

Apparent grazing losses of Labyrinthulomycetes protists in oceanic and coastal waters: An experimental elucidation

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Abstract

Labyrinthulomycetes are unicellular eukaryotes known for their ability to synthesize polyunsaturated fatty acids that are essential for growth and development of zooplankton. But relationship of these microorganisms with microzooplankton and their trophic status in food chain remains unexplored. Hence grazing losses of these protists were studied in coastal and oceanic waters. Traditional dilution plot experiments to study consumption of natural phytoplankton (herbivory) and bacteria (bacterivory) by microzooplankton were carried out, incorporating a modification to unravel their preying on Labyrinthulomycetes. It was incorporation of fluorescently-labeled Labyrinthulomycetes cells. Experiments in oceanic waters displayed grazing on phytoplankton and bacteria at all the three times of study while that on Labyrinthulomycetes at only one time out of the three. There was no significant grazing on phytoplankton in coastal waters and microzooplankton grazed on Labyrinthulomycetes and bacteria. When grazing was studied with respect to time, significant grazing on bacteria occurred in the first 6 h and on Labyrinthulomycetes occurred towards the end of 24 h of incubation. Grazing on natural Labyrinthulomycetes population instead of fluorescently-labeled cells also revealed that they were grazed. Overall, the grazing experiments demonstrated that microzooplankton can exert positive grazing pressure on Labyrinthulomycetes, though during certain times only, signaling the probable ‘top-down control’ of Labyrinthulomycetes by microzooplankton during those times. Labyrinthulomycetes may therefore constitute an important part of microbial loop in the marine ecosystem by utilizing dissolved and particulate organic matter due to osmoheterotrophic mode of nutrition and in turn supplying it to the next trophic level viz., the microzooplankton.

Key Words Labyrinthulomycetes, thraustochytrid, aplanochytrid, microzooplankton grazing, dilution plot

Introduction

Labyrinthulomycetes are heterotrophic fungoid protists that are ubiquitous in marine environment. These unicellular eukaryotic organisms have been isolated from water column, sediments as well as detrital matter (Raghukumar 2002). They produce extensions of plasma membrane called ectoplasmic net (EN) elements that help them to attach to any substrate and absorb nutrition by producing various enzymes through them, and functioning as remineralizers in the environment (Raghukumar & Damare 2011). Falling into 3 distinct groups, viz., labyrinthulids, thraustochytrids and aplanochytrids, these are differentiated from each other based on their cell wall composition and dispersal of spores. The aplanochytrid cells glide over the EN elements whereas the thraustochytrids don't (Porter 1990; Leander et al. 2004). These protists are occasionally found in high numbers in the water column and contribute to a large extent to the microbial carbon biomass of proportions equaling that of bacteria (Damare and Raghukumar 2008).

These organisms have gained ecological as well as biotechnological importance in the recent years due to the production of polyunsaturated fatty acids (PUFAs) (Bajpai et al. 1991; Raghukumar 2002; Raghukumar 2008). PUFAs are required for cell membrane functioning and optimal physiological performance in higher organisms including zooplankton (Persson and Vrede 2006; Kattner et al. 2007; Mariash et al. 2011). These are not present in most bacteria but are found in flagellates and ciliates. It is through these microzooplankton that the essential fatty acids are transferred from the microbial loop to the classical food chain (Zhukova and Kharlamenko 1999). As the zooplankton cannot synthesize PUFAs *de novo*, they have to depend on dietary supplements for incorporation of these fatty acids into their biomass and Labyrinthulomycetes serve as one of them (Goulden and Place 1993; Veloza 2005; Damare et al. 2013). Once Labyrinthulomycetes are fed to the larvae, they cause trophic upgradation of the food quality (larvae) to be utilized in aquaculture attributable to the presence of these PUFAs (Alonzo et al. 2005; Castillo et al. 2009; Naylor et al. 2009). Thus they remain important suppliers of essential nutrients to the mesozooplankton and indirectly to fish. Whether the microzooplankton consume Labyrinthulomycetes as a source of PUFAs, apart from some dinoflagellates and certain bacteria, in natural environment also, is not known. Also, their exact position in the classical food chain is unclear.

So far the microzooplankton grazing studies comprised either herbivory or bacterivory. The importance of Labyrinthulomycetes as their prey has not been elucidated. Neither do we know on the abundance and

reproduction of Labyrinthulomycetes under predator pressures. Therefore this study is an attempt to find out the effect of microzooplankton grazing on Labyrinthulomycetes abundance. Seawater dilution experiments were designed to monitor changes in growth or abundance of these organisms along with phytoplankton and bacteria by the impact of microzooplankton grazing.

Methods

Organisms used

One thraustochytrid isolate, DP7 isolated from surface waters of Dona Paula Bay, Goa, India (Lat: 15°27'00"N, Long: 73°48'00"E) during March 2005 by pine pollen baiting technique (Raghukumar 1979) and one aplanochytrid isolate S2124 obtained from faecal pellets of zooplankton from equatorial Indian Ocean during the cruise #SK 212 of the research vessel ORV Sagar Kanya in October 2004 were used in this study. Briefly, the procedure for isolation of aplanochytrid from the faecal pellets was as follows. The zooplankton collected in the multiple plankton net (200 µm mesh size; Hydrobios, Germany) were transferred to sterile seawater and incubated at room temperature for 1 hour. The seawater in which the zooplankton were incubated was then filtered through 100 µm mesh bolting silk and the filtrate was collected. One hundred micro liter of this filtrate was plated on the Modified Vishniac (MV) Agar medium (0.001 % liver infusion extract, 0.01 % yeast extract, 0.15 % peptone, 0.4 % glucose, 0.8 % agar) supplemented with antibiotics and foetal bovine serum (Damare and Raghukumar 2006) and incubated at room temperature for 7-14 days till visible colonies were obtained. Both the cultures, DP7 and S2124 were maintained on MV agar plates and were sub-cultured every 10 days. The former was used for coastal studies and the latter for oceanic studies.

Fluorescent-labeling of aplanochytrids and thraustochytrids

Grazing studies were carried out based on the 'fluorescently labeled bacteria' short term uptake technique of Sherr and Sherr (1993) as follows. Both isolates S2124 and DP7 were grown separately in MV broth under shaking conditions (125 rpm) at room temperature. Cells were harvested after 5 days of growth, washed with sterile 0.22 µm-filtered aged seawater and suspended in 100 mL of the same seawater. Cells were then stained for 2 hours at 50° C with 0.2 mg mL⁻¹ final dye concentration. Dye solution was prepared by dissolving the stain DTAF (5-(4,6-dichlorotriazin-2-yl) aminofluorescein; Sigma) in 0.05 M Na₂HPO₄-NaCl solution, adjusted to pH 9.0. Cells were centrifuged, washed thrice with sterile 0.22 µm filtered seawater, suspended in 5 % sucrose, aliquoted and frozen immediately

under liquid nitrogen. These were lyophilized and then either stored in refrigerator or maintained at -20° C. The fluorescently-labeled cells were enumerated by filtering an aliquot over $0.45\ \mu\text{m}$ black polycarbonate filter paper (Millipore) and observing under blue excitation light using a BX60 Olympus epifluorescence microscope equipped with a 100W Hg lamp. Microscopic fields possessing clumps with tightly packed cells were ignored during counting.

Grazing experiments

The dilution plot technique of Landry and Hassett (1982) was employed for estimating microzooplankton grazing on Labyrinthulomycetes, bacteria and phytoplankton. Seawater was filtered through $200\ \mu\text{m}$ mesh to remove mesozooplankton and added to experimental bottles (Nalgene) in various concentrations. It was diluted with $0.22\ \mu\text{m}$ filtered sea water of the same location in order to make up the final volume to 2 L in each bottle. Bottles with different concentrations of natural microzooplankton (20, 40, 60, 80 and 100 % represented as fractions of undiluted seawater 0.2, 0.4, 0.6, 0.8 and 1 in the graphs) were thus set up. Nutrients were added into experimental bottles at concentrations of $0.5\ \mu\text{M NH}_4\text{Cl}$, $0.03\ \mu\text{M NaH}_2\text{PO}_4$, $1.0\ \text{nM FeSO}_4$ and $0.1\ \text{nM MnSO}_4$ to avoid nutrient limiting conditions and ensure that nutrients are amply available to phytoplankton at all dilutions. Additionally, an experimental treatment with 100 % seawater was prepared in 3 bottles without nutrients (unenriched bottles) to check the effect of nutrient limitation, if any, during the experimental period on phytoplankton growth and eventually on microzooplankton grazing behaviour. Grazing on Labyrinthulomycetes was examined by incorporating fluorescently-labeled thraustochytrids in case of coastal waters or fluorescently-labeled aplanochytrids in case of oceanic waters in the dilution bottles. Before addition of the cells in the incubation bottles, the fluorescently-labeled cells were vortexed briefly and pipetted in and out several times to get uniform suspension. The decrease in the number of fluorescent cells was estimated from formalin-fixed bottles after the end of incubation period. Three control bottles containing $0.22\ \mu\text{m}$ filtered sea water and equal proportions of fluorescent cells as that in experimental bottles served as control.

To estimate the abundance of bacteria and fluorescent Labyrinthulomycetes cells in the experimental bottles at the end of the incubation period, 100 mL samples were withdrawn from the bottles and fixed with formalin up to 3 % final concentration. These samples were stored under refrigeration until the actual estimations were carried out (within one month). Grazing on bacteria was studied by estimating their numbers through acridine orange direct count (AODC) technique at the start and end of the

experiment (Parsons et al. 1984). Grazing on phytoplankton was examined by estimating the concentration of chlorophyll in each bottle. Chlorophyll was estimated in duplicates or triplicates by filtering 950 mL or 500-700 mL of water, respectively, by spectrophotometric method (Parsons et al. 1984). The contribution of chlorophyll to organic carbon was determined from the chlorophyll concentration ($\mu\text{g L}^{-1}$) using the conversion factor of $45 \mu\text{g C}/\mu\text{g chl a}$ (*sensu* Christian and Karl 1994). Linear regression analysis between the concentration of unfiltered seawater and apparent growth rates of different food sources was carried out to establish the impact of grazing on all three food types.

The grazing on 3 different groups of organisms was determined separately by calculating their apparent growth rates (k) according to the equation: $k = (\ln C_f - \ln C_0) / (\text{incubation time})$; where C_0 and C_f are the concentrations at the beginning (time=0) and end (time=final) of the incubation, respectively (Landry et al. 1995; Murrell and Hollibaugh 1998). The k values obtained were plotted against different dilutions of the seawater. The downward (negative) slope indicated mortality rate (g) due to grazing and the Y-intercept indicated the instantaneous growth rate in the absence of grazing pressure. Upward (positive) slope indicated no net grazing.

Preliminary experiment for evaluation of grazing on fluorescently-labeled thraustochytrids with respect to time

To examine the effect of time on the behavior of fluorescently-labeled thraustochytrids in seawater, with respect to grazing, an initial experiment was carried out with surface waters collected from Dona Paula Bay (coastal waters). The changes occurring in the population of Labyrinthulomycetes and bacteria with respect to time were studied. Seawater was filtered through $200 \mu\text{m}$ mesh and filled in three 2 L Nalgene bottles. These bottles were amended with nutrients and fluorescently-labeled thraustochytrids (FLT). Water samples (200 mL) were taken out from all bottles at regular interval of 6 hours up to 24 hours and fixed with formalin for enumeration of FLT and total bacterial counts (TBC). To check the changes in fluorescence of the FLT with respect to time, a control was maintained that contained no microzooplankton i.e. the bottle was filled with $0.22 \mu\text{m}$ filtered seawater and amended with equal amounts of FLT as that in experimental bottles. The FLT were thus not subjected to grazing pressure in the control bottle and were enumerated at 0 and 24 h i.e. in the beginning and towards the end of the experiment. One way repeated ANOVA (using IBM SPSS v21.0) was carried out to demonstrate the effect of grazing on the population of these organisms.

Evaluation of grazing in oceanic waters

Microzooplankton grazing was studied at 3 different locations in the equatorial Indian Ocean using fluorescently-labeled aplanochytrids. For this the surface waters were collected from 77° E, 83° E and 93° E along the equator during cruise SK 228 in September 2006 (Fig. 1). Fluorescently-labeled aplanochytrid cells (FLA) were added into all the bottles and incubated *in situ* seawater for 24 hours at the respective locations. Initial numbers of FLA were noted. All the experimental bottles with nutrients (all concentrations) and without nutrients (only 100 %) as well as control bottles were tied together and deployed *in situ* for 24 hours. At the end of the incubation period, the contents in the bottles were vigorously mixed and 100 mL aliquots were fixed in 3 % formalin and stored in refrigerator to measure bacteria and fluorescent aplanochytrid cells. From this as much as 10 mL aliquots were used to measure bacterial counts and 25 mL for FLA counts. Chlorophyll from these bottles was also estimated in triplicates by filtering 500-700 mL sample through GF/F filter paper (Whatman).

Evaluation of grazing in coastal waters- for a fixed time period

The experiments using water samples from Dona Paula Bay were carried out almost similar to that described for oceanic waters. These were done on three different days (02, 08 and 12th February 2007 designated as Coastal I, Coastal II and Coastal III, respectively) with FLT as tracer, instead of FLA. The experimental bottles were incubated for 24 hours in open tanks filled with seawater and placed in an open area outside the laboratory to match light intensity at depth of sample collection. After 24 h, the contents in the bottles were vigorously mixed and 100 mL aliquots were fixed in 3 % formalin and stored in refrigerator to measure bacteria and fluorescent thraustochytrid cells. From this as much as 10 mL aliquots were used to measure bacterial counts and 25 mL for FLT counts. Chlorophyll from these bottles was also estimated in triplicates by filtering 500-700 mL sample through GF/F filter paper (Whatman).

Evaluation of grazing in coastal waters- with respect to time

After examining grazing in coastal waters for a fixed time period of 24 h, the grazing pattern of microzooplankton within those 24 h was studied by carrying out grazing experiment in Dona Paula Bay as described above except for a modification. Unlike the final fixation after 24 hours of incubation, samples for various estimations (TBC, FLT, chl a) were drawn periodically (at 6 h, 12 h and final at 24 h). This experiment (referred as Coastal IV) was conducted on 23rd May 2012 at 10:30 am during bright

day light and the first sample of 900 mL was drawn after 6 hours (nearing sunset). From this an aliquot of 100 mL was fixed with formalin for enumeration of TBC and FLT in duplicates and the remaining 800 mL was used for chlorophyll estimation in quadruplets. After 12 h of incubation only 100 mL sample was withdrawn for enumeration of TBC and FLT and chlorophyll estimation was not done at this time due to practical constraints (low volume of sample left in experimental bottles for estimation in replicates). The last sample was taken after 24 hours from the start of the experiment and measurements similar to that made for 6 h samples were carried out.

Evaluation of grazing in coastal waters- on natural Labyrinthulomycetes population

This experiment (referred as Coastal V) was different from the previous experiments in coastal waters in that fluorescent cells were not incorporated in the bottles, rather microzooplankton grazing was examined on natural Labyrinthulomycetes population instead of artificially-added one. This experiment was a comparison with other dilution experiments (Coastal I-IV) to understand how the natural Labyrinthulomycetes population respond to grazing by microzooplankton or in other words to understand whether fluorescent thraustochytrid cells follow the natural ones during grazing studies. This was carried out on 29th May 2012 for a fixed time period of 24 h using waters of Dona Paula Bay. At the beginning of the experiment, direct counts of Labyrinthulomycetes from the same water collected for the experiment were done by acriflavine direct detection (AfDD) technique ([Raghukumar and Schaumann 1993](#)). During this experiment fluorescently-labeled cells were not added in the experimental bottles, instead the abundance of Labyrinthulomycetes cells occurring constitutively in seawater of each experimental bottle after dilution was measured at the end of the incubation period. Chlorophyll from these bottles was also estimated in triplicates as mentioned before. Hundred mL aliquots were fixed in 3 % formalin and refrigerated till the enumeration of bacteria and Labyrinthulomycetes by AODC and AfDD, respectively, was carried out. From this as much as 10 mL aliquots were used to measure bacterial counts and 30 mL for Labyrinthulomycetes counts. After the enumeration of bacteria and Labyrinthulomycetes, the formalin-fixed samples of only experimental bottles containing 100 % seawater were also visualized under the microscope for enumeration of various phytoplankton and microzooplankton groups ([Gauns et al. 1996](#)). For this, the samples (triplicates) were left undisturbed and allowed to settle for 48 h. These were concentrated to 10 mL by siphoning out the supernatant. One mL sample was taken using Sedgwick-Rafter counting chamber and observed under inverted microscope (Olympus, IX51).

Results

Preliminary experiment for evaluation of grazing on fluorescently-labeled thraustochytrids with respect to time

Fluorescent thraustochytrids decreased substantially from 552×10^3 to 192×10^3 cells L^{-1} in experimental bottles as compared to 422×10^3 cells L^{-1} in control bottle in 24 hours (Fig. 2). There was major decrease in bacterial numbers also from 55×10^6 to 5.18×10^6 cells L^{-1} . Mauchly's test indicated that the assumption of sphericity had been violated, $\chi^2(2) = 115.409$, $P = 0.000$ ($P < 0.05$), therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\epsilon = 0.500$). The results showed that there was no significant effect of time on decrease in cell numbers of FLT in experimental and control bottles as well as bacteria in experimental bottles, $F(1,12) = 17.012$, $P = 0.001$. This suggested that the marked decrease in the number of cells in experimental bottles as compared to control bottles was due to grazing.

Evaluation of grazing in oceanic waters

In the dilution experiments carried out along the equator during cruise SK 228, the FLA decreased from 1400×10^3 to as low as 76.7×10^3 cells L^{-1} at $77^\circ E$, to 67.1×10^3 cells L^{-1} at $83^\circ E$ and to 19.2×10^3 cells L^{-1} at $93^\circ E$. Although there was apparent fall in the numbers of FLA, no significant grazing on them was evident at $77^\circ E$ and $83^\circ E$ due to positive grazing rate (Fig. 3, Table 1). Grazing on FLA was evident only at $93^\circ E$ as seen by the negative value of the grazing rate (g) of $0.014 d^{-1}$. Significant relationship was found between apparent growth rate of Labyrinthulomycetes and concentration of unfiltered seawater at this station ($R^2 = 0.5$, $P = 0.0067$; Table 2). As compared to fluorescent aplanochytrids, grazing on phytoplankton was evident at all the 3 stations by decrease in the concentration of chlorophyll *a* with increasing microzooplankton concentration and hence negative grazing rates. The grazing rates were 0.027 , 0.024 and $0.01 d^{-1}$ at 77° , 83° and $93^\circ E$, respectively (Fig. 3, Fig. S1, Table 1). The C: chl *a* ratio varied from 0.1 - $1.4 \mu g C L^{-1}$. The unenriched bottles showed that phytoplankton were not nutrient limited in these experiments as their apparent growth rates were either equivalent to or higher than that in enriched bottles. Bacterioplankton were also grazed with the grazing rates of 0.03 , 0.04 and $0.014 d^{-1}$ at 77° , 83° and $93^\circ E$, respectively. Phytoplankton as well as bacterioplankton displayed significant relationship with concentration of unfiltered seawater or in other words, with microzooplankton.

Evaluation of grazing in coastal waters- for a fixed time period

Dilution experiments carried out in coastal waters displayed two scenarios; one in which there was grazing on fluorescent thraustochytrids (g were 0.014 and 0.02 d^{-1} , P value 0.0003 and 2.039E-06, respectively) and no grazing on phytoplankton (significant positive slope) and the other in which there was no grazing on fluorescent thraustochytrids (positive but insignificant slope) and slight grazing on phytoplankton ($g = 0.002 d^{-1}$, P value = 0.0423) (Fig. 4, Fig. S1). The former situation was observed in Coastal I and Coastal III experiments and the latter in the Coastal II experiment. The unenriched bottles however showed that phytoplankton were nutrient limited in all the experiments (Table 1). On the other hand bacterioplankton were grazed upon by microzooplankton in all the 3 experiments. This can be recognized from the significantly negative grazing rates between 0.05-0.06 d^{-1} with R^2 0.84-0.91 (P values < 0.001 ; Tables 1-2, Fig. 4, Fig. S1) in all the three experiments. The carbon contributed by phytoplankton biomass (deduced from chlorophyll values) in the experimental bottles was more in the coastal waters than in oceanic waters. The C: chl a ratio ranged from 3.0 to 54.3 $\mu g C L^{-1}$.

Evaluation of grazing in coastal waters- with respect to time

During the first 6 h of incubation, microzooplankton did not graze upon fluorescent thraustochytrids as well as phytoplankton (Fig. 5). Rather they grazed significantly upon bacteria ($g = 6.97 d^{-1}$, $R^2 = 0.53$, P value = 0.017; Table 1-2). At the end of 12 h of incubation, although microzooplankton showed grazing on fluorescent thraustochytrids as well as bacteria ($g = 0.49$ and 0.11, $R^2 = 0.02$ and 0.01 for thraustochytrids and bacteria, respectively), their grazing was insignificant (P value = 0.71 and 0.83 respectively). After 24 h of incubation, negative g values marked grazing on fluorescent thraustochytrids but insignificant (P value = 0.34). As compared to this, no grazing was significantly manifested on phytoplankton (P value = 1.67E-07) and to some extent on bacteria (P value = 0.29). The C:chl a ratio during this experiment ranged between 1.3 and 21.5 $\mu g C L^{-1}$.

Evaluation of grazing in coastal waters- on natural Labyrinthulomycetes population

The mean counts of Labyrinthulomycetes in natural seawater at zero time were 175.8 (± 49.6) $\times 10^3$ cells L^{-1} . Their numbers in the experimental bottles ranged from 55.2 to 220.8 $\times 10^3$ cells L^{-1} . Implicit grazing on these organisms was discernible by the negative g value ($g = 0.005 d^{-1}$, P value = 0.079) (Fig. 6). Bacteria were also grazed upon by microzooplankton ($g = 0.003 d^{-1}$, P value = 0.0045). The positive g value with phytoplankton suggested no grazing upon them (P value = 7.52E-05). The C: chl a ratio

ranged between 77.1 to 265.5 $\mu\text{g C L}^{-1}$. Microscopic examination of the formalin-fixed samples under low power, revealed high abundance of large phytoplankton like *Thalassiosira* ($118 \times 10^3 \text{ L}^{-1}$) and *Chaetoceros* ($30 \times 10^3 \text{ L}^{-1}$) followed by *Coscinodiscus* (3000 L^{-1}), *Skeletonema*, *Navicula*, *Pleurosigma* and *Thalassionema*. Amongst microzooplankton communities, ciliates were more abundant ranging up to $29 \times 10^3 \text{ L}^{-1}$, followed by 10×10^3 tintinnids L^{-1} . Rotifers (1000 L^{-1}) and nauplii (1000 L^{-1}) were also observed.

Discussion

Apparently the microzooplankton grazed upon the thraustochytrids and aplanochytrids that were introduced in the incubation bottles. However in all the experiments the grazing pattern was highly variable. Although significant grazing on these organisms was noticed at certain times, non-significant or no net grazing was also observed during other times. The oceanic waters displayed grazing on these organisms at only one station of 93° E . In spite of the apparent fall in their numbers, no significant grazing on them at 77° E and 83° E might be due to comparatively more number of FLA at higher concentrations of seawater (and thus microzooplankton) than the lower concentrations. These waters, in general, were more favorable to grazing on phytoplankton than the Labyrinthulomycetes. Phytoplankton didn't seem to be nutrient limited in these experiments as compared to the experiments in coastal waters. The coastal waters on the other hand, exhibited microzooplankton grazing on Labyrinthulomycetes instead of phytoplankton. Non-significant grazing on phytoplankton is the most common feature in productive waters (York et al. 2011) as the growth rate of phytoplankton surpasses their mortality rate from natural death and grazing (Landry et al. 1995). Generally coastal waters are more productive than oceanic waters mostly due to terrestrial run-off (Munn 2011). The coastal water in the present study, i.e. Dona Paula Bay is also rich in nutrients (approximately $5 \mu\text{M}$ nitrate and up to $3 \mu\text{M}$ phosphate during summer as observed by Bhaskar 2003) and support good growth of phytoplankton (Patil and Anil 2011). Presence of large diatoms like *Thalassiosira* and *Chaetoceros* as well as high C:chl a ratios observed in these waters in the present study confirms the prevalence of large phytoplankton supporting the growth of suspension-feeding mesozooplankton (Landry et al. 1998; Patil and Anil 2011). Larger the C:chl a ratio, larger is size of the phytoplankton cell that escape preying by the microzooplankton (Calbet and Landry 2004). The microzooplankton would therefore search for alternate prey like bacteria and Labyrinthulomycetes in the present case. The larger cell size of phytoplankton leads to faster

consumption of nutrients due to high demand and hence poor growth during nutrient-limiting conditions like in unenriched bottles (Finkel et al. 2010)

On the other hand, the low C:chl a ratios in oceanic waters points towards preponderance of small-sized phytoplankton cells over the large ones (Dawson 2002; Chen et al. 2012). Microzooplankton dominate mesozooplankton as primary consumers of phytoplankton production in open oceans (Calbet and Landry 2004) averting the need to prey on Labyrinthulomycetes. Such differences in trophic habits of microzooplankton are due to differences in their community structure which change as per the location (First et al. 2007; Burian et al. 2013) as well as season (Asha Devi et al. 2010). Assessing the community structure of microzooplankton and phytoplankton was not the primary focus of this study and hence was not carried out for all experiments except one where fluorescently-labeled Labyrinthulomycetes cells were not added. Types of microzooplankton and phytoplankton were assessed in coastal V experiment only as the first four coastal experiments demonstrated consistent grazing on Labyrinthulomycetes leading inquisitively the need to know the community structure of their predators. Generally the oligotrophic oceanic waters inhabit small phytoplankton and ciliate grazers whereas the more productive coastal regions favour large diatoms, heterotrophic dinoflagellates and ciliates (Paterson et al. 2007). The latter was confirmed in the present study. The coastal experiments were carried out during pre-monsoon season which is generally characterized by mixed bloom of large diatoms (Patil and Anil 2011). This period is also characterized by highest number of ciliate species and crustacean nauplii as observed in the present study (Asha Devi et al. 2010). Prevalence of ciliates signifies ample availability of nutrition that includes nano- and pico-sized cells (Fileman and Leaky 2005; Sato et al. 2007). Vegetative cells of Labyrinthulomycetes measure 4-20 μm in diameter (Raghukumar 2002). This alludes to Labyrinthulomycetes as the probable prey along with bacteria in the coastal region.

It appears that Labyrinthulomycetes and bacteria that form microbial portion of the food web serve as the potential feed to microzooplankton during the times and places where microzooplankton do not consume majority of the phytoplankton production (Rivkin et al. 1999; Calbet and Landry 2004). For instance, certain suspension-feeding zooplankton prefer protists in their diet (Stoecker and Capuzzo 1990). The no net grazing on Labyrinthulomycetes during other times might have been the result of their proclivity to exist in clumps and to be associated with particles (Raghukumar et al. 2001; Lyons et al. 2005). It was seen that few DTAF-stained cells occurred in clumps (Fig. S2). If predators would have

encountered such clumps that would pack their energy requirements, then they would not have searched and fed on new particles. In such cases dilution with particle-free water would not have reduced the grazing pressure in a direct linear fashion (Murrell and Hollibaugh 1998) leading to no net grazing response. The breaking of such clumps by sloppy feeding (Putland 2000) during grazing would have led to release of some cells from the clumps, that would probably not have been ingested and hence accountable, distorting the initial counts.

Present study characterizes the importance of Labyrinthulomycetes in the microbial loop as this microbial portion is responsible for transfer and upgradation of carbon to zooplanktonic communities (Masclaux et al. 2011). Labyrinthulomycetes play a significant role in food web of marine environment owing to their ubiquitous occurrence and sizeable biomass (Kimura et al. 1999). Apart from their role as remineralizers (Raghukumar and Damare 2011), they serve also as a food source to the mesozooplankton (Damare et al. 2013). This is the first study that points out their function as prey to the microzooplankton who in turn play a key role in shaping the structure of pelagic food webs (Calbet and Landry 2004). Thus Labyrinthulomycetes also play an important role in the food web by forming a part of microbial loop. These utilize dissolved as well as particulate organic matter for growth and in turn supply this organic matter to high trophic level, the microzooplankton. Probably grazing by microzooplankton might be one of the reasons for low abundance of Labyrinthulomycetes in coastal regions during certain conditions like phytoplankton blooms (Raghukumar 2002). The factors responsible for controlling the population of Labyrinthulomycetes in the water column during the blooms might not only be the metabolites produced by living phytoplankton that are inhibitory for the growth of these protists (Raghukumar 2002) but also the ‘top-down control’ on these protists by microzooplankton

Conclusion

Labyrinthulomycetes seemed to be the preferred food source of microzooplankton over the phytoplankton in the coastal waters as compared to oceanic waters. The positive grazing pressure of microzooplankton on Labyrinthulomycetes in one oceanic and four coastal experiments implies that these organisms may serve as a food source to the microzooplankton during certain times only or to a selective group of microzooplankton only. This finding canvasses new directions for microzooplankton grazing studies in future. As microzooplankton grazing is one of the major factors structuring the plankton communities, this study reveals an important and indirect role of Labyrinthulomycetes in food

dynamics of marine ecosystems. Further studies for in-depth exploration of their trophic relationships should investigate whether any particular species of Labyrinthulomycetes is preferred by the microzooplankton and whether the unfavoured Labyrinthulomycetes species possess any defense mechanisms to evade the grazing pressure.

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Legend to Figures

Figure. 1 Sampling stations in the equatorial Indian Ocean during cruise SK 228 in September 2006 and at Dona Paula Bay, Goa, India. Stars represent sampling points.

Figure. 2 Changes in abundance of fluorescently-labeled thraustochytrids (FLT) and bacteria due to microzooplankton grazing with respect to time.

Figure. 3 Net apparent growth rate of *Labyrinthulomycetes* (a, d, g), bacteria (b, e, h) and phytoplankton (c, f, i) with respect to the fraction of undiluted oceanic water at 3 different regions along the Equator namely, 77° E, 83° E and 93° E, in the equatorial Indian Ocean. Panels a, b and c represent 77° E; d, e, and f represent 83° E and g, h and i represent 93° E station. Experimental bottles with nutrients are represented by filled circles and experimental bottles without nutrients are represented by open squares. Control bottles containing 0.22 µm filtered seawater with nutrients for checking natural loss of fluorescence in fluorescently-labeled aplanochytrids (FLA) are denoted by open triangle.

Figure. 4 Net apparent growth rate of *Labyrinthulomycetes* (a, d, g), bacteria (b, e, h) and phytoplankton (c, f, i) during fixed time period with respect to the fraction of undiluted coastal water from Dona Paula Bay. Experimental bottles with nutrients are represented by filled circles and experimental bottles without nutrients are represented by open squares. Control bottles containing 0.22 µm filtered seawater with nutrients for checking natural loss of fluorescence in FLT are denoted by open triangle.

Figure. 5 Net apparent growth rate of *Labyrinthulomycetes* (a), bacteria (b) and phytoplankton (c) with respect to time and the fraction of undiluted coastal water from Dona Paula Bay during coastal water experiment.

Figure. 6 Grazing rates of microzooplankton on *Labyrinthulomycetes* (a), bacteria (b) and phytoplankton (c) during standard dilution experiment (Coastal V) in the coastal waters. Experimental bottles with nutrients are represented by filled circles and experimental bottles without nutrients are represented by open squares.

Legend to Supplementary Figures

Figure. S1 Grazing rates of microzooplankton on *Labyrinthulomycetes*, bacteria and phytoplankton during modified dilution experiments at all the sampling points.

Figure. S2 Photomicrograph of DTAF-stained cells of isolate S2124 as seen under blue light of fluorescence microscope with the arrow pointing towards the clump of cells. Bar represents 20 µm.

Fig. 1

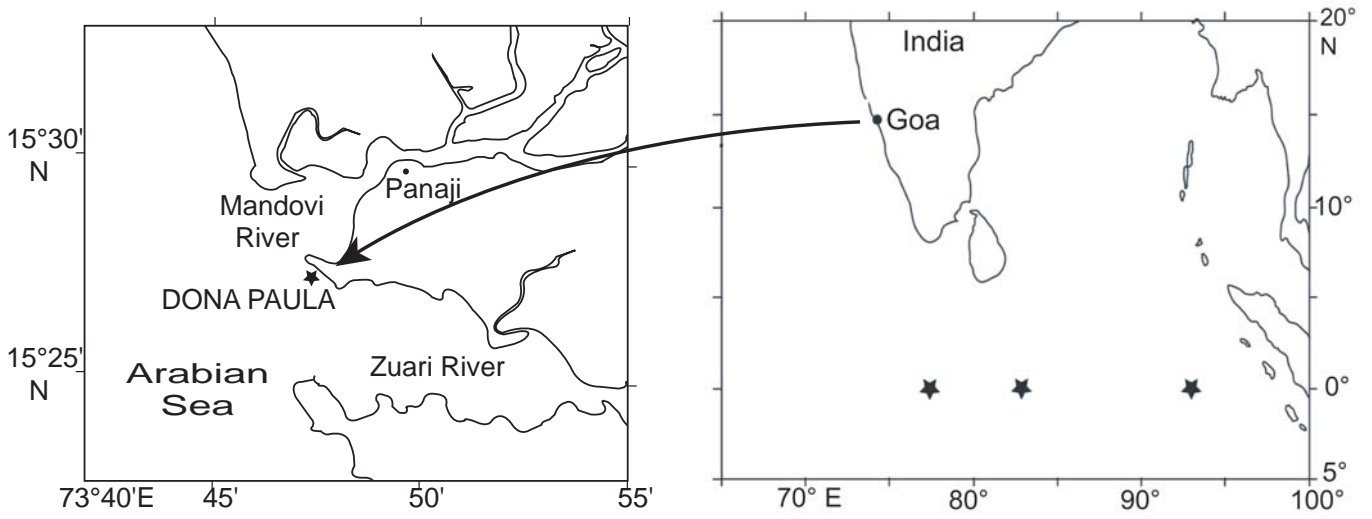


Fig. 2

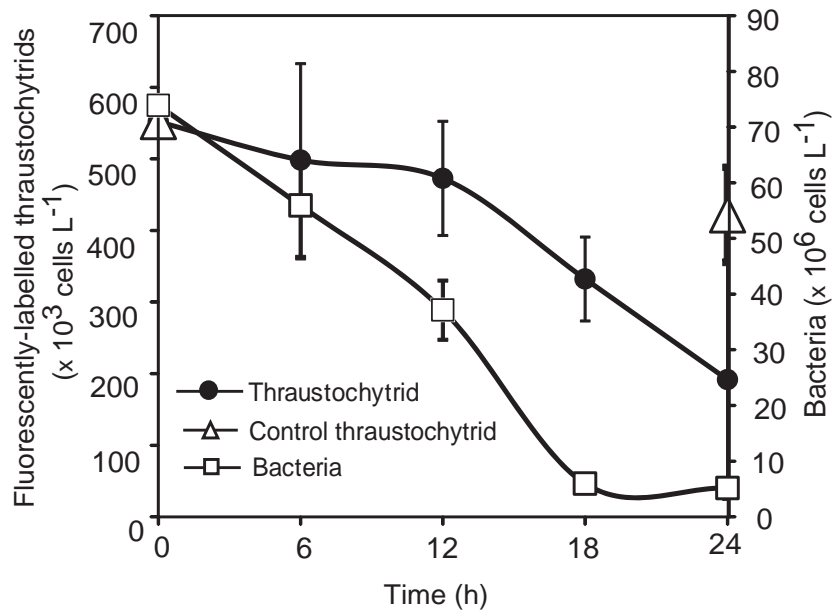


Fig. 3

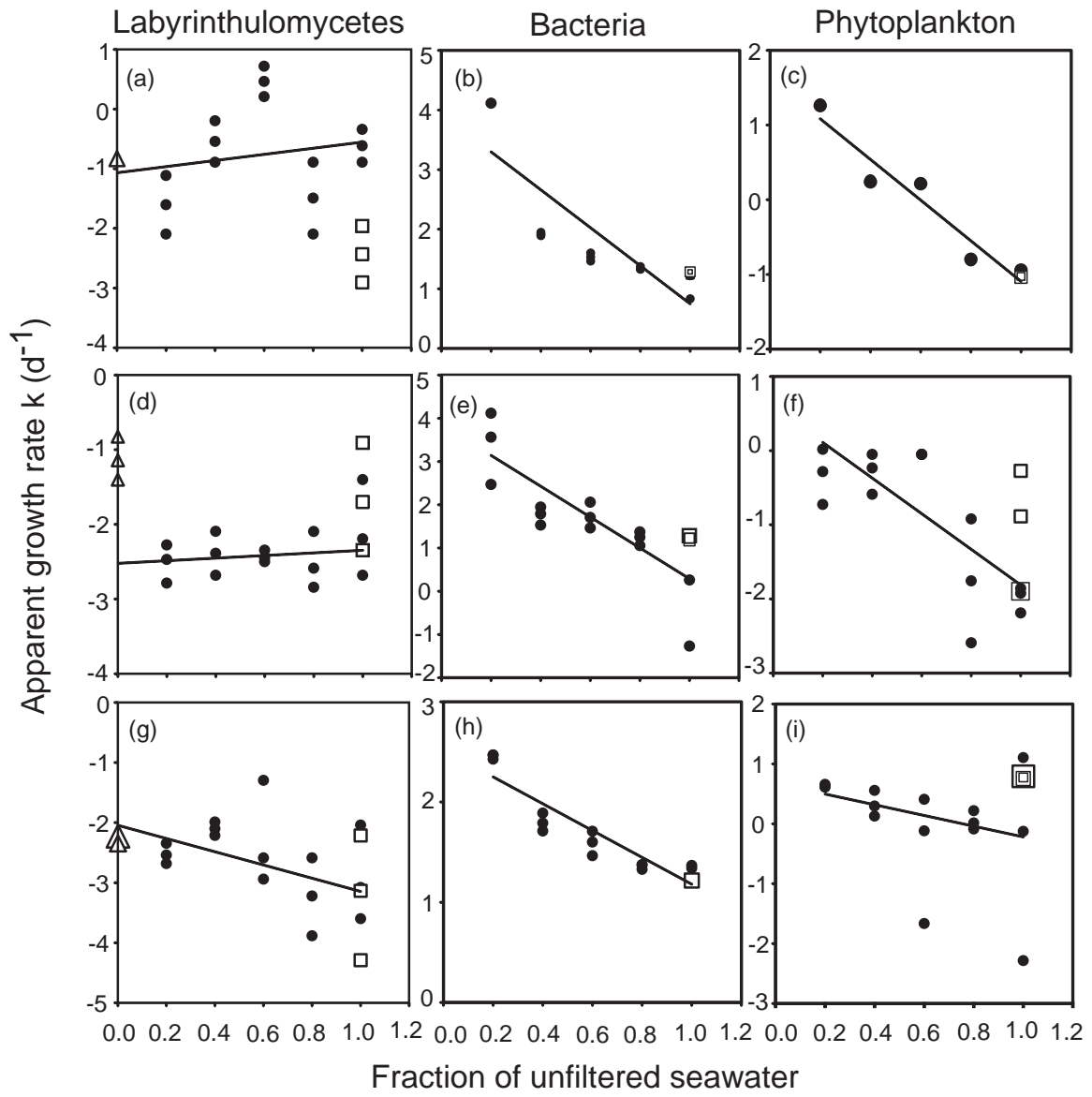


Fig. 4

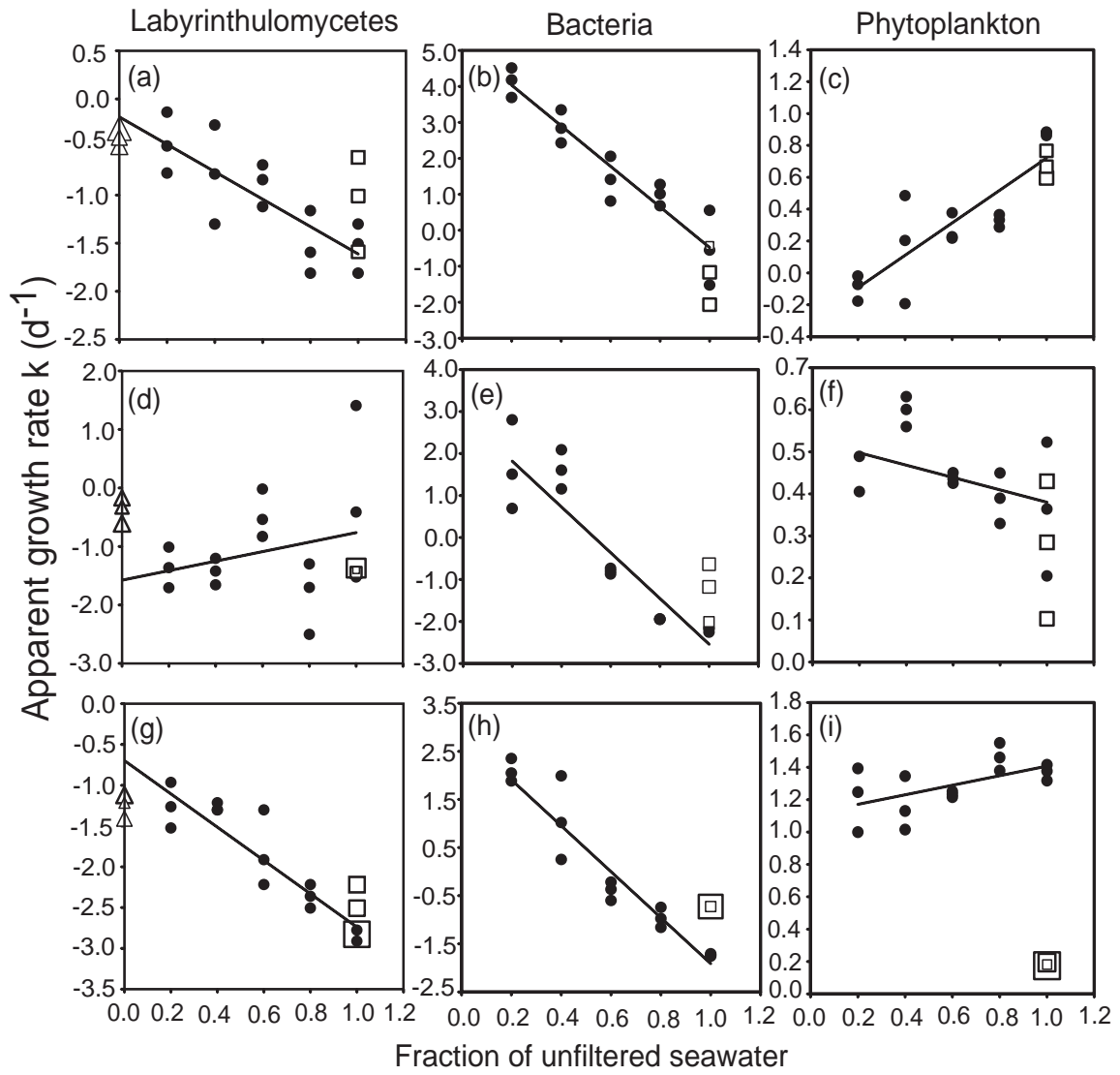


Fig. 5

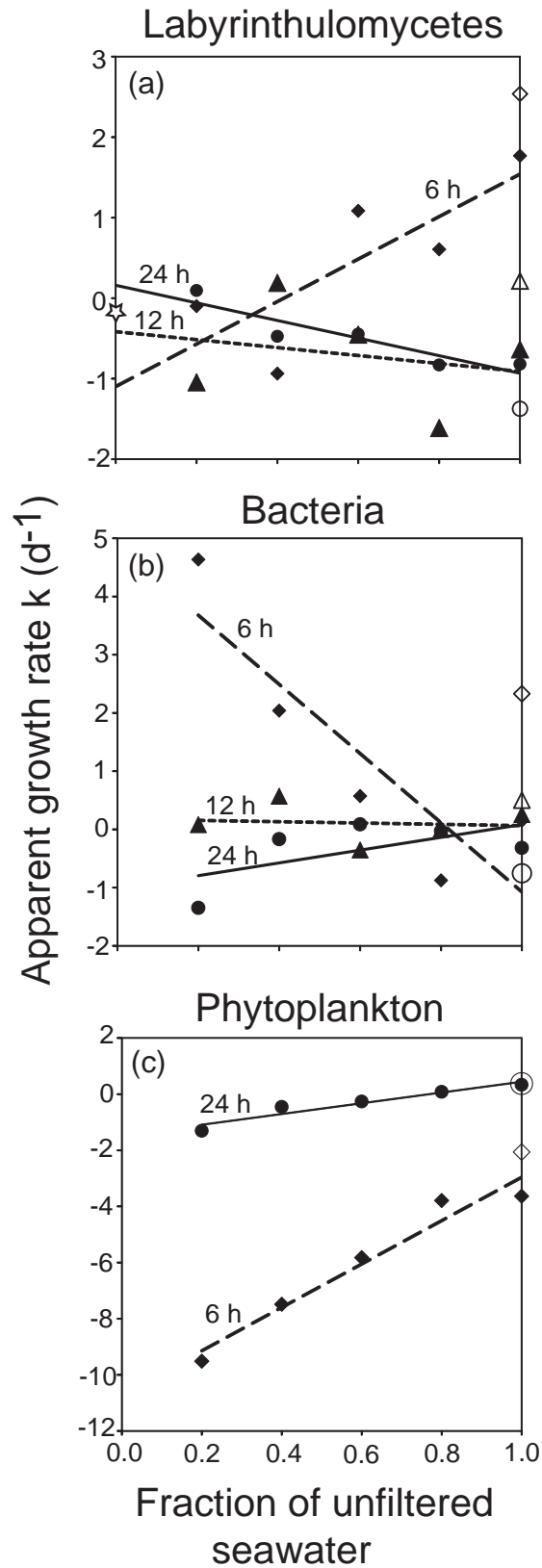


Fig. 6

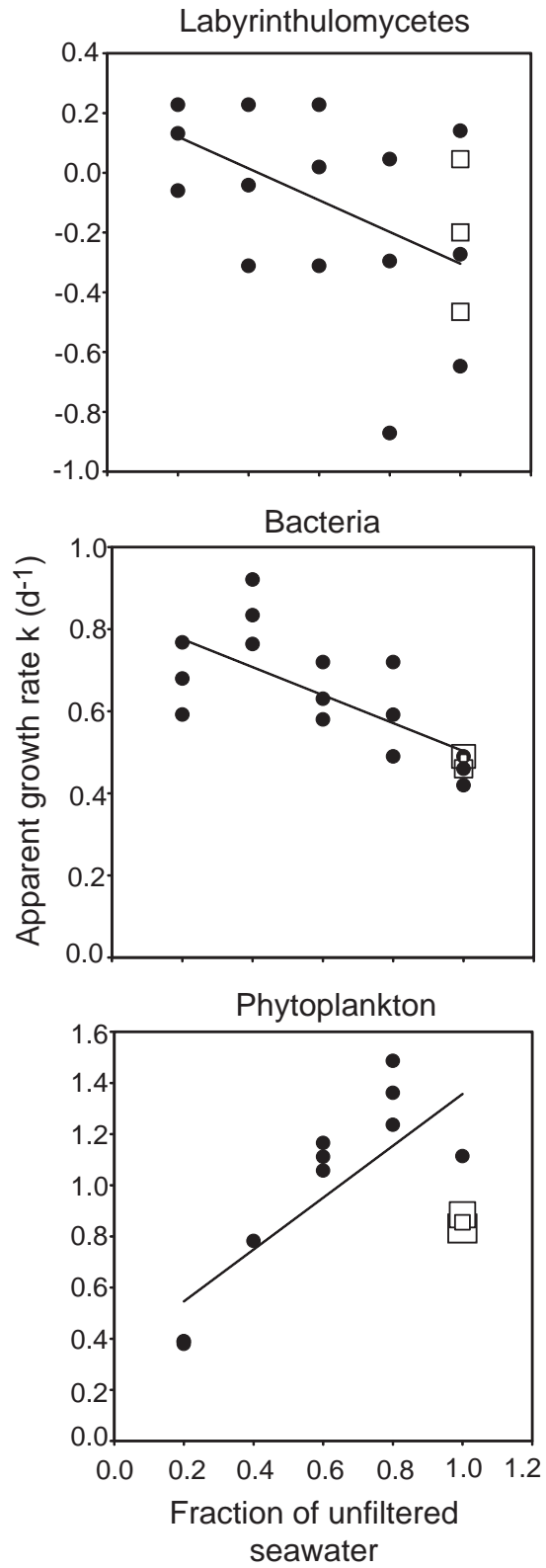


Fig. S1

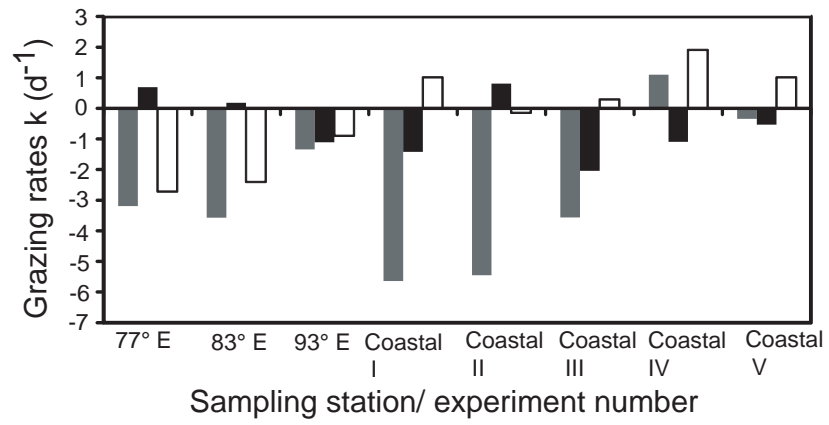


Fig. S2

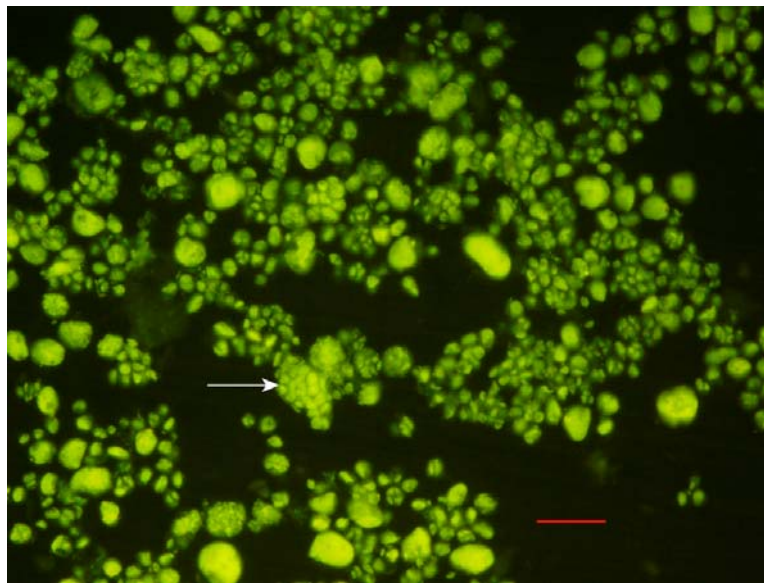


Table 1. Summary of the microzooplankton grazing experiments by modified dilution method. Average values of growth rate at 100 % unfiltered seawater concentration with and without nutrients are given below. Microzooplankton grazing rate (g) is derived from the slope of the equation and instantaneous growth rate (c) in the absence of grazing pressure is derived from the Y-intercept. (nd = not determined). Slopes significantly different than zero: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Sampling station/ experiment no.	Growth rate k (d ⁻¹) of Labyrinthulomycetes		'g' (d ⁻¹) on Labyrinthulomycetes	c (d ⁻¹) of Labyrinthulomycetes	Growth rate k (d ⁻¹) of bacteria		'g' (d ⁻¹) on bacteria	c (d ⁻¹)	Growth rate k (d ⁻¹) of phytoplankton		'g' (d ⁻¹) on phytoplankton	c (d ⁻¹) of phytoplankton
	100 % with nutrients	100 % without nutrients			100 % with nutrients	100 % without nutrients			100 % with nutrients	100 % without nutrients		
77 °E	-0.61	-2.43	0.0051	-1.070	1.22	1.28	-0.030***	3.98	-0.94	-1.03	-0.027***	1.64
83 °E	-2.19	-1.70	0.0040	-2.600	0.26	1.23	-0.040***	3.78	-1.92	-0.89	-0.024***	0.55
93 °E	-3.08	-3.14	-0.0140**	-1.960	1.34	1.23	-0.014***	2.52	-0.13	0.78	-0.010***	0.75
Coastal I	-1.51	-1.01	-0.0140***	-0.170	-0.55	-1.17	-0.060***	5.13	0.88	0.67	0.011***	-0.32
Coastal II	2.83	1.83	0.0130	-1.700	-2.17	-1.18	-0.060***	3.05	0.36	0.28	-0.002*	0.58
Coastal III	-2.78	-2.83	-0.0200***	-0.670	-1.73	-0.73	-0.050***	2.93	1.38	0.18	0.003*	1.10
Coastal IV 6 h	1.77	2.54	2.1000	8.200	0.15	2.33	-6.970**	5.07	-3.64	-2.06	7.610***	-10.60
Coastal IV 12 h	0.64	0.21	-0.4900	4.190	0.26	0.50	-0.110	0.18	nd	nd	nd	nd
Coastal IV 24 h	0.82	1.37	-0.6700	2.170	-0.32	-0.75	1.100	-1.02	0.33	0.38	1.850***	-1.42
Coastal V	-0.27	-0.2	-0.0050	0.196	0.46	0.49	-0.003**	0.85	1.11	0.86	0.010***	0.34

Table 2. Summary of regression analysis of growth rates of Labyrinthulomycetes, bacteria and phytoplankton (chlorophyll a) in all experimental bottles with respect to concentration of unfiltered seawater in the present study. Slopes significantly different than zero are marked as * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$. (df = degrees of freedom)

Experiment	Parameters	df	R ²	Adjusted R ²	P value
77° E	Labyrinthulomycetes	1,13	0.030	-0.043	0.5290
	Bacteria	1,13	0.730	0.710	4.72E-05***
	Chlorophyll a	1,13	0.922	0.916	1.4E-08***
83° E	Labyrinthulomycetes	1,13	0.086	0.015	0.2900
	Bacteria	1,13	0.699	0.676	0.000103***
	Chlorophyll a	1,13	0.578	0.545	0.001006***
93° E	Labyrinthulomycetes	1,12	0.502	0.456	0.0067**
	Bacteria	1,13	0.850	0.840	1.2E-06***
	Chlorophyll a	1,12	0.690	0.660	0.0008***
Coastal I	Labyrinthulomycetes	1,13	0.643	0.615	0.00033***
	Bacteria	1,13	0.890	0.880	1.36E-07***
	Chlorophyll a	1,13	0.743	0.723	3.59E-05***
Coastal II	Labyrinthulomycetes	1,12	0.203	0.136	0.1060
	Bacteria	1,13	0.850	0.840	1.03E-06***
	Chlorophyll a	1,12	0.301	0.243	0.0423*
Coastal III	Labyrinthulomycetes	1,13	0.834	0.821	2.039E-06***
	Bacteria	1,13	0.919	0.912	1.85E-08***
	Chlorophyll a	1,13	0.330	0.280	0.0246*
Coastal IV (6 h)	Labyrinthulomycetes	1,8	0.080	-0.04	0.4320
	Bacteria	1,8	0.530	0.470	0.0170**
	Chlorophyll a	1,17	0.786	0.774	4.27E-07***
Coastal IV (12 h)	Labyrinthulomycetes	1,8	0.020	-0.100	0.7100
	Bacteria	1,8	0.006	-0.120	0.8300
Coastal IV (24 h)	Labyrinthulomycetes	1,8	0.120	0.004	0.3400
	Bacteria	1,8	0.140	0.030	0.2900
	Chlorophyll a	1,17	0.810	0.797	1.67E-07***
Coastal V	Labyrinthulomycetes	1,13	0.220	0.160	0.0792
	Bacteria	1,13	0.480	0.440	0.0045**
	Chlorophyll a	1,13	0.710	0.690	7.52E-05***