

Occurrence of thraustochytrid fungi in corals and coral mucus

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Occurrence of thraustochytrid fungi in corals, fresh coral mucus and floating and attached mucus detritus from the Lakshadweep islands in the Arabian Sea was studied. *Corallochytrium limacisporum* Raghukumar, *Thraustochytrium motivum* Goldstein, *Labyrinthuloides minuta* (Watson and Raper) Perkins, *L. yorkensis* Perkins and *Ulkenia visurgensis* (Ulken) Gaertner were isolated. *C. limacisporum* was the most common and was detected in the polyps of 3 corals using immunofluorescence. Mucus detritus from Kalpeni island harboured 1.9×10^6 thraustochytrids. g^{-1} dry weight and fresh mucus from *Acropora* sp. 12 and 20 ml^{-1} . The number of thraustochytrids in detrital mucus incubated with sterilised lagoon water increased significantly than when incubated with unsterilised water, suggesting grazing by protozoans. Fresh mucus may be further colonised by thraustochytrids once liberated into the water. Ciliates could be maintained in the laboratory on cells of *C. limacisporum*.

The unicellular and obligate marine fungi, thraustochytrids¹, have received little attention despite the fact that these are regularly recorded from water, sediments and algae^{2,3}. This has resulted in only a partial evaluation of the role of fungi in marine processes. The thraustochytrid *Corallochytrium limacisporum* Raghukumar occurs abundantly in the waters of the coral reef lagoons in the Lakshadweep islands of the Arabian Sea^{4,5}. In the present study, occurrence of thraustochytrids in corals and coral mucus and their dynamics in terms of numbers in the latter substrate have been studied.

Materials and Methods

This study was carried out in the Lakshadweep group of islands in the Arabian Sea (lat. $10^{\circ}30' - 11^{\circ}30'$ N, long. $72^{\circ} - 73^{\circ}$ E). Immunofluorescence technique was used to detect *C. limacisporum* in coral polyps^{6,7}. Antiserum was prepared against *C. limacisporum*, isolate (NIOCC 77), which was obtained from the lagoon waters of the Kavaratti island. The fungus was grown in a liquid nutrient medium containing 0.25% glucose, 0.25% peptone, vitamins according to Vishniac⁸, and seawater. Cells were harvested after a week's growth, suspended in phosphate-buffered saline and the protein was estimated⁹. About 2 mg protein was administered into New Zealand White Rabbits as 4 weekly intramuscular injections. The

first 2 were given with Freund's complete adjuvant and the subsequent ones with incomplete adjuvant. Antiserum was collected, purified and conjugated with fluorescein isothiocyanate. In order to detect thraustochytrid in polyps, bits of living coral were first rinsed in sterile seawater, crushed and the coarse calcareous bits discarded. The homogenised polyp tissue was stained with a 1:4 dilution of the antiserum and examined under an Olympus BH2 RFL epifluorescence microscope, using a 495 nm excitation filter and 515 nm barrier filter.

Either of the following methods was used to isolate and enumerate thraustochytrids: (1) The substratum was homogenised in a known amount of sterile seawater and 0.1 ml aliquots plated onto a nutrient seawater agar medium containing 0.05 % glucose, 0.05% peptone, vitamin mix⁸ and 50 mg streptomycin sulphate and 20,000 units sodium benzyl penicillin per 100 ml. Dry weights of aliquots of the suspension were determined independently. Numbers of thraustochytrids were estimated from the number of thraustochytrid colonies per plate and the corresponding dry weight of material plated. (2) Numbers were also enumerated by the MPN method^{10,11}. Five replicates of 4 dilutions of the above suspension, 1, 0.5, 0.25 and 0.13 ml were added to 5 ml of sterile seawater in petri dishes and sterilised pine pollen added as bait. Numbers were calculated

on the basis of presence or absence in each series of dilution and the dry weight of the homogenate.

Growth of thraustochytrids in mucus under 3 different conditions, (1) absence of protozoans and bacteria, (2) absence of protozoans alone and (3) presence of both protozoans and bacteria, was investigated. Three samples were studied. Two of these consisted of fresh, liquid mucus of *Acropora* sp. from Agatti island. Coral pieces were placed over a funnel inverted over a bottle and covered with a plastic bag under sterile conditions. The third sample consisted of flocculated mucus detritus attached to coral rocks in the lagoons, which was sucked up from the substrates below the water using sterile, 50 ml Millipore syringes.

All samples were distributed in 9 glass tubes each, bearing 200 μm perforations. The liquid, fresh mucus samples were distributed in 1 ml amounts and the mucus detritus as aliquots of a suspension containing 1 mg dry wt (as determined later from the suspension). All the tubes were covered with dialysis tubing to allow the diffusion of dissolved organic matter and to prevent its buildup in unnaturally high concentrations. Sets of 3 tubes each were subjected to 3 different treatments: (1) 10 ml of sterile seawater with penicillin and streptomycin in concentrations mentioned earlier; (2) sterile seawater without antibiotics and (3) unsterilised seawater collected fresh from the lagoon. Each tube was immersed in flasks containing 750 ml seawater corresponding to the treatment the tube was undergoing. The water in the flasks was replaced daily till the end of the 8 to 10 d experiment with fresh seawater corresponding to the treatments. Number of thraustochytrids was estimated every 2 d starting from day 0.

The results were analysed using a one-way analysis of variance¹² (ANOVA). Separate analyses were carried out for values between different incubation days of each treatment (days) and between 3 treatments together for each day of incubation (treatments).

C. limacisporum was grown on 5 ml of liquid nutrient medium in 5 cm petri dishes. After a week, the medium was drained off and the cells attached to the bottom of the dish rinsed with sterile seawater. Sterile seawater (5 ml) or a 50 or 100% liquid medium (0.05% glucose, 0.05% peptone, vitamin mix⁸) containing streptomycin and penicillin was added and inoculated with a loopful of ciliates. Ciliates were obtained from the setup in which mucus in unsterile seawater had been baited with pine pollen. The number of ciliates in the inoculum was estimated by microscopically enumerating 10 replicates of 1 loopful each. Number of ciliates in 25 microscope

field areas of the water column of the petri dish were counted and the values extrapolated to the total petri dish volume.

Results

Immunofluorescence—Specificity of antiserum was tested against 4 isolates of thraustochytrids, namely, *Thraustochytrium motivum* Goldstein (NIOCC 84), *Thraustochytrium* sp. isolated from mucus in the coral reef lagoons, *Schizochytrium* sp. isolated from algae in the Zuari estuary, Goa and *Ulkenia visurgensis* (Ulken) Gaertner from hydroid polyps from the Zuari estuary, Goa. A mold, *Aspergillus* sp. and yeasts and bacteria which were present in the polyps themselves also served as controls. No cross reaction of the antiserum with any of these species was observed and none of the species tested revealed fluorescence with the FITC-conjugated antiserum of *C. limacisporum*.

Three species of corals, an unidentified species of *Acropora*, *Acropora secale* and *Porites* sp. from the Kadamat island were examined for the presence of *C. limacisporum*. Homogenised polyp tissue of all 3 species revealed cells which fluoresced bluish-green with the antiserum confirming the presence of the thraustochytrid within the polyps. The numerous zooxanthellae present revealed reddish-orange autofluorescence of the chlorophyll and an yellowish fluorescing pyrenoid. The number of *C. limacisporum* cells was low in *Porites* sp. and *Acropora secale*. However, *Acropora* sp. revealed numerous cells of the thraustochytrid, which were 3.5-4.5 μm in diam., often containing prominent globules. Average counts from 100 microscope fields revealed the presence of 1 cell of *C. limacisporum* for every 23 zooxanthellae in this coral.

Isolation of thraustochytrids—Thraustochytrids were isolated from fresh polyps of *Acropora* sp. and *Pocillopora* sp. from Kadamat island, polyps of *Acropora* sp. from Agatti island, fresh mucus from *Acropora variabilis*, *A. arbuscula* and *Acropora* sp. from Minicoy, Kalpeni and Agatti islands, floating mucus detritus from Kalpeni island and mucus adhering to corals from Kalpeni, Kavaratti, Chatlet and Kadamat islands. *Corallochytrium limacisporum* was isolated from 9 of these samples. The other species isolated were *Labyrinthuloides yorkensis* Perkins, *L. minuta* (Watson and Raper) Perkins, *Thraustochytrium motivum* Goldstein, *Ulkenia visurgensis* (Ulken) Gaertner and yeasts. Fresh mucus from *Acropora* sp. from Agatti island and mucus adhering to corals from Kavaratti island yielded 3 species of thraustochytrids.

Growth in coral mucus—Mucus detritus attached to corals yielded thraustochytrids $1.9 \times 10^6 \cdot g^{-1}$ mucus (Table 1), whereas the 2 samples of fresh mucus yielded 12 and $20 \cdot ml^{-1}$ mucus (Tables 2 and 3). On incubation, the number of thraustochytrids increased significantly by the 6th day for all 3 treatments in the case of mucus detritus (Table 1) and by the 5th or 8th day for sample 1 fresh mucus (Table 2). Numbers subsequently declined in all cases.

Highest numbers of thraustochytrids occurred when the samples were incubated in sterile seawater with antibacterial antibiotics. In the case of mucus detritus and fresh mucus sample 1, the numbers were distinctly lower when unsterilised lagoon water was used for incubation (Tables 1 and 2).

Mucus detritus harboured *C. limacisporum* (1.4×10^6 cells) and *Labyrinthuloides minuta* (0.5×10^6). No other species appeared when

Table 1—Total numbers of thraustochytrids (no. g^{-1}) mucus detritus incubated under 3 different conditions

Days	Treatments*			One-way ANOVA 'F' values (treatments) (df = 2, 6)
	1	2	3	
0	1.9×10^6	1.9×10^6	1.9×10^6	—
2	15.0×10^6	2.7×10^6	1.7×10^6	35.1 ^c
4	29.0×10^6	14.8×10^6	5.0×10^6	249.0 ^c
6	71.4×10^6	25.5×10^6	12.5×10^6	32.6 ^c
8	27.7×10^6	7.0×10^6	1.6×10^6	1560.4 ^c
One-way ANOVA values (df 4, 10)	736.0 ^c	5.6 ^a	10.6 ^b	

* 1—Sterile seawater with streptomycin and penicillin, 2—sterile seawater without antibiotics, 3—unsterile seawater from lagoon. Significant at: ^a 5.0%; ^b 1% and ^c 0.1% level

Table 2—Total numbers of thraustochytrids (no. ml^{-1}) fresh mucus (sample 1) from *Acropora* sp. incubated under 3 different conditions

Days	Treatments*			One-way ANOVA df and 'F' values (treatments)
	1	2	3	
0	12	12	12	
2	6730	67	100	2, 6 (6.8 ^b)
5	11270	5833	4066	2, 6 (10.6 ^b)
8	—	1400	4130	1, 4 (26.7 ^a)
10	—	300	200	1, 4 (0.4 ^{ns})
One-way ANOVA df and 'F' values (days)	3, 8 (71.8 ^b)	5, 12 (241.0 ^b)	5, 12 (10.3 ^b)	

* 1—Sterile seawater with streptomycin and penicillin, 2—sterile seawater without antibiotics, 3—unsterile seawater from lagoon. Significant at: ^a 1% and ^b 0.1% level, NS—Not significant

Table 3—Total numbers of thraustochytrids (no. ml^{-1}) fresh mucus (sample 2) from *Acropora* sp. incubated under 3 different conditions

Days	Treatments*			One-way ANOVA df and 'F' values (treatments)
	1	2	3	
0	20	20	20	
2	0	0	1000	2, 6 (81.0 ^b)
4	0	0	1120	2, 6 (12.6 ^a)
6	3250	87	370	2, 6 (57.9 ^b)
8	—	0	183	1, 4 (30.3 ^a)
10	—	4000	105	1, 4 (236.0 ^b)
One-way ANOVA df and 'F' values (days)	3, 8 (71.8 ^b)	5, 12 (241.0 ^b)	5, 12 (10.3 ^b)	

* 1—Sterile seawater with streptomycin and penicillin, 2—sterile seawater without antibiotics, 3—unsterile seawater from lagoon. Significant at: ^a 1% and ^b 0.1% level

incubated in unsterile seawater (Fig. 1). Fresh mucus sample 1 yielded only *L. minuta* in the 0 d isolations, but apparently harboured *C. limacisporum* and *Thraustochytrium motivum*, because these were isolated when the mucus was further incubated in sterile seawater (Fig. 2). The other sample of fresh *Acropora* mucus (sample 2) yielded only *C. limacisporum* (Fig. 3). However, when incubated in unsterile lagoon water, this mucus became colonised also by *L. minuta* and *Thraustochytrium motivum*.

Growth of ciliates—In order to confirm that ciliates do graze on thraustochytrids, feeding experiments were carried out using *C. limacisporum*. In 2 separate experiments, initial numbers of 2 and 40 ciliates increased in numbers to 623 and 2701 in 4 and 5 d respectively, when fed on the thraustochytrid (Fig. 4). Their numbers subsequently declined. When inoculated onto a 50 or 100% liquid medium

containing actively growing cells of *C. limacisporum*, ciliates increased in numbers to 41690 and 103920 in 3 and 4 respectively.

Discussion

Thraustochytrid fungi seem to form a common microbial component of the coral reef ecosystem, while the presence of thraustochytrids in the coral reef lagoon waters has been demonstrated⁴, the

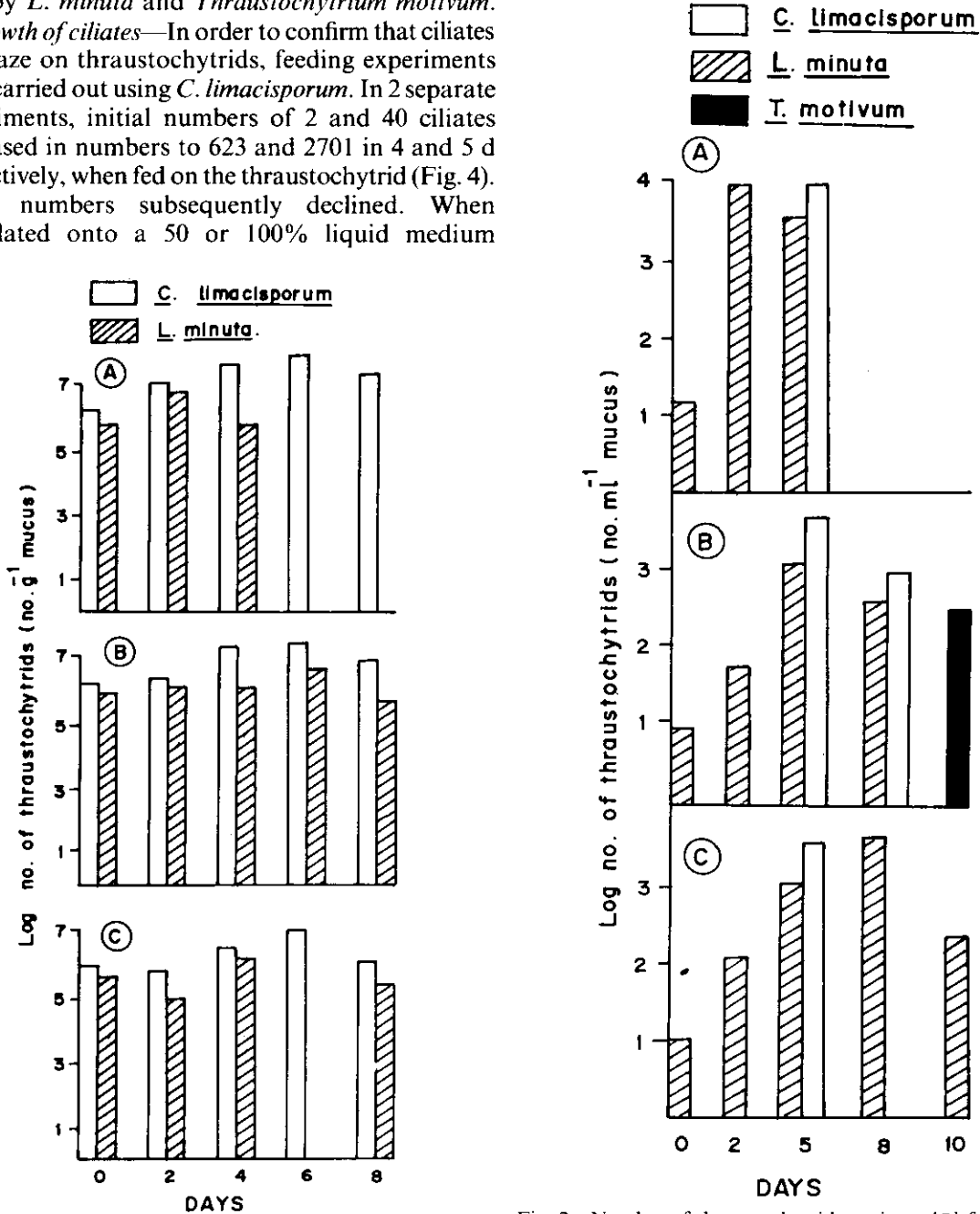


Fig. 1—Number of thraustochytrid species.g⁻¹ mucus detritus incubated in (A) sterile seawater with antibiotics; (B) sterile seawater without antibiotics and (C) unsterile seawater

Fig. 2—Number of thraustochytrid species. ml⁻¹ fresh mucus (sample 1) from *Acropora* sp. incubated in (A) sterile seawater with antibiotics, (B) sterile seawater without antibiotics and (C) unsterile seawater

presence of a thraustochytrid in the tissues of a coral polyp has been directly demonstrated in the present study using immunofluorescence. Thraustochytrids have also been demonstrated inside sponges^{13,14}, echinoderms¹⁵ and hydroids¹⁶ and may therefore be commonly associated with invertebrates.

Presently available culture techniques might underestimate the actual numbers present since clumps of cells would be represented as one colony. Besides, all the cells present in a sample may not form colonies on nutrient media. Therefore, the values presented here would only be conservative estimates.

Mucus detritus harboured thraustochytrids of $1.9 \times 10^6 \text{ g}^{-1}$ dry weight. However, it could

potentially support much higher numbers of thraustochytrids, since on incubation in seawater, the number of thraustochytrids in mucus detritus as well as fresh mucus increased for a while, followed by a decline. It is possible that an increase in the number of grazing protozoans by this time was responsible for such a decline in the number of thraustochytrids. In this study, amoebae and ciliates were observed in treatments involving incubations of all 3 mucus samples in sterile and especially in unsterile seawater. Ingestion of thraustochytrid cells could be frequently observed by light microscopy. A high number of grazing protozoans in unsterile seawater incubations probably was the reason for the much lower numbers of thraustochytrids in mucus detritus in this treatment when compared to that in sterile seawater.

The number of thraustochytrids in mucus detritus in nature may be controlled by grazers. The increase in number of ciliates when fed cells of *C. limacisporum* confirmed that this is possible (Fig. 4).

Fresh coral mucus from corals is already seeded with thraustochytrids (Tables 2 and 3), which probably multiply once the mucus is liberated into the surrounding water. Ducklow and Mitchell¹⁷ came to the same conclusion regarding bacteria in coral mucus. Once liberated into water, additional species may colonise mucus. Thus, the sample of mucus (sample 2) which initially harboured only *C. limacisporum*, was colonised by *L. minuta* and *T. motivum* when incubated in unsterile lagoon water (Fig. 3).

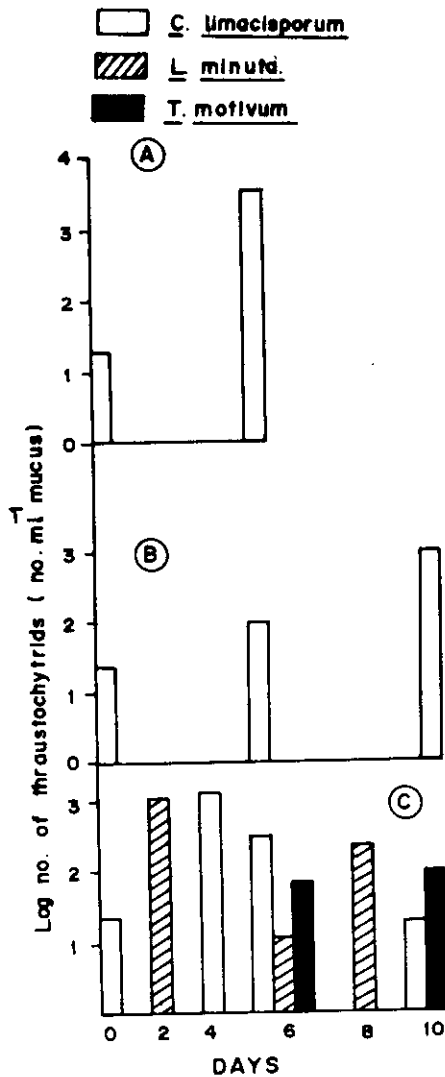


Fig. 3- Number of thraustochytrid species ml^{-1} fresh mucus (sample 2) from *Acropora* sp. incubated in (A) sterile seawater with antibiotics (B) sterile seawater without antibiotics and (C) unsterile seawater

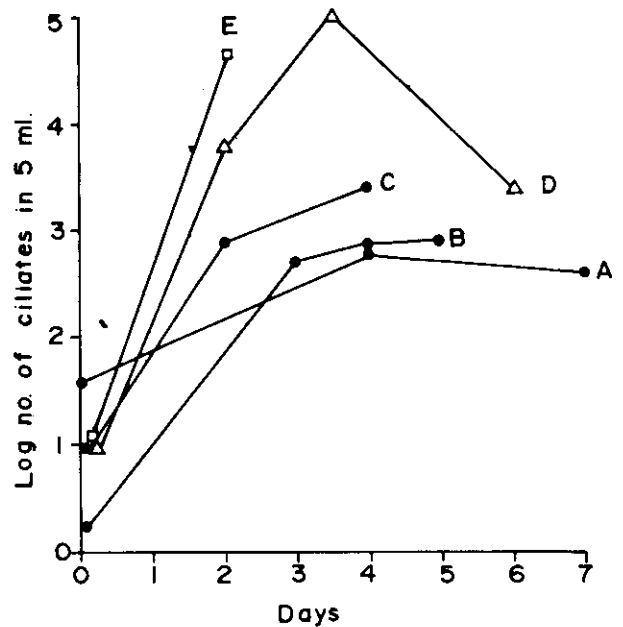


Fig. 4—Number of ciliates grown in the presence of *Corallochytrium limacisporum* cells suspended in 5 ml sterile seawater (A, B, C) and 50% (D) and 100% (E) liquid medium

Microorganisms in detritus are thought to play a vital role as links in trophic web through the intermediary of ciliates¹⁸⁻¹⁹ which are in turn preyed upon by copepods and other grazers²⁰. Coral mucus contributes substantially to detritus and therefore detritus based foodweb in coral reef ecosystems. Studies on bacteria associated with coral mucus¹⁷ have suggested that microorganisms may have a key role in mobilising the mucus for consumption by other organisms. Present results indicate that thraustochytrids may form an important component of the microbial assemblage in coral mucus. The importance of this group of protists in transformation of mucus detritus and as food for lower animals, either in terms of quantity of food or as providers of specific nutrients will have to be worked out in future.

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