

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

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to the

**GOA UNIVERSITY**



by

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## **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.

**Varada S. Damare**

## **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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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

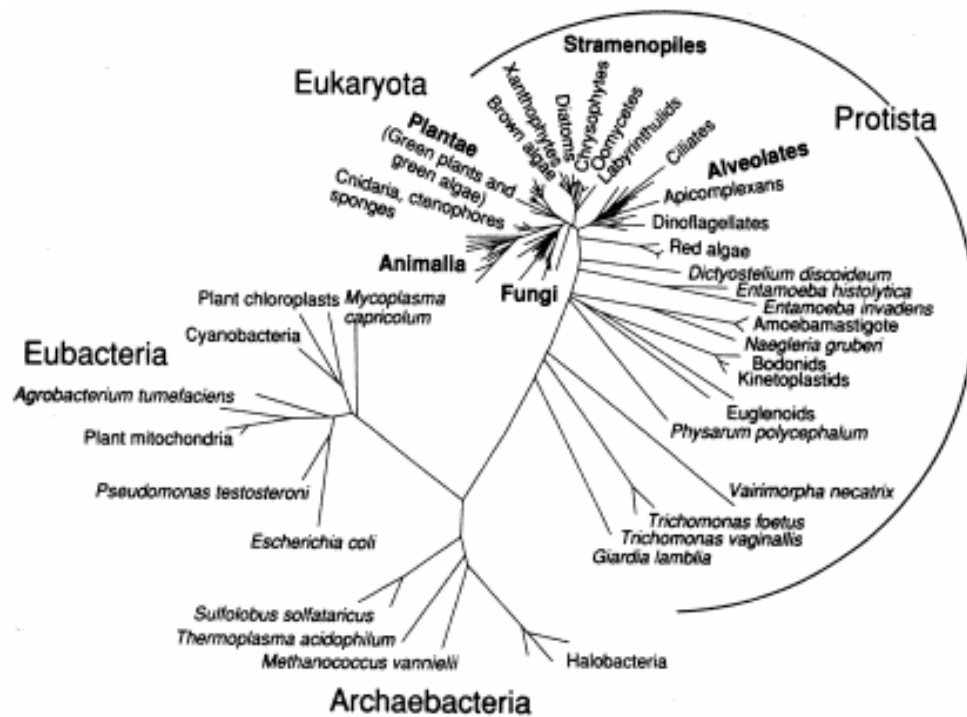
Thraustochytrids and aplanochytrids are eukaryotic, unicellular organisms belonging to the Kingdom Chromista or Stramenipila (also called Stramenopila), which also includes diatoms, brown algae, the oomycetan fungi and a variety of flagellates ([Mozley \*et al.\*, 2003](#)) (Fig. 1). They are further 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

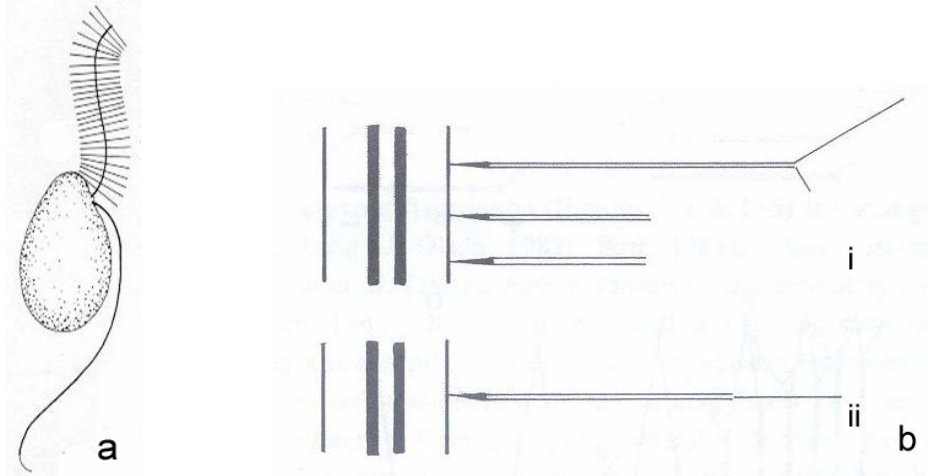
the sagenogenetosome or bothrosome (Porter, 1990) (Fig. 3). Besides helping them in absorbing nutrients from the environment, the EN also help cells to attach to a substratum (Raghukumar, 2002).

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

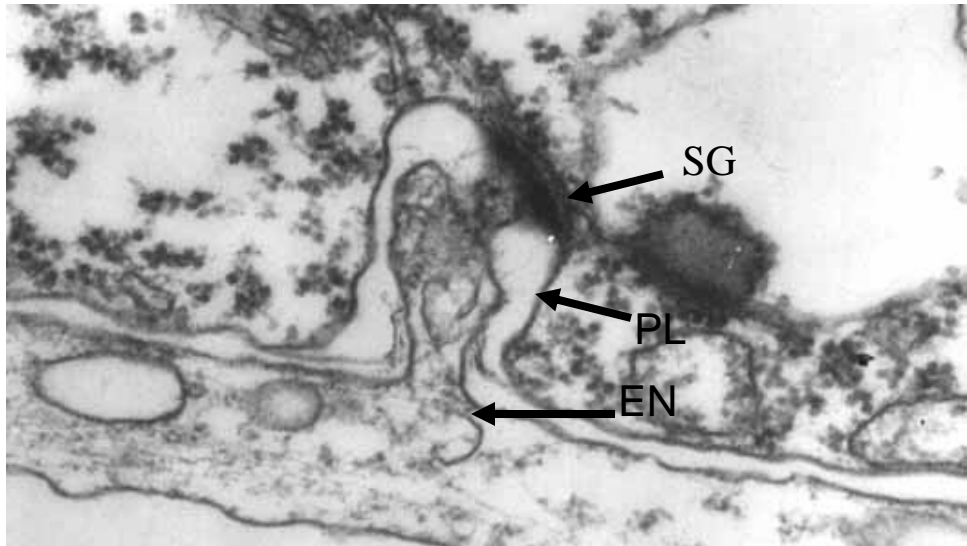




**Fig. 2. (a) A typical, straminipilan, heterokont zoospore characteristic of Labyrinthulomycetes, showing a whiplash and a tinsel flagellum. (b) Arrangement of tripartite hairs on the tinsel flagellum in other straminipiles (i) and Labyrinthulomycetes (ii) (From Dick, 2001)**



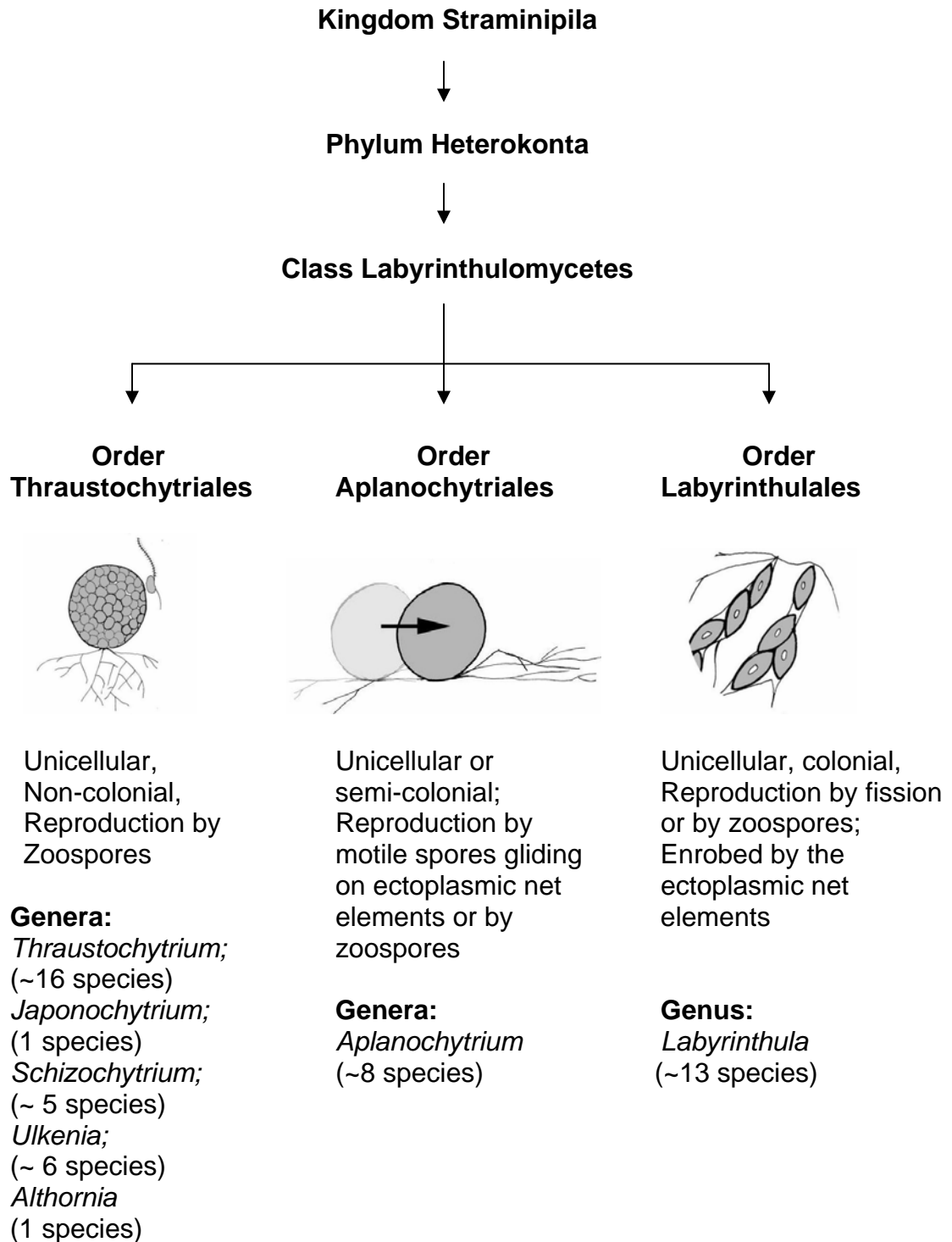
**Fig. 3. Electron micrograph of sagenogenetosome or bothrosome of Labyrinthulomycetes (SG) and the invaginated plasmalemma (PL) that extends out as the ectoplasmic net element (EN). (From <http://www.botany.uga.edu/zoosporicfungi/amgz.htm>)**



## 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.**



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<sup>+</sup> ions which cannot be replaced by K<sup>+</sup>. 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 ( $\omega$ -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 19<sup>th</sup> 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; Bongiorno & 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 detrital 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.**

<b>Location</b>	<b>Reference</b>
Thraustochytrids in decaying seaweed, <i>Fucus serratus</i> , England	Miller & Jones, 1983
<i>Labyrinthula</i> in the seagrass <i>Zostera marina</i>	Muehlstein <i>et al.</i> , 1988
<i>Aplanochytrids</i> in marine algae	Leander <i>et al.</i> , 2004
Thraustochytrids and aplanochytrids in brown algal detritus of <i>Sargassum cinereum</i> , India	Sathe-Pathak <i>et al.</i> , 1993
Labyrinthulomycetes in decaying mangrove leaves	Findlay <i>et al.</i> , 1986
Thraustochytrids in phytoplankton detritus, North Sea	Raghukumar & Schaumann, 1993
Thraustochytrids in faecal pellets of salp <i>Pegea confoederata</i> , 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 <i>et al.</i> , 2005
Thraustochytrids in water column, of the Arabian Sea	Raghukumar <i>et al.</i> , 2001
Water column, Seto Inland Sea, Japan and Hyuga-nada area, Japan	Kimura <i>et al.</i> , 2001
Water column, Seto Inland Sea, Japan and Hyuga-nada area, Japan	Kimura <i>et al.</i> , 1999
Water column, Seto Inland Sea, Japan	Naganuma <i>et al.</i> , 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.

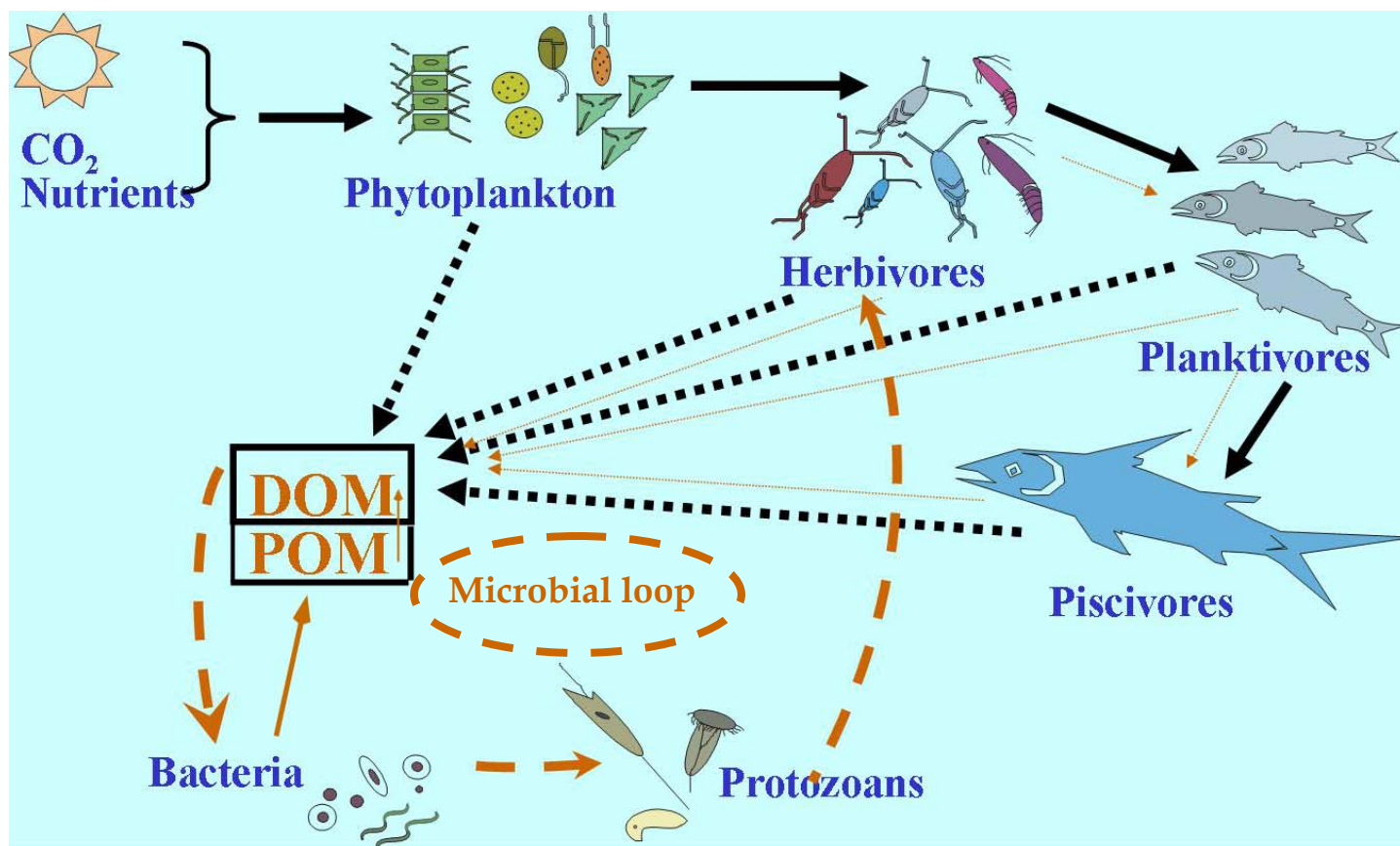
### **1.5 Occurrence in the water column**

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<sup>-1</sup>. 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  $\mu\text{m}$ ), 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.