

Chapter 6.

Detection of aplanochytrids in zooplankton using molecular probes

6.1 Introduction

There have been many earlier reports on the association of the Labyrinthulomycetes, thraustochytrids and aplanochytrids with invertebrates in the sea. These associations may be parasitic or symbiotic (mutualistic or commensalistic). Among parasitic associations, the 'QPX quahog parasite unknown' (Whyte *et al.*, 1994; Smolowitz & Leavitt, 1997) and the abalone parasite *Aplanochytrium haliotidis* (Bower, 1987; Bower *et al.*, 1989) have been studied in detail. Symbiotic associations have been reported in oysters, mussels, tunicates, corals etc. (see Table 1, Chapter 2). Despite numerous non-parasitic invertebrate associations of Labyrinthulomycetes, the nature of their relationship, namely whether they are commensalistic or mutualistic, is not clear. Detection of the exact location of thraustochytrids or aplanochytrids within their host tissues will provide a clue to this. For example, Raghukumar (1988) detected *Ulkenia visurgensis* in coelenteron and hydranth of hydroid using immunofluorescence, an observation that strongly suggests a mutualistic association between the invertebrate and the thraustochytrid.

Direct detection of cells of a given organism is possible by several methods, such as the use of lectins, antibodies, and molecular probes (Anderson *et al.*, 2005; Bullock, 2005).

- Lectins are carbohydrate-binding proteins which occur on cell surfaces and therefore act as recognition molecules. Because of their ability to differentiate between various carbohydrate structures, they

can be used as probes to differentiate amongst morphologically similar species (Hou *et al.*, 2008). However, some problems are associated with their use. Several different types of lectins have to be used for a single sample for comparison. This can be labor intensive when large numbers of samples have to be handled. Secondly the signal intensity depends upon the concentration of lectin receptors on the surface and therefore epifluorescence microscope for detection of the signal will not be sufficient if the signal is very low. Therefore there is a need for spectrofluorometry or flow cytometry to quantify the signal which makes their use expensive.

- Antibodies which are the host glycoproteins secreted by B cells in response to any foreign molecule, can be used for detection by conjugating with fluorophores or an enzyme which after reaction with a chromogenic substrate produces detectable colour reaction (Lipman *et al.*, 2005). Polyclonal antibodies are preferred over monoclonal antibodies as they bind to multiple epitopes and detection using immunofluorescence is widely used.

The use of antibodies is also beset with problems like nonspecific background staining. Frequent cross reactivity is seen unless monoclonal antibodies are used in place of polyclonal antibodies.

- Oligonucleotide probes have emerged in the recent years as the most powerful tools to detect microorganisms in their natural environment. Oligonucleotide probes are short sequences of nucleotides from any

gene of an organism and are designed based on sequences that are found specifically only in that individual strain, species or other higher taxonomic levels, such that they bind to complementary sequences of RNA or DNA.

The most useful gene for designing oligonucleotide probes is the small-subunit rRNA gene. There are several advantages in using this gene for designing probes:

- The rRNA gene is present as tandem repeats in the genome. In the case of their RNA, numerous copies are present in the ribosomes. Therefore it can produce abundant signal.
- The small-subunit rRNA gene (see Chapter 3) is a highly conservative gene and sequences therein are specific to different phylogenetic levels, namely prokaryotes or eukaryotes, different kingdoms, phyla and genera. Oligonucleotide probes to detect bacteria in natural environments have extensively targeted the small-subunit, 16SrRNA gene and that for eukaryotes, the 18SrRNA gene. The ITS portion of this gene is highly variable between species and often even strains. Specific sequences from the ITS region can be used to design probes to detect at species level.

A probe should preferably be of a length of about 17-28 nucleotides with GC content of around 50-60 %. It should end in C or G and should not form primer dimers. It should not be self complementary i.e. should not form hairpins ([Innis & Gelfand, 1990](#)).

Labeled oligonucleotide probes can be used in different ways for detection of the corresponding organism.

- Dot-blot hybridization: This is done by extracting the DNA of the organism and carrying out a PCR of the small-subunit rDNA using appropriate primers. The amplified products from agarose gel are transferred on to a nitrocellulose or nylon membrane and immobilized on the membrane. Bands on the membrane containing the DNA sequence of interest are detected by hybridization with the labeled probe.
- *In situ* hybridization (ISH): This is another common method to directly detect the cells of the organism in question (Biegala *et al.*, 2003). *In situ* hybridization, first described by Gall and Pardue (1969) and John *et al.* (1969), localizes a specific DNA or RNA sequence in a portion or section of tissue or in the entire tissue with the help of a labeled oligonucleotide probe complementary to it and detecting it under a microscope (Schwarzacher, 2003).

Oligonucleotide probes, whether for dot-blot or *in situ* hybridization, can be labeled in many ways. Radioactively labeled probes have been used for many years but they have certain disadvantages like half-life of the isotopes and disposal problem of radioactive waste. Therefore non-radioactively labeled probes were brought into use. These can be labeled by direct or indirect method. Direct method involves covalent attachment of a detectable label to the probe by chemical means or enzymatic means.

Indirect method involves attachment of a hapten to the probe which is then detected by various methods depending on the choice of the hapten. Biotin or digoxigenin or fluorescein can be used as the hapten. The hapten is covalently attached to the probe. It is then detected using a specific binding protein, such as anti-biotin, avidin or streptavidin, anti-digoxigenin or anti-fluorescein, which itself is conjugated to a fluorophore or to an enzyme. In case of the latter the substrates that are used are chemiluminescent or chromogenic.

In biotin-streptavidin system, streptavidin conjugated to enzyme alkaline phosphatase binds to the hapten biotin, which itself is attached to the probe. The enzyme is then detected with the help of 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) substrates that gives a coloured reaction. A blue precipitate of 5,5'-dibromo-4,4'-dichloro-indigo is formed after sequential dephosphorylation of BCIP and oxidation of NBT. The advantages of this system are good signal amplifications and long-term hybridization probe stability ([Paragas et al., 1997](#)).

In situ hybridization may have problems if the sequence to be determined is lower in concentration within the cell or is masked by an associated protein. A typical ISH procedure therefore comprises 5 steps.

1. Permeabilization is carried out in such a way that the structural integrity of the cell is not destroyed.

2. Prehybridization or pretreatment is carried out preferably when probe detection methods involve enzymes for visualization of the label. It reduces the background signal by neutralizing the endogenous tissue enzymes which could result in very high background.
3. Hybridization is carried out under stringent conditions for proper annealing of the probe to the target sequence ([Bottari et al., 2006](#)).
4. Washing is carried out to remove the unbound probe.
5. Detection is made incubating with an appropriate substrate, followed by microscopy.

ISH has been widely used for cultivation-independent assessment of microbial communities from various aquatic and soil habitats. Not only free-living microbial systems, but also symbiotic associations between organisms have been studied by ISH. Amongst the microorganisms, bacteria and archaea are extensively studied using this technique, especially with the help of fluorescently-labeled probes. In addition to these prokaryotes, various eukaryotic microorganisms have also been studied, some examples of which are listed in Table 1.

Table 1. Examples of organisms detected using molecular probes.

Organism	Group	Detection system	Detection from	Reference
Phytophthora	Fungus	Radioactive probe	Infected potato tubers	Tooley et al., 1997
Aureobasidium	Fungus	FITC probe	Apple leaf surfaces	Li et al., 1997
Karenia brevis	Dinoflagellate	Fluorescein, carboxytetramethylrhodamine probe	Red tide samples from around Florida	Gray et al., 2003
Amoebophrya	Dinoflagellate	Texas Red, fluorescein probe	Dinoflagellate <i>Akashiwo sanguinea</i>	Gunderson et al., 2001
Symbiodinium	Dinoflagellate	FITC probe	Corals, sea anemones	Loram et al., 2007
Heterosigma	Dinoflagellate	FITC probe	Algal bloom	Chen et al., 2008
Alexandrium	Dinoflagellate	FITC probe	Scottish coastal waters	John et al., 2003
		FITC, Texas Red, Cy3 probe	Water samples from Gulf of Maine	Anderson et al., 2005
		FITC lectin	Chinese coastal waters	Hou et al., 2008
Pseudo-nitzschia	Dinoflagellate	FITC lectin	New Zealand coastal waters	Rhodes et al., 1995; Rhodes, 1998

In the present study, aplanochytrids were isolated in culture from a number of zooplankton specimens collected from the water column of the equatorial Indian Ocean indicating a strong relationship between the two. But however, the knowledge about this relationship is not clear, since the actual location of the cells in relation to the zooplankton hosts cannot be studied by mere isolation in culture. The use of molecular probes can therefore be very helpful to study the associations of aplanochytrids with the zooplankton (see Chapter 2). An 18S rRNA targeted probe has recently been designed against thraustochytrids to facilitate their detection and enumeration in natural environment (Takao *et al.*, 2007). Such probes, however, work more efficiently at taxonomic levels above the genus. ITS-based probes are more useful to detect individual species in the ecosystem. Eight isolates from zooplankton were identified as belonging to the *Aplanochytrium yorkensis* complex based on morphology, life cycle and 18S rRNA genes.

In view of the above, the following objectives were defined:

- (1) To design an oligonucleotide probe against some isolates of aplanochytrids isolated from zooplankton of equatorial Indian Ocean in order to use it for their detection
- (2) To check the specificity of the probe in order to avoid binding to other organisms and thus avoid false positive reactions.
- (3) To standardize the hybridization technique with the cell control in order to obtain high signal.

(4) To apply the probe on specimens of zooplankton collected from the equatorial Indian Ocean in order to locate the aplanochytrids in them.

6.2 Materials and Methods

6.2.1 Alignment of ITS sequences: A total of 13 ITS sequences were aligned by the Multalin software, version 5.4.1. This consisted of 7 isolates of aplanochytrids obtained from zooplankton of equatorial Indian Ocean, whose ITS regions were sequenced in the present study (Chapter 3; Appendix 1) and 6 sequences of other organisms belonging to Kingdom Straminipila, downloaded from the website <http://www.ncbi.nlm.nih.gov/>. The list of all the 13 organisms used for the analysis are presented in Table 2.

6.2.2 Designing of the probes: Based on multiple alignment of the 7 aplanochytrids and 6 other stramenopiles, sequences which showed consensus regions only among the aplanochytrids and minimal or nil similarity with other organisms were chosen for designing probes for the aplanochytrids. These consensus sequences were analysed using Vector NTI 10 software (Invitrogen, USA). This software analyses the sequences for probes based on the formation of primer dimer, melting temperatures, GC content, salt concentration for various concentrations of primers, binding regions of primer to DNA and tentative length of PCR products to be formed. The software analysis showed that it was feasible

to design probes for two isolates, namely S1961 and S2124. A total of four probes, consisting of one forward and one reverse primer for each isolate could be designed. These were designated S61F1 and S61R1 for the isolate S1961 and 138F1 and 138R1 for the isolate S2124. Details of the primers are given under the 'Results' section. The primer oligonucleotides were obtained from Sigma Genosys, Bangalore. All primers were biotinylated at the 5' end.

Table 2. List of microorganism strains used for multiple alignment.

Kingdom	Phylum	Strain	Sequence length	Obtained from
Stramenopila	Labyrinthulomycota	S1961	556	Equatorial Indian Ocean (Isolated in the present study)
		S1963	503	
		S19610	503	
		S2122	552	
		S2124	447	
		S2128	572	
	S2129	562		
		Labyrinthulid quahog parasite QPX DQ641197	434 bp	NCBI database
Bacillariophyta	<i>Sellaphora pupula</i> AJ544676	706 bp		
	<i>Nitzschia compressa</i> AY574377	706 bp		
Fungi	Chytridiomycota	<i>Spizellomyces</i> sp. OSU AY349099	626 bp	NCBI database
		<i>Rozella allomycis</i> AFTO-IO297 AY997087	628 bp	
	Blastocladiomycota	<i>Catenophlyctis</i> sp. JEL298 AY997034	635 bp	

6.2.3.1 Confirmation of probe specificity: DNA was extracted from the different isolates of aplanochytrids obtained from zooplankton of equatorial Indian Ocean as well as of thraustochytrid cultures CW-1, Z7 and DP7 present in NIO culture collection, using the method described in Chapter 3. Polymerase chain reaction (PCR) amplification was carried out for each set of DNA using the forward and reverse primer probes designed for the two aplanochytrids, namely S61F1-S61R1 and 138F1-138R1 respectively. Amplification was carried out with final concentrations of 7.5 pM primer each, 100 μ M dNTP mix, 1X PCR buffer containing 1.5mM MgCl₂, and 1U Taq polymerase in BioRad Thermal Cycler (S. No. AL100938). The annealing temperature conditions were optimized for both the primer sets by carrying out a gradient PCR in Peltier Thermal Cycler (MJ Research, PTC-200, S. No. AL083846). The temperature gradient that was checked, ranged from 45° to 65° C. The optimal conditions for S61F1-S61R1 primer set were 94° C/ 4 min, followed by 30 cycles of denaturation at 94°C/40s, annealing at 56°C/50s and extension at 72° C/ 1min, with a final elongation step of 72° C/ 6 min. Best conditions for the 138F1-138R1 primer set were 94° C/ 4 min, followed by 30 cycles of denaturation at 94° C/40s, annealing at 60°C/50s and extension at 72° C/min, with a final elongation step of 72° C/ 6 min.

6.2.3.2 Southern hybridization: PCR amplification of the DNA of the aplanochytrid isolates was carried out using both primer sets and optimal

conditions, as above. Agarose gel electrophoresis of the PCR products was carried out at 60 Volts. The gel was blotted on to a nylon membrane (Bangalore Genei) by electroblotting, using electroblotter (ETS-5, supplied with Southern Hybridization Kit, Bangalore Genei). Hybridization and post-hybridization reactions were carried out on the membrane as well as on the gel to check if all the bands had blotted over the membrane as per the instructions in the Southern Hybridization Kit (Bangalore Genei). The bands were then hybridized with the probes S61F1 for S1961, S1963 and S19610 isolates and 138F1 for S2122, S2124, S2128 and S2129 isolates. Hybridization was carried out at 45°C for 12 hours followed by 3 washing steps each with wash buffer A and B. This was followed by incubation for 1 hour in blocking buffer with continuous rocking and then incubation with streptavidin-conjugated alkaline phosphatase (Bangalore Genei) for 20 mins with rocking. Three washing steps each with wash buffer C and D were carried out followed by incubation with 1X substrate solution (BCIP-NBT) provided with the kit till blue bands appeared. The reaction was stopped by transferring the membranes in distilled water.

6.2.4 Standardization of *In situ* hybridization (ISH) of whole aplanochytrid cells:

Two aspects of *in situ* hybridization (ISH) were standardized using cells of isolates S1961 and S19610. These were permeabilization and the actual hybridization. Five day old cells of cultures of the isolates grown in

MV medium (see Chapter 2) were harvested and used for the experiments.

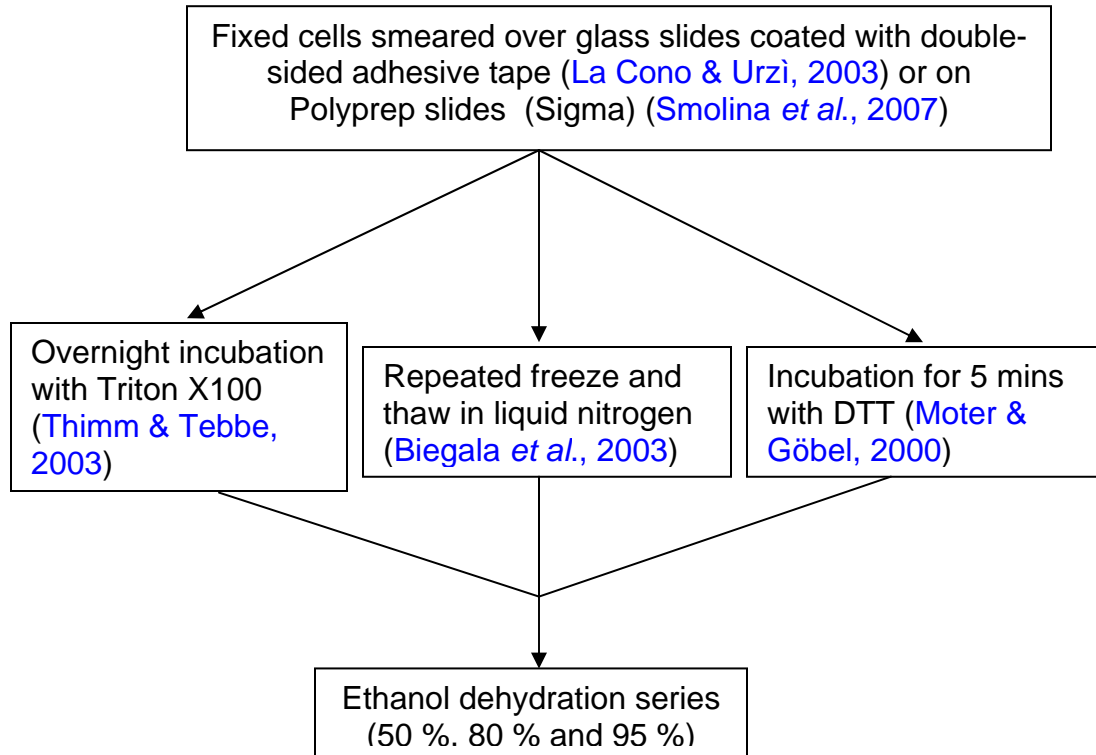
In order to optimize permeabilization, cells were suspended in phosphate-buffered saline containing formalin up to 2 % concentration and stored under refrigeration till further use. Different permeabilization techniques that were tried are shown in Fig.1.

Electroporation was carried out by transferring cells suspended in sorbitol to the cuvette and providing an electric pulse of 2500 V for ~5 milliseconds in Gene Pulser Xcell™ (BioRad, S. No. 617BR105105) as per the protocol mentioned for eukaryotic cells in the manual of Gene Pulser Xcell™.

Hybridization of the permeabilized cells was optimized as follows. The Southern Hybridization Kit (Bangalore Genei) was used for the purpose. Permeabilized cells were incubated with prehybridization buffer provided with the kit at 45°C for 45 minutes. After discarding the buffer, the cells were hybridized with hybridization buffer containing S61F1 probe at a final concentration of 5 pM/μL. Two separate reactions, one containing the forward primer and one containing the reverse primer were used individually. Hybridization was carried out at 45° C for 12 hours. This was followed by the post-hybridization steps, as provided in the Southern Hybridization Kit (Bangalore Genei). The cells were washed thrice with wash buffers A and B and incubated with blocking buffer for one hour with

Fig. 1. Protocols for different permeabilization techniques carried over the cells of aplanochytrid isolates S1961 and S19610.

Protocol 1-



Protocol 2-

Incubation of fixed cells with 1 M DTT for 5 mins.



Washing with distilled water



Final suspension of cells in 1 M sorbitol



Electroporation

gentle rocking. The cells were then incubated with streptavidin-ALP conjugate for 20-30 min, washed with wash buffers C and D thrice and finally incubated in 1X substrate solution.

6.2.5 *In situ* hybridization of field samples using probes:

6.2.5.1 Zooplankton samples fed with aplanochytrids: In order to test if the standardized methodology on permeabilization and hybridization using aplanochytrid cells as above was also efficacious together with zooplankton samples, the following experiment was carried out. Mesozooplankton were sampled from the Dona Paula Bay using a zooplankton net of mesh size 200 μm . Approximately 7-8 live specimens of mesozooplankton were kept in 5 separate beakers each under aeration in laboratory. They were starved for a period of 6 hours. Cells of the aplanochytrid S1961 were fed to each experimental beaker. Cells were added up to twice the concentration found in natural waters of Dona Paula Bay as estimated independently using epifluorescence microscopy (232×10^3 cells L^{-1}). A beaker each was sampled at 4, 6, 10, 15 and 24 hours each. The entire contents of each beaker were fixed with 2 % formaldehyde to preserve the specimens and in order to check if the aplanochytrids had been grazed and were present in their guts. The specimens from each beaker were permeabilized as per protocol 2 (mentioned in Fig. 1). These were transferred to microcentrifuge tubes, treated with 1M DTT for 5 mins, washed twice with distilled water and

once with 1 M sorbitol and finally suspended in 1 M sorbitol. Electroporation was carried out using Gene Pulser Xcell™ with the same pulse conditions as that for whole aplanochytrid cells. After electroporation, *in situ* hybridization was carried out in the microcentrifuge tubes using the same procedure which was followed for the whole aplanochytrid cells as above.

6.2.5.2 *Natural zooplankton population from equatorial Indian Ocean:*

Zooplankton samples collected using the multiple plankton net during the two cruises on board ORV Sagar Kanya SK 212 (October 2004) and SK 228 (September 2006) from the water column of the equatorial Indian Ocean were preserved in formalin up to 2 % concentration in refrigerator. They were subjected to *in situ* hybridization using S61F1 and 138F1 probes individually. The pretreatment as well as hybridization was carried out as mentioned above for the experiment on zooplankton specimens fed with aplanochytrids.

6.2.6 *Detection and analysis:* The post-hybridization steps like washing, incubation with blocking buffer, conjugate and substrate were carried out as per the protocol used for aplanochytrid cells given above. After incubation with substrate solution for 1-2 hours, the specimens were transferred to distilled water and then on to a glass slide and observed under microscope (Olympus BX60) using bright field microscopy. Specimens with blue coloration were noted as positive. The photographs

of those specimens showing positive reaction were captured using Olympus Digital DP70 camera.

6.2.7 *In situ* PCR of chaetognath specimens: Specimens of chaetognaths from the Indian Ocean collected and fixed as above were washed with phosphate-buffered saline (PBS) and either dried under vacuum or suspended in 1M sorbitol and electroporated before carrying out *in situ* PCR. A total of 50 μ l of PCR buffer was added to each specimen in the PCR tube. *In situ* PCR using the two different sets of primers, i.e., S61F1-S61R1 and 138F1-138R1 was carried out separately, using BioRad Thermal Cycler (S. No. AL100938). PCR cycles consisted of denaturation at 94° C for 40 s, primer annealing (56° C for S61F1-R1 primer set or 60° C for the 138F1-R1 primer set) for 50 s, and extension at 72° C for 60 s. Amplification was repeated for 30 cycles with a thermal cycler. After *in situ* PCR, ISH was carried on the same specimens, following the same methods as those for the zooplankton fed with aplanochytrids as mentioned above.

6.2.8 *In situ* hybridization of aggregates collected from the equatorial Indian Ocean water column:

Marine aggregates that came up along with the multiple net deployed to collect mesozooplankton were fixed along with the zooplankton with formalin up to 2 % concentration and later separated

from the zooplankton using a Pasteur pipette. Two different initial methodologies were followed for *in situ* hybridization. In the first, the samples were electroporated and in the other they were not.

For electroporation, the fixed aggregates were suspended in PBS, incubated with DTT for 5 min., washed with distilled water and finally suspended in 1M sorbitol in a microcentrifuge tube. They were then transferred to a cuvette and given an electric pulse as described under standardization of ISH for aplanochytrid cells. After this pretreatment, *in situ* hybridization on those aggregates was carried out using S61F1 probe and maintaining the same conditions as mentioned above for the experiment on zooplankton specimens that were fed with aplanochytrids.

Alternatively, the aggregates were air dried over polyprep slides (Sigma) instead of subjecting them to electroporation. They were then passed through a series of ethanol dehydration (50 %, 80 % and 95 %) and dried again. ISH was performed on these aggregates over the glass slides using S61F1 probe. The protocol was the same as mentioned for the experiment on zooplankton specimens that were fed with aplanochytrids.

6.3 Results

Alignment of the ITS sequences and designing of probes

The partial-aligned ITS sequences of the 7 aplanochytrid isolates, as well as the six other stramenopiles are shown in Appendix 1. A cluster analysis of the seven aplanochytrids has been presented in Chapter 3 showing isolates S1961, S19610 and S1963 from the SK196 cruise on the one hand and isolates S2122, S2124, S2128 and S2129 from the SK212 cruise on the other clustering together. Probe design analysis using Vector NTI 10 resulted in choosing two isolates as the best possible candidates for the probe, these being S1961 from the SK196 cruise and the other S2124 from the SK212 cruise. Two sets of primers each, one forward and the other reverse, (a total of four) were designed against the two isolates (Table 3). Thus, altogether 4 probes, 2 specific for each group of aplanochytrids, were designed. Regions of binding of the probes is shown in Appendix 1.

Table 3. Sequence and type of the designed probes for the aplanochytrids from zooplankton of the equatorial Indian Ocean.

Isolate No.	Probe	Length	Sequence	Type
S1961	S61F1	19	5'-TAATCCATTTCTAATTCCG	Forward
	S61R1	19	5'-TACATAAAAACCCAAAATC	Reverse
S2124	138F1	20	5'-GCTGTGCGCCTGTTCTGATTG	Forward
	138R1	20	5'-ACATACTTGCGTACATATTG	Reverse

Confirmation of probe specificity

PCR amplification with the designed set of primers showed that primer set S61F1-S61R1 amplified DNA of not only isolate S1961, but also other isolates obtained from the September 2003 cruise, namely S1963 and S19610. However, they did not amplify the DNA of the other isolates obtained from the October 2004 cruise (Fig. 2). Similarly primer set 138F1-138R1 designed for isolate S2124 amplified DNA of not only this isolate, but also that of S2122, S2128 and S2129, which had been isolated during October 2004 cruise (Fig. 3). This primer set did not amplify the DNA of the isolates obtained from the September 2003 cruise.

Southern hybridization carried out using S61F1 probe on the amplified product of the isolates S1961, S1963 and S19610 for the first primer set (Fig.4, 5a) and using 138F1 probe on the amplified product of the isolates S2122, S2124, S2128 and S2129 for the second primer set gave clear positive signals (Fig. 5b).

Fig. 2. Agarose gel electrophoresis of PCR-amplified products of aplanochytrids from the present study and thraustochytrids from NIO culture collection using S61F1-S61R1 primer set.

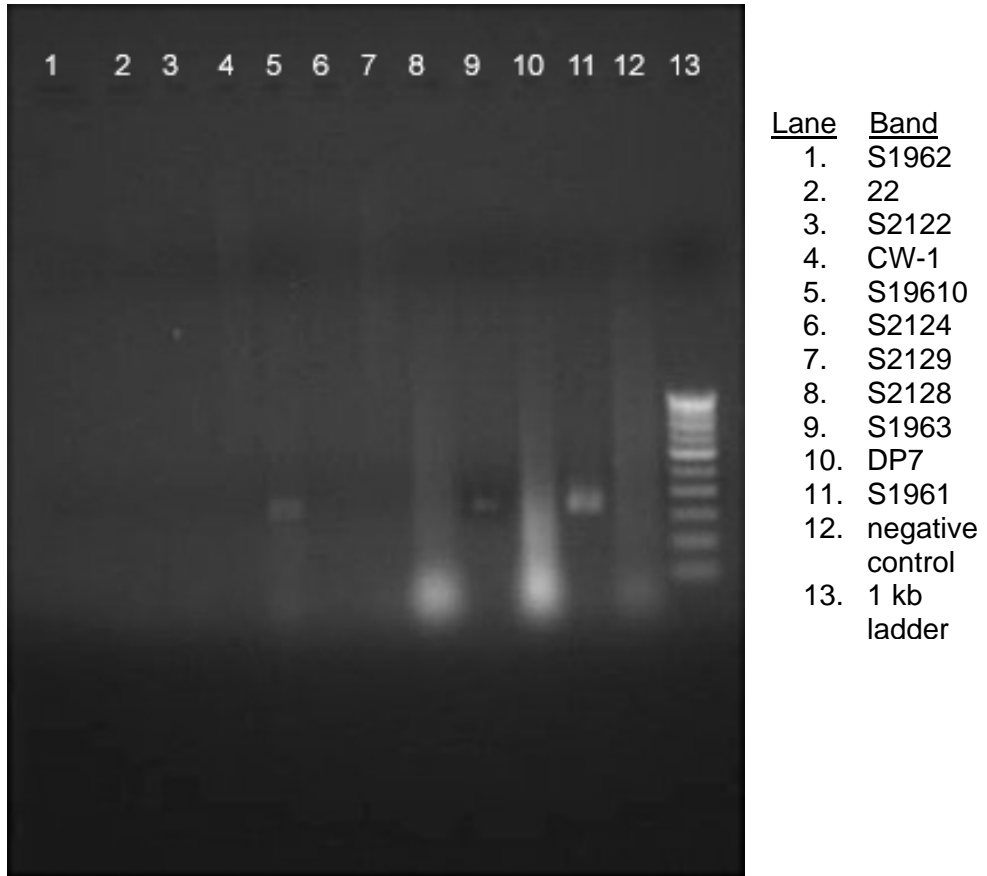
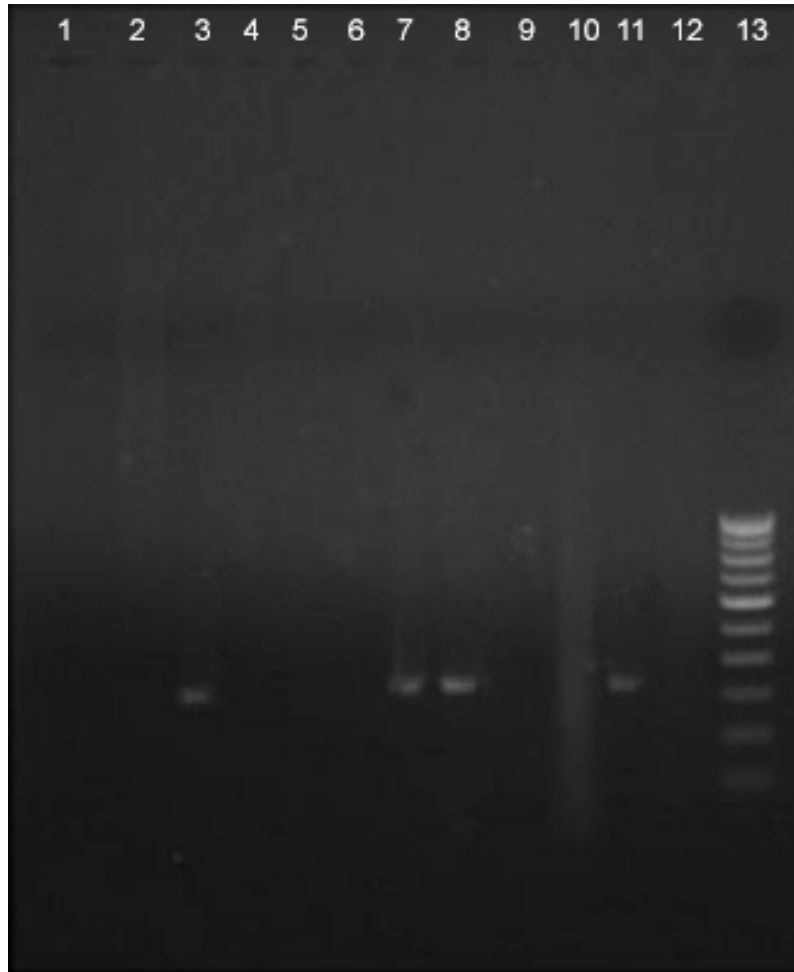


Fig. 3. Agarose gel electrophoresis of PCR-amplified products of aplanochytrids from the present study and thraustochytrids from NIO culture collection using 138F1-138R1 primer set.



<u>Lane</u>	<u>Band</u>
1.	S1962
2.	22
3.	S2122
4.	CW-1
5.	S19610
6.	S1961
7.	S2129
8.	S2128
9.	S1963
10.	DP7
11.	S2124
12.	negative

Fig. 4. Southern hybridization of S1961 amplified product using S61F1-S61R1 primer set as seen stepwise (i) amplified PCR product of approximately 350 bp in length in agarose gel electrophoresis, (ii) portion of the gel containing the band cut prior to electroblotting and (iii) band blotted on nylon membrane visualized by hybridization reaction.

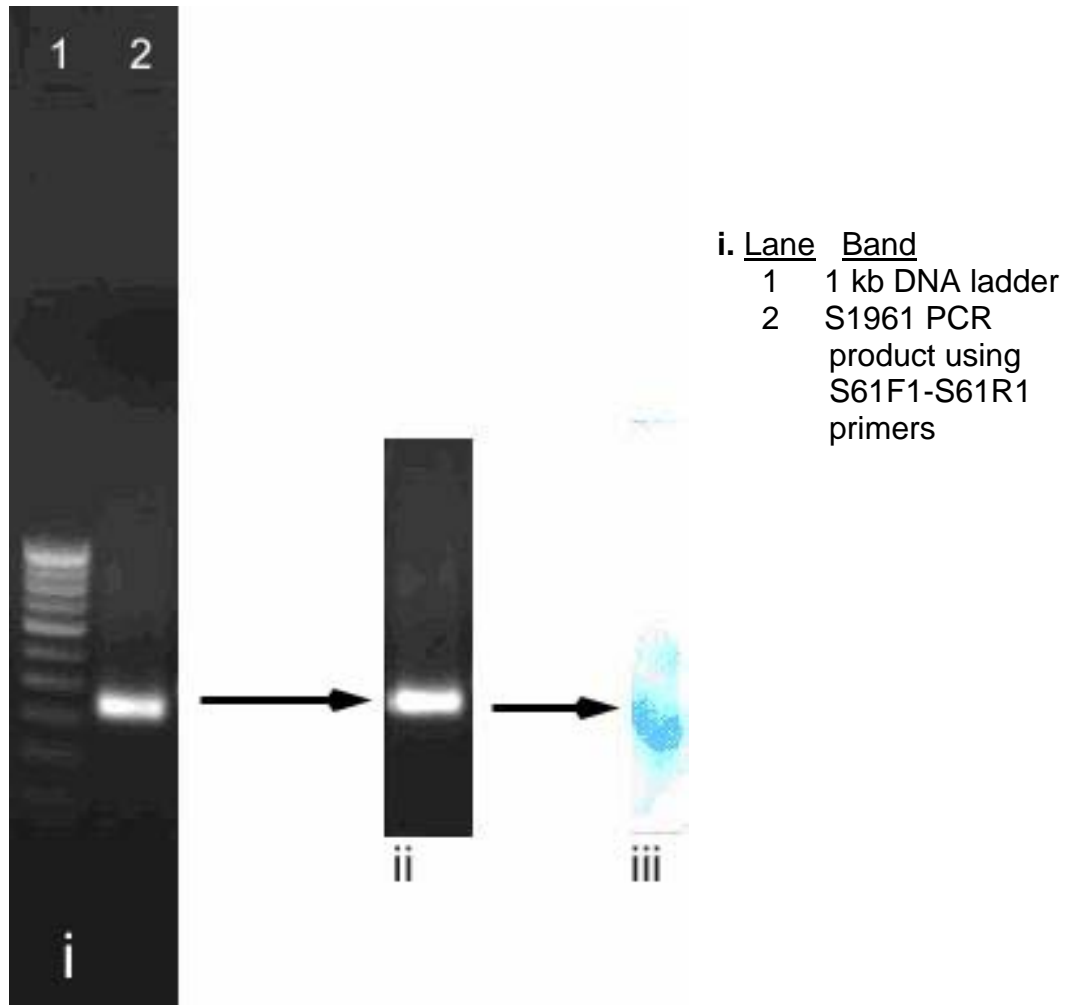
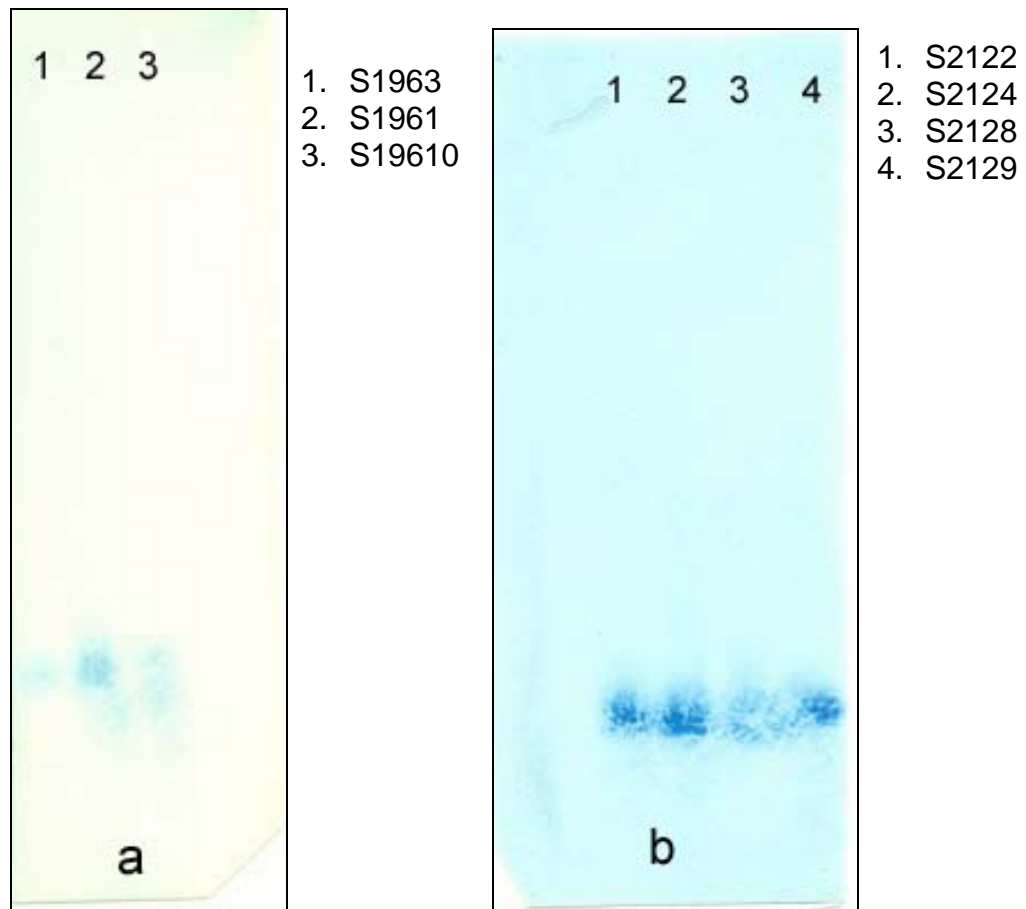


Fig. 5. Nylon membranes as seen after performing Southern hybridization using (a) S61F1 probe and (b) 138F1 probe



Standardization of In situ hybridization (ISH) of whole aplanochytrid cells

All the different permeabilization protocols were positive for *in situ* hybridization reaction of the cells. However, the results varied depending on the protocol used. Thus, cells smeared on slides were mostly washed off. Besides, not all cells pretreated in this way showed the positive blue colour after reaction with streptavidin-conjugated alkaline phosphatase and substrate (Fig. 6a). Electroporation of cells to allow permeabilization provided more consistent results. Thus, all the cells treated in this way showed blue colour upon treatment with streptavidin-ALP. However, the cells were stained blue to different degrees (Fig. 6b). Thus, young cells stained dark as compared to older ones.

In situ hybridization of field samples using probes

Zooplankton samples fed with aplanochytrids: Zooplankton specimens that were fed with cells of the aplanochytrid S1961 for different time intervals were subjected to ISH, following permeabilization using electroporation. No reaction to ISH and, therefore, no development of blue colour that is characteristic of the alkaline phosphatase- BCIP-NBT test was detected in the tissue of the control zooplankton specimens that were not fed with aplanochytrids. On the contrary, aplanochytrid cells that reacted to ISH were detected in the hind gut of several of the zooplankton specimens towards the end of 4 hours of feeding (Fig. 7).

Fig. 6. The aplanochytrid S1961 showing a positive reaction for *in situ* hybridization using biotinylated probes and streptavidin-conjugated alkaline phosphatase. Bar represents 10 μ m.

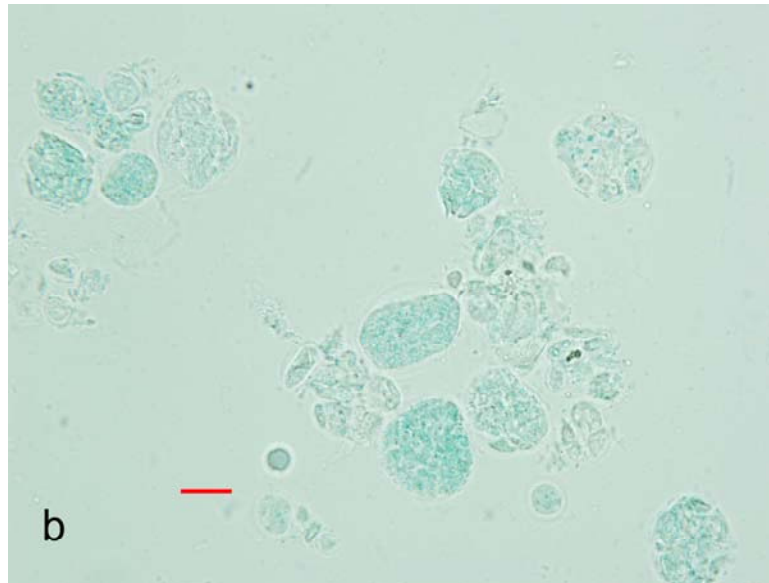
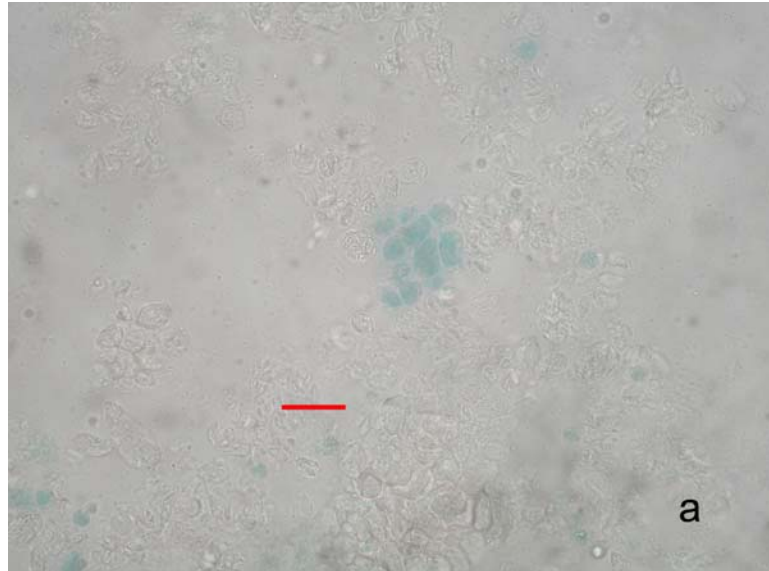


Fig. 7. Zooplankton fed with the aplanochytrid S1961 showing positive reaction in their guts for *in situ* hybridization using probes for the aplanochytrid isolate. Fig. b and d are high power photographs of a and c respectively. Bar represents 100 μm in a and c and 50 μm in b and d.



No aplanochytrid cells were detected in the specimens after 6 hours and more of feeding. However, blue colouration owing to reaction to alkaline phosphatase used in the ISH was noticed in different parts of the body of zooplankton (Fig. 8). The colour intensity decreased after 15 hours of feeding.

In situ hybridization of aggregates: Aggregates collected from the water samples of the equatorial Indian Ocean were subjected to different pretreatments, namely electroporation and air-drying over Polyrep slides prior to ISH with probes for S1961 as well as S2124. Both treatments revealed the presence of aplanochytrid probe-positive cells attached to the aggregates (Fig. 9). These cells were globose and measured 5 to 20 μm in diameter. The cells were generally present scattered in the aggregates.

Natural zooplankton population from equatorial Indian Ocean: *In situ* hybridization using the probes developed for the aplanochytrids was carried out on zooplankton samples collected from different depths of the water column from the equatorial Indian Ocean during the October 2004 and September 2006 cruises, using electroporation and hybridization methods outlined earlier. Specimens from both the cruises showed positive reaction towards probes developed for the isolate S1961 obtained from the September 2003 cruise, as well as those designed for S2124 isolated from the October 2004 cruise (Table 4 and 5). The use of S61F1

Fig. 8. Blue reaction in different parts of the zooplankton body to the alkaline phosphatase- BCIP- NBT following *in situ* hybridization using probes for the aplanochytrid S1961.

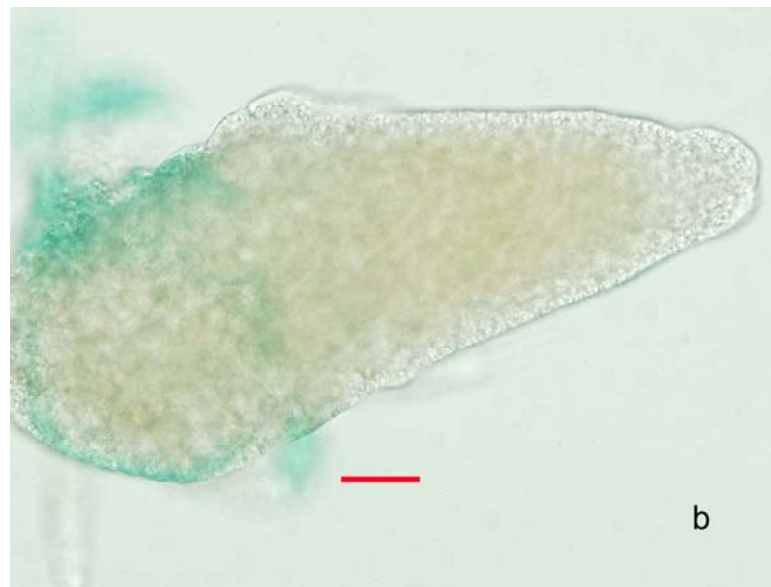
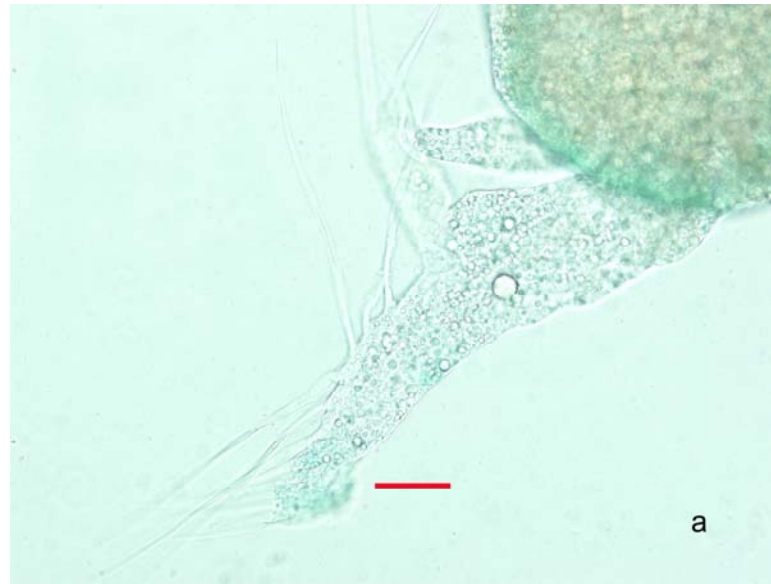
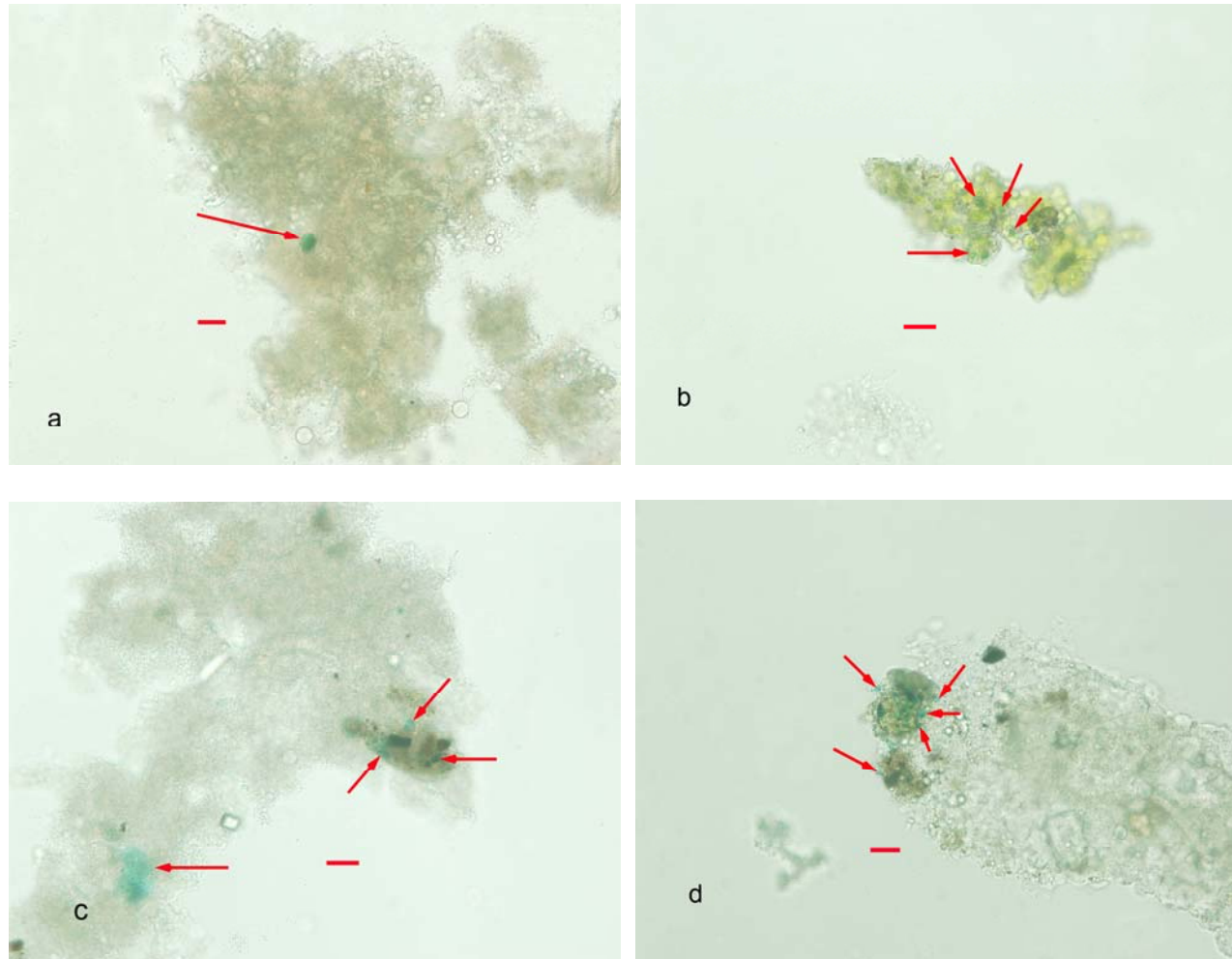


Fig. 9. *In situ* hybridization of aggregates from the equatorial Indian Ocean using biotinylated ITS-probes developed against the aplanochytrid S1961 and S2124. Bar represents 10 μ m.



probe yielded positive reactions from 1.4 - 27.6 % zooplankton specimens sampled during October 2004 cruise, while the use of 138F1 probe yielded 0 – 34.9 % positive reactions (Table 4). ISH-positive specimens of zooplankton were found in all the depths at all nine stations sampled. On the contrary, fewer depths and fewer of a total of 6 stations yielded positive specimens during the September 2006 cruise, SK 228 (Table 5). Only 0 – 12.5 % of the zooplankton specimens were positive to the S61F1 probe during the cruise, and only 0 – 9.3 % were positive to the 138F1 probe (Table 5). No distinct differential response to the two probes was noticed either between the cruises or the depths. Photographs of two specimens are shown in figure 10. Although positive reactions were seen to hybridization, no distinct aplanochytrid cells were detected in the specimens. The reaction was often generalized and found in various parts of the zooplankton.

While generally only a few copepods were positive for hybridization, all the chaetognath specimens took up the blue colour when hybridized with the two probes separately (Fig. 11). In one instance, an aggregate-like structure in the vicinity of the mouth part of the chaetognath reacted positively to ISH (Fig. 11a). Positive reactions were mostly concentrated surrounding the gut. The contents inside the mouth parts of many specimens also showed positive reaction (Fig. 11b and c). Several globose, scattered cells that reacted positively to ISH were observed within the body of the chaetognaths (Fig. 11d).

Fig. 10. *In situ* hybridization of zooplankton specimens from the equatorial Indian Ocean using biotinylated ITS-probes developed against the aplanochytrid S1961 and S2124. Bar represents 10 μ m.

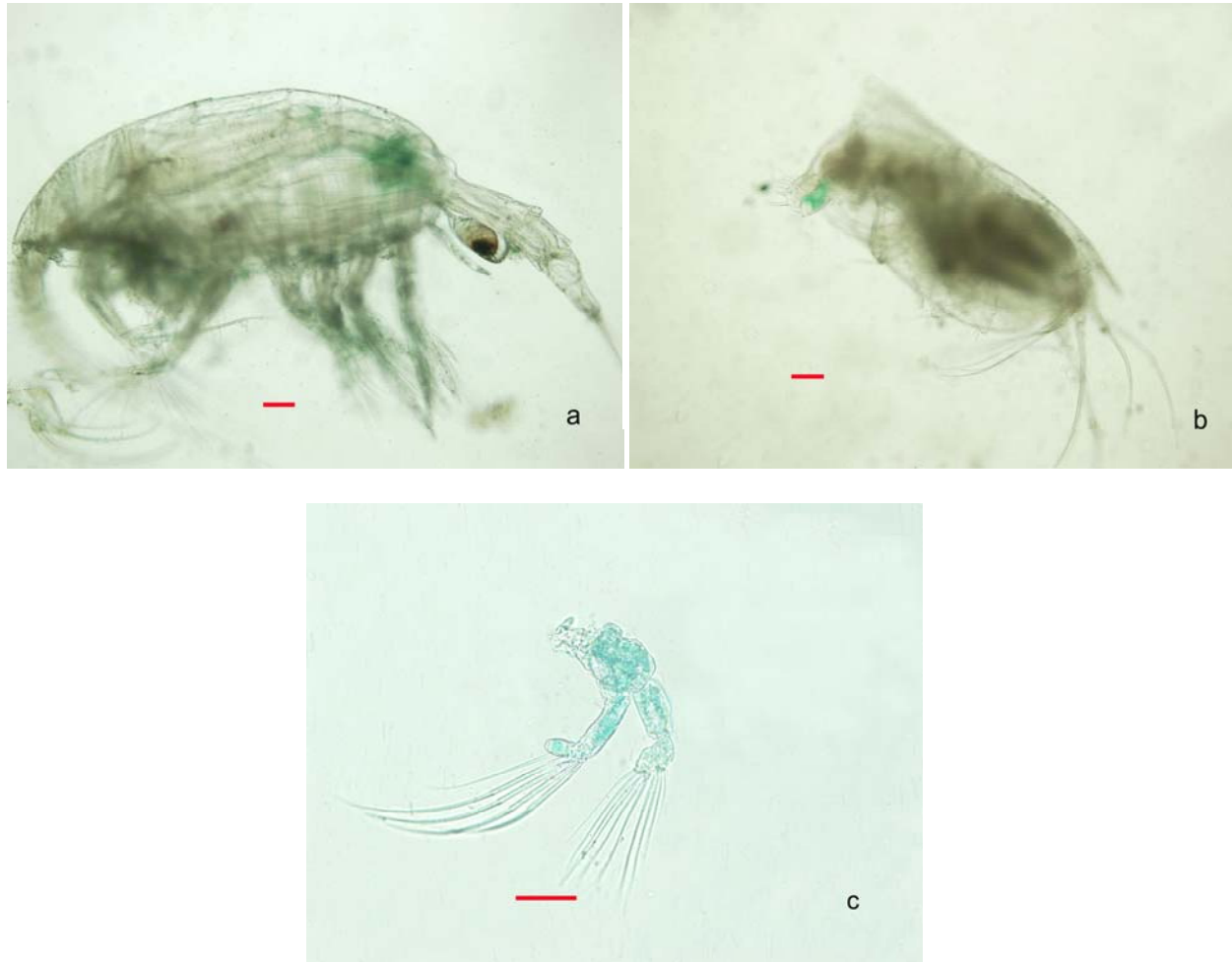


Fig. 11. *In situ* hybridization of chaetognaths from equatorial Indian Ocean using biotinylated ITS-based probes developed against the aplanochytrid S1961 and S2124. Bar represents 100 μ m in a and b and 10 μ m in c and d.

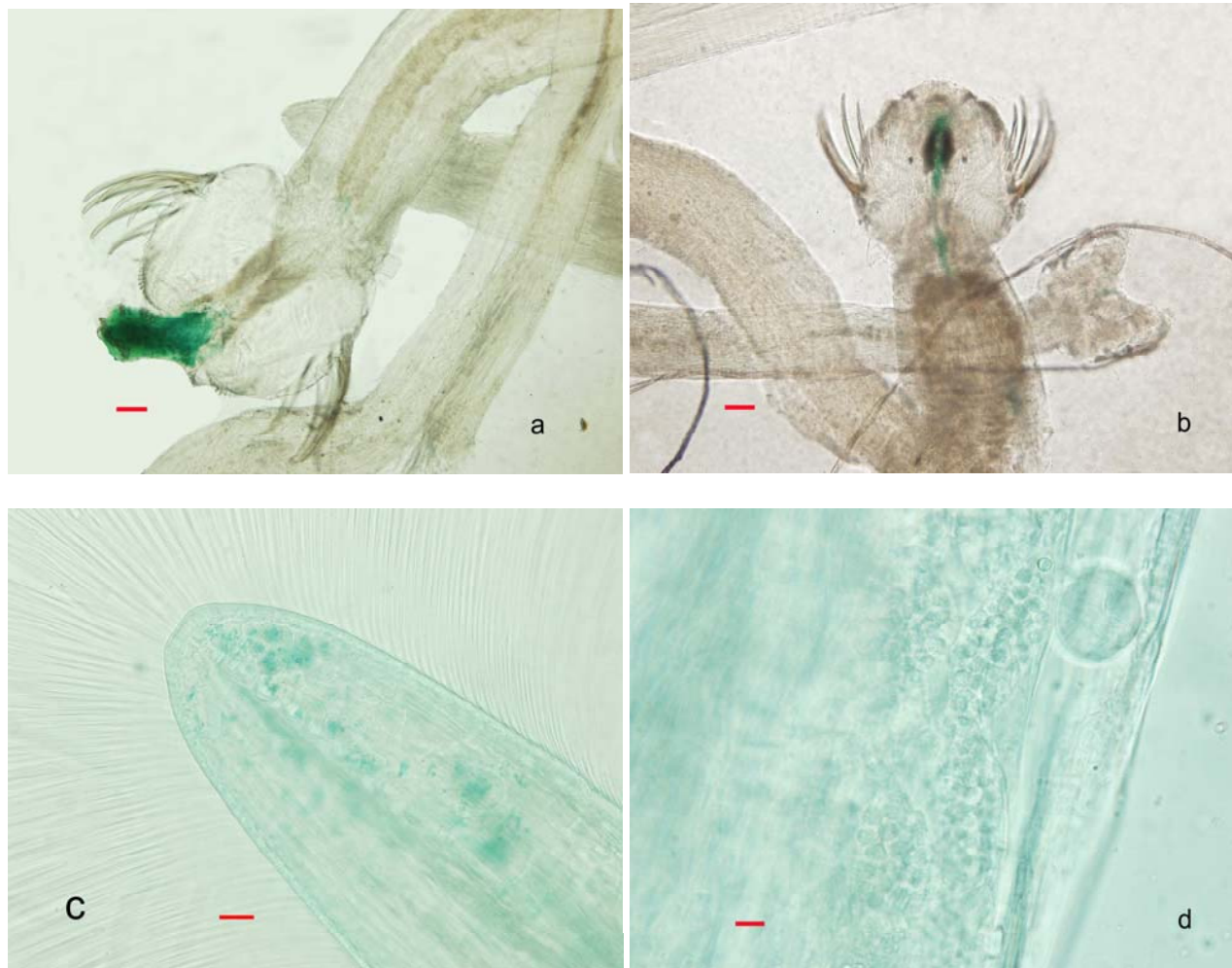


Table 4. Zooplankton from October 2004 cruise (SK 212) positive to both probes. The higher values amongst the two probes are marked in bold.

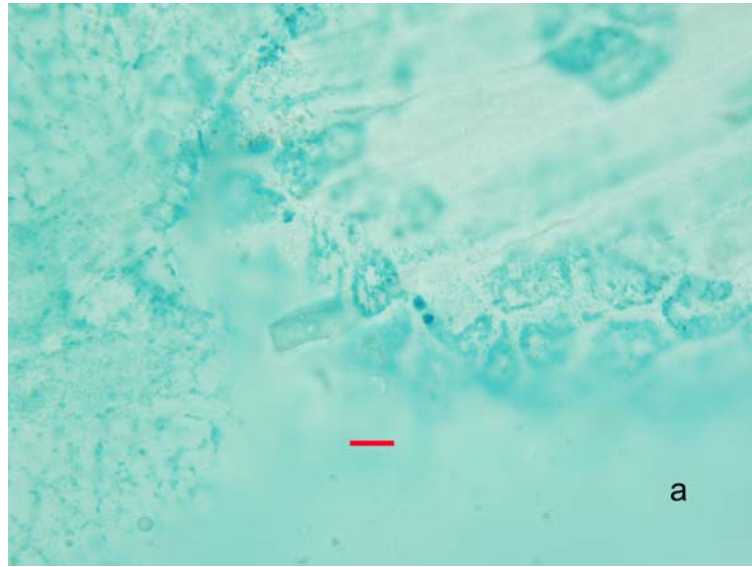
Station	Depth	S61F1 probe			138F1 probe		
		Total specimens	Total positive	% positive	Total specimens	Total positive	% positive
1°N 80.5°E	200-30	63	3	4.8	43	15	34.9
0°Eq 80.5°E	200-38	63	4	6.3	135	4	9.3
0°Eq 80.5°E	500-200	59	5	8.5	54	6	14.0
0°Eq 83°E	1000-500	40	3	7.5	64	0	0.0
0°Eq 83°E	500-300	22	3	13.6	29	5	11.6
0°Eq 83°E	30-0	58	16	27.6	77	7	16.3
1°S 80.5°E	1000-500	73	1	1.4	68	0	0.0
1°S 80.5°E	500-200	61	3	4.9	52	7	16.3
0°Eq 77°E	200-60	50	2	4.0	56	2	4.7

Table 5. Zooplankton from September 2006 cruise (SK 228) positive to both probes. The higher values amongst the two probes are marked in bold.

Station	Depth	S61F1 probe			138F1 probe		
		Total specimens	Total positive	% positive	Total specimens	Total positive	% positive
0°Eq 77°E	500-200	17	0	0.0	23	3	7.0
0°Eq 77°E	200-0	17	0	0.0	31	1	2.3
0°Eq 83°E	500-200	21	0	0.0	36	0	0.0
0°Eq 83°E	200-0	9	1	11.1	9	0	0.0
0°Eq 93°E	500-200	7	0	0.0	5	0	0.0
0°Eq 93°E	200-0	24	3	12.5	24	4	9.3

In situ PCR of chaetognath specimens: *In situ* PCR using primers S61F1-S61R1 and 138F1-138R1 was carried out separately on chaetognath specimens in order to increase signals, followed by electroporation and ISH using the probes S61F1 and 138F1 respectively. This procedure revealed fewer ISH-positive cells within the tissue of chaetognaths compared to the procedure where only electroporation was used prior to ISH. However, the application of *in situ* PCR resulted in cells which stained much brighter (Fig. 12). Cells that reacted positively to *in situ* PCR-ISH were mostly ellipsoidal to irregular in shape, similar to cells of aplanochytrids in culture (Fig. 12 a; see Chapter 2). Such aplanochytrid-positive cells were present lining the gut.

Fig. 12. *In situ* hybridization of chaetognaths from the equatorial Indian Ocean following *in situ* PCR, using biotinylated ITS-probes developed against the aplanochytrid S2124. Bar represents 10 μm in fig. a and 100 μm in fig. b.



6.4 Discussion

ITS-based probes for two aplanochytrid isolates from the equatorial Indian Ocean were successfully developed and used in this study. This is only the third instance where molecular probes have been used for detecting members of the Labyrinthulomycetes. Earlier Lyons *et al.* (2005) generated two 18S rRNA targeting probes against Quahog Parasite Unknown (QPX). They used these probes to detect the presence of the parasite in the marine aggregates. Takao *et al.* (2007) have also designed an 18S rRNA targeted probe against thraustochytrids, especially *Aurantiochytrium*, formerly *Schizochytrium*. Though they have optimized the FISH protocol using this probe, they have not used it for detection in natural samples.

The ITS-based probe designed here was meant to study individual strains of the aplanochytrids, belonging to the *A. yorkensis* complex. Molecular probes can be designed using different labels, such as fluorescent ones like FITC, rhodamine, enzyme-linked labels like biotin, digoxigenin using alkaline phosphatase (ALP) or hydrogen peroxidase (HRP). However, fluorescent *in situ* hybridization (FISH) probes were avoided in this study because zooplankton, on which the probes were intended to be used, have a chitinous exoskeleton, which autofluoresces and can cause severe interference in detecting the required organism. Therefore, it was decided to use a bright-field microscopy based probe which can react with chromogenic substrate to give a coloured reaction.

A biotin-labelled probe was used with alkaline phosphatase conjugated to streptavidin.

Two probes, based on two specific primers were developed for two individual isolates each in the present study. Interestingly, although both isolates apparently belonged to the *Aplanochytrium yorkensis* complex, being closest to that species, the ITS primers designed for the isolate from the SK196 cruise, S1961 was specific only to it and not to S2124 isolated from the SK212 cruise. This shows that the probes might be specific to strains or individual populations of *A. yorkensis* occurring in the waters of the equatorial Indian Ocean. ITS sequences are known to be highly variable amongst species ([White et al., 1990](#); [Martin & Rygiewicz, 2005](#)). Although the probe for S1961 did not react with S2124, it amplified the DNA of other isolates obtained from that cruise, namely S19610 and S1963. Likewise, the primers for S2124 amplified the DNA of other isolates obtained from that cruise, namely S2122, S2128 and S2129. The first three were isolated during September 2003 and the latter four during October 2004. This indicates that different populations of *A. yorkensis* might be associated with zooplankton at different periods in the equatorial Indian Ocean.

The probes could be applied successfully to detect the aplanochytrid in aggregates collected from the water column (Fig. 9). [Lyons et al. \(2005\)](#) also demonstrated that the thraustochytrid, QPX parasite, colonized marine aggregates, by using 18S rRNA probes. There

is increasing evidence to show that thraustochytrids might actively colonize and grow on marine aggregates. The use of the AfDD epifluorescence technique to detect thraustochytrids and aplanochytrids has indicated that these organisms might be related to particulate organic matter, particularly marine aggregates in the water column. Epifluorescence detection methods have a limitation because they might not be entirely specific like molecular probes. Secondly zoospores which lack the cell wall cannot be detected by epifluorescence. The use of ITS-based, species-specific probes for the aplanochytrids shows that marine aggregates are important substrates for these protists, as discussed in Chapter 4.

Microorganisms inhabiting aggregates could form an important source of nutrients for zooplankton feeding on the aggregates (Kjørboe, 2001; Grossart *et al.*, 2003). *In situ* hybridization carried out on chaetognaths often yielded positive reaction in the foregut part of the organism, suggesting the presence of the aplanochytrids in that region, probably through feeding. Thraustochytrids are now believed to be an important source of the omega-3 polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA) to zooplankton. There are no detailed reports of PUFAs in aplanochytrids. If these too are rich in DHA, they would then play an important source of the PUFA to zooplankton.

The presence of aplanochytrids in the guts of zooplankton after feeding was also verified through an experiment in which cells of the

aplanochytrid were added to living zooplankton, after which ISH using the probe for the aplanochytrid was attempted. This experiment was carried out to ensure that the aplanochytrids can be detected when associated with zooplankton. Cells added in this manner are likely to be eaten by the copepods. None of the copepods showed a positive blue reaction to alkaline phosphatase- BCIP-NBT substrate in the control set where aplanochytrid cells were not added. On the other hand, many copepods showed a blue colouration in their hind guts 4 hours after the cells of the aplanochytrid were added. This not only confirmed that the probes were effective but also that the cells were eaten and had passed into the hind gut.

ISH preceded by *in situ* PCR showed distinct, angular to amoeboid cells within the tissues of the chaetognath, typical of *A. yorkensis* on a nutrient agar medium. These results suggest that aplanochytrids might live within the body parts of these zooplankton, probably in a mutualistic association. [Rinkevich & Rabinowitz \(1993\)](#) have also found thraustochytrid cells circulating in blood system of tunicate *Botryllus schlosseri*. They are found in all body parts of tunicates, corals, sponges and therefore the major contaminants in cell lines of these animals ([Rinkevich & Rabinowitz, 1997](#); [Rinkevich, 1999](#)).

The *in situ* hybridization carried out in this study often showed a positive reaction from many body parts of both copepods and the chaetognaths. For example, the setae of copepods, or the ovaries and tail

of chaetognaths also showed a positive reaction (Fig. 10c and Fig. 12c, d). There are two possible explanations for this. (1) The aplanochytrids could have been attached to the exterior of various body parts. The diffused nature of the colouring and the absence of distinct cells of the aplanochytrid probably resulted from the destruction of the aplanochytrid cells during processing. (2) Alkaline phosphatase is known to be present in the body tissues of zooplankton. The enzyme is often associated with cell surfaces (Cembella *et al.*, 1984). It is produced when phosphate concentrations are depleted intracellularly or extracellularly. Zooplankton secrete this enzyme in the environment (Jamet & Boge, 1998; Gambin *et al.*, 1999; Boge *et al.*, 2006). It is reported to be produced by adults as well as juveniles of copepods and branchipods (Lespilette *et al.*, 2007). This could be the endogenous alkaline phosphatase giving positive reaction to the substrates. Care was taken for inhibition of the endogenous ALP activity by pre-treatment with dithiothreitol (DTT), a strong reducing agent. In addition, the specimens had been fixed in formalin, which destroys endogenous alkaline phosphatase activity (Bussolati & Leonardo, 2008). Despite these precautions, false positive reactions could have occurred, owing to the presence of alkaline phosphatase produced by the zooplankton. Alternatively, the positive reactions could therefore be due to insufficient penetration of both the reagents. The use of a biotin-labelled probe for use with alkaline phosphatase, therefore, could have a handicap. The use of other labels and substrates for *in situ* hybridization,

such as the use of horse-radish peroxidase as the colour indicator, or the use of digoxigenin-labelled probes will confirm the findings of this study.

6.5 Conclusions

ITS-based probes were successfully designed against aplanochytrids isolated from mesozooplankton of the equatorial Indian Ocean. *In situ* hybridization using cells of the aplanochytrids was standardized using biotinylated probes, together with streptavidin-conjugated alkaline phosphatase for detection. Laboratory experiments using the ISH suggested that aplanochytrids added to living zooplankton were consumed by the animals. Aplanochytrids were detected in marine aggregates collected from the equatorial Indian Ocean. ISH also suggested the presence of the aplanochytrids in the guts of chaetognaths. Copepods from natural samples also reacted positively to ISH. Various body parts of the zooplankton also reacted positively to alkaline phosphatase used together with the probes during ISH, suggesting either that cells of the aplanochytrid were attached to the body surfaces or that false positive reactions occurred. Further use of the probes with other labels will help to refine the method by avoiding possible false positives and to reconfirm the results.