

Marine aggregates and transparent exopolymeric particles (TEPs) as substrates for the stramenopilan fungi, the thraustochytrids: Roller table experimental approach.

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Abstract

In order to understand the kind of organic substrates utilized by thraustochytrids in the coastal water column, their growth in the presence of Transparent Exopolymeric Substances (TEPS) and aggregates generated in roller table experiments was studied. Thraustochytrids appeared to be inhibited in the presence of microphytoplankton. However, they grew well on diatom polysaccharides in the absence of living diatom cells, but not in the presence of bacterial polysaccharides. TEPs formed in the presence of only nanoplankton and bacteria in the water column were highly favourable for the growth of thraustochytrids. The relationship between bacteria and thraustochytrids varied in the experiments. Aggregates formed in water, stimulated by an initial addition of antibiotics, were extremely favourable for the growth of not only thraustochytrids, but also mycelial fungi. This study demonstrates that thraustochytrids could multiply on TEPs and aggregates formed in situ in seawater, even in the absence of additional nutrients.

Introduction

Thraustochytrids are marine, stramenopilan fungi and are found in a wide variety of habitats, like coastal and oceanic waters, soils, mangroves, seagrasses (Raghukumar, 2002). Recently they have gained biotechnological importance due to the production of polyunsaturated fatty acids, especially ω -3 docosahaexanoic acid (DHA), carotenoids and enzymes (Raghukumar, 2008). Several studies have shown that thraustochytrids play an important role in ecological processes of the sea (Raghukumar & Damare, 2011). Relation of thraustochytrids with particulate organic matter in the sea suggests that marine aggregates could be one of the potential habitats of thraustochytrids in the water column.

Marine aggregates or 'marine snow' are the particles that resemble snow flakes. These comprise detrital and inorganic particles, faecal pellets and cadavers of zooplankton and microorganisms. These 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). Formation of these aggregates is a complex process. Several aquatic organisms release copious amounts of dissolved organic carbon in the form of extracellular polymeric substances (EPS) (Verdugo *et al.*, 2004). Heterotrophic bacterial processes on the biologically labile organic carbon of the EPS gradually convert them into increasingly refractory material. This coagulates to form fibrillar, sticky mucopolysaccharides. These are light and transparent in nature and called "transparent exopolymeric particles" or TEPs (Alldredge *et al.*, 1993; Beauvais *et al.*, 2003).

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 leading to the formation of large aggregates, the marine snow (Passow *et al.*, 2001; Engel *et al.*, 2004; Verdugo *et al.*, 2004).

TEPs and marine aggregates are potential substrates for microbial growth. Various studies being carried out on microbial dynamics, microbial degradation of organic carbon and nitrogen on TEPs, mostly focus on the role of bacteria in degradation (Passow, 2000; Grossart & Plough, 2001; Kiorboe, 2001). Little is known about its relation with other microbes. Raghukumar *et al.* (2001) found dense numbers of thraustochytrids on aggregates in Arabian Sea. TEPs have been generated in laboratory from various sources using devices like rolling tables and Couette flocculators (see Damare *et al.*, 2011). The present study focuses on the relationship of thraustochytrid fungi with TEPs and aggregates.

Material and Methods

All experiments were carried out using a roller table to generate TEPs and aggregates (Shanks & Edmondson, 1989). The roller table device used in this study consisted of four 1 litre sampling bottles housed in a cage made up of 4 aluminium rods mounted horizontally on 2 end flanges and also one centre flange to firmly secure the bottles, The entire cage was mounted on a steel frame and was driven by an electric motor using a pulley and belt arrangement at a speed of 23 rpm (Damare *et al.*, 2011). Sunpet bottles with screw capped lids were used as sampling bottles and were surface sterilized with ethanol prior to use.

Formation of TEPs/aggregates from coastal waters of Goa.

Experiments were carried out using surface seawater collected from Dona Paula Bay. Four bottles were filled with water. 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 meso- and micro- plankton, but retaining nano- and pico-plankton. The other set (C and D) was filled with 200 µm-filtered seawater to exclude mesoplankton, but to retain micro- and nano- plankton, 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 on the roller table. Samples were withdrawn from each bottle on day 1, 3, 4, 5, 6, 7, 9, 11, 13 and 15.

The samples were preserved with formaldehyde at a final concentration of 2 % and stored at 5° C. Total bacteria, thraustochytrids and TEPs were analyzed. 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). 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). A BX60 Olympus epifluorescence microscope was used for all observations. TEPs were estimated 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 787nm 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).

Analysis of carbohydrate chemistry of TEPs

Carbohydrate chemistry of the TEPs was studied using FITC-labeled lectins, as follows: (1) Concanavalin A, which is specific for D-mannose, D-glucose and fructofuranose residues, found in the cell wall or membranes of most biological systems like algae, yeasts, fungi; (2) Limulin which is specific for n-acetylneuraminic acid, glucuronic acid and phosphorycholine analogs, found in bacteria; and *Ulex europaeus* lectin which is specific for fucose as found in diatoms, aplanochytrids. The methodology of Khandeparker *et al.* (2003) was followed. A total of 100 microscope fields were examined under an epifluorescence microscope and the numbers of samples positive for each lectin were calculated.

Epifluorescence observations of aggregates generated in roller table experiment

The aggregates formed in bottles were stained with acriflavine and calcofluor separately to check out for thraustochytrids and fungi. In case of thraustochytrids, acriflavine direct detection (AfDD) staining was carried out on the aggregate by placing it 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.

Growth of thraustochytrids on TEPS of diatom origin

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.

Growth of thraustochytrids on TEPs of bacterial origin

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. A 5 % inoculum concentration was used. 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 stirred ultrafiltration cell (Amicon, USA). EPS was precipitated at 4° C overnight by adding absolute ethanol to a final concentration of 70 %. The precipitate was 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 55 mL volume of the EPS was stored at -20° C till further use. 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.

Results

TEPs from natural waters

Thraustochytrids attained significantly higher numbers in experimental bottles A and B in which phytoplankton greater than 20 µm had been excluded (20 µm filtered seawater), than in those where they were present (bottles C and D). Among the two, their numbers were greater when an initial dose of antibiotics was added (treatments B). The highest number of thraustochytrids was found in treatment B, containing 20 µm filtered water to which antibiotics had been added, their numbers ranging from below detectable limits to 766.7×10^3 cells L⁻¹ (Fig. 1). Least number of thraustochytrids were found in water containing phytoplankton of the size greater than 20 µm (in 200 µm filtered water) without an initial dose of antibiotics (treatment C). Thraustochytrids showed one or more peaks during the entire experiment.

Bacterial numbers were generally high in all treatments. However, their numbers were significantly higher in microplankton-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 (B and D) showed the presence of large visible aggregates measuring 0.04 to 6 mm (Fig. 2). 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 seawater containing no microplankton and no initial dose of antibiotics (treatment A), where the maximum concentration of TEPs reached a value of 96.3 mg eq AA L⁻¹.

Aggregates from treatments B and D revealed several spindle-shaped cells, resembling those of the labyrinthulid genus *Labyrinthula* (Fig. 3b), in addition to numerous cells of thraustochytrids (Fig. 3a). Calcofluor, stained aggregates revealed numerous fungal hyphae (Fig. 3c). These were not visible using bright field microscopy (Fig. 3d). Although bacteria, thraustochytrids and TEPs varied between treatments and days, none of these was statistically related to each other (**Table 1**).

Lectin-based carbohydrate study showed that TEPs were mostly positive for D-mannose, D-glucose and fructofuranose (Concanavalin). The overall the percentage response ranged from 50 to 95 % (Fig. 4). Percentage positive response to fucose (lectin from *Ulex europaeus*) was generally the least, being distributed unevenly in all the treatments. Response to n-acetylneuraminic acid, glucuronic acid and phosphorylcholine analogs (Limulin) was next to that of Concanavalin. Two-way ANOVA (**Table 2**) 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 and the least in bottle C (Fig. 4). The changes were not significant between the different days within a treatment.

TEPs from diatom origin

There was a significant positive correlation between TEPs and thraustochytrids in this experiment ($r=0.6$, $p=0.02$) (**Table 3**; Fig. 5). 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 10³ cells L⁻¹. Bacteria attained maximum density of 3484 x 10⁶ 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. TEPs concentration in these bottles reached a peak on Day 5 as against Day 3 in the experimental bottles. The maximum concentration of TEPs in control bottle was 78.5 mg eq AA L⁻¹.

TEPs from bacterial origin

Thraustochytrid numbers steadily decreased throughout the experiment. Their numbers decreased from 1141 to 122.7 x 10³ cells L⁻¹ (Fig. 6). 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 4**).

TEPs reached a peak of 161.2 mg eq AA L⁻¹ on Day 5. Bacteria increased steadily reaching maximum density of 3003 x 10⁶ cells L⁻¹ on Day 7. Bacterial numbers increased steadily in the control bottle also 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 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.

Discussion

Thraustochytrids multiplied in the roller table bottles in all the experiments where *in situ* coastal water from Dona Paula, Goa was used, without any additional nutrient or inoculum (Fig. 1). TEPs concentration also increased in those bottles and macroaggregates were formed in a few. This indicates that TEPs might serve as a substrate for thraustochytrids for settlement and growth.

Thraustochytrids may be inhibited in the actual presence of live phytoplankton. Thus, their numbers were much less in experiments containing 200 µm filtered seawater than 20 µm filtered seawater (Figs. 1). Phytoplankton between the size range of 20-200 µm appear to be dominant over the smaller ones in coastal waters of Goa (Garg & Bhaskar, 2000). Raghukumar *et al.* (2001) 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) observed that with respect to macroalgae, their populations increase only after senescence and death of the macroalgae.

Chemistry of the TEPs might be an important determinant as a substrate for thraustochytrids. TEPs generated from diatoms might be an excellent nutrient source for thraustochytrids. Thus, they grew well when a diatom polysaccharide was added to the experimental bottles in the absence of phytoplankton cells (Fig. 5). Therefore, while live phytoplankton might be inhibitory, phytoplankton polysaccharides and their TEPs are probably utilized well by thraustochytrids for their growth and multiplication.

On the other hand, thraustochytrids displayed poor growth in the presence of bacterial polysaccharides (Fig. 6). Generally bacterial extracellular polysaccharides (EPS) are relatively less labile as compared to phytoplankton EPS (Bhaskar & Bhosle, 2005). Bacterial EPS are rich in glucose, galactose and uronic acids while phytoplankton EPS are rich in rhamnose, mannose and xylose and are relatively poor in uronic acids (<5 % as against 20-50 % in bacterial EPS). The poor growth of thraustochytrids on bacterial EPS may be the result of the poor capability of thraustochytrids to utilise uronic acids present in the bacterial EPS as seen by Bahnweg (1979).

Thraustochytrids multiplied in numbers despite dense bacterial populations in all experimental bottles. However, their growth was poor in experimental bottles that were not amended with antibiotics, suggesting that bacteria might compete with them for nutrients. Large fractions of bacteria (42 %) are attached to TEPs (Alldredge *et al.*, 1993). Hence bacteria would be strong competitors against thraustochytrids for nutrients.

TEPs play a major role in the formation of aggregates. Plenty of aggregates were formed in experiments carried out with coastal water to which antibiotics had been added initially. 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. Epifluorescence microscopy of these aggregates revealed abundant bacteria, thraustochytrids, and fungi. In addition, ellipsoidal cells resembling the labyrinthulid genus *Labyrinthula* were also abundant. Labyrinthulids are a related group to thraustochytrids, that are usually colonial and glide over their network of ectoplasmic net elements (plasma membrane extensions) (Porter, 1990).

In conclusion, roller table experiments to generate TEPS from coastal waters showed that thraustochytrids and bacteria could multiply under conditions that stimulate TEPs and aggregate formation, even in the absence of additional nutrients. Aggregates formed in water, stimulated by an initial addition of antibiotics, were extremely favourable for the growth of not only thraustochytrids, but also fungi.

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Legends to figures

- Fig. 1. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using 20 and 200 μm filtered coastal seawater, with and without antibiotics.
- Fig. 2. Photograph of macroaggregates formed on day 4 inside an experimental bottle on the roller table during Expt 2. Scale represents 1 cm.
- Fig. 3. 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.
- Fig. 4. 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.
- Fig. 5. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using culture filtrate of diatom.
- Fig. 6. Distribution of thraustochytrids, bacteria and TEPs with respect to time in the roller table experiment conducted using EPS of diatom-associated bacteria.

Table 1. Correlation analysis between thraustochytrids, bacteria and TEPs from roller table bottles containing coastal seawater (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

Table 2. 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 microphytoplankton) 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

Table 3. Correlation analysis between thraustochytrids, bacteria and TEPs in the roller table experiment using diatom culture filtrates in the bottles.

	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

Table 4. Correlation analysis between thraustochytrids, bacteria and TEPs in the roller table experiment using bacterial EPS in the bottles.

	TEPs generated from bacteria associated with diatom (n=15)	
	r	P
Thraustochytrids-bacteria	-0.67	0.008
Thraustochytrids-TEPs	-0.42	0.12
Bacteria-TEPs	0.17	0.55

Fig. 1

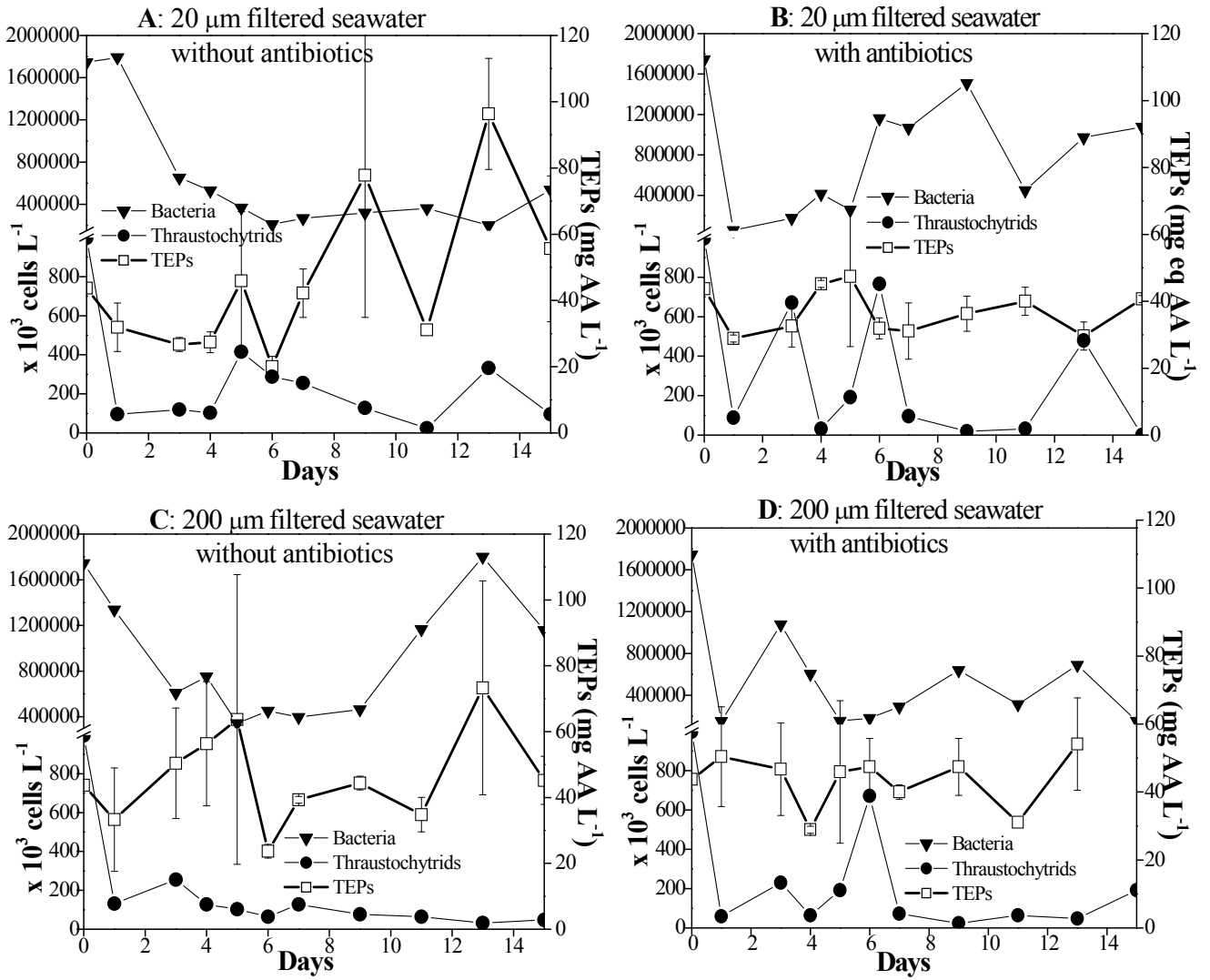


Fig. 2



Fig. 3

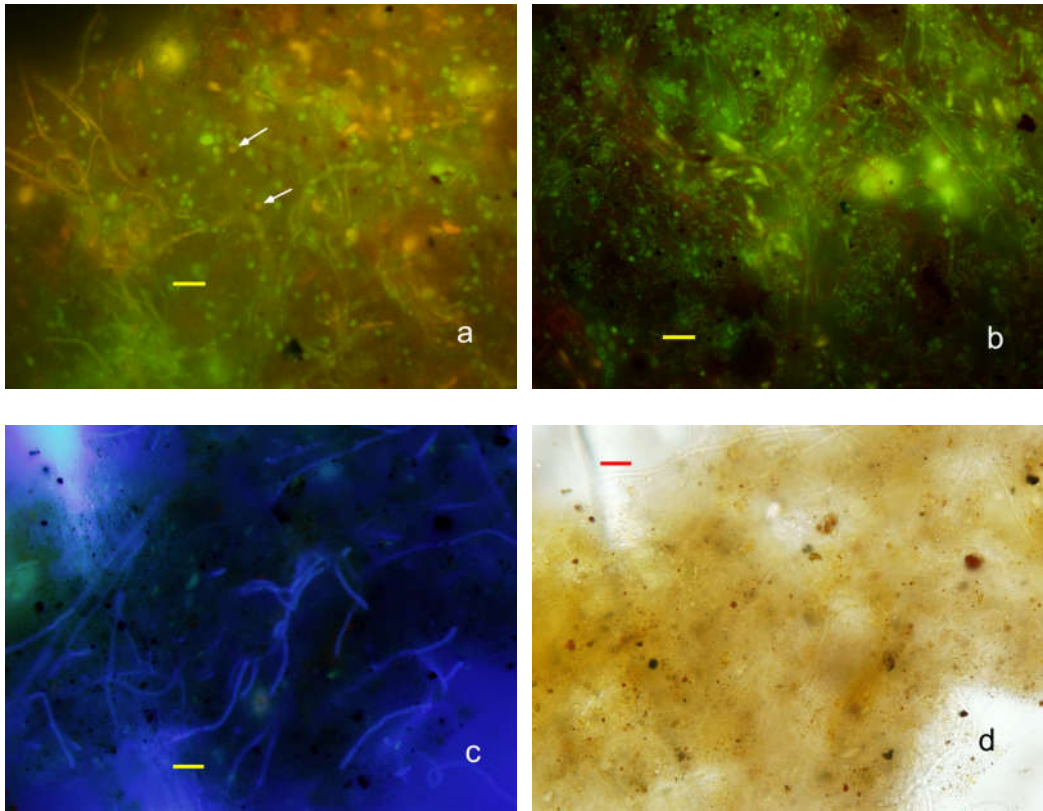


Fig. 4

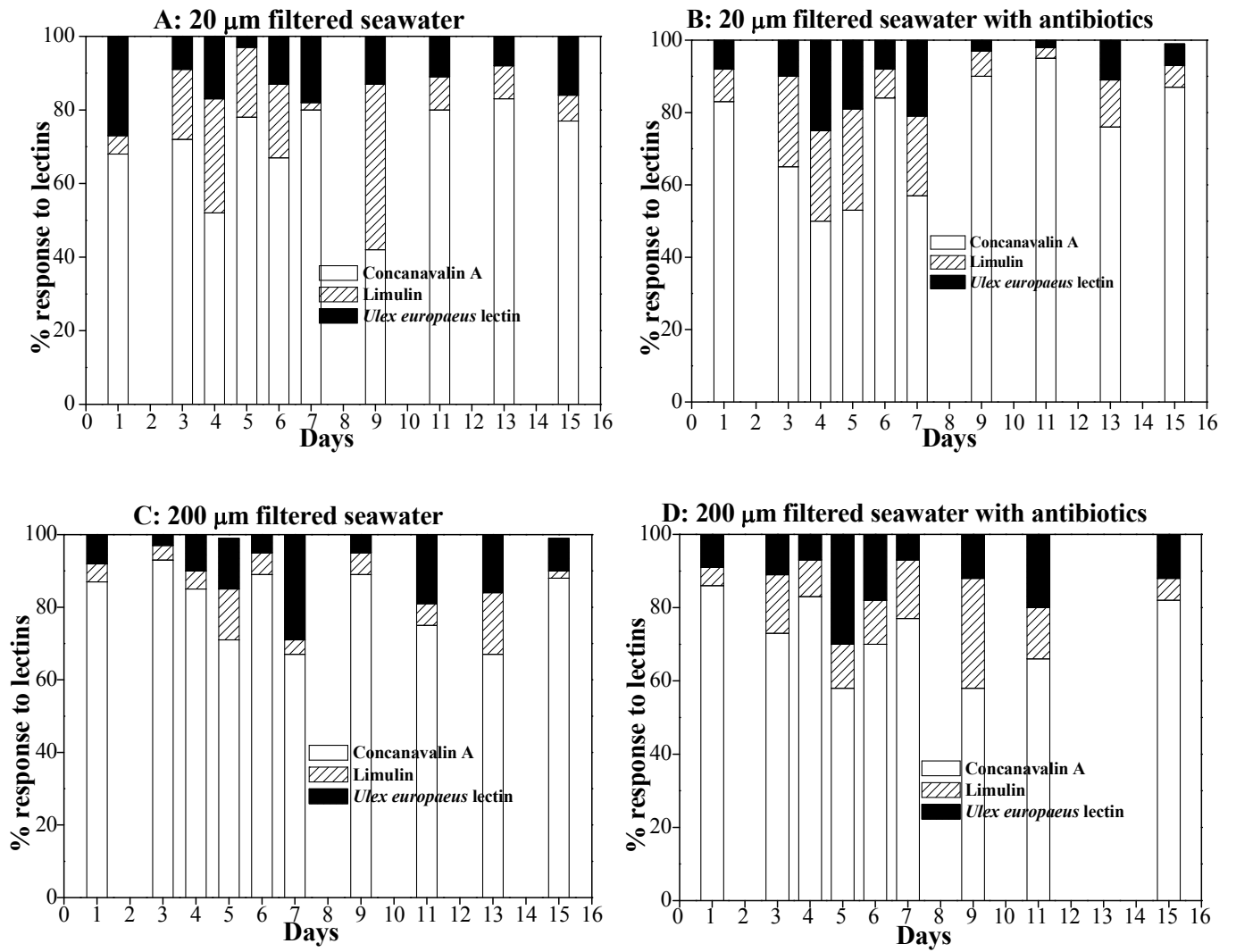


Fig. 5

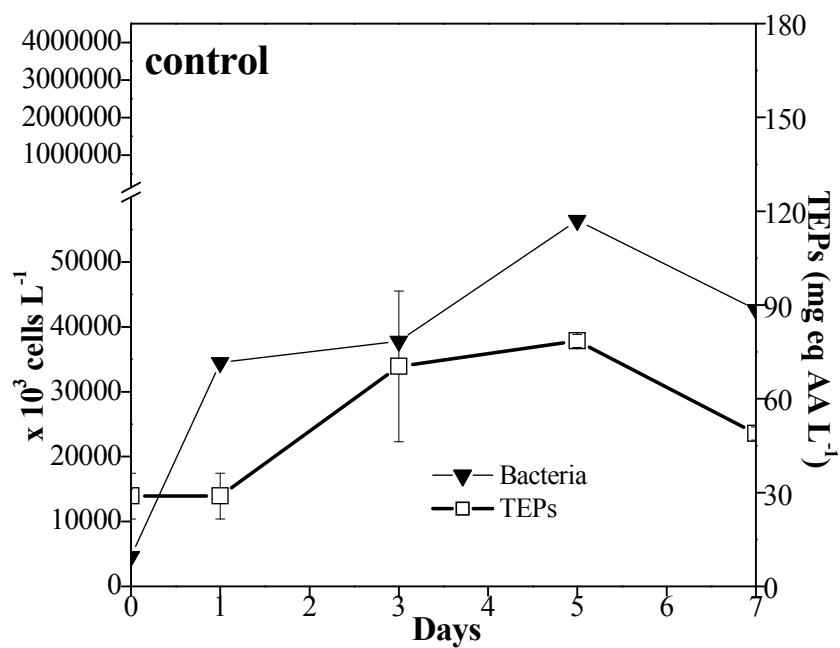
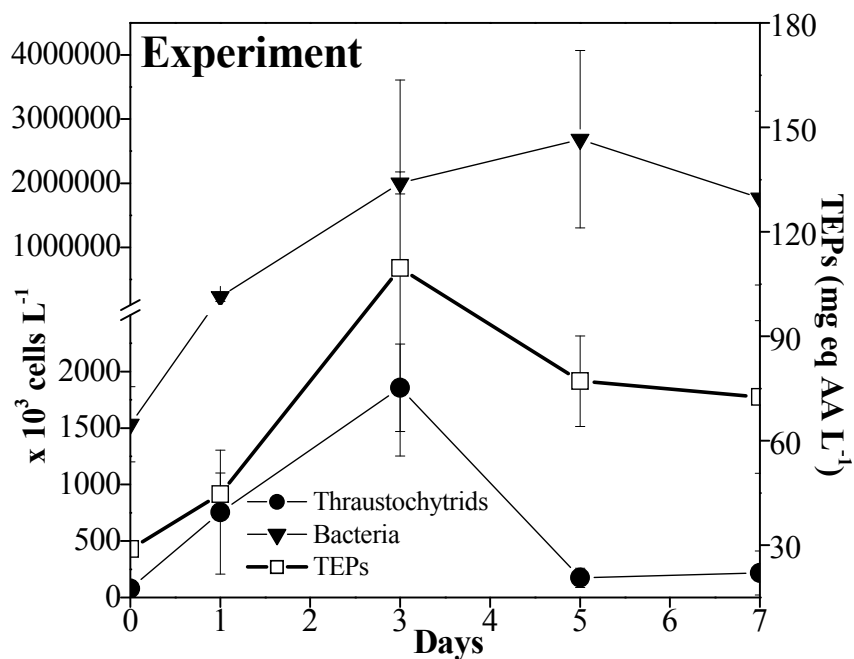


Fig. 6

