 5 μm.

Table 4—Protease activity of 14 isolates of aplanochytrids isolated from equatorial Indian Ocean, as observed by the zone of clearance of milk protein.

Isolate no.	Zone of clearance of milk protein (mm)
S1961	16.5
S1962	nil
S1963	18.125
S19610	10.75
S19615	-
S2121	nil
S2122	nil
S2123	2
S2124	13.25
S2125	11.5
S2126	2.625
S2127	3.5
S2128	10.75
S2129	7.75

The phenogram generated using carbon and nitrogen requirements (Fig. 10B) separated out S2121 in a cluster. This isolate preferred glucose as a carbon source and lysine and threonine as a nitrogen source. Within the second cluster, isolates S1961, S1963 and S19615, which utilized the pentose sugar ribose were clearly separated from the rest. Trehalose-utilizing isolates, S1962, S2125, S2127 and S2128 also stood out as a separate cluster.

Discussion

Aplanochytrids have been isolated mostly from coastal marine habitats[3,6]. They have also been isolated from subantarctic marine waters and the Ross Sea of Antarctica[4,12]. This is the first detailed report of aplanochytrids from oceanic water column. Aplanochytrids were isolated from zooplankton of various depths at 15 out of 19 locations during this study

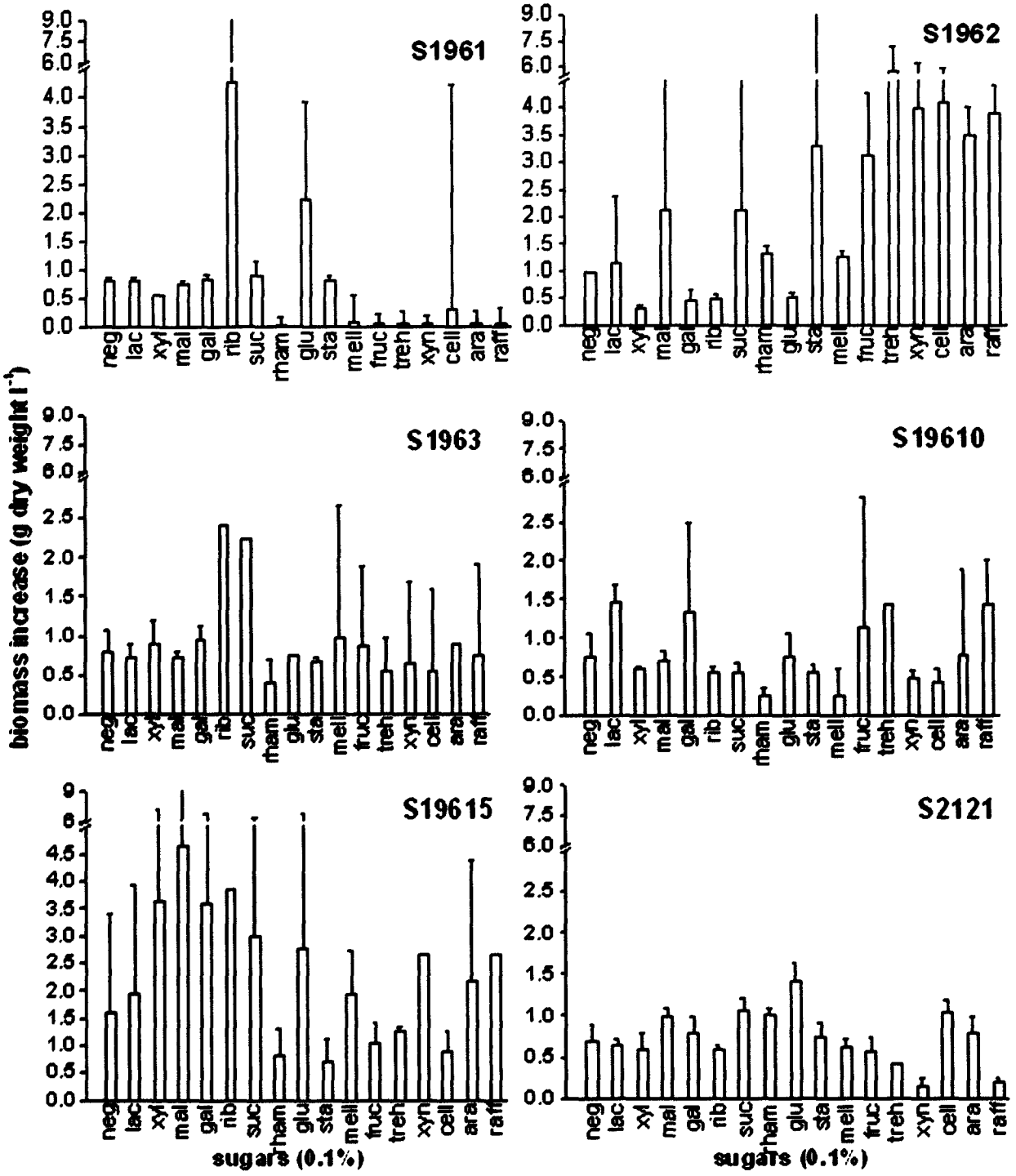

Fig. 4—Growth of aplanochytrid isolates S1961, S1962, S1963, S19610, S19615 and S2121 on various carbon sources. Key: neg- negative control, lac- lactose, xyl- xylose, mal- maltose, gal- galactose, rib- ribose, suc- sucrose, rham- rhamnose, glu- glucose, sta- starch, mell- mellibiose, fruc- fructose, treh- trehalose, xyn- xylan, cell- cellobiose, ara- arabinose and raff- raffinose.

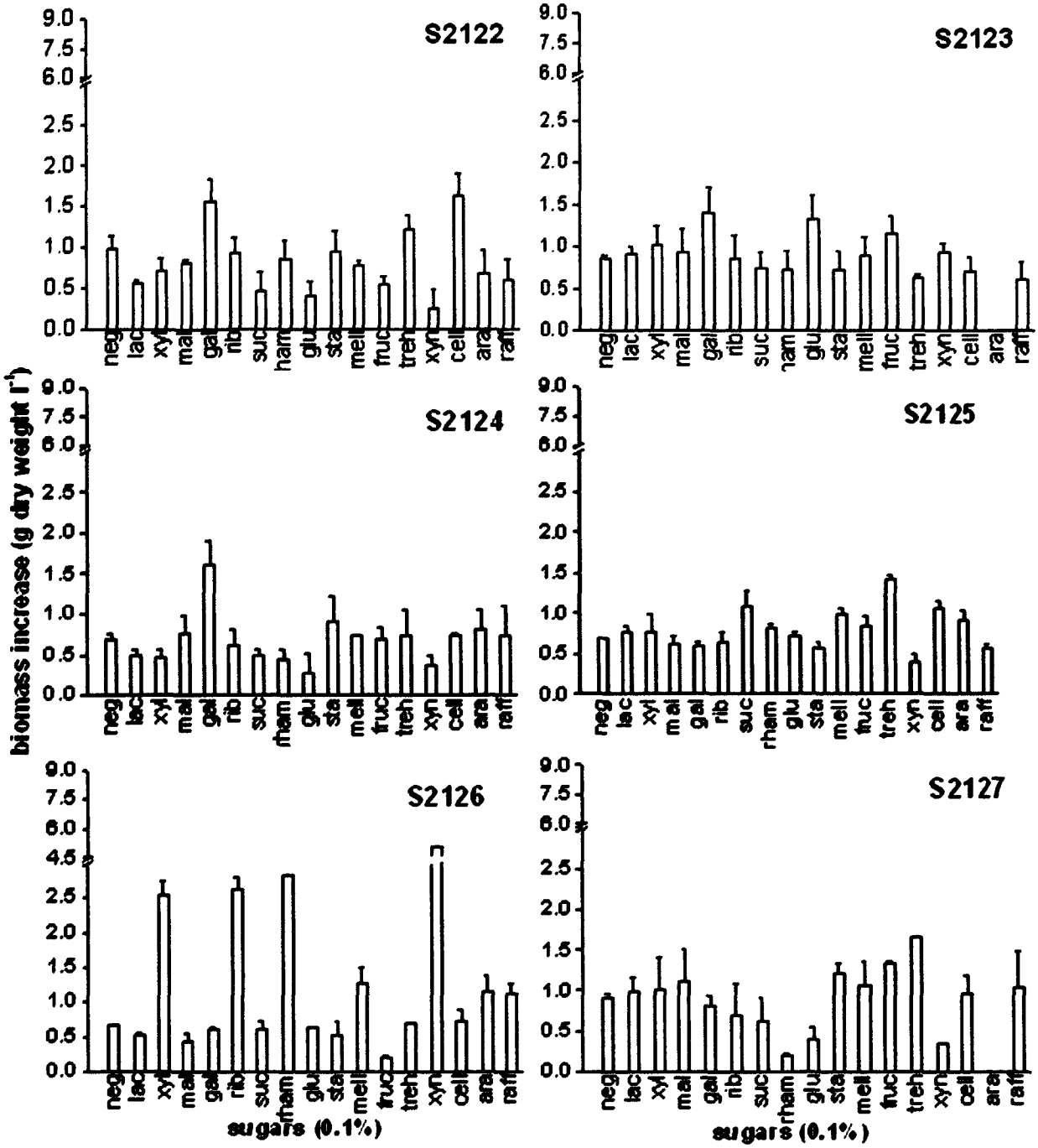

Fig. 5—Growth of aplanochytrid isolates S2122, S2123, S2124, S2125, S2126 and S2127 on various carbon sources.

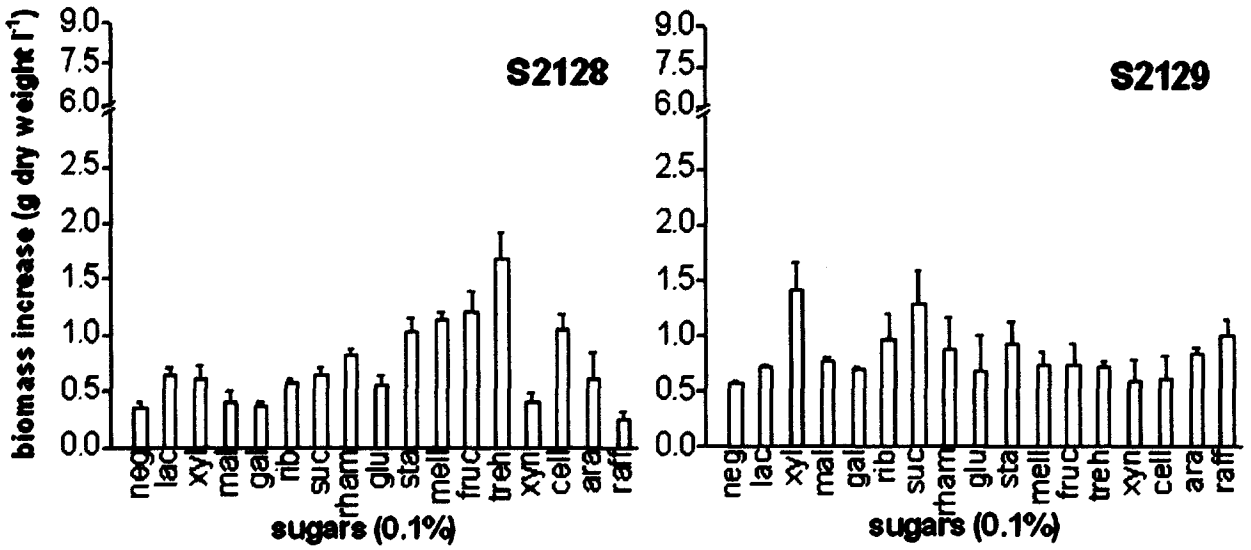

Fig. 6—Growth of aplanochytrid isolates S2128 and S2129 on various carbon sources.

(Fig. 1). About 45 % of the total number of samples yielded aplanochytrids. Surprisingly none of the samples yielded thraustochytrids in culture. A similar observation was made by Raghukumar[7] who isolated mostly *Aplanochytrium yorkensis* (previously named *Labyrinthuloides yorkensis*) and sometimes *Ulkenia amoeboidea* from most of the samples of the offshore waters in the Arabian Sea. It will be interesting to find out if aplanochytrids are more abundant than thraustochytrids in oceanic waters. The epifluorescence technique devised by Raghukumar & Schaumann [13] for direct detection of these organisms does not differentiate between aplanochytrids and thraustochytrids as both reveal orange-to-red fluorescing cell wall due to presence of sulphated polysaccharides and yellow-to-green fluorescing nuclei. Therefore, many of the cells enumerated as thraustochytrids in seawater samples from various parts of the world[3] could have also been aplanochytrids.

Like thraustochytrids, aplanochytrids survive on organic substrates and might degrade complex organic compounds[3]. The aplanochytrids that we isolated from zooplankton may survive on them as commensals, mutualists or degraders. Since physiological characters might throw light upon their ecological behaviour, we studied the production of different degradative enzymes, as well as carbon and nitrogen requirements of aplanochytrids. Thraustochytrids are known to produce a variety of degradative enzymes[14]. Chitin is a structural part of the exoskeleton of zooplankton[15]. Yet, none of the isolates produced chitinase therefore aplanochytrids may not play a significant role in degradation of zooplankton exoskeleton. This is in accordance with observations made by Bahnweg[16] on *Aplanochytrium yorkensis*. However, the production of protease by most of the isolates studied indicates that aplanochytrids might be involved in the degradation of complex proteinaceous compounds of zooplankton cadavers (Table 4).

All the equatorial Indian Ocean isolates generally preferred pentoses and disaccharides to glucose (Fig. 4-6). On the contrary, the isolate of *Aplanochytrium yorkensis* studied by Bahnweg[16] preferred hexoses and their derivatives too along with pentoses and disaccharides. All the isolates displayed poor growth in liquid medium containing different amino acids showing almost insignificant differences in dry weight of biomass amongst the isolates and therefore they were plated on solid media containing different amino acids and their colony diameter was measured. Most of them showed maximum growth with glutamic acid but some preferred proline, lysine and alanine (Fig. 7-9). Microorganisms utilize glutamic acid for synthesis of glutamine, proline or aspartic acid[17,18]. This might be true also of aplanochytrids. Many preferred proline next to glutamic acid. Since proline cannot replace glutamic acid for growth[18], there generally is a higher requirement for glutamic acid followed by proline. However, Bahnweg[19] observed the opposite wherein growth in the presence

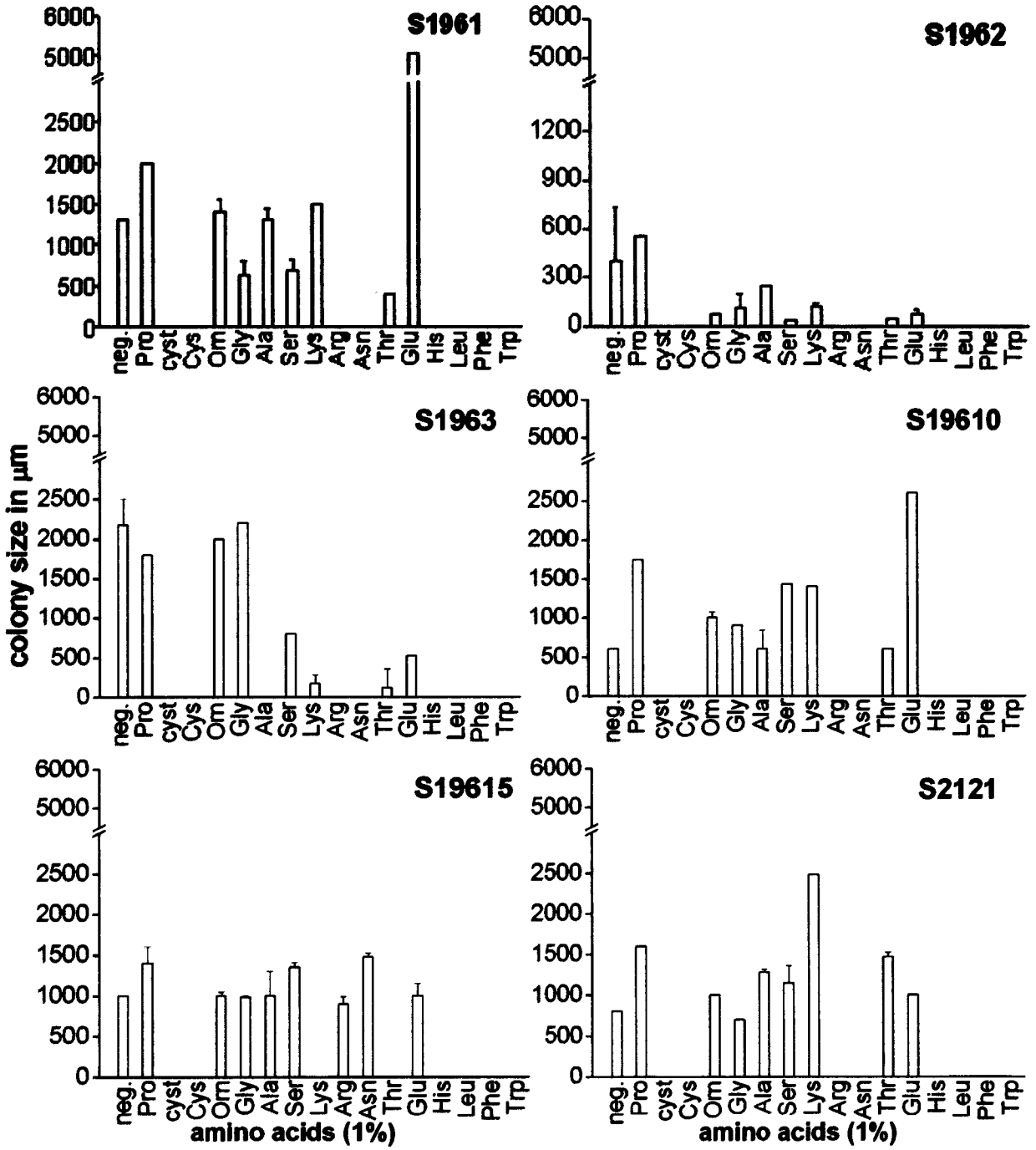

Fig. 7—Growth of aplanochytrid isolates S1961, S1962, S1963, S19610, S19615 and S2121 on various nitrogen sources. Key: neg- negative control, Pro- proline, cyst- cystine, Cys- cysteine, Orn- ornithine, Gly- glycine, Ala- alanine, Ser- serine, Lys- lysine, Arg- arginine, Asn- asparagine, Thr- threonine, Glu- glutamic acid, His- histidine, Leu- leucine, Phe- phenylalanine and Trp- tryptophan.

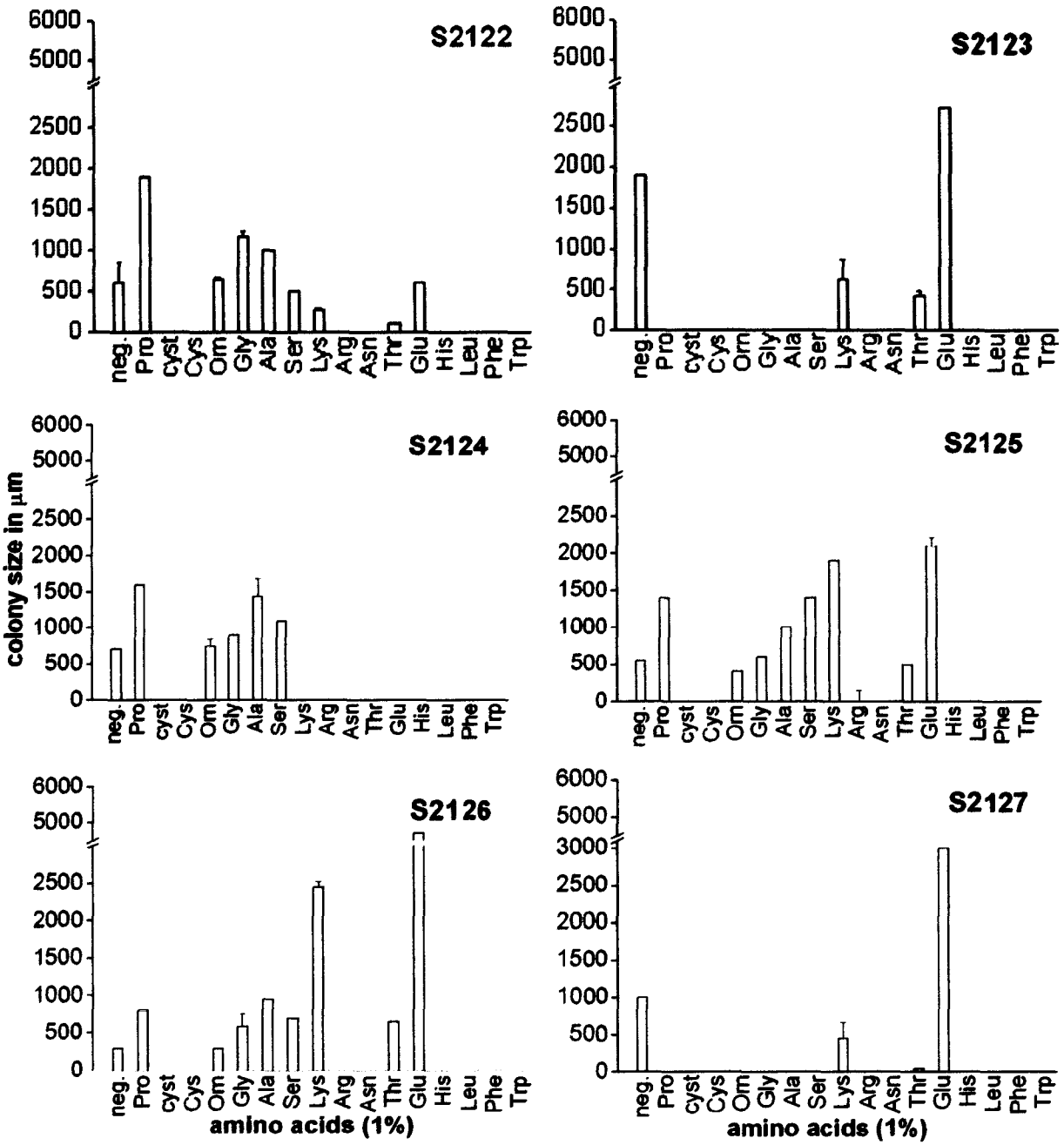

Fig. 8—Growth of aplanochytrid isolates S2122, S2123, S2124, S2125, S2126 and S2127 on various nitrogen sources.

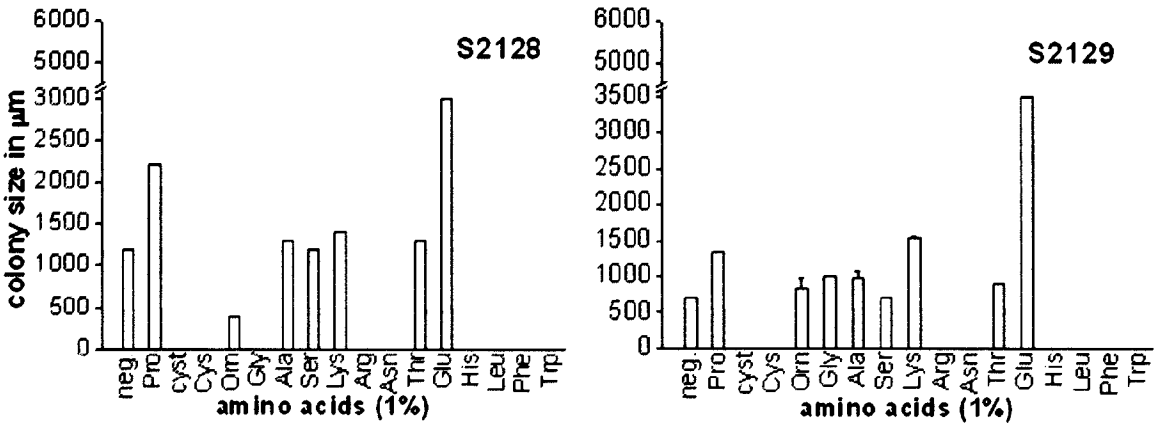

Fig. 9—Growth of aplanochytrid isolates S2128 and S2129 on various nitrogen sources.

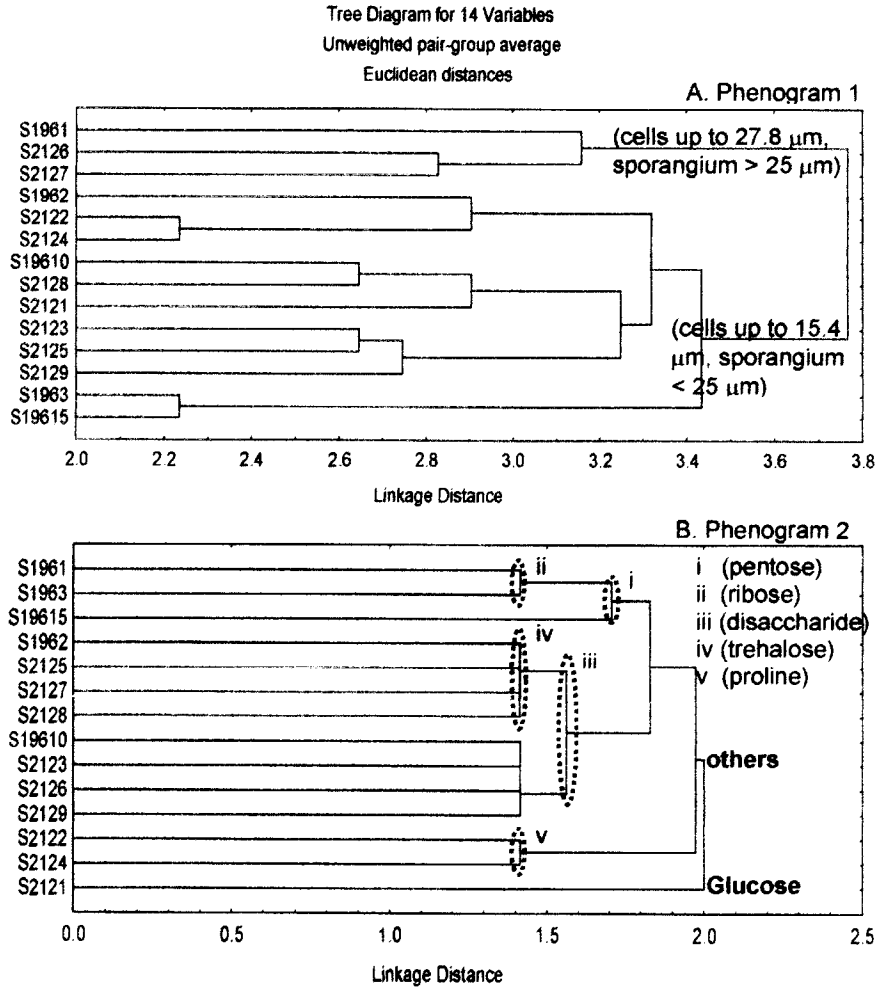

Fig. 10—Phenograms showing similarities amongst various isolates of aplanochytrids isolated from equatorial Indian Ocean: A) Phenogram 1is based on morphological as well as physiological characteristics and B) Phenogram 2 is based on physiological characteristics only.

of proline was more than that in the presence of glutamic acid. It will be thus interesting to study the isolate S2124 in details as it also showed maximum growth in the presence of proline but no growth in the presence of glutamic acid. This isolate might synthesize glutamic acid from proline via the formation of pyrroline–5–carboxylic acid[20] rather than direct incorporation from the medium. The high requirement for proline might also be due to its ATP-synthesizing ability by driving mitochondrial oxidative phosphorylation[21]. None of the isolates studied here showed any growth on histidine, arginine, asparagine, cystine, cysteine, leucine, phenylalanine and tryptophan (Fig. 7-9). Therefore, aplanochytrids might synthesize most other amino acids *in vivo* from glutamic acid and proline rather than incorporate them from their substrates.

Raghukumar & Raghukumar[22] have isolated thraustochytrids from fecal pellets of salp *Pegea confoederata*. We also recovered aplanochytrids from samples of zooplankton fecal pellets (Table 1). Aplanochytrids may be commensals in the guts of zooplankton. Alternatively, their presence in faecal pellets might reflect that zooplankton ingest particles containing aplanochytrids and passively egest them out of their body. Thraustochytrids are known to be associated with particulate matter in the water column[3,23]. Members of Labyrinthulomycetes produce high amounts of polyunsaturated fatty acids (PUFAs) like docosahexanoic acid (DHA)[24]. Zooplankton are incapable of producing them but require for their growth and reproduction[25, 26]. They thus incorporate essential fatty acids in their body through nutrition[27-30]. Therefore zooplankton might be feeding on aplanochytrids to fulfill their requirement of DHA[31,32]. Recently Alonzo *et al.*[33] reported that DHA was most efficiently incorporated in krill fed on thraustochytrids. Whether aplanochytrids are consumed by zooplankton and their lipids incorporated in their body is not known. This aspect needs further investigation to elucidate the role of aplanochytrids in the water column, especially in oceanic waters with relation to zooplankton.

About eight species of *Aplanochytrium* described so far have been classified on morphological and developmental characters some of which are listed in Table 3. Leander *et al.* [6] noticed that most of these characters were highly plastic and ambiguous. In the present study, all the isolates could be broadly classified as *Aplanochytrium yorkensis* (Perkins) Leander & Porter. Although differences were observed in cell size, spore morphology, the nature of the ectoplasmic net elements, the presence or absence of cell wall following spore liberation and the presence or absence of amoeboid stages (Table 3), it is not clear which of these characters is taxonomically reliable. In view of the present taxonomic scenario of aplanochytrids, Leander *et al.*[6] carried out a detailed study to evaluate the reliability of such characters, together with the molecular phylogeny of *Aplanochytrium* species. Their study suggests that the most useful morphological characters, in descending order of importance, are as follows. (1) Possession of small, oblong cells as in *Aplanochytrium minutum* versus presence of large spherical cells, as in *Aplanochytrium yorkensis*; (2) Colonies possessing radial rays of cells or their absence; (3) Presence or absence of penetration of the agar medium by cells. We attempted to group our isolates using cluster analysis, using (1) morphological criteria and carbon and nitrogen requirements (Fig. 10A); (2) carbon and nitrogen requirements alone (Fig. 10B) and (3) morphological characters alone. Phenograms using morphological characters alone and those using morphology and C and N nutrition were similar, suggesting that morphological characters predominated in importance over C requirements. All 14 of our isolates belonged to the *Aplanochytrium yorkensis* type, a likely result of their prevalence in oceanic waters and a paucity of *A. minutum* group therein. Phenograms generated using morphology and C and N requirements did not differentiate between isolates producing colonies with rays and those that did not, or those in which cells penetrated agar or lacked this character. This is different from the observations of Leander *et al.*[6]. On the contrary, the isolates were divided into two groups, one with distinctly large cells and the other with smaller cells (Fig. 10A). No further distinct groups, based either on morphology or C and N requirements were noticed using this cluster analysis. Phenograms based on C and N requirements alone divided the isolates into two groups (Fig. 10B). The most significant clustering in this analysis was of those isolates that utilized ribose most efficiently and isolates that utilized disaccharides, including trehalose.

We suggest that the different morphological and physiological traits that we studied are merely variations within a single species and that our isolates represent populations of *Aplanochytrium yorkensis*.

The fact that all our isolates were obtained from zooplankton from the same area, namely the equatorial Indian Ocean (77° E, 80.5° E and 93° E), emphasizes this view. These characters may thus be inherent variations of a species and may not be reliable tools to distinguish species within the genus *Aplanochytrium*. It will further be interesting to examine if various combinations of these characters, as observed in this study are the result of sexual reproduction within the species. Although no sexual reproduction has been fully confirmed in any of the Labyrinthulomycetes, this cannot be ruled out.

Acknowledgement

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RESEARCH ARTICLE

Abundance of thraustochytrids and bacteria in the equatorial Indian Ocean, in relation to transparent exopolymeric particles (TEPs)

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thraustochytrids; bacteria; TEPs; equatorial Indian Ocean.

Abstract

Thraustochytrid protists are often abundant in coastal waters. However, their population dynamics and substrate preferences in the oceanic water column are poorly understood. We studied the abundance and distribution of thraustochytrids, bacteria and TEPs in the equatorial Indian Ocean waters during September 2003, October 2004 and September 2006. Thraustochytrids and bacteria were abundant, suggesting high biological productivity of the region. Thraustochytrids were positively related to bacteria during October 2004 but not at other times, suggesting overlapping or varying substrate preferences at different times. Thraustochytrid and bacteria were positively related to TEPs only in a few stations during October 2004, but were mostly positively related to TEPs generated from *in situ* water in a roller table experiment. TEPs from natural samples during October 2004 had a much greater affinity to the lectin Concanavalin A than to Limulin compared with those in September 2006 and from the roller tank experiments. The chemical composition of TEPs might explain their relationship with thraustochytrids. Thraustochytrids averaged a higher biomass than bacteria in two of the three cruises, but were less frequent and more patchily distributed compared with bacteria.

Introduction

Thraustochytrids, the single-celled, marine stramenopilan protists, are widespread in the sea. Because they are similar to bacteria in their osmoheterotrophic mode of nutrition (Porter, 1990; Raghukumar, 2002), they are likely to play a role in remineralization of particulate and dissolved organic matter, as bacteria do. Within the context of this larger role, however, it might be expected that these heterotrophic protists occupy a distinct ecological niche in the sea, in order to avoid competition from the ubiquitous bacteria. Comparative studies on the relative abundance and biomass of bacteria and thraustochytrids in different habitats might throw light on this problem. Only a few studies, namely those of Raghukumar & Schaumann (1993) in the North Sea, Naganuma *et al.* (1998) and Kimura *et al.* (1999, 2001) in the Seto Inland Sea, Japan, Raghukumar *et al.* (2001) in the Arabian Sea and Bongiorni *et al.* (2005) in the Mediterranean, have dealt with the abundance and biomass of these organisms in marine habitats. These studies have demonstrated that (1) thraustochytrids in coastal waters or the land-locked Arabian Sea often attain densities of a few hundred thousand cells per liter seawater and (2) thraustochytrids may occasionally attain biomass values up to 50% of the bacteria. Kimura *et al.* (1999, 2001) postulated that thraustochytrid biomass was related to riverine inputs of organic material. Therefore, it is not clear whether these protists are also prevalent in oceanic waters and, if so, what their relationships with bacteria are. One of the potential habitats of thraustochytrids in the water column are the transparent exopolymeric particles (TEPs). TEPs are fibrillar mucopolysaccharides formed through coagulation of the increasingly refractory dissolved organic matter left behind after the action of heterotrophic bacterial processes on the biologically labile organic carbon of dissolved polysaccharide exudates released by phytoplankton and bacteria (Alldredge *et al.*, 1993; Beauvais *et al.*, 2003). They act as the sticky matrix for 'marine snow' or 'marine aggregates', comprising detrital and inorganic particles, fecal pellets and cadavers of zooplankton and microorganisms (Simon *et al.*, 1990; Passow & Alldredge, 1994).

The aim of this study was to examine the relative abundance and distribution of thraustochytrids in relation to bacteria and TEPs in open oceanic waters. We studied

these aspects in the waters of the equatorial Indian Ocean along two parallel tracts during two cruises at the end of the southwest monsoon in September and the transition period of the northeast monsoon in October. In another cruise, only bacteria and thraustochytrids were studied. The equatorial Indian Ocean comprises the southern limit of one of the three major circulation systems of the Indian Ocean, namely the seasonally changing monsoon gyre (Wyrtki, 1973a, b; Reddy, 2001). This region has received little attention with regard to both bacterial and thraustochytrid dynamics, although several papers have examined bacterial dynamics in the adjacent northern Indian Ocean, namely the Arabian Sea (Ducklow, 1993; Ramaiah *et al.*, 1996; Wiebinga *et al.*, 1997; Pomeroy & Joint, 1999).

Materials and methods

Sampling stations

This study was carried out in the equatorial Indian Ocean during three cruises on board *ORV Sagar Kanya* during September 2003 (Cruise # SK 196), October 2004 (Cruise # SK 212) and September 2006 (Cruise # SK 228) (Fig. 1). Altogether, nine stations between 2°N and 2°S were studied for each cruise, at a longitude of 77°E during September 2003 and 80.5°E during October 2004 and September 2006.

Sample collection

Water samples were collected with clean 5- or 10-L Niskin bottles attached to a Sea Bird CTD rosette sampler from the surface, 10, 20, 40, 60, 80, 100, 120 and 200 m in all the three cruises (a total of nine depths). Two additional depths of 500 and 1000 m were sampled during October 2004 and September 2006 (a total of 11 depths).

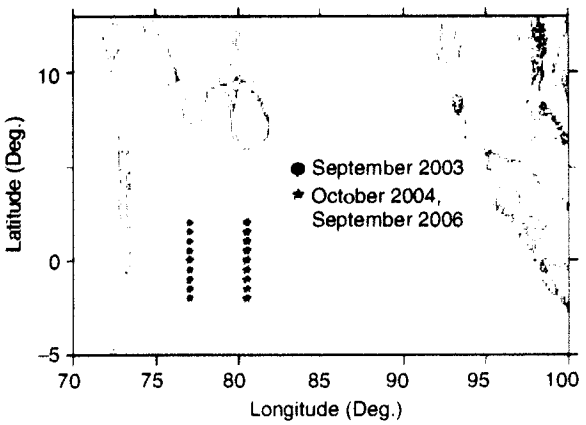

Fig. 1. ORV Sagar Kanya station locations in the equatorial Indian Ocean during cruises SK 196 (September 2003), SK 212 (October 2004) and SK 228 (September 2006) where water samples were collected.

Estimation of bacterial and thraustochyrid abundance

Water samples collected during both the cruises were preserved with formaldehyde at a final concentration of 2% and stored at 5 °C. Ten milliliters of water was filtered over 25 mm, 0.22-μm black polycarbonate Isopore membrane filters (Millipore) and stained for bacteria using the acridine orange direct count (AODC) method (Parsons *et al.*, 1984). The filters were mounted on microscope slides in a non-fluorescent immersion oil (Olympus Optical Co. Ltd) and observed under blue excitation light using a BX60 Olympus epifluorescence microscope equipped with a 100 W Hg lamp. Ten different fields were observed for counting.

Thraustochytrids were enumerated by filtering 25 mL of water over a 0.4-μm black polycarbonate Isopore membrane filter (Millipore). The filters were then stained according to the acriflavine direct detection (AfDD) technique (Raghukumar & Schaumann, 1993). Thraustochytrid cells were counted from 100 different fields under a blue excitation filter and counterchecked for photosynthetic picoplankton under a green filter.

Determination of biomass

Kimura *et al.* (1999) and Raghukumar *et al.* (2001) used a value of 20.6 pg of C per thraustochytrid cell, considering an average cell diameter of 5.0 μm. The average estimated size from 100 counts in our study corresponded to 4.7 μm. Therefore, our biomass estimations were also based on an average cell diameter of 5.0 μm as above. A factor of 20.0 fg of C per average cell from oceanic waters was used for bacteria (Ducklow, 2000).

Determination of TEPs concentration

TEPs were estimated in two of the three cruises (October 2004 and September 2006), following the method of Passow & Alldredge (1995). Ten to twenty-five milliliters of water samples were filtered through 0.4-μm polycarbonate filter papers and stained with alcian blue (0.02% w/v in 0.06% acetic acid, pH 2.5). The filter papers were then transferred into beakers and soaked in 80% sulfuric acid for 2 h. The absorbance of the solution was read at 787 nm against distilled water as a reference. The concentrations of TEPs was determined in duplicate or triplicate for all the samples and were calculated using the formula described by Passow & Alldredge (1995) and expressed as milligram equivalent of alginic acid (AA) per liter (mg eq AA L^{-1}) (Ramaiah *et al.*, 2000).

The relationship among bacteria, thraustochytrids and TEPs during the cruises was analyzed using a correlation matrix (STATISTICA 5.0).

Roller table experiment to study TEPs, bacteria and thraustochytrids

Surface seawater collected at 2°N 80.5°E during cruise # SK 228 in September 2006 was used to generate TEPs in a roller table based on the design of Shanks & Edmondson (1989). The water was filtered through a 200-μm mesh to remove mesozooplankton. Four bottles of 1 L each were filled with the water and incubated for 2 weeks at 24 r.p.m. in the laboratory on board under diffuse light and at a temperature of 25–27 °C. The bottles were divided into two sets of two each. Samples from one set were drawn on Days 0, 5 and 9 and from the other on days 3, 7 and 14. A total of 50 mL sample was drawn each time. The volume of the bottles was maintained constant by replacing the sample amount of 50 mL with 0.22 μm filtered seawater collected from the same station. The samples were fixed with formalin at a final concentration of 2% and kept under refrigeration till enumeration of TEPs, bacteria and thraustochytrids was performed in the laboratory on land. Samples were analyzed in duplicate.

Chemistry of the TEPs using lectins

Two lectins were used to examine the presence of different sugars in TEPs in water samples from representative stations, as well as the water sample in the roller table experiment. These were fluorescein isothiocyanate (FITC)-labelled Concanavalin A that binds to D-mannose, D-glucose and fructofuranose residues and FITC-labelled Limulin that binds to *n*-acetylneuraminic acid, glucuronic acid and phosphorycholine analogs. The lectins were obtained from Sigma-Aldrich Chemicals Pvt Ltd. A total 1 mL of water sample was taken in 2-mL Eppendorf tubes and 50 μL of Concanavalin A at a concentration of 2.5 mg mL^{-1} or 25 μL of Limulin at a concentration of 1 mg mL^{-1} was added separately and the samples incubated for 30 min. They were then filtered over 0.22-μm black polycarbonate Isopore membrane filters and examined using an epifluorescence microscope at 450–490 nm (blue excitation filter). A total of 100 microscope fields were examined and the number of samples positive for each lectin were calculated.

Results

The isothermal mixed layer during September 2003 was present up to 40-m depth at the equator and north, becoming shallower (20 m) towards the south. The thermocline was deeper at all the locations during October 2004 and September 2006 (60–80 m during October 2004 and 45–65 m during September 2006). A subsurface salinity maximum at a depth of 40–80 m was observed in the three cruises.

Distribution of thraustochytrids, bacteria and TEPs

The results on thraustochytrid and bacterial densities and the concentration of TEPs for six out of nine stations sampled during the cruises in October 2004 and September 2006 are presented in Figs 2 and 4. Data for all nine stations were included for the correlation analyses presented in Tables 1 and 2 and Figs 3, 5 and 6. During October 2004, both thraustochytrids and bacteria showed a prominent peak of abundance both in the mixed layer between 0 and 40 m southwards of the equator and below the mixed layer between 100 and 120 m northwards of the equator. Thraustochytrids in the mixed layer up to 40 m ranged from below detection levels to 674.6×10^3 cells L^{-1} (Fig. 2), corresponding to a maximum biomass of 13.9 μg C L^{-1}. Bacterial densities ranged from 1.84 to 759.0×10^6 L^{-1}, corresponding to biomass values of 0.04–15.2 μg C L^{-1}. The two groups attained maximum densities and biomass at 120 m at 1°N, corresponding to a thraustocytrid biomass of 15.8 μg C L^{-1} and a bacterial biomass of 16.1 μg C L^{-1}. The maximum thraustochytrid biomass contribution to the total of bacterial and thraustochytrids C occurred at 0.5°S (10 m), where thraustochytrids comprised 99.4% of the total. Thraustochytrids at this station numbered 276×10^3 cells L^{-1}, bacteria amounting to a total of 1.84×10^6 L^{-1}. The numbers of both thraustochytrids and bacteria were generally less below 120 m, the former ranging from negligible to 73.6×10^3 cells L^{-1} and the latter from 19.32 to 103.73×10^6 cells L^{-1}. Thraustochytrids and bacteria generally showed very similar trends of distribution in the water column and were positively related in five of the nine stations (Table 1). There was an overall, highly significant positive correlation between the two groups (Fig. 3). TEPs ranged from 5.3 to 451.1 mg equivalent AA L^{-1}. Peak TEPs values varied between the stations, occurring in the mixed layer (40–60 m), at 100–120 m or at 200 m. Their distribution did not relate to either bacteria or thraustochytrids on an overall basis (Fig. 3). However, a significant positive correlation of the two groups with TEPs was observed at two stations (at 1°N and 2°S; Fig. 2; Table 1). TEPs from six of the samples examined from this cruise showed varying response to Concanavalin A and Limulin, percentage response to the latter ranging from 12% to 75% of the samples (Table 3). TEPs from 1°N and 2°S, which showed a positive relationship with thraustochytrids as well as bacteria, generally seemed richer in *n*-acetylneuraminic acid (Limulin positive) than those from the equator, where the distribution of TEPs appeared to have a negative trend relative to both bacteria and thraustochytrids. The distribution trend of TEPs at 1.5°N from 0 to 120 m was similar to that of thraustochytrids and bacteria, but displaced downwards by 20 m (Fig. 2). A similar observation

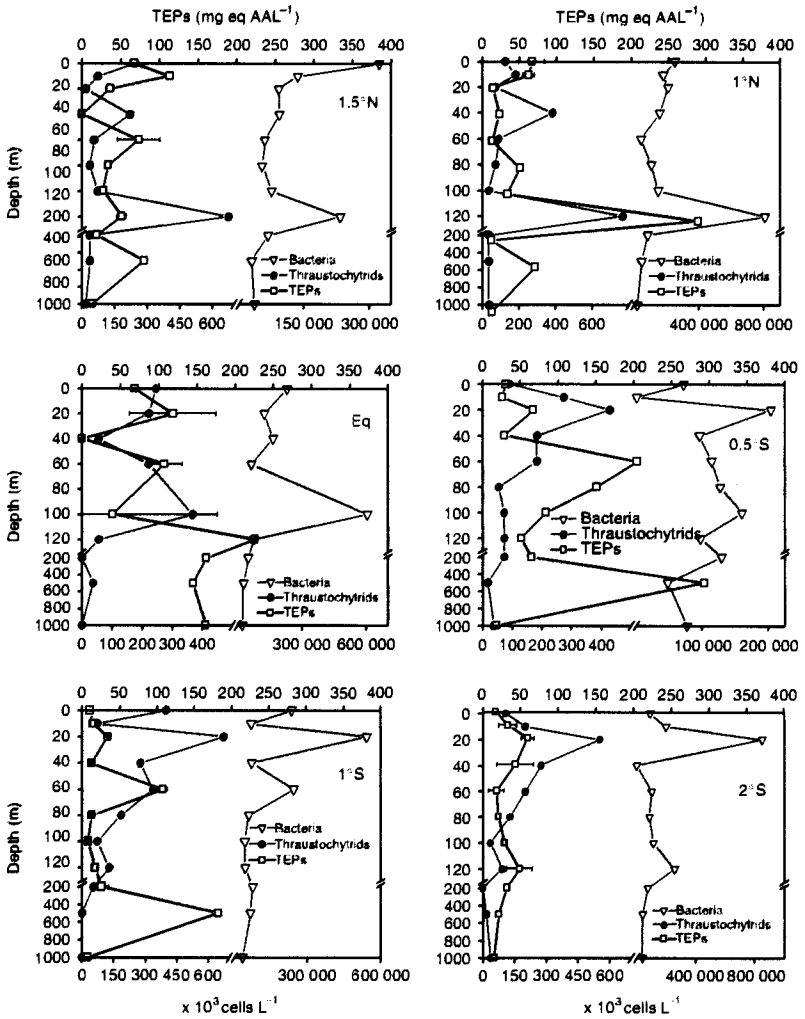

Fig. 2. Distribution of thraustochytrids, bacteria and TEPs in the equatorial Indian Ocean during October 2004.

was also made at 2°N, whose data are not shown in the figure.

The abundance of both thraustochytrids and bacteria was much less during September 2006 (Fig. 4). Thraustochytrids in the mixed layer up to 80 m ranged from below detection levels to 255.6×10^3 cells L^{-1}, while bacteria varied from 0.81 to 183.4×10^6 cells L^{-1}. Thus, thraustochytrids contributed up to 5.3 μg L^{-1}, while bacterial biomass amounted to 0.016–3.6 μg C L^{-1}. The maximum thraustochytrid biomass contribution to the total of bacterial and thraustochytrid C occurred at 0.5°S (200 m), where thraustochytrids comprised 93% of the total. Thraustochytrids at this station numbered 76.7×10^3 cells L^{-1}, bacteria amounting to a total of 6.4×10^6 L^{-1}. The maximum density of thraustochytrids was found at 10 m at 1°N. Bacterial numbers at this station were also some of the highest noticed. Both were also often abundant at 100–1000 m, thraustochytrids ranging from below detection levels to 76.7×10^3 cells L^{-1} and bacteria from 2.6 to 104.9×10^6 cells L^{-1}. Unlike during October 2004, thraustochytrids and bacteria showed dissimilar trends of distribution in the water column during September 2006 and no positive relations between their numbers were found in any of the nine stations (Table 2). TEPs ranged from 7.5 to 339.3 mg equivalent AA L^{-1} (Fig. 4). Maximum levels of TEPs were often observed below 200-m depth. Their distribution in the water column did not correspond either with that of the bacteria or thraustochytrids and no significant relations were noticed in any of the sampling stations, or on an overall basis (Table 2; Fig. 5). TEPs from the four samples examined from this cruise showed a much greater response to Concanavalin A (mannose) than to Limulin (*n*-acetylneuraminic acid), the response to the latter lectin being in the range of 9–39% (Table 3). However, the distribution trend of TEPs in the water column closely corresponded with that of thraustochytrids at the equator and with that of bacteria at 1°S, but displaced

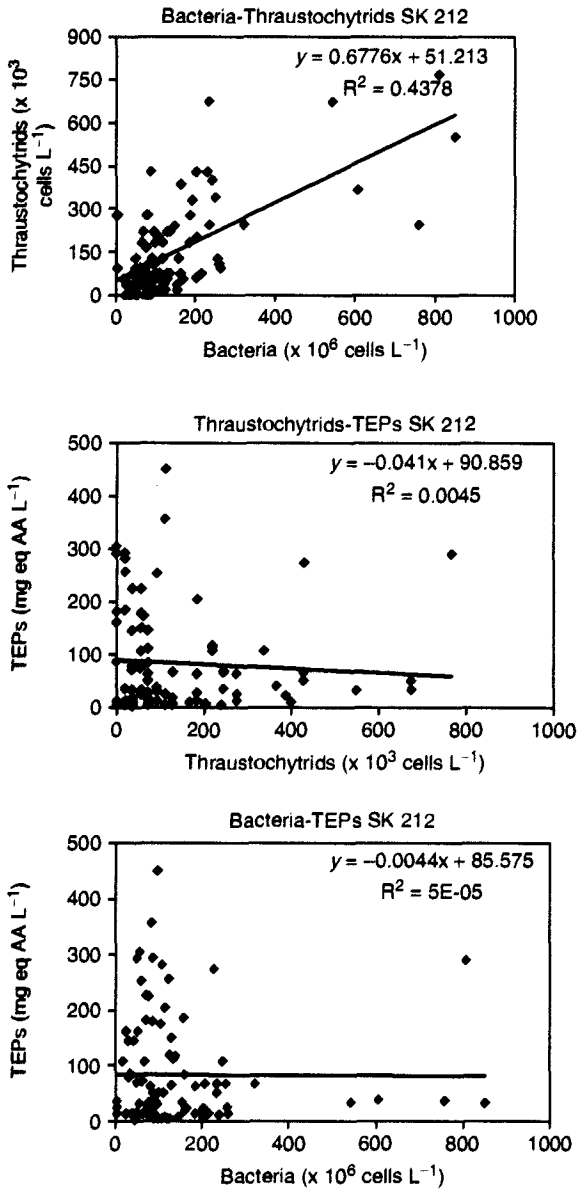

Fig. 3. Regression analyses for thraustochytrid numbers against bacteria and TEPs as well as bacteria against TEPs in the water column during October 2004.

downwards by 20 m. When the analyses were carried out at these stations after shifting the values of these organisms 20 m downwards, a statistically significant relation was noticed between TEPS on the one hand and thraustochytrids and bacteria on the other.

Thraustochytrids and bacteria, but not TEPs, were estimated during the first cruise in September 2003 (SK 196). Thraustochytrids at this time ranged from below detection levels to 598×10^3 cells L^{-1}, contributing up to 12.3 μg L^{-1} C, while bacteria ranged from 49 to 3533×10^6 cells L^{-1}, contributing a maximum of 67.5 μg L^{-1} C. The maximum thraustochytrid biomass contribution to the total of bacterial and thraustochytrid C occurred at 60 m at 0.5°N, where thraustochytrids comprised 79.1% of the total. Thraustochytrids at this station numbered 184×10^3 cells L^{-1}, bacteria amounting to a total of 50×10^6 L^{-1}. The two groups showed no correlations in terms of abundance (Fig. 6).

Roller table experiment to study TEPs, bacteria and thraustochytrids

Experimental results on the association of bacteria and thraustochytrids with TEPs using a roller table carried out on board during the cruise in September 2006 showed that TEPs increased steadily over time up to 12 days (Fig. 7). This was also accompanied by a steady increase of thraustochytrids, the maximum densities reaching up to 400×10^3 cells L^{-1}. These levels corresponded to those occurring in the natural water column. Bacteria showed a much more rapid increase in numbers up to 2 days, followed by a decline. Their numbers, however, increased substantially towards the end of the experiment when TEPs values were high, reaching values of about 200×10^6 cells L^{-1}, also similar to levels found in the water column. Thraustochytrids showed a positive trend with TEPs from day 5 onwards. TEPs from these experiments contained high amounts of mannose (Concanavalin positive), as well as *n*-acetylneuraminic acid (Limulin positive), the positive percentage response to Concanavalin A ranging from 32 to 67 and that to Limulin from 33 to 68 (Table 3).

Discussion

Kimura *et al.* (1999, 2001) suggested that thraustochytrids were associated with allochthonous particles in coastal waters, rather than phytoplankton- and bacterial-derived particulate organic carbon (POC). Raghukumar *et al.* (2001) showed that they were often related to chlorophyll 'a' and POC in the Arabian Sea, suggesting that thraustochytrids might be important in degradation of autochthonous oceanic material. This study unequivocally demonstrates the abundant presence of thraustochytrids in the oceanic waters of the equatorial Indian Ocean far removed from coastal influences, suggesting that they also play a role in the oceans, by utilizing autochthonous sources of nutrients.

The densities of thraustochytrids in the present study corresponded to earlier reports of up to a few hundred thousand cells per liter seawater (Naganuma *et al.*, 1998; Kimura *et al.*, 1999, 2001; Raghukumar *et al.*, 2001; Bongiorni *et al.*, 2005). Bacterial densities, likewise, corresponded to values reported for the adjacent Arabian Sea (Ducklow, 1993; Ramaiah *et al.*, 1996; Wiebinga *et al.*, 1997; Pomeroy & Joint, 1999; Prasanna Kumar *et al.*, 2001). Such

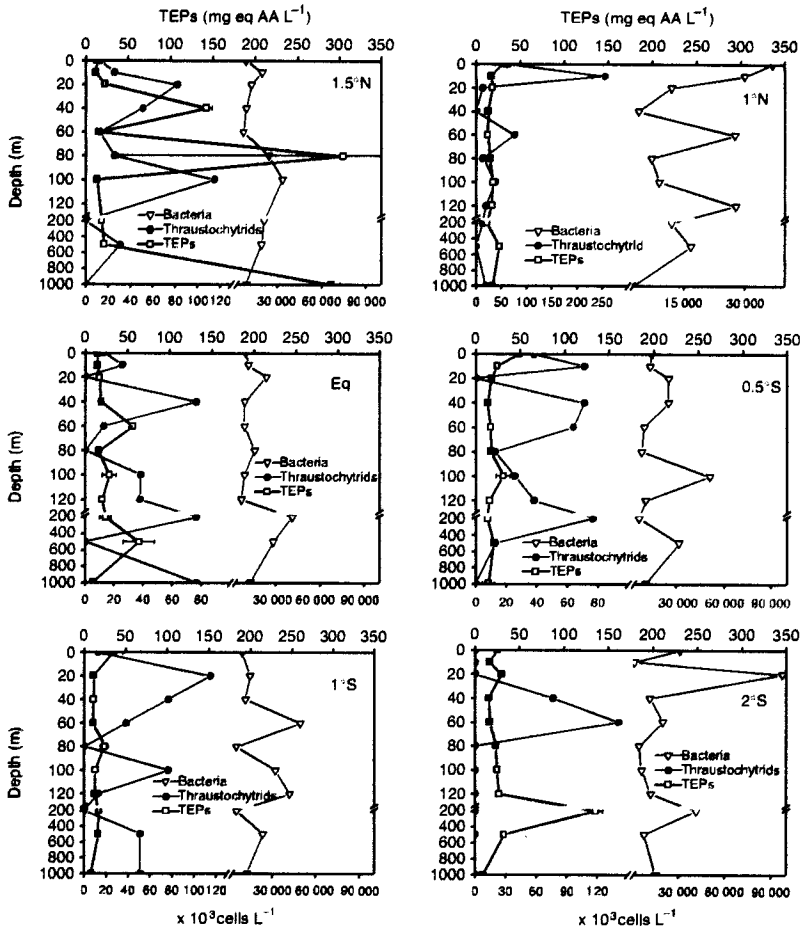

Fig. 4. Distribution of thraustochytrids, bacteria and TEPs in the equatorial Indian Ocean during September 2006.

high densities suggest that the equatorial Indian Ocean may be a biologically productive region.

Each of the three cruises revealed different relations between bacteria and thraustochytrids (Fig. 8). (1) At one end of the spectrum, namely September 2006, thraustochytrids were detected only in 72% of the samples and had the lowest average biomass value of the three cruises (0.74 μg C L^{-1}). This average biomass value was higher than that of the bacterial biomass of 0.53 μg C L^{-1}. However, thraustochytrids showed a high variability in their populations and, therefore, their biomass (SD 0.9; Fig. 8). Thraustochytrid and bacterial abundances were not related. (2) During September 2003, thraustochytrids were found in all the samples (100% frequency of occurrence) and their average biomass in the water column was 2.5 μg C L^{-1}. Bacterial biomass was nearly 10 times that of the thraustochytrids. Bacteria and thraustochytrids showed no relations among each other. (3) Thraustochytrids were found in 98% of the samples during October 2004. Their average biomass was the highest of all three cruises (3.8 μg C L^{-1}) and again exceeded the average bacterial biomass of 3.4 μg C L^{-1}, but with a high variability (SD 3.4; Fig. 8). Thraustochytrids and bacteria showed a highly significant positive relationship. The above results provide various clues and questions regarding thraustochytrids.

(1) Thraustochytrids in the water column may occur in patches of very high density, as also reported by Raghukumar *et al.* (2001) for the Arabian Sea and Bongiorni *et al.* (2005) in fish farm-impacted seagrass sediments. An average thraustochytrid cell of 5.0-μm diameter, 65.41 μm^3 biovolume and 20 pg C (Kimura *et al.*, 1999; Raghukumar *et al.*, 2001) would correspond to 1000 bacterial cells, each of 0.5-μm diameter, 0.065 μm^3 and 20 fg biomass C. Therefore, patches of thraustochytrids would constitute 'hot spots' of nutrition for microbivorous protists, particularly by providing the ω-3 fatty acid, docosahexaenoic acid (DHA), a key essential fatty acid in the growth and maturation of crustaceans (Veloza *et al.*, 2006).

(2) Kimura *et al.* (2001) reported a positive correlation between thraustochytrids and bacteria for waters of Seto

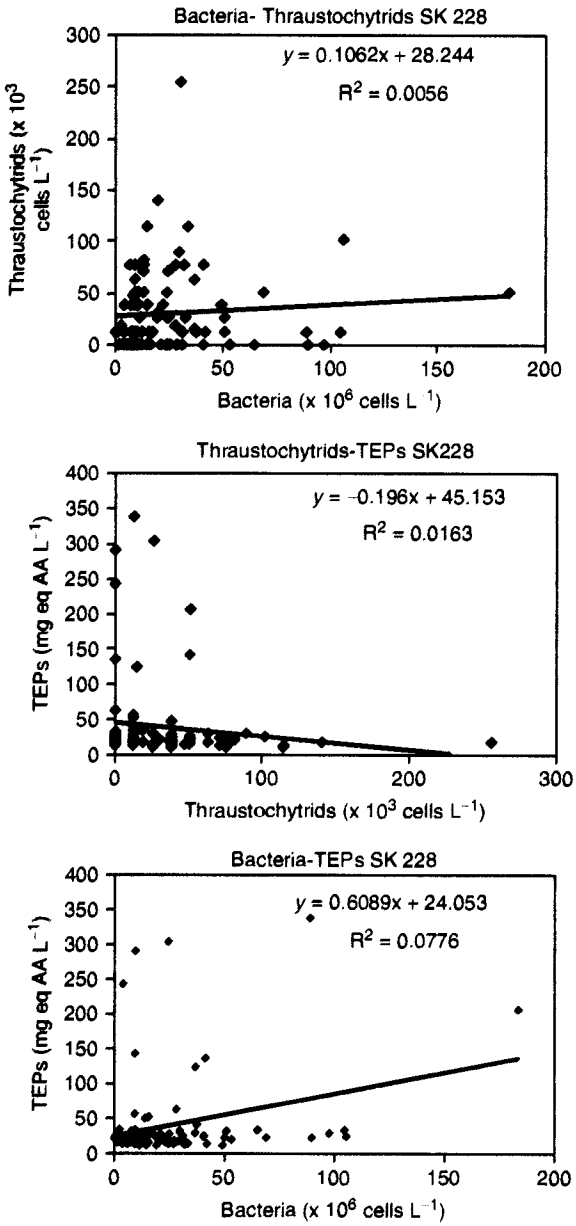

Fig. 5. Regression analyses for thraustochytrid numbers against bacteria and TEPs as well as bacteria against TEPs in the water column during September 2006.

Inland Sea of Japan. Bongiorni *et al.* (2005) also noticed a weakly positive correlation between bacterial and thraustochytrid abundance in fish farm sediments. However, the relationship between bacteria and thraustochytrids in the equatorial Indian Ocean waters was highly variable as found by Raghukumar *et al.* (2001) for the water column of the Arabian Sea. Therefore, thraustochytrids and bacteria may depend on the same or different nutrient sources in the water column.

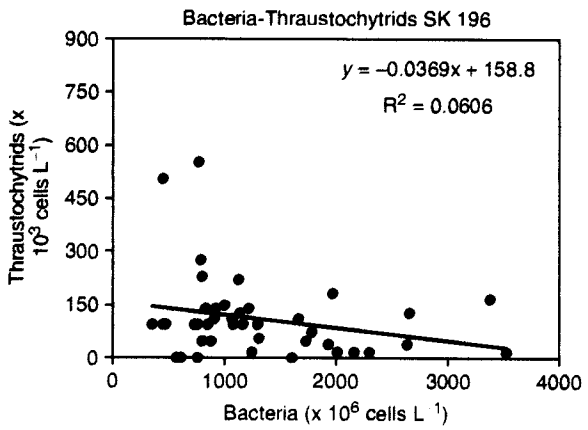

Fig. 6. Regression analyses for thraustochytrid numbers against bacteria in the water column during September 2003.

What were the possible nutrient sources for thraustochytrids? The general patchiness of distribution of thraustochytrids in the water column, both in terms of their frequency of occurrence, as well as abundance, suggested that they were particle dependent. Raghukumar *et al.* (2001) had earlier noticed dense populations of thraustochytrids in association with diatom mucus particles in the water column at the end of the southwest monsoon in the Arabian Sea. Lyons *et al.* (2005) reported the dense presence of the thraustochytrid pathogen QPX in marine aggregates and suggested that aggregates might provide a means for survival and transport of the pathogen. Kimura *et al.* (2001) and Raghukumar *et al.* (2001) found that thraustochytrid abundance was significantly related to POC concentration in the water column. In view of the above, we were prompted to examine the relationship of thraustochytrids and bacteria with TEPs in the water column. We frequently observed the association of thraustochytrids with TEPs in the water column, these too staining with acriflavine used for direct detection of cell wall-sulfated polysaccharides of thraustochytrids (Fig. 9). However, except for a few individual stations, thraustochytrids and TEPs did not show a positive relationship. Therefore, we attempted to study them under experimental conditions using a roller tank. The results indicated a similar trend in changes among thraustochytrids, bacteria and TEPs, suggesting that these relationships are variable, probably depending on the chemistry of TEPs.

Because TEPs may be formed as a result of bacterial activity on phytoplankton exudates (Sugimoto *et al.*, 2007), or even without bacterial intervention directly from diatom exudates (Grossart *et al.*, 2006), the chemistry of TEPs may be important in understanding the relationship of thraustochytrids with them. TEPs are responsible for the formation of larger aggregates in the water column. We could not study the chemistry of the aggregates because none were

Table 1. Results of correlation analyses ('r') values between abundance of thraustochytrids, bacteria and TEPs to each other during SK Cruise # 212 in October 2004

	80.5°E Longitude								
Parameters	2°N	1.5°N	1°N	0.5°N	0°Eq.	0.5°S	1°S	1.5°S	2°S
Thraustochytrids vs.	0.17;	**0.70;**	**0.9;**	0.54;	**0.82;**	0.28;	**0.92;**	0.13;	**0.8;**
bacteria	$P=0.63$	$\mathbf{P=0.02}$	$\mathbf{P<0.001}$	$P=0.11$	$\mathbf{P=0.01}$	$P=0.4$	$\mathbf{P<0.001}$	$P=0.75$	$\mathbf{P=0.003}$
Thraustochytrids vs.	−0.54;	0.09;	**0.85**	−0.11;	**−0.85;**	−0.27;	−0.11;	−0.2;	**0.67;**
TEPs	$P=0.11$	$P=0.78$	$\mathbf{P<0.001}$	$P=0.75$	$\mathbf{P=0.007}$	$P=0.42$	$P=0.74$	$P=0.6$	$\mathbf{P=0.02}$
Bacteria vs. TEPs	−0.14;	0.29;	**0.93;**	−0.51;	**−0.75;**	−0.027;	0.07;	−0.19;	**0.73;**
	$P=0.69$	$P=0.42$	$\mathbf{P<0.001}$	$P=0.1$	$\mathbf{P=0.03}$	$P=0.94$	$P=0.83$	$P=0.63$	$\mathbf{P=0.01}$

Significant values are given in bold.

Table 2. Results of correlation analyses ('r') values between abundance of thraustochytrids, bacteria and TEPs to each other during SK Cruise # 228 in September 2006

	80.5°E Longitude								
Parameters	2°N	1.5°N	1°N	0.5°N	0°Eq.	0.5°S	1°S	1.5°S	2°S
Thraustochytrids vs.	0.19;	0.5;	0.56;	−0.08;	0.07;	−0.27;	0.09;	0.41;	−0.1;
bacteria	$P=0.58$	$P=0.15$	$P=0.07$	$P=0.82$	$P=0.85$	$P=0.42$	$P=0.80$	$P=0.22$	$P=0.78$
Thraustochytrids vs.	−0.22;	−0.27;	−0.09;	−0.20;	−0.31;	−0.22;	−0.57;	−0.009;	−0.23;
TEPs	$P=0.52$	$P=0.46$	$P=0.79$	$P=0.57$	$P=0.35$	$P=0.52$	$P=0.09$	$P=0.98$	$P=0.5$
Bacteria vs. TEPs	−0.2;	−0.08;	0.05;	0.18;	0.21;	0.21;	−0.51;	0.59;	0.29;
	$P=0.57$	$P=0.82$	$P=0.89$	$P=0.61$	$P=0.54$	$P=0.54$	$P=0.13$	$P=0.06$	$P=0.4$

Table 3. Percentage response of TEPs in different water samples to the lectins Concanavalin A and Limulin

Period	Station	Depth (m)	% Positive response to ConA : Limulin	Thraustochytrid, TEPS relation
October 2004	1°N	10	88 : 12	Low TEPs levels; few thraustochytrids
	1°N	120	50 : 50	Overall positive relation
	Eq	60	78 : 22	No overall correlation
	Eq	120	77 : 23	No overall correlation
	2°S	20	54 : 42	Overall positive relation
	2°S	120	25 : 75	Overall positive relation
September 2006	0.5°N	500	83 : 17	No overall correlation
	1.5°N	40	71 : 29	No overall correlation
	1.5°N	1000	91 : 9	No overall correlation
	2°S	200	67 : 33	No overall correlation
Roller table experiment at	Day 3	–	56 : 44	Positive from day 5 onwards
different days during	Day 5	–	32 : 68	
September 2006 (SK 228)	Day 7	–	54 : 46	
	Day 9	–	56 : 44	
	Day 14	–	67 : 33	

detected in the natural water samples, probably owing to the sampling techniques. Nor were aggregates generated in the roller table experiment. Therefore, we examined the TEPs for a few sugars using lectins. Michael & Smith (1995) have used lectins to describe chemically complex carbohydrate moieties and the heterogeneous structure of microbial biofilms on glass. Wigglesworth-Cooksey & Cooksey (2005) reported that extracellular polymeric substances (EPS) of diatoms stained with Concanavalin A and not with a lectin specific for fucose. Khandeparker *et al.* (2003) noticed that EPS from bacteria stained positive with limulin for *n*-acetylneuraminic acid, glucuronic acid and phosphorycholine and not for *n*-acetyl-D-galactosamine, suggesting that the latter was more part of the bacterial capsular polysaccharides. We chose Concanavalin A and Limulin for studies based on the above. Our preliminary results suggest that thraustochytrids were generally positively related to TEPs concentrations that were relatively higher in

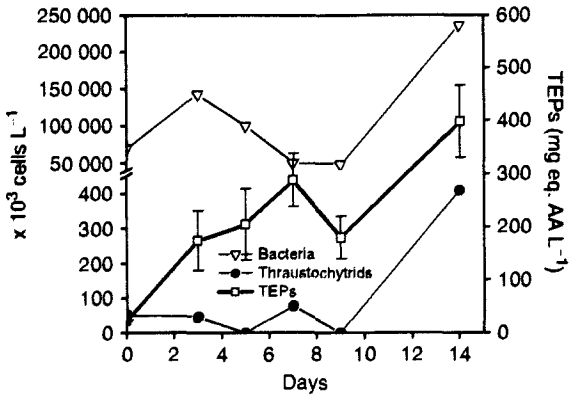

Fig. 7. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted on board cruise # SK 228 during September 2006.

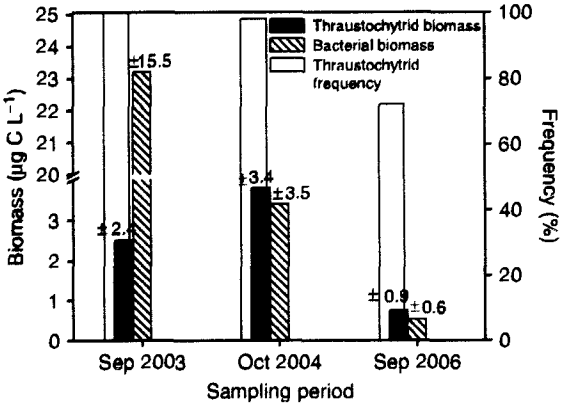

Fig. 8. Thraustochytrid frequency and biomass with respect to bacterial biomass during three cruises. SD values (±) are given above the columns.

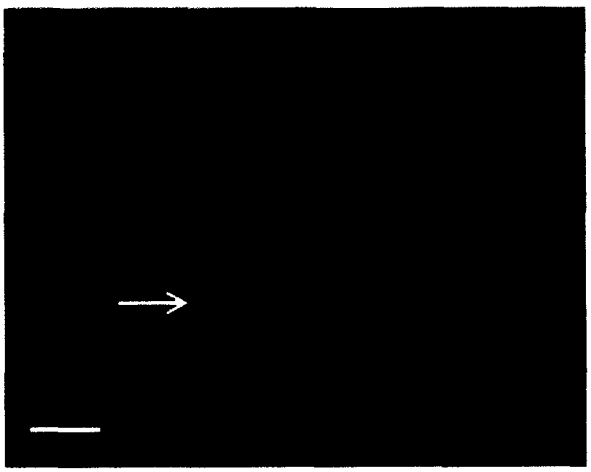

Fig. 9. Photomicrograph of an acriflavine-stained thraustochytrid cell (marked by arrow) from a natural sample on a black filter paper along with other stained particles. Scale bar represents 10 µm.

n-acetylneuraminic acid (limulin positive) as in two stations during October 2004 and TEPs generated in the laboratory (Fig. 7). TEPs from the September 2006 cruise that were richer in mannose and glucose (Concanavalin positive) showed no definite relationship with thraustochytrids. Further studies along these lines might shed more light on thraustochytrids.

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