

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

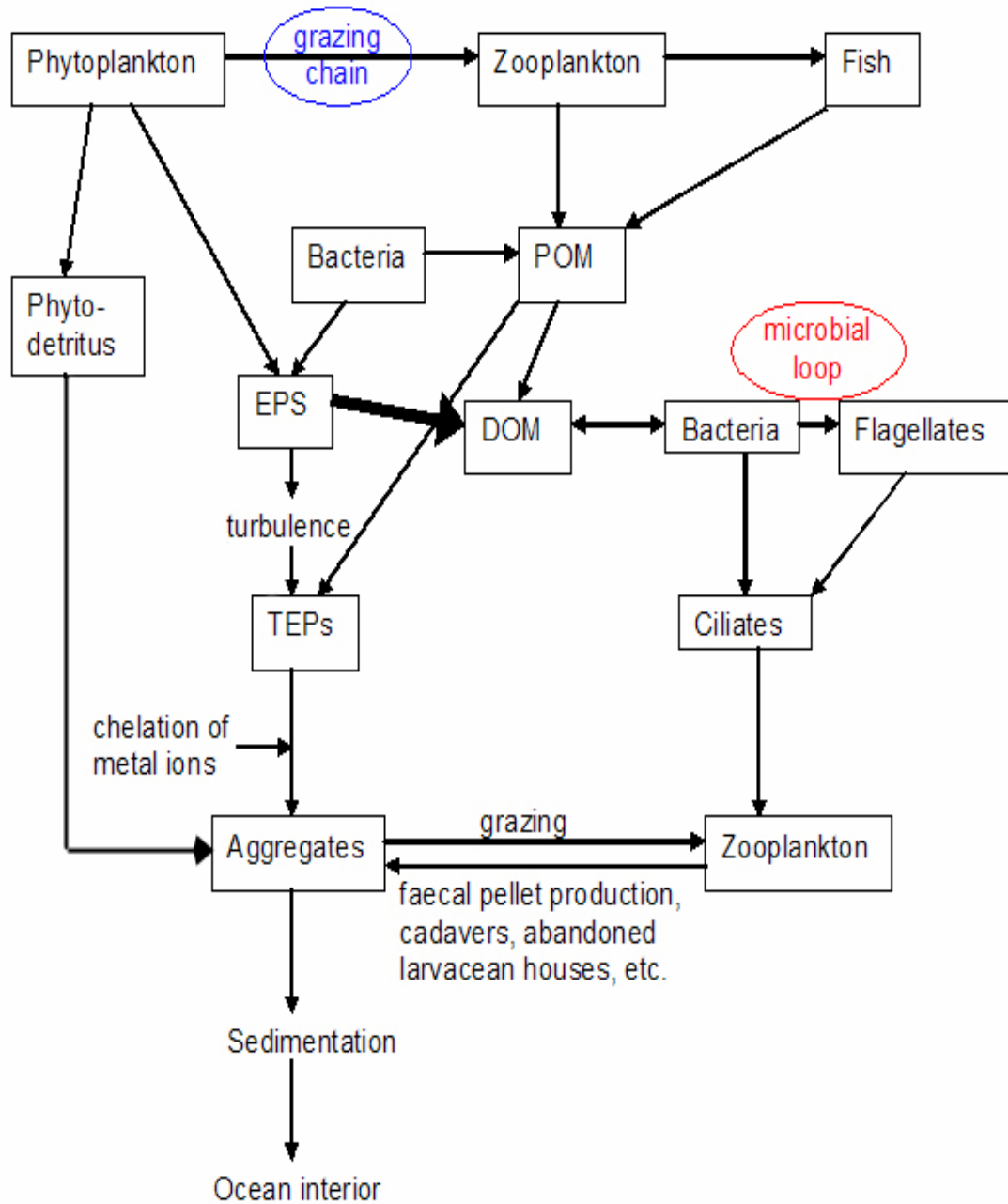
are light and transparent in nature and therefore called “transparent exopolymeric particles” or TEPs (Aldredge *et al.*, 1993; Beauvais *et al.*, 2003). Bacteria, phytoplankton and also zooplankton contribute directly or indirectly to formation of TEPs via the excretion of dissolved organic matter (Aldredge *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 ([Aldredge 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 (Aldredge *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; Bongiorno *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).

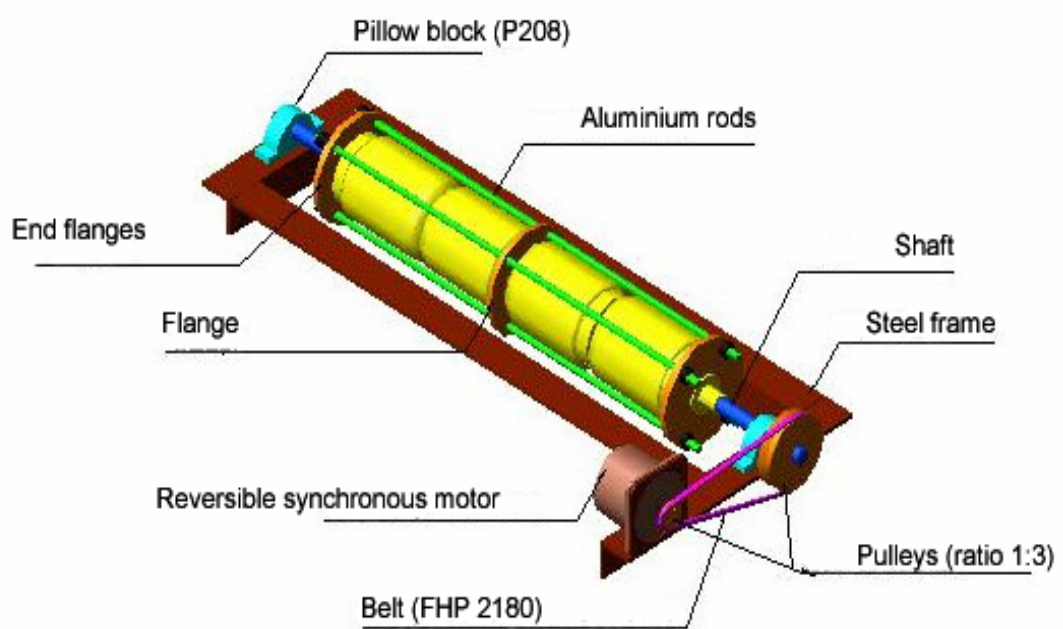
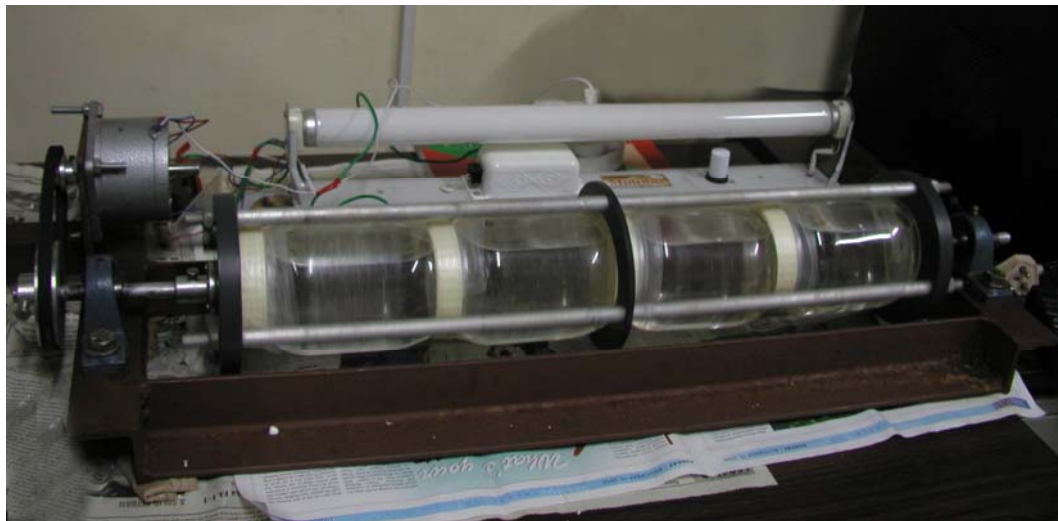


Fig. 2b. Photograph of bottles mounted on the roller table (plankton wheel)



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 Zobell 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^o 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^o 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 calcofluor 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.

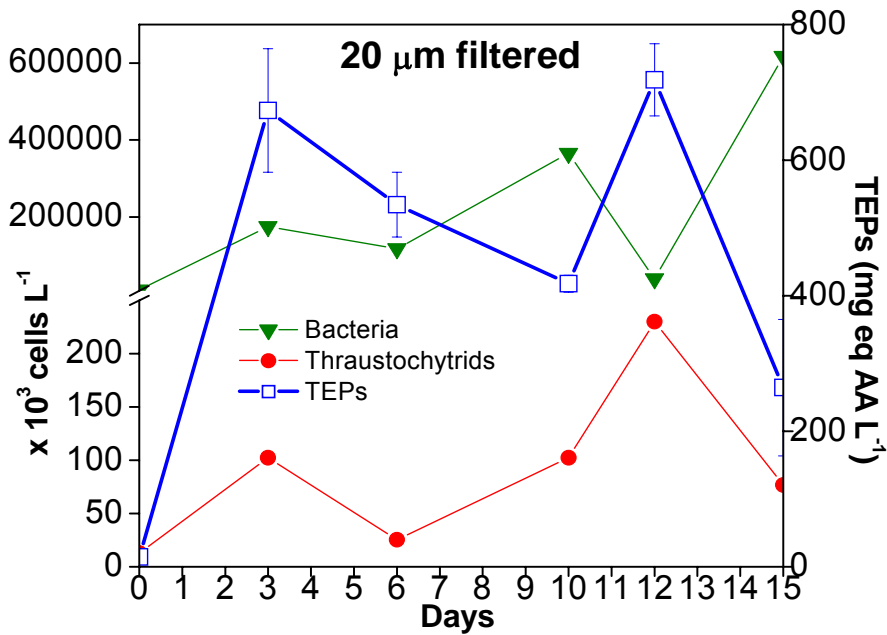
Calcofluor staining was carried out by incubating the aggregate in 0.5 % calcofluor for 1 min, draining the stain and washing by sterile distilled water to wash off excess stain (Damare *et al.*, 2006). The aggregate was then mounted on a glass slide and observed under UV by epifluorescence microscope.

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 25.5×10^3 cells L^{-1} on day 6 to 230×10^3 cells L^{-1} on

Day 12. Bacterial numbers ranged from 39.6×10^6 cells L^{-1} , as seen on Day 12, to 617.8×10^6 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 400×10^3 cells L^{-1} by Day 14, while bacteria reached values of about 200×10^6 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.

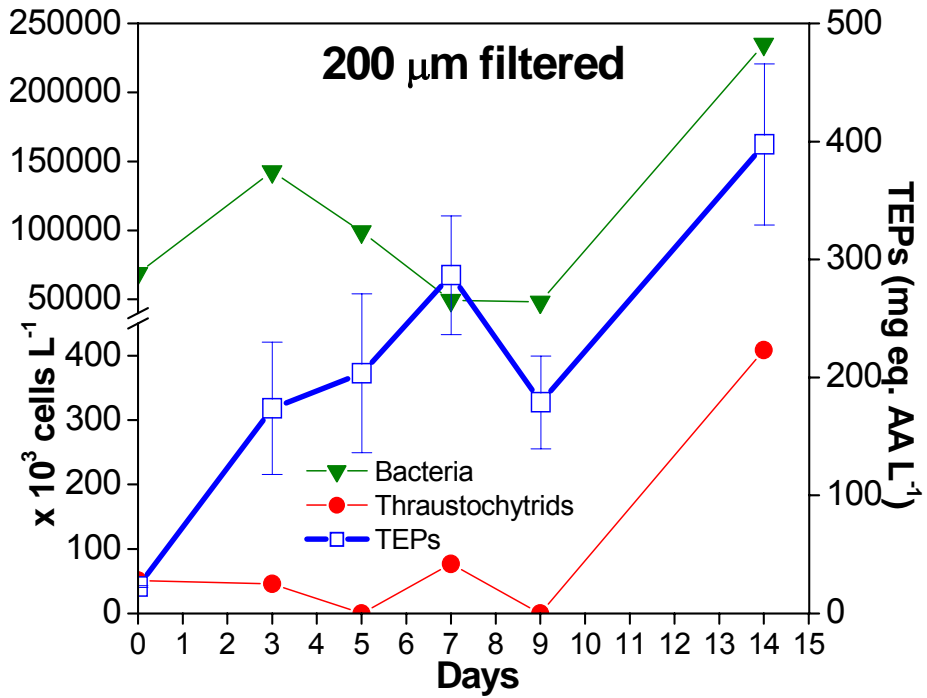
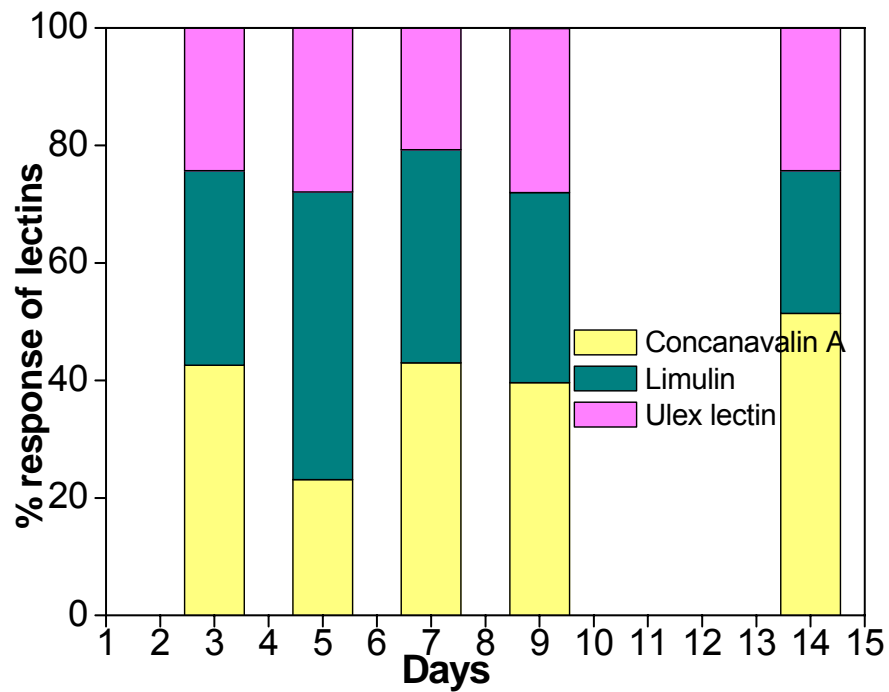


Fig. 5. Percentage response of TEPs to various lectins with respect to time in the roller table experiment conducted using 200 μm filtered equatorial Indian Ocean water on board cruise # SK 228 during September 2006.



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 408.9×10^3 cells L^{-1} on Day 15, while bacterial numbers reached a peak of 1757.7×10^6 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 μ m filtered coastal seawater.

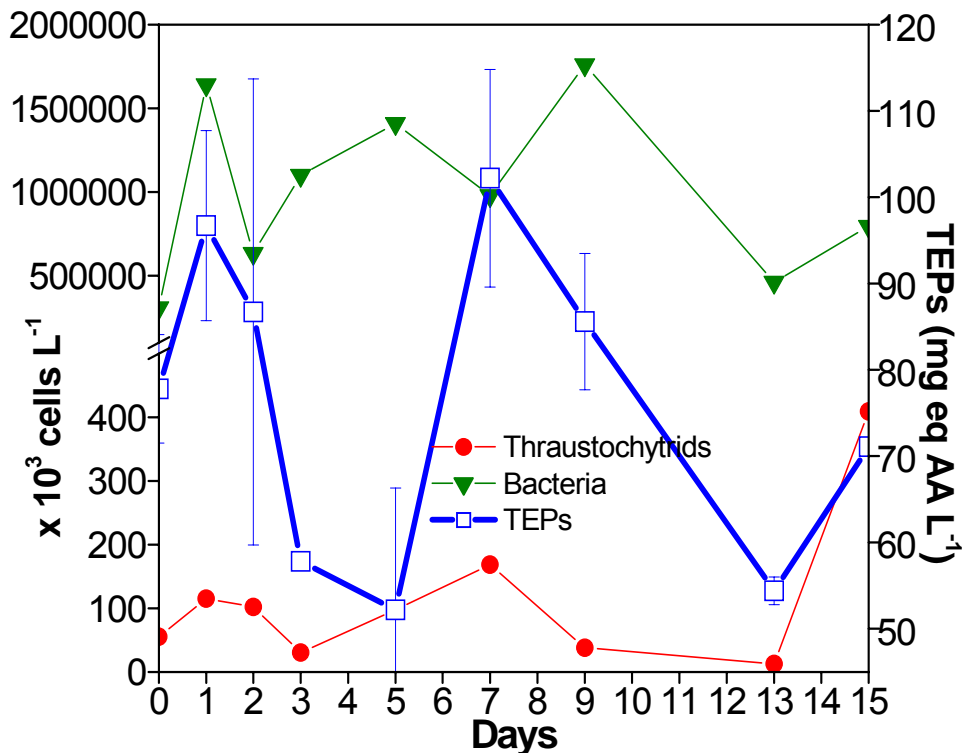


Table 1. Correlation analysis of thraustochytrids, bacteria and TEPs with each other from roller table experiment conducted using 20 μm filtered coastal seawater.

	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

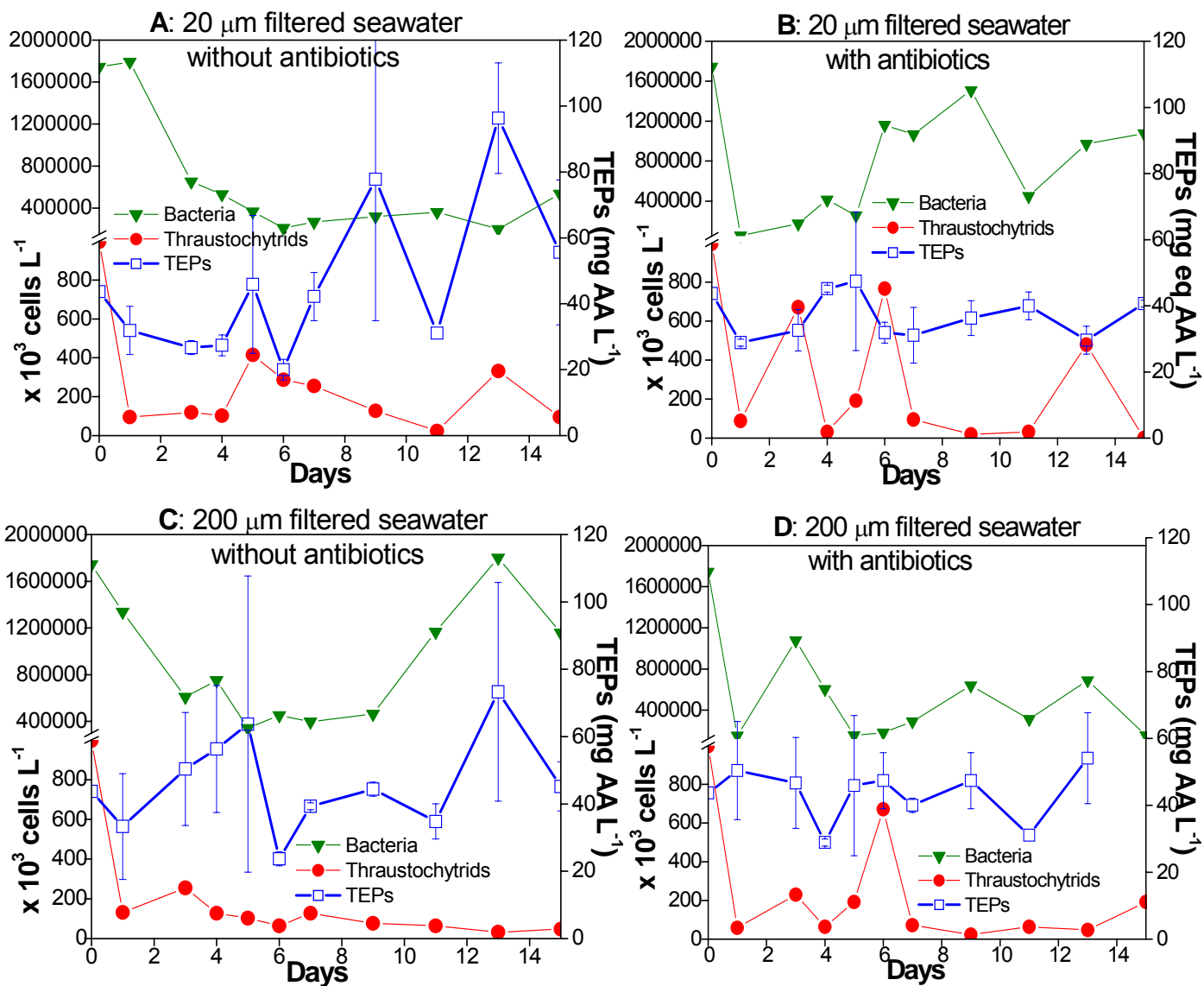
Experiment 4. TEPs from the coastal waters of Goa, in the presence and absence of bacteria and phytoplankton:

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 (Bottle 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 766.7×10^3 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 calcofluor, 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.

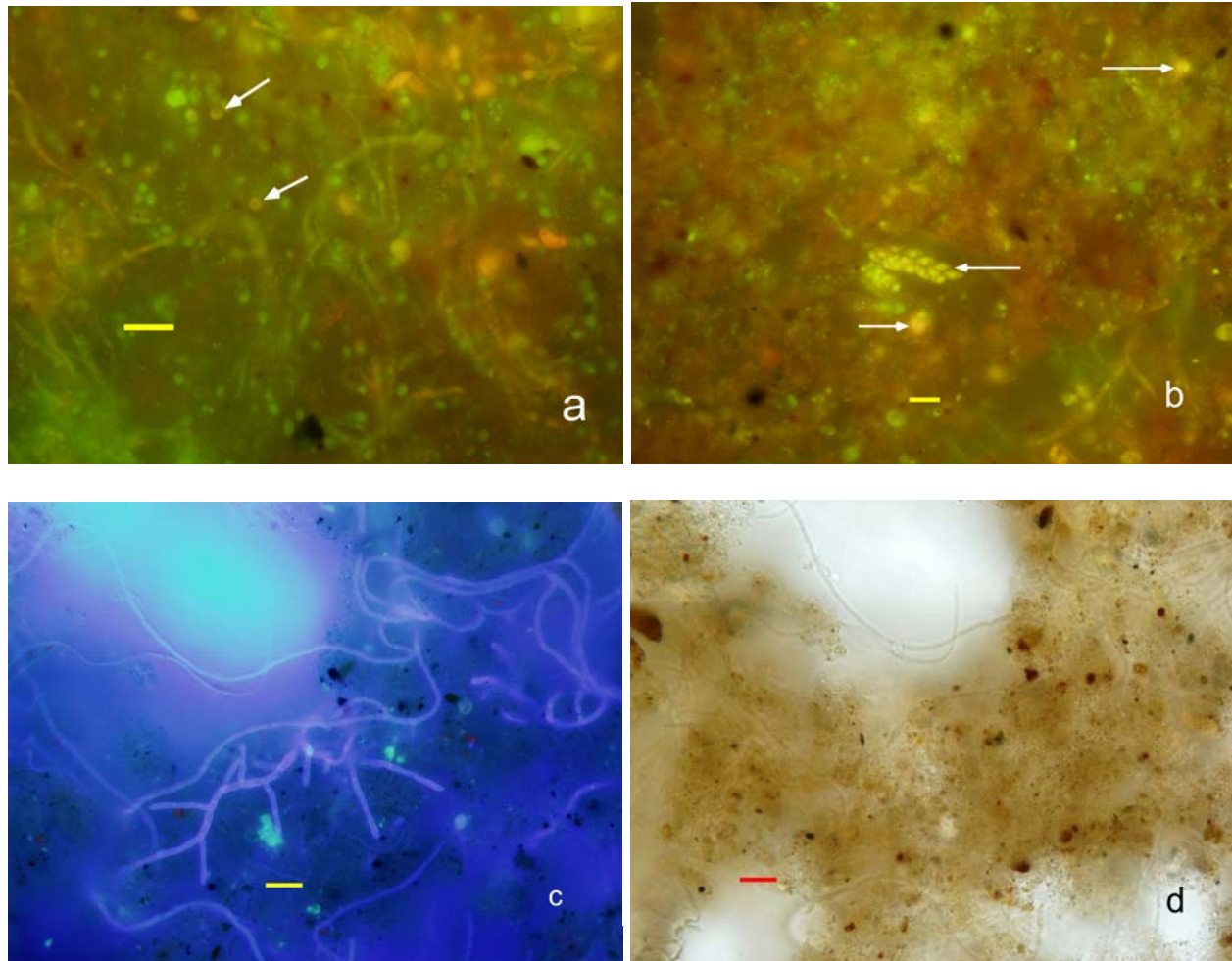


Table 2. Correlation analysis between thraustochytrids, bacteria and TEPs of experiment 4 (N=11).

	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.

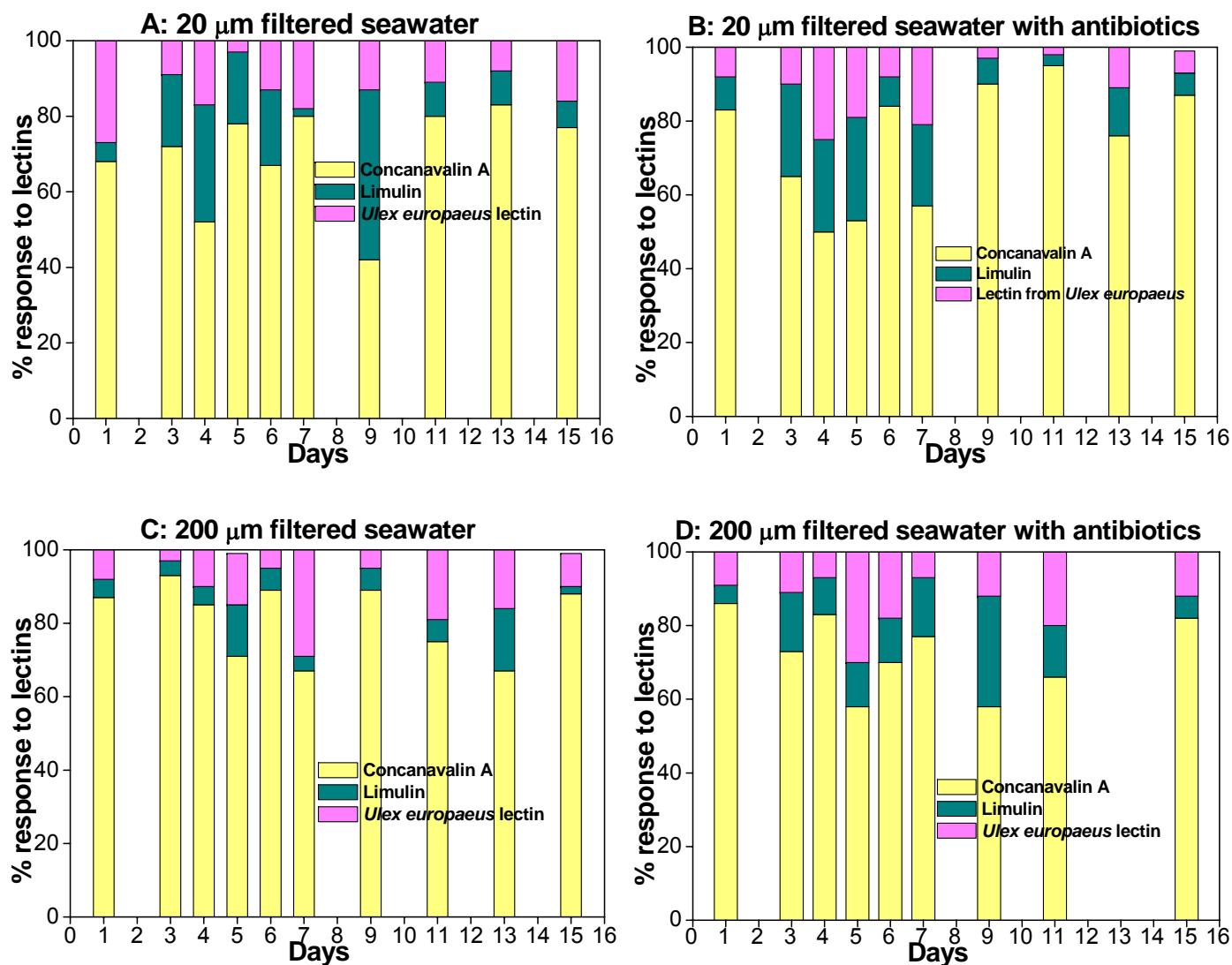


Table 3. Results of two-way ANOVA of percentage response of TEPs from coastal waters to 3 lectins. The water samples were subjected to four different treatments (presence or absence of bacteria and phytoplankton) and incubated for 16 days.

Conditions tested	% response of Concanavalin A		% response of Limulin		% response of lectin from <i>Ulex europaeus</i>	
	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 1705×10^3 cells L^{-1} . Bacteria attained maximum density of 3484×10^6 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.

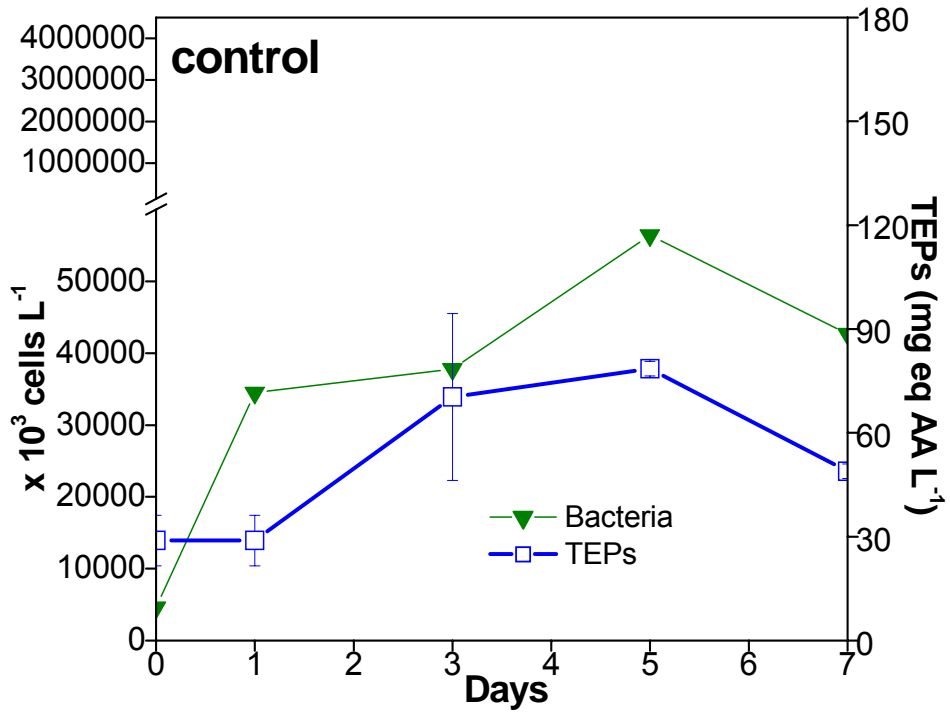
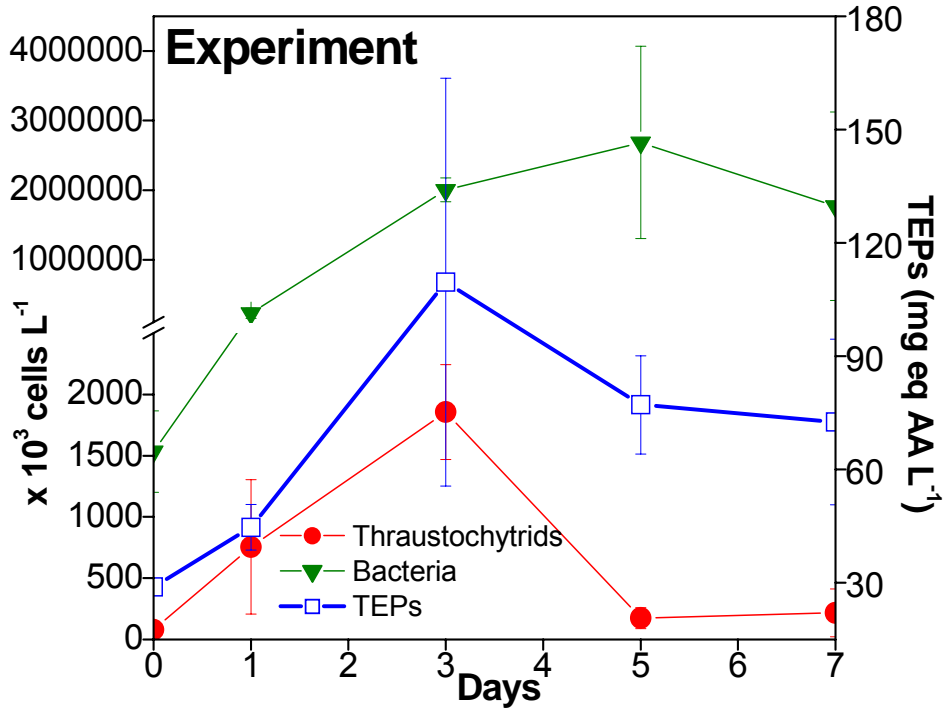


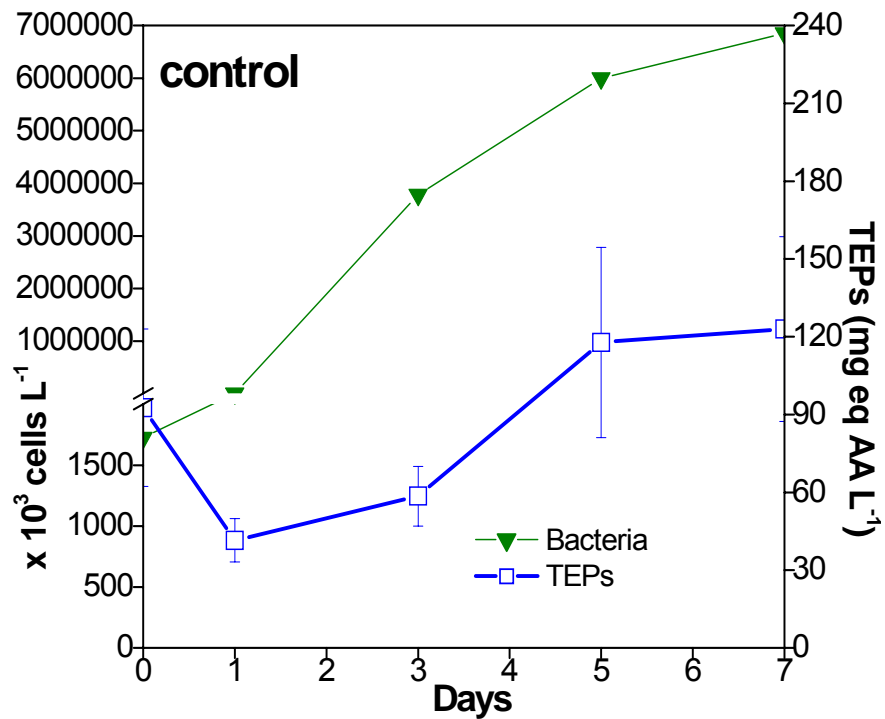
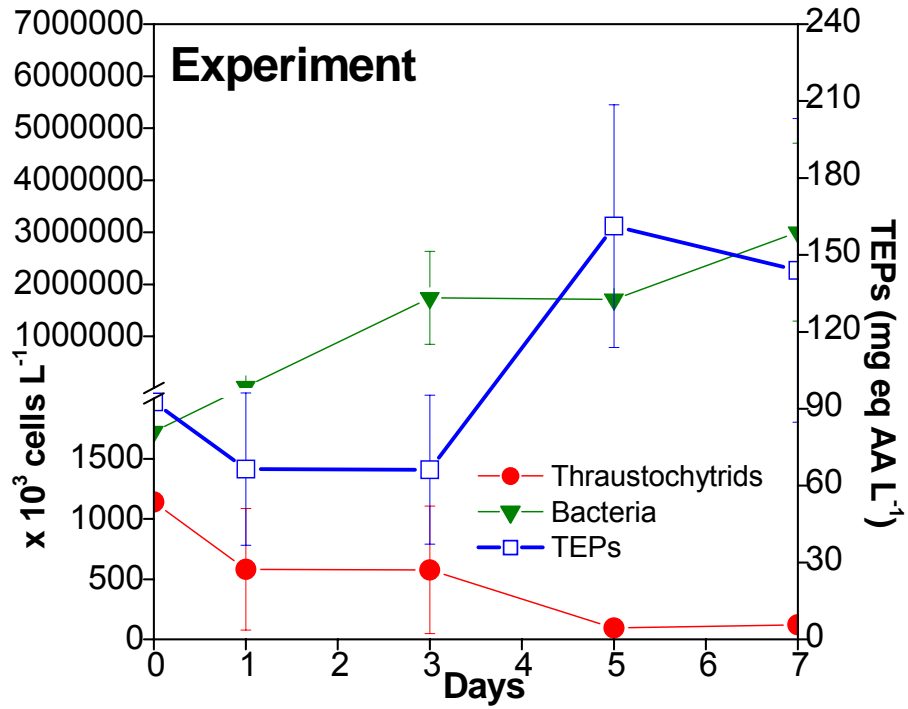
Table 4. Correlation analysis between thraustochytrids, bacteria and TEPs in the roller table experiment using diatom culture filtrates in the bottles.

	Experiment 5 (TEPs generated from the diatom <i>Chaetoceros</i>) (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 122.7×10^3 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 3003×10^6 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 microzooplankton 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*-acetyl 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 filtered 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.