

Thraustochytrid protists degrade hydrocarbons*[†]

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Although thraustochytrid protists are known to be of widespread occurrence in the sea, their hydrocarbon-degrading abilities have never been investigated. We isolated thraustochytrids from coastal waters and sediments of Goa coast by enriching MPN isolation tubes with crude oil. Three isolates tested showed positive hydrophobicity of cell walls as judged by the Microbial Adhesion to Hydrocarbons (MATH) assay. Addition of Bombay High crude oil to nutrient broth slightly enhanced growth of the protists as compared to unenriched controls. Autoclaved crude oil added to sediments was degraded by 2 thraustochytrids to a much greater extent than non-autoclaved oil. Tarballs supported excellent growth of thraustochytrids when added to a peptone broth. Inoculation of thraustochytrids to tarball-enriched sediment resulted in a decrease of up to 71% of tarball contents after a month. Up to 30% of tarballs added to peptone broth was degraded by thraustochytrids in 7 days, as estimated by gravimetry and gas chromatography. Fractions above the retention time for 20°C aliphatics were degraded to a much greater degree than those below 20°C. Thraustochytrids appear to play a definite role in tar ball degradation in sediments.

Thraustochytrids are unicellular, zoosporic, marine fungi belonging to chromistan protists and are ubiquitous in the sea. They occasionally attain biomass that parallels that of bacteria, and have been isolated from a wide variety of habitats, including seawater and sediments, macrophytic detritus, live algae, animal guts and live animals¹⁻⁷. In spite of this, their ecological role in the sea has been poorly investigated. Bahnweg^{8,9} has shown that thraustochytrids have the capability to utilize a wide range of organic nitrogen and carbon compounds for their nutrition. However, the capability of these protists to degrade hydrocarbons has not been examined so far.

Hydrocarbons occur in seawater either through natural means such as seepage from oil wells in the seafloor, by chronic pollution in harbours and oil tanker routes or as a result of acute, periodic inputs such as oil tanker accidents¹⁰. A wide variety of microorganisms, particularly bacteria, fungi and cyanobacteria are known to be important hydrocarbon degraders in the sea¹¹. Being regular components of the marine microbial consortia, the thraustochytrids are also presented with an opportunity to degrade and utilize hydrocarbons for their nutrition.

Hydrocarbon-degrading microbes may be selective in utilizing a few of the hundreds of fractions in natural crude oil, broadly classified into (a) the saturates or aliphatic fraction, including paraffins (*n*-alkanes and branched alkanes) and naphthanes (cycloalkanes); (b) the aromatic fraction and (c) the asphaltenes¹²⁻¹⁵. A more complete degradation of oil in the marine environment is possible with mixed populations of microbes^{11,15}. Therefore, it is of intrinsic interest to study the different components of the natural assemblages of microorganisms in order to understand hydrocarbon degradation in nature. Information gathered therefrom can also be utilized to construct artificial microbial inocula which can be applied in the field to clean up catastrophic spills¹¹. The present study was carried out to examine the hydrocarbon-degrading potentials of thraustochytrids isolated from the coast of Goa.

Materials and Methods

All the thraustochytrid cultures used in this study were isolated from six sites along the coast of Goa (15°25'N; 73°95'E), which were exposed to chronic oil pollution due to the presence of boat jetties, dockyard or harbour – Dona Paula jetty; Betim jetty; Cortalim boatyard; Malim jetty; Marmagoa harbour and Siridao beach.

Isolation and enumeration—A modification of the Most Probable Number (MPN) technique, as described by Gaertner¹⁶ was used. For each water

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sample, four replicates of four dilutions each, namely 20, 10, 5 and 2.5 ml were added to sterile, flat-bottomed, wide-mouthed, screw-capped bottles (total of 16 tubes). The final volume in each tube was adjusted to 20 ml using sterile seawater. A small amount of pine pollen sterilized at 90-100°C for 48 h was added as a bait to aid growth. For each sample, one set of MPN tubes was enriched with 0.05 ml of Bombay High crude oil, while another set of 16 tubes was unenriched. A similar technique was used for sediment samples, whereby a suspension containing a known amount of soil was added in dilutions of 2, 1, 0.5 and 0.25 ml to sterile seawater in tubes as above, with the final volume made up to 20 ml with sterile seawater and baited with pine pollen. One enriched and one unenriched sets were maintained. All tubes were microscopically examined after 1 week for growth of thraustochytrids on pine pollen and their numbers per litre were calculated using Thomas formula¹⁷. Axenic cultures were obtained from the tubes by inoculating a loopful of the initial culture onto 5 ml sterile seawater containing 0.05 g streptomycin and 1000 units of penicillin. After 5 days, the bacteria-free cultures were streaked onto plates containing Modified Vishniac's agar¹⁸. Bacteria-free colonies from the plates were subsequently transferred to seawater / pine pollen in tubes and maintained therein. Isolates were subcultured every 20 days.

Growth in the presence of hydrocarbons—Two basal and 3 organically rich media were used. These were: (1) basal salts medium containing thiamine, pyridoxine and biotin¹⁹; (2) basal organic medium with the above organic compounds and 0.1% glutamate; (3) Oppenheimer-Zobell-Gaertner (OZG) medium containing 1% glucose, 1% peptone, 2% yeast extract, 0.002% FeCl₃ and 0.125% Na₂HPO₄ in seawater; (the medium was adjusted to pH 4.5 prior to autoclaving and subsequently adjusted to pH 8.0 with sterile, 0.1 N NaOH (ref. 20)); (4) Modified Vishniac's (MV) medium and (5) 0.1% peptone in seawater. Media 1-4 were dispensed in 50 ml volumes in 250 ml flasks and enriched with 0.15 g Bombay High crude oil. For growth of thraustochytrids on tarballs, 100 mg of tarball dissolved in 2 ml of hexane was added to 20 ml of medium 5 in 100 ml flasks (0.5% tarballs, w/v). These flasks were inoculated only after 3 days to allow evaporation of hexane. Inocula were raised in MV broth and 0.5 ml of the culture was used as inoculum for media 1 to 4. In the case of medium 5 containing tarballs, the inoculum was prepared as

follows: Cells grown in MV broth were harvested by centrifugation and resuspended in sterile seawater to yield an optical density of 0.7. About 8 ml of this was added to 12 ml of peptone in seawater to yield a final concentration of 0.1% peptone. Three replicates were maintained for each treatment. Growth was estimated after 7 days by centrifuging the cells and resuspending in distilled water. The suspension was added to the original flask and total proteins were estimated according to Lowry's method²¹.

Cell wall hydrophobicity, as expressed by the fraction of adherence to hexadecane was estimated by the MATH (Microbial Adhesion to Hydrocarbons) assay, according to Rosenberg²².

Degradation of hydrocarbons in sediment—A known weight of intertidal sediment (silt-sand) from Dona Paula beach, Goa was autoclaved in 5 cm acid-washed Petri dishes. To these were added: 0.15 g of non-autoclaved crude oil; 0.15 g of autoclaved crude oil; or 2 ml hexane containing 100 mg of tarballs. In the last case, the dishes were left at 45°C overnight to allow the hexane to evaporate.

The plates were inoculated with 1 ml cell suspension of thraustochytrids in seawater, obtained from MV broth after harvesting and rinsing the cells in sterile seawater. Three replicates and uninoculated controls were maintained. The plates were sealed with adhesive tape to retain moisture. Following incubation for a month, the percent degradation of tarballs was estimated using 1 or more of 3 different methods: Fluorimetry: Degradation of Bombay High crude oil and tarballs in sediments was estimated as follows. The sediment was placed in 100 ml Erlenmeyer flasks and extracted thrice in 5 ml of double-distilled hexane, for 1 h each on a shaker. The extracts were pooled, a pinch of sodium sulphite added to remove moisture and filtered through a GF/C filter. Fluorescence²³ was read using F-2000 fluorescence spectrophotometer (Hitachi) with an excitation at 310 nm and emission at 360 nm. The values were read against standard curves made up with 0.2-0.5 µg/ml of Bombay High crude oil in double-distilled hexane. Simultaneously, the total carbohydrates and nitrogen in the sediment were estimated using sulphuric acid extraction²¹ and Kjeldahl analysis, respectively. Degradation of tarballs in peptone broth by thraustochytrids was estimated by gravimetry and gas chromatography. (1) Gravimetry: Tarballs were extracted from experimental flasks with 10 ml of carbon tetrachloride, the extract transferred to pre-weighed Petri dish and the solvent allowed to

evaporate at 37°C for 24 h. The final weight of the dishes was measured and percentage of tarballs degraded was calculated. (2) Gas chromatography: The tarball fraction from the experimental flasks was extracted in 10 ml of analytical grade carbon tetrachloride and analysed using a Chemito Gas chromatograph, fitted with a capillary column. The analyses were carried out from 60°C to 320°C at a temperature ramp of 6°C/min. Percentages of degradation were calculated based on the total areas of peaks of control and experimental chromatograms. The retention times of individual peaks were compared against those of standard alkanes of various carbon numbers.

Results

Densities of thraustochytrids in water and sediments—Thraustochytrids ranged from 50 to 750 / l in the water samples (Fig. 1A). Crude oil addition to isolation tubes resulted in much less numbers in 2 sites, but not in others. Thraustochytrids in sediments ranged from 1 to 30 / g at Betim and Malim respectively (Fig. 1B). Much higher numbers were

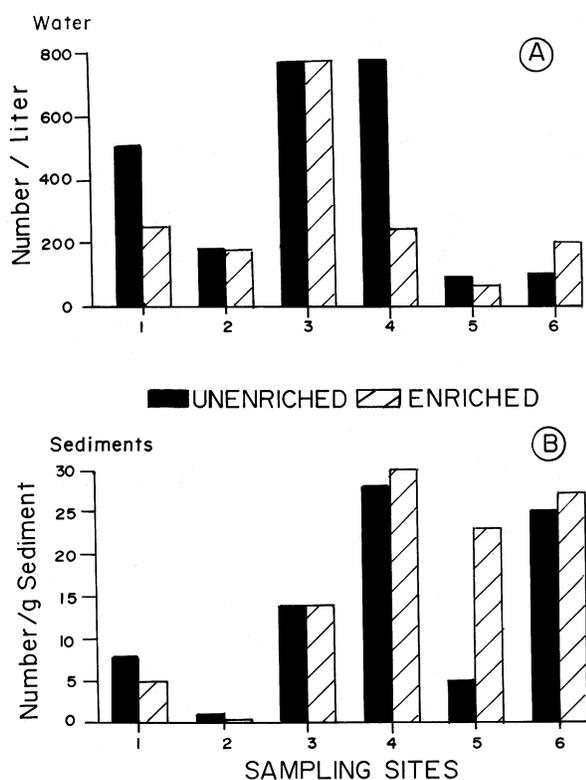


Fig. 1—Abundance of thraustochytrids in water and sediments from 6 different sites along the coast of Goa, as estimated under conditions of crude oil enrichment in MPN tubes and its absence. 1) Dona Paula jetty; 2) Betim jetty; 3) Cortalim boatyard; 4) Malim jetty; 5) Marmagoa harbour and 6) Siridao beach

recovered from harbour sediments when crude oil was added to isolation tubes. No distinct differences between crude oil enrichment or its absence was observed for other sites (Fig. 1B).

MATH assay—The maximum fraction of adherence for the 3 isolates tested ranged from 0.2-0.42 (Fig. 2). Adherence was very poor or absent when the assay was carried out at 5°C.

Growth in the presence of oil and tarballs—All the thraustochytrid isolates tested grew very poorly or not at all in the basal salts and the basal organic medium, whether enriched with oil or not. On the contrary, excellent growth was observed in 20% OZG and MV media (Figs. 3,4). Growth in OZG medium was slightly enhanced in 3 out of 5 isolates tested when crude oil was added. Growth in MV medium was distinctly higher in the presence of crude oil. Microscopic examination revealed thraustochytrid cells on the surface of oil droplets or embedded within them (Fig. 5). Addition of tarballs to the peptone broth significantly improved growth of the thraustochytrids tested (Fig. 6). In the case of isolate # 21, increase in growth in tarballs was more than 100% when compared with controls.

Degradation of crude oil and tarballs in sediments—Two isolates were grown on autoclaved and non-autoclaved crude oil mixed with sterile sediment (Fig. 7). After a period of 1 month, no discernible decrease in extractible crude oil was observed when non-autoclaved oil was used. However, autoclaved crude oil added to sediments was degraded to a considerable extent.

Inoculation of 3 thraustochytrids onto sediment containing tarballs resulted in a marked decrease in the concentration of the extractible fraction of tarballs (Table 1). Isolate # 67 showed a high degree of

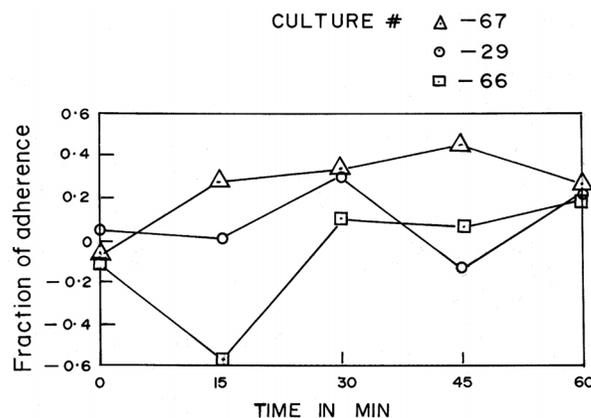


Fig. 2—Fraction of adherence of 3 thraustochytrids, as estimated by the MATH (Microbial Adhesion to Hydrocarbons) assay

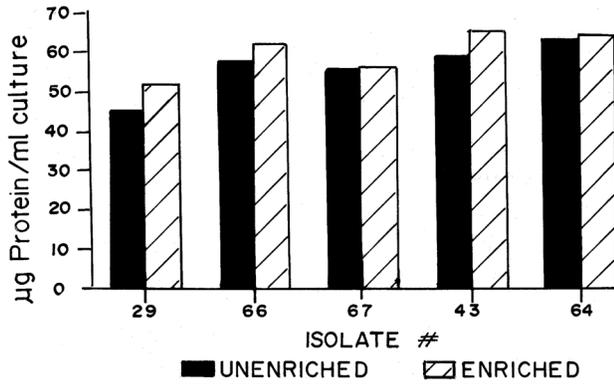


Fig. 3—Growth of thraustochytrids in OZG medium under conditions of crude oil enrichment and its absence

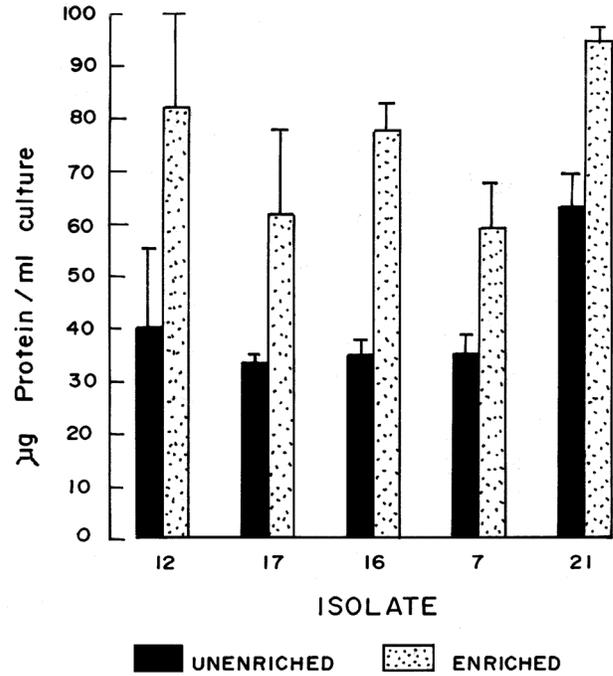


Fig. 6—Growth of thraustochytrids in peptone broth under conditions of tarball enrichment and its absence

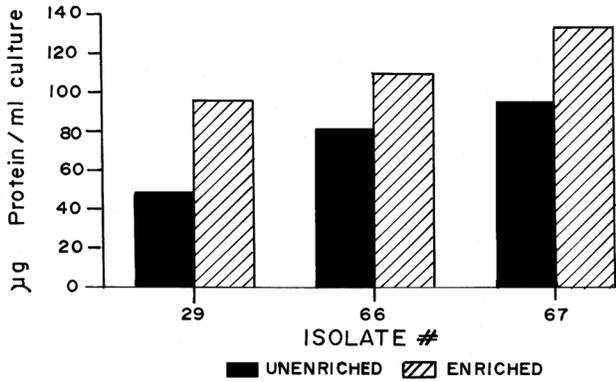


Fig. 4—Growth of thraustochytrids in MV medium under conditions of crude oil enrichment and its absence



Fig. 5—Thraustochytrid cells adhering to an oil droplet. Bar represents 10 µm (x 3,300)

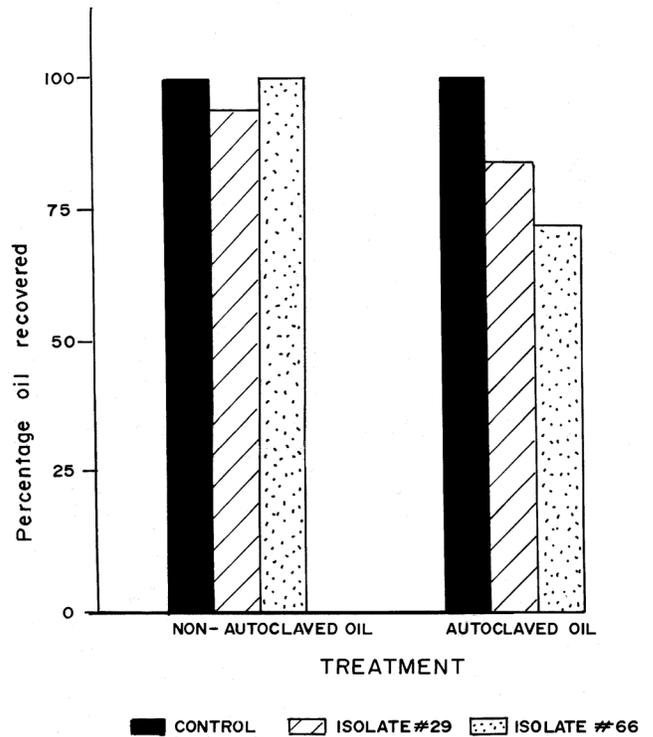


Fig. 7—Percentage of crude oil recovered from sediments after growth of thraustochytrids for 1 month

reduction, equalling 71%, followed by # 66 (58%) and # 29 (48%). Concomitantly, total nitrogen in the sediment showed a distinct decrease (Table 1). Growth of 2 thraustochytrids (# 29 and # 66) was more pronounced in sediments enriched with tarballs than in the control.

Gravimetry and gas chromatography revealed that thraustochytrids removed 15-30% of the tarballs incorporated into the nutrient broth in 7 days (Fig. 8). The GC profiles are given in Fig. 9. Hydrocarbons above C 20 were removed to a greater extent than those below C 20.

Discussion

According to Rosenberg¹⁰, microorganisms capable of predominantly utilizing soluble hydrocarbons may not adhere to oil and have little need for hydrophobicity of cells. On the contrary, organisms that adhere to high molecular weight hydrocarbons by virtue of their hydrophobic cell walls are potential degraders of hydrocarbons that have a poor solubility in water. The MATH assay has commonly been used to test cell wall hydrophobicity²². Judging by this assay, all 3 thraustochytrids tested appeared to be potential hydrocarbon degraders, with fractions of adherence ranging from 0.3 to 0.4 (Fig. 2). Bacteria which degrade lighter crudes were shown to have MATH values of >0.3 and those capable of degrading heavier crudes, >0.4. Often, the optical densities in the MATH assays, after mixing with hexadecane and allowing 20 min for phase separation, were higher than those of the cell suspensions alone. This leads us to suspect that in the present assays, many thraustochytrid cells remained in the aqueous phase along with adhered hexadecane droplets and did not segregate into the upper organic phase, apparently by virtue of the high relative densities of the cells. In such cases, fractions of adherence of the three isolates tested could have been higher than that reported here. It is possible that thraustochytrids, as a group, have hydrophobic cell walls.

Table 1—Degradation of tarballs by thraustochytrids in sediment after 1 month

Parameters analyzed	Treatment			
	Uninoculated control	Inoculated with # 29	Inoculated with # 66	Inoculated with # 67
% Tarballs recovered	100	51.7	41.7	30.8
mg N / g sediment	2.5	2.08	2.1	1.94

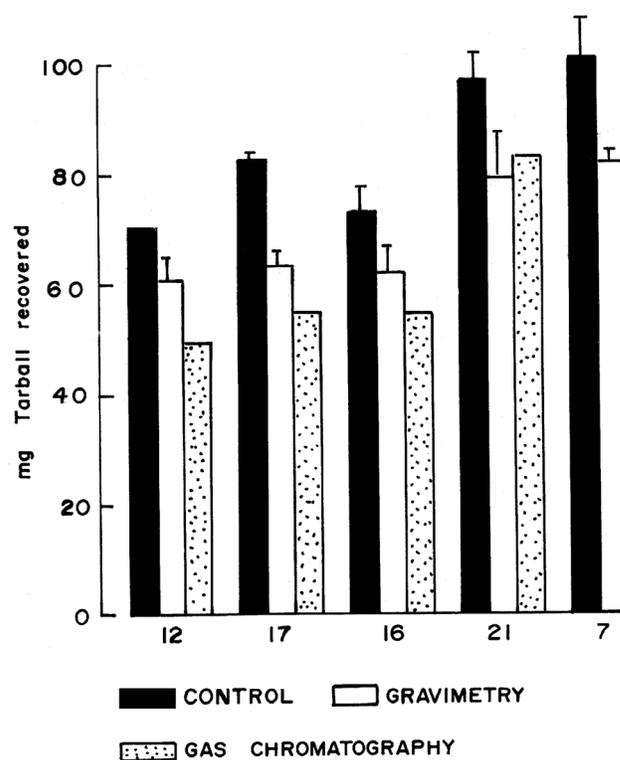
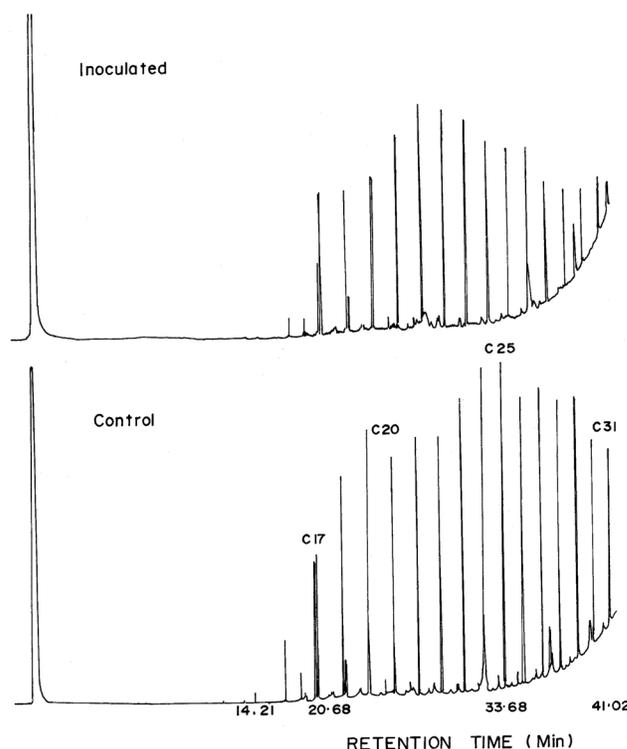


Fig. 8—Amount of tarballs recovered from peptone broth after growth of thraustochytrids, based on gravimetric and gas chromatographic analyses



9—Gas chromatograph profile of tarballs extracted after growth of thraustochytrids, along with control profile

The thraustochytrids tested showed no growth when crude oil was incorporated into basal media. On the contrary, excellent growth was achieved when media rich in organic nitrogen were used (Figs. 3,4), indicating the need for such a nitrogen source for hydrocarbon degradation by thraustochytrids. Bahnweg⁷ has shown earlier that thraustochytrids are inefficient in using inorganic nitrogen. Since organic nitrogen in quantities sufficient for growth of thraustochytrids is more likely to be present in the sediment than in the water column, we presume that these protists are more efficient in degradation of hydrocarbons in the former habitat than in the latter. The following sequence of logic and observations appear to corroborate our views.

1) While crude oil distinctly enhanced growth of thraustochytrids in MV medium, it did so only marginally when OZG medium was employed (Figs. 3,4). It is likely that toxic fractions of crude oil inhibited a more pronounced increase in growth. Several low molecular soluble aromatics are considered toxic to many microorganisms²⁴ and heating the oil volatilizes and removes these¹⁵. The much greater degradation of autoclaved crude oil mixed with sediments, when compared with non-autoclaved oil (Fig. 7) indicates that weathered oil, bereft of many possibly toxic aromatics may support growth of thraustochytrids than crude oil *per se*.

2) Weathered oil, in the form of tarballs, eventually sinks to the bottom and is relatively resistant to microbial attack¹³. Thraustochytrids evinced appreciable growth on tarballs *in vitro* (Fig. 6).

3) Thraustochytrids degraded high proportions of tarballs from sediments, accompanied by a decline of organic nitrogen (Table 1). However, since tarballs undergo a strong binding to sediments making them very recalcitrant to degradation, our estimations of tarball degradation are restricted to fractions extractable by the methods that we have used in this study. Nevertheless, our results strongly suggest that thraustochytrids may play a definite role in the degradation of weathered oil in sediments.

As estimated by gravimetry and gas chromatography (Fig. 8), up to 30% of tarballs was degraded in nutrient broth. Fractionation of tarballs has shown that these contain approximately 32% aliphatics, 5% aromatics, 11% polar compounds and 53% asphaltenes. At the moment, the nature and extent of these fractions degraded by thraustochytrids is not clear. Broadly, however, fractions above the retention time for C 20 saturates were preferentially

and to a very high degree degraded (Fig. 9), suggesting that possible affinity of thraustochytrids to long chain aliphatics. This is interesting, since for bacteria, in general, *n*-alkanes are less susceptible²⁵ to degradation as the carbon length increases from 16 to 32. Mixed bacterial populations have been shown to degrade significant proportions of asphaltenes and resins in tarballs^{11,26}. The asphaltene fraction contains a mixture of high boiling compounds, a part of which is called the unresolved complex mixture (UCM) and cannot be resolved by gas chromatography. The capability of thraustochytrids to degrade these fractions, as well as other recalcitrant hydrocarbons such as the branched alkanes, pristane and phytane, cycloparaffins and polycyclic aromatics^{14,24,27} merits further investigation.

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